CpG methylation participates in regulation of hepatitis B virus gene expression in host sperm and sperm-derived embryos

Aim: This study was undertaken to investigate relationship between hepatitis B virus (HBV) CpG methylation and HBV gene transcription in sperm and sperm-derived embryos. **Methods:** HBV-infected patient sperm and HBV plasmid-transfected donor sperm were subjected to interspecific *in vitro* fertilization, methylation-specific PCR, bisulfite sequencing PCR, reverse transcription PCR and real-time quantitative PCR. **Results:** Positive methylation bands for CpG islands II and III in the HBV genome were observed in patient sperm but not in controls, and methylation percentages of CpG sites varied among different patient sperm samples. After fertilization, CpG sites were highly demethylated in embryos. Transcriptional levels of HBV X and S genes increased with decrease in CpG site methylation percentages. **Conclusion:** HBV CpG sites can be methylated in patient sperm before maturation. Methylation of CpG islands II and III participates in transcriptional regulation of HBV X and S genes, respectively, in sperm and sperm-derived embryos.

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Keywords: hepatitis B virus • methylation and gene expression • sperm and embryos

Hepatitis B virus (HBV) can invade the human male germline, and its DNA has been detected at various stages of spermatogenesis in patients with chronic HBV infection [1,2]. In 1985, Hadchouel *et al.* found presence of integrated HBV DNA sequences in patient sperm and assumed the possibility of true vertical transmission of HBV via the germline [3]. Thereafter, some studies detected that HBV DNA is able to integrate into sperm chromosomes of the patients and such HBV DNA-carrying sperm can enter oocyte to achieve normal fertilization [4]. After fertilization, the sperm-introduced HBV genes remain their function in replication and expression in embryonic cells [5–7]. It is worth mentioning that using FISH, the positive signals for HBV DNA were detected in both nuclei of individual two-celled embryo derived from the sperm carrying HBV genes, which suggested that the sperm-introduced HBV genes

are able to replicate concomitantly with the host embryo genome, and the two viral gene copies segregated normally into the daughter cells during the first cleavage [5]. This mode of replication is semiconservative and completely different from the canonical replication mode of HBV particles (virion) through its life cycle including cccDNA formation [8,9]. Recently, Hu *et al.* collected 578 embryos from couples with at least one HBsAg seropositive partner. The FISH results showed that HBV DNA was present in 14.4% (83/578) of embryos, and the rates of HBV DNA positive embryos among the couples, in whom the woman, man or both partners were HBsAg seropositive, were 13.1% (57/436), 21.3% (16/75) and 14.9% (10/67), respectively [10]. The increasing studies demonstrated that not only HBV but also the other viruses, such as HIV-1 and hepatitis C virus (HCV) are able to integrate into sperm genome of the patients and vertically transmit

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to embryos [10–18]. Because HBV has a potentially negative effect on male reproductive function and because dangerous infections can be transmitted to partners and newborns [19,20], the mechanism underlying the regulation of HBV gene expression in sperm and embryos has attracted the attention of researchers.

The HBV genome contains four partially overlapping open reading frames (*pre-S/S*, *core/e*, *X* and *P* genes) that are under the transcriptional control of four promoters (sp1, sp2, cp and xp) and two enhancer elements (Enh I and Enh II). These effectors modulate the transcription and translation of seven different HBV proteins using distinct in-frame start codons [8,9]. DNA methylation, an important form of epigenetic modification, plays a crucial role in the repression of gene transcription, which is estimated to occur at approximately 70–80% of CG dinucleotides throughout the genome [21]. The remaining unmethylated CG dinucleotides are grouped into clusters (CpG islands) that are predominantly located in or near gene promoters [21]. A 3.2-kb partially double-stranded circular HBV genome was found to contain three CpG-rich regions spanning the ATG start site for surface antigen gene (island I), Enh I with X-protein gene promoter (island II) and the ATG start site of polymerase gene (island III) [22–26]. It is well recognized that hypermethylation of CpG islands is involved in gene silencing at the transcriptional level [27]. However, the following points remain unclear. First, it is unknown what occurs when HBV genes are introduced into human sperm. HBV DNA is methylated in certain human somatic tissues, such as non-neoplastic liver tissues and HBV-associated hepatocellular carcinoma tissue [26,28], but it is not known whether it is methylated in human male germ cells. Next, in mammals, there are at least two developmental periods, in germ cells and in preimplantation embryos, in which the methylation patterns are reprogrammed throughout the genome [29,30]; thus, it is important to identify changes in methylation patterns that occur at CpG sites in the HBV genome during transmission of the virus gene from sperm to sperm-derived embryos. Finally, among the three CpG islands in the HBV genome, islands II and III are of particular interest because, in contrast with island I that is absent from approximately 50% of HBV sequences isolated from patients residing in 48 different countries, these islands are conserved across genotypes and are present in all HBV sequences [23–25]. Island II encompasses Enh I and the *X* gene promoter, and island III is located upstream of the sp1 and sp2 promoters [23–25]. Whether CpG islands II and III participate in the regulation of the HBV *X* and *S* gene expression in sperm and sperm-derived embryos remains to be elucidated.

The aim of this study was to answer the aforementioned questions to increase understanding of the potential involvement of epigenetic modifications of HBV DNA in a novel mechanism of regulation of viral gene expression in human sperm and sperm-derived embryos.

Materials & methods

Subjects

Eleven patients ranging in age from 22 to 38 years who sought fertility treatment at the hospital were clinically diagnosed as chronic HBV carriers by antigen–antibody combination assay (Supplementary Table 1). Eleven healthy donors ranging in age from 22 to 38 years were negative for HBV markers and selected as controls.

Preparation of sperm & oocyte, insemination & postinsemination cultures

Preparation of human motile sperm and zona-free hamster ova, insemination and post-insemination cultures were performed as described previously [5]. Inseminated oocytes were incubated for 24 h until formation of two-celled embryos.

Plasmid methylation

Unmethylated and methylated pBR322-HBV plasmids containing the full-length HBV genome were used in this study. Methylation of the plasmid was performed in a 50 µl reaction mixture containing plasmid DNA (0.5 µg/µl, 5 µl), 10× NEBuffer (5 µl; New England BioLabs, Beijing, China), S-adenosylmethionine (SAM; 1.6 mM, 5 µl), CpG methyltransferase (M. SssI, 4U/µl, 2 µl; New England Biolabs) and H₂O (33 µl). After incubation at 37°C for 1 h, the reaction was stopped by heating at 65°C for 20 min. DNA was purified by phenol–chloroform extraction and ethanol precipitation.

Exposure of human sperm to plasmids

Donor sperm samples were transfected with the unmethylated or methylated plasmid. Briefly, 100 µl of a transfection solution containing 2 µg plasmid and 3 µl FuGENE® HD Transfection Reagent (Roche, Guangzhou, China) was prepared in sterile water and kept at room temperature for at least 15 min. Sperm cells were collected and incubated in fresh Biggers–Whitten–Whittingham medium supplemented with 1.5% bovine serum albumin for capacitation. One hour later, the plasmid (final concentration of 1.5 µg/ml) was added to capacitating medium. After exposure to the plasmid and an additional 3 h of incubation for capacitation, the sperm cells were washed five-times to remove the plasmid and were then used to fertilize zona-free hamster ova *in vitro* to obtain two-celled embryos.

Detection of plasmid transfection efficiency into sperm

To measure the transfection efficiency of the unmethylated and methylated plasmids into sperm, a Nick Translation Kit (Roche, Basel, Switzerland) and Fluorescein-12-dUTP (Thermo Fisher Scientific, MA, USA) were employed. The two plasmids were labeled with fluorescein-12-dUTP by nick translation according to the manufacturer's instructions. Transfected sperm were detected by their green fluorescence and counted under a fluorescence microscope.

CpG sites in islands II & III in the HBV genome

As reference sequences, three normal HBV sequences were obtained from GenBank of the National Center for Biotechnology Information (NCBI; accession numbers: EU871997, AY206384 and AY123424). The schematic structure of the HBV genome, including the CpG island distribution in the representative HBV genomic sequence, is shown in [Supplementary Figure 1](#). Island II is located at nucleotides 1170–1669, which encompasses Enh I and the X gene promoter and part of the X gene. Island III is located at nucleotides 2298–2446, which is upstream of the sp1 and sp2 promoters. Primer pairs were designed using MethPrimer [31] to amplify a segment of island II (nt1249–1380) containing seven CpG sites and a segment of island III (nt2369–2480) containing eight CpG sites. The seven CpG sites in island II were located in the X promoter region near enhancer I, specifically at -20, -42, -48, -68, -74, -100 and -112 nucleotides, respectively, from the X promoter. The eight CpG sites in island III were located upstream of the sp1 and sp2 promoters, specifically at -278, -296, -299, -301, -303, -316, -322 and -327 nucleotides, respectively, from the sp1 promoter ([Supplementary Table 2, Supplementary Figures 1 & 2](#)).

Detection of methylation statuses of CpG sites in islands II & III in the HBV genome

A Universal Genomic DNA Extraction Kit (TaKaRa, Dalian, China) was used to extract DNA from sperm transfected with plasmids, as well as from sperm from the patients and controls and from sperm-derived embryos, according to the manufacturer's protocol. Bisulfite conversion of DNA was performed using an EZ DNA Methylation-Gold™ Kit (for sperm) or an EZ DNA Methylation-Direct Kit (for embryos; ZYMO, Orange, CA, USA) according to the manufacturers' instructions.

The PCR mixture contained bisulfite-modified DNA (2 µl), Taq™ Hot Start (12.5 µl; TaKaRa), the forward and reverse primers (10 µM, 0.5 µl each) and ddH₂O (9.5 µl). The PCR conditions were as follows: denaturation at 95°C for 3 min; 39 cycles at 94°C for

30 s, 55°C for 30 s and 72°C for 1 min; a final extension at 72°C for 5 min; and storage at 4°C. For methylation-specific PCR (MSP), bisulfite-modified DNA was amplified using a methylation-specific or unmethylation-specific primer pair ([Supplementary Table 3](#)) that had been previously confirmed not to amplify nonbisulfite-modified DNA. For bisulfite sequencing PCR (BSP), bisulfite-treated DNA was amplified using BSP primers ([Supplementary Table 3](#)), and the amplified products were cloned into a pMD®18-T vector (TaKaRa). Fourteen colonies for each sample were randomly chosen for sequencing.

For the sperm from the patients and controls, the BSP-amplified products were divided into two aliquots: one was subjected to electrophoresis through a 1.5% agarose gel and stained with ethidium bromide, and the other was cloned into a pEASY-T3 vector (TransGen Biotech, Beijing, China), and 16 colonies for each sample were randomly chosen for sequencing.

Detection of the HBV X & S gene transcription using reverse transcription PCR & real-time quantitative PCR

Total RNA was extracted from sperm transfected with plasmids, as well as from sperm from the patients and controls and from sperm-derived embryos, using TRIzol reagent (Life Technologies, CA, USA). The cDNA was synthesized with a PrimeScript™ RT Reagent Kit with gDNA Eraser (Cat. #RR047A; Takara Biotech, Dalian, China) according to the manufacturer's protocol.

Reverse transcription PCR (RT-PCR) was performed in a 20 µl reaction volume using a QuantiFast™ SYBR® Green PCR Kit (Qiagen, Hilden, Germany). Human/mouse *β-actin* genes were used as internal control genes for the sperm/embryo samples, respectively. The primer pairs used for *β-actin* and the HBV X and S genes are shown in [Supplementary Table 3](#). The cycling conditions were as follows: 5 min at 95°C, followed by 40 cycles of 15 s at 95°C and 30 s at 60°C. The amplified PCR fragments were subjected to electrophoresis through a 1.5% agarose gel and stained with ethidium bromide.

Real-time quantitative PCR (qRT-PCR) was performed using a QuantiFast SYBR® Green PCR Kit (QIAGEN, Shanghai, China) according to the manufacturer's instructions. *GAPDH* was used as an internal control gene. The 20 µl reaction mixture contained cDNA template (2 µl), 2× QuantiFast SYBR® Green PCR Master Mix (10 µl), the forward and reverse primers (10 mM, 0.4 µl each) and RNase-free water (7.2 µl). The primer pairs used for *GAPDH* and the HBV X and S genes are shown in [Supplementary Table 3](#). The real-time qPCR conditions were as follows: 50°C for 2 min

and 95°C for 5 min, followed by 40 cycles of 95°C for 15 s and 60°C for 30 s. Data analysis for singleplex assay was performed using an ABI 7300 PCR Thermal Cycler and ABI 7300 SDS software v1.4.0 (ABI, CA, USA).

Treatment with SAM

After fertilization of zona-free hamster oocytes with patient sperm, inseminated ova were divided into two groups. In the treatment group, SAM (8 mM, 1 μ l) was added to a droplet (50 μ l each) of ovum culture medium (Flow Laboratories, Germany) containing the inseminated ova at the beginning of postinsemination culturing. For the control group, no SAM was added. After incubation for 24 h, two-celled embryos were collected to detect the methylation statuses of the CpG sites in islands II and III using BSP and to assess the transcriptional levels of the HBV *X* and *S* genes using real-time qRT-PCR and the $2^{-\Delta\Delta C_t}$ method.

Statistical analysis

The Spearman's correlation analysis and graphing were performed using GraphPad Prism 7 software to assess the associations between the transcriptional

levels of the HBV *X* and *S* genes and the methylation percentages of CpG sites in islands II and III in the HBV genome, respectively, in the sperm and sperm-derived embryos. The Wilcoxon signed rank test for paired samples was performed using SPSS 16.0 software to identify significant differences in the average methylation percentages of CpG sites in islands II and III between the sperm and sperm-derived embryos. A *p*-value of <0.05 was considered significant.

Results

HBV genes in plasmid-transfected sperm & sperm-derived embryos

Methylation statuses of CpG sites in islands II & III in the HBV genome

Transfection efficiency

Human sperm samples were transfected with a plasmid, pBR322-HBV, which was either unmethylated or methylated. The transfection efficiencies of the unmethylated and methylated plasmids into the sperm were $36.8 \pm 2.67\%$ and $33.9 \pm 1.86\%$, respectively. There was no significant difference in transfection efficiency between the two plasmids (*p* > 0.05).

Figure 1. CpG site methylation in islands II and III in the hepatitis B virus genome in sperm transfected with an unmethylated plasmid and in sperm-derived embryos, as detected by bisulfite sequencing PCR. (A) Detection of CpG site methylation in island II. (A1) in plasmid; (A2) in sperm; and (A3) in sperm-derived embryo. (B) Detection of CpG site methylation in island III. (B1) in plasmid; (B2) in sperm; and (B3) in sperm-derived embryo. The methylation statuses of 14 clones for each sample are presented; each column represents one CpG position in island II or III, and each circle in the column indicates either cytosine (open circles) or methyl cytosine (filled circles).

Figure 2. CpG site methylation in islands II and III in the hepatitis B virus genome in sperm transfected with a methylated plasmid and in sperm-derived embryos, as detected by bisulfite sequencing PCR. (A) Detection of CpG site methylation in island II. (A1) in plasmid; (A2) in sperm; and (A3) in sperm-derived embryo. (B) Detection of CpG site methylation in island III. (B1) in plasmid; (B2) in sperm; and (B3) in sperm-derived embryos.

Sperm transfected with the unmethylated plasmid

The BSP results showed that CpG sites in islands II and III in the HBV genome were hypomethylated in the unmethylated plasmid and in the sperm and sperm-derived embryos; the methylation percentages were 2.04, 3.06 and 2.04% for island II and 0.89, 1.78 and 0.89% for island III, respectively (Figure 1).

Sperm transfected with the methylated plasmid

The methylation statuses of CpG sites in islands II and III in the HBV genome were analyzed by MSP. For the methylated plasmid, the M product, which was amplified using a methylation-specific primer, was observed in islands II and III but not in the negative control. The U product, which was amplified using an unmethylated-specific primer, was not detected in either island (Supplementary Figure 3A). In the sperm, only the M product was observed in island II, but both the M and U products were observed in island III. Neither the M nor the U product was detected in the negative control (Supplementary Figure 3B). In the

sperm-derived embryos, only the U product was observed in both islands but not in the negative control (Supplementary Figure 3C).

The methylation statuses of CpG sites in islands II and III in the HBV genome were further analyzed by BSP. These sites were hypermethylated in the methylated plasmid, with methylation percentages of 96.93 and 95.5%, respectively (Figure 2A1 & 2B1). In the transfected sperm, CpG sites in island II were also hypermethylated, with a methylation percentage of 95% (Figure 2A2), but some sites in island III were demethylated, with a decrease in the methylation percentage from 95.5 to 82.14% (Figure 2B2). In the sperm-derived embryos, most CpG sites in both islands II and III were demethylated, with methylation percentages of 3.06 and 1.78%, respectively (Figure 2A3 & 2B3).

Transcription of the HBV X and S genes in transfected sperm & sperm-derived embryos

A positive band for the internal control gene (β -actin) was observed by RT-PCR for both sperm and sperm-derived embryos. However, only a positive band for

Figure 3. Transcription of the HBV X and HBV S genes in sperm transfected with a methylated plasmid and in sperm-derived embryos. (A) In sperm. M: marker DL2000; 1: X gene; 2: S gene; 3: -RT; 4: positive control; 5: β -actin; and 6: -T. **(B)** In sperm-derived embryos. M: marker DL2000; 1: X gene; 2: S gene; 3: -T; 4: -RT; 5: positive control; and 6: β -actin.

the S gene was observed for the sperm (Figure 3A), and positive bands for both the X and S genes were detected for the sperm-derived embryos (Figure 3B).

The effects of CpG site methylation on the transcriptional levels of the X and S genes in human sperm and sperm-derived embryos were further assessed by real-time qRT-PCR and the $2^{-\Delta\Delta CT}$ method. The transcriptional levels of these genes in the sperm transfected with the methylated plasmid were significantly lower than those in the sperm transfected with the unmethylated plasmid. The transcriptional levels of the X and S genes decreased by 8333- and 11.63-fold, respectively, in the sperm transfected with the methylated plasmid compared with that transfected with the unmethylated plasmid (Table 1). These levels were very similar between embryos derived from sperm transfected with the methylated plasmid and those derived from sperm transfected with the unmethylated plasmid (Table 1), with no significant differences detected ($p > 0.05$).

HBV genes in patient sperm & sperm-derived embryos

Methylation statuses of CpG sites in islands II & III in the HBV genome

Gel electrophoresis of the BSP-amplified products revealed the presence of methylation-specific bands for CpG sites in islands II and III in the sperm from the 11 HBV patients but not in that from the 11 controls. The sizes of the amplified products were 132 and 112 bp for islands II and III, respectively (Figure 4).

The sequencing results for the BSP-amplified products showed variable methylation percentages of CpG sites in islands II and III in the sperm from the 11 HBV patients, with mean values of $56.66 \pm 35.27\%$ (range of 9.82 to 95.54%) and $62.43 \pm 32.42\%$ (range of 10.16 to 96.09%), respectively. After fertilization, the CpG sites in islands II and III were highly demethylated, with mean values of $3.33 \pm 2.37\%$ (range of 0.89 to 8.04%) and $3.20\% \pm 2.48\%$ (range of 0.78 to 7.81%) in the sperm-derived embryos, respectively (Supplementary Figure 4; Table 2).

Table 1. Effects of the methylation statuses of CpG sites on the transcriptional levels of the hepatitis B virus X and S genes in human sperm transfected with an unmethylated and a methylated plasmids and in sperm-derived embryos, respectively.						
Samples	HBV X ($2^{-\Delta\Delta CT}$)			HBV S ($2^{-\Delta\Delta CT}$)		
	M	Un-M	FCV	M	Un-M	FCV
Sperm	$1.2 \times 10^{-4***}$	1.00	-8333	0.086***	1.00	-11.63
Sperm-derived embryo	0.96*	1.00	0.96	0.96*	1.00	0.96
* $p > 0.05$. *** $p < 0.0001$. Real-time qRT-PCR and the $2^{-\Delta\Delta CT}$ methods were used to evaluate effects of the methylation statuses of CpG sites on the transcriptional levels of the X and S genes in human sperm transfected with an unmethylated or a methylated plasmid and in sperm-derived embryos, respectively. The data were presented as an FCV in transcription of the X and S genes in the M normalized to internal control gene (<i>GAPDH</i>) and relative to those in the Un-M. The individual data were converted into a linear form using $2^{-\Delta\Delta CT}$ calculation and then subjected to a paired-sample t test to determine whether there was a significant difference in average transcriptional levels of the X and S genes between the methylated and unmethylated groups. A p-value of less than 0.05 was considered significant. FCV: Fol change value; M: Methylated group; Un-M: Unmethylated group.

Figure 4. For sperm from the hepatitis B virus-infected patients and controls, aliquots of the bisulfite sequencing PCR amplified products were subjected to electrophoresis through a 1.5% agarose gel and stained with ethidium bromide. Methylation-specific bands for CpG sites in island II (A) and island III (B) in the hepatitis B virus genome were observed for the patient sperm but not for the control sperm. C1–C11: Sperm from controls; M: Marker; P1–P11: Sperm from the patients.

The mean values significantly differed between the sperm and sperm-derived embryos ($p < 0.01$).

Transcriptional levels of the HBV X & S genes

The transcriptional levels of the *X* and *S* genes increased with the decreases in the methylation percentages of CpG sites in islands II and III, respectively, in the sperm from the 11 patients and in the sperm-derived embryos (Figure 5 & Table 2). A strong negative correlations was detected between the methylation percentages and transcriptional levels for both the sperm ($r = -1$, $p < 0.0001$ for *X* gene; $r = -0.9909$, $p < 0.0001$ for *S* gene) and sperm-derived embryo ($r = -0.9623$, $p < 0.0001$ for *X* gene; $r = -0.9745$, $p < 0.0001$ for *S* gene).

Effects of SAM on the methylation statuses of CpG sites in islands II & III & on the transcriptional levels of the HBV X & S genes

The methylation percentages of CpG sites in islands II and III in SAM-treated embryos were markedly increased (61.61 and 55.47%) compared with those in untreated embryos (1.79 and 0.78%) (Figure 6). Further, the transcriptional levels of the *X* and *S* genes decreased by 2.13- and 2.04-fold, respectively, in the SAM-treated embryos compared with the untreated embryos (Table 3).

Discussion

In the current study, two measures were taken to ensure reliable and accurate results. First, combined bioinformatic and experimental analysis was performed. Given that HBV DNA contains three conventional CpG islands (I, II and III) [23–26], we searched GenBank of the NCBI and retrieved three normal HBV sequences (accession numbers: EU871997, AY206384 and AY123424) for use as reference sequences. We then verified the locations and sizes of islands I, II and III in the representative HBV genomic sequences. The relationships between CpG site methylation in island II and III and the transcriptional levels of the *X* and *S* genes in sperm and sperm-derived embryos were then investigated using experimental methods. Next, combined *in vitro* and an *in vivo* experiment was performed. For the *in vitro* experiment, sperm from healthy donors was transfected with either an unmethylated or a methylated plasmid, pBR322-HBV, containing the full-length HBV genome. This allowed for determination of differences in the methylation statuses of CpG sites in the HBV genome and for assessment of the effects of such differences on the regulation of HBV gene expression in sperm and sperm-derived embryos. For the *in vivo* experiment, we performed analysis using sperm cells from HBV-infected

Table 2. Methylation percentages (mCpG%) of CpG sites in islands II and III, and the transcriptional levels of the hepatitis B virus X and S genes in the patient sperm and sperm-derived embryos.								
Patients	CpG sites in island II/HBV X gene				CpG sites in island III/HBV S gene			
	Sperm		Two-celled embryos		Sperm		Two-celled embryos	
	mCpG%	2 ^{-ΔΔCT}	mCpG%	2 ^{-ΔΔCT}	mCpG%	2 ^{-ΔΔCT}	mCpG%	2 ^{-ΔΔCT}
2#	95.54	1.00	8.04	1.00	96.09	1.00	7.81	1.00
5#	91.96	1.35	6.25	1.16	93.75	1.32	6.25	1.08
7#	89.29	1.81	5.36	1.17	92.19	1.59	5.47	1.13
8#	88.39	2.10	4.46	1.18	89.84	1.93	4.69	1.15
1#	83.93	2.47	2.68	1.20	86.72	2.25	3.13	1.15
10#	64.29	4.58	2.68	1.21	71.09	4.21	2.34	1.17
11#	44.64	6.38	1.79	1.22	55.47	5.76	1.56	1.17
3#	25.89	12.56	1.79	1.23	40.63	11.67	1.56	1.18
4#	17.86	21.66	1.79	1.23	25.00	20.59	0.78	1.18
9#	11.61	24.04	0.89	1.23	24.22	19.57	0.78	1.19
6#	9.82	34.81	0.89	1.23	10.16	28.56	0.78	1.19
Mean	56.66 ± 35.27	10.25	3.33 ± 2.37	1.19	62.43 ± 32.42	8.95	3.20 ± 2.48	1.14
Using real-time qRT-PCR and the 2 ^{-ΔΔCT} methods, the data were presented as a fold change in transcriptional levels of the X or S genes in the sperm from the 11 patients and in the sperm-derived embryos normalized to an endogenous reference gene (<i>GAPDH</i>) and relative to those of the X or S gene in the sperm and sperm-derived embryo with the highest level of CpG sitemethylation, respectively. HBV: Hepatitis B virus.								

patients to obtain more accurate results regarding CpG site methylation and HBV gene transcription in these patients. Furthermore, the interspecific *in vitro* fertilization was employed in the current study to obtain two-celled embryos. Although using embryo produced by fertilization between human oocyte and human sperm that carries HBV genes would be an ideal model, such a model presents major moral, ethical and legal problems, and is not allowed to use. The interspecific *in vitro* fertilization between human sperm and zona-free golden hamster ova is highly associated with human *in vitro* fertilization as well as avoids the aforementioned problems. Thus this model has been widely used in the research area of reproductive biology and medicine [4–7,15–18,32–36] and included in the “WHO laboratory manual for the Examination and processing of human semen” [37], and recognized by the academic circles.

Methylation statuses of CpG sites in the HBV genome in transfected sperm
CpG sites in islands II and III in the HBV genome were hypomethylated in the unmethylated plasmid (2.04 and 0.89%). Transfection of the plasmid into sperm did not result in a significant change in the methylation status (3.06 and 1.78%). These findings suggest that there is no mechanism for *de novo* methylation of HBV CpG sites in mature sperm, which may be related to that mature sperm lose most of their cytoplasm, as

well as DNA methyltransferases during the process of spermatogenesis [38,39].

CpG sites in islands II and III in the HBV genome were hypermethylated in the methylated plasmid (96.93 and 95.5%). However, interestingly, when this plasmid was transfected into sperm, the CpG sites in island II were still hypermethylated (95%), but those in island III showed a decrease in the methylation percentage from 95.5 to 82.14%. These findings suggested that some CpG sites in island III were demethylated in the mature sperm, which are in accordance with the results of MSP assay. Using MSP assay, only the methylation-specific primer successfully amplified a methylated product in island II. In contrast, both the methylation- and unmethylation-specific primers successfully amplified methylated and unmethylated products, respectively, in island III. Similar results have been observed in a previous study [40]. Further investigation is required to determine whether a demethylation mechanism exists for some viral CpG sites in mature sperm.

Methylation statuses of CpG sites in the HBV genome in patient sperm & sperm-derived embryos
CpG sites in the HBV genome can be methylated in sperm cells before maturation
In the current study, positive methylation bands for CpG sites in islands II and III in the HBV genome were detected in all sperm samples from the 11 HBV-

infected patients but not in those from the 11 controls. CpG islands are normally unmethylated [21,41]; therefore, these results indicate that CpG sites in the HBV genome can be methylated by the cellular DNA methylation machinery in human male germ cells, as observed in human liver tissues [26–28]. However, as mentioned above, CpG sites in the HBV genome could not be

methylated in mature sperm. Thus we speculated that CpG site methylation in the HBV genome in patient sperm might occur prior to the elongation stage in the spermatid nucleus not in mature sperm.

The DNA methylation level is associated with multiple factors, including the induction of DNA methyltransferase activity, short hairpin RNA, and

Figure 5. Correlation between the methylation percentages (mCpG%) of CpG sites in islands II and III in the hepatitis B virus genome and the transcriptional levels ($2^{-\Delta\Delta CT}$) of the hepatitis B virus X and S genes in patient sperm and sperm-derived embryos. The transcriptional levels of the X and S genes increased with decreases in the methylation percentages of CpG sites in islands II and III, respectively, and strong negative correlations were observed. (A) X gene/island II correlation in sperm (Spearman's $r = -1$; $p < 0.0001$); (B) X gene/island II correlation in sperm-derived embryos (Spearman's $r = -0.9623$; $p < 0.0001$); (C) S gene/island III correlation in sperm (Spearman's $r = -0.9909$; $p < 0.0001$); and (D) S gene/island III correlation in sperm-derived embryos (Spearman's $r = -0.9745$; $p < 0.0001$).

Figure 6. Effects of S-adenosylmethionine on the methylation of CpG sites in islands II and III in embryos. (A1) island II with S-adenosylmethionine (SAM); (A2) island II without SAM; (B1) island III with SAM; and (B2) island III without SAM. The results showed that the methylation percentages of CpG sites in islands II and III in the embryos markedly increased following treatment with SAM.

methyl-binding domain-containing proteins and DNA glycosylases activities [21,42]. The activities of such factors varied among the HBV-infected patients, which might be the reason for the differences in the methylation percentages of the CpG sites in the HBV genome among the 11 patients.

Methylation patterns of CpG sites in the HBV genome during transmission from patient sperm to sperm-derived embryos

In male germ cells, methylation begins in prospermatogonia and persists throughout spermatogenesis [43,44]. After fertilization, active demethylation of the paternal genome in the zygote occurs before DNA replication. This process is followed by passive demethylation during the cleavage stage, and *de novo* methylation has been proposed to occur after implantation [45–47]. In the current study, after fertilization, the CpG sites in islands II and III in the HBV genome in both the patient sperm and HBV-transfected sperm were significantly demethylated ($p < 0.01$). These results suggest that the demethylation of CpG sites in islands II and III in the HBV genome is synchronous with active demethylation of the host genome in

sperm-derived embryos. In the host genome, the preferential demethylation of paternal DNA in zygotes is a general phenomenon. One study has reported that the components of the elongator complex (Elp1, Elp3 and Elp4) are important for paternal DNA demethylation [46]. Notably, further studies are needed to determine whether there is any correlation between these factors and the mechanism underlying demethylation in the HBV genome in zygotes and preimplantation embryos.

Relationship between transcription of the HBV X & S genes & methylation of CpG sites in islands II & III in host sperm & sperm-derived embryos

In the current study, the results of the *in vitro* and *in vivo* experiments showed that the transcriptional levels of the X and S genes increased with decreases in the methylation percentages of CpG sites in islands II and III, respectively, in both the sperm and sperm-derived embryos. In addition, strong negative correlations were observed between the methylation percentages and transcriptional levels. These findings indicate that CpG site methylation in islands II and III in the HBV

Table 3. Transcriptional levels of the hepatitis B virus X and S genes in the embryos treated and untreated with S-adenosylmethionine.			
Genes	Treatment with SAM $2^{-\Delta\Delta CT}$	Treatment without SAM $2^{-\Delta\Delta CT}$	Fold change value
HBV X gene	0.47 (0.43–0.51)	1 (0.67–1.33)	-2.13
HBV S gene	0.49 (0.45–0.53)	1 (0.74–1.26)	-2.04

Real time qRT-PCR and the $2^{-\Delta\Delta CT}$ methods were used to evaluate the effects of SAM treatment on expression of the X and S genes in the embryos. The data were presented as the fold change value in expression of the X and S genes in SAM-treated group normalized to internal control gene (*GAPDH*) and relative to those in SAM-untreated group.
HBV: Hepatitis B virus; SAM: S-adenosylmethionine.

genome plays roles in transcriptional regulation of the *X* and *S* genes, respectively, in sperm and sperm-derived embryos.

SAM, the classical methyl donor of numerous methylation reactions, inhibits active demethylation [48]. In the current study, the treatment of embryos with SAM induced the hypermethylation of CpG sites in islands II and III in the HBV genome and correspondingly decreased the transcriptional levels of the *X* and *S* genes by 2.13- and 2.04-fold, respectively, compared with the control treatment. These results further confirm that CpG site methylation in islands II and III in the HBV genome regulates transcription of the *X* and *S* genes, respectively.

To date, it remains uncertain which CpG island contributes to the regulation of surface gene expression. Island I spans the ATG start sites of surface antigen genes, and theoretically, it should be associated with surface gene expression. However, it has been reported that approximately 50% of the HBV sequences isolated from patients residing in 48 different countries lack island I [23]; this island has been detected only in 19.7% of the sequences isolated from Chinese patients with HBV genotype C [24], and it has not been detected in those isolated from European patients with genotype E and G [25]. Nonetheless, the regulation of surface gene expression still occurs in such patients lacking island I. Thus, it is unclear which island participates in regulating surface gene expression. In the current study, the results of the *in vitro* and *in vivo* experiments revealed a strong negative correlation between the transcriptional levels of surface genes and CpG site methylation in island III in sperm and sperm-derived embryos. These findings not only indicate that island III participates in the regulation of surface gene expression but also potentially explain the regulation mechanism of surface gene expression in HBV genomes lacking island I.

Conclusion

This study provides the first conclusive evidence that CpG sites in islands II and III in the HBV genome are methylated in sperm cells before maturation and are synchronously demethylated with active demethylation of the host genome in sperm-derived embryos. Transcriptional regulation of the HBV *X* and *S* genes is closely associated with the methylation of CpG sites in islands II and III in the HBV genome, respectively, which potentially explain the regulation mechanism of surface gene expression in HBV genomes lacking island I.

Supplementary data
To view the supplementary data that accompany this paper please visit the journal website at: <http://www.futuremedicine.com/doi/full/10.2217/epi-2016-0129>

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Financial & competing interest disclosure

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Ethical conduct of research

Semen samples were collected from healthy donors and patients with chronic HBV infection. Written informed consent was obtained from all subjects, permitting the use of their sperm samples for research purposes. All protocols involving human and animal subjects that were used in this study 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 University Medical College (Approval number: SUMC-00-0031), according to the recommended guidelines of the NIH involving human subjects and animal care and the 1975 Declaration of Helsinki.

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Executive summary

- CpG sites in the hepatitis B virus (HBV) genome can be methylated in patient sperm cells before maturation.
- The demethylation of CpG sites in the HBV genome is synchronous with active demethylation of the host genome during transmission from patient sperm to sperm-derived embryos.
- Treatment of embryos with S-adenosylmethionine induced the hypermethylation of CpG sites in the HBV genome and correspondingly decreased the transcriptional levels of the HBV genes.
- Islands II and III participate in the regulation of the HBV *X* and *S* gene transcription, respectively, which potentially explain the regulation mechanism of surface gene expression in the HBV genome lacking island I.

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