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Revival, characterization, and hepatitis B virus infection of cryopreserved human fetal hepatocytes

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A B S T R A C T

Primary human hepatocytes are considered the ideal cellular model for *in-vitro* studies of liver-specific pathology, such as hepatitis B virus (HBV) infection. However, poor accessibility, limited cell numbers, and lot-to-lot variation of primary human hepatocytes limit their broad application. Human fetal hepatocytes were isolated from postmortem embryonic liver tissues by two-step collagenase perfusion and cryopreserved. A monolayer of cryopreserved human fetal hepatocytes was established by optimizing such conditions as cell density and viability and purification of viable cells by Percoll. Finally, revived human fetal hepatocytes were characterized and infected with HBV. A large number of viable human fetal hepatocytes could be isolated and cryopreserved, with seeding density and viability being critical for the establishment of a compact monolayer culture. Using low-viability cryopreserved human fetal hepatocytes, a typical monolayer was established by purification with Percoll. The revived cells were actively proliferative, showed identical morphologic characteristics to non-cryopreserved cells, and had a typical hepatic gene expression profile. Moreover, this optimized model was susceptible to HBV infection and could be used to screen entry inhibitors against HBV infection. In conclusion, these methods can be used on human fetal hepatocytes to provide a cell bank for studies of the early stages of HBV infection.

1. Introduction

Primary human hepatocytes are currently considered to be the ideal cellular model for *in-vitro* studies of liver-specific pathology, such as hepatitis B virus (HBV) and hepatitis C virus, owing to their closest physiological state to hepatocytes *in vivo* (Ochiya et al., 1989; Gripon et al., 1993; Galle et al., 1994; Sheahan et al., 2010). However, poor accessibility, limited cell numbers, and lot-to-lot variations are drawbacks that tremendously limit the large-scale application of freshly explanted primary human hepatocytes. Cryopreservation is believed to be a promising alternative solution.

Numerous studies have been performed to improve cryopreservation and revival methods of primary human adult hepatocytes (Loretz et al., 1989; Adams et al., 1995; Li et al., 1999; Alexandre et al., 2002; Katenz et al., 2007; Li, 2007). However, the efficiency of revival so far reported is relatively low and varied. An alternative to primary human hepatocytes is human fetal hepatocytes, which are the premature stage of human adult hepatocytes, with relatively high viability and proliferative ability (Malhi et al., 2002; Lazaro et al., 2003). These characteristics endow them the potential to reconstitute and form a compact monolayer even after harsh treatment during cryopreservation. Moreover, primary human adult hepatocytes are generally isolated from biopsies of pathologic tissues of patients, the small mass of which means that only limited numbers of cells can be obtained (Alexandre et al., 2002; Schulze-Bergkamen et al., 2003), whereas human fetal hepatocytes are isolated from human fetal livers, which provide a large number of cells with relative high viability. There are a few reports of preliminary studies for the cryopreservation and revival of human fetal hepatocytes (Zuckerman et al., 1968) and for HBV infection using human fetal hepatocytes (Zuckerman et al., 1972; Zuckerman, 1975). To date, there are no reports concerning methods of cryopreservation and revival of human fetal hepatocytes to support the entire HBV lifecycle efficiently.

Previously, it was found that human fetal hepatocytes co-cultured with non-parenchymal hepatic cells could be maintained

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for up to 4 months without losing susceptibility to HBV infection within 72 days, providing a valuable approach to the long-term maintenance of primary human hepatocytes for studies of HBV infection for prolonged periods (Zhou et al., 2013). However, this approach cannot solve the limitations of primary human hepatocytes mentioned previously, and co-cultured human fetal hepatocytes showed only about 5% HBV-positive cells when infected with HBV-positive patient serum. This study aims to establish an approach for the cryopreservation and revival of human fetal hepatocytes, and further, to characterize revived human fetal hepatocytes and investigate their susceptibility to HBV infection.

2. Materials and methods

2.1. Cell isolation and culture

Human fetal hepatocytes were isolated from the embryonic livers of postmortem premature neonates (estimated gestational age, 10–24 weeks) provided by Zhongnan Hospital of Wuhan University, with informed written consents from all participating families. The neonates died of natural causes and the mothers were HBV or hepatitis C virus sera-negative (unpublished data). Ethical approval for the study was granted by the institutional bioethics committee of Wuhan Institute of Virology, Chinese Academy of Sciences (approval number WIVH24201101). Embryonic liver tissues were perfused using a two-step collagenase perfusion (Berry et al., 1992; Zhou et al., 2013) within 3 h of the death of the neonates. The prolonged time between the death of the neonates and the actual perfusion of the liver tissue caused a dramatic decrease in cell viability (data not shown). Typical yields were $>1 \times 10^9$ cells and cell viability was 70% to 95%, as determined by trypan blue dye staining using Counter star (Inno-Alliance Biotech, Shanghai, China). Non-cryopreserved and cryopreserved cells were cultured as described elsewhere (Lazaro et al., 2003). Briefly, the non-cryopreserved and cryopreserved human fetal hepatocytes were seeded with the indicated seeding density in six-well tissue culture plates (NEST, Shanghai, China) pre-coated with collagen (Rat tail type I collagen, Becton-Dickinson, Franklin Lakes, USA) in seeding medium containing 5% v/v fetal bovine serum (Gibco, Grand Island, USA) for 4–6 h, followed by refreshing medium using serum-free seeding medium containing 2% v/v DMSO. To stimulate the proliferation of human fetal hepatocytes, 20 ng/ml EGF (Becton-Dickinson) was added to serum-free seeding medium. The cells were maintained at 37 °C in a 5% CO_2 incubator with the medium changed every 2–3 days, depending on the specific experiment. All related chemical agents, if not specially mentioned, were purchased from Sigma-Aldrich (Sigma, Shanghai, China).

2.2. Cell cryopreservation and revival

After cell isolation, human fetal hepatocytes were resuspended at a density of 10^7 viable cells/ml in William's E medium (Gibco, Grand Island, USA) supplemented with 45% fetal bovine serum and 10% DMSO and transferred to a cryovial in a freezing container (Nalgene™ Cryo 1 °C, Rochester, USA) to achieve a cooling rate of −1 °C/min. After being kept at −80 °C overnight in the freezing container, the cryovials containing human fetal hepatocytes were transferred to liquid nitrogen and stored for up to 24 months without affecting revival efficiency and susceptibility to HBV infection. Successful revival required rapid thawing of the cryopreserved human fetal hepatocytes in a 37 °C water bath. Cryopreservation and revival of these highly viable human fetal hepatocytes resulted in a decrease of cell viability of 10–20% (data not shown). Typically, highly viable cells (viability >70%) were seeded at 3.5×10^5 viable cells/cm^2 in seeding medium containing 5% v/v FBS. Low-viability human fetal hepatocytes (viability <70%) were purified Percoll (Sigma) and seeded as described previously. Details are shown next.

2.3. Percoll purification of viable cryopreserved cells

Percoll solutions were prepared following the manufacturer's protocol. In brief, a Percoll working solution (100%) was prepared by mixing 10 ml 10× PBS with 90 ml Percoll mother solution. Other densities were prepared by diluting the 100% Percoll solution using 1× PBS (for example, 100 ml 70% Percoll needed 70 ml 100% Percoll and 30 ml of 1× PBS). Layers of 2 ml of different Percoll densities (70% Percoll, 50% Percoll, and 30% Percoll) were carefully loaded into a 15 ml centrifuge tube to form a discontinuous gradient. Low-viability cryopreserved cells with a viability of 40–70% were washed twice with PBS and resuspended in PBS before being carefully loaded onto the gradient of Percoll and centrifuged at $350 \times g$ for 30 min at 4 °C. As shown in Table 1, three visible layers in the gradient (30% Percoll, 50% Percoll, and 70% Percoll) were formed: an upper layer (the interface between the loading sample and the 30% Percoll layer), an intermediate layer (the interface between the 30% Percoll layer and the 50% Percoll layer), and a lower layer (the

Table 1
Optimization of viable cell purification by Percoll.

Condition	Density (%)	Medium[a]	Layer	Viable cells (10^6)[b]	Viability (%)[b]	Recovery (%)[c]
1	30	PBS	Upper[d]	2.83 ± 0.56	40.89 ± 2.54	34.04 ± 4.92
	50		Intermediate[e]	2.48 ± 0.32	63.32 ± 3.54	
	70		Lower[f]	2.74 ± 0.37	78.85 ± 4.37	
2	30	DMEM, FBS-free	Upper	3.25 ± 0.33	46.92 ± 5.78	24.28 ± 0.24
	50		Intermediate	2.83 ± 0.24	67.74 ± 2.78	
	70		Lower	1.95 ± 0.20	75.13 ± 2.49	
3	50	PBS	Upper	6.81 ± 0.42	50.40 ± 2.37	17.15 ± 1.16
	70		Intermediate	No layer	No layer	
	90		Lower	1.41 ± 0.18	71.62 ± 1.31	
4	50	DMEM, FBS-free	Upper	6.54 ± 0.51	57.35 ± 5.17	17.42 ± 0.20
	70		Intermediate	No layer	No layer	
	90		Lower	1.38 ± 0.20	73.27 ± 2.51	

[a] Cell resuspension solution loaded onto Percoll.
[b] Mean of three independent experiments ± standard deviation.
[c] Ratio of viable cells recovered from the lower layer divided by the total number of cells recovered from all three layers; mean of three independent experiments ± standard deviation.
[d] Interface between loading sample and 30% Percoll.
[e] Interface between 30% Percoll and 50% Percoll.
[f] Interface between 50% Percoll and 70% Percoll.

Table 2
Name, sequence, and reaction setting for primers.

Primers	Sequence (5′ → 3′)	Reaction setting[a]
cccDNA-F	TGCACTTCGCTTCACCT	Denaturation 95 °C, 10 s; annealing 61 °C, 20 s;
cccDNA-R	AGGGGCATTTGGTGGTC	elongation 72 °C, 40 s
HBVp-F	ACCAATCGCCAGTCAGGAAG	Denaturation 95 °C, 30 s; annealing 60 °C, 30 s;
HBVp-R	ACCAGCAGGGAAATACAGGC	elongation 72 °C, 30 s
NTCP-F	CTCAAATCCAAACGGCCACAATAC	Denature 95 °C, 30 s; annealing 60 °C, 20 s;
NTCP-R	CACACTGCACAAGAGAATGATGATC	elongation 72 °C 30 s
HNF4-F	GGCCAAGTACATCCCAGCTT	Denature 95 °C, 30 s; annealing 60 °C, 20 s;
HNF4-R	TCATTGCCTAGGAGCAGCAC	elongation 72 °C, 30 s
β-Actin-F	ATCGTGCGTGACATTAAGGAG	Denature 95 °C, 30 s; annealing 60 °C, 20 s;
β-Actin-R	GGAAGGAAGGCTGGAAGAGT	elongation 72 °C, 30 s

[a] For 40 reaction cycles.

interface between the 50% Percoll layer and the 70% Percoll layer). However, only two visible layers were formed in the gradient of 50% Percoll, 70% Percoll, and 90% Percoll: an upper layer (the interface between the loading sample and the 50% Percoll layer) and a lower layer (the interface between the 50% Percoll layer and the 70% Percoll layer). Harvested cells from these visible layers were washed twice with PBS again and resuspended in seeding medium containing 5% v/v FBS, and were subsequently plated and cultured as described previously.

2.4. Electron microscopy

Specimens were prepared for electron microscopy as described elsewhere (Lazaro et al., 2003). In brief, for transmission electron microscopy, human fetal hepatocytes cultured in collagen-coated 60 mm dishes were fixed with Karnovsky fixative, and then post-fixed in 1% osmium tetroxide before dehydration and embedding in EPON. For scanning electron microscopy, human fetal hepatocytes cultured on collagen-coated glass cover slips were fixed with 4% paraformaldehyde containing 2.5% glutaraldehyde in 0.2 mol/l PBS, and then post-fixed in 1% osmium tetroxide before dehydration, critical-point dried using liquid CO_2, and coated with 5 nm of vacuum drying gold. Finally, well-prepared specimens were subjected to transmission electron microscopy (Tecnai, Osaka, Japan) or scanning electron microscopy (TESCAN, Brno, Czech Republic).

2.5. Western blotting

Cultured human fetal hepatocytes were lyzed and Western blotting was performed according to standard protocols. In brief, the cultured cells were washed twice with PBS and lyzed in 200 μl lysis buffer (50 mM Tris–HCl, pH 8.0; 150 mM NaCl; 0.1% SDS; 1% NP40; 0.5% deoxycholic acid; 0.5% sodium azide; 100 μg/ml PMSF) per well of a six-well plate. The same volume of each sample was loaded on 10% SDS-PAGE gels. Subsequently, proteins were electro-transferred to a polyvinylidene fluoride membrane (Minipore, Bedford, USA), which was then blocked at room temperature by 5% milk in PBS, and incubated overnight at 4 °C with the diluted primary antibodies. The primary antibodies, including rabbit anti-human albumin, rabbit anti-human cytokeratin 18 (CK18), rabbit anti-human α-fetoprotein (AFP), rabbit anti-human cytochrome P450 3A4 (CYP 3A4), and rabbit anti-human multi-drug resistance protein 2 (MRP2), and the corresponding HRP-conjugated secondary antibodies applied were purchased from Proteintech™ (Chicago, USA). The result was visualized using an Alpha Fluorchem HD2 Chemiluminescent imaging system (ProteinSimple, Santa Clara, USA).

2.6. Enzyme-linked immunosorbent assay

Medium was collected from the human fetal hepatocyte cultures at the indicated time points, to measure human albumin (Bethyl Laboratories, Montgomery, USA) and AFP (Linc-Bio Science, Shanghai, China). Secreted HBsAg was detected in medium samples collected from HBV infected human fetal hepatocytes using an HBsAg ELISA Kit (KHB, Shanghai, China), according to the manufacturer's instruction, and the results were measured using a Multiskan MK3 microplate spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA). The data are shown as means ± standard deviation, and represent the average of three-time independent experiments with triplicate samples.

2.7. Quantitative PCR analysis

Hepatitis B virus covalently closed circular DNA (cccDNA) was extracted by the standard method (Hirt, 1967). The cccDNA-specific primers (Glebe et al., 2003; Zhou et al., 2013) were applied for semi-quantitative analysis. A 10-fold dilution series (10^3–10^7 copies/ml) of plasmid pGEM-3Z-1.3 × HBV (subtype: adw) (Doitsh and Shaul, 2003) was served as the standard, while DNA extracted from HepG2.117 served as the positive control with DNA from HepG2 and HBV virus particles as the negative controls. Table 2 lists detailed primer sequences and PCR reaction settings. Hepatitis B virus DNA from the infected supernatant was isolated using a genomic DNA isolation kit (Qiagen, Hilden, Germany). Quantitative real-time PCR was performed on a real-time PCR system (Stratagene Mx3000P; Agilent Technologies, Santa Clara, CA, USA) using SYBR Master Mix (TAKARA, Dalian, China) with primers HBVp-F and HBVp-R (Table 2). A series of 5-fold dilutions (10^5–10^8 copies/ml) of plasmid pGEM-3Z-1.3 × HBV was used as the template to generate the standard curve. The total intracellular RNA at the indicated culture time was isolated using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA), and cDNA was synthesized using the PrimeScript RT reagent kit with gDNA Eraser (TaKaRa, Dalian, China), following the manufacturer's directions. The cDNA products were subjected to real-time PCR with the primers for the NTCP and HNF4α. β-Actin served as the internal control for sample normalization. Relative NTCP and HNF4α mRNA levels were calculated using the ΔΔCT method (calibrator: HepG2). All samples were measured in triplicate and all experiments were repeated independently three times.

2.8. HBV infection of revived human fetal hepatocytes

The cryopreserved human fetal hepatocytes were reconstituted as described previously. The infection medium was William's E medium containing 10% (v/v) HBV-positive serum (HBV copies: 2×10^9 copies/ml) supplemented with 2% (v/v) DMSO and 4% (m/v) PEG8000. Multiplicities of infection in all experiments were estimated to be 100. For competing infection assays, reconstituted human fetal hepatocytes were pre-incubated with different concentration of PreS/2-48^{myr} (Myristoyl-GTNLSVPNPL-GFFPDHQLDPAFGANSNNPDWDFNPNKDHWPEANKVG) at 37 °C for 30 min in a 5% CO_2 incubator, after which infectious medium

(containing PreS/2-48^{myr}) was added. After incubation for 24 h, the infectious medium was discarded and fresh maintenance medium containing 2% DMSO was added. The medium was changed every 2 days.

2.9. Detecting intracellular core protein by immunofluorescence assay

Twelve days after the HBV infection, the cells were fixed with 4% paraformaldehyde for 15 min and permeabilized with 0.25% (v/v) Triton X-100 in PBS for 45 min at room temperature. After blocking with 2% BSA (m/v), 0.25% Triton X-100 in PBS for 1 h and a brief wash, the cells were incubated with polyclonal rabbit anti-HBcAg (Dako, Glostrup, Denmark) for 2 h and DyLight-488 conjugated goat anti-rabbit IgG (Earthox, San Francisco, USA) for 1 h. Nuclear staining was performed with DAPI (Roche, Basel, Switzerland). A fluorescence microscope (Leica, Wetzlar, Germany) was used for imaging.

2.10. Detection of HBV DNA by Southern blotting

The HBV infected cells were harvested at the indicated time points. Intracellular HBV replicative intermediates were extracted as described by Hirt (1967). Viral DNA was separated by 1.5% agarose gel electrophoresis and transferred to a charged nylon membrane by capillary action. The membrane was then dried for 1 h at 65 °C and pre-hybridized for at least 1 h at 65 °C in a rolling hybridizing oven using PerfectHyb Hybridization Solution (TOYOBO, OSAKA, Japan). Hybridization was performed for 6 h at 65 °C by employing random-primed ^{32}P DNA probes specific for the whole HBV genome. Next, the nylon membrane was successively washed twice for ten min, in each of 3× SSC buffer, 1× SSC buffer, and 0.3× SSC buffer. Finally, HBV intracellular replicative intermediates were detected by PerkinElmer Cyclone Plus (PerkinElmer, Massachusetts, USA). The DNA extracted from HepG2.117 (Sun and Nassal, 2006) and HepG2 served as a positive and negative control, respectively.

3. Results

3.1. Plating density and cell viability are critical to the formation of monolayers

It has been reported that cell density ($1–1.5 \times 10^5$ viable cells/cm^2) is critical for freshly isolated primary human hepatocytes to form compact monolayers (Schulze-Bergkamen et al., 2003). In this study, the optimal cell density of revived human fetal hepatocytes following cryopreservation to form compact monolayers has been investigated. As shown in Fig. 1A–F, the most suitable density for compact monolayer formation was $3–4 \times 10^5$ viable cells/cm^2, which was more than twice the reported density. This may be a result of the stress that occurred in cryopreservation, which greatly decreased the attachment ability of cryopreserved human fetal hepatocytes.

In addition to plating density, cell viability was found to be critical for the formation of a confluent and compact monolayer. A critical plating density (3.5×10^5 viable cells/cm^2) was applied to compare cryopreserved human fetal hepatocytes of different viabilities. The results indicated that only cells with high viability (71.3% in Fig. 1H and 84.5% in Fig. 1I) could form typical monolayers, and cells with a viability less than 70% easily underwent dedifferentiation and could not establish a compact monolayer (data not shown). In some cases, a compact monolayer could be established using low viable cryopreserved human fetal hepatocytes by prolonged culturing in seeding medium and proliferation medium. However, cell cultures established by this method were not susceptible to HBV infection (data not shown).

To reconstitute the low-viability cryopreserved human fetal hepatocytes, Percoll was utilized to purify viable cells. As shown in Fig. 1J–L and Table 1, cells harvested from the interface between 50% Percoll and 70% Percoll exhibited the highest viability (over 70%), and were suitable for the formation of a typical monolayer. Notably, including 30% Percoll into the Percoll gradient and applying PBS for cell resuspension were critical for a high recovery rate of viable cells, possibly due to easier entrance into the Percoll gradient. Hence, condition 1 in Table 1 was applied for further experiments.

3.2. Characterization of revived human fetal hepatocytes following cryopreservation

Under phase contrast microscopic examination, reconstituted cryopreserved human fetal hepatocytes (Fig. 2B) showed the same morphology as fresh isolated human fetal hepatocytes (Fig. 2A), both having spherical bright nuclei and a polarized shape. Notably, bright canaliculi were observed (Fig. 2A and B, black arrow), which are reported to be an important relevant marker for susceptibility to HBV infection of primary human adult hepatocytes and hepatocytes, such as HepaRG (Gripon et al., 2002). Periodic acid–Schiff staining also indicated that glycogen was rich in fresh isolated human fetal hepatocytes (data not shown) and revived human fetal hepatocytes but not in HepG2 cells (Fig. 2C). The detailed micro-structure was revealed by high resolution scan electron microscopy and transmission electron microscopy. A sporadic distribution of microvilli could be observed both on the cell membrane (Fig. 2D, black arrow) and in the canaliculi (Fig. 2E, black arrow). Simultaneously, mitotic-phase nuclei of cryopreserved human fetal hepatocytes were also captured (Fig. 2F), which might reflect a proliferative mode of human fetal hepatocytes *in vitro* and *in vivo*. In addition, the glycogen granules, numerous mitochondria, dot-like ribosomes, Golgi bodies, and endoplasmic reticulum could also be easily recognized in the revived human fetal hepatocytes (Fig. 2E). All of these were typical of hepatic morphology; these characteristics indicated that the revived cryopreserved human fetal hepatocytes maintain the hepatocyte characteristics.

To characterize the cryopreserved human fetal hepatocytes further, the typical hepatic proteins were analyzed by Western blotting in revived cryopreserved human fetal hepatocytes at different time points. As shown in Fig. 3A, human fetal hepatocytes expressed multi-drug resistance protein 2 (MRP2), cytochrome P450 3A4 (CYP 3A4), cytokeratin 18 (CK18), and albumin, which are all key features of hepatocytes. The α-fetoprotein (AFP) level decreased a little with culture time, which was quite different from further results for AFP secretion. The concentrations of secreted albumin and AFP were further measured by ELISA. As shown in Fig. 3B, in the absence of DMSO, the albumin levels reached maximum secretion on the fourth day and rapidly decreased further, while inclusion of DMSO postponed the peak time to day 14. Albumin secretion of human fetal hepatocytes cultured in medium containing 2% DMSO, correlated with the intracellular albumin level, as shown in Fig. 3A, and decreased more slowly than that in medium containing 1% DMSO. This result indicated that DMSO was harmful to cryopreserved human fetal hepatocytes at the early culturing stage but beneficial in maintaining hepatocytic hallmarks further. In contrast with albumin secretion, AFP secretion kept decreasing in the culture, independent of DMSO concentration (Fig. 3C), and was undetectable after 10 days, while intracellular AFP expression was still detectable. Notably, downregulation of AFP expression may indicate the maturation of primary human fetal hepatocytes from 'fetal' to 'adult' stage (Smith et al., 1971).

In addition, cryopreserved human fetal hepatocytes exhibited high proliferative ability, as determined by EdU incorporation

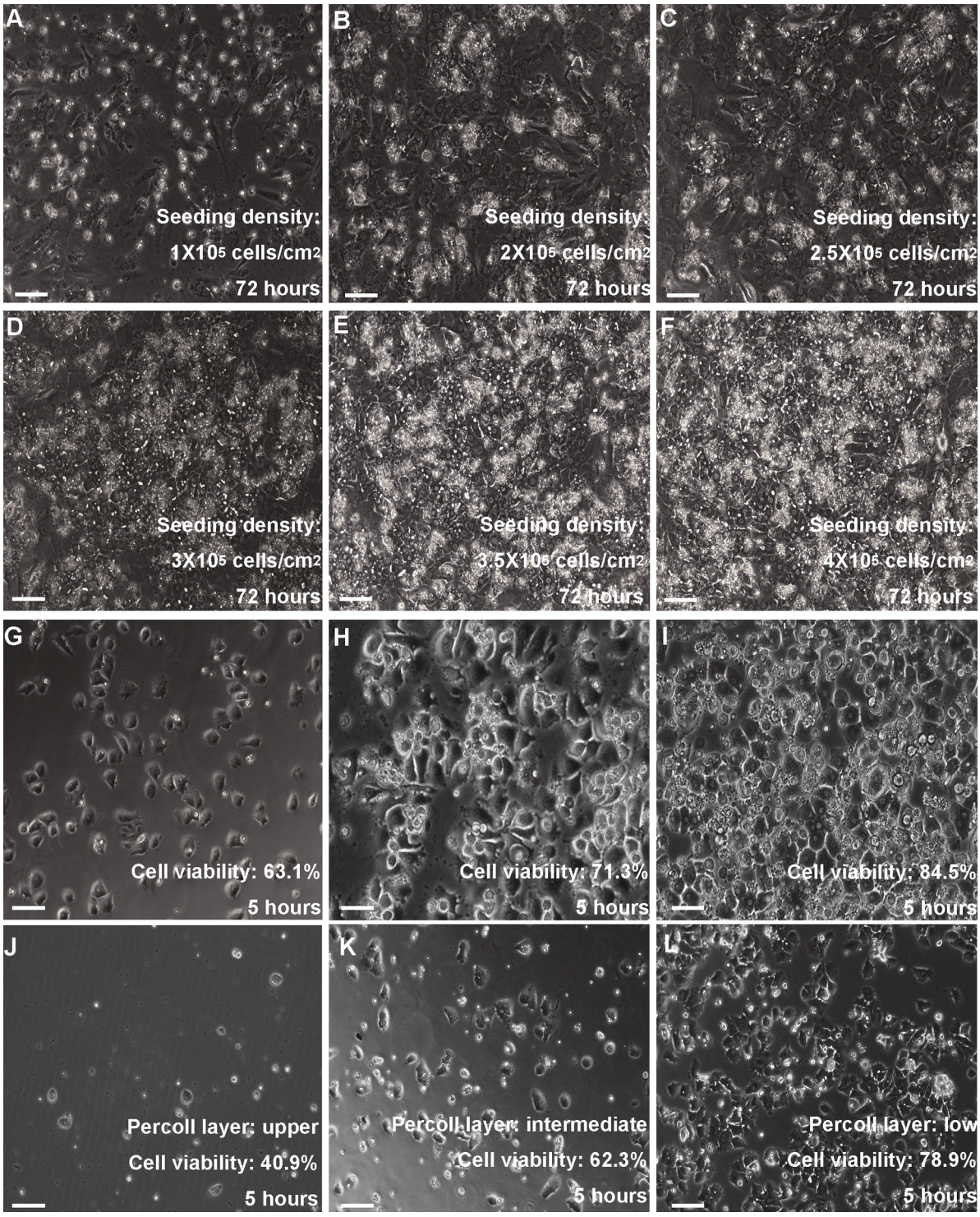

Fig. 1. *Plating density and cell viability are critical in forming monolayers.* (A)–(F) Various cell densities of cryopreserved human fetal hepatocytes with cell viability of 82.7% were plated; images were recorded 72 h later. The following viable cell densities were used: (A) 1×10^5 cells/cm^2; (B) 2×10^5 cells/cm^2; (C) 2.5×10^5 cells/cm^2; (D) 3×10^5 cells/cm^2; (E) 3.5×10^5 cells/cm^2; (F) 4×10^5 cells/cm^2. Seeding density and time of recording image are indicated. (G)–(I) Cryopreserved human fetal hepatocytes with viabilities of (G) 63.1%, (H) 71.3%, and (I) 84.5% were cultured at a seeding density of 3.5×10^5 cells/cm^2; images were recorded 5 h later. Cell viability and time of recording image are indicated. (J)–(L) Low-viability cryopreserved human fetal hepatocytes with a viability of 52% were purified by Percoll and seeded at a density of 3.5×10^5 cells/cm^2. Percoll layering, cell viability, and time of recording image are indicated. A phase contrast microscope was used for imaging. Scale bar, 50 μm.

assay. As shown in Supplementary Fig. 1, the proliferating cell ratio was significantly higher in the presence of epidermal growth factor (EGF) than in its absence, indicating that EGF could enhance cell proliferation of cryopreserved human fetal hepatocytes (Supplementary material 1). However, the fact that (10.58% ± 1.84%) cells were EdU-positive at 48 h in the absence of EGF revealed the proliferative ability of the human fetal hepatocytes themselves. This observation well explained why a sub-confluent monolayer of cryopreserved human fetal hepatocytes formed a typical compact monolayer. A confluent and compact monolayer could maintain hepatocytic features longer than a sub-monolayer (Enat et al., 1984; Sheahan et al., 2010), and only a confluent and compact monolayer

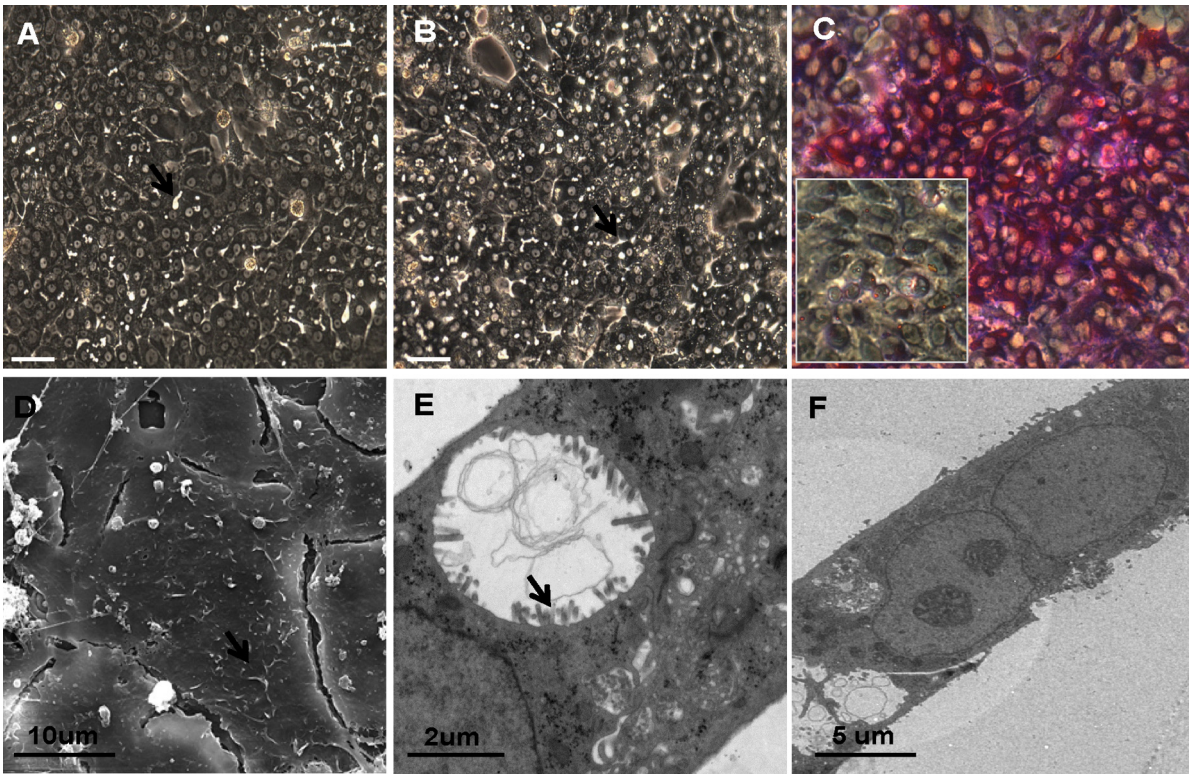

Fig. 2. *Morphology of cryopreserved human fetal hepatocytes.* (A) Freshly isolated and (B) cryopreserved human fetal hepatocytes with the same viability (80.6%) were cultured at a density of 3.5×10^5 cells/cm^2, and images were taken 3 days post plating using a phase contrast microscope. Canaliculi structure are indicated by black arrows (scale bar, 50 μm). (C) Reconstituted human fetal hepatocytes were stained with Periodic acid–Schiff (red) for intracellular glycogen. The left lower corner shows staining of HepG2 cells as a negative control. (D)–(F) Reconstituted human fetal hepatocytes examined by (D) scanning electron and ((E) and(F)) transmission electron microscopy. Black arrow indicates microvilli. Split nucleolus hepatocytes in the mitotic phase are also shown (F). All images are representative of at least three independent experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

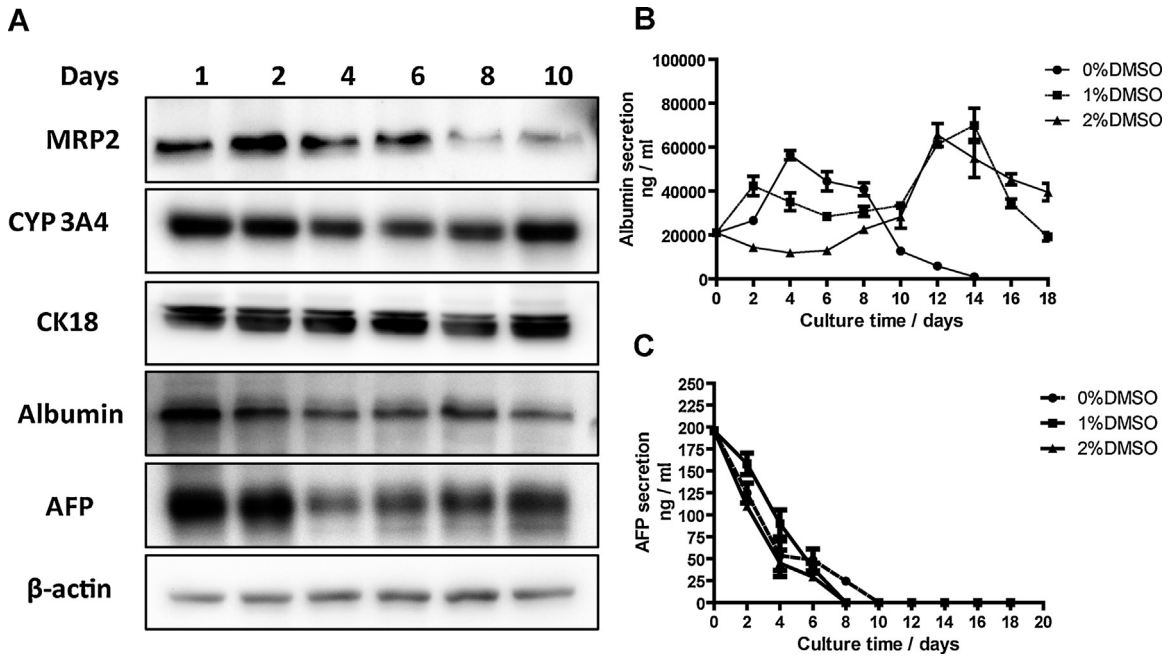

Fig. 3. *Expression of typical hepatocyte proteins in cryopreserved human fetal hepatocytes.* Cryopreserved human fetal hepatocytes were cultured at a density of 3.5×10^5 cells/cm^2. Cells and culture medium were collected at the indicated time points. (A) Western blotting detected the indicated proteins, including MRP2, CYP 3A4, CK18, AFP, and albumin. β-Actin served as an internal control. Albumin (B) and AFP (C) secretion were measured by ELISA in the absence or presence of DMSO (1% and 2%).

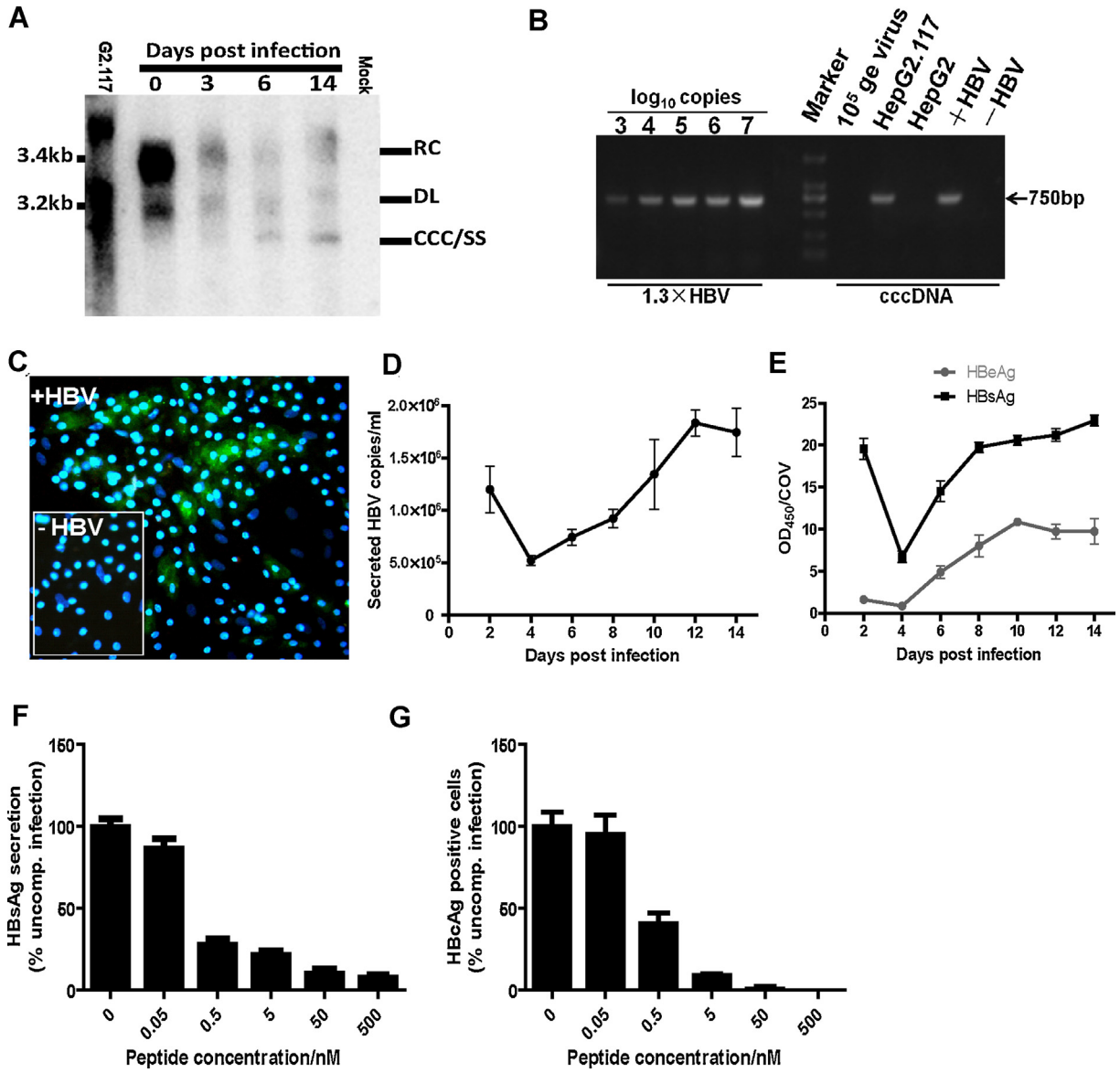

Fig. 4. *HBV infection of cryopreserved human fetal hepatocytes*. Cryopreserved human fetal hepatocytes were cultured and infected with HBV. (A) Southern blotting detected intracellular HBV replicative intermediates at the indicated time points. DNA from HepG2.117 and HepG2 was extracted and served as positive and negative controls for HBV DNA, respectively. (B) The existence of cccDNA was confirmed by semi-quantitative PCR using cccDNA-specific primers; a 1.3 × HBV plasmid served as standard. DNA extracted from HepG2.117 served as the positive control, while DNA extracted from HepG2 and HBV virus particles served as the negative controls. Target bands are indicated. (C) Immunofluorescence examination of HBcAg (Green) and DAPI (Blue), 12 days post HBV infection. The lower left corner shows mock-infected cells. Images were taken at a magnification of 10 × 20. (D) Released HBV viral particle load was measured by real-time quantitative PCR. (E) Secretion of HBsAg and HBeAg was measured by ELISA. (F) and (G) In the competition assay in the presence of different concentrations of PreS/2-48^{myr}, HBsAg secretion was measured by ELISA (F), and12 days post infection intracellular HBcAg was stained by immunofluorescence and counted using ImageJ image-processing software (G). CCC, covalently closed circular DNA; DL, double stranded DNA; RC, relax circular DNA; SS, single strand DNA; 'uncomp. infection', HBV infection without PreS/2-48^{myr}. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

could survive during HBV infection, owing to the addition of 4% PEG8000 (data not shown).

3.3. HBV infection of cryopreserved human fetal hepatocytes

The revived cryopreserved human fetal hepatocytes maintained the key features and characteristics of hepatocytes, and then their susceptibility to HBV infection was tested. For this purpose, pooled serum from treatment-free HBV patients served as a viral source (HBV copy 2×10^9 copies/ml). Highly viable cryopreserved human fetal hepatocytes were cultured and infected with HBV, as described in Section 2 (multiplicity of infection = 100). Southern blotting detected HBV replicative intermediates, as shown in Fig. 4A. A strong signal of HBV DNA was detected after 24-h incubation, presumably corresponding to entered or attached HBV particles. On the third day post infection, the HBV DNA signal was lessened, probably because of the anti-viral effect of the cell's innate immune system (Gripon et al., 2002; Lucifora et al., 2010). From day 6 to day 14 post infection, relax circular DNA and double stranded DNA concentrations increased gradually, which indicated the replication of HBV. Notably, cccDNA or single strand DNA were undetectable on the third day, but could be clearly observed from the sixth day post infection. The existence of cccDNA was further confirmed by cccDNA-specific semi-quantitative PCR. As shown in Fig. 4B, HBV infected cryopreserved human fetal hepatocytes showed an extensive cccDNA signal, whereas extraction

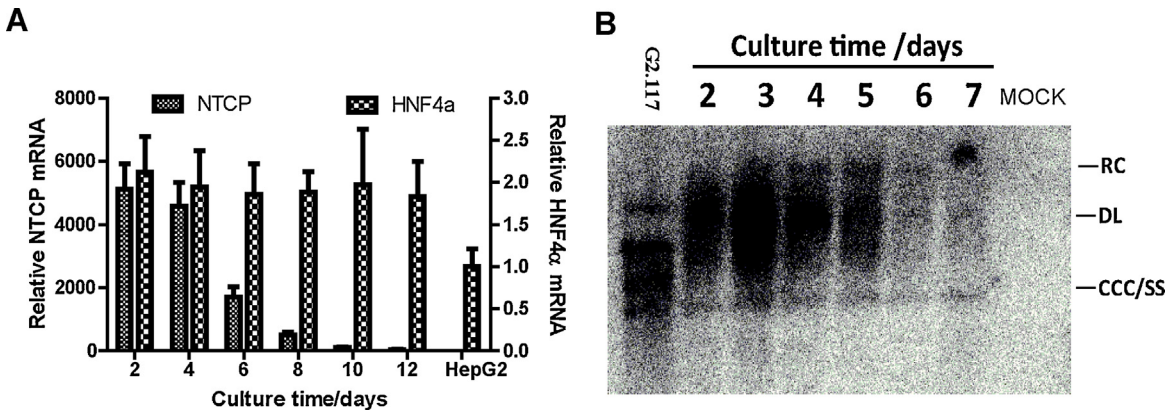

Fig. 5. *Rapid loss susceptibility to HBV infection due to NTCP downregulation.* (A) Relative mRNA of NTCP and HNF4α at the indicated time points was detected by real-time quantitative PCR. Relative mRNA levels were calculated using the ΔΔCT method (calibrator: HepG2). (B) Intracellular HBV replicating intermediates were detected by Southern blotting. Cryopreserved human fetal hepatocytes were cultured for the indicated times and infected by HBV. Infected cells were harvested 4 days post infection. DNA from HepG2.117 and HepG2 were extracted and served as positive and negative controls for HBV DNA, respectively.

of virus genomic equivalent, HepG2, and uninfected human fetal hepatocytes exhibited no signal. According to the analysis based on intracellular HBcAg immunofluorescence results, over 30% of cryopreserved human fetal hepatocytes were infected with HBV (Fig. 4C). To measure the secreted viral particles, numbers of HBV copies were determined by real-time quantitative PCR (Fig. 4D). The number of HBV copies decreased at 4 days post infection, possibly owing to unspecific attachment at 2 days post infection, and reached a peak at 12 days post infection. The secretion pattern of viral antigens was quite similar to that of the secreted viral particles, as shown in Fig. 4E. PreS/2-48^{myr} has been previously proven to inhibit HBV infection completely at a concentration of 100 nM (Glebe et al., 2005; Gripon et al., 2005; Engelke et al., 2006; Schulze et al., 2010). In this study, the data clearly showed HBV entry inhibition by PreS/2-48^{myr} with a dose-dependent manner, and correlating with published results, 100 nM PreS/2-48^{myr} blocked HBV infection totally in cryopreserved human fetal hepatocytes (Fig. 4F and G). Hence, cryopreserved human fetal hepatocytes could be specifically infected by HBV, with relative high infection efficiency, and this cellular HBV infection model could be utilized to screen HBV entry inhibitors.

It has been reported that Na^+/taurocholate cotransporting polypeptide (NTCP) (Yan et al., 2012) and hepatocyte nuclear factor 4-α (HNF4α) (He et al., 2012; Tang and McLachlan, 2001) are the key elements for viral entry and replication, respectively. To further characterize the susceptibility to HBV infection of cryopreserved human fetal hepatocytes, mRNA of NTCP and HNF4α at indicated time points were measured by real-time PCR, in parallel with HBV infection detected by Southern blotting. As shown in Fig. 5A and B, intracellular HBV replicating intermediates 4 days post infection decreased dramatically along with the culture time before HBV infection, which was well correlated with the mRNA level of NTCP rather than HNF4α. Hence, NTCP is the major cause of rapid loss susceptibility of cryopreserved human fetal hepatocytes to HBV infection after being revived and cultured.

4. Discussion

Currently, the best *in-vitro* platform for liver pathology study remains primary human hepatocytes. However, owing to such shortcomings as poor accessibility or lot-to-lot variation, the broad application of primary human hepatocytes has been limited and poor experimental reproducibility occurs. Cell cryopreservation is known as a promising solution to these limitations. A large number of studies of methods of cryopreservation have been reported, aimed at providing a cell bank for fundamental studies, such as metabolism and cytotoxicity of xenobiotics, and clinical applications such as cell transplantation for patients suffering from acute or chronic hepatic failure (Rijntjes et al., 1986; Chesne et al., 1993; Coundouris et al., 1993; Li et al., 1999; Alexandre et al., 2002; Garcia et al., 2003; Terry et al., 2005; Jouin et al., 2006; Hang et al., 2009). According to preliminary experiments, cryopreserved primary human adult hepatocytes could not be well revived and cultured in our system (unpublished data).

An alternative to primary human hepatocytes is human fetal hepatocytes.

A preliminary study of the recovery of human fetal liver cells after storage in liquid nitrogen is reported, but lacks further characterization and application cases (Zuckerman et al., 1968). In this study, the maximum cell yields for successful isolation could reach over 10^9 viable cells with over 90% viability, which was perfect for cryopreservation and revival. Cryopreserved human fetal hepatocytes could be well reconstituted by optimizations of: (1) seeding density (Fig. 1A–F); (2) cell viability used (Fig. 1G–I); and (3) Percoll purification (Fig. 1J–L). This approach to cryopreservation and the revival of human fetal hepatocytes greatly facilitates researchers in ensuring the accessibility of primary human hepatocytes, and has overcome, to a large extent, shortages of primary human hepatocytes, such as lot-to-lot variation, improving experimental reproducibility and making them possible for large-scale studies on liver pathology.

There are few reports on infection models utilizing human fetal hepatocytes (Zuckerman et al., 1972; Zuckerman, 1975; Ochiya et al., 1989; Lin et al., 2007) and no reports utilizing cryopreserved human fetal hepatocytes, possibly due to a lack of efficient recovery methods. According to these data, human fetal hepatocytes, cryopreserved and revived using the optimized approach, could be efficiently infected with HBV (Fig. 4A–E). In general, this approach provides an ideal cellular model for studies of HBV pathology: (1) there is reduced lot-to-lot variation of cell source, and hence the reproducibility of experiments is theoretically enhanced; (2) the cells are highly susceptible to HBV infection. Recently, Li et al. reported that NTCP is the functional receptor for HBV and hepatitis D virus entry (Yan et al., 2012), which confers HepG2 and Huh7 cells susceptible to HBV or hepatitis D virus infection. However, for cell-line-based infection systems, the infection rate is relatively low, suggesting that NTCP alone might not be enough for efficient HBV infection *in vitro*. Therefore, cryopreserved human fetal hepatocytes, with the closest physiological status to hepatocytes *in vivo*, are useful for studying the as yet not well-known entry step of HBV infection, including virus-receptor recognition, internalization, and intracellular trafficking. In addition, NTCP rather than HNF4α, has

been found to be correlated with a rapid loss of susceptibility to HBV infection, indicating that rapid lost susceptibility of cryopreserved human fetal hepatocytes to HBV infection is caused by rapid loss of NTCP mRNA levels. Hence, infection experiments performed on this model should be undertaken within 6 days post seeding to ensure high susceptibility to HBV infection and experimental reproduction.

In conclusion, cryopreserved human fetal hepatocytes revived provide a valuable tool for fundamental research, such as studies of HBV infection.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jviromet.2014.06.015.

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