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Basic Study

Human liver chimeric mouse model based on diphtheria toxin-induced liver injury

Xiao-Nan Ren, Rong-Rong Ren, Hua Yang, Bo-Yin Qin, Xiu-Hua Peng, Li-Xiang Chen, Shun Li, Meng-Jiao Yuan, Chao Wang, Xiao-Hui Zhou

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Author contributions: Ren XN and Zhou XH designed the experiments; Ren XN, Ren RR and Yang H performed the majority of experiments; Ren XN and Zhou XH analyzed the data; Qin BY, Peng XH, Chen LX, Yuan MJ and Wang C contributed to genotyping; Ren XN and Zhou XH wrote the paper; Li S revised the paper.

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Abstract

AIM

To establish an inducible liver injury mouse model and transplant human hepatocytes to obtain liver-humanized mice.

METHODS

We crossed three mouse strains, including albumin (Alb)-cre transgenic mice, inducible diphtheria toxin receptor (DTR) transgenic mice and severe combined immune deficient (SCID)-beige mice, to create Alb-cre/DTR/SCID-beige (ADSB) mice, which coincidentally harbor Alb-cre and DTR transgenes and are immunodeficient. As the Cre expression is driven by the liver-specific promoter Alb (encoding ALB), the DTR stop signal flanked by two loxP sites can be deleted in the ADSB mice, resulting in DTR expression in the liver. ADSB mice aged 8-10 wk were injected intraperitoneally (i.p.) with diphtheria toxin (DT) and liver damage was assessed by serum alanine aminotransferase (ALT) level. Two days later, mouse livers were sampled for histological analysis, and human hepatocytes were transplanted into the livers on the same day. A human ALB enzyme-linked immunosorbent assay was

performed 7, 14, 21 and 28 d after transplantation. Human CD68 immunohistochemistry was performed 30 and 90 d after transplantation.

RESULTS

We crossed Alb-cre with DTR and SCID-beige mice to obtain ADSB mice. These mice were found to have liver damage 4 d after i.p. injection of 2.5 ng/g bodyweight DT. Bodyweight began to decrease on day 2, increased on day 7, and was lowest on day 4 (range, 10.5%-13.4%). Serum ALT activity began to increase on day 2 and reached a peak value of 289.7 ± 16.2 IU/mL on day 4, then returned to background values on day 7. After transplantation of human liver cells, peripheral blood human ALB level was 1580 ± 454.8 ng/mL (range, 750.2-3064.9 ng/mL) after 28 d and Kupffer cells were present in the liver at 30 d in ADSB mice.

CONCLUSION

Human hepatocytes were successfully repopulated in the livers of ADSB mice. The inducible mouse model of humanized liver in ADSB mice may have functional applications, such as hepatocyte transplantation, hepatic regeneration and drug metabolism.

Key words: Liver disease; Liver injury; Diphtheria toxin; Liver chimeric mouse model

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Core tip: We established a novel liver chimeric mouse model following liver damage caused by intraperitoneal injection of diphtheria toxin (DT), and transplanted human hepatocytes to obtain liver-humanized mice. After 28 d, human albumin was detected in these mice. Human hepatocytes were successfully repopulated in the livers of triple-crossed albumin-cre transgenic mice, inducible DT receptor transgenic mice and severe combined immune deficient-beige mice [*i.e.*, Alb-cre/DTR/SCID-beige (ADSB) mice]. Our inducible mouse model of humanized liver in ADSB mice may have functional applications, such as studies on hepatocyte transplantation, hepatic regeneration and drug metabolism.

Ren XN, Ren RR, Yang H, Qin BY, Peng XH, Chen LX, Li S, Yuan MJ, Wang C, Zhou XH. Human liver chimeric mouse model based on diphtheria toxin-induced liver injury. *World J Gastroenterol* 2017; 23(27): 4935-4941 Available from: URL: <http://www.wjgnet.com/1007-9327/full/v23/i27/4935.htm> DOI: <http://dx.doi.org/10.3748/wjg.v23.i27.4935>

INTRODUCTION

Liver diseases are a serious global health issue, particularly viral hepatitis infection and related diseases. The hepatitis B virus (HBV) and hepatitis C virus (HCV)

are representative hepatotropic viruses. HBV is the prototype of the hepadnaviridae family of hepatotropic, partially double-stranded DNA viruses^[1], while HCV is a single-stranded RNA virus^[2]. Although different at the molecular level, they share many similarities as pathogens, and both infections can be acute or chronic^[3]. Persistent HBV and HCV infections can lead to cirrhosis and/or hepatocellular carcinoma^[4,5]. Despite the availability of vaccines and drugs, a huge number of patients suffer from the liver diseases related to these viral infections.

Animal models play a critical role in immunological or therapeutic drug development. The narrow spectrum of species that accommodate HBV and HCV infections restricts preclinical studies. Although chimpanzees have played an important role in studying HBV and HCV infections, there are few studies on chimpanzees due to high costs, ethics and their limited availability^[6-8]. Other hepadnaviruses that infect woodchucks^[9], ducks^[10] and ground squirrels^[11] harbor limitations due to genetic heterogeneity.

Fundamental questions regarding hepatotropic pathogen biology *in vivo* need to be addressed. However, this requires a suitable small animal model to guide the challenging and expensive studies. The transgenic 1.2 or 1.3 copy of the HBV genome in mice shows immunological tolerance to HBV antigens. Adenovirus-associated virus-based transduction or hydrodynamic transfection of mouse liver by the 1.2 or 1.3 copy of the HBV genome has also been used to study HBV immunobiology, but does not support viral replication for re-infection in the cycle. Human liver chimeric mouse models are useful in human liver disease research.

In this study, severe combined immune deficient (SCID)-beige mice were crossed with transgenic albumin (Alb)-cre mice which expressed cre enzyme^[12] under the control of a liver cell-specific Alb promoter, and diphtheria toxin receptor (DTR)^[13,14] transgenic mice, in which the DTR transgene is located in the ubiquitous *gt(ROSA26)Sor(R26)* locus after a loxp-flanked transcriptional stop sequence.

The resulting Alb-cre/DTR/SCID-beige (ADSB) mice specifically expressed DTR in the liver. Following administration of diphtheria toxin (DT), these mice developed liver injury. We further generated humanized liver in ADSB mice by the transplantation of human hepatocytes. The human hepatocytes were repopulated in the mouse liver, which were functional and secreted human albumin. Human Kupffer cells were also found to chimerize in the mouse liver.

Thus, we developed a novel animal model to investigate hepatocyte proliferation^[15-17] and hepatotropic viruses.

MATERIALS AND METHODS

Generation of ADSB transgenic mice

To generate the ADSB mice, we crossed Alb-cre

mice (a gift from Dr. Qiang Deng, Institute Pasteur of Shanghai, Chinese of Academy Sciences, Shanghai, China) with DTR mice (a gift from Dr. Yue-Lei Shen, Beijing Biocytogen Co., Ltd, Beijing, China) to obtain Alb-cre/DTR mice. Transgenic mice were selected from the offspring by genomic PCR of tail DNA, and then Alb-cre/DTR transgenic mice were crossed with SCID-beige mice (purchased from the Shanghai SLAC Laboratory Animal Co., Ltd, Shanghai, China), and ADSB mice were selected by genomic PCR of tail DNA.

Transaminase activity in the blood

DT (Sigma-Aldrich, St. Louis, MO, United States) was intraperitoneally administered (2.5 ng/g) to 8-10-wk-old ADSB mice, and blood was collected from these mice at different time points after DT administration. Samples were centrifuged at $600 \times g$ for 15 min to separate the serum. Serum alanine aminotransferase (ALT) activity was measured with a commercially-available kit according to the manufacturer's instructions (Roche, Basel, Switzerland). Serum ALT activity levels in mice used for hepatocyte transplantation were measured 3 d after DT injection.

Histological assessments

The livers were fixed with 4% formaldehyde for 24 h and stored in 75% ethanol. They were then embedded in paraffin and serial sections were cut and stained with hematoxylin and eosin (H and E).

Humanization protocol

We found that a single DT dose of 2.5 ng/g body-weight was the maximum dose tolerated with a 100% survival. Using this dose, serum ALT activity levels were determined prior to cell transplantation. Human cryopreserved hepatocytes (Bioreclamation IVT, Baltimore, MD, United States) were thawed and the cryopreservation solution was removed by centrifugation at $100 \times g$ for 5 min at 4 °C followed by resuspension in Dulbecco's modified Eagle's medium (DMEM). The resuspended hepatocytes were diluted 1:1 in trypan blue and then centrifuged again at $100 \times g$ for 5 min at 4 °C and reconstituted in hepatocyte culture medium at 1×10^7 cells/mL, and 1×10^6 viable hepatocytes suspended in 100 μ L DMEM were injected into the inferior splenic pole.

Human ALB ELISA

Starting 1 wk after transplantation, human ALB levels were monitored. Blood samples (10 μ L) were collected and centrifuged at $600 \times g$ for 15 min. Serum samples were assayed using the Quantitative Human Albumin ELISA Quantitation Kit (Bethyl Laboratory, Montgomery, TX, United States) according to the manufacturer's protocol.

Immunohistochemistry

At the time of harvest, the liver was fixed in 4% formaldehyde for 24 h and stored in 75% ethanol.

Sections were then prepared and incubated with primary human CD68 antibody (1:200 dilution; Servicebio, Shanghai, China) and were used to detect specific Kupffer cells in the chimeric mice, and then incubated with horseradish peroxidase-goat anti-rabbit secondary antibody (1:200; Servicebio).

Statistical analysis

Statistical analyses were performed using Prism 5.0 software (GraphPad Software, San Diego, CA, United States). A *P* value of < 0.05 was considered significant.

RESULTS

Experimental design and PCR analysis of Alb-cre/DTR transgenic mice

The experimental design is outlined in Figure 1. In this study, we crossed Alb-cre with DTR and SCID-beige mice to obtain ADSB mice. In PCR used to identify the Alb-cre gene, Tg1, Tg2 and Tg4 mice were cre-positive (Figure 1B) and in PCR for the *DTR* gene, Tg1, Tg2 and Tg5 were found in homozygous DTR mice, and Tg3 and Tg4 in heterozygous DTR mice (Figure 1C). Genotyping of SCID-beige mice was performed as previously described^[18]. The mice were then injected intraperitoneally with DT to induce liver injury, and adult human hepatocytes were transplanted to obtain chimeric mice.

Specific inducible liver injury in ADSB mice by DT

To examine the liver damage caused by DT, ADSB mice and non-transgenic mice (C57BL/6) were injected intraperitoneally with 2.5 ng/g bodyweight of DT in 200 μ L phosphate-buffered saline. Both groups of mice were sacrificed 4 d later. The livers of non-transgenic mice appeared normal and dark red (Figure 2A), whereas the livers from ADSB mice were pale and almost white (Figure 2B). Liver sections from both types of mice were stained with H and E. Microscopically, the liver sections from non-transgenic mice were of normal histological appearance, the structure of the hepatic lobule was complete, the hepatic cord and hepatic sinusoid were appropriately arranged, and degeneration or necrosis of hepatocytes was not observed (Figure 2C). Hepatocyte nucleus fragmentation disappeared in ADSB mice, suggesting that ADSB mice had characteristic histological hepatocellular injury (Figure 2D).

Kinetic study of bodyweight and liver injury

ADSB mice and non-transgenic mice were injected intraperitoneally with 2.5 ng/g bodyweight of DT. At different time points, bodyweight was recorded and blood samples were collected to determine ALT activity. In ADSB mice, after DT injection, bodyweight began to decrease on day 2, was regained on day 7, and was lowest on day 4 (range, 10.5%-13.4%). No weight reductions were found in non-transgenic mice (Figure 3A). Serum ALT activity in ADSB mice

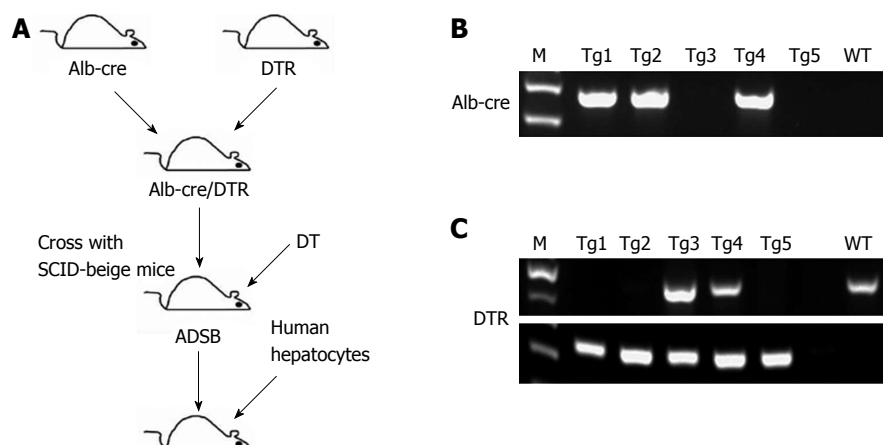


Figure 1 Experimental design and PCR analysis of Alb-cre/diphtheria toxin receptor transgenic mice. A: Experimental design used to characterize DT liver injury in ADSB mice, which were used for human hepatocyte transplantation; B: PCR analysis of the Alb-cre gene, Tg1, Tg2 and Tg4 mice are cre-positive; C: PCR analysis of the DTR gene, Tg1, Tg2 and Tg5 are homozygous DTR mice, and Tg3 and Tg4 are heterozygous DTR mice. ADSB: Triple-crossed albumin (Alb)-cre transgenic mice, inducible diphtheria toxin receptor (DTR) transgenic mice and severe combined immune deficient-beige mice; DT: Diphtheria toxin.

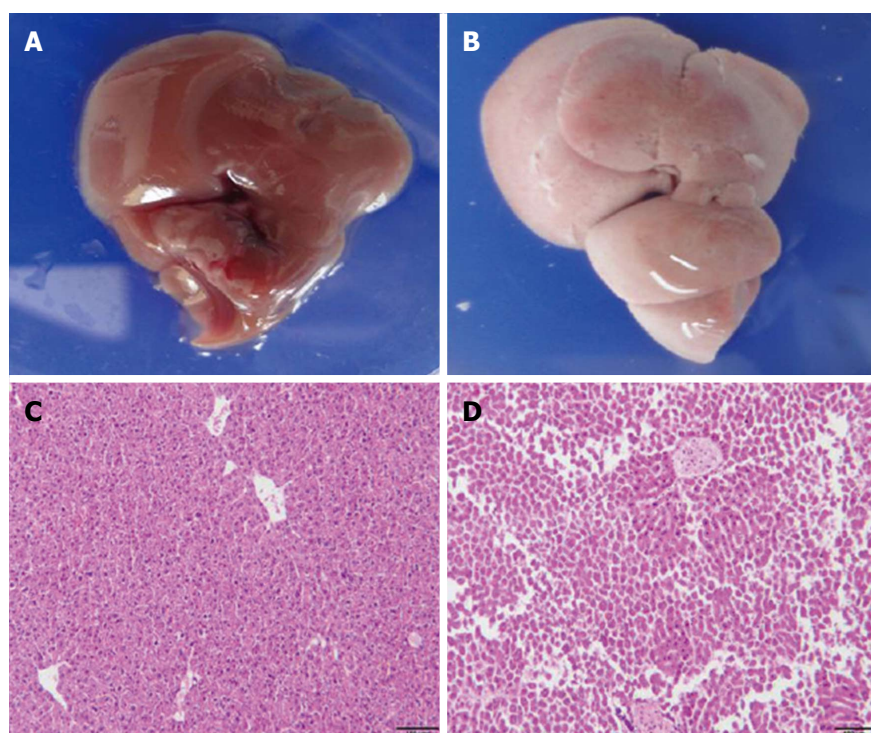


Figure 2 Histological analysis of liver injury. Two days after DT treatment, liver sections from non-transgenic mice and ADSB mice were stained with H and E. A: The liver from non-transgenic mice (C57BL/6); B: The liver from ADSB mouse; C: A representative liver section from non-transgenic mice showing normal histological appearance; D: A representative liver section from ADSB mice showing liver injury. ADSB: Triple-crossed albumin (Alb)-cre transgenic mice, inducible diphtheria toxin receptor (DTR) transgenic mice and severe combined immune deficient-beige mice; DT: Diphtheria toxin; H and E: Hematoxylin and eosin.

began to increase on day 2, reached a peak value of 289.7 ± 16.2 IU/mL on day 4, and then returned to background values on day 7 (Figure 3B). In non-transgenic mice, ALT activity remained at basal levels (< 50 IU/mL). Therefore, from day 2 to day 7 after DT injection liver damage occurred, demonstrating that proliferation of transplanted hepatocytes took place in this mouse model.

Human hepatocyte reconstitution in ADSB mice

ADSB mice were transplanted 3 d after DT injection,

and then peripheral blood ALB levels were determined on days 7, 14 and 21 after hepatocyte transplantation (Figure 4A). Serum levels of human ALB in ADSB mice are shown on days 7, 14, 21 and 28 after hepatocyte transplantation. Before 28 d, no human ALB was detectable either in ADSB mice or the non-transgenic mice. However, 28 d after transplantation we detected serum human ALB in ADSB mice at the level of 1580 ± 454.8 ng/mL (range, 750.2-3064.9 ng/mL), and no human ALB was detected in non-transgenic mice (Figure 4B). These results demonstrated that human

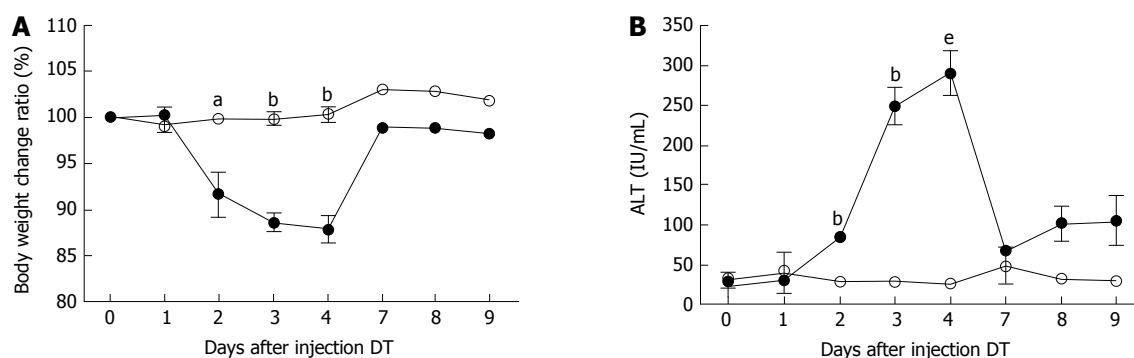


Figure 3 Body weight and alanine aminotransferase analysis after diphtheria toxin injection. ADSB mice (filled circle) and non-transgenic mice (open circle) were injected with 2.5 ng/g bodyweight DT. A: The bodyweight change ratio in the two groups of mice after injection of DT; B: Analysis of serum ALT activity in the mice. Data are shown as the mean of each group, and error bars represent SD ($n = 3$); $^aP < 0.05$, $^bP < 0.01$, $^eP < 0.001$. ADSB: Triple-crossed albumin (Alb)-cre transgenic mice, inducible diphtheria toxin receptor (DTR) transgenic mice and severe combined immune deficient-beige mice; ALT: Alanine aminotransferase; DT: Diphtheria toxin.

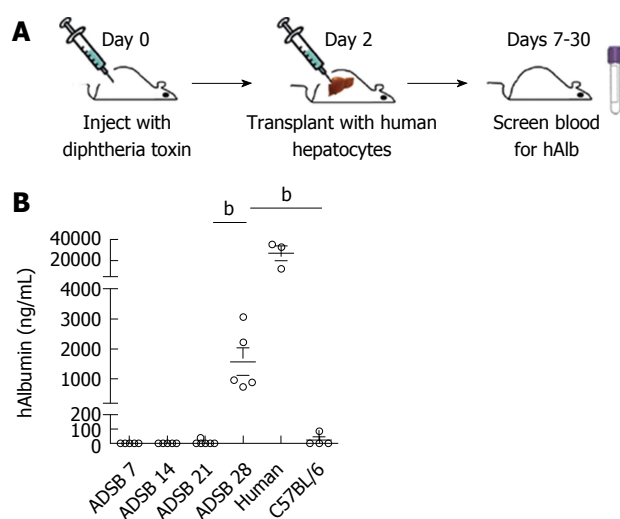


Figure 4 Human albumin plasma concentration in Alb-cre/DTR/SCID-beige mice after adult hepatocyte transplantation. A: Schematic of liver humanization. Two days after the intraperitoneal injection of DT, serum was collected for ALT assay. Human hepatocytes were transplanted into these mice on the same day; B: Serum levels of human albumin are shown for ADSB mice ($n = 5$) on day 7, 14, 21 and 28 after hepatocyte transplantation as detected by enzyme-linked immunosorbent assay. Results are mean \pm SEM ($n \geq 3$); $^bP < 0.01$. ADSB: Triple-crossed albumin (Alb)-cre transgenic mice, inducible diphtheria toxin receptor (DTR) transgenic mice and severe combined immune deficient-beige mice; ALT: Alanine aminotransferase; DT: Diphtheria toxin.

ALB was expressed at least 4 wk after hepatocyte transplantation.

Human Kupffer cells in the livers of ADSB mice

CD68 is considered a specific marker for activated Kupffer cells. Kupffer cells are essential for many hepatic functions and play a major role in inflammatory responses in this organ^[19-21]. CD68 immunohistochemistry was used to measure CD68 expression in Kupffer cells. In ADSB mouse liver sections, CD68⁺ cells were present 4 wk and 12 wk after transplantation, and more CD68⁺ cells were found at 12 wk after transplantation than at 4 wk after transplantation (Figure 5).

DISCUSSION

Human liver chimeric mouse models are useful in human liver disease research. The urokinase-type plasminogen activator (uPA) transgenic mouse^[22], was the first reported liver humanized mouse model; however, uPA mice have low breeding efficiency, are unhealthy and die due to hypofibrinogenemia; thus, the transplant time for uPA mice is limited. Two reports showed successful engraftment based on genetic knockout of the fumarylacetoacetate hydrolase (Fah) genes^[23,24]. Fah is the last enzyme in the tyrosine breakdown pathway and its deficiency leads to lethal type I hypertyrosinemia in humans and liver failure in mice. However, Fah mice also have mouse health problems, and 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexanedione controls liver injury so that impacts its application in drug metabolism. More recently, two additional transgenic models have been developed, the TK-NOG^[25] and the AFC8^[26] models, which express active caspase 8 fused with the FK506 binding domain and has inducible suicidal activity in mouse liver under Alb promoter control, but its repopulation rate of human liver cells is only 30%. The FRG model was then developed. FRG^[23] mice are immune-deficient, Fah knockout mice crossed with mice lacking the *Rag-2* gene and the common gamma chain of the interleukin receptor.

We report here a novel ADSB mouse model which can be efficiently repopulated with human hepatocytes. The transplanted human hepatocytes can reside in the mouse host's natural environment and maintain normal functions. Theoretically, these mouse models can be infected with HBV and HCV in a reproducible manner.

In this model, recipient mouse hepatocytes were destroyed by DT, and the transplanted human mature hepatocytes had a selective advantage in the mouse liver. We confirmed that these mice have the ability to engraft adult human hepatocytes, and the liver can harbor human Kupffer cells. Thus, this model provides

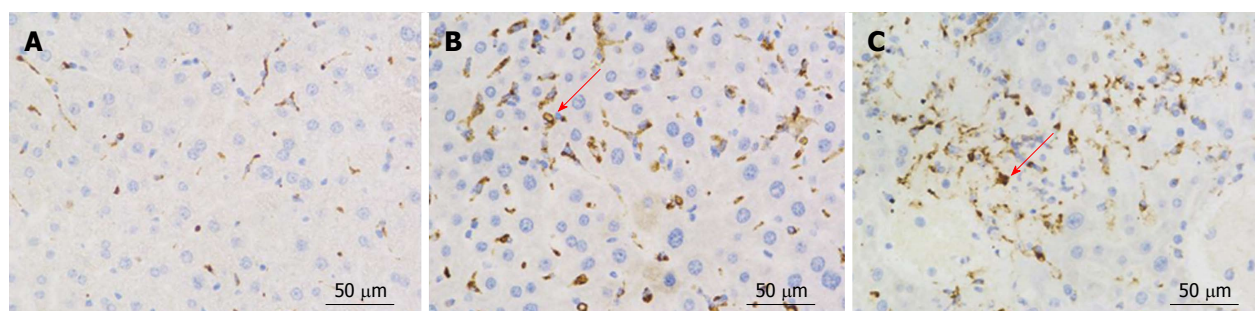


Figure 5 Human Kupffer cells in the liver of Alb-cre/DTR/SCID-beige mice. Human Kupffer cells stained with CD68, showing the high degree of liver chimerism. A: Non-transplanted C57BL/6 mice; B: ADSB mice 4 wk after transplantation; C: ADSB mice 12 wk after transplantation. Red arrows exhibit positive staining. Scale bar = 50 µm, × 400. ADSB: Triple-crossed albumin (Alb)-cre transgenic mice, inducible diphtheria toxin receptor (DTR) transgenic mice and severe combined immune deficient-beige mice.

a platform for basic biology in liver regeneration research and liver disease development.

Our mouse model has distinct advantages over the other chimeric models. First, ADSB mouse breeding is not as difficult as for uPA mice, making it possible to obtain sufficient ADSB mice for experiments. In addition, these mice are healthy and long-lived, and can be used for long-term transplantation studies. Second, the transplantation time points are flexible following DT injection to induce murine liver injury. Furthermore, we determined the appropriate dose of DT to be 2.5 ng/g bodyweight, which can sustain acute liver injuries with only one dose of DT, resulting in no death of mice, and can efficiently support the proliferation of transplanted hepatocytes.

In conclusion, this study introduced a new *in vivo* mouse model, which will serve as a promising tool for research into the interaction between host and virus *in vivo*, and in the development of new treatment approaches. This model is convenient for studies on hepatocyte transplantation, human drug metabolism research and drug-drug interactions^[27,28]. Our model achieved the establishment of human liver without hemopoietic reconstitution. In a future study, we will attempt to establish human liver/immune dual chimeric mice in order to investigate HBV or HCV infections in these chimeric animals.

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COMMENTS

Background

Hepatitis B virus and hepatitis C virus are hepatotropic viruses that represent

a serious global health issue. Humanized mouse models are useful in human liver disease research. However, mouse models have disadvantages and need to be improved.

Research frontiers

Recently, many humanized mouse models have been reported, such as the AFC8 mouse and Fah mouse models. However, these mouse models have disadvantages, such as low breeding efficiency, limited time window for transplantation and low repopulation rate.

Innovations and breakthroughs

In the present study, the authors developed a novel liver-chimeric mouse model. Liver failure was induced by diphtheria toxin (DT) and then human hepatocytes were transplanted and repopulated in the mice.

Applications

The results of this study suggest that the liver chimeric mouse model based on triple-crossed albumin-cre transgenic mice, inducible DT receptor (DTR) transgenic mice and severe combined immune deficient-beige mice (*i.e.*, ADSB mice) may provide a more stable platform for human drug metabolism research and viral hepatitis infections.

Terminology

A liver chimeric mouse is established using transgenic or knockout techniques to cause liver failure and human liver cells are transplanted to construct a chimeric mouse. In order to avoid host immune rejection following human hepatocyte transplantation, the mice used are usually immunodeficient.

Peer-review

The researchers provide a novel mouse model of human liver chimeric based on DTR transgenic mice, in which liver injury can be induced by DT injection. This model could serve as a promising tool for research on the interaction between host and hepatitis virus *in vivo*, and in the development of new treatment approaches against related liver diseases.

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