Establishment of Cre-mediated HBV recombinant cccDNA (rcccDNA) cell line for cccDNA biology and antiviral screening assays

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1. Introduction

Chronic Hepatitis B virus (HBV) infection remains as one of the most prevalent viral infection in human beings. There are approximately 257 million chronic hepatitis B (CHB) patients worldwide with a significantly increased risk for the development of cirrhosis, liver failure, and hepatocellular carcinoma (HCC) (Global Hepatitis Report, 2017). The efficacy of currently available drugs for CHB including interferon α (IFN-α) or nucleotide(s)ide analogues are far from being satisfactory. More importantly, current therapies rarely achieve the inactivation or elimination of covalently closed circular DNA (cccDNA) pool persistent in the infected hepatocytes (Ahmed et al., 2015; Levrero et al., 2016; Revill and Locarnini, 2016).

HBV cccDNA, as the template of HBV transcription, plays a key role in the life cycle of the virus and permits the persistence of infection. HBV cccDNA is exclusively produced from rcDNA, either from incoming virions or newly formed nucleocapsids. Once formed, cccDNA persists as a minichromosome in the nucleus of infected cells and the regulation of its transcription occurs through epigenetic modulations (Chen et al., 2015; Seeger and Mason, 2015). Most present knowledge on cccDNA is derived from the duck-DHBV model, however, little is known regarding the exact mechanism and factors that may be involved in HBV cccDNA formation, fluctuation and degradation (Guo and Guo, 2015; Lucifora and Protzer, 2016). One of the key obstacles of cccDNA research is the lack of a reliable and quantifiable in vitro culture system that can generate authentic HBV cccDNAs (Nassal, 2015; Li et al., 2017).

The current in vitro systems for cccDNA-orientated studies include non-infection-based systems and in vitro infection systems, both of which have been extensively used, leading to many important discoveries. However, in non-infection-based systems, such as HepG2.2.15 (Sells et al., 1987), HepAD38 (Ladner et al., 1997; Zhou et al., 2006), and HepDE519 (Guo et al., 2007), cccDNA generation relied on the
recycled newly formed nucleocapsids and was very inefficient. cccDNA formation are more efficient in Primary human hepatocytes (PHHs) (Schulze-Bergkamen et al., 2003), stem cell derived hepatocytes (Xia et al., 2017), human liver-derived HepaRG (Gripon et al., 2002; Hantz et al., 2009) and stable HepG2/Huh7-hNTCP cell lines (Yan et al., 2012), but the cccDNA in these infection systems was still difficult to be visualized by Southern blotting, the gold standard for cccDNA detection. Besides, difficult to handle and lack of suitable assay systems for these cell lines restrict their use in high-throughput screening (Chen et al., 2015).

Recently, several recombinant cccDNA (rcccDNA) systems based on site-specific DNA recombination technique were developed (Guo et al., 2016; Li et al., 2016; Yan et al., 2017; Qi et al., 2014). These rcccDNA could be generated in large quantity, served as cccDNA surrogates both in vitro and in vivo, and so provide a new useful model for HBV research and drug discovery. In the present study, based on the Cre/loxP-mediated recombinant HBV rcccDNA system (Qi et al., 2014), we reported the development of stable rcccDNA-producing cell lines termed HepG2-HBV/loxP. In brief, loxP sites flanking monomeric linear HBV genomes were integrated into HepG2 cells using the sleeping beauty transposons system (Kowarz et al., 2015). In the presence of Cre, 3.3-kb rcccDNAs bearing a chimeric intron were produced by Cre/loxP-mediated DNA excision. The chimeric intron could be removed from viral transcripts through RNA splicing, thus would not affect the function of pgRNA and viral replication. The production of HBsAg was cccDNA dependent and served as a surrogate marker of cccDNA. HepG2-HBV/loxP cell lines will provide suitable models both for studying the molecular mechanism of cccDNA regulation and maintenance, as well as for screening of new therapeutic agents to silent and/or eliminate HBV cccDNA.

2. Materials and methods

2.1. Plasmid and viruses

The original SB vector pSBbi-GP was a gift from Eric Kowarz (Addgene plasmid # 60511) and the Transposase pCMV(CAT)7-SB100 was a gift from Zsuzsanna Izsvak (Addgene plasmid # 34879). pSBbi-rcccDNA was created by fusion of the loxP sites flanking monomeric linear HBV sequence (GenBank accession no. V01460.1) from prcccDNA plasmid (Qi et al., 2014) to the pSBbi-GP vector. pAdPLDest-Cre was constructed by cloning Cre sequences into pAdPLDest vector. pAdPLDest-Cre was digested by PscI and then transfected into HEK293 cells to produce pAd-Cre adenoviruses. HBV inoculum was prepared from the culture media of HepAD38 cells.

Fig. 1. Establishment of HepG2-HBV/loxP cell lines. (A) Schematic illustration of HepG2-HBV/loxP cell lines establishment and rcccDNA production. The loxP sites flanking monomeric linear HBV genome were integrated into HepG2 cells by transposon system. In the presence of Cre, the 3.3 kb rcccDNA can be produced by Cre/loxP-mediated DNA excision. The expression of HBsAg is a marker of rcccDNA formation. (B) HBV genome copy number for each clone was detected by quantitative PCR assays. 37 clones were analyzed. (C, D) 1 × 10⁷ Clone 3, 6 and 8 cells were transduced with pAd-Cre at MOI 2 separately. The level of HBsAg in the supernatant was detected by ELISA at indicated time (C). The cccDNA in each clone was extracted by Hirt extraction at 4 days after pAd-Cre transduction. DNA was left untreated or digested with EcoRI restriction nuclease, followed by Southern blotting (D).
2.2. Cell culture

HepG2-HBV/loxP cell lines were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1 μg/ml puromycin. For generating rcccdNA, cells were incubated with pAd-Cre at multiplicity of infection (MOI) 2 for 12 h and maintained in the medium containing 2% FBS. HepDES19 cells were cultured with DMEM/F12 medium supplemented with 10% FBS, plus 400 μg/ml G418, 1 μg/ml tetracycline (tet). For detecting cccDNA in HepDES19, cells were cultured in tet-free medium containing 2% FBS and 0.5% DMSO for 10 days before harvest. Maintenance and HBV infection of HepG2-NTCP cells was performed according to the method described previously (Chen et al., 2016).

2.3. Transposition assays

1 × 10^6 HepG2 cells were co-transfected with 2 μg DNA (1.8 μg pSbBi-rcccdNA + 0.2 μg pCMV-SB100) using PEI (Polysciences, Warrington, PA, USA) (Grabundzija et al., 2010). 2 days post-transfection, 10% of the transfected cells were replated to 10 cm dish and subjected to 1 μg/ml tetracycline (tet). For detecting cccDNA in HepDES19, cells were cultured with tet-free medium containing 2% FBS and 0.5% DMSO for 10 days before harvest. Maintenance and HBV infection of HepG2-NTCP cells was performed according to the method described previously (Chen et al., 2016).

2.4. ELISA and detection of HBV DNA

The HBeAg and HBsAg in the supernatant were examined by ELISA (Kehua, Shanghai, China) according to the manufacturer's instructions. The IFN-β was measured by human IFN-β ELISA (PBL, Piscataway, NJ, USA). HBV DNA in the supernatant was determined by Q-PCR using Hepatitis B viral DNA Quantitative Fluorescence Diagnostic Kit (Shengxiang, Hunan, China).

2.5. Whole-cell DNA extraction and rcccdNA quantitative analysis

Whole-cell DNA was extracted according to the method described previously with minor modifications (Belloni et al., 2012). The cells were lysed in lysis buffer A (10 mM Tris-HCl [pH 8.0], 50 mM NaCl, 1 mM EDTA, 0.1% NP-40) for 10 min at 4°C. After centrifugation, pelleted nuclei were resuspended in lysis buffer B (10 mM Tris-HCl [pH 8.0], 150 mM NaCl, 10 mM EDTA, 0.5% SDS, 0.5 mg/ml Protease K) and incubated overnight at 56°C. Nucleic acids were purified by phenol-chloroform extraction and ethanol precipitation. For rcccdNA estimation, the precipitated DNA was deproteinized with plasmid-safe DNase (Epicentre, Madison, WI, USA) for 1 h at 37°C and then primers P3 and P4 (Qi et al., 2014) (Table S1) were used for Q-PCR amplification. For the detection of remaining HBV DNA integrations, we performed gel electrophoresis to separate the low molecular weight fraction comprising cccDNA molecules from the high molecular weight fraction prior to Q-PCR using primers P5 and P6. The chromosome 19 sequence was amplified as the normalization (Kowarz et al., 2015).
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2.6. Southern/Northern blotting

Hirt’s procedure was used to purify cccDNA (Cai et al., 2013). Briefly, cells from 10 cm dish were lysed in 8 ml SDS lysis buffer (10 mM Tris-HCl [pH 7.5], 10 mM EDTA and 0.5% SDS), mixed with 2 ml of 5 M NaCl, and incubated at 4 °C for at least 16 h. The lysate was then centrifuged at 14,500 g for 30 min at 4 °C. Protein-free DNA was further extracted with phenol and chloroform, followed by precipitation using ethanol. HBV replicative intermediates from intracellular core particles and HBV transcripts were extracted according to the published protocols (Li et al., 2010). HBV DNA and RNA were detected by Southern/Northern blotting according to the method described previously (Chen et al., 2016; Zhang et al., 2016). The Hirt’s DNA was digested with EcoRI restriction nuclease and mitochondrial gene Cox1 was hybridized as the loading control for cccDNA.
HBV pgRNA and core particle DNA were extracted and quantitatively detected according to the method described previously (Zhang et al., 2017). The quantifications of HBV pgRNA and core particle DNA were normalized to 18s rRNA and genomic gapdh separately.

2.8. Immuno fluorescence staining

HepG2-HBV/loxP cells were seeded on the Lab-Tek® Chamber Slide™ (Thermo, Waltham, MA, USA). 3 d post pAd-Cre transduction, cells were fixed with 4% paraformaldehyde, permeabilized and blocked with 0.1% Triton X-100, 10% FBS in PBS. Cells were then incubated with Anti-HBsAg and Anti-HBcAg antibody (ZSGB-BIO, Beijing, China) at 4 °C overnight, washed, and incubated with Alexa Fluor 594 Goat Anti-Mouse IgG or Alexa Fluor 488 Goat Anti-Rabbit IgG (ZSGB-BIO, Beijing, China) for 1 h at room temperature. Cell nuclei were stained with Hoechst. Images were collected with TCS SP5 II Confocal Microscope (Leica, Buffalo Grove, IL, USA).

2.9. Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed according to the method described previously (Chen et al., 2016). Anti-H3ac (39139), anti-H4ac (39926), anti-H3K4me3 (39916) and anti-H3K27ac (39134) antibodies were purchased from Active motif (Carlsbad, CA, USA). Chromatin immunoprecipitates and inputs were quantified by Taqman PCR amplification (ToYoBo, Osaka, Japan) using primers P5 and P6 and probe Pb-1 (Table S1).

2.10. cccDNA quantification

CccDNA was extracted by Hirt’s procedure, pre-treated with plasmid-safe DNase and analyzed by ToYoBo Taqman PCR amplification. For total cccDNA quantification, primers P5 and P6 and probe Pb-1 were used. For simultaneous quantification of rcccDNA and wt cccDNA, a dual Q-PCR system were designed with primers P7 and P8 and probes Pb-2 and Pb-3 targeting the rcccDNA and wt cccDNA separately (Table S1 and Fig. S6). The quantifications of cccDNA were...
normalized to mitochondrial gene Cox1.

3. Results

3.1. Establishment of HepG2-HBV/loxP cell line

We established the HBV rcccDNA producing cell line based on the Cre/loxP-mediated rcccDNA system (Fig. 1A) (Qi et al., 2014). Briefly, the loxP sites flanking monomeric linear HBV genome were integrated into HepG2 cells by transposon system. After puromycin selection, we successfully obtained 37 clones. The copy numbers of HBV genome integrated in all 37 clones were examined by Q-PCR experiments. The majority of clones had insertions in a range of 10–50 copies (28 in 37), 4 clones had insertions less than 10, 5 clones had more than 50 copies of monomeric linear HBV genome integration (Fig. 1B). Since the linear HBV genome breaks at the HBsAg gene reading frame (Qi et al., 2014), HepG2-HBV/loxP cells cannot express HBsAg. We then used adenoviral transduction to achieve the Cre induced-generation of rcccDNA in HepG2-HBV/loxP cells (Fig. S1). In the presence of Cre, 3.3-kb rcccDNAs bearing a chimeric intron can be produced by Cre/loxP-mediated DNA excision. As HBsAg could only be transcribed from the newly generated rcccDNA, the expression of HBsAg is therefore rcccDNA-dependent and can be a marker of rcccDNA formation. As expected, we detected differentially expression levels of HBsAg and rcccDNA in clones after pAd-Cre transduction. Here, we showed the results of clone 3, 6 and 8, which have ∼20 copies, ∼60 copies or ∼2 copies HBV genome integration respectively (Fig. 1C and D). We did pAd-Cre transduction at a low MOI to avoid the activation of innate immune system. Our data showed that no significant interferon-β was induced in HepG2-HBV/loxP cells after pAd-Cre transduction at MOI 2 (Fig. S2).

3.2. Rapidly generation and persistence of rcccDNA in HepG2-HBV/loxP cells

Here, we chose clone 6 as an example to show the characteristics of HepG2-HBV/loxP cell lines. 4 days after transduction by pAd-Cre, a clear supercoiled form of the protein-free DNA band could be detected by Southern blotting. Its supercoiled nature was further confirmed by its resistance to T5 exonuclease and its linearization with EcoRI (Fig. 2A). The copy numbers of the integrated HBV genome and the rcccDNA generated were quantified by Southern blotting. Its supercoiled nature was further confirmed by its resistance to T5 exonuclease and its linearization with EcoRI (Fig. 2A). The copy numbers of the integrated HBV genome and the rcccDNA generated were quantified by Q-PCR. As shown in Fig. 2B, 3-4 days after pAd-Cre transduction, HBV sequence remained in host genome was barely detectable, and copy number of rcccDNA in each cell reached ∼60 copies. We continued to monitor the rcccDNA level for a further 8 days. The level of rcccDNA remained stable for at least 12 days after transduction (Fig. 2B–C). We further did the ChIP assay using primers P3 and P4 (Table S1) to investigate the epigenetic modification of rcccDNA. Four histone modifications with active transcription, H3ac, H4ac, H3K4me3 and H3K27ac were all observed (Fig. 2D). Moreover, the levels of these epigenetic modifications in HepG2-HBV/loxP cells were similar to those in HBV infected HepG2-NTCP cells (Fig. S3). Above results suggested that rcccDNA epigenetically organized as a nascent minichromosome.

3.3. RcccDNA-dependent HBV transcription, replication and expression in HepG2-HBV/loxP cells

To determine the transcription and replication competency of rcccDNA, we first analyzed HBV transcription and replication in HepG2-HBV/loxP cells by Northern blotting and Southern blotting. The secretion of viral antigen and DNA in the culture supernatant was also detected by ELISA and Q-PCR. In clone 6 of HepG2-HBV/loxP cell, RNA transcripts transcribed from the HBV basal core promoter of the integrated HBV genome were detected, which presented as a smear of bands of different molecular weights (Fig. 3A). A Trace expression of HBeAg was also observed (Fig. 3E). While no viral replicative intermediates, both within the capsids and in the supernatant, or HBsAg was detected (Fig. 3B–D). After pAd-Cre transduction, a time-dependent kinetics of synthesis and accumulation of viral transcripts and replicative intermediates were observed (Fig. 3A–B). The rcccDNA-dependent viral replication was similar to the level in HBV infected HepG2-NTCP cells, both of which were lower than HepDES19 cells (Fig. 3C and S4). The synthesis and accumulation of HBsAg in the supernatant could be detected (Fig. 3D) and the expression level of HBeAg was substantially increased after rcccDNA generation (Fig. 3E). The expression of HBsAg and HBeAg were also checked by Immunofluorescence staining (Fig. 3F). After pAd-Cre transduction, HBsAg could be detected in the cytoplasm, while HBeAg was detected in both the cytoplasm and nucleus, reflecting its nucleo-cytoplasmic translocation feature. We further investigated whether there were wild type cccDNA (wt cccDNA) generated through recycling pathway in our HepG2-HBV/loxP cells. Since the wt cccDNA is only 140 bp shorter than rcccDNA, which cannot be separated by Southern blotting, we used the primers P1 and P2 flanking the chimeric insertion, which would amplify a 451 bp fragment from rcccDNA and a 291 bp fragment from wt cccDNA (Qi et al., 2014). Weak bands of 291 bp could be detected at the later time of pAd-Cre transduction, suggesting recycling pathway also exists in the HepG2-HBV/loxP cells (Fig. 3G). The proportion of rcccDNA and wt cccDNA was determined by dual Q-PCR system (Fig. S6A–B). A time-dependent generation of wt cccDNA was observed. On 12 days after transduction, the wt cccDNA accounted for less than 20% of the rcccDNA.

3.4. Inhibitory efficacies of anti-HBV agents in HepG2-HBV/loxP cells

Our above data demonstrated the existence of rcccDNA-dependent HBV replication and expression in HepG2-HBV/loxP cells. Thus, this cell model may permit the evaluation of anti-HBV drugs with different mechanisms of action. To test the usefulness of this system, clone 6 of HepG2-HBV/loxP cells were transduced with pAd-Cre and further cultured 3 days for rcccDNA generation. Then the cells were mock-treated or treated with IFN-α or Adefovir. As APOBEC3A (A3A) was reported to induce cccDNA degradation (Lucifora et al., 2014), we added another group of cells to over-express A3A from lentiviral vector plasmid pLent6.3 (Fig. 4A). The results showed that, while Adefovir treatment efficiently reduced the HBV replicative intermediates in intracellular core particles (Fig. 4E), IFN-α treatment efficiently reduced the HBV pgRNA (Fig. 4D) and Core particle DNA at later time points (Fig. 4E). These results were consistent with the action mechanisms of IFN-α and Adefovir. In addition, A3A substantially reduced HBsAg (Fig. 4B), HBeAg (Fig. 4C) and cccDNA (Fig. 4F). The reduction of cccDNA by A3A was further confirmed by Southern blotting (Fig. 4G). PCR amplification of the Hirt DNA extraction from each group clearly shown that while IFN-α and ADV treatment could efficiently reduced the wt cccDNA generation, A3A was the only one reducing the rcccDNA (Fig. 4H and Fig. S6C). These findings demonstrate that the efficacies of different types of anti-HBV drugs, such as direct-targeting cccDNA agents and transcription or replication inhibitors, can easily be evaluated using this model. These results indicate that HepG2-HBV/loxP cells are valuable platform for anti-HBV drug discovery.

4. Discussion

HBV cccDNA plays a central role during HBV persistent infection and is the main obstacle for curative therapy. Lacking of a robust, reliable and quantifiable in vitro culture system that can generate authentic HBV cccDNA greatly hindered the cccDNA biology research and anti-HBV drug discovery process. Recently, several HBV cccDNA models were reported (Guo et al., 2017; Lucifora et al., 2017). ZH Qi et al. reported an HBV cccDNA system through Cre/loxP-mediated site-specific DNA recombination (Qi et al., 2014). Here, combined the Cre/
loxp and sleeping beauty transposons system, we established HepG2-derived cell lines integrated with different copies of monomeric HBV genome flanked by loxp sites, termed HepG2-HBV/loxp. In the present of Cre recombinase, 3.3-kb rcccDNAs can be produced and could be easily detected by Southern blotting using the digoxigenin (DIG) probe system. The cccDNA-dependent HBsAg is a specific surrogate marker for cccDNA. Such strategy could also be applied to conveniently establish new cell lines integrated different HBV genotypes and mutations.

We found several characteristics and advantages of our cell system for HBV cccDNA study. First, rcccDNA is quickly generated in our cell lines. 3–4 days post pAd-Cre transduction, the Cre/loxp-mediated recombination was completed and the cccDNA pool was stable. While in the current HBV stable cell lines, such as tetrahymena-cyclinducible (tet-off) HepAD38 and HepDE19/DES19 cells, cccDNA is generated through the intracellular nuclear re-importing of the newly synthesized nucleocapsids, thus at least 8–10 days are required for accumulating a detectable cccDNA pool (Zhou et al., 2006; Guo et al., 2007). In the infection system, 7–10 days waiting are usually required (Hantz et al., 2009; Iwamoto et al., 2014). However, notably a recently reported C3AINTC0 cell line, in which the cccDNA can be detected at 2 h post-infection by Q-PCR analyses and at 24 h by Southern blotting, thus would be a powerful system for HBV antiviral therapy and basic research (Guo et al., 2017).

Second, the copy number of rcccDNA generated in HepG2-HBV/loxp cells is predictable and quantifiable. As the copy numbers of HBV genome integration in the cell line could be accurately measured, how many rcccDNA would be produced is predictable. The rcccDNA contains a chimeric intron, and thus could be accurately quantified by real-time PCR using primers targeting the intron sequences, avoiding the interference signal from the viral replication intermediates. This characteristic makes our cell lines a more stable and accurate model than the previously transient transfection system as well as several in vitro recombination and transfection system which were recently reported (Cai et al., 2013; Li et al., 2016). Furthermore, we have dozens of clones which could produce different copies of rcccDNA. These clones will be good models for quantitative analysis of cccDNA maintenance and activity.

Third, in our cell lines, no HBV sequences will remain in the genome of cells after Cre excision, thus all viral RNAs can only be transcribed from cccDNA, just like the infection system. This characteristic makes our HepG2-HBV/loxp system is more suitable for identification of factors that regulate cccDNA metabolism and transcription by comparison with the current HBV stable cell lines in which the transfected plasmid, rather than cccDNA, serves as the dominant transcription template.

Finally, in our cell lines, HBsAg is the surrogate marker of cccDNA. Compared to HepAD38 and HepDE19/DES19 cells which take HBsAg as the cccDNA surrogate marker, HBsAg is a better readout signals for high throughput screening (Cai et al., 2016).

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