Modeling HBV infection and therapy in immunodeficient NOD-Rag1-/-IL2RgammaC-null (NRG) fumarylacetoacetate hydrolase (FAH) knockout mice with human chimeric liver

Animal Models

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Introduction

- Besides human, only a few species have been reported to be permissive for Hepatitis B virus (HBV) infection(1-3). Human liver chimeric mouse models have been developed to overcome these limitations. Currently, three mouse models are available for modeling HBV replication and therapy: urokinase-plasminogen activator (Alb-uPA), fumarylacetoacetate hydrolase knockouts ($FAH^{-/-}$), and herpes simplex virus type 1 thymidine kinase (TK) transgenic mice(4-9). In these strains, transplanted human hepatocytes have a selective growth advantage. However, $FAH^{-/-}$ and TK transgenic mice provides a good alternative to the fertility defect of uPA mice(10).
- Knockout of FAH results in the hepatic accumulation of toxic tyrosine metabolic intermediates and subsequent death of mouse hepatocytes which can be controlled by administration/withdrawal of 2-(2-nitro-4-fluoromethylbenzoyl)-1,3-cyclohexanedione (NTBC)(4, 11, 12). Here we describe a detailed protocol about 1) maintenance and genotyping of NRG-$FAH^{-/-}$ mice, 2) injection of human hepatocytes, 3) NTBC drug cycle, 4) human albumin, and 5) HBV infection and detection.
- This protocol was originally published in Hepatitis B Virus: Methods and Protocols (see reference 12)

Materials and Reagents

A. Solution

1. 5% sodium bicarbonate.
2. NTBC, 2-(2-Nitro-4-trifluoromethylbenzoyl)-1, 3-cyclohexanedione (Sigma-Aldrich).
3. Povidone-Iodine.
4. KAPA Express Extract Kits (Kapa Biosystems, Inc.).
5. ABsolute qPCR SYBR green ROX Mix (ref. #AB1163A, Thermo scientific).
6. Human Albumin ELISA Quantitative Set (ref. #E80-129, Bethyl).
7. ELISA Coating Buffer, 0.05 M Carbonate-Bicarbonate, pH 9.6 (ref. #E107, Bethyl)
8. ELISA Wash Solution, 50 mM Tris, 0.14 M NaCl, 0.05% Tween 20, pH 8.0 (ref. #E106 , Bethyl).
9. ELISA Blocking Solution, 50 mM Tris, 0.14 M NaCl, 1% BSA, pH 8.0 (ref. #E104, Bethyl).

10. Sample/Conjugate diluent, 50 mM Tris, 0.14 M NaCl, 1% BSA, 0.05% Tween 20
11. Enzyme Substrate, TMB One Component Substrate (ref. #E102, Bethyl).
12. ELISA Stop Solution, 1 M H_2SO_4 (ref. #E115, Bethyl).
13. HBV patient serum (SeraCare Life Science).
14. Freshly made Avetin. Mix 0.25 g of 2,2,2-Tribromoethanol, 99% (Alfa Aesar) with 0.5 ml of tert-amyl alcohol (SIGMA-ALDRICH) and dissolving it in 20 ml of water at 50°C. Filter-sterilize through 0.2-micron filter.
15. Bleach (10%).
16. ddH20 Molecular biology grade (Sigma Aldrich).
17. 70% ethanol.
18. 1 X DPBS (Gibco).

B. Equipment:

1. Surgery tools: Scissors, scalpel, curve blunt end forceps, wound clips/clip hold, suture.
2. Water bath.
3. 22 µm filter Millex® Syringe Filters (Millipore).
4. Realtime PCR machine (7000 System, 7300 System, 7700 System, 7900HT System, StepOne™ System, StepOnePlus™ System).
5. 5ml and 1ml Insulin syringe.
6. Hair shaver.

Experimental Procedures

Preparation of NTBC drug solution (1000X).

1. Dissolve 8 g of NTBC in 1 liter of 0.5% sodium bicarbonate at 65°C for 30 min. Shake every 3-5 min until the entire compound dissolved (see Note 1).
2. Filter the solution with a 0.22 um filter. Aliquot and store at -20°C.
3. NRG/F mouse genotyping and colony maintenance.
4. Mouse genomic DNA extraction.
5. Genomic DNA is extracted from mouse-tail tips using the KAPA Mouse genotyping Kit according to the instructions. (See Note 2).
6. For one sample, mix the 10X KAPA Express Extract Buffer (5 µl /sample), 1 U/µl KAPA Express Extract Enzyme (1 µl /sample) and ddH2O (44 µl /sample). The volume can be scaled up accordingly for more samples.
7. Heat at 75 °C for 15 min, and inactivate the enzyme at 95 °C 5 min (see Note 2).
8. The sample can be stored at 4 °C for 1 week, or frozen at -20°C for long term.

Quantitative PCR detection of $FAH^{-/-}$

1. Genomic DNA extract is diluted at a ratio of 1:20 (5 µl to 95 µl ddH2O) and use 5 µl as template.
2. For the $FAH^{-/-}$ detection, use the following primers, FAH-QF (TTGAATCTTGAAGAATGGTTTGAGC), FAH-QR (AATGCCAACATGATCATCCTAGG), with amplicon size 148 bp. Mix both primers for a final concentration of 10 µM.
3. For the mouse genome DNA control, use the following primers, mGAPDH (450+26) (AACCACGAGAAATATGACAACTCACT) and mGAPDH (584-20) (GGCATGGACTGTGGTCTAGA), amplicon size 134 bp. Mix both primers for final concentration of 10 µM.
4. Set up of qPCR reaction: 2 X SYBR buffer 7.5 µl, primers mix (10 µm) 0.105 µl, ddH2O 2.4µl, and diluted sample 5µl.
5. Program for qPCR: step 1, 50°C 2 min; step 2, 95°C 15 min; step 3, 95°C 15 sec; Step 4, 60 °C 1 min; go to step 3 for 35 cycles.
6. Data analysis: normalize the $FAH^{-/-}$ to mGAPDH. No $FAH^{-/-}$ signal should be detected in

negative controls. Set heterozygous mice to 1 and the homozygous mice should be around 2.
7. NRG/F homozygous mice maintenance.

NRG/F homozygous mice must be maintained with 100% NTBC (8 µg/ml). The NTBC drug is pretty stable up to 3 weeks in the cage. **Transplantation with human adult hepatocytes.**

1. Hepatocyte preparation.
2. Suspensions of freshly isolated or cryopreserved primary human hepatocytes (PHH) could be used for liver humanization. Our PHH are usually obtained from the Research Triangle following the instructions of the supplier.
3. Centrifuge cells at 100 g for 10 min.
4. Carefully discard most of supernatant, keeping less than 0.5ml residual medium.
5. Resuspend and count the cells, and determine the cell viability with Trypan blue usually around 80%.
6. Adjust the volume with medium to $1x10^6$ cells/50 µl per mouse.
7. Intrasplenic injection.

Throughout the procedure, standard sterile surgical techniques are used, including 70% ethanol sterilization of instruments between animals. The entire surgical procedure must be performed under biosafety cabinet with a laminar flow and should not exceed 10 minutes per mouse because the viability of PHH could decrease during the procedure. If splenic bleeding occurs, use sterile swab to exert slight pressure on the spleen and close only if the bleeding stops. After surgery, mice must be kept in a warm environment such as post-surgery warming pads to facilitate a better recovery. Mice should wake up in about 2 hours.

1. Mouse anesthesia is performed with intraperitoneal injection of 500 µl of Avetin. Depending on the weight of the mouse. It will take about 2 min to fully anesthetize the mouse. Checked toe pinch reflex before surgery.
2. Shave the left side abdomen around the spleen.
3. Lay the mouse down on surgical board and sterilize the shaved region by sequentially using Povidone-Iodine and 70% ethanol on the body surface with swab.
4. Open the skin and the muscle of abdomen with scissors. The incision size is about 1 cm.
5. Gently pull out the fat pad under the spleen using a curved blunt-ended forceps.
6. Slowly inject $1x10^6$ PHH in 50 µl into the lower pole of the spleen.
7. After injection, pull out the needle very slowly to prevent bleeding (see Note3).
8. Close abdominal muscle layer with muscle suture.
9. Close skin layer with skin staples.
10. Wound clips should be removed within 10 days after surgery.

NTBC cycling. The timing of NTBC cycling is very important, since NTBC controls the level of liver damage (see Note 4). The cycle is listed below and summarized in table 1. The remaining NTBC water can be reused at each step for further dilution. Cycle 1:

- Day 0, right after surgery, change water to 25% of NTBC (2 µg/ml).
- Day 2, change water bottle to 12% NTBC (0.96µg/ml).
- Day 4, change water bottle to 6% NTBC (0.48µg/ml).
- Day 7, change water to 0 % NTBC.
- Day 21, Change the water to 100% NTBC.

Cycle 2:

- Day 28, change water to 25% of NTBC (2µg/ml)
- Day 30, change water bottle to 12% NTBC (0.96µg/ml).
- Day 32, change water bottle to 6% NTBC (0.48µg/ml).

- Day 35, change water to 0 % NTBC.
- Day 49, Change the water to 100% NTBC.

Cycle 3:

- Day 56, change water to 25% of NTBC (2µg/ml)
- Day 58, change water bottle to 12% NTBC (0.96µg/ml).
- Day 60, change water bottle to 6% NTBC (0.48µg/ml).
- Day 63, change water to 0 % NTBC.
- Day 77, change water to 100% of NTBC (2µg/ml)

Cycle 4:

- Day 84, change water to 25% of NTBC (2µg/ml)
- Day 86, change water bottle to 12% NTBC (0.96µg/ml).
- Day 88, change water bottle to 6% NTBC (0.48µg/ml).
- Day 91, change water to 0 % NTBC.

Table 1: Schedule of NTBC drug recycling (see Note 4).

Cycle	**NTBC concentration**			
	25%	12%	6%	0%
1	Day0	Day2	Day4	Day7-21
2	Day28	Day30	Day32	Day35-49
3	Day56	Day58	Day60	Day63-77
4	Day84	Day86	Day88	Day91-

Generation of NRG/FAH-hu HEP mice with 4 cycles of NTCP reduction

Human Albumin quantitation Adapted from a protocol of Bethyl Laboratory human Albumin ELISA Quantitative Set (see Note 5).

1. Plate coating: Coating antibody dilution 1:250 (see Note 5). Add 100 µl of diluted coating antibody to each well. Note: Run each standard or sample in duplicate. Incubate overnight at 4°C. Wash plate 5 times.
2. Add 250 µl of Blocking Solution to each well. Incubate at room temperature for 30 minutes. Wash plate 5 times.
3. Dilute the plasma/serum samples during step 1 and step 2. Fold dilution, 100 during week 3-7, and 1000 thereafter. Standard dilution: to 400, 200, 100, 50, 25, 12.5, 6.25, 3.125 ng/ml.
4. Add 100 µl of standard or sample per well. Incubate at room temperature for 1 to 2 hours. Wash plate 5 times.
5. Prepare 1:30000 dilution of detection antibody dilution (see Note 5). Add 100 µl of diluted HRP detection antibody to each well. Incubate at room temperature for 1 hour. Wash plate 5 times.
6. Add 100 µl of TMB Substrate Solution to each well. Develop the plate in the dark at room temperature for 15 minutes. Stop reaction by adding 50 µl of Stop Solution to each well. Measure absorbance with a plate reader at 450 nm.

HBV infection and detection.

1. Inoculate NRG/F-hu HEP mice with HBV

For infection studies, NRG/F HuHep mice with hAlb. > 0.5 mg/ml are subjected to a short anesthesia, Isoflurane, then inoculated through retro-orbital route with $1x10^7$ ge of HBV derived from patient or *in vitro*.

1. Detection of HBV DNA in mouse serum/plasma (See Note 6)

Serum or plasma was obtained from tail vein or retro-orbital bleed and HBV viral DNA was extracted with QIAamp® MinElute® Virus spin Kit according to the instruction. Elute HBV DNA with 50 µl elution buffer.

- The following primers are used to quantitate HBV viral load. Forward primer: GTTGCCCGTTTGTCCTCTAATTC; Reverse primer: GGAGGGATACATAGAGGTTCCTTGA
- Setup of the q-PCR reaction: 2 X SYBR Green ROX buffer 7.5µl; mixed primers (10 µM) 0.25 µl; ddH2O 2 µl; standards and sample 5µl. Prepare duplicate wells for each sample.
- HBV standard is performed with HBV 1.3 mer plasmid with dilution from 10^8 to 10^1 copies/reaction.
- Program for q-PCR. Step 1: 50°C 2 min, Step 2: 95°C 15 min, Step 3: 95°C 15 sec, Step 4: 60 °C 1 min, go to step3 for 40 cycles.

Notes

1. The NTBC can be dissolved in 100% DMSO, in which case it does not need to be heated. If dissolved with DMSO, special filters resistant to DMSO should be used.
2. Foot, finger, and ear tissue can also be used. The KAPA express extract takes much less time. Other methods to extract genomic DNA using Protease K digestion can also be used to extract genomic DNA. Unlike digestions with Proteinase K, KAPA Express Extract does not completely degrade the tissue. There will be intact tissue visible in the tube after lysis. This does not have a negative impact on the downstream PCR.
3. Ligation is required sometimes, but it is not necessary if a small volume of cells is injected and the needle is pulled out very slowly. We only use 1 million cells in the NRG/F mice. Up to 5 million cells have been used in published reports [20]. Our experience is that large volume (100 µl) injection with a high concentration of cells usually causes bleeding.
4. If the surgery is not successful, mice will soon die (day 2 to day 10). At around day 21 in the first cycle and day 49 in the second cycle, the NTBC must be put back to 100%. Otherwise, the majority of the transplanted mice will be lost. We have observed a big loss of mice at week 3 and week 7 after surgery if NTBC is not adjusted back to 100%. The time of cycling also relies on the amount and proliferation capacity of human hepatocytes injected. If more cells (> 1million cells) are used, the interval of NTBC 0 % can be extended. If the transfected adult hepatocytes are highly proliferative, the interval of NTBC 0% can be extended. However, this requires consistent checking of the mouse health status.
5. Coating antibody is used at 1:100 dilution in the original manufacturer's protocol. However, 1:100 dilution usually gives a high background. Use of the coating antibody at 1:250 dilution will not only reduce the background but also greatly reduce the cost.
6. We usually use quantitative PCR to detect the HBV viral DNA. HBsAg ELISA can be used to detect HBsAg.

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