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Host genes regulate transcription of sperm-introduced hepatitis B virus genes in embryo

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ABSTRACT

Hepatitis B virus (HBV) can invade the male germline, and sperm-introduced HBV genes could be transcribed in embryo. This study was to explore whether viral gene transcription is regulated by host genes. Embryos were produced by *in vitro* fertilization of hamster oocytes with human sperm containing the HBV genome. Total RNA extracted from test and control embryos were subjected to SMART-PCR, SSH, microarray hybridization, sequencing and BLAST analysis. Twenty-nine sequences showing significant identity to five human gene families were identified, with *CSH2*, *EIF4G2*, *PCBD2*, *PSG4* and *TTN* selected to represent target genes. Using qRT-PCR, when *CSH2* and *PCBD2* (or *EIF4G2*, *PSG4* and *TTN*) were silenced by RNAi, transcriptional levels of HBV *s* and *x* genes decreased (or increased). This is the first report that host genes participate in regulation of sperm-introduced HBV gene transcription in embryo, which is critical to prevent negative impact of HBV infection on early embryonic development.

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1. Introduction

Hepatitis B is a potentially life-threatening liver infection caused by the hepatitis B virus (HBV). Transmission of HBV through blood

transfusion [1], body fluids [2], intrauterine infection [3], cell, tissue and organ transplantation [4], and others including hemodialysis units or intravenous drug injection or occupational exposure [5,6] have been documented. In recent years some studies reported a true vertical transmission of HBV via germline and found that HBV has a negative effect on sperm motility *in vivo* and that couples in which the male is infected with HBV have a high risk of a low fertilization rate after *in vitro* fertilization (IVF) [7]. Huang et al. provided direct evidence that HBV DNA is able to integrate into the chromosomes of patient sperm, and that such HBV-carrying sperm can fertilize oocytes [8]. After fertilization, the sperm-introduced HBV *c*, *s* and *x* genes retain their functions of replication and expression in embryonic cells [9,10]. Subsequently, some studies showed that the HBV S protein can cause a series of apoptotic events resulting in reduced sperm motility, loss of sperm membrane integrity, sperm dysfunction, decreased fertility, and sperm death [11–13]. Hu et al. collected 578 embryos from couples with at least one HBsAg seropositive partner, and found that HBV DNA was present in 14.4% (83/578) of embryos [14]. Thus the effects of HBV infection on human reproduction and early embryonic development and their mechanisms have attracted the attention of researchers.

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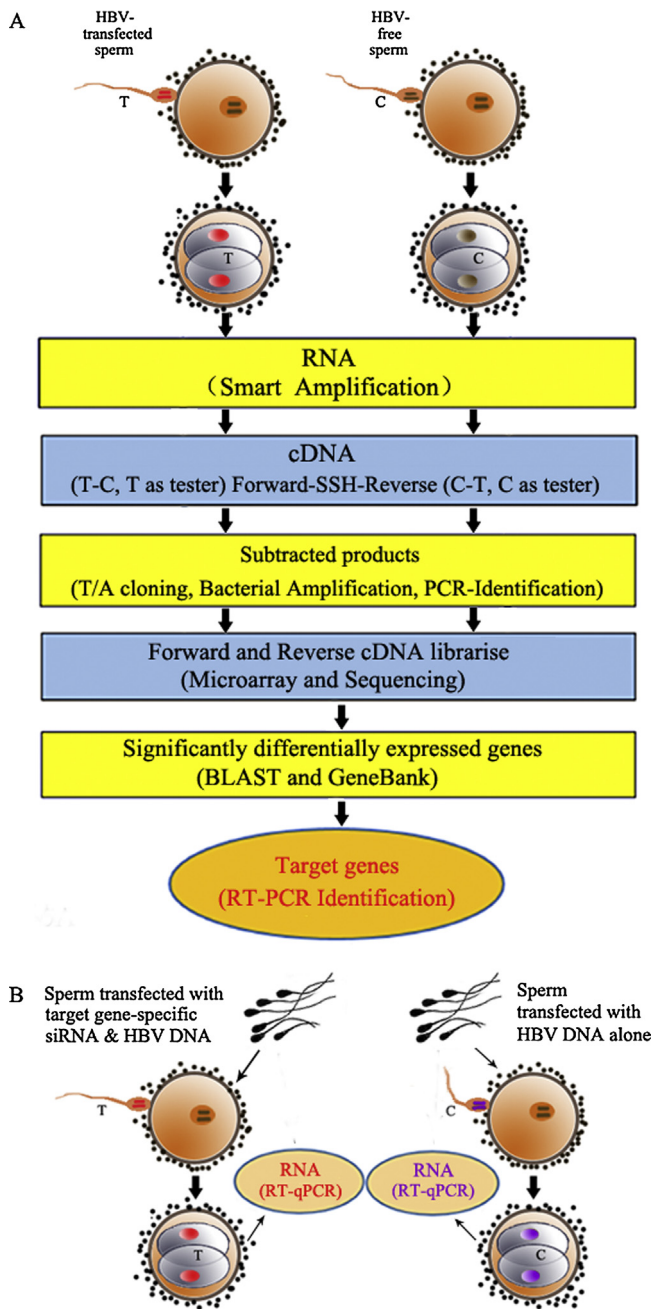


Fig. 1. Experimental flowchart. **A:** Sperm samples from healthy donors were divided into two groups: the test group was transfected with plasmid containing the full-length HBV genome (T), and the control group was non-transfected (C). Zona-free hamster ova were fertilized by transfected or non-transfected sperm to obtain two-cell embryos. Total RNA was extracted from T- and C-sperm-derived embryos, respectively and then subjected to SMART amplification, followed by SSH in both forward (T as tester) and reverse (C as tester) directions to enrich for up-regulated genes (T-C amplicon) or down-regulated genes (C-T amplicon). After T-A cloning and bacterial amplification, the forward and reverse libraries were cloned by PCR to obtain single insert-containing clones that were used for microarray assay. Clones with a fold change value greater than 2 or less than 0.5 when compared with the average Cy5/Cy3 intensity ratio, the differential expression of which was statistically significant, were selected for sequencing. For the acquired sequences, BLAST was used to search for sequences homologous to human genes in the GenBank nucleotide database. Of the sequences, five were selected as the target genes and their expression in two-cell embryos was confirmed by RT-PCR. **B:** Sperm in the test group was co-transfected with plasmid containing the full-length HBV genome and the target gene-specific shRNA/siRNA. Sperm in the control group was transfected with plasmid containing the full-length HBV genome alone. The transcription levels of HBV s and x genes in the two-cell embryos were assessed by real time qRT-PCR to identify whether the target genes participated in regulation of HBV gene expression. Same procedures listed above were

Hepatitis B infection and disease pathogenesis are known to be influenced by a number of factors, including host genetic factors [15]. Many cellular proteins that possibly regulate HBV gene transcription in hepatocytes have been identified, including liver-enriched proteins, such as HNF1, HNF3, C/EBP, and KLF15, ubiquitous factors, such as Sp1, RFX1, NF-Y, and AP1, and members of the nuclear receptor superfamily, such as HNF4, RXRa, PPARa and COUP-TFs [16–19]. Hepatotropism is a prominent feature of HBV infection, and virus infection appears to be restricted to hepatocytes [20]. Because sperm-derived embryos differ from hepatic cells, this study was undertaken to explore whether host genes participate in regulation of HBV gene transcription in embryonic cells, which is critical to reveal the regulation mechanism of viral gene transcription in these cells and to prevent the negative impact of HBV infection on early embryonic development.

Although using embryo produced by fertilization of human oocyte with human sperm carrying HBV genes would be an ideal model, such a model presents major moral, ethical and legal problems. The interspecific IVF between human sperm and zona-free golden hamster ova is highly associated with human *in vitro* fertilization [21] and has no the aforementioned problems, which has been included in “WHO laboratory manual for the examination and processing of human semen” [22] and widely used in the research of reproductive biology and medicine [23–25], thus the interspecific IVF was employed to obtain two-cell embryos in the current study.

As HBV DNA has been detected in patient sperm [7,8], donor sperm transfected with recombinant plasmid containing the full-length HBV genome was used instead of patient sperm to explore whether host genes participate in regulation of sperm-introduced HBV gene transcription in embryos. Three measures were taken to ensure reliable and accurate results (Fig. 1). First, switching mechanism at 5' end of RNA template (SMART) amplification was employed because, unlike cells from other tissues, limited number of two-cell embryos made recovery of significant amounts of RNA very difficult. SMART amplification allows the synthesis of high-quality cDNA for suppression subtractive hybridization (SSH) from sub-microgram levels of RNA [26]. Combined with T-A cloning and bacterial amplification, we obtained sufficient subtraction products for array probe preparation, microarray assay and cDNA sequencing. Next, we combine SSH with microarray to obtain the cDNAs highly enriched for differentially expressed genes of both high and low abundance and greatly reduce tedious work for screening of subtraction libraries, as well as the likelihood of false-positive clones enriched via SSH [27]. Finally, we silenced the target genes and a control gene by RNA interference (RNAi) to detect effects of the silencing of these genes on transcriptional level of HBV genes to determine whether host genes participate in regulation of HBV gene transcription.

2. Materials and methods

2.1. Ethical approval

Semen samples were obtained from healthy donors. Written, informed consent was obtained from all study subjects who allowed their sperm samples to be used for research. All protocols used in the current study involving human subjects were approved by the Institutional Ethical Review Boards of Chengdu Jingjiang Hospital for Maternal and Child Health Care (approval number: CJHMCHC-0010) and by the Ethics Committee of Shantou Univer-

also used to isolate and identify a control gene with a fold change value greater than 0.5 and less than 2 when compared with the average Cy5/Cy3 intensity ratio, the differential expression of which was not statistically significant.

sity Medical College (Approval No. SUMC-00-0031) according to the World Medical Association Declaration of Helsinki: ethical principles for medical research involving human subjects [28].

The animal protocol was designed to minimize pain or discomfort. The animals were acclimatized to laboratory conditions (23 °C, 12 h/12 h light/dark, 50% humidity, ad libitum access to food and water) for two weeks prior to experimentation. All animals were euthanized by barbiturate overdose (intravenous injection, 150 mg/kg pentobarbital sodium) for oocyte collection, and then their bodies were subjected to harmless treatment. All procedures involving animals were reviewed and approved by the Medical Animal Care & Welfare Committee in Shantou University Medical College (IACUC protocol number: SUMC2015-152) according to the recommended guide for the care and use of laboratory animals published by the National Research Council (US) [29].

2.2. Sperm preparation

Semen samples were incubated in a humidified incubator (37 °C, 5% CO₂ in air) for 30 min to allow liquefaction. Motile sperm were selected using the swim-up method in Biggers-Whittem-Whittingham (BWW) medium supplemented with 0.3% bovine serum albumin (BSA) [30]. After washing, these sperm samples were used for subsequent experiments.

2.3. Construction and transfection of recombinant plasmid and its efficiency

Recombinant plasmid pBR322-HBV containing the full-length HBV genome was kindly provided by Professor Yi-Ping Hu (The Open Laboratory for Molecular Genetics, The Second Military Medical University, China). Construction and transfection of plasmid pIRES2-EGFP-HBV (Fig. 2) was performed as described previously [31]. Briefly, a full-length cDNA of HBV was isolated from pBR322-HBV by digestion of the restriction enzyme *EcoRI*, and purified using purification extraction kit (TaKaRa Biotechnology (Dalian) Co., Ltd, China). Plasmid pIRES2-EGFP vector (Promega, Madison, WI, USA) was linearized and mixed with the HBV cDNA at 1:3–1:10 molar ratio in a ligation reaction and incubated at 16 °C overnight. The ligation mixture was used directly to transform the *E. coli* Competent Cells DH5 α according to the manufacturer's instruction (TaKaRa). The right orientation and authenticity of the HBV coding sequence were confirmed by restriction digestion.

Each sperm sample from a healthy donor was divided into two groups: a test group and a control group. A 100- μ l aliquot of a mixture comprised of 1 μ l of plasmid pIRES2-EGFP-HBV (1.5 μ g/ μ l) containing the full-length HBV genome, 6 μ l of Fugene HD, and 93 μ l of HEPES-buffered saline was incubated at room temperature for 15 min, and then added to the sperm in the test group, which was incubated for 1.5 h. The sperm in the control group were not transfected.

To measure the transfection efficiency, a Nick Translation Kit (Roche, Basel, Switzerland) and Fluorescein-12-dUTP (Thermo Fisher Scientific, Waltham, MA, USA) were used. The plasmid was labeled with fluorescein-12-dUTP by nick translation according to the manufacturer's instructions. Transfected sperm were detected by green fluorescence and counted under a fluorescence microscope. In our preliminary experiment, the transfection efficiency was $39.51 \pm 2.72\%$, and the proportion of embryos derived from HBV transfected sperm was 27.66%.

2.4. Oocyte preparation, insemination and embryo grouping

Oocyte preparation and insemination were performed as described in reference [22]. Briefly, zona-free hamster ova were fertilized by sperm from the test and control groups and then incu-

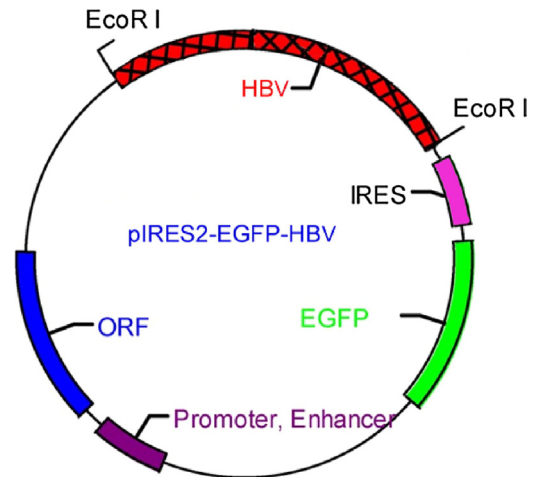


Fig. 2. Recombinant plasmid pIRES2-EGFP-HBV. It contains a full-length HBV genome and an enhanced green fluorescent protein (EGFP) gene as a reporter for gene expression.

bated in a humidified incubator (37 °C, 5% CO₂ in air) for 24 h to obtain two-cell embryos. Two-cell embryos exhibiting green fluorescence, derived from the HBV-transfected sperm (the test group), and those without green fluorescence, derived from the non-transfected sperm (the control group), were collected under a fluorescence microscope (Dmi1 Led, LEICA, Germany). After washing, total RNA from the two-cell embryos was extracted for cDNA library construction by SMART amplification.

2.5. SMART amplification and SSH

SMART amplification and SSH were carried out using SMARTer™ PCR cDNA Synthesis Kit and Clontech PCR-Select™ cDNA Subtraction Kit (Clontech Laboratories, Inc, CA, USA) according to the manufacturer's instructions. Control mouse liver total RNA (the positive group), deionized H₂O (the negative group) and the primers (3' SMART CDS Primer II A and 5' PCR Primer II A) were provided by the kit. Briefly, 3.5 μ l (50 ng) total RNA extracted from two-cell embryos of the test and control groups were successively subjected to first-strand cDNA synthesis, amplified by long-distance PCR, purified by column chromatography, digested with *RsaI*, diluted to a final concentration of 300 ng/ μ l in 1X TNE buffer, and subjected to adaptor ligation.

SSH materials consisted of cDNA from the test two-cell embryos as the tester and cDNA from the control two-cell embryos as the driver for forward subtraction, and vice versa for reverse subtraction. After the first and second hybridizations, hybridized samples 1 and 2 were mixed and then incubated at 68 °C overnight, followed by a primary PCR and a secondary PCR. After an agarose/ethidium bromide gel analysis, the products were used for DNA ligation. The same procedures listed above were also performed for reverse subtraction and control subtraction.

2.6. T/A cloning, bacterial amplification and identification of subtraction products

T/A cloning and amplification of subtraction products were performed using pGEM®-T Easy Vector System (Promega) according to the manufacturer's instructions. Briefly, ligation reactions containing pGEM®-T Easy vector, 2X Rapid Ligation Buffer, T4 DNA Ligase and the secondary PCR products of SSH were incubated overnight at 4 °C. Two microliters of ligation reaction was added into 50 μ l of JM109 high efficiency competent cells, which was placed on ice for 20 min followed by heat-shocking at 42 °C for 45 s, putting

on ice for 2 min, and then adding into 950 μ l of Luria-Bertani (LB) medium and incubation at 37 °C for 1.5 h with shaking. One hundred microliters of the transformation culture was plated onto LB/ampicillin/IPTG/X-Gal plates followed by incubation overnight at 37 °C. The 174 white (positive) colonies obtained were separately resuspended in 1.5 ml LB medium with ampicillin (100 μ g/ml) and grown at 37 °C for 6 h. PCR amplifications were carried out using 0.3 μ l bacterial suspension and Premix Taq™ (TaKaRa), and 5 μ l from each PCR reaction was subjected to 1% agarose gel electrophoresis. Positive bands were detected for 152 of 174 colonies.

2.7. Microarray hybridization and data analysis

Microarray hybridization and data analysis were performed by TaKaRa Biotechnology (Dalian) Co., Ltd. Briefly, the PCR products for differentially expressed genes from 152 clones, obtained from the subtraction libraries, were purified using 2-propanol precipitation and then spotted in triplicate onto TaKaRa glass slides (TaKaRa) using an Affymetrix Arrayer 417 (TaKaRa). The PCR products from the forward- and reverse-subtracted libraries (2 μ g each) were labeled with Cy3 and Cy5 fluorescent dyes and separately used to probe the glass slides containing the PCR-amplified cDNA. The slides were hybridized overnight at 65 °C with labeled purified probes. Quality control of the microarray chips was performed according to TaKaRa's method and standard.

Array slides were scanned using an Affymetrix 428 Array Scanner (Affymetrix, Buckinghamshire, UK). The measured intensities are expressed as a ratio of Cy5/Cy3 intensities, which were background-corrected and normalized to the average Cy5/Cy3 ratio. The ratios were \log_2 -transformed, and the following clones were selected for sequencing: 1. clones with a fold change value greater than 2 or less than 0.5 when compared with the average Cy5/Cy3-intensity ratio, the differential expression of which was statistically significant, were used to select target genes; 2. A clone with a fold change value greater than 0.5 and less than 2, the differential expression of which was not statistically significant, was chosen as the control [32].

2.8. DNA sequencing and analysis

DNA sequencing and analysis were performed by Beijing Genomics Institute Shenzhen Co., Ltd. (Shenzhen, China) using a 3730 XL DNA Analyzer (Applied Biosystems, CA, USA). Sequencing reactions were carried out with BigDye v3.1 Mix and POP-7™ polymer (Applied Biosystems).

2.9. Selection of target genes and confirmation of their transcription in embryonic cells

For the acquired sequences, basic local alignment search tool (BLAST) was used to search for sequences homologous to human genes in the GenBank nucleotide database. Of the sequences, five with statistically significant differential expression were selected as the target genes, and a non-statistically significant sequence without statistical significance of differential expression was selected as a control. The transcriptions of both target and control genes in the two-cell embryos were confirmed using RT-PCR. The extraction and reverse transcription of total RNA was performed using an Ambion Cells-to-cDNATM II Kit (Life Technologies, CA, USA) according to the manufacturer's instructions. PCR amplification was performed in a 25 μ l reaction mixture containing cDNA (5 μ l), premixed Taq polymerase (12.5 μ l) (TaKaRa), forward and reverse primer (10 μ M, 0.5 μ l each) and ddH₂O (6.5 μ l), and the β -actin was co-amplified as an internal control [33]. The gene-specific primers were designed using Primer-BLAST (Table 1). Cycling conditions were as follows: 2 min at 94 °C; 35 cycles of 30 s at 94 °C

and 30 s at 57 °C; and one cycle of 1 min at 72 °C. The amplified PCR fragments were subjected to 1.5% agarose gel electrophoresis and stained with ethidium bromide.

2.10. Identification of target genes regulating transcription of sperm-Introduced HBV genes in embryo

Eighteen healthy donors were randomly divided into six groups, and their sperm samples were used to fertilize zona-free hamster oocytes *in vitro* and assess the effects of the silencing of five target genes (*CSH2*, *EIF4G2*, *PCBD2*, *PSG4* and *TTN*) and a control gene (*ESRRG*) on transcription of HBV *s* and *x* genes by real-time quantitative (q)RT-PCR. The sperm samples from three donors were individually used for assaying each gene, and each assay was repeated three times.

Gene-specific short hairpin (sh)RNA or short interfering (si)RNA used for silencing the target and control genes, and non-interfering scrambled oligonucleotides used as the negative controls, were designed and synthesized by Shanghai GenePharma Co., Ltd. or QIAGEN China (Shanghai) Co., Ltd, respectively (Table S1). Each sperm sample from a healthy donor was divided into two groups: a test group, which was co-transfected with plasmids containing the full-length HBV genome and the gene-specific shRNA/siRNA or a non-interfering scrambled oligonucleotide; and a control group, which was transfected with the plasmid containing the full-length HBV genome alone. Real-time qRT-PCR was performed using an ABI 7300 Real-Time PCR System (Applied Biosystems, CA, USA) to compare the transcriptional level of HBV genes in the two-cell embryos of the test and control groups, with *GAPDH* as an internal control [33]. Total RNA was extracted from the two-cell embryos and cDNA synthesis was performed as described previously [34]. A total volume of 20 μ l of reaction mixture contained cDNA (2 μ l), 2 x QuantiFast SYBR Green PCR Master Mix (10 μ l) (Qiagen, Hilden, Germany), forward and reverse primers (10 μ M, 0.2 μ l each) for *s* and *x* genes and *GAPDH* (Table 1), and RNase-free water (7.6 μ l). Cycling conditions were as follows: 5 min at 95 °C; 40 cycles of 15 s at 95 °C and 30 s at 60 °C; and one cycle of 15 s at 95 °C, 2 min at 55 °C, and 15 s at 95 °C. The data was analyzed quantitatively using the $2^{-\Delta\Delta C_t}$ method.

2.11. Statistical analysis

In the $2^{-\Delta\Delta C_t}$ analysis, individual data were converted to a linear form using the 2^{-C_t} calculation [35] and then subjected to a paired-sample *t*-test using SPSS 16.0 software to determine a significant differences in average transcription levels of *s* and *x* genes between the test and control groups. A *P*-value of less than 0.05 was considered statistically significant.

3. Results

3.1. Smart amplification, SSH, T/A cloning, bacterial amplification and identification

SMART amplification showed that dispersed positive bands were detected in the test, control and positive groups, and not in the negative group, indicating successful amplification. After SSH, T/A cloning and bacterial amplification, 174 white (positive) colonies from subtracted libraries (123 from the forward and 51 from the reverse SSH libraries) were amplified by PCR, resulting in 152 positive insert-containing clones (103 from the forward- and 49 from the reverse-subtracted libraries). The insert sizes ranged from 500 to 1500 bp, and the majority of insert sizes was approximately 1000 bp (Fig. 3), in agreement with the statistical prediction of *RsaI* digestion.

Table 1

The primers were used to amplify the target genes, a control gene, an internal control genes and HBV S and X genes.

Genes*	Primers	
	Forward	Reverse
<i>CSH2</i>	5'- ACCACGCTATGCTCCAAGCC-3'	5'- CCTTCCTCTAGGTCCTTTAGGAGG-3'
<i>EIF4G2</i>	5'- TCATAGTCGCCACGGACTT-3'	5'- TGGTTTGAGGGGATGGATCC-3'
<i>PCBD2</i>	5'- TCATAGTCGCCACGGACTT-3'	5'- AGCGAGCCTCAITGTGTGTG-3'
<i>PSG4</i>	5'- TGACTTCACACTGTGGACAG-3'	5'- CTTCTCTGCAAACACACAGGC-3'
<i>TTN</i>	5'- AGTATGCCTCAGTGGGTTGAG-3'	5'- CAAAGGCATTGGCTGCACTC-3'
<i>ESRRG</i>	5'- GTGTGTGGTGACATCGCTTC-3'	5'-TCAGGTATGGGCTGTTCTCC-3'
β -actin	5'-GAGACCTTCAACACCCAGC-3'	5'-ATGTCACGCACGATTTCCC-3'
HBV S	GTGTCTGCGGCGTTTATCA	GACAAACGGGCAACATACCTT
HBV X	CGTCTGTGCCTTCTCATCTGC	GGTCGGTCGTTGACATTGCT
<i>GAPDH</i>	5'-GGTGTCTCCTCTGACTCAACA-3'	5'-GGTGTCTAGCCAAATTCGTTGT-3'

**CSH2*, chorionic somatomammotropin hormone 2 gene; *EIF4G2*, eukaryotic translation initiation factor-4 γ , 2 gene; *PCBD2*, pterin-4 α -carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 α (TCF1)2 gene; *PSG4*, pregnancy-specific β -1-glycoprotein 4 gene; *TTN*, titin gene; *ESRRG*, estrogen receptor-related receptor gene; β -actin, β -non-muscle actin gene; *GAPDH*, glyceraldehyde-3-phosphate dehydrogenase gene; *HBVs*, s gene of hepatitis B virus and *HBVx*, x gene of hepatitis B virus. RT-PCR and real-time qRT-PCR were performed to confirm transcription of the target genes (*CSH2*, *EIF4G2*, *PCBD2*, *PSG4* and *TTN*) in embryonic cells and to determine whether they regulate transcription of HBV s and x genes, respectively. *ESRRG* that was uncorrelated to target genes was chosen as control. Both β -actin and *GAPDH* were used as the internal controls for RT-PCR and real-time qRT-PCR assays, respectively [33].

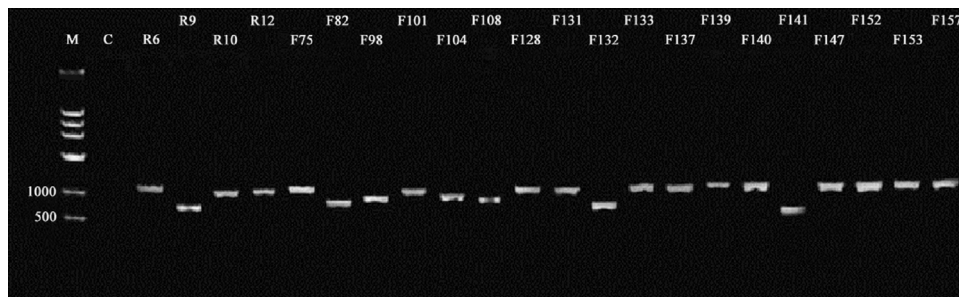


Fig. 3. Representative insert sizes of cloned cDNA from the forward (F) and reverse (R) SSH libraries. After SSH, the subtraction products were cloned into pGEM[®]-T Easy Vector followed by bacterial amplification, 174 white (positive) colonies (123 from forward and 51 from reverse SSH libraries) were selected from the Luria-Bertani (LB) medium and subjected to PCR assay for eliminating a false positive result, and the blank LB medium was as control. It resulted in 152 positive insert-containing clones (103 from forward and 49 from reverse subtracted libraries). M: marker DL 500–15,000; C: control; R6 ~ F157: the insert sizes ranged from 500 to 1500 bp, with the majority being approximately 1000 bp.

3.2. Microarray analysis

In the current study, the two-fold average ratio of Cy5/Cy3 intensities was 5.740, and the 0.5-fold average ratio of Cy5/Cy3 intensities was 1.4349. Both are the thresholds for determining whether gene differential expression is statistically significant. Of 152 positive insert-containing clones, 29 showed fold change values greater than 2 or less than 0.5, and 123 exhibited fold change values greater than 0.5 and less than 2. These 29 clones and one of the 123 clones were selected for sequencing (Table S2).

3.3. DNA sequencing, selection and identification of target genes in embryonic cells

BLAST analysis revealed 29 acquired sequences showing significant identity to five human gene families (average identity = 94.59%, ranging from 82% to 100%, E-value: 0.0) (Table S2). Of these, five representative genes (one from each family, identity: \geq 96%, E-value: 0.0) were selected as target genes that were chorionic somatomammotropin hormone 2 (*CSH2*), eukaryotic translation initiation factor-4 γ , 2 (*EIF4G2*), pterin-4 α -carbinolamine dehydratase/dimerization cofactor of hepatocyte nuclear factor 1 α (TCF1)2 (*PCBD2*), pregnancy-specific β -1-glycoprotein 4 (*PSG4*) and titin (*TTN*) (Table 2). The selection criteria were as follows: First, three sequences showed significant identity to three human gene families, and each gene family had one sequence only, thus they (*EIF4G2*, *PSG4* and *TTN*) were chosen as representative genes in these gene families. Next, seven sequences showed significant identity to two members (*NADH* and *PCBD2*) of a human gene fam-

ily. Because *NADH* is located within mitochondria and not within cytoplasm, it is difficult to determine entry efficiency of *NADH*-specific shRNA into mitochondria and to evaluate its silencing effects, thus *PCBD2* was chosen as representative gene in this gene family. Finally, 19 sequences showed significant identity to one human gene family, in which a sequence from the clone F123 with the highest ratio of Cy5/Cy3-intensities showed the highest identity (99%) to the members (*CSH1* and *CSH2*) of this gene family, thus the *CSH2* was chosen as representative gene. In addition, a sequence (F110) from 123 clones showed significant identity to human estrogen receptor-related receptor (*ESRRG*) family and was chosen as a control gene (Table 2, Table S2). Their transcriptions in the two-cell embryos were confirmed by RT-PCR (Fig. 4).

3.4. Identification of target genes regulating transcription of sperm-introduced HBV genes in embryo

The transcriptional levels of HBV s and x genes in two-cell embryos after the silencing of target genes and a control gene by RNA interference (RNAi) are shown in Table 3. When *CSH2* and *PCBD2* were silenced, s and x genes expression in two-cell embryos was down-regulated. *CSH2* knockdown reduced the transcription of s and x genes by 11.1- and 8.33-fold in two-cell embryos, respectively, as compared to those in the control group. Knockdown of *PCBD2* reduced s and x gene transcription by 4.35- and 3.70-fold, respectively. When *EIF4G2*, *PSG4* and *TTN* were silenced, s and x genes expression in two-cell embryos was up-regulated between 2.52- to 14.94-fold. There was no significant difference in the levels

Table 2

The clones were selected as the target genes and a control gene.

Library ID	Ratio of Cy5/Cy3-intensities	FCV ^{§1}	Homologous genes	Accession No.	Identity ^{§2} (%)	E value ^{§3}
F123	1.1015	<0.5	<i>CSH2</i> *	NM_020991.3	688/694 (99%)	0.0
R5	7.6808	>2	<i>EIF4G2</i> *	NM_001042599.2	1001/1043 (96%)	0.0
F12	0.6321	<0.5	<i>PCBD2</i> *	NT_034772.6	569/590 (96%)	0.0
F14	1.4166	<0.5	<i>PSG4</i> *	NM_213633.1	526/536 (98%)	0.0
F11	5.8078	>2	<i>TTN</i> *	NM_133437.3	768/781 (98%)	0.0
F110	3.0834	>0.5, <2	<i>ESRRG</i> ¶	NG_029784.1	740/744 (99%)	0.0

^{§1} **FCV**: fold change value. In the current study, the two-fold average ratio of Cy5/Cy3 intensities was 5.740, and the 0.5-fold average ratio of Cy5/Cy3 intensities was 1.4349. Both are the thresholds for determining whether gene differential expression is statistically significant. ^{§2} **Identities**: the extent to which two nucleotide sequences have the same residues at the same positions in an alignment, often expressed as a percentage [36]. ^{§3} **E value**: the Expectation value or Expect value represents the number of different alignments with scores equivalent to or better than S that is expected to occur in a database search by chance. The lower the E value, the more significant the score and the alignment [36]. * The target genes. ¶ The control gene.

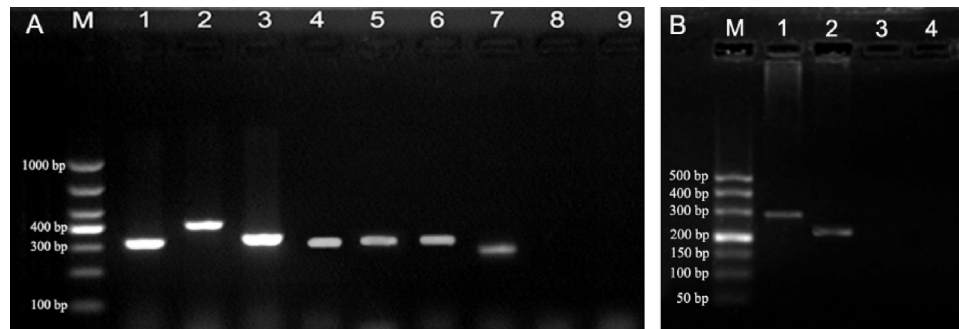


Fig. 4. Confirmation of the target and control gene transcription in two-cell embryos. **A:** RT-PCR results for the target genes. M: marker DL100–1000 bp; 1: *PCBD2* (313 bp); 2: *TTN* (404 bp); 3: *ND*(328 bp); 4: *PSG4* (300 bp); 5: *EIF4G2* (315 bp) and 6: *CSH2* (328 bp). Positive band for each target gene was observed, indicating the transcription of the target genes in the two-cell embryos. 7: β -actin (263 bp) as the internal control; 8: –RT; 9: –T. **B:** RT-PCR results for the control gene. M: marker DL50–500 bp; 1: *ESRRG* (280 bp), positive band for the control gene was observed, indicating the transcription of *ESRRG* in the two-cell embryos; 2: β -actin (263 bp) as the internal control; 3: –RT; 4: –T.

Table 3

Effects of the silencing of the target and control genes on transcriptional levels of HBV S and X genes in two-cell embryos.

Host genes	S gene ($2^{-\Delta\Delta CT}$)			X gene ($2^{-\Delta\Delta CT}$)		
	T	C	FCV	T	C	FCV
<i>CSH2</i>	0.09 ± 0.01*	1	–11.1	0.12 ± 0.04*	1	–8.33
<i>PCBD2</i>	0.23 ± 0.15*	1	–4.35	0.27 ± 0.08*	1	–3.70
<i>EIF4G2</i>	2.52 ± 0.10*	1	2.52	4.01 ± 0.84*	1	4.01
<i>PSG4</i>	2.86 ± 0.35*	1	2.86	3.62 ± 0.92*	1	3.62
<i>TTN</i>	5.16 ± 1.76*	1	5.16	14.94 ± 5.32*	1	14.94
<i>ESRRG</i>	1.02 ± 0.21¶	1	1.02	1.02 ± 0.04¶	1	1.02

Eighteen healthy donors were randomly divided into six groups, and their sperm sample were used to fertilize zona-free hamster oocytes *in vitro* and assess the effects of the silencing of five target genes (*CSH2*, *EIF4G2*, *PCBD2*, *PSG4* and *TTN*) and a control gene (*ESRRG*) on transcription of HBV S and X genes in two-cell embryos using real time qRT-PCR and $2^{-\Delta\Delta CT}$ method. The sperm samples from three donors were individually used for assaying each gene, and each assay was repeated three times.

Each sperm sample from a donor was divided into two groups: a test group (T), which was co-transfected with plasmids containing the full-length HBV genome and the gene-specific shRNA or siRNA; and a control group (C), which was transfected with plasmid containing the full-length HBV genome alone.

The data were presented as a fold change value (FCV) in transcription of S and X genes in the test group normalized to internal control gene (*GAPDH*) and relative to those in the control group. Individual data were converted to a linear form using 2^{-CT} calculation [35] and then subjected to a paired-sample *t*-test using 16.0 software to determine a significant difference in average transcriptional levels of S and X genes between the test and control groups. A *P*-value of less than 0.05 was considered statistically significant.

* $P < 0.05$; ¶ $P > 0.05$.

of *s* and *x* gene transcription in two-cell embryos silenced for *ESRRG* compared to non-silenced two-cell embryos (Table 3).

In the current study, two non-interfering scrambled oligonucleotides (NISO) were used as the negative controls. There was no significant difference in the levels of *s* and *x* gene transcription in the two-cell embryos between the test (NISO treated) and control (NISO non-treated) groups (Table 4).

4. Discussion

RNAi is a biological process in which RNA molecules inhibit gene expression or translation, by neutralizing targeted mRNA molecules. Oligonucleotides are short DNA or RNA molecules, of which siRNA and shRNA are central to RNAi. It has been demon-

strated that hamster and human sperm have a strong tendency to interact with exogenous DNA and are able to transfer DNA to oocytes [37]. Zhang et al. injected plasmid RNAi-Ready-pSIREN-RetroQ-ZsGreen, which was constructed for interrupting the *Zfx* gene, into testis in the test group mice, and then the male mice were mated individually with females, resulting in $78.75 \pm 7.50\%$ of the male offspring, significantly higher than the offspring derived from the control groups ($p < 0.01$), suggesting that RNAi could be as a tool to control the sex ratio of mouse offspring by interrupting *Zfx/Zfy* genes in sperm cells [38]. In the current study, the two-cell embryos derived from sperm co-transfected with plasmids containing the full-length HBV genome and gene-specific shRNA/siRNA for interrupting the target genes were as the test group, and those derived from sperm transfected with plasmid containing the full-length

Table 4
Effects of the non-interfering scrambled oligonucleotides on transcriptional levels of HBV S and X genes in two-cell embryos.

NISO	S gene ($2^{-\Delta\Delta CT}$)			X gene ($2^{-\Delta\Delta CT}$)		
	T	C	FCV	T	C	FCV
NISO1 (shRNA)	1.01 ± 0.07 π	1	1.01	1.11 ± 0.22 π	1	1.11
NISO2 (siRNA)	0.98 ± 0.18 π	1	0.98	1.02 ± 0.23 π	1	1.02

Each sperm sample from a donor was divided into two groups: a test group (T), which was co-transfected with plasmids containing the full-length HBV genome and a non-interfering scrambled oligonucleotide (NISO); and a control group (C), which was transfected with plasmid containing the full-length HBV genome alone.

The data were presented as a fold change value (FCV) in transcription of S and X genes in the test group normalized to internal control gene (*GAPDH*) and relative to those in the control group. Individual data were converted to a linear form using 2^{-CT} calculation [35] and then subjected to a paired-sample *t*-test using 16.0 software to determine a significant difference in average transcriptional levels of S and X genes between the test and control groups. *AP*-value of less than 0.05 was considered statistically significant. $\pi P > 0.05$.

HBV genome alone were as the control group. The transcription levels of HBV genes between the test and control groups are significantly different ($P < 0.05$), which suggested that in the test group the target genes have been silenced by RNAi and participated in transcriptional regulation of HBV genes, causing the change of HBV gene transcription levels.

We found that *CSH2*, *PCBD2*, *EIF4G2*, *PSG4* and *TTN* to be involved in transcriptional regulation of HBV *s* and *x* genes in two-cell embryos. The protein encoded by *CSH2* is a member of the somatotropin/prolactin family of hormones and plays an important role in growth control [39]. *PCBD2* is a protein coding gene and associated with tetrahydrobiopterin biosynthesis [39]. Silencing of *CSH2* and *PCBD2* by RNAi decreased the levels of *s* and *x* gene transcription in two-cell embryos, indicating that both genes up-regulated transcription of *s* and *x* genes. The protein encoded by *EIF4G2* is a subunit of EIF4F, a cap binding protein complex that mediates translation initiation by specific recognition [39]. *PSG4* is a member of the carcinoembryonic antigen (CEA) gene family and may play a role in regulation of the innate immune system [39]. *TTN* encodes a large abundant protein that is a key component in the assembly and functioning of vertebrate striated muscles, and in non-muscle cells, seems to play a role in chromosome condensation and chromosome segregation during mitosis [39]. When *EIF4G2*, *PSG4* and *TTN* were silenced by RNAi, the transcriptional levels of *s* and *x* genes in two-cell embryos increased, suggesting that these genes down-regulate *s* and *x* gene transcription. These results suggest that certain host genes participate in regulating HBV gene transcription in two-cell embryos. However, the interplay between the virus and host cells is complex, and it remains largely unknown how these genes interact with each other or act independently to allow differential transcription of HBV genes. Some clues may help in understanding their interaction. First, certain previously identified host HBV-regulatory genes share high similarity in function and signaling pathways with genes (identified herein) that regulate HBV gene transcription. For example, Sp1 is a ubiquitous factor that binds to GC-rich motifs in many promoters, can regulate transcription of HBV genes [16,17], and is involved in many cellular processes, including cell differentiation, growth, and apoptosis. The protein encoded by *CSH2* also plays an important role in growth control and provides avenues for developmental regulation and tissue specificity. Both genes function in the prolactin signaling pathway, suggesting possible regulation of HBV gene transcription by a similar mechanism. Next, some host genes might indirectly participate in regulating HBV gene transcription through general activation of transcription regulators or factors. For example, HNF1 is a liver-enriched transcription factor that can regulate transcription of HBV genes [16,17]. *PCBD2*, identified herein, regulates dimerization of HNF1 α and enhance its transcriptional activity. Finally, our identified genes might directly contribute to regulation of HBV gene expression. For example, *EIF4G2*, besides down-regulating transcription of *s* and *x* genes, might repress viral RNA translation by forming translationally inactive complexes [39].

There are still significant questions. Human embryonic genome activation (EGA) occurs at the 4–8 cell stage [40,41], why host genes can regulate HBV gene expression in two-cell embryos? Some studies offered the useful clues, which may help shed light on this question. First, it has been demonstrated that EGA in mouse occurs at the two-cell stage, which is controlled by maternally deposited RNAs and proteins. Yu et al. identified lately that oocyte-expressed yes-associated protein is a key activator of EGA in mouse [42]. In the current study, the two-cell embryos were produced by IVF of zona-free golden hamster oocyte with human sperm. EGA in golden hamster also occurs at the two-cell stage [43], whether certain activator (s) deposited in golden hamster oocyte activate human genome? Next, in development of human preimplantation embryo, there are four unique embryonic stage-specific patterns (ESSPs) of gene expression [44]. ESSP1 (maternally inherited oocyte mRNAs) were expressed at high levels at the zygote stage and declined during development to the blastocyst stage. ESSP2 includes embryonic-activated genes, first transcribed at approximately the 8-cell stage. ESSP3 comprises genes not expressed until the blastocyst stage. ESSP4 includes persistent transcripts that maintained stable expression from the zygote to blastocyst stages [41]. It is unknown whether the target genes identified in the current study are included in ESSP4? Undoubtedly, clarification of these questions would explore many mysteries in early embryonic development.

In addition to the above question, HBV *s* gene encodes HBsAg, which packages the viral components. HBV *x* gene encodes HBx protein, whose activity is absolutely required for *in vivo* replication and spread of the virus [44]. Can the target genes affect virion assembly, or affect replication and spread of the virus through regulating viral transcription to block its transmission? Moreover, outcome of HBV infection is markedly heterogeneous, varying from acute asymptomatic self-limiting infection to fulminant hepatic failure or to decompensated cirrhosis and hepatocellular carcinoma. It has been recognized that host genetic background influences the outcome of viral hepatitis infection [45]. In the current study, we found that some host genes upregulate or downregulate transcription of *s* and *x* genes. Can we assume that these host genes together function to maintain homeostasis? Disturbance of such homeostasis could decrease or increase host susceptibility to HBV infection or allow infection to develop in different directions, leading to different clinical outcomes. These questions will be evaluated in future clinical studies.

Human embryo development is more fragile than that of many other species [41]. Human fecundity rates are relatively low, largely due to pre- and post-implantation embryo loss [41]. *In vitro*, 50–70% of IVF embryos fail to reach the blastocyst stage [41,46]. Certain factors that may contribute to abnormal development before EGA include inherited genetic mutations, aneuploidy, environmental insult to germ cells, events during fertilization and sperm-related factors [41]. In our previous study, it was detected that HBV infection induces various chromosomal abnormalities in patient sperm, including aneuploidy, acentric fragment and deletion, ring chromo-

some, triradial, dicentric chromosome and pulverization [8]. Sperm with these chromosomal abnormalities can achieve normal fertilization and introduce these aberrations into the embryo, which may increase the risk of abortion, stillbirth, or birth defects [8]. In a recent clinical literature, HBV mRNA was found in abandoned IVF embryos of HBV-infected fathers, which confirmed that HBV could not only enter early cleavage embryos via sperm but also replicate in embryos, resulting in early abortion [47]. The aforementioned findings suggested that HBV may interfere with early embryonic development and thus affect pregnancy outcome. Therefore it is very important to investigate HBV gene transcription and its regulation mechanism in embryos.

5. Conclusions and perspectives

This study, for the first time, provides experimental evidence that transcription of HBV genes occurs in early embryonic cells and is regulated by host genes. It is worth mentioning that, besides HBV, there are more emerging infectious diseases virus, such as hepatitis C virus (HCV), human immunodeficiency virus (HIV), severe acute respiratory syndrome virus (SARS), Ebola virus and Zika virus (ZIKV), which pose a serious threat to human health [48–52]. Lately, some studies begin to investigate the true vertical transmission of HCV and HIV via germline [34,53–56], but the research on such transmission of SARS, Ebola and ZIKA viruses is still a blank. To clarify whether the aforementioned viral genes are transmitted via germline and their expressional regulation in embryo would make a great contribution to exploring the interplay mechanism between the viruses and host cells and to maintaining human reproductive health.

Conflicts of interest

The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, and in the decision to publish the results.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.reprotox.2017.08.009>.

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