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Establishment of a Mouse Model of Chronic Hepatitis B Virus Infection and Purification of Hepatic Parenchymal and Non-Parenchymal Cells

Yan Yan and Chantsalmaa Davgadorj

Abstract

The use of replication-competent hepatitis B virus (HBV) DNA to construct a mouse model will help explore antiviral treatment strategies for more than 240 million patients infected with HBV worldwide. Eradication of chronic HBV infection can effectively block the adverse consequences of HBV-induced hepatic cirrhosis, failure and carcinoma. The core reason that HBV is difficult to eradicate is that most of infected people develop chronic HBV infection due to the establishment of immune tolerance. Here, we introduce a mouse model of adeno-associated virus (AAV)-HBV transfection, which produces HBV surface antigen (HBsAg) that can be maintained for more than 6 months. During virus replication, intermediates, transcripts, and proteins can be detected in peripheral blood. At the same time, the prerequisite for studying liver disease formation and immunotherapy through *in vitro* experiments is to isolate hepatic subgroup cells. Here, we describe a cell sorting method based on liberase perfusion technology combined with low-speed centrifugation and magnetic bead antibody labeling to purify hepatic parenchymal cells (PCs) and non-parenchymal cells (NPCs) step by step from murine liver, such as hepatic sinusoidal endothelial cells (LSECs) and Kupffer cells (KCs), which will help accelerate the study of the genetic and clearance mechanistic of chronic HBV infection.

Keywords: hepatitis B virus, CD8⁺ T cell, hepatic parenchymal cell (HPC), hepatic sinusoidal endothelial cell (LSEC), Kupffer cell (KC)

1. Introduction

Hepatitis B virus (HBV) has an extremely narrow host-range, such as humans [1], chimpanzees [2], Mauritian cynomolgus monkey [3], treeshrew [4, 5] and woodchuck [6, 7]. These model thereby can be exploited to investigate viral-host interaction and pathogenesis including acute hepatitis, chronic hepatitis, hepatic fibrosis, hepatic cirrhosis and hepatic carcinoma. There are certain limitations using of these models, due to expensive facilities required, difficult to care and ethics restriction, and the shortage of reagents for measuring host factors and for investigating host immunopathogenesis, such as chimpanzees. Mouse is considered to be a less expensive

alternative animal model, but its liver lacks the sodium taurocholate cotransporting polypeptide (NTCP) needed for HBV infection. Although human-NTCP (huNTCP) transgenic mice are considered to be able to construct HBV-infected mice, the results failed to support the original hypothesis by measuring HBV DNA [8].

Studies on the HBV mouse models have gone through several generations, involved in HBV DNA transgenic mice [9], HBV DNA transfected mice [10], HBV rc-cccDNA transfected mice [11], HBV-infected liver chimeric humanized mice and CRISPR/Cas9 technological NRG/Fah^{-/-} immune deficient mice [12]. Their advantages and disadvantages are as follows, separately: (1) 1.3-fold HBV genome transgenic mice support the expression of viral RNA and viral proteins in the liver, and can develop complete pgRNA, viral assembly and viral secretion during in the viral cycle, and support endosomal antiviral [13]. Due to the integration of the HBV genome in the host chromosome, the disadvantages of transgenic mice include the undetectable HBV cccDNA in mouse hepatocytes, the innate immune tolerance to HBV antigens (Ags), and the inability to study Ag-related immune activation. (2) The 1.2-fold HBV genomic DNA hydrodynamic injection (H.I.) model is one major breakthrough and first developed to meet the requirements of mouse nonintegrated viral genomes [14]. Different from immunodeficient mice such as liver humanized mice, the mice can express HBV Ags, and the immune system can also recognize the Ags to stimulate virus-specific immune responses. This model system can be used to test HBV cure strategies and study HBV immunology [15, 16]. However, this mode has limitation of mouse category. For example, experiments have shown that male C3H and C57BL/6 J are more effective, and the success rate varies in mouse models [17, 18]. The operation of H.I. also requires a high level of technical proficiency. The AAV-HBV vector has minimal AAV genome, only the essential AAV inverted terminal repeat sequence (ITR), is used to analyze the virus packaging and does not encode any AAV viral proteins. Therefore, this vector provides a clean background when analyzing HBV-specific immune responses. In our chapter, we will introduce how to establish this mouse model, the detectable HBsAg protein persists in blood or liver more than 6 months and has been widely used as chronic infection model [19]. (3) HBV replication is not detectable and HBV cccDNA non-formation is suggested to be restricted in mouse model [20]. HBV cccDNA is a symbol of continuous virus replication and an indispensable component for HBV to cause liver damage and fibrosis [21]. The half-life of HBV cccDNA is long in the human liver, which is a difficult problem for clinical cure of hepatitis B. Construct a transfected recombinant minicircle cccDNA achieving long-term maintenance in C3H mouse model contributes to investigating HBV cccDNA-related biology and for evaluating anti-HBV drugs [11]. In order to overcome the shortcoming of cccDNA lasting only a few weeks, a mouse model was constructed using adenovirus vectors and the linear HBV cccDNA genome was delivered to Cre transgenic mouse [22]. (4) Humanized immunodeficient mice have been used to generate HBV-infected mice [23]. However, these animals lack a functional immune system, hindering research on immunological issues related to HBV infection and immunotherapy.

At present, AAV-HBV plasmid transfection can obtain an ideal HBV chronic infection mouse model, and it has been applied in a number of studies [14, 24, 25]. In the clinical recovery process of chronic hepatitis B, there are strict rules on the selectivity of anti-viral therapy for patients to ensure that interferon combined with nucleoside analog therapy produces better results. Therefore, it is necessary to further understand the relationship between drugs, HBV and hepatocyte immunity. As our previous studies have shown, TLRs [26, 27] and chemokines [15, 25] can promote the clearance of HBV by regulating the status of hepatic parenchymal cells (PCs) and non-parenchymal cells (NPCs), and also clarifies the cellular mechanism. Hepatic PCs and NPCs play a key role in mediating liver immune tolerance and mediating early

innate and adaptive immune responses. Small molecule drugs or cytokines activate these cells to promote virus clearance. The specific mechanism will be verified using isolated mouse hepatocytes and the corresponding mechanism will be explained.

2. Materials

2.1 Materials for transformation and extraction of plasmids

1. Plasmid: The plasmid pAAV/HBV1.2 (ampicillin resistance) was kindly provided by Prof. Chen Pei-Jer (Graduate Institute of Clinical Medicine, College of Medicine, National Taiwan University, Taiwan), containing 1.2 times of the full-length DNA HBV genome [28].
2. Sterilized 100 mL liquid Luria Broth (LB) bacterial medium and 50 mL Luria Agar (LA) (1.5–2% agar, 0.75–1 g) solid bacterial medium [29]. LB medium: Tryptone 1 g/L, Yeast extract 0.5 g/L, NaCl 1 g/L, pH 7.4. The final volume of the solution is up to 100 mL.
3. 50 μ L competent *E.coli* DH5 α strain bath in broken ice (Laboratory preparation or purchase).
4. Sterilized ddH₂O, 1 mol/L CaCl₂, 0.6 mol/L MgCl₂ and sterile 50 μ g/mL ampicillin (Amp) (Invitrogen).
5. Water bath at 42°C.
6. A horizontal shaker with constant temperature at 37°C.
7. 5% CO₂, 37°C incubator for bacterial culture.
8. NucleoBond Xtra Maxi EF, Maxi kit for endotoxin-free plasmid DNA kits (Macherey-Nagel).
9. 4% paraformaldehyde: 4 g paraformaldehyde powder is dissolved in PBS at 56°C, stored at room temperature and protected from light.

2.2 Materials, reagents and culture media for hepatocyte extraction and culture

1. Mice: 6–8 weeks old male C57BL/6 J are bred and maintained under specific pathogen-free (SPF) conditions in the Animal Research Center.
2. Water bath at 37°C.
3. Sterile 50 mL conical centrifuge tubes, 25 mL disposable plastic pipettes and adjustable pipette device.
4. Disposable 70 μ m filters or reusable stainless steel meshes.
5. Sterile 10-cm bacteria or cell petri dishes.
6. A 50 mL beaker containing surgical instruments soaked in 75% alcohol, several pairs of ophthalmological scissors and fine-pointed ophthalmological tweezers.

Perfusion buffer and medium	Reagent ingredient	Total amount	Proportion
Liberase digestive medium (LDM)	Liberase powder	50 mg	−20°C
	GBSS buffer	7.14 mL	300 µL/tube (aliquot)
Liver perfusion medium (LPM)	LDM	300 µL/tube	−20°C
	GBSS buffer	30 mL	
Liver cell digestion medium (LCDM)	DMEM (High glucose)	500 mL	
	Hepes (1 mol/L)	7.5 mL	15 mmol/L
	Penicillin (100 ×)	5 mL	1%
Hepatocyte culture medium (HCM)	Williams' Medium	500 mL	Sigma-Aldrich
	Fetal bovine serum	50 mL	10%
	100 × L-glutamine (Glu)	5 mL	1%
	Hepes (1 mol/L)	2.5 mL	5 mmol/L
	Penicillin (100 ×)	5 mL	1%
	DMSO	10 mL	2%
	Hydrocortisone		0.05 nM
	Insulin		5 µL/mL
	100 × None essential amino acid (NEAA)	5 mL	1%
LSEC/KC medium	DMEM (High glucose)	500 mL	
	Fetal bovine serum	50 mL	10%
	100 × L-Glu (glutamine)	5 mL	1%
	Hepes (1 mol/L)	2.5 mL	5 mmol/L

Table 1.
Formulas of perfusion and hepatocyte culture media.

- 7.75% ethanol, adjustable-speed peristaltic pump and perfusion connection line device (27 gauge 1.25 inch needles).
- 8.24-well and 48-well culture plates pre-coated with mouse collagenase type II (Sigma-Aldrich), rinse 3–4 times with PBS before use.¹
- 9.Sterilized 1 L phosphate buffered saline (PBS, calcium- and magnesium-free): 7.9 g NaCl, 0.2 g KCl, 0.24 g Na₂HPO₄ and 1.8 g K₂HPO₄, pH 7.4.

¹ Dilute 5 mg rat tail collagen type II with 1 mL 0.006 mol/L (0.36 g/L) sterile acetic acid solution, and dissolve 240 µL of collagen II (5 mg /mL) to 95 mL sterile acetic acid solution, and finally dilute to 100 mL. Dissolve the rat tail collagen type II solution used for coating at 5–8 µg/cm² (cm² refers to the bottom surface area of the culture well) (12 µg/mL). Add 300 µL to each well of 24 well plate, and add 150 µL to each well of 48 well plate, then dry overnight under ultraviolet light. The dried plates are kept sealed and can be stored stably for several months (>3 months) at 4–25°C.

10. Sterilized Gey's balanced salt solution (GBSS, calcium-free): 0.14 mM NaCl, 5 mM KCl, 0.3 mM MgSO₄, 1 mM Na₂HPO₄, 3 mM NaHCO₃, 0.2 mM KH₂PO₄, 1 mM MgCl₂, 5.5 mM glucose, pH 7.4.
11. The formulas of digestive enzymes, culture media and drugs are shown in **Table 1**.
12. 0.2% trypan blue, a hemacytometer, a cover glass and a large amount of absorbent tissues.
13. MACS buffer: 1 × PBS adds a total volume of 0.05% bovine serum albumin (BSA) and 2 ml EDTA·Na₂ (0.5 M), pH 7.2.
14. PE rat anti-mouse CD146 antibody (Ab), biotin anti-mouse-F4/80 Ab, anti-PE magnetic beads and streptavidin magnetic beads.
15. BD IMag™ Cell Separation Magnet (BD Biosciences) or MACS separator plus LS Columns (Miltenyi Biotec).
16. 5% CO₂, 37°C incubator for cell culture.
17. Optional: Pentobarbital.

3. Method

3.1 Plasmid enlargement culture and extraction from bacteria

1. 2 µL pAAV/HBV1.2 mix with 45 µL ddH₂O, 2.5 µL CaCl₂ (1 mol/L), 2.5 µL MgCl₂ (0.6 mol/L), then added 50 µL competent *E.coli* DH5α strain, and bathe in ice. Transformation conditions: ice bath 40 min, 42°C heat shock 1.5 min, ice bath 2 min. Add 0.8 mL LB medium, shake for 1 h at 37°C, then centrifugation at 6000 r/min for 5 min, discard the supernatant and inoculate 200 µL transformation bacteria on LB solid medium (Amp⁺), and incubate approximate 16 h at 37°C.
2. The target plasmids are extracted as directed by the Macherey-Nagel manufacturer's instructions. The user manual can be downloaded from the website: <https://www.mn-net.com/nucleobond-xtra-maxi-kit-for-transfection-grade-plasmid-dna-740414.50?c=3889>. Plasmid DNA is divided and stored below −20°C. Detection of DNA concentration before use.²

3.2 Mouse model

1. 10 µg of pAAV/HBV1.2 plasmid are injected (H.I.) into the mouse tail veins in a volume of PBS equivalent to 10% of their body weights (g) (v/w) is completed within 5 s [10, 30].
2. HBV replication in the injected mouse serum: Blood specimens are taken from the cheeks of H.I. mice and used to detect hepatitis B surface antigens (HBsAg), hepatitis B e antigens (HBeAg), hepatitis B surface antibodies (anti-HBs), or hepatitis B core antibodies (anti-HBs) at 2 weeks post injection [10].

² Send the diluted 20 µL DNA for sequencing or real-time PCR to verify accuracy.

Serum levels of HBsAg, HBeAg and HBsAb are detected with ELISA according to the reagent instructions (Jingmei Biotechnology).

3. HBV transcription in the liver: The mouse liver is used to preserve in 4% polyformaldehyde for the next immunohistochemical analysis [10, 25] (Another alternative detection method.³

3.3 Perfusion and digestion of mouse liver

1. Biosecurity cabinet and perfusion labs for pre-ultraviolet disinfection.
2. Warm up the PBS and LPM at 37°C, at least 15 mL per mouse per reagent.
3. Install the needle at one end of the perimeter pump line, rinse the line with 75% ethanol for 20 min, then rinse with PBS to remove residual ethanol.
4. Prepare a 10 cm sterile petri dish containing 10 mL of LCDM for liver cell separation and cleaning in the biosecurity cabinet, and an ice box to place cell samples from liver separation on ice. The spare LCDM is also placed in the ice box.
5. Anesthetize or kill the mouse. In the reclining position, according to the previous description [31], the limbs of the mouse are secured with tape on the foam work platform and operated under sterile conditions.⁴
6. Clean the outer surface of the mouse's chest and abdomen with alcohol. The whole process is fast and leaves no blank spots. The skin is the main source of contamination. Use surgical instruments to cut the mouse's abdominal fur in a semicircle below shape, turn it upside down and secure the peritoneum with a surgical instrument.
7. Find the inlet vein of the liver, check the pipes for bubbles, insert a fine needle from the distal end of the inlet vein, and secure the position of the needle. Perfuse the liver tissue with PBS at a speed of 3-5 mL/min, and then cut off the inferior cavity vein after a few seconds. After removing the thrombosis and completing the complete PBS perfusion and 15 mL LPM continuous perfusion.^{5,6,7}

³ Detect the level of nucleocapsid HBV DNA extracted from 60 mg of murine liver by real-time PCR on the ABI PRISM 7500 Sequence Detection System (ABI PRISM™). Use an All-In-One DNA/RNA/Protein Mini-preps kit (Sangon Biotech) to purify total DNA from serum. According to the manufacturer's instructions, use careHBV PCR Assay V3 reagents (QIAGEN, China) for HBV DNA detection. This type of model chronic HBV mice has no liver damage and abnormal serum alanine aminotransferase (ALT).

⁴ Using tape instead of puncture fixation can reduce the pain of mice and also prevent the mice from waking up from anesthesia as soon as possible.

⁵ Before starting priming, carefully check and remove any visible air bubbles in the pump system. Properly activating the pump and piercing the needle into the portal vein to remove any invisible air in the needle tip area may block the microcapillaries and cause insufficient perfusion of certain liver lobes.

⁶ This is a non-circulatory perfusion, and all liquid will flow out of the inferior vena cava. Therefore, it is necessary to lay some absorbent tissues on the foam workbench where the mouse is fixed.

⁷ Perfusion and digestion are very important steps. During the digestion process, digestive enzymes are injected and the severed inferior vena cava is pressed to close the vascular output, so that the liver is fully perfused and expanded, which can prolong the effective digestion time. After ideal digestion, the liver will turn from red to yellow-white, and the tissues will become soft after digestion. The more complete the liver digestion, the higher the ratio of single cells to live hepatic parenchymal cells, and the higher the survival rate in culture.

8. After perfusion, clamp the digested liver tissue with ophthalmic tweezer, cut off the connective tissue around it with slight operation, and place it in the dish containing LCDM in biosecurity cabinet.
9. Firstly, peel off the gallbladder. Using two sets of disinfection ophthalmic tweezers to gently scrape the liver capsule, gently shake the liver tissue to release the digested single cells, and 10 mL LCDM becomes muddy, only a small amount of fibrous tissue remaining is the ideal digestion process.⁸

3.4 Isolation and culture of hepatocytes

1. Transfer the hepatocyte suspension to a new 50 mL centrifuge tube via a 70 μ m filter. Wash the 10 cm petri dish 2–3 times with 15 mL LCDM and transfer to the 50 mL conical centrifuge tube via the same 70 μ m filter.
2. **Hepatic PCs:** After isolating all mouse hepatocytes, the hepatocyte suspension can be placed on ice.
 - i. Spin the cells at 50 g at 4°C for 5 min, 3 times, and wash with hepatocyte culture medium (HCM); Centrifuge has 9 speeds of acceleration and 9 speeds of decelerates (i.e., acceleration of 9, deceleration of 9).
 - ii. Aspirate the supernatant into a new 50 mL centrifuge tube. The supernatant contains hepatic non-parenchymal cells (NPCs).⁹ The precipitated cells are hepatic PCs.
 - iii. Inoculate hepatic PCs on a 24-well plates covered with collagen II, 360 μ L/well (Use HCM to dilute PCs to 3.5×10^6 cells/ml before inoculation).
 - iv. Incubate at 37°C, 5% CO₂, and shake at the crossover level every 15 min for 1 h to suspend the hepatocytes with lower viability. Incubate overnight, wash with PBS 3 times, change the medium and continue culturing. The morphology of hepatic PCs is shown in **Figure 1**.¹⁰

4. Hepatic NPCs (LSECs/KCs)

- i. Spin hepatic NPCs at 4°C, 300 g for 10 min; Accelerate by 9, decelerate by 9; Discard the supernatant and gently scrape the bottom to allow the cells to

⁸ Do not damage the gallbladder during the entire process of liver separation, as this will reduce the production and viability of hepatic parenchymal cells. After the liver is digested, only a small amount of fibrous tissue remains, which means it is in the best digestion state.

⁹ The supernatant of hepatic PCs of the same mouse each time washing may contain target hepatic NPCs, all of which are mixed together for subsequent cell sorting.

¹⁰ Experimental results show that the survival rate of mouse liver parenchymal hepatocytes needs to reach more than 70%, and a better survival rate can be obtained after culture. Mature hepatocytes mostly have two nuclei and three nuclei. In addition, even if washed with PBS, dead cells will still exist.

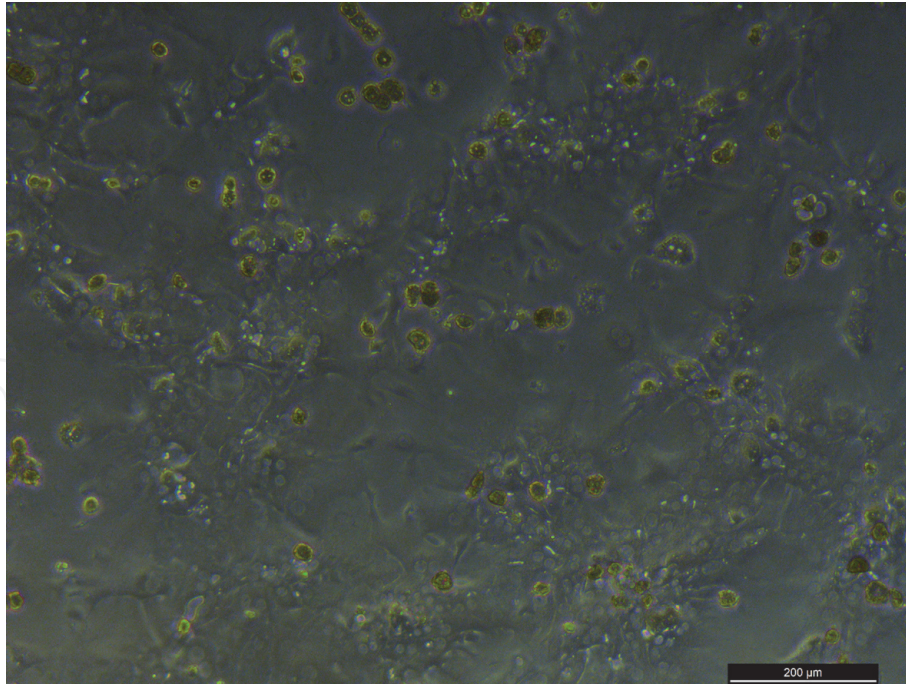


Figure 1.
hepatic PCs cultured for 48 h

- suspend; Wash the NPCs with 50 mL MACS buffer, pick out if there are dead cells or tissue clumps, centrifuge as above, discard the supernatant, and gently scrape the bottom.
- ii. Add the selected PE rat anti-mouse CD146 Ab solution (2.5 μ L Ab +250 mL MACS per liver) to the cell suspension for labeling LSECs, protected from light.
 - iii. Incubate at 4°C for 20 min or at room temperature for 30 min.
 - iv. Add more than 10 times the volume of MACS buffer to the incubated cells for washing.
 - v. Spin NPCs at 4°C, 300 g for 10 min; Accelerate by 9, decelerate by 9; Discard the supernatant and gently scrape the bottom to allow the cells to suspend.
 - vi. Add 20 μ L of anti-PE magnetic bead to 180 μ L of MACS buffer in each liver specimen.
 - vii. Incubate at 4°C for 15 min, add 10 times the volume of MACS buffer, and spin at 4°C, 300 g for 10 min.
 - viii. Discard the supernatant, supplement with MACS until the number of cells is $1-8 \times 10^7$ cells/mL, put it in one or more flow cytometry tubes and immediately place them on the BD IMag™ Cell Separation Magnet for 8–10 min to attract the cells labeled with magnetic beads; keep the flow tubes on the magnetic stand, and carefully aspirate the supernatants to a new 50 mL tube. Using the same suction method, suspend and clean the old tube twice with MACS. The negative cells in the supernatants can be collected in a new tube.

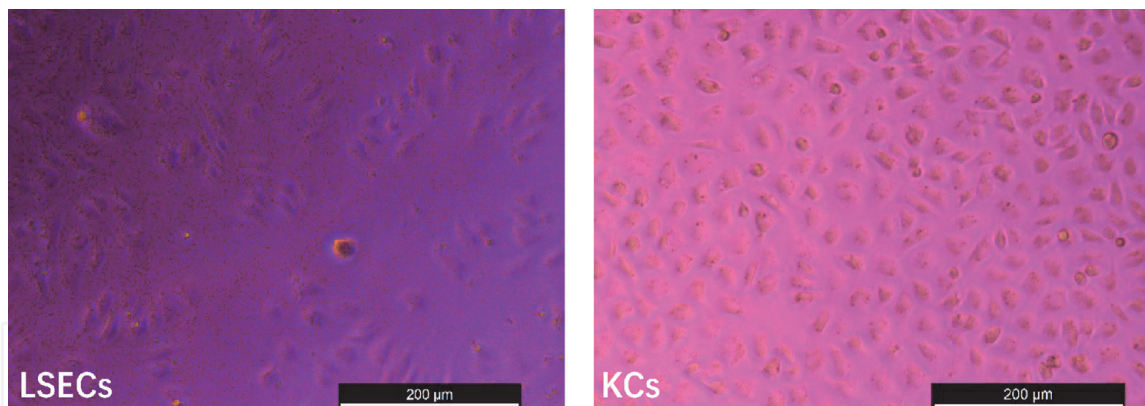


Figure 2.
hepatic NPCs cultured for 48 h.

- ix. Add 1 mL LSEC culture medium to suspend the cells. After counting, inoculate the diluted LSECs ($1 \times 10^6/\text{mL}$) into a 48-well plate pre-coated with collagen II, and inoculate 500 μL per well.
- x. Spin the liquid collected in step viii with a 50 mL tube at 4°C and 300 g for 10 min, and discard the supernatant. Gently scrape the bottom to suspend the cells and add 2.5 μL biotin anti-mouse-F4/80 Ab MACS solution (2.5 μL Ab +250 mL MACS per liver).
- xi. Refer to steps iii-v for antibody incubation and washing, and add 20 μL streptavidin magnetic bead to 180 μL MACS buffer in each liver specimen.
- xii. Incubation and magnetic attraction are the same as steps vii-viii to obtain F4/80^+ KCs. Add 1 mL KC culture medium to suspend the cells. After counting, inoculate the diluted KCs ($1 \times 10^6/\text{mL}$) into a 96-well plate with 200 μL per well.
- xiii. Both LSECs and KCs need to be cultured overnight in a 5% CO_2 37°C incubator. The next day, wash with PBS 3 times, and add DMEM complete medium the next day. The cell morphology of LSECs and KCs is shown in **Figure 2.**¹¹

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¹¹ The isolated NPCs can be verified by flow cytometry in a small amount: the cell surface markers of NPCs are stained for flow cytometry analysis as described previously [31]. Briefly, LSEC (CD146^+) and KCs (CD45^- , IA/IE^+ , and F4/80^+) can be detected by flow cytometry assay. All Abs are purchased from BD biosciences and eBioscience. The flow test results are analyzed using FlowJo 11.0.

Conflict of interest

The authors declare no conflict of interest.

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