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—Review—

Tupaia Belangeri as an Experimental Animal Model for Viral Infection

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Abstract: Tupaias, or tree shrews, are small mammals that are similar in appearance to squirrels. The morphological and behavioral characteristics of the group have been extensively characterized, and despite previously being classified as primates, recent studies have placed the group in its own family, the Tupaiidae. Genomic analysis has revealed that the genus *Tupaia* is closer to humans than it is to rodents. In addition, tupaias are susceptible to hepatitis B virus and hepatitis C virus. The only other experimental animal that has been demonstrated to be sensitive to both of these viruses is the chimpanzee, but restrictions on animal testing have meant that experiments using chimpanzees have become almost impossible. Consequently, the development of the tupaia for use as an animal infection model could become a powerful tool for hepatitis virus research and in preclinical studies on drug development.

Key words: genome, HBV, HCV, *Tupaia*, virus

Taxonomic Classification

Tupaia belangeri belongs to the family Tupaiidae, which consists of four genera and 19 extant species (Table 1) [13, 19]. The members of *Tupaia*, which are colloquially referred to as tree shrews, were first recorded in a sketch by William Ellis on a voyage with Captain Cook in 1780 [7]. With a body weight ranging between 45–350 g (Table 1), members of the genus *Tupaia* are similar in appearance to squirrels (Fig. 1). The natural habitat of *Tupaia* spp. consists of the tropical rainforest in South East Asia where they feed on fruits, insects and small vertebrates [7].

Similarities between *Tupaia* spp. and primates were first reported in the 1920s; for example, Le Gros Clark proposed that tree shrews and primates were closely related based on brain anatomy [20]. However, recent molecular studies have separated tupaias from the primates and placed them in the order Scandentia and within the grandorder Euarchonta, which also contains the Primates and Dermoptera [17].

Handling of Tupaia

Tupaia is active during daytime, and animal rooms are illuminated from 7:00 am to 9:00 pm with a relative humidity of 50–60%, and temperature at 26°C. Their foods are CMS-1M (CREA, Japan) 20 g, apple, banana and boiled egg, everyday. They usually slip into the boxes as soon as somebody enters the room, then we can catch them by net. We can bleed approximately 0.5 ml from the tail or leg vein once in 2 weeks. Tupaias can be

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Table 1. Composition of family Tupaiidae [1]

Taxa	Morphological characteristics	Reproductive characteristics	Weaning and longevity	Distribution
Family: Tupaiidae				
Genus: *Tupaia*				
*Species: *belangeri*	BW: 50–270 g	GP: 41–55 d	W: ca. 30 d	
Subspecies:	HBL: 12–21 cm	L: 1–5	L: 9–12 yr	
belangeri chinensis	NN: 1–3 pairs	NBW: 6–10 g		
**Species:*				Tropical forests
chrysogaster, dorsalis, glis,				in Southeast Asia
gracilis, javanica, longipes,				
minor, moellendorffi,				
montana, nicobarica,				
palawanensis, picta,				
splendidula, tana				
Genus: *Anathana ellioti*	BW: 180 g	UK	UK	
	HBL: 19 cm			
	NN: 3 pairs			
Genus: *Dendrogale melanura,*	BW: 60 g	GP: 41–55 d	W: ca. 30 d	
murina	HBL: 13 cm	L: 1–5	L: 9–12 yr	
	NN: 1 pair	NBW: 6–10 g		
Genus: *Urogale everetti*	BW: 220–359 g	GP: 30 d	W: ca. 30 d	
	HBL: 20 cm	L: 1–4	L: 6 yr	
	NN: 2 pairs	NBW: 10 g		

*BW: body weight; HBL: head-body length; NN: number of nipples; GP: gestation period; L: litter size; NBW: Newborn body weight; W: weaning; L: life span; UK: unknown.

Fig. 1. Adult female tupaia (*Tupaia belangeri*) maintained at the Department of Animal Hygiene, Kagoshima University.

breeding after 6–9 months age and easily to give average 4 babies after approximately 45 days of pregnancy. Tupaia usually possesses few health problems, but sometimes shows diarrhea by *Escherichia coli*, *Klebsiella pneumonia* or protozoa, which can be checked by quarantine. The inbred tupaia has not been established yet.

Genetic Characteristics of Tupaia Spp

Evolutionary characterization of 7S RNA-derived short interspersed elements (SINEs) revealed that 7S RNA is a component of the cytoplasmic signal recognition particle [33] in primates [5], tupaia [25] and rodents [18], i.e. all of the members of the placental mammalian order Supraprimates and the superorder Euarchontoglires. The fossil *Alu* monomer was previously considered to be the oldest common ancestor of all 7S RNA-derived SINEs [27], and was thought to be restricted to primates [17]. *Tupaia* possesses specific, chimeric, Tu-type II SINEs, which may share a common ancestor with rodent B1 SINEs [27]. Phylogenetic analysis of 7S L RNA-derived SINEs has shown that tupaias can be grouped with primates and Dermoptera in the Euarchonta, while the Rodentia and Lagomorpha can be grouped with the Glires [17].

Whole-genome analysis by several groups ([8], Tsukiyama-Kohara *et al*., *in preparation*) revealed a genetic relationship between tupaias and humans. Similarly, phylogenetic analysis based on whole genome sequences showed that humans are closer to tupaias than they are to mice (Fig. 2). Further, several of the same highly conserved and variable genes have been identified

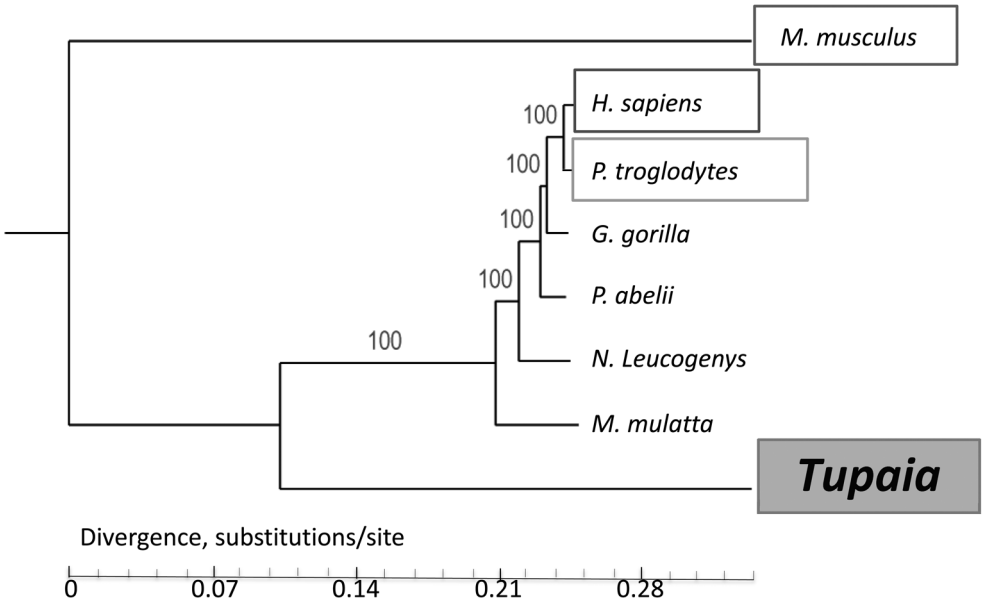

Fig. 2. Dendrogram showing relationships between primates, tree shrews and rodents. Phylogenetic tree constructed using orthologous genes at 4-fold degenerate sites by the maximum likelihood method. Branch lengths represent the neutral divergence rate and blue characters indicate bootstrap values.

in both tupaia and humans. For example, relatively high homology has been observed between human and *Tupaia* hepatitis C virus (HCV) viral receptor CD81 (Fig. 3A), scavenger receptor class B member I (SR-BI), the tight junction proteins claudin I and occludin I [16], as well as the hepatitis B virus (HBV) receptor, sodium-taurocholate cotransporting polypeptide (NTCP) (Fig. 3B) [38], particularly in the receptor and virus envelope surface glycoprotein regions that interact with the transmembrane proteins. It is possible that these highly conserved molecules could be a missing link during the evolution of tupaia, and detailed analysis of this hypothesis is currently underway.

Tupaia as an Experimental Animal Model

The high degree of genetic homology between several neuromodulator receptor proteins in tree shrews and primates has meant that *Tupaia* has been extensively utilized in preclinical research, particularly in the areas of toxicology and virology [10]. Although adult male tupaias exhibit strong territoriality in their natural habitat, the coexistence of two males in visual and olfactory contact in the laboratory leads to the establishment of a stable dominant-subordinate relationship, with subordinates showing distinct stress-induced alterations to behavior, physiology and central nervous activity [9]. These alterations exhibited by the subordinate male tupaias are similar to those observed in depressed human patients, and could be applicable to preclinical research of antidepressant drugs [11]. Various aspects of human behavior, infant development, communication and social structure could also potentially be studied in tupaia [22, 23].

Tupaia as Viral Hepatitis Model

Tupaia have also been employed in studies of viral infection, especially on hepatitis B and C viruses (HBV and HCV) [12]. For these viruses, the only existing natural-infection animal model is the chimpanzee. However, because chimpanzees are long-lived (>50 years), very expensive, and subject to stringent animal welfare regulations, several groups have attempted to develop *Tupaia* for use as an animal infection model. Pathogenesis of HCV was characterized using various transgenic mouse animal models and they can develop chronic hepatitis, liver cirrhosis and hepatocellular carcinoma [30], however natural infection is difficult to be established in these mice. HCV can successfully establish infection in the humanized chimeric mice liver [15, 24], but they do not have immune response, therefore, patho-

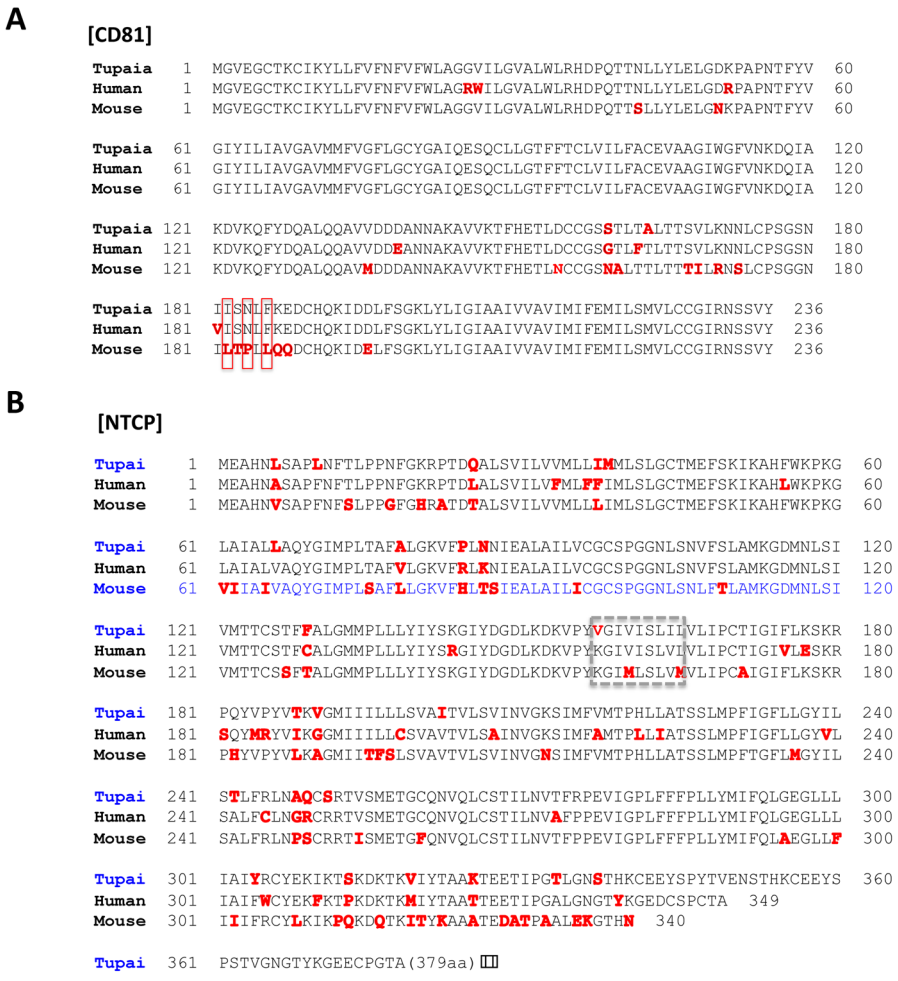

Fig. 3. Alignment of amino acid sequences of viral receptors. (A) Alignment of CD81 amino acid sequences from tupaia, human and mouse. Different amino acids were indicated with red colour. Significant amino acids for binding to HCV E2 protein were surround by square (Ile182, Asn184 and Phe186) [14, 6]. (B) Alignment of NTCP amino acid sequences from tupaia, human and mouse. Different amino acids were indicated with red colour. HBV pre-S1 binding region [37] was surrounded by break line box.

genicity of HCV could not be characterized.

We previously conducted infection experiments using HCV in *Tupaia* and characterized the pathogenesis in this animal [2]. Chronic HCV infection, which manifests as liver cirrhosis and hepatocellular carcinoma, is easily established [1]. Currently, approximately 170 million people around the world may be infected with HCV [35]. The current standard therapy for chronic hepatitis C is a combination of pegylated interferon (IFN) alpha-2a and nucleoside analog ribavirin. Recently, IFN-free combinations of direct-acting antiviral agents have been tested for clinical use and can achieve significant antiviral activity [29]. However, no vaccines against HCV infection have been developed to date, mainly because of the lack of suitable animal experimental systems.

We injected tupaias with serum from a chronic hepatitis C patient (HCR6; 3.7×10^4 50% chimpanzee infectious dose/ml) or reconstituted virus (RCV; genotype 1b). Inoculation with patient serum caused marked fluctuations in the serum alanine aminotransferase (ALT) concentrations − from 2–5 fold in both tupaias − suggesting acute hepatitis (Figs. 4 and 5). Quantitation of viral RNA by reverse transcription PCR revealed HCV viremia in *Tupaia* (Tup. 5 and 6, Fig. 5A). Inoculation with RCV showed sustained viremia for up to 10 weeks (Tup. 4 and 8; Fig. 5B). Histological examination re-

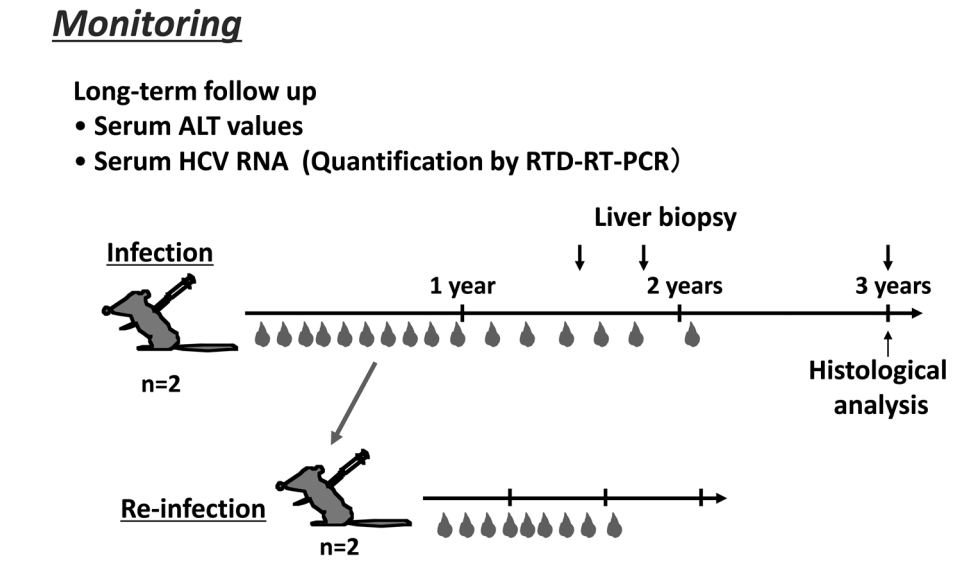

Fig. 4. Experimental design of HCV infection and re-infection of tupaia.

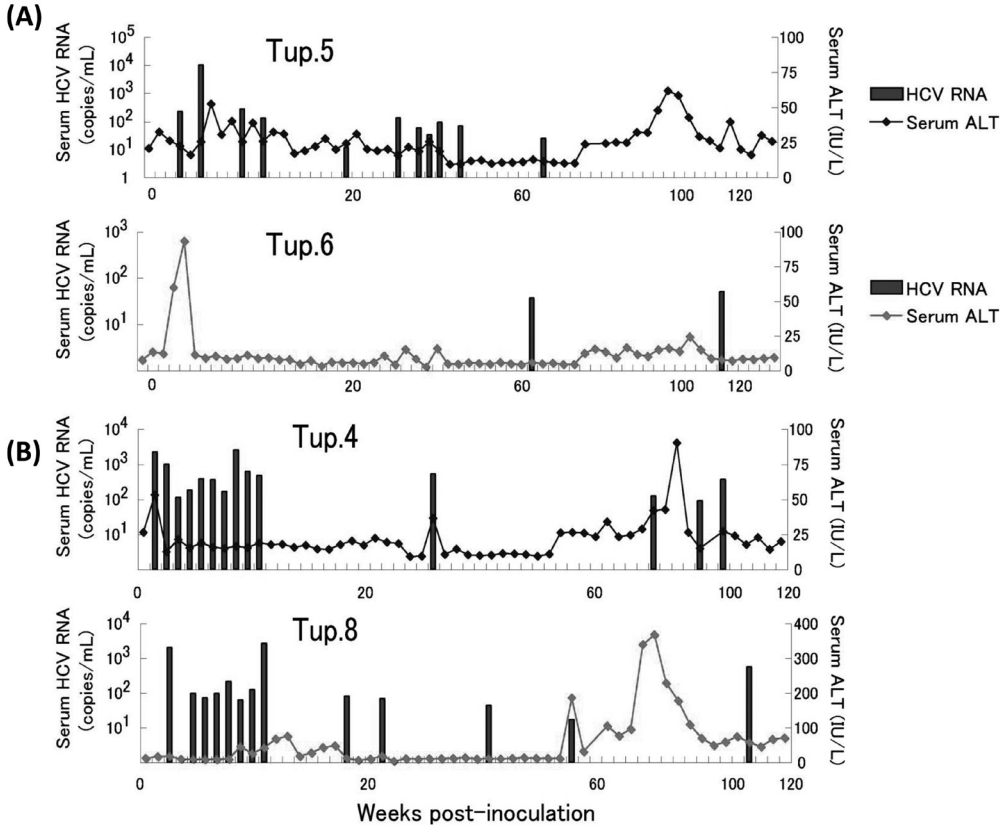

Fig. 5. Course of HCV infection in tupaia. (A) Tupaias No. 5 and 6 were inoculated with patient serum HCR6. Serum ALT (IU/ml) and viral loads, measured as amount of HCV RNA (copies/ml), were measured for over 120 weeks. Set point for serum ALT in untreated tupaias was 22.3 IL/ml (n=23). Negative control animals showed no significant ALT fluctuations for more than 2 years (n=3). No HCV RNA was detected in the negative controls after more than 2 years (n=3). (B) Tupaias No. 4 and 8 were inoculated with RCV as for the HCR6 inoculated animals.

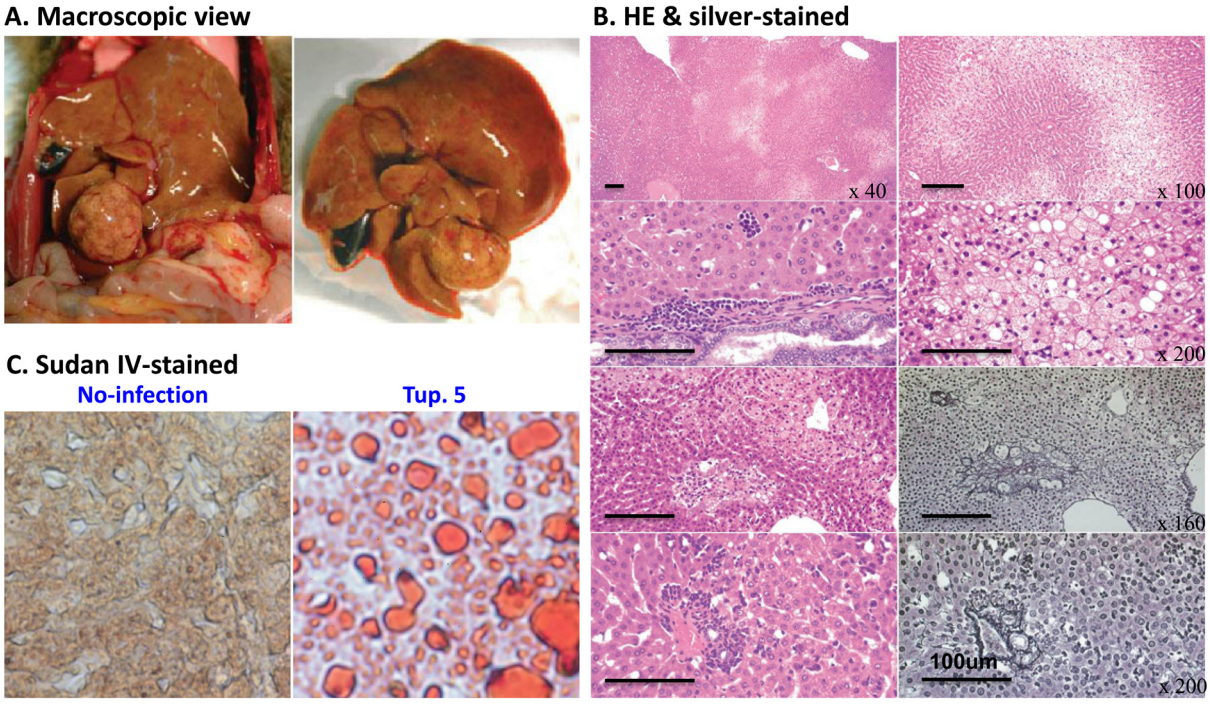

Fig. 6. (A) Macroscopic view of liver inoculated with patient serum HCR6 after 2 years (Tup.5, serum ALT value was 25 IU/l at autopsy). (B) HE staining (×40, ×100, ×200; scale bars indicate **) and silver staining (×160, ×200) of liver tissue (Tup 5) were indicated. Lymphocytic infiltration, steatosis and fibrogenesis were observed. (C) Sudan IV staining of the liver tissue of Tup5 (right) and non-infection (left).

vealed that HCV caused chronic hepatitis, fibrosis and cirrhosis (Fig. 6), with progressive lipid degeneration observed in tupaias over the course of infection. Macroscopic observations also indicated that liver cirrhosis worsened and large surface nodules were observed (Fig. 6). Transmission of viral RNA-positive serum to naïve animals reproduced acute hepatitis and viremia, indicating that HCV infection could reproduce the pathogenesis typically associated with acute and chronic hepatitis in tupaia. However, sustained seroconversion was not observed in tupaia and production of HCV and antibody only occurred at specific time points. To increase the susceptibility of tupaia to HCV infection and to develop a sensitive HCV infection model, these differences between HCV infection in tupaias and humans should be examined in future. HCV infection studies in tupaias have been examined using x-rays [41] and metabolic analysis [31], and the efficacy of natural products for treating HCV-infected tupaia has also been evaluated [39].

Several groups have successfully infected tupaias with HBV, as follows. In culture medium, infection by HBV has been shown to produce HBs antigen (Ag) and HBeAg. HBV infection in newborn and adult tupaias induced the production of HBsAg, HBsAb, HBcAb and HBeAb; all of the adults were successfully infected [34]. Experimental infection of tupaias with HBV was successful in approximately 55% of the animals inoculated [38]. HBV infection and aflatoxin B1 exhibited a synergistic effect in hepatocarcinogenesis [21]. To establish chronic infection by HBV, newborn tree shrews were infected with HBV [36]. Six of 46 newborn babies were found to be susceptible to HBV infection at 48 weeks post inoculation. Histological analysis of liver tissues from infected tupaias revealed chronic hepatitis symptoms, such as hydropic, fatty and eosinophilic degeneration of hepatocytes, lymphocytic infiltration, and hyperplasia of small bile ducts in the portal area [28]. One tupaia infected with HBV for more than 6 years showed multiple necrotic areas [28]. These findings show that although the efficacy of infection needs to be improved in future, tupaias are potentially well suited for use as a model for HBV infection.

Tupaias have also been reported to be infected by

specific viruses, such as tupaia herpes virus, which induces tumorigenicity [4], and potentially with nonpathogenic tupaia paramyxovirus [32]. Tupaias have also been infected with TTV [26], tupaia adenovirus [3], and influenza virus [40].

Conclusion

Tupaia shares considerable genetic homology with both humans and primates, and is considered to be well suited for use as a model for studies on viral infection and preclinical drug development. At present, difficulties associated with maintaining and handling tupaia are major factors limiting the widespread adoption of this animal for use in infection studies. However, optimizing these issues will facilitate the use of tupaias as an experimental animal. In addition, development of genetic methods for modifying the tupaia genome would also increase the potential value of tupaia as a model animal, as this would facilitate detailed studies of virus pathogenesis and drug evaluation.

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