Infection of Human Hepatocyte-chimeric Mice with HBV and *in vivo* Treatment with εRNA

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[Abstract] Hepatitis B virus (HBV) can cause both acute and chronic disease in human liver with potentially high risk of cirrhosis and liver cancer. The host range of non-human primates susceptible to this virus is limited. Therefore, experimental studies with human hepatocyte-chimeric mice provide an invaluable source of information regarding the biology and pathogenesis of HBV. This section describes the protocol for infection of the human hepatocyte-chimeric mice with HBV. In addition, it has recently been shown that HBV replication can be suppressed by exogenous expression of viral epsilon RNA (εRNA; Sato *et al*., 2015), which serves as an encapsidation signal (Bartenschlager *et al*., 1992). Based upon this finding, we also describe the protocol for the liposome-mediated delivery of a plasmid encoding εRNA to liver in these chimeric mice.

Materials and Reagents

1. 0.1-10 µl pipet tips (Thermo Fisher Scientific, catalog number: QSP#TF104)
2. 1-200 µl and 100-1,000 µl pipet tips (Corning, catalog number: 4845 and 4846, respectively)
3. 0.2 ml 8 strips PCR tubes and caps (NIPPON Genetics, catalog number: FG-028DC)
4. 1.5 ml and 2.0 ml microcentrifuge tubes (Corning, catalog number: MCT-150-A and MCT-200-C, respectively)
5. 15 ml and 50 ml centrifuge tubes (Corning, catalog number: 352096 and 352070, respectively)
6. 96-well fast plate (NIPPON Genetics, catalog number: 38801)
7. 1 ml syringe (MonotaRO Co., NIPRO Genetics, catalog number: 08-010)
8. Human hepatocyte-chimeric mice (PhoenixBio Co.)
   Note: Chimeric mice are intravenously infected with 100 µl of HBV-C in saline solution (10^6 copies per mouse) derived originally from patient with chronic hepatitis (Sugiyama et al., 2006).
9. HBV (genotype C; HBV-C) (Dr. Yasuhito Tanaka, Department of Virology and Liver Unit, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan; Sugiyama *et al.*, 2006)

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10. Sodium chloride (Nacalai tesque, catalog number: 31320-05)
11. YSK lipid (a pH-sensitive cationic lipid) (Faculty of Pharmaceutical Sciences, Hokkaido University, Sapporo, Hokkaido, Japan) (Sato *et al*., 2012)
12. Cholesterol (Avanti Polar Lipid, catalog number: 57-88-5)
13. 1, 2-dimyristoyl-sn-glycerol methoxypolyethyleneglycol (PEG-DMG) (NOF Corporation, catalog number: GM-020)
14. Nuclease free-H_2O
15. Citrate buffer (12.5 mM citrate, 500 mM NaCl)
16. pCpGfree-mcs vector (Invivogen)
17. Primers for vector construction
    pLKO.1 Fw SpeI: CCCACTAGTTTTCCCATGATTCCTTCATATTT
    pLKO.1 Rv BglII: CCCAGATCTAAAATTGTGGATGAATACTGCC
18. TaqMan Universal PCR Master Mix (Life Technologies, catalog number: 4304437)
    Note: Currently, it is "Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4304437".
19. Primers and probe for quantification of HBV DNA from HBV-infected chimeric mice sera:
    Forward Primer: SF2: 5'-CTTCATCCTGCTGCTATGCCT-3'
    Reverse Primer: SR2: 5'-AAAGCCCAGGATGATGGGAT-3'
    Probe: SP2: FAM-ATGTTGCCCGTTTGTCCTCTAATTCCAG-TAMRA
20. MISSION® pLKO.1-puro Empty Vector Control Plasmid DNA (Sigma-Aldrich, catalog number: SHC001)
21. DNA oligo nucleotides (5'-3')
    Sense:
    CCGGTGTACATGTCCCACTGTTCAAGCCTCCAAGCTGTGCCTTGGGTGGCTTTG GGGCATGGACATTTTTG
    Antisense:
    AATTCAAAAATGTCCATGCCCCAAAGCCACCCAAGGCACAGCTTGGAGGCTTGA ACAGTGGGACATGTACA
22. Oligo nucleotides (see Recipes)

Equipment

1. Biosafety hood in a biosafety level 3 (BSL3) facility (HITACHI, catalog number: SCV-1303 ECIIB)
2. Pipettes (PIPETMAN P2, P20 and P1000) (Gilson Scientific, catalog number: F144801, F123600 and F123602, respectively)
3. qPCR adhesive seal (NIPPON Genetics, catalog number: 4Ti-0560)
4. Applied Biosystems Veriti Thermal Cycler (Life Technologies, catalog number: 4375786)

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Note: Currently, it is "Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4375786".

5. ABI StepOnePlusTM Real-Time PCR Systems (Life Technologies, catalog number: 4379216)

   Note: Currently, it is "Thermo Fisher Scientific, Applied Biosystems™, catalog number: 4379216".

Procedure

A. εRNA-MEND preparation

1. The oligo nucleotides are annealed and inserted into an AgeI-EcoRI doubly digested pLKO.1-puro vector. With the ligated vector, the U6 promoter and εRNA-coding fragment are amplified through PCR by using a pair of primers (pLKO.1 Fw SpeI: CCCACTAGTTTTCCCATGATTCCTTCATATTT; pLKO.1 Rv BglII: CCCAGATCTAAAATTGTGGATGAATACTGCC), the PCR product is digested with SpeI and BglII and inserted into pCpGfree-mcs vector. This final construct is hereinafter called p-εRNA.

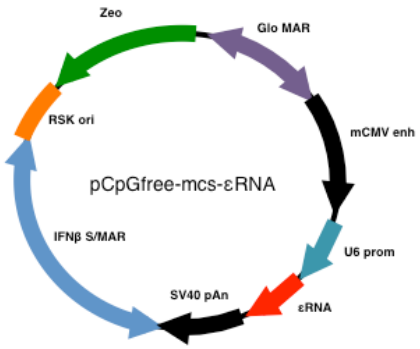

Figure 1. A plasmid map of U6 promoter-driven εRNA expression vector (p-εRNA)

2. The preparation of the p-εRNA loaded in a liposome carrier is based on the procedure that was previously described (Sato *et al.*, 2012): p-εRNA or empty pCpGfree-mcs vector is formulated into lipid nanoparticles (MEND). 80 μg of p-εRNA or empty vector (in H_2O) is dissolved in 120 μl of citrate buffer (12.5 mM citrate, 500 mM NaCl). Addition of this solution to 480 μl of the tertiary butanol containing YSK lipid (2,100 nmol), cholesterol (900 nmol), and 1, 2-dimyristoyl-sn-glycerol, methoxypolyethyleneglycol (150 nmol) leads to spontaneous formulation of liposomal particles (εRNA-MEND or control-MEND). The prepared εRNA-MEND is stocked at 4 °C until use.

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B. Infection of human hepatocyte-chimeric mice with HBV

Note: All procedures involving the manipulation of HBV infectious materials should be conducted within biological safety cabinets (BSL3).

Chimeric mice are intravenously infected with 100 µl of HBV-C in saline solution (10^6 copies per mouse) derived originally from patient with chronic hepatitis (Sugiyama *et al*., 2006). Three weeks after HBV infection, the sera are prepared by collecting blood samples from the tail vein and the efficiency of infection is confirmed by measuring the number of viral genome copies in the sera of HBV-infected chimeric mice by qPCR analysis as below:

1. Prepare HBV DNA standard sample:

   The HBV plasmid (pUC19-HBV, genotype A) is subjected to a 10-fold serial dilutions in Nuclease free-H_2O ranging from 1 x 10^3 to 1 x 10^9 copies/ml, and use 10 µl of this diluted sample (ranging from 1 x 10 to 1 x 10^7 copies/assay) as standard to quantification of HBV DNA.

2. Set up qPCR reaction mixtures as follows (for one sample):

   DNA samples from sera of HBV-infected chimeric mice:

Nuclease free-H_2O	6 µl
TaqMan Universal PCR Master Mix	12.5 µl
Probe SP2 (10 µM)	0.5 µl
Forward primer SF2 (10 µM)	0.5 µl
Reverse primer SR2 (10 µM)	0.5 µl
HBV DNA sample (from sera)	5 µl
Total	25 µl

Standard DNA samples:

Nuclease free-H_2O	1 µl
TaqMan Universal PCR Master Mix	12.5 µl
Probe SP2 (10 µM)	0.5 µl
Forward primer SF2 (10 µM)	0.5 µl
Reverse primer SR2 (10 µM)	0.5 µl
Standard DNA:	10 µl
Total	25 µl

Note: Use 10 µl of nuclease free-H_2O instead of standard DNA as negative control. Each sample is prepared in triplicate.

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3. Start the real-time PCR following the program as below:

Holding stage	95 °C	10 min		1 cycle
Cycling stage	95 °C	15 sec		45 cycles
	60 °C	1 min	Data collection	
Final holding stage	4 °C	∞		

C. *In vivo* treatment with εRNA

At 4-week postinfection, εRNA-MEND or control-MEND, is administered intravenously at a dose of 0.5 mg/kg of body weight (n=3 per group) every two days for 14 days. Serum and liver samples can be subjected to qPCR for the quantification of DNA copy numbers of HBV or other analyses.

Recipes

1. DNA oligonucleotides used for the p-εRNA plasmid construction

	Sequence (5'→3')
Sense	CCGGTGTACATGTCCCACTGTTCAAGCCTCCAAGCTGTGCCTTGGG TGGCTTTGGGGCATGGACATTTTTG
Antisense	AATTCAAAAATGTCCATGCCCCAAAGCCACCCAAGGCACAGCTTGG AGGCTTGAACAGTGGGACATGTACA

Acknowledgments

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