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Humanized Murine Model for HBV and HCV Using Human Induced Pluripotent Stem Cells

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Abstract

Infection of hepatitis B virus (HBV) and hepatitis C virus (HCV) results in heterogeneous outcomes from acute asymptomatic infection to chronic infection leading to cirrhosis and hepatocellular carcinoma (HCC). *In vitro* models using animal hepatocytes, human HCC cell lines, or *in vivo* transgenic mouse models have contributed invaluablely to understanding the pathogenesis of HBV and HCV. A humanized mouse model made by reconstitution of human primary hepatocytes in the liver of the immunodeficient mouse provides a novel experimental opportunity which mimics the *in vivo* growth of the human hepatocytes. The limited access to primary human hepatocytes necessitated the search for other cellular sources, such as pluripotent stem cells. Human embryonic stem cells (hESCs) have the features of self-renewal and pluripotency and differentiate into cells of all three germ layers, including hepatocytes. Human-induced pluripotent stem cells (iPSCs) derived from the patient's or individual's own cells provide a novel opportunity to generate hepatocyte-like cells with the defined genetic composition. Here, we will review the current perspective of the models used for HBV and HCV study, and introduce the personalized mouse model using human iPSCs. This novel mouse model will facilitate the direct investigation of HBV and HCV in human hepatocytes as well as probing the genetic influence on the susceptibility of hepatocytes to HBV and HCV.

Keywords

HBV; HCV; Humanized model; iPSCs; Reprogramming

INTRODUCTION

Hepatitis B virus (HBV) and hepatitis C virus (HCV) are hepatotropic viruses that can cause acute and chronic diseases in liver after infection. It is estimated that more than 2 billion people have been infected with HBV and the annual death toll due to HBV infection is more than 600,000 (Ganem and Prince, 2004). The global HCV infection was estimated to be 3% or 170 million individuals, and more than 350,000 fatalities are due to HCV-related liver

disease each year (Te and Jensen, 2010; Yang and Roberts, 2010). People chronically infected with HBV or HCV have a higher risk of liver cirrhosis or hepatocellular carcinoma (HCC). According to a WHO report, 90% of infants infected with HBV during their first year of life develop chronic infection. 30 to 50% of children infected between the ages of one to four years develop a chronic condition, while about 90% of healthy adults infected with HBV will recover and be completely devoid of the virus within six months. 10 to 30% of the individual infected with the acute HCV recover, while the rest may develop into chronic carriers after the acute stage (Afdhal, 2004). Chronic HBV and HCV infection has huge economic burdens in countries with endemic infection (Yang and Roberts, 2010).

The distribution of HBV or HCV occurrence is varied geographically. The endemic levels are within 8 to 15% in many areas including China, South-East Asia, most of Africa, most Pacific Islands, parts of the Middle East and the Amazon Basin. Meanwhile less than 2% of the population are infected with HBV or HCV in the United States, Western Europe and Australia (Te and Jensen, 2010). There are not only cultural differences among these countries, but also differences in the population-level genetic background that have been proposed to be responsible for the difference. Upon infection, individuals present a spectrum of symptoms from an acute symptomatic illness to chronic conditions, which lead to cirrhosis and HCC. According to the epidemiological study, there are individual and regional differences in terms of the susceptibility to acquire HBV or HCV. Hepatitis B vaccine has been available since 1982 and is 95% effective in preventing infection. However, most chronic carriers of HBV are threatened by the development of cirrhosis and HCC. At present, there are no effective Hepatitis C vaccines available, partially due to the rapid mutation rate of the HCV genome and the limited knowledge of HCV pathogenesis. Understanding the detailed mechanism of entry into and propagation in cells is imperative for the development of effective vaccines and antivirals for HCV (Washburn et al., 2011).

Research on HBV or HCV has been hampered by the difficulty in culturing human primary hepatocytes. They tend to differentiate and lose hepatic function after a limited period of *in vitro* culture. Thus, alternative models have been used. Animal hepatocytes, HCC cell lines, or transgenic mouse models have contributed to understanding the pathogenesis of HBV and HCV. Despite the success, there are shortcomings in those models, such that they do not properly model *in vivo* human hepatocytes. Other alternative cellular sources have been sought to make a model closer to the human primary hepatocytes. Human embryonic stem cells (hESCs) have the capacity of self-renewal and pluripotency (Murry and Keller, 2008). The pluripotency allows for generation of theoretically all cell types in the body, including hepatocytes. The indefinite self-renewing feature of hESCs promises the continuous supply of hepatocyte with the same genetic composition. The recent development of human induced pluripotent stem cells (iPSCs) even provides cells of the defined genetic background from any patients or individuals (Hanna et al., 2010). In this review, we will give an overview of the model systems used in studying the HBV and HCV and will discuss the novel model based on the human pluripotent stem cells.

MODELS TO INVESTIGATE HBV OR HCV PATHOGENESIS

Models using cell lines or animals have been developed for *in vitro* and *in vivo* investigation of HBV or HCV (Tables I and II). Despite the limitations, each model contributes to understanding the fundamentals of HBV and HCV pathogenesis and to the development of vaccines for HBV. The duck HBV (DHBV) primary hepatocyte model aided the discovery of key features of HBV such as virus structure, genome and mechanisms of replication (Yokosuka et al., 1988; Seigneres et al., 2001). Furthermore, this model facilitated the development of the first oral antiviral drug for HBV – lamivudine (Lee et al., 1989; Fischer and Tyrrell, 1996; Tomita et al., 2000). However, the DHBV model has shortcomings in

modeling human HBV, because DHBV does not express Protein X found in human hepadnaviruses, which is presumed to be critical for the development of HCC by human HBV (Feitelson and Miller, 1988).

Models biologically more relevant to the human system have been established using HCC cell lines including HepG2, Chang, Hep3B and Huh7. These cell lines have been useful *in vitro* models for production of the infective HBV virions (Sells et al., 1988) and drug screening (Sun and Nassal, 2006). However, there are limitations with these models as well. They are refractory to HBV infection due to the loss of features of primary human hepatocytes, such as the expression of the specific receptors for HBV (Glebe and Urban, 2007). Therefore, these cell lines are not the optimal models for investigating early steps of human HBV infection (Garcia et al., 2002; Mee et al., 2009). In addition, these cell lines are derived from HCC that had already become malignant and may not be a suitable model to study the progressive development of HCC caused by either HBV or HCV.

Animal models based on the expression of HBV in the transgenic mouse have been useful for investigating HBV pathogenesis and for developing antiviral drugs. However, HBV replication is minimal in HBV transgenic mice (Araki et al., 1989). They also present an acute phenotype rather than the chronic disease due to the transgene tolerance (Moriyama et al., 1990). By providing syngeneic unprimed splenocytes, scientists have developed an improved transgenic model for the chronic HBV in immunodeficient mice (Larkin et al., 1999).

Primary human hepatocytes may represent the model biologically most relevant in investigating HBV or HCV pathogenesis. However, human primary hepatocytes are genetically diverse and show a large variation among isolates. In addition, obtaining and maintaining them for a long time in *in vitro* cultures is challenging. For example, they tend to lose the susceptibility to HBV infection after culturing *in vitro*. Thus, there have been many attempts to reconstitute mouse liver with human primary hepatocytes to maintain the pristine hepatocyte-like features. In order to give growth advantage to donor human hepatocytes, urokinase-type plasminogen activator (uPA) transgenic or fumarylacetoacetate hydrolase (Fah)-deficient mouse models have been developed. Albumin-promoter driven expression of uPA is toxic to the hepatocytes, which undergo continual necrosis, leading to an insensitive stimulus for liver regeneration (Sandgren et al., 1991). Likewise, the absence of the tyrosine catabolic enzyme, fumarylacetoacetate hydrolase (Fah), causes liver failure in Fah^{-/-} mouse due to the accumulation of toxic compounds in blood and urine. Feeding Fah^{-/-} mice with NTBC (2-(2-nitro-4-trifluoromethylbenzoyl)-1,3-cyclohexanedione) prevents the liver failure (Dandri et al., 2001; Mercer et al., 2001; Bissig et al., 2007). By crossing the Fah^{-/-} mouse with immunodeficient Rag2^{-/-} (recombination activating gene 2) and IL2rg^{-/-} (IL-2 receptor common γ -chain) mice, Bissig et al. have generated a Fah^{-/-}Rag2^{-/-}IL2rg^{-/-} mouse, which lacks mature B and T lymphocytes as well as NK cells (Bissig et al., 2007). These triple KO mice make it possible for more efficient engraftment of human hepatocytes.

PLURIPOTENT STEM CELLS FOR HEPATIC DISEASE MODELING

hESCs are derived from cells of inner cell mass of blastocysts of human embryos. Like inner cell mass cells, they have features of self-renewal and pluripotency. The success of reprogramming human somatic cells to generate induced pluripotent stem cells (iPSCs) represents a novel approach for generating patient-specific pluripotent stem cells. Overexpression of four transcription factors (Oct4, octamer-binding transcription factor 4; Sox2, sex determining region Y-box 2; Klf4, Kruppel-like factor 4 and Myc, cellular homologue of avian myelocytomatosis virus oncogene) epigenetically reprograms somatic

cells to acquire the pluripotent features. A more detailed description of the reprogramming can be found in recent reviews (Takahashi et al., 2007; Yu et al., 2007; Zwi et al., 2009). iPSCs have the similar features as hESCs and demonstrate self-renewing and pluripotent capacity (Nizzardo et al., 2010; Si-Tayeb et al., 2010; Sullivan et al., 2010). Reprogramming avoids the ethical issues attributed to hESCs. Additionally, iPSCs can be derived from a wide range of individuals with different ethnicities and disease states, facilitating the development of personalized models of disease and autologous regenerative medicine. Human iPSCs are readily used to model genetic and non-genetic diseases, especially for those that lack patient tissue or suitable disease models. iPSCs for a series of neuronal, hematopoietic and complex diseases have been generated by multiple groups (Park et al., 2008; Marchetto et al., 2010; Zhang et al., 2010). *In vitro* neuronal disease models using iPSCs have been well explored, such as familial dysautonomia, Rett syndrome and spinal muscular dystrophy (Lee et al., 2009; Marchetto et al., 2010; Chang et al., 2011).

Many efforts were made to generate cells of different lineages, including hepatocytes. Initially, sodium butyrate was used to initiate hepatic programming in hESCs differentiated as embryoid bodies (EBs; Rambhatla et al., 2003). Subsequent studies used growth factors that are critical to endodermal tissue development. The use of Activin A and Wnt3a showed an increase in efficiency of generating hepatocyte-like cells (HLCs) in differentiating cells (Hay et al., 2008). Use of BMP4, which facilitates the formation of mesoendodermal cells, also showed an increase in HLC formation (Gouon-Evans et al., 2006; Zhao et al., 2009). Validation of the function of *in vitro* derived hepatocyte-like cells has been limited. Expression of hepatic markers, such as albumin, AFP and HNFs in *in vitro* differentiated cells has been used to show hepatic differentiation *in vitro*. Faithful functional recovery in liver from mice transplanted with *in vitro* derived hepatocytes or HLCs should be true validation of function (Duncan et al., 2009). Like in primary human hepatocytes, uPA transgenic mice were used for the successful engraftment of *in vitro* derived hepatocytes (Agarwal et al., 2008).

Human iPSCs have been tested for their potency of differentiating into cells of different lineages, including the endodermal hepatic lineage. We are among the first groups that reported the successful differentiation of human HLCs from normal iPSCs as well as iPSCs from a diabetic patient (Sullivan et al., 2010). Si-Tayeb et al. also described the generation of HLCs from human iPSCs by using hypoxic conditions which produced over 80% HLCs (Si-Tayeb et al., 2010). Recently, the iPSCs from patients with an alpha-1-anti-trypsin mutation were generated (Rashid et al., 2010). Alpha-1-antitrypsin (A1AT), a serine protease inhibitor secreted primarily from hepatocytes, irreversibly inhibits neutrophil elastase, cathepsin G and proteinase 3. Mutations in glutamate 342 to lysine causes the accumulation of insoluble aggregations in the endoplasmic reticulum of hepatocytes, leading to autophagy, oxidative stress and apoptosis (Goopu and Lomas, 2009). iPSCs from patients with A1AT mutations mimic the patient's phenotypes and will provide a platform to drug discovery.

A HUMANIZED MOUSE MODEL FOR HBV OR HCV

The application of personalized human iPSCs has a number of advantages with respect to development of *in vitro* and *in vivo* models for HBV or HCV research. As discussed in the previous section, human hepatocytes show diverse responsiveness to HBV or HCV, depending on the genetic composition of the individual. Differences in the expression of human leukocyte antigen (HLA) loci, tumor necrosis factor alpha (TNF- α) and mannose binding protein among individuals has been associated with the propensity for the development chronic HBV infection (Thursz et al., 2011).

Because iPSCs can be derived from any individual, their response to HBV and HCV, or even to the drugs can be monitored in iPSC-derived hepatocytes. These patient specific iPSC-derived HLCs can be used to reconstitute the *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} mouse livers that will support the efficient engraftment of human hepatocytes. This humanized mouse liver model can be used to investigate the pathogenesis of HBV and HCV at a personalized level in addition to identifying new therapeutic targets (Fig. 1). Comparative analysis between two groups of people who show differences in susceptibility to HBV or HCV can be directly performed using the *in vivo* reconstituted cells.

CONCLUSION

Here, we have discussed the advantages and shortcomings of current models in HBV and HCV research and a novel chimera model using human iPSCs from people with different reactions to HBV or HCV infection. The success of the humanized mouse model of HBV and HCV would still need further improvement in areas, including the optimization of hepatic differentiation protocols and engraftment of iPSC-derived hepatocytes. Despite these needs, this model would provide a preclinical humanized mouse model for HBV and HCV investigation. The response of two different groups of hepatocytes to HBV or HCV in this mouse model will provide important information about the progressive change in these hepatocytes. In addition, the effect of known or new drugs for HBV or HCV can be tested for their efficacy in this model before being tested in patients. This novel personalized hepatitis mouse model will provide the opportunity to clarify the still unclear pathogenic mechanisms of HBV or HCV infection and to develop a platform for antiviral drug screening.

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Fig. 1.

Strategies for developing a personalized mouse model for HBV and HCV using personalized human iPS cells. A preclinical humanized mouse model can be generated in a step-wise manner. In stage 1, among the individuals who have a history of HBV or HCV infection, those with the chronic infection and those who recovered after asymptomatic infection can be determined. In stage 2, somatic cells from those patients can be used to produce iPSCs, which further differentiate into hepatocyte-like cells *in vitro* in stage 3. The *in vitro* differentiated hepatocyte-like cells can be transplanted to the appropriate immunodeficient recipient mice, such as *Fah^{-/-}Rag2^{-/-}Il2rg^{-/-}* mice in stage 4. Finally, mice will be infected with HBV or HCV and examined for a response in hepatocytes from susceptible individuals, or resistant individuals (stage 5).

Table I

HBV *in vitro* and *in vivo* models

HBV Model	Features	Shortcomings	Important Findings/ highlights	References
Primary human hepatocytes	Closest to natural status for virus infection; Good system for searching real HBV receptors	Difficult to obtain and maintain; lose the function of human primary hepatocytes soon	Suitable for elucidating the entry process of HBV; test anti-HBV drug <i>in vitro</i>	(Ochiya et al., 1989; Ren and Nassal, 2001; Schulze-Bergkamen et al., 2003)
Human hepatoma cell lines	Easy to culture <i>in vitro</i> and obtain the stable results	Lack the characteristics of primary human hepatocyte; malignant cells	HepG2.2.15 with full-length HBV is capable of replicating the viral genome and producing progeny Virus; Useful in virion production and drug screening	(Sells et al., 1988; Chouteau et al., 2001; Sun and Nassal, 2006)
Chimpanzee model	Human HBV can infect chimpanzee liver and replicate in it	High expenditure, ethical problems	Served as a useful means to study the mechanism of hepatitis B viral progression to chronic liver disease	(Pancholi et al., 2001; Kamili et al., 2009)
Duck HBV primary hepatocyte culture model	Duck HBV has similar biological features of human HBV	Lack of Protein X of human hepadnaviruses	Helpful in investigating virus structure, genome, and mechanisms of replication; accelerated the discovery of first anti-HBV drug	(Feitelson and Miller, 1988; Yokosuka et al., 1988; Fischer and Tyrrell, 1996; Tomita et al., 2000; Seigneres et al., 2001)
Transgenic HBV mouse model	Can investigate HBV <i>in vivo</i>	Low virus replication, transgene tolerance	Helpful in chemotherapy and drug screening for HBV	(Kajino et al., 1997; Chemin et al., 1999; Larkin et al., 1999; Weber et al., 2002; Barone et al., 2006; Yu et al., 2011)
Tupaia model	Tupaia are squirrel-sized animals closely related to primates	Low infection rates <i>in vivo</i> ; does not show a persistent HBV infection <i>in vitro</i>	Improved the understanding of the fulminant clinical course associated with HBV mutations	(Walter et al., 1996; Glebe et al., 2003; Baumert et al., 2005; Glebe et al., 2005)
A chimeric mouse model with human hepatocytes	Maintain the pristine human-hepatocyte-like features	Low repopulation ratio, limited resource of human hepatocytes; lack of an immune system	A useful tool for the study of HBV virology and evaluation of anti-HBV drugs	(Tsuge et al., 2005; Sugiyama et al., 2007; Tabuchi et al., 2008; Tanaka et al., 2008; Robinet and Baumert, 2010; Lutgehetmann et al., 2011)

Table II

HCV *in vitro* and *in vivo* models

HCV Model	Advantages	Shortcomings	Important Findings/ highlights	References
Primary human hepatocytes	Closest model for the natural host cell of HCV	Difficult to obtain and maintain, lose the function of human primary hepatocytes soon	This model allows production of infectious HCV	(Nahmias et al., 2006; Jouan et al., 2010; Podevin et al., 2010)
The established human hepatoma cell lines	Easy to culture <i>in vitro</i> and obtain the stable results	Lack the characteristics of primary human hepatocyte; malignant cells	Identify binding proteins to HCV or elements interact with HCV protein; A break-through in HCV research was achieved in 2005 when Huh-7 infected with the cloning of JFH1, a genotype 2a-HCV produce workable titers	(Kalkeri et al., 2001; Garcia et al., 2002; Lindenbach et al., 2005; Mee et al., 2009)
Chimpanzee model	HCV replication was detectable in this surrogate model; The only validated <i>in vivo</i> model for testing the infectivity of HCV and studying the natural history of HCV	High cost, ethical problems	Played a critical role in the discovery of HCV; currently, the immunogenicity and efficacy of vaccine candidates against HCV can be tested only in chimpanzees.	(Lu et al., 2001; Mizukoshi et al., 2002; Youn et al., 2005; Puig et al., 2006)
Transgenic HCV mouse model	Can investigate HCV <i>in vivo</i>	Usually use only part of HCV elements to generate this model	Valuable in studying the biology of HCV and evaluating antiviral compounds.	(Renard et al., 2000; Wedemeyer et al., 2001; Perlemuter et al., 2003; Shuai et al., 2008)
Tupaia model	Tupaia are squirrel-sized animals closely related to primates	Low infection rates <i>in Vivo</i>	A potential practical experimental model for studies of HCV infection.	(Barth et al., 2005; Amako et al., 2010)
A chimeric mouse model with human hepatocytes	Maintain the pristine human-hepatocyte-like features, susceptible to infection with native HCV	Low repopulation ratio, limited resource of human hepatocytes; lack of an immune system.	Useful tool for evaluating the effect of anti-HCV drugs such as IFN, protease inhibitors and polymerase inhibitors.	(Mercer et al., 2001; Kneteman et al., 2006; Robinet and Baumert, 2010; Washburn et al., 2011)