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Property of hepatitis B virus replication in *Tupaia belangeri* hepatocytes

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ABSTRACT

The northern treeshrew (*Tupaia belangeri*) has been reported to be an effective candidate for animal infection model with hepatitis B virus (HBV). The objective of our study was to analyze the growth characteristics of HBV in tupaia hepatocytes and the host response to HBV infection. We established primary tupaia hepatocytes (3–6-week old tupaia) and infected them with HBV genotypes A, B and C, and all the genotypes proliferated as well as those in human primary hepatocytes (>10^5 copies/ml in culture supernatant). We next generated a chimeric mouse with tupaia liver by transplantation of tupaia primary hepatocytes to urokinase-type plasminogen activator cDNA (cDNA-uPA)/severe combined immunodeficient (SCID) mice and the replacement ratio with tupaia hepatocytes was found to be more than 95%. Infection of chimeric mice with HBV (genotypes B, C, and D) resulted in HBV-DNA level of 10^4-10^6 copies/ml after 8 weeks of infection, which were almost similar to that in humanized chimeric mouse. In contrast, serum HBV level in adult tupaia (1-year-old tupaia) was quite low (<10^3 copies/ml). Understanding the differences in the response to HBV infection in primary tupaia hepatocytes, chimeric mouse, and adult tupaia will contribute to elucidating the mechanism of persistent HBV infection and viral eradication. Thus, *T. belangeri* was found to be efficient for studying the host response to HBV infection, thereby providing novel insight into the pathogenesis of HBV.

1. Introduction

Hepatitis B virus (HBV), a member of the family *Hepadnaviridae*, causes acute and chronic hepatitis, which is one of the major health concerns in the world. Chronic HBV infection is strongly associated with an increased risk of cirrhosis, which in turn leads to hepatocellular carcinoma [1,2]. Worldwide, 2 billion people have been reported to be infected with HBV, and more than 350 million have been reported with chronic infection [3].

Currently, clinically available HBV drugs are nucleoside or nucleotide analogs that inhibit the viral reverse transcriptase (RT), but these drugs have recently been reported to result in viral resistance [4]. Moreover, RT inhibitors only control HBV replication, and cannot cure HBV infection completely. One factor that impedes the development of therapy for HBV elimination is the lack of a suitable animal model to study HBV pathogenesis. To date, the chimpanzee is the only existing natural infection animal model, but

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because of longevity (>50 years), high investigation costs, and stringent animal welfare regulations, it is difficult to use it [5].

Northern treeshrew (*Tupaia belangeri*), which belongs to the family Tupaiidae, is a small mammal that has a body weight of about 100–150 g, and it is genetically close to primates [5,6]. Tupaia is susceptible to hepatitis viruses such as HBV and hepatitis C virus (HCV) [7,8]. Since HBV causes hepatitis and persistent infection in tupaia like that in humans, it could be used as an animal model for studying HBV infection. However, the infection mode in tupaia and the host response to HBV infection is unknown.

Chimeric urokinase-type plasminogen activator transgenic (uPA)/severe combined immunodeficient (SCID) mice with humanized liver [9] are immune-deficient and thus are suitable hosts for HBV infection within hepatocytes [10,11]. To expand the potential of the tupaia animal model for the characterization of HBV growth, we established chimeric mice with tupaia hepatocytes based on urokinase-type plasminogen activator cDNA (cDNA-uPA)/SCID mouse in this study, and assessed the HBV propagation in primary tupaia hepatocytes, chimeric mouse with tupaia hepatocytes, and adult tupaia.

2. Materials and methods

2.1. Ethics statement

This study was carried out in strict accordance with the *Guidelines for Animal Experimentation of the Japanese Association for Laboratory Animal Science* and the recommendations in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. All protocols were approved by the regional ethics committee.

2.2. Animals

Northern treeshrews (*T. belangeri*) were purchased from Kunming Institute of Zoology, Chinese Academy of Sciences.

2.3. HBV inocula

The HBV inoculum was the serum of chimeric mouse with humanized liver infected with HBV genotype A (A2_JP1, A2_JP2, A2_JP4; now submitting to GENBANK), B (Bj_JPN35; accession number: AB246341.1), C (C_JPNAT; accession number: AB246345.1), D (D3_MR1; now submitting to GENBANK), or J (JRB34; accession number: AB486012.1).

2.4. Isolation and culture of primary tupaia hepatocytes

Tupaia hepatocytes (tupaia hepatocytes #1 and #2) were isolated using a two-step collagenase perfusion method which was modified based on a previously described method [12] (Fig. 1A). Tupaia (3–6 weeks old) liver was perfused at 38 °C for 7 min at 3 ml/min with Hank's balanced salt solution (HBSS) supplemented with 1 mg/ml D-glucose, 200 μg/ml ethylene glycol tetraacetic acid, 10 μg/ml gentamicin, and 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES). The liver was then perfused at 37 °C for 11.5–16 min at 3 ml/min with HBSS containing 0.05% type IV collagenase (Sigma Aldrich, St. Louis, MO, USA), 140 μg/ml $CaCl_2$, 10 μg/ml gentamicin, 10 mM HEPES, and 100 μg/ml trypsin inhibitor. The perfused liver was separated and disaggregated with maintenance medium as Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 U/ml penicillin G, and 100 μg/ml streptomycin, 44 mM $NaHCO_3$. The disaggregated cells were collected and centrifuged at 50 × *g* for 2 min thrice. The cell pellet was suspended in the maintenance medium and cultured on a collagen-coated 48-well plate with 2.0×10^5 cells/well.

2.5. HBV infection in primary tupaia and human hepatocytes

Primary human hepatocytes (PXB-cells) were purchased from PhoenixBio (Hiroshima, Japan). Primary tupaia and human hepatocytes were infected with HBV at 5-genome equivalents/cell with 4% polyethylene glycol 8000 (Promega, Madison, WI, USA). At 1 and 2 days postinoculation (dpi), the inoculated cells were washed, and the culture supernatant was collected every 5 days.

2.6. HBV-DNA quantification

Viral DNA from serum or culture supernatant was extracted using SMItest EX-R&D kits (Nippon Genetics Co., Ltd., Tokyo, Japan) according to the manufacturer's instructions. HBV-DNA was quantified by real-time detection PCR, as previously described [13]. The primers and probes for the S gene consisted of a forward primer HB-166-S21 (nts 166–186): 5′-CACATCAGGATTCCTAGGACC-3′, a reverse primer HB-344-R20 (nts 344-325): 5′-AGGTTGGTGAGTGATTGGAG-3′, and Taq Man probe HB-242-S26FT (nts 242–267): 5′-CAGAGTCTACACTCGTGGTGGACTTC-3′.

2.7. Generation of chimeric mouse with tupaia liver

Primary tupaia hepatocytes from 3-week-old tupaia (primary tupaia hepatocytes #2) were transplanted into the liver of cDNA-uPA/SCID mice (5.0×10^5 cells/animal) via the spleen [14]. At 8 weeks post transplantation, sera were collected to measure the tupaia albumin level.

2.8. Histological analysis

Liver tissues of the chimeric mice were fixed with 10% phosphate-buffered formalin and embedded in paraffin. The samples were then sectioned and stained with hematoxylin and eosin.

2.9. Evaluation of the replacement of tupaia hepatocytes

Hepatocytes were isolated from the chimeric mice by using a two-step collagenase perfusion method as described above. Thereafter, the hepatocytes were incubated with anti-mouse hepatocyte-specific antibodies (IgG, IY0266Z, Institute of Immunology, Tokyo, Japan) labeled with Dynabeads (Thermo Fisher Scientific, Waltham, MA, USA) for 30 min on ice. Only mouse cells were labeled with the beads. The number of viable tupaia cells without beads and viable mouse hepatocytes with beads were counted by the trypan blue exclusion test, and the ratio of tupaia hepatocytes to that of the mouse hepatocytes was calculated [12].

2.10. Measurement of serum albumin level

The albumin levels of the sera from the chimeric mice with tupaia hepatocytes were measured using the monkey albumin ELISA kit (Immunology Consultants Laboratory, Inc., Portland, OR, USA) according to the manufacturer's instructions.

2.11. HBV infection to chimeric mouse with tupaia hepatocytes

Fourteen-week-old (11 weeks after transplantation) chimeric mice with tupaia hepatocytes were inoculated intravenously with 1.0×10^6 viral DNA copies of HBV. Sera were collected every 2 weeks for 10 weeks and stored at −80 °C until use.

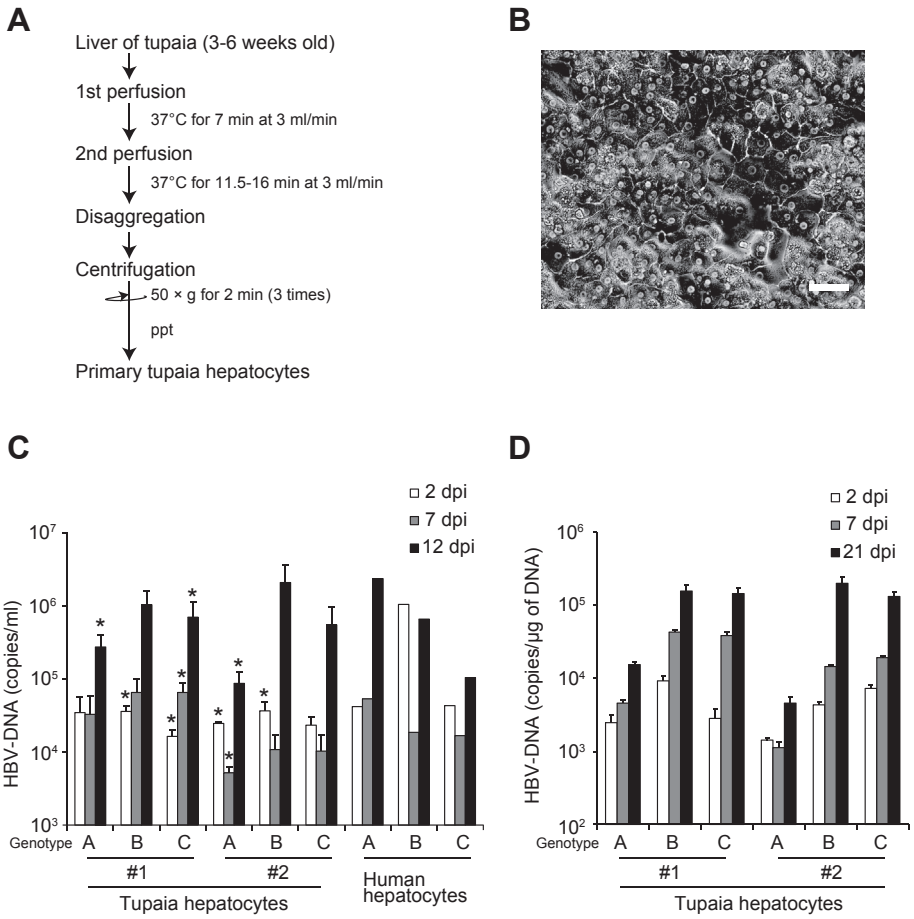

Fig. 1. (A) Procedure for the isolation of tupaia hepatocytes. (B) Morphology of the tupaia hepatocytes at 1 day after seeding. Bar, 50 μm (C) Viral DNA titer in the culture fluid of tupaia or human hepatocytes infected with HBV. Error bars represent standard deviation. Asterisks represent significant difference between tupaia hepatocytes and human hepatocytes. (D) Viral DNA in tupaia hepatocytes infected with HBV.

2.12. HBV infection to tupaia

One-year-old tupaias (n = 14) were inoculated intravenously with 1.0×10^6 viral DNA copies of HBV. Sera were collected every 2 weeks and stored at −80 °C until use.

2.13. Measurement of serum alanine aminotransferase (ALT) activity

Serum ALT activity in tupaia was determined using Transaminase CII-test Wako (Wako Pure Chemical Industries, Osaka, Japan).

2.14. HBs quantification

Serum HBsAg levels in tupaia were measured by the two-step sandwich assay principle with a fully automated chemiluminescent enzyme immunoassay system (Lumipulse G 1200, Fujirebio, Tokyo, Japan) [15].

2.15. Statistical analyses

Student's *t*-test was used to conduct statistical analyses of the data. *p* values lower than 0.05 were considered to be significant.

3. Results

3.1. HBV-DNA levels in primary tupaia hepatocytes and primary human hepatocytes

To evaluate the ability of tupaia hepatocytes to support HBV infection, we isolated tupaia primary hepatocytes (Fig. 1A). Isolated cells exhibited epithelial cell morphology and distinctly round nuclei with prominent nucleoli, which is typical of a hepatocyte (Fig. 1B). We infected HBV genotypes A, B, and C to primary tupaia hepatocytes and primary human hepatocytes, and measured HBV-DNA level in culture supernatant at 2, 7, and 12 dpi (Fig. 1C) and in the hepatocytes at 2, 7, and 21 dpi (Fig. 1D). All HBV genotypes infected and propagated in both tupaia hepatocytes #1, #2, and primary human hepatocytes. In culture supernatants at 12 dpi, the viral DNA titer of genotypes B and C in tupaia hepatocytes were equally or significantly higher than those in human hepatocytes (Fig. 1C). The genotype A in the viral titers in both tupaia hepatocytes was significantly lower than that in human hepatocytes at 12 dpi. The HBV-DNA levels in tupaia hepatocytes also increased gradually and reached 10^3-10^6 copies/μg of DNA at 21 dpi (Fig. 1D).

3.2. Replacement of chimeric mice hepatocytes with tupaia hepatocytes and HBV infection

To evaluate the ability of tupaia hepatocytes to support HBV propagation *in vivo*, we next transplanted primary tupaia hepatocytes #2 to cDNA-uPA/SCID mice (Fig. 2A), and carried out HBV

infection after 11 weeks. After transplantation, the mice survived at least 5 months in spite of the presence of the uPA gene. No gross pathology was observed like white liver disease due to hepatocyte injury (Fig. 2B). Hematoxylin and eosin staining showed that the transplanted hepatocytes engrafted into the liver were organized at the liver plate (Fig. 2C). The efficiency of the replacement was evaluated by measuring the serum albumin levels in chimeric mice. ELISA showed the tupaia serum albumin levels, but not mouse serum albumin levels (Fig. 2D). At 8 weeks post-transplantation, the OD values of the chimeric mouse sera were similar or more than those of tupaia. The tupaia hepatocytes replaced the chimeric mouse hepatocytes at a rate of 96.2% (#5) and 97.6% (#8) (Fig. 2E). These data show that tupaia hepatocytes had sufficiently replaced the chimeric mouse hepatocytes.

To assess the growth of HBV in the chimeric mice with tupaia hepatocytes, we infected chimeric mice with HBV (HBV genotypes B, C, and D). The HBV-DNA from the sera was detected from 4 weeks post-infection (4 wpi) (genotypes B and C; Fig. 2F), or from 6 wpi (genotype D), and the levels were found to increase gradually. At 10 wpi, the viral DNA levels in the chimeric mouse sera were 10^4–10^7 copies/mL.

3.3. HBV infection in adult tupaia

To compare the HBV infection mode between adult tupaia and chimeric mouse with tupaia hepatocytes, we infected adult tupaia with HBV and analyzed the viral titer (Fig. 3A). After HBV infection, the HBV-DNA positive ratio peaked at 2 wpi (Table 1). The HBV-DNA positive ratio then decreased, but in some adult tupaias, HBV-DNA was detected intermittently during the observation period. The viral DNA level in the serum was less than 10^3 copies/ml, which was lower than that in the serum samples from chimeric

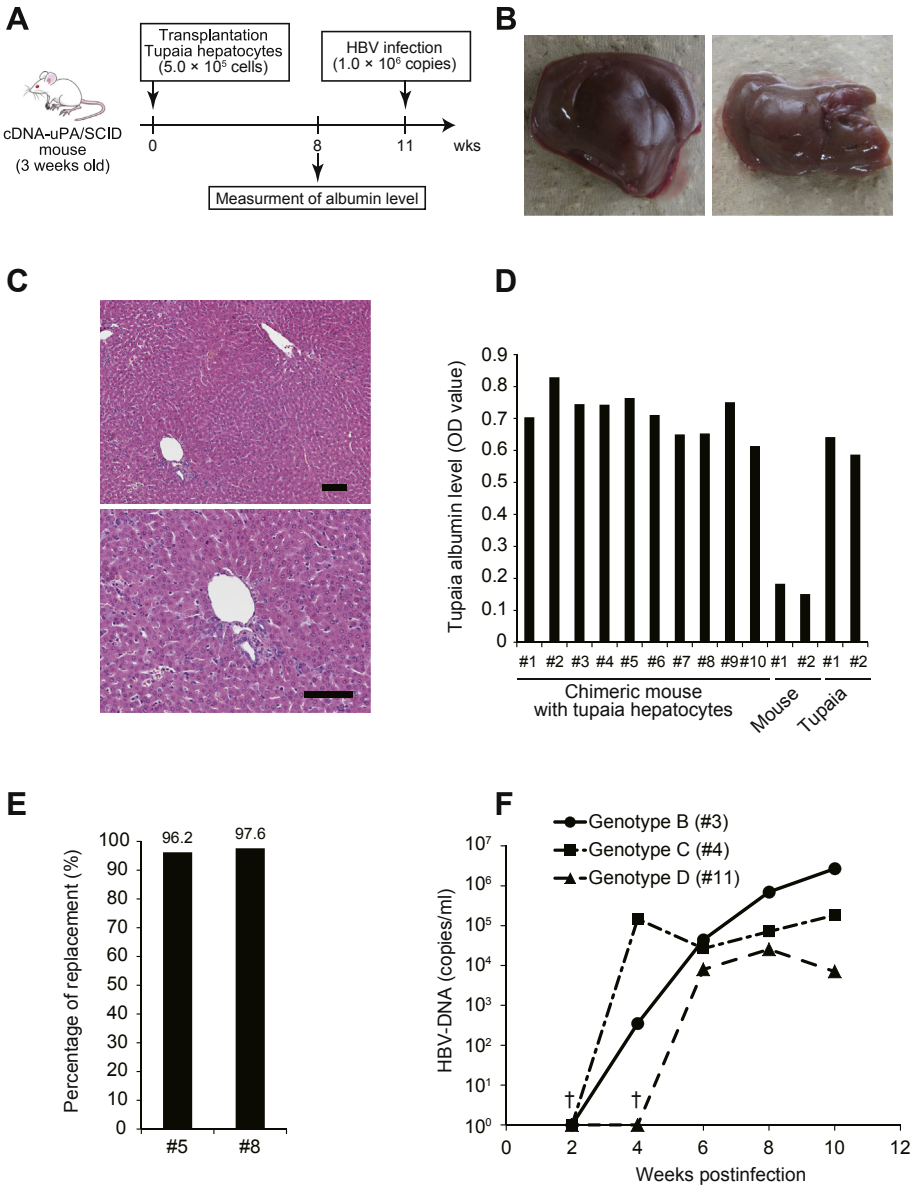

Fig. 2. (A) Experimental schedule for the production of chimeric mouse with tupaia hepatocytes and HBV infection. (B) Gross image of the liver of chimeric mouse with tupaia hepatocytes. (C) Histological analysis of the liver from chimeric mouse with tupaia hepatocytes. Bar, 100 μm (hematoxylin-eosin staining) (D) Tupaia albumin level in serum by ELISA after 8 weeks of transplantation. (E) Percentage of replacement of tupaia hepatocytes from livers of chimeric mice with tupaia hepatocytes. (F) Viral DNA titer in serum of HBV-infected chimeric mice with tupaia hepatocytes. Daggers represent undetectable.

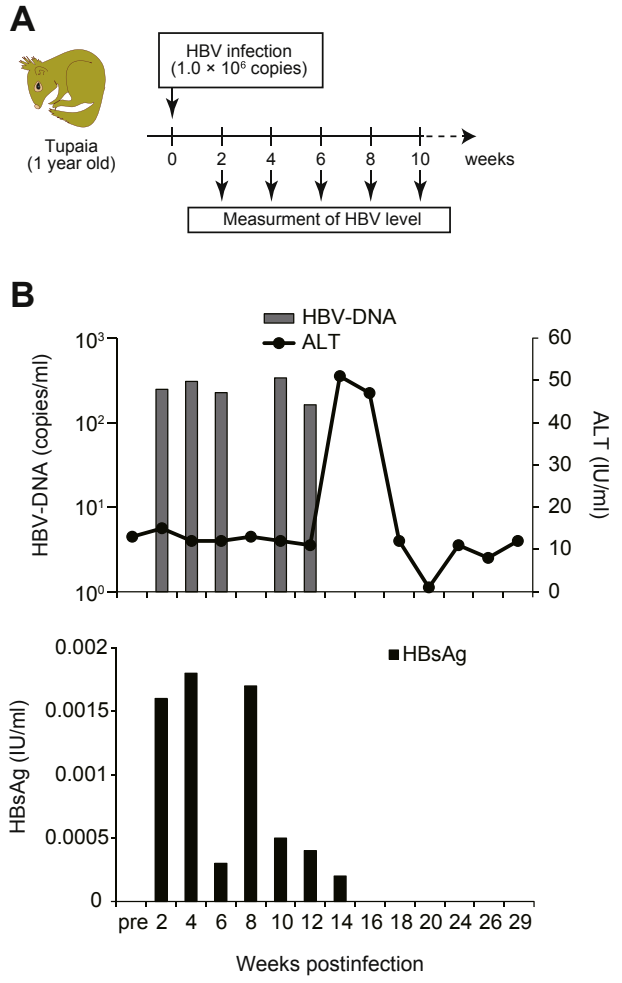

Fig. 3. (A) Experimental schedule of HBV infection in adult tupaia. (B) Viral DNA titer, ALT level (upper), and HBs Ag level (lower) in the serum of adult tupaia (ID: #98) infected with HBV genotype J.

mice with tupaia hepatocytes. Tupaia #98 infected with HBV genotype J showed HBV-DNA positive with high frequency, especially in the early phase of HBV infection. For detailed growth characterization, the ALT level, which is the marker of liver damage, and the HBsAg levels were analyzed (Fig. 3B). Similar to the kinetics of HBV-DNA, HBsAg was detected persistently for 14 weeks. At 16–18 wpi, the ALT level increased abruptly, after which the HBV-DNA and HBsAg were undetectable.

4. Discussion

The lack of efficient experimental animal models that are susceptible to HBV infection has hampered the study of HBV pathogenesis. HBV infection in tupaia has shown to result in hepatitis and persistent infection like that in humans [16,17]. Therefore, tupaia is expected to be a suitable animal model for HBV infection. However, the infection mode in tupaia and the host response against HBV infection is unknown.

Several studies have shown that chimeric mouse with humanized liver based on uPA/SCID supports hepatitis infections caused by HBV and HCV [9,18]. This mouse is immune deficient and thus a suitable host to address the response to viral infection in hepatocytes. To address the tupaia response in hepatocytes precisely, we developed a chimeric mouse with tupaia hepatocytes. We

Table 1
Viral DNA titer (copies/ml) in sera from tupaia infected with HBV.

Individual number	Sex	Genotype	Weeks postinfection												
			2	4	6	8	10	12	14	16	18	20	22	24	26
84	Male	A (A2_JP1)	2.8×10^1	–	–	–	–	–	–	–	–	–	–	–	–
85	Male	A (A2_JP1)	–	–	–	–	–	–	–	2.7×10^1	–	–	–	–	–
86	Male	A (A2_JP2)	4.5×10^1	–	–	–	–	–	7.2×10^2	4.5×10^1	–	–	–	6.3×10^1	–
87	Male	A (A2_JP2)	4.1×10^1	–	–	–	–	–	–	–	1.8×10^1	–	–	–	–
88	Male	A (A2_JP4)	4.1×10^1	–	–	–	–	–	–	–	–	–	–	–	4.0×10^1
89	Male	A (A2_JP4)	–	–	–	1.7×10^2	–	–	–	1.5×10^2	–	–	–	–	–
92	Female	A (A2_JP1)	–	–	–	–	–	–	1.2×10^2	6.8×10^1	–	–	–	–	–
93	Female	A (A2_JP1)	1.4×10^2	–	–	–	–	–	–	–	–	ND	ND	ND	–
94	Female	A (A2_JP2)	–	–	6.9×10^0	–	–	–	–	–	–	ND	–	–	–
95	Female	A (A2_JP2)	–	–	–	–	–	–	–	–	–	–	–	–	–
96	Female	A (A2_JP4)	1.6×10^2	5.1×10^1	–	–	–	–	–	–	–	–	–	–	–
97	Female	A (A2_JP4)	–	9.1×10^1	2.2×10^0	–	–	–	–	–	–	–	3.9×10^2	–	–
90	Male	J	6.5×10^1	–	–	–	–	–	–	–	–	–	–	–	–
98	Female	J	2.5×10^2	3.1×10^2	2.3×10^2	–	3.4×10^2	1.6×10^2	–	–	–	–	ND	–	–

-, Under the detectable level; ND, Not done.

succeeded in developing a chimeric mouse with tupaia hepatocytes with high replacement with tupaia hepatocytes (>95%), thus making it a powerful tool to analyze host innate immunity response against various pathogens and not only HBV. This replacement level is higher than that previously reported in chimeric mouse with tupaia liver [19] and same level as chimeric mouse with humanized mouse based on cDNA-uPA/SCID mouse [14].

In the present study, we examined the ability of tupaia hepatocytes to support HBV infection *in vitro* and *in vivo*. Primary tupaia hepatocytes showed high efficiency for HBV replication and chimeric mice with tupaia hepatocytes produced 10^4–10^6 copies/ml. A previous study showed that after HBV infection, chimeric mice with humanized liver could support HBV infection efficiently (~1.5×10^6 copies/ml of serum) [20]. These data indicate that like human hepatocytes, tupaia hepatocytes can also support HBV replication. In contrast, the HBV-DNA levels in adult tupaia were less than 10^3 copies/ml of serum. The level of HBV replication in adult tupaia was much lower than that in primary tupaia hepatocytes and chimeric mouse with tupaia hepatocytes. This suggests that the immune system of tupaia may have a critical factor that suppresses HBV infection. A recent study indicated that TNFα [21] and IFNγ strongly suppressed HBV replication by targeting the covalently closed circular DNA [22]. The expression levels of these factors in HBV-infected tupaia and chimeric mice needed to be compared to analyze the essential factors required for the regulation of HBV replication.

Chronic HBV infection is defined by HBV persistence for more than 6 months with detectable HBsAg and HBV titers (>2000 IU/ml) [23]. In adult tupaia, not all but 1 individual out of 2 (genotype J) or 4 out of 12 tupaias (genotype A) showed chronic HBV infection. In humans, mother-to-child-transmission is one of the major routes of HBV infection, and such infected infants develop chronic HBV infection [24]. Thus, inoculation in neonatal tupaia may accelerate the chronic infection rate.

HBV showed different propagation rates in primary tupaia hepatocytes, chimeric mouse with tupaia hepatocytes, and adult tupaia. This study shows the differences in the host response to HBV infection and the mechanism of persistent infection. In addition, the use of primary hepatocytes and chimeric mouse strongly highlights the potential of using tupaia animal model for HBV research. Thus, antiviral therapies can be developed in the future using this tupaia model by analyzing the expression profile of the factors regulating the HBV infection and by performing a comprehensive analysis of the HBV genome.

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Conflicts of interest

The authors have declared no conflict of interest.

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