

## Fumarylacetoacetate Hydrolase Knock-out Rabbit Model for Hereditary Tyrosinemia Type 1 Postprint

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### Abstract

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## Full Text

## Preamble

### **Fumarylacetoacetate Hydrolase Knockout Rabbit Model for Hereditary Tyrosinemia Type 1**

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**Running title:** Fumarylacetoacetate hydrolase knockout rabbits

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## ABSTRACT

Hereditary tyrosinemia type 1 (HT1) is a severe human autosomal recessive disorder caused by deficiency of fumarylacetoacetate hydrolase (FAH), an enzyme catalyzing the last step in the tyrosine degradation pathway. Lack of FAH causes accumulation of toxic metabolites (fumarylacetoacetate and succinylacetone) in blood and tissues, ultimately resulting in severe liver and kidney damage with onset that ranges from infancy to adolescence. This tissue damage is lethal but can be controlled by administration of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), which inhibits the generation of toxic metabolites upstream of fumarylacetoacetate and succinylacetone. Notably, in animals lacking Fah, transient withdrawal of NTBC can be used to induce liver damage and a concomitant regenerative response that stimulates the growth of healthy hepatocytes. Among other things, this model has raised tremendous interest for the in vivo expansion of human primary hepatocytes in these animals and exploring cell-based therapy. Here, we report the generation of Fah knockout rabbits via pronuclear-stage embryo microinjection of transcription activator-like effector nucleases (TALENs). Fah<sup>-/-</sup> rabbits exhibit phenotypic features of HT1 including liver and kidney abnormalities, but additionally develop frequent ocular manifestations likely caused by tyrosine accumulation upon NTBC administration. We also show that allogeneic transplantation of wild-type rabbit primary hepatocytes into Fah<sup>-/-</sup> rabbits enables highly efficient liver repopulation and prevents liver insufficiency and death. Due to advantages over rodents

and their ease of breeding, maintenance, and manipulation compared to larger animals including pigs, Fah / rabbits are an attractive alternative for modeling the consequences of HT1 and testing experimental therapies.

## INTRODUCTION

Animal models bearing mutations in genes causing human disease are essential to understand disease mechanisms, identify new diagnostic procedures, and develop therapies. In some cases, these animals have also proven valuable for unexpected applications (1). One such example is Fah / mice (2), which lack fumarylacetoacetate hydrolase (FAH), an enzyme responsible for catalyzing the last step in the degradation of the amino acid tyrosine (3). In humans, FAH deficiency causes hereditary tyrosinemia type 1 (HT1), a rare autosomal recessive condition characterized by accumulation of toxic metabolites including fumarylacetoacetate and succinylacetone in blood and tissues, which ultimately causes liver failure and renal tubular dysfunction as the most prominent manifestations (4,5). Liver failure can develop acutely in the first few months after birth or be more progressive. Treatment of HT1 consists mainly of 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC), which inhibits the conversion of 4-hydroxyphenylpyruvate to homogentisic acid by 4-hydroxyphenylpyruvate dioxygenase, the second step in the tyrosine degradation pathway, thus preventing accumulation of fumarylacetoacetate and succinylacetone (6). Yet, if patients are diagnosed too late or do not comply with NTBC treatment, there is risk of end-stage liver disease with hepatocellular carcinoma due to chronic damage.

Notably, in Fah / mice, controlled withdrawal of NTBC can be used to cause liver damage in a desired manner, inducing a regenerative response that allows repopulation of the damaged liver by the remaining normal hepatocytes after NTBC is added back (2,7). When Fah / mice are simultaneously deficient in key genes regulating the immune response (e.g., Rag2 and/or Il2rg), the same principle allows liver repopulation with transplanted heterologous primary hepatocytes (9), stem cell-derived hepatocytes (9), or transdifferentiated hepatocytes (10,11) without immune rejection.

For a long time, genetic engineering of mammals has been mostly restricted to mice. This is because of the availability of mouse embryonic stem cells (ESCs) and the relative ease of manipulating these cells with traditional gene modification techniques. Genetically modified ESCs can then be injected into mouse blastocysts to produce chimeric animals with germ line transmission (12). Somatic cell modification and nuclear transfer have traditionally been an alternative for genetic engineering of species for which bona fide ESCs are not available, but the procedure is inefficient (13). More recently, the development of designer nuclease technologies (zinc-finger nucleases, transcription activator-like effector nucleases [TALENs], and clustered regularly interspaced short palindromic repeats [CRISPR]/CRISPR-associated protein 9 [Cas9]) (14-20) has significantly expanded the repertoire of species amenable to routine genetic engineering (e.g.,

rats, rabbits, dogs, pigs, sheep, and cattle) and made the procedure less time-consuming; bona fide rat ESCs were also isolated recently (21,22). Accordingly, several groups have reported the generation of Fah / pigs (23,24) and rats (25,26), which offer some advantages over Fah / mice.

Rabbits are widely used in animal experimentation. Their physiology is closer to humans than rodents, and they have relatively low maintenance costs and are easy to breed (27,28). Herein, we report the generation of Fah / rabbits by injecting TALENs into the cytoplasm of rabbit pronuclear-stage embryos. These Fah / rabbits display liver and kidney phenotypic features analogous to Fah / rodents and pigs, but also develop frequent ocular manifestations including corneal keratitis. We further show that allogeneic transplantation of wild-type rabbit primary hepatocytes achieves efficient liver repopulation and improves liver function and survival rate of Fah / rabbits. These results demonstrate that genetically engineered Fah / rabbits are an attractive choice for modeling the consequences of HT1.

## RESULTS

### Construction of TALENs and generation of Fah knockout rabbits

We designed a pair of TALENs targeting exon 2 of the rabbit Fah gene (Figure 1A [Figure 1: see original paper]), and assembled them according to the Golden Gate method with the following codes: NI for adenine, NG for thymine, HD for cytosine, and NN for guanine. We injected different concentrations (10, 20, 30, 50, and 100 ng/ L) of in vitro transcribed TALEN-coding mRNAs into the cytoplasm of rabbit pronuclear-stage embryos (20,27), and then transferred the embryos into surrogate mothers. NTBC was administered to pregnant rabbits from day 15 of pregnancy to prevent intrauterine death (2). With the highest concentration of TALEN mRNAs, all foster mothers miscarried and no rabbits were born (Table I). The pregnancy was not affected with the other TALEN mRNA concentrations; a total of 31 rabbits were born and the ear tissue of each animal was collected for genotyping. With the lowest concentration of TALEN mRNAs, all 6 newborn rabbits were negative for gene targeting. Yet, we detected Fah mutations using 20 and 30 ng/ L, and the targeting efficiency was 100% (4 out of 4 newborns) using 50 ng/ L (Table I). These Fah mutant rabbits were mostly mosaic, with different indels (insertions and/or deletions) within the Fah locus, as shown in (Figure 1B). Through breeding of Fah mutant founder (F0) animals, we obtained a total of 9 Fah / first filial generation (F1) rabbits (Figure 1C) and 8 Fah / rabbits, which appeared healthy at birth. These animals were bred further to produce additional filial generations.

To ascertain the importance of NTBC administration in preventing death of Fah / rabbits, we prepared 13 Fah / rabbits and divided them into 3 groups, each with different NTBC administration modes (Figure 1D). The rabbits were closely monitored to collect blood before death and to store the tissues in good condition. In the first group, no NTBC was administered at any time after

birth, and these animals died shortly (within 1 week after birth) (Figure 1E). In the second and third groups, NTBC was continuously administered until the rabbits were 2 weeks or 1 month old, respectively. Rabbits in the second group survived no longer than 3 weeks after birth, whilst rabbits in the third group were able to live as long as 4 months after birth (Figure 1E).

Western blot analysis of liver tissue lysates from 2 dead Fah / rabbits confirmed that FAH was undetectable when compared with wild-type (Figure 1F). Likewise, heterozygous knockout (Fah / ) rabbits expressed half of the amount of protein of the wild-type. Thus, we have generated Fah / rabbits that can only be maintained alive with NTBC, and we next proceeded to study whether these animals develop characteristic phenotypic features of HT1.

### **Fah / rabbits have liver and kidney phenotypic characteristics of HT1**

We dissected the bodies of dead Fah / rabbits belonging to the above-mentioned groups, and observed prominent liver swelling, hemorrhage, and yellow/green discoloration suggestive of both cholestasis and liver necrosis, in contrast to the normal aspect of their wild-type counterparts (Figure 2A [Figure 2: see original paper]). We also collected and sectioned the liver and kidney tissues for immunohistological analysis with FAH antibodies. As expected, FAH protein was completely absent in Fah / livers and kidneys, 2 tissues that normally express it at high level, but displayed strong staining in wild-type rabbits (Figure 2B). In addition, hematoxylin and eosin staining showed diffuse hepatocellular injury with dysplastic hepatocytes and tubule-interstitial nephritis in Fah / rabbits only (Figure 2C), both of which are hallmarks of HT1. Moreover, picosirius red staining (29) revealed mild fibrosis in the liver of Fah / rabbits but not in wild-type (Figure 2D), which is in agreement with Fah / mice but contrasts with the development of cirrhosis in Fah / rats and pigs (25,30). In addition, we extracted serum from blood samples and analyzed the levels of alanine aminotransferase (ALT), aspartate aminotransferase (AST), and triglycerides, all of which are elevated as a consequence of liver damage (23). Significant increase of all 3 parameters was observed in Fah / rabbits maintained without NTBC compared to wild-type rabbits (Figure 2E). We also detected that tyrosine levels had increased in Fah / rabbits maintained without NTBC, illustrating that, as expected, deletion of Fah blocks the tyrosine metabolic pathway (2,5). Altogether, these results confirm that Fah / rabbits develop features similar to HT1 patients and rodent/pig models (5,7,23-26).

### **Ocular manifestations in Fah knockout rabbits**

Ocular involvement is not frequent in HT1 patients but in those rare cases corneal keratitis is the main manifestation (31,32). This has been attributed to inflammation produced by local tyrosine deposition in the form of crystals, which is caused by low compliance with a low-protein diet and the secondary effect of using NTBC on tyrosine accumulation. Interestingly, in the course of our study, we noticed that Fah knockout rabbits develop frequent ocular

manifestations too. This problem was observed in all third filial generation Fah / rabbits and a small proportion of Fah / rabbits. Of note, although the latter were not treated with NTBC at any time after birth, it was administered to their mothers during pregnancy. We performed ophthalmological analysis of 2 Fah / rabbits compared to a wild-type rabbit. Through direct eye inspection, we discovered keratoleukoma accompanied by edema and opacity in both eyes of the 2 Fah / rabbits (Figure 3A [Figure 3: see original paper]). Using slit lamp imaging, we also noticed deeper chamber depth and lens opacification accompanied with dilated pupil and posterior synechia of the iris in the right eye of the first Fah / rabbit (Figure 3B), implying cataract and iritis. Moreover, in the same rabbit there was a pathogenic high intraocular pressure of 48.33 mm Hg in the left eye (Figure 3C), indicating secondary glaucoma, whilst intraocular pressure in the right eye was normal. Conversely, chamber depth and intraocular pressure were normal in the second Fah / rabbit (Figure 3B and C), and no significant cataract was found. In addition, hematoxylin and eosin staining showed edema and thickening in the corneal epithelium and stroma of both Fah / rabbits, and the corneal surface became irregular particularly in the left eye of the second Fah / rabbit (Figure 3D). Moreover, swelling, condensed nuclei, and fragmented bodies could be seen in corneal epithelial and stroma cells in both Fah / rabbits, indicating necrocytosis (Figure 3D). Therefore, there is frequent ocular involvement, mostly manifested as corneal keratitis, in Fah knockout rabbits.

### **Allogeneic hepatocyte transplantation rescues liver damage of Fah / rabbits**

We then studied whether transplantation of Fah-competent hepatocytes could rescue the liver phenotype and prevent death of Fah / rabbits untreated with NTBC. First, to assess feasibility, we induced acute liver damage in wild-type rabbits with Concavalin A (33), which causes a rapid regenerative response that facilitates engraftment of transplanted cells, and 24 hours later injected 10 rabbit primary hepatocytes extracted from a healthy wild-type intrasplenically. To assist with their identification, transplanted hepatocytes were labeled with DiI, as this fluorescent compound can last as long as 1 month *in vivo* (34,35). The recipient animals were immunosuppressed with Cyclosporin A starting 24 hours before transplantation. We observed a large number of DiI-positive cells in liver sections 3 weeks after transplantation, proving the efficacy of the approach (Figure 4A [Figure 4: see original paper]).

Next, we transplanted wild-type rabbit primary hepatocytes into 2 Fah / rabbits that had been treated with NTBC after birth. A wild-type rabbit and a non-transplanted Fah / rabbit treated with NTBC were used as controls. NTBC was gradually decreased in the 3 Fah / rabbits, and completely withdrawn 1 week after the transplantation (Figure 4B). One month after NTBC withdrawal, a small liver biopsy was obtained from one of the transplanted Fah / rabbits for histological analysis. Approximately 30% of liver cells in the transplanted Fah /

rabbit were DiI-positive at this time point (Figure 4C). Likewise, FAH immunohistochemistry confirmed significant engraftment of FAH-positive hepatocytes in the same Fah / rabbit, and these hepatocytes displayed normal morphology in contrast with damaged cells in non-engrafted areas (Figure 5A [Figure 5: see original paper]). We also measured body weight in these Fah / rabbits and the wild-type control over the post-transplantation period. We noticed that, 11 weeks after NTBC withdrawal the Fah / rabbit that did not receive transplantation showed a 40% decrease in body weight and died shortly after, whilst the wild-type and Fah / rabbits receiving allogeneic hepatocyte transplantation had similar weight and appeared healthy (Figure 5B and C).

In addition, we performed serum biochemical analysis over the post-transplantation period to see whether liver function can be recovered in Fah / rabbits compared to the control. Indeed, ALT and AST values of wild-type and a transplanted Fah / rabbit were alike at week 10 post-transplantation, whilst the non-transplanted Fah / rabbit showed significantly higher values (Figure 5D and E). Interestingly, we also observed more engrafted FAH-positive cells at three months in transplanted Fah / rabbits (Figure 5F and G), indicating long-term stability of the engrafted cells and suggesting *in vivo* proliferation. These data prove the utility of Fah / rabbits as tools for testing experimental methodologies involving liver cell transplantation.

## DISCUSSION

Patients with HT1 are treated with NTBC and dietary restrictions but this is not curative and, besides, a number of individuals fail to respond and require liver transplantation (7). Because shortage of donors limits organ transplantation, other therapeutic strategies (e.g., stem cell-based or gene therapy approaches) are urgently needed to treat this disease population. Appropriate preclinical animal models are required for testing these experimental therapies and although Fah / mice present many phenotypic features of HT1, their physiology (e.g., inflammatory responses (36)) differs significantly from humans. Likewise, their small size and short life span pose a limitation for analytical studies and long-term assessments. Aiming to solve these issues, Fah / rats (25,26), and in particular pigs (23,24), have been generated recently. However, whilst pigs have many advantages over rodents for HT1 disease modeling, their handling and breeding is laborious and costly.

Rabbits are excellent animals for state-of-the-art experimentation. They are closer phylogenetically to primates than rodents and have a longer life span (8-10 years), whilst their medium size, short pregnancy period (1 month versus 4 months in pigs), and relatively straightforward husbandry requirements facilitate production of large cohorts at relatively low cost (27,28). Like pigs, rabbits also have a more diverse genetic background than rodents, a situation that is closer to that in humans. Notably, the first transgenic rabbits were generated over 3 decades ago (37,38), but the lack of bona fide rabbit ESCs for more complex genetic engineering and the inefficiency of rabbit somatic cell nuclear

transfer (39) hampered the development of the field until the arrival of highly efficient designer nuclease technologies (20,40-42).

Our work presented here constitutes the first description of genetically engineered Fah / rabbits. We used TALEN mRNA microinjection into pronuclear-stage rabbit embryos (20) rather than the CRISPR/Cas9 system (40,43) because we envisaged that for embryo injections the toxicity and off-target effects of the former are easier to control (44), though recent improvements of the CRISPR/Cas9 technique could solve this issue (45). Fah / rabbits display liver and kidney manifestations of the human genetic disorder but also have frequent ocular alterations (mostly corneal keratitis). Ocular involvement is rare in patients with HT1 but frequent in patients with tyrosinemia type II (46), which is produced by mutations in tyrosine transaminase, the enzyme undertaking the first step of tyrosine catabolism. As with tyrosinemia type II, corneal keratitis in HT1 patients, and possibly Fah knockout rabbits too, is caused by enhanced local accumulation of tyrosine, which is boosted by NTBC (31,32). The frequency with which ocular manifestations happen in Fah / rabbits potentially makes them a useful model for studying how to prevent and treat this potential complication in HT1 patients. Fah / rabbits did not develop cirrhosis in our study, although it is likely that varying the NTBC administration/withdrawal routine and allowing more chronic damage would induce it. Besides being an excellent choice for modeling HT1 and studying chronic liver damage, Fah / rabbits could also be employed for gene therapy approaches, which have proved successful in Fah / mice and pigs but require additional studies to evaluate long-term safety and efficacy (24). In addition, Fah / rabbits could be exceptional bioreactors for growing human hepatocytes for in vivo human disease modeling (e.g., hepatitis), potential xenotransplantation, or for in vitro studies, but this would require producing Fah / Rag2 / Il2rg / rabbits to avoid immune rejection. Such triple knockout animals would be valuable too as preclinical models for experimental stem cell-based therapies, including the transplantation of hepatocyte-like cells derived from induced pluripotent stem cells (11) or produced through transdifferentiation (10,11). Given the time and cost of pursuing this approach in pigs, rabbits offer an attractive option.

In summary, we have demonstrated that Fah / rabbits are a promising alternative for modeling HT1 and for developing therapeutic strategies aiming to cure this disease. From a wider perspective, our work also shows that genetically engineered rabbits offer a powerful approach to recapitulating human disease.

## EXPERIMENTAL PROCEDURES

### Animals and ethics statement

New Zealand white rabbits were obtained from the Laboratory Animal Centre of Southern Medical University (Guangzhou, China). All rabbit experiments were conducted under the approval of the Animal Care and Use committee of the Guangzhou Institutes of Biomedicine and Health (ID 2012040) and the Depart-

ment of Science and Technology of Guangdong Province (ID SYXK 2005-0063). Animals were observed at least once daily for clinical signs and symptoms consistent with acute liver failure. To obtain Fah / and Fah / F1 rabbits, we bred F0 animals with each other, and then we bred F0 with F1 and F1 with F1, and so on, for producing other filial generations. For details of which rabbits were used in each experiment and their respective genotypes see Supplemental Table I. Pregnant rabbits giving birth to Fah / rabbits received NTBC 7.5 mg/L per 200 mL of drinking water per day beginning on day 15 of pregnancy. The same dose was used for maintenance of Fah / rabbits. Ophthalmological evaluation was performed in Zhongshan Ophthalmic Center, Sun Yat-sen University.

### **TALEN preparation, embryo microinjection, and embryo transfer**

TALENs were designed and assembled according to the Golden Gate assembly method (27). In vitro transcribed TALEN mRNAs were prepared using mMES-SAGE mMACHINE® T7 Kit (Ambion, Austin, TX, USA) and purified using RNeasy Mini Elute Cleanup Kit (Qiagen, Valencia, CA, USA). The TALEN microinjection procedure was essentially as described previously (27). Briefly: 6-8 months old female rabbits were induced to superovulate with 50 IU FSH every 3 hours for 3 days, mated with male rabbits about 72 hours later, and injected with 100 IU of human chorionic gonadotropin. Female rabbits were euthanized 18 hours after mating, and oviducts were flushed with pre-warmed embryo manipulation medium for collecting embryos at the pronuclear stage. Mixed in vitro transcribed TALEN mRNAs were microinjected into the cytoplasm, and then embryos were transferred to EBSS medium for in vitro culture in a 5% CO incubator at 38.5°C with 100% humidity. Approximately 15-30 good quality (as judged by microscope inspection) injected embryos were transferred into unilateral pavilions of the oviducts for each recipient mother.

### **Genotyping**

DNA of newborn rabbits was extracted from a small piece of ear tissue using a Hipure Tissue DNA Mini Kit (Magen, Beijing, China) following the manufacturer' s protocol. PCR products spanning the TALEN target sites were amplified with KOD-Plus-Neo DNA Polymerase (TOYOBO, Tokyo, Japan) with the primers: 5' -GCACTTGAGCCATCGTCCGT (FAH-F) and 5' -ACCAGCAGCAGGCAATCCCA (FAH-R), and then sequenced.

### **Western blot assay**

For Western blotting, liver tissue samples were homogenized in RIPA buffer and centrifuged at 14,500 g at 4°C for 30 minutes; supernatants were then collected and protein concentration quantified. Lysates were subjected to 10% polyacrylamide-SDS gel electrophoresis, followed by immunoblotting onto a polyvinylidene fluoride membrane (Millipore, Temecula, CA, USA). Membranes were blocked with 5% evaporated milk in TBST for 2 hours and incubated overnight at room temperature with primary antibodies against FAH (ab140167,

Abcam, Cambridge, UK). Membranes were then incubated with a secondary HRP-conjugated anti-rabbit antibody (sc-2004, Santa Cruz, CA, USA) for 60 minutes at room temperature and imaged using a SuperSignal™ West Pico Chemiluminescence Substrate (Thermo, Rockford, USA). ACTIN antibody (sc-47778, Santa Cruz, CA, USA) was probed as loading control.

### **Histology**

Liver and kidney tissues were fixed with 4% neutral buffered paraformaldehyde for 48 hours and processed for paraffin embedding and sectioning. For immunohistochemistry staining, antigen retrieval was performed in a 1 M citrate buffer (pH 6.0) bath for 20 minutes. Tissues were immunostained with anti-FAH primary antibodies (ab140167) and visualized using VECTASTAIN Elite ABC HRP Kit (Vector Laboratories, CA, USA). Hematoxylin and eosin staining and picrosirius red staining were prepared using standard protocols.

### **Serum analysis**

Blood was obtained via puncture of the ear vein using heparinized tubes. Serum was separated by centrifugation at 900 g for 20 minutes and stored at -80°C prior to analysis. Concentration of ALT, AST, and triglycerides was measured with a CL-8000 Hitachi 7180 automatic biochemical analyzer (Shimadzu, Kyoto, Japan). Tyrosine was quantified by liquid chromatography mass spectrometry using the ABI 3200 Q TRAP LC-MS/MS system (Applied Biosystems, Foster City, CA, USA).

### **Allogeneic hepatocyte transplantation**

Fresh hepatocytes were isolated from wild-type rabbit livers by in situ collagenase perfusion as previously reported (47). Briefly, the liver was perfused with calcium- and magnesium-free Hank's Balanced salt solution (Thermo, Rockford, USA) supplemented with 0.5 mM EGTA for 10 minutes and 10 mM HEPES for 3 minutes. The solution was changed to EBSS supplemented with 0.1 mg/ml collagenase IV (Sigma, St Louis, MO, USA) for 10 minutes. The liver was then gently minced in the second solution and filtered through 150  $\mu$ m, 75  $\mu$ m, 50  $\mu$ m, and 37.5  $\mu$ m nylon mesh, sequentially. After centrifugation at 50 g for 5 minutes, the pellet was washed with 4.5 g/mL DMEM 3 times at 50 g for 2 minutes. The number and viability of cells was assessed by trypan blue. 10<sup>6</sup> viable cells were then incubated in a 20<sup>6</sup> M DiI solution (Sigma, St Louis, MO, USA) for 5 minutes at 37°C and for 15 minutes at 4°C for labeling, and washed in 4.5 g/mL DMEM. Eventually, 10<sup>6</sup> hepatocytes in 2.5 mL Clonetics® Hepatocyte culture medium (Lonza, Walkersville, MD, USA) were injected into the spleen of recipient rabbits via a small flank incision. Concavalin A and Cyclosporin A were purchased from Sigma.

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## CONFLICT OF INTEREST

None.

## AUTHOR CONTRIBUTIONS

M.A.E. and LX.L. had the idea; L.L., LX.L., and M.A.E. designed the experiments; L.L. and M.A.E. analyzed the data; L.L. conducted most of the experiments; all other authors contributed to the experiments or provided critical advice; M.A.E. and LX.L. provided funding; M.A.E. and L.L. wrote the manuscript, and M.A.E. and LX.L. approved the final version for submission.

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## FOOTNOTES

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**Table I: Generation of Fah knockout rabbits using TALENs.**

TALEN mRNA concentration (ng/ L)	No. embryos transferred	No. new-borns	No. mutant rabbits (%)
10	6 (12)	6	0 (0)
20	12 (31)	9	3 (33)
30	9 (28)	8	3 (38)
50	4 (11)	4	4 (100)
100	3 (25)	0	0 (miscarriage)

## Figure Legends

**Figure 1: Preparation of TALENs and generation of Fah knockout rabbits.** A) Design of TALENs targeting exon 2 of rabbit Fah gene. Bases in red indicate the TALEN recognition sequences. B) Sanger sequencing of the targeted region in the Fah locus in F0 rabbits; \* represents rabbits in which wild-type Fah sequence was detected. C) Sanger sequencing of the targeted region in the Fah locus in Fah / F1 rabbits. D) Schematic of the 3 groups of Fah / rabbits used to study the dependence on NTBC for long-term survival

(ON: NTBC is administered; OFF: NTBC withdrawal). E) Survival curve for the same 3 groups of Fah / rabbits. F) Western blotting analysis confirms that Fah / rabbits (-/-) are negative for FAH protein expression in liver tissue lysates, whilst Fah / (+/-) rabbits express reduced amount of FAH. Band intensities (FAH/ACTIN) were quantified using ImageJ software and shown as relative to wild-type (+/+) rabbits.

**Figure 2: Fah / rabbits develop progressive liver failure in the absence of NTBC.** A) Severe necrosis in the liver of a Fah / rabbit (right) belonging to group 3 of NTBC administration (as in Figure 1D), in contrast to a healthy wild-type rabbit. B) Immunohistochemistry (IHC) of liver and kidney sections of a Fah / rabbit belonging to group 2 of NTBC administration show no expression of FAH, in contrast to a wild-type rabbit. Scale bars: 50  $\mu$ m. C) Hematoxylin and eosin (HE) staining shows abnormal tissue architecture in liver and kidney sections of the same Fah / rabbit in B, in contrast to a wild-type rabbit. In the Fah / rabbit, diffused hepatocellular injury with dysplastic hepatocytes and tubular epithelial injury of kidney were observed. Scale bars: 50  $\mu$ m. D) Picrosirius red staining shows the existence of mild interstitial fibrosis in the liver of the same Fah / rabbit in A but not in the wild-type rabbit. Scale bars: 50  $\mu$ m. E) Serum biochemical parameters indicate liver damage in Fah / rabbits compared to a wild-type rabbit. TG stands for triglycerides. Data were presented as mean  $\pm$  standard error of the mean (SEM; n = 3 replicate measurements). \* corresponds to P < 0.01 according to Student's t-test.

**Figure 3: Ocular manifestations in Fah knockout rabbits.** A) Photographs of right (OD) and left (OS) eyes of 2 Fah / rabbits show corneal abnormalities; a wild-type (WT) rabbit was used as control. B) Slit lamp photographs of the same 3 rabbits show abnormalities of chamber depth and lens opacification in Fah / rabbit number 1 compared to the wild-type. C) Measurement of intraocular pressure using ICAR tomometer (Icane, Finland) in the same 3 rabbits. Only the left eye of Fah / rabbit number 1 shows pathogenic levels. D) Hematoxylin and eosin staining shows corneal structural abnormalities (edema, thickening, cellular defects, and disorganization of the corneal epithelium and stroma) in the left eye of Fah / rabbit number 2 compared to the wild-type. Scale bars: 50  $\mu$ m.

**Figure 4: Hepatocyte transplantation of Fah / rabbits.** A) Transplantation of wild-type rabbit primary hepatocytes into the liver of a wild-type rabbit treated with Concavalin A (ConA; 5.0 mg/kg, intrasplenic injection) to induce acute liver damage. Engraftment of DiI-labeled hepatocytes can be observed under the fluorescence microscope at 3 weeks post-transplantation. DAPI was used to stain nuclei. Scale bars: 50  $\mu$ m. B) Schematic showing the allogeneic transplantation procedure in Fah / rabbits. NTBC was administered for another 2 days after the first liver biopsy. C) Engraftment of DiI-labeled cells observed under the fluorescence microscope in a Fah / rabbit without NTBC at 1 month post-transplantation. Scale bars: 50  $\mu$ m.

**Figure 5: Wild-type primary hepatocyte transplantation rescues liver**

**architecture and prevents death of Fah / rabbits.** A) Top panels: FAH immunohistochemistry shows wild-type hepatocyte repopulation of the liver of a Fah / rabbit 1 month post-transplantation compared to the control (non-transplanted Fah / rabbit). Lower panels: hematoxylin and eosin staining shows restoration of liver architecture in the same transplanted Fah / rabbit compared to the non-transplanted Fah / rabbit. Scale bars: 50  $\mu$ m. B) Body weight changes of the 2 Fah / rabbits in A (with or without transplantation) and a control wild-type rabbit. \* corresponds to  $P < 0.01$ . C) Photographs of a transplanted Fah / rabbit at 3 months post-transplantation and a non-transplanted Fah / rabbit. The non-transplanted Fah / rabbit appears weak. D and E) Serum levels of ALT and AST in a wild-type and 2 Fah / rabbits (with or without transplantation). Data are presented as the means  $\pm$  SEM ( $n = 3$  replicate measurements). \* corresponds to  $P < 0.01$ . F) FAH immunohistochemistry shows extensive wild-type hepatocyte repopulation of the liver of a Fah / rabbit 3 months post-transplantation compared to the non-transplanted Fah / rabbit. Scale bars: 50  $\mu$ m. G) Right panel: hematoxylin and eosin staining shows restoration of liver architecture (circled by dashed lines) in the same transplanted Fah / rabbit at 3 months post-transplantation. Left panel: adjacent liver section of the transplanted Fah / rabbit showing co-localization of areas with normal structure and positive FAH staining. Scale bars: 50  $\mu$ m.

*Note: Figure translations are in progress. See original paper for figures.*

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