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·*Original Article*·

Expression of hepatitis B virus genes in early embryonic cells originated from hamster ova and human spermatozoa transfected with the complete viral genome

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Abstract

Aim: To detect the expression of hepatitis B virus (HBV) genes (HB *S* and *C* genes) in early embryonic cells after introducing motile human sperm carrying HBV DNA into zona-free hamster oocytes via the *in vitro* fertilization (IVF) technique. **Methods:** Human sperm-mediated HBV genes were delivered into zona-free hamster oocytes by the IVF method. Polymerase chain reaction (PCR) was used to detect HB *S* and pre-Core/Core (*pre-C/C*) coding genes both in one- and two-cell embryos. Reverse transcription-PCR (RT-PCR) analysis was used to study the expression of the two genes. Fluorescence in situ hybridization (FISH) analysis using the full-length HBV DNA as the hybridization probe was performed to confirm the integration of viral DNA in the host embryonic genome. **Results:** Both HB *S* and *pre-C/C* coding genes are present and transcribed in one- and two-cell embryos originated from hamster ova IVF with human spermatozoa carrying HBV DNA sequences. **Conclusion:** Sperm-mediated HBV genes are able to replicate and express themselves in early embryonic cells. These results provide direct evidence that HBV DNA could transmit vertically to the next generation via the male germ line. *(Asian J Androl 2006 May; 8: 273–279)*

Keywords: hepatitis B virus; gene expression; hamster ovary; human spermatozoa; *in vitro* fertilization

1 Introduction

Hepatitis B virus (HBV) antigens were detected in human semen [1], and it is now well-established that this biological fluid is a vector for the spread of hepatitis B [2–4]. However, few studies have tried to identify the contaminated cells within semen. It has been reported that HBV DNA was integrated into the DNA of spermatozoa in two of three patients with acute hepatitis, suggesting that there may be true transmission of HBV via the germ line [5]. Another study of chronic HBV antigen carriers showed that HBV DNA was present in all of the semen samples tested, with the infected cells being both spermatozoa and mononuclear cells. Persistent free HBV DNA has also been detected in the semen of patients with no markers of viral replication in serum, indicating that the genital tract may act as a reservoir and that these

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patients may transmit the virus sexually.

HBV is an enveloped virus and causes acute self-limited and chronic infections in human [6]. HBV belongs to the hepadnavirus family, containing a small (3.2 kb), circular, double-stranded DNA genome. The minus strand includes at least four open reading frames (ORF), of which S-ORF is divided into *pre S1*, *pre S2* and *S* gene. The HB *S* gene contains three regions and encodes for three different glycoproteins, which differ only in the length of their N-terminus. Hepatitis B surface antigen (HBsAg) encoded by the *S* gene is the major component of hepatitis B vaccine. The *pre-C/C* gene codes the core-protein (HBcAg), which is a part of the viral nucleocapsid or the HBeAg, which is secreted into the blood, respectively [7].

It is well-known that the ability of sperm to deliver exogenous DNA into oocytes at the time of fertilization has attracted considerable interest and controversy [8, 9]. Spermatozoa of a wide variety of species can fuse with zona-free hamster oocytes. Zona-free hamster oocytes were inseminated with spermatozoa of other species such as mouse, guinea pig and human. Interspecific *in vitro* fertilization (IVF) has been widely used by investigators for studying morphological and molecular details of sperm–egg interactions [8, 9].

Our previous study has provided the first direct evidence that the HB *X* gene could be expressed in one- and two-cell embryos originated from golden hamster ova *in vitro* fertilized with human spermatozoa [10]. The objective of the current study was to detect the expression of other HBV genes (HB *S* and *C* genes) in early embryonic cells after introducing motile human sperm carrying HBV DNA into zona-free hamster oocytes via the IVF technique.

2 Materials and methods

2.1 General

The Institutional Review Board of Shantou University Medical College approved all experiments in the present study. The following materials were used: (i) recombinant plasmid, pBR322-HBV; (ii) semen sample, given voluntarily and taken from a healthy (HBV-negative) donor (a member of the laboratory); (iii) mature female golden hamsters (*Mesocricetus auratus*, 8–12 weeks old); (iv) Biggers–Whitten–Whittingham (BWW) medium supplemented with 0.3% human serum albumen (HSA, Sigma Chemical, St Louis, MO, USA) for human sperm preparation, oocyte collection, insemination and subsequent handling; and (v) ovum culture medium (OCM, from Flow Laboratories, Germany) containing 10% heat-inactivated fetal bovine serum for post-insemination culture of ova.

The IVF assay of zona-free hamster oocytes was performed according to Yanagimachi *et al.* [11]. For a broader discussion of the technique, including the treatment of the semen sample, super ovulation, egg processing, insemination and post-insemination culture, please refer to references [12–14].

2.2 Semen preparation and exposure to HBV DNA

Semen samples were kept in a CO_2 incubator (37ºC, 50 mL/L CO_2 in air) for 30 min in order to be liquified. The most highly motile spermatozoa were recovered from the semen with a ''swim-up'' method. The sperm suspension thereby obtained was centrifuged at 600 × *g* for 5 min. The pellet was resuspended in fresh BWW and centrifuged again. The washed spermatozoa were suspended in 5 mL 10 μmol/L Ca^{2+} Ionophore (Sigma Chemical, St Louis, MO, USA) solution for 8 min in the same incubator to facilitate the capacitation. The treated spermatozoa were centrifuged and washed twice with fresh BWW and then suspended in the capacitation medium with 3.5% HSA and incubated for 4 h to allow capacitation of the spermatozoa. Three hours after the beginning of capacitation, human spermatozoa were exposed to the pBR322-HBV plasmid. In brief, a total of 100 μL mixture containing 1 μL pBR322-HBV plasmid (1.5 μg/mL), 6 μL liposome, and 93 μL Hepes buffered saline (HBS) was incubated at room temperature for 15 min, and then added to the capacitation media containing spermatozoa and kept in the incubator for 1 h. After exposure to HBV DNA, the spermatozoa were washed five times in 5 mL fresh BWW via centrifugation at 600 × *g* for 5 min to remove excess HBV DNA.

2.3 Hamster egg preparation

Mature female golden hamsters (8–12 weeks old) were housed in groups under standard lighting conditions with free access to water and food. The animals were induced to super-ovulate by i.p. injection of 30 IU of pregnant mare serum gonadotrophin (PMSG, Ningbo Hormone Product, Ningbo, China) on day 1 of their oestrous cycle, followed 72 h later by administration of 30 IU of human chorionic gonadotrophin (hCG, Ningbo Hormone, Ningbo, China). They were anesthetized and

killed by cervical dislocation 17 h after hCG injection, and then oocytes were collected from the ampullar region of oviducts and freed from cumulus cells by gentle pipetting in 0.1 % hyaluronidase (Sigma Chemical, St. Louis, MO, USA). Cumulus-free oocytes were washed twice in fresh BWW medium and treated with 0.1 % trypsin (Sigma Chemical, St. Louis, MO, USA) to remove the zona pellucida, and then washed twice immediately in fresh BWW.

2.4 Insemination and post-insemination culture

As soon as the zona pellucida is removed, the zona-free oocytes must be inseminated with spermatozoa to prevent their degradation. Insemination was performed with the sperm suspensions at a concentration of approximately 10^6 /mL. The oocytes soon began to rotate anti-clockwise due to flagellar movement of the spermatozoa attached to the egg surface. The oocytes were kept in the sperm suspension for only 20 min. The inseminated oocytes were washed twice in fresh BWW in order to remove the excess sperm, then transferred to fresh BWW under mineral oil (Sigma Chemical, St. Louis, MO, USA) and incubated for another 1 h to ensure sperm penetration. After washing twice in fresh OCM each five oocytes were cultured in a droplet (50 μL for each) of OCM under oil in a plastic Petri dish kept in a CO_2 incubator (37ºC, 50 mL/L CO_2 in air) for 24 h.

2.5 Microscopic investigation

Twenty-four hours after insemination, all embryos were investigated under the microscope (LeicaDM IRE2, Leica Microsystem, Wetzlar, Germany) at ×400 magnification. A total of 290 golden hamster oocytes were classified into the following groups: (i) normal one-cell embryo: having both male and female pronucleus and the second polar body; (ii) unfertilized egg: containing only female pronucleus; fertilization rate was calculated based on the total number of eggs used in the present study; (iii) normal two-cell embryo: containing two blastomeres with one nucleus each; and (iv) abnormal two-cell embryo: containing two or more nuclei in each blastomere.

Photographs were taken under the same microscope, using a Minolta camera (Minolta X-700, Japan).

2.6 Embryo preparation

Each normal one- or two-cell embryo was picked up from the culture and washed three times in cold 1 × phosphate-buffered saline (PBS) to remove the medium serum. Twenty two-cell embryos were used for chromosomal preparations to perform fluorescence *in situ* hybridization (FISH). Each embryo remaining was transferred into 200 μL polymerase chain reaction (PCR) tube using 0.5 μL 1 × PBS, making it possible to store these embryos or to perform PCR or reverse transcription-PCR (RT-PCR).

2.7 Polymerase chain reaction (PCR) amplification

Approximately 4.5 μL of cell lysis buffer was added to each sample, mixed, and then incubated at 70ºC for 10 min. The cell lysate of each single embryo (one- or two-cell embryo) was used as a DNA template. The pBR322-HBV DNA and water were used as positive and negative controls, respectively.

The primers used in the present study were synthesized by Sangon (Shanghai, China) and designed according to the known HBV genome sequences and the main popular subtype (adr) in China. The HB *C* gene was amplified using the following primers: forward, 5'-ACCTCGAGGAGTTGGGGGAGGAGATTAGG-3'; and reverse, 5'-GTTCTA GAATAAAGCCCAGTAAAG TTTCC-3' specific to the region 774 bp. According to Okamoto *et al*. [15], these primers covered the entire *Pre-C/C* region (639 bp), 1814–2452, numbering and a part of the *X* and polymerase genes. Forty-five microliter PCR reaction containing 40 pmol of each primer was added to the cell lysate (5 μL) of each sample. The mixture was heated to 94ºC for 5 min followed by 30 cycles consisting of 94ºC for 30 s, 55ºC for 30 s and 72ºC for 1 min and finally 72ºC for 10 min in a Peltier Thermal Cycler (PTC 100; USA). The HB *S* gene was amplified using the following primers: forward, 5'-TATCGCTGGATGTGTCTGC-3'; reverse, 5'-AGACT TGGCCCCCAATACC-3' specific to region 403 bp according to Huang *et al*. [16]. The PCR reaction and conditions were the same as aforementioned except for the primers. The amplification products were visualized after staining with ethidium bromide (EB), after electrophoresis on 1% agarose gel. This experiment was repeated under the same conditions four times.

2.8 Reverse transcription–polymerase chain reaction (RT-PCR)

To study expression of HB *S* and *C* genes in one- and two-cell embryos, two-step RT-PCR was carried out using the cells-to-cDNA II kit (Ambion, USA) as follows:

49.5 μL ice-cold Cell Lysis II Buffer was added to each sample and mixed by pipetting, then incubated for 10 min at 75ºC. Approximately 0.12 U DNase I was added to the sample, then mixed gently and incubated at 37 ºC for 30 min. To deactivate DNase, samples were incubated at 75ºC for 5 min. Reverse transcription was performed according to the kit protocol. PCR amplifications were carried out using 5 μL from an RT reaction as a template for each sample and the specific primer pair of each gene separately. To ensure that the amplified bands originated from cDNA, and not genomic DNA, two negative controls (minus template and minus reverse transcriptase) were included for each sample. The minus template PCR should have all the PCR components, with water substituted for the RT reaction aliquot. The amplification step was carried out using the same conditions of normal PCR to amplify each gene as aforementioned. Approximately 20 μL of each RT-PCR product was made visible by staining with EB, after electrophoresis on 2% agarose gel. This experiment was repeated several times under the same condition.

2.9 Fluorescence in situ hybridization protocol

The full length HBV DNA (3.2 kb) was amplified by the routine PCR method, and then it was purified using Uniqu-10-PCR product purification kit (Sangon, Guangdong, China). The 3.2 kb HBV DNA probe was labeled with Digoxigenin-11-2-deoxy-uridine-5-triphosphate (Dig-11-dUTP) (Roche Diagnostics, Germany) by nick translation (BioNick DNA Labeling System, GIBCO, Germany). Slides containing chromosomes from two-cell embryos were treated with 100 μg/mL RNase (Sigma Chemical) for 1 h at 37ºC, 200 μg/mL pepsin (Sigma Chemical) in 0.01 mol/L HCl at 37ºC for 10 min, and 1% paraformaldehyde in PBS for 10 min at room temperature in succession. Chromosomes were denatured at 75ºC for 4 min in 73% formamide in 2 × saline-sodium citrate (SSC). *In situ* hybridization with the denatured DNA probe was performed with a modification of the procedure described by Huang *et al*. [17]. Briefly, 10 μL hybridization buffer (50% deionized formamide, 10% dextran sulfate, and 2 × SSC) containing 50 ng/mL Dig-11-dUTP-labeled HBV DNA probe and 500 ng/mL sheared salmon sperm DNA was placed on the slide. A coverslip (18 × 18 mm) was applied and sealed with rubber cement. The slides were then incubated in a moist chamber at 37ºC for 19 h. Post-hybridization washes were performed according to Korenberg and Chen [18], first in 50% formamide 2 × SSC for 15 min at 42ºC, then twice in 2 × SSC for 10 min each at 42ºC. Hybridization signals were detected with Anti-Digoxigenin-Rhodamine (Boehringer, Germany), washed with 4 × SSC in 0.01% Tween 20 three times for 5 min each. In order to reduce the non-specific binding, slides were pre-incubated in 4 × SSC with 15% non-fat dry milk at 37ºC for 15 min. Chromosomes were counterstained with propidium iodide (PI, Sigma) and (4,6)-diamidion-2-phenylindole (DAPI, Sigma), 2 mg/mL each in PBS/glycerol (1:9, v/v) containing 0.2 % (1,4)-diazobicyclo-(2,2,2)octane (DABCO, Sigma Chemical) as an anti-fade agent. Photographs were taken under a fluorescence microscope (BX51 TRF, Olympus, Japan) with the G and U excitation filters (DAPI-Rhodamine 1000 X).

3 Results

3.1 Fertilization rate

The data showed that 232 of a total 290 oocytes (80%) were fertilized. One hundred and sixty and 32 of the fertilized eggs (68.96% and 13.79%) were able to develop to normal and abnormal two-cell embryos, respectively. The percentages of normal one-cell embryos and unfertilized eggs was 17.24% (40/232) and 20% (40/290), respectively (Figure 1).

3.2 Polymerase chain reaction(PCR) amplification

All PCR products from each single (one- or two-cell) embryo were visible with EB staining after agarose gel electrophoresis as a single band at the expected sizes (403 bp and 774 bp) for HB *S* and *C* genes, respectively. No amplification was detected in the negative control reactions (Figure 2). All amplification products were found to be reproducible when reactions were repeated using the same reaction condition.

3.3 Reverse transcription–polymerase chain reaction (RT-PCR)

The 403 bp and 774 bp RT-PCR products corresponding to the amplified HB *S* and *C* gene fragments, respectively, were observed in each one- and two-cell embryo. No RT-PCR products of the aforementioned genes were detected in the control (Figure 3). The results show that both HB *S* and *pre-C/C* coding genes could be expressed in early embryonic cells.

3.4 Fluorescence in situ hybridization

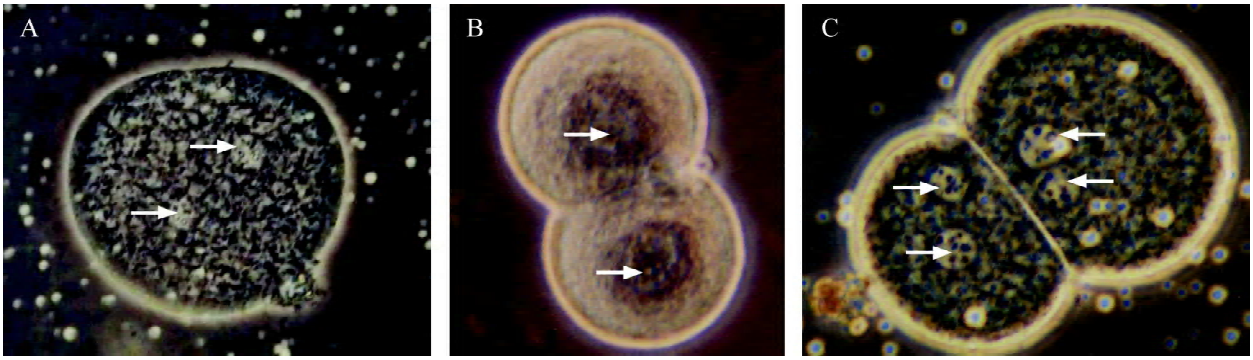

Figure 1. Morphology of normal and abnormal embryos at 24 h after insemination. (A): Normal one-cell embryo showing the male and female pronuclei (arrows). (B): Normal two-cell embryo showing a nucleus (arrows). in each. (C): Abnormal two-cell embryo showing multiple nuclei in each (arrows).

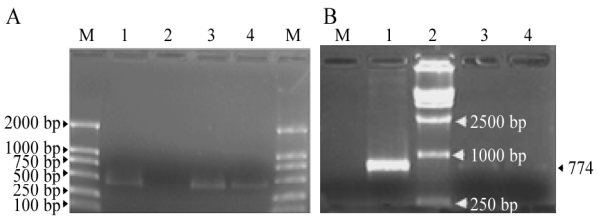

Figure 2. Ploymerase chain reaction (PCR) product. (A): Hepatitis B virus (HBV) S gene. Lane M, DNA marker (DL 2 000); lane 1, positive control; lane 2, negative control (minus template); lanes 3 and 4 were from one- and two-cell embryos, respectively. (B): *Pre-C/C* coding gene. Lane 1, negative control (minus template); lane 2, positive control; lane M, DNA marker (DL15 000); lanes 3 and 4,were from one- and two-cell embryos, respectively.

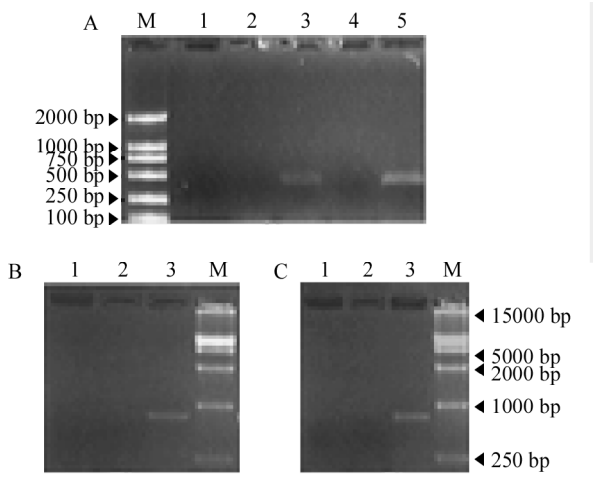

Figure 3. Reverse transcription–polymerase chain reaction (RT-PCR) products. (A): Hepatitis B (HB) *S* gene (403 bp); lane M, DNA marker (DL 2,000); lane 1, negative control (minus template); lanes 2 and 3, minus RT (–RT) and RT from one-cell embryo; lanes 4 and 5, -RT and RT from two-cell embryo. (B, C): HB *C* gene. Lane M, DNA marker (DL 2000); lane 1, negative controls (minus template); lane 2, –RT, and lane 3, RT from one- and two-cell embryo, respectively.

To perform FISH, full-length HBV DNA was used as a specific probe. Positive and clear signals were observed on chromosomes from two-cell embryos (Figure 4).

4 Discussion

In the present study, human spermatozoa take up an HBV-containing construct and deliver it to hamster oocytes in IVF assays. Using PCR and RT-PCR analysis we showed that the HBV DNA is present and is transcribed in one- and two-cell embryos. FISH analysis using full-length HBV DNA as the hybridization probe shows signals in the chromosomes of the one-cell embryo, suggesting that the exogenous sequences are integrated in the host embryo genome. The study on replication and expression of HBV gene (s) in the human embryo would be an ideal model but such a system presents major logistical, moral, and ethical problems. Thus, it is crucial to establish a model system for such study. In the present study, interspecific *in vitro* fertilization between human sperm and zona-free hamster ova made it possible.

The results showed that the high fertilization rate (80%) might be due to the optimization of the IVF method in our lab. The percentage (69%) of two-cell embryos obtained in this study is similar with that of our previous

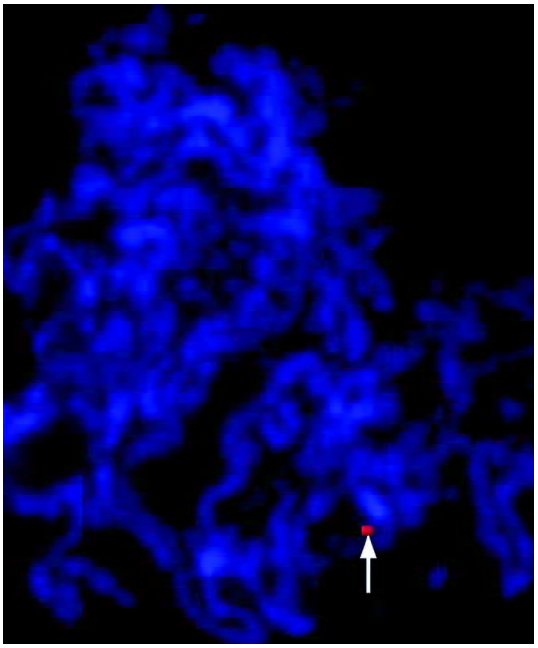

Figure 4. Detection of hepatitis B virus (HBV) sequences in chromosomes by fluorescence *in situ* hybridization with biotinylated whole-length HBV DNA probe. Positive signal onto chromosomes of two-cell embryo (arrow).

report [10]. In contrast, the timing of the events during early development appears to be critical to a successful outcome. In particular, the time of the first cleavage division after insemination *in vitro* is highly correlated to the ability of the embryo to reach the blastocyst stage [19]. Concerning the percentage of abnormal two-cell embryos (13.79%), it might be caused by polyspermy (Figure 1). Our preliminary experiments showed that the excessive polyspermic penetration might be due to at least one of the following reasons. The final concentration of spermatozoa that actively moved exceeded 10^6/mL; the large number of spermatozoa that attached firmly to the egg surface; or the insemination being over a long period (2–3 h). To minimize the chance of polyspermy in our experiments, therefore, the sperm suspension was often diluted more, and the oocytes in the fertilization medium were periodically observed under a dissecting microscope and those bound by approximately 20–30 spermatozoa were transferred to the sperm-free medium to incubate for approximately 30 min to ensure the higher rate of monospermic penetration.

Single-cell PCR provides a valuable tool for genetic characterization using a limited amount of starting material. A single embryo at the one- or two-cell stage was used for amplification of HB S and C genes from genomic DNA as well as for RT-PCR. The results showed that HB S and C genes were already detected both in one- and two-cell embryos. The PCR products were obtained as a clear single band at the predicted size in all of the experiments (Figure 2). However, neither specific nor non-specific bands were detected in the negative control reactions (minus template). Our results indicate that the foreign DNA could be integrated into the genome of the progeny. Single-cell RT-PCR is shown to be reliable for detecting HBV gene(s) expression. Clear positive bands in the samples from one- and two-cell embryos were detected, respectively (Figure 3). However, no positive bands were detected in two controls. This result demonstrated that human sperm-mediated HBV genes are able to be expressed in early embryonic cells. Further, this result confirms the possibility of HBV vertical transmission via germ line. The result of the present study is in agreement with the finding of our previous report [10]; they support each other and confirm the possibility of the HBV vertical transmission via the male germ cell.

Using the full-length HBV DNA as a probe, FISH analysis showed a positive signal on the chromosomes of two-cell embryos (Figure 4). It provided direct evidence that HBV DNA was integrated into the host genome. In our previous report, we found that clear signals of HBV DNA integrated into the male pronucleus, chromosomes of one-cell embryo, and each nucleus of the two-cell embryo [10]. Furthermore, our preliminary observations showed that HBV integration into sperm chromosomes does occur [17], thereby further demonstrating the possibility of vertical transmission of HBV via germ cells [5]. As a whole, the results in this work support the conclusion that human sperm cells can act as vectors for the vertical transmission of HBV genes to the progeny.

The present study demonstrated that the HBV genes were integrated into the sperm genome and introduced into the zygote of a normal oocyte via IVF with spermatozoon. The sperm-mediated HBV genes also are able to be expressed in early embryonic cells. It may well have far-reaching implications for not only human health but also genome reshaping evolutionary processes. However, further studies such as protein expression and the contribution of HBV DNA methylation for exploring the

mechanism of HBV vertical transmission is needed as a part of our future program.

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