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• VIRAL HEPATITIS •

A vaccinia replication system for producing recombinant hepatitis C virus

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Abstract

AIM: To develop a cell culture system capable of producing high titer hepatitis C virus (HCV) stocks with recombinant vaccinia viruses as helpers.

METHODS: Two plasmids were used for the generation of recombinant HCV: one containing the full-length HCV cDNA cloned between T7 promoter and T7 terminator of pOCUS-T7 vector, and the other containing the HCV polyprotein open reading frame (ORF) directly linked to a vaccinia late promoter in PSC59. These two plasmids were co-transfected into BHK_{21} cells, which were then infected with vTF7-3 recombinant vaccinia helper viruses.

RESULTS: After 5 d of incubation, approximately 3.6×10^7 copies of HCV RNA were present per milliliter of cell culture supernatant, as detected by fluorescence quantitative RT-PCR (FQ-PCR). The yield of recombinant HCV using this cell system increased 100- to 1 000- fold compared to *in vitro*-transcribed HCV genomic RNA or selective subgenomic HCV RNA molecule method.

CONCLUSION: This cell culture system is capable of producing high titer recombinant HCV.

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INTRODUCTION

Hepatitis C virus (HCV) is a causative agent of acute and chronic liver diseases that infect millions of people worldwide[1]. There is no vaccine for prevention of HCV infection, and interferon is the only known therapeutic drug, yet it is only 10-20% effective alone and less than 50% effective when used in combination with ribavirin[2]. Overall, despite the epidemic significance of HCV infection, the development of anti-HCV drugs and vaccines has been impeded by the lack of functional cell culture systems capable of growing the virus on a large scale.

Several approaches have been taken for the propagation of HCV in cell cultures. One approach focuses on the identification of primary cells or cell lines that support replication of the virus. It has been reported that HCV isolated from infected patients is capable of infecting human primary hepatocytes[3-5], peripheral blood mononuclear cells[6], and cell lines such as human T cell line HPBMa10-2, Daudi B cell line[7-9] and various hepatocyte cell lines[10-12]. However, HCV replication in these cell culture systems is generally transient and very inefficient. A second approach is to transfect *in vitro*-transcribed HCV genomic RNA into human hepatoma cell line, Huh7[13]. Although it was reported that the transfected cells produced infectious viral particles, the replication efficiency of this method was very poor, and moreover, many research groups have reported failure to generate detectable HCV virions using this method. Recently, Lohmann *et al*. recovered high levels of selective subgenomic HCV RNA molecules from transfected Huh-7 cells. These replicons were derived from a cloned full-length HCV (genotype 1b) consensus sequence with the C-p7 or C-NS2 regions removed and a neomycin phosphotransferase gene (neo) inserted downstream of the HCV internal ribosome entry site IRES[14-17]. However, HCV particles generated by this system may differ from the native virions, due to the alteration of full-length HCV genomic RNA. Thus, researchers continue to search for a HCV replication system that will produce large-scale quantities of native virions.

Compared to the infection of cell lines with HCV-containing patient serum, the introduction of cloned viral genomes was superior because the inoculum was well-defined and could be generated in high quantities[18]. Here, we used recombinant vaccinia viruses as helper viruses to produce high-titer cloned HCV stocks in a cell culture system. This method has the advantages of being simple, highly efficient, and capable of producing large quantities of high-fidelity HCV particles.

MATERIALS AND METHODS

Plasmid construction

Plasmid pBRT703'X (NIHJ1) (generously provided by T. Suzuki), containing the full-length HCV cDNA, was cut with Hind III and inserted into vector pOCUS-T7 (plasmid pOCUS containing a T7 terminator) between *Eco*R I and Hind III sites, resulting in insertion of the HCV sequence between bacteriophage T7 promoter and terminator sequences to create plasmid pT7HCV. Next, plasmid O. pMKC1A (HCV) was cut with *Eco*R I and Hind III and inserted into PSC59 between *Eco*R I and Stu I sites to generate plasmid pVHCV, which contained the HCV polyprotein open reading frame (ORF) linked to a vaccinia late promoter. The correct sequences of pT7HCV and pVHCV were confirmed by restriction enzyme digestion and DNA sequencing.

HCV production

One day prior to use, BHK_{21} cell cultures (5×10^5 cells per well) were seeded in 6-well plates and incubated in Dulbecco's modified Eagle's medium (DMEM) with high levels of glucose (4.5 g/L) supplemented with 100 mL/L fetal calf serum and antibiotics (100 U/mL penicillin and 100 U/mL streptomycin). Cultures were incubated overnight in a 37 °C CO_2 incubator. Cells were then transfected with either 5 μg pT7HCV, 5 μg pT7HCV plus 5 μg pVHCV, 5 μg pVHCV or control plasmid (pOCUS-T7) using the LIPOFECTAMINE™ 2000 reagent (Invitrogen) followed by infection with vTF7-3 vaccinia viruses[19].

Two hours after inoculation, the inoculum was removed and the cells were cultured in a fresh medium in a 30 °C CO_2 incubator for 2-5 d.

Determination of HCV virions in cell culture supernatants

HCV virion RNA was used as an indicator for HCV virion production. Accordingly, cell culture supernatants were filtered through a 0.22 μm filter to remove residual cells and vaccinia viruses. Ten units of RNase A and 20 units of DNase I were added to 200 μL of each filtrate, and samples were incubated at 37 °C for 1 h for complete digestion of RNA and DNA. Protease K was then added to a final concentration of 100 μg/mL, and extraction of HCV genomic RNA was carried out using the high pure viral RNA kit (Roche). The resultant RNA was treated with DNase I and used as a template for RT-PCR (GIBCO BRL). For detection of positive strand HCV genomic RNA, a 416 bp fragment of the 5' region (nt 346 to 761) and a 488 bp fragment of the 3' region (nt 9378 to 8891) were amplified by RT-PCR. The primers used for reverse transcription and 5' and 3' region PCR reactions are shown in Table 1.

PCR amplification consisted of 30 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s. Amplified fragments were confirmed by 10 g/L agarose gel electrophoresis, ethidium bromide staining and visualization under UV light.

Immunofluorescence and Western blot analysis of HCV proteins

For immunofluorescence, BHK_{21} cells were grown on glass coverslips for 48 h and fixed after washed 3 times with PBS in an ice-cold mixture of 9:1 acetone and methanol. After 10 min of incubation at -20 °C, the cells were washed 3 times with PBS and incubated for 1 h in 1F buffer (PBS, 30 g/L bovine serum albumin and 1 g/L Triton X-100) at 4 °C. A mixture of NS3- and NS5a-specific mouse monoclonal antibodies (Biodesign) was added at a dilution of 1:100 in 1F buffer. After 1 h, the cells were washed 3 times with PBS followed by incubation with a mouse-specific antibody conjugated with fluorescein isothiocyanate (Sigma) in 1F buffer. Coverslips were washed 3 times with PBS and mounted on glass slides, and the cells were examined under a fluorescence microscope.

For Western blot analysis, cells were lysed with a buffer containing 10 g/L sodium deoxycholate, 1 g/L sodium dodecyl sulfate (SDS), 10 g/L Triton X-100, 10 mmol/L Tris, 140 mmol/L NaCl, 1 mmol/L phenylmethylsulfony1 fluoride (PMSF), 2 μg of aprotinin per mL, and 2 μg of leupeptin per mL (pH 8.0). The cell lysate was cleared of cell debris and nuclei by low-speed centrifugation (15 min at 15 000 *g*, 4 °C). A fraction of the supernatant was denatured by heating for 4 min at 100 °C in SDS sample buffer. The samples were electrophoresed on SDS-100/L polyacrylamide gels and transferred to nitrocellulose membranes. The membranes were then incubated overnight in blocking buffer (PBS containing 5 mL/L Tween 20 and 50 mL/L milk powder), and the mixture of NS3- and NS5a-specific mouse monoclonal antibodies was added at a dilution of 1:100 in blocking buffer for 1h. After washed 3 times with 5 mL/L Tween 20 in PBS, the membranes were then incubated with a mouse-specific antibody conjugated with peroxidase (Sigma) in blocking buffer for 1 h, washed 3 times as described above, and bound antibodies were detected by DAB.

Immunoelectron microscopy

Transfected cells were grown in 6-well plates for 48 h. After washed 2 times with ice-cold PBS (pH 7.4), the cells were fixed in Petri dishes for 30 min in a mixture of 40 g/L formaldehyde, 2.5 g/L glutaraldehyde, and 2 mL/L picric acid in PIPES buffer. The fixed cells were washed 3 times with PBS, then cell pellets were embedded in 40 g/L melted agarose and cut into small blocks, which were stained in 10 g/L uranyl acetate dissolved in water for 30 min at 4 °C. The blocks were further dehydrated in ethanol and embedded in Epon-Araldite. Ultrathin sections were mounted on nickel grids without supporting films. All labeling was conducted on grids according to the method of Sparkman and White[20]. Grids were etched with 10 g/L sodium periodate in water for 15 min, washed 3 times with PBS, and incubated with 30 g/L BSA in PBS for 30 min. The grids were then incubated for 1 h with a mixture of NS3- and NS5a-specific mouse monoclonal antibodies (diluted 1:100 in 10 g/L BSA-PBS) or in 10 g/L BSA-PBS only. After washed 5 times with PBS, the sections were treated for 1 h with goat anti-mouse antibody conjugated with colloidal gold (10-nm diameter, diluted 1:100 in PBS). Samples were rinsed five times with PBS. After counterstained with uranyl acetate and lead citrate, the sections were examined with an electron microscope.

Quantification virion RNA copy numbers

Fluorescence quantitative RT–PCR (FQ-PCR) was used to determine the amount of HCV virion RNA in cell culture supernatants treated with RNase A and DNase I as described above. Hepatitis C virus fluorescence polymerase chain reaction (PCR) diagnostic kit (Da Am Company, China) was used as directed to quantify virion RNA. Samples from ten transfections were detected by FQ-PCR.

RESULTS

Recombinant plasmids pT7HCV and pVHCV were confirmed by restriction endonuclease digestion. As expected, pT7HCV generated two fragments of 6.7 kb and 4.6 kb when digested with *Eco*R I, and pVHCV generated two fragments of 8 kb and 4.4 kb when digested with *Xho* I. DNA sequencing confirmed that the 3' and 5' sequences were as expected.

RT-PCR was used to detect HCV virion RNA in the cell culture supernatant as a measure of virion production. To ensure that the detected HCV RNA was the product of virions, we used RNase A treatment to remove HCV RNA that might release into the culture medium, as the virion envelope could protect the desired HCV RNA from digestion. To ensure that the fragments detected by RT-PCR did not come from the HCV DNA, the cell culture supernatants were also treated with DNase I. To ensure that the detected HCV RNA included the entire genome, the 5' and 3' ends of HCV genome were both amplified by RT-PCR. Positive samples amplified a 416 bp DNA fragment from the 5' UTR and a 488 bp DNA fragment from the 3' UTR . Transfection of BKH_{21} cells with either pT7HCV or pT7HCV plus pVHCV resulted in samples that were positive for both the 3' and 5' fragments amplified by RT-PCR but not PCR alone. HCV RNA was not detected following transfection with pVHCV alone or with the control plasmid. These results confirmed that HCV virions existed in the culture supernatant of BKH_{21} cells

Table 1 Primers for RT-PCR

Oligodeoxynucleotide	5' region	3' region
Reverse transcription primers	agccctggcagcgccccctaggggggcgcc (nt 791 to 762)	gggggatggcctattggcctggagtgt (nt 9414 to 9388)
PCR primer 1	gcacaaatccaaaaccccaaagaaaaa (nt 346-372)	cgctcatcggttggggagcaggtagatg (nt 9378 to 9351)
PCR primer 2	gacgagcggaatgtaccccatgaggtcggc (nt 761-732)	ccatccttctagcccaggagcaacttga (nt 8891 to 8918)

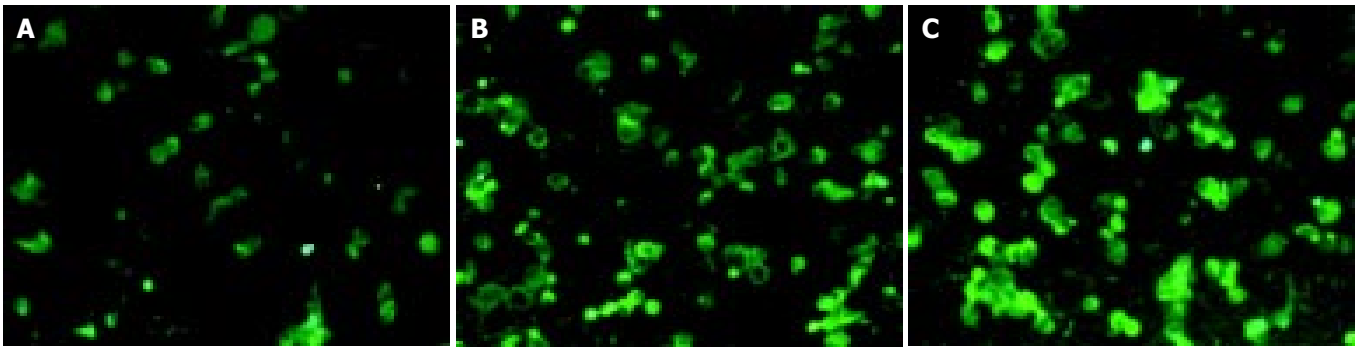

Figure 1 Immunofluorescent analysis of HCV nonstructural proteins expressed in BHK_{21} cells using a mixture of NS3- and NS5a-specific mouse monoclonal antibodies. A: cells transfected with pT7HCV; B: cells cotransfected with pT7HCV and pVHCV; C: cells transfected with pVHCV.

transfected with pT7HCV or pT7HCV plus pVHCV.

Expression of HCV nonstructural proteins in the infected cells was detected by immunofluorescence and Western blot analysis using NS3- and NS5a-specific mouse monoclonal antibodies. BKH_{21} cells transfected with pT7HCV, pT7HCV plus pVHCV or pVHCV were positive for immunofluorescence, though to varying degrees. Cells transfected with pVHCV showed the strongest signal, and cells transfected with pT7HCV showed the weakest (Figure 1). Expression of HCV nonstructural proteins in BKH_{21} cells was further confirmed by Western blot analysis. Nonstructural proteins NS3 and NS5a were detected as bands of -70 and 56 ku, respectively. Western blot analysis also showed that the expression of nonstructural proteins was highest in cells transfected with pVHCV and lowest in cells transfected with pT7HCV (Figure 2).

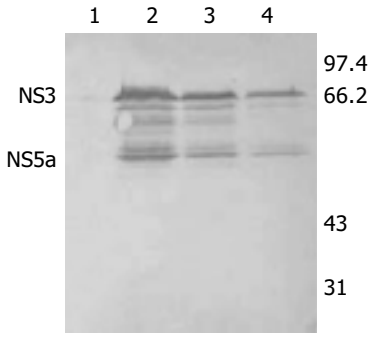

Figure 2 Western blotting of HCV gene products expressed in BHK_{21} cells probed with a mixture of NS3- and NS5a-specific mouse monoclonal antibodies. 1: cells transfected with control plasmid (pOCUS-T7); 2: cells transfected with pVHCV; 3: cells cotransfected with pT7HCV and pVHCV; 4: cells transfected with pT7HCV.

NS3- and NS5a-specific mouse monoclonal antibodies were used to determine the subcellular localization of HCV proteins in cells transfected with pT7HCV plus pVHCV. Specific, though weak, signals were found in cytoplasm and vesicles (Figure 3), which were consistent with the results of Pietscmann *et al*.[16], who reported that antisera monospecific for NS3, NS4b, NS5a or NS5b resulted in weak specific signals that were difficult to interpret. Our immunoelectron microscopy results identified positively stained small particles, about 40 to 60 nm in diameter, in cytoplasm and vesicles (Figure 3). The morphology and size of these particles were consistent with previous predictions regarding the HCV particles[21-24]. No virion-like structures were identified in cells transfected with control plasmid and/or infected with vTF7-3 vaccinia alone.

Next, FQ-PCR was used to quantify the virion production in supernatants of cells transfected with pT7HCV, pT7HCV plus pVHCV, pVHCV or the control plasmid. The copy number of HCV RNA per milliliter of culture supernatant 2, 3, 4 and 5 days after inoculation are shown in Table 2.

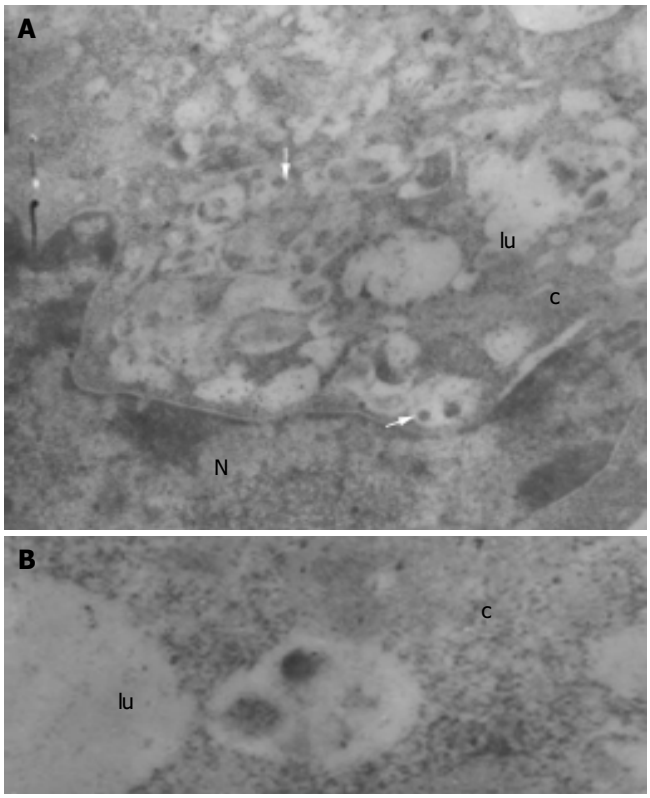

Figure 3 Immunoelectron microscopy of HCV-like virions in BHK21 cells cotransfected with pT7HCV plus pVHCV and stained with mixed NS3- and NS5a-specific mouse monoclonal antibodies. Virions about 40 to 60 nm in diameter (arrows) were identified in cytoplasm and vesicles. Bar: 500 nm (A) and 200 nm (B). c: cytoplasm; lu: lumen; n: nucleus.

Table 2 Virion RNA quantitation in cell culture supernatants as measured by fluorescence quantitative RT–PCR (RNA copies per mL)

Culture time	RNA copies			
	PT7HCV	PT7HCV+ PvHCV	PvHCV	Control plasmid
48 h	$1.12\pm0.18\times10^5$	$3.76\pm0.22\times10^5$	0	0
72 h	$4.08\pm0.20\times10^5$	$8.00\pm0.28\times10^5$	0	0
96 h	$1.28\pm0.27\times10^6$	$7.68\pm0.22\times10^6$	0	0
120 h	$3.24\pm0.25\times10^6$	$3.60\pm0.18\times10^7$	0	0

DISCUSSION

Since HCV virions contain only genomic RNA and structural proteins, transfecting host cells with *in vitro* synthesized HCV viral genomic RNA could result in generation of HCV virions if the host cells were able to translate mRNA and properly processed the resulting polyprotein[13]. However, because of low transfection efficiency, the short lifetime of viral RNA in the cytoplasm, and poor translation efficiency, viral production was generally very low by the RNA transfection method.

In this study, we used the vaccinia viral replication machinery to produce HCV virions in cell culture. The vaccinia expression system was previously tried for the production of HCV virions in cell culture. Selby *et al*. transfected Ost7-1 cells with a plasmid containing a cDNA of HCV genomic RNA downstream of a T7 promoter. The transfected cells were then infected with a recombinant vaccinia virus containing a T7 polymerase gene. Although HCV polyprotein was synthesized in Ost7-1 cells and correctly processed into individual viral proteins, no HCV virions were generated[25], perhaps because the researchers did not place a T7 terminator downstream of HCV cDNA. Without terminator, transcripts synthesized by T7 RNA polymerase were heterogeneous concatemers that were too large to be packaged into a HCV virion. To correct this problem, Mizuno *et al*. cloned HCV cDNA between a T7 promoter and a T7 terminator[21], resulting in the expression of both structural and nonstructural proteins in HeLa G cells, and the appearance of HCV core antigen-positive particle-like structures in cytosol and cisternae of the endoplasmic reticulum (ER). However, these particles were not tested for the presence of HCV RNA.

For identification of recombinant HCV virions, we detected the expression of HCV nonstructural proteins NS3 and NS5a in the supernatant of transfected cells. This has been reported by Mizuno *et al*. who detected the expression of structural proteins in HeLa G cells transfected with the full-length HCV genome sequence[21]. Next, we used RT-PCR to detect the presence of positive strand HCV genomic RNA. Following digestion of HCV RNA from blocked cells, and residual plasmid DNA, RT-PCR of fragments from the 5' (nt 346 to 761) and 3' (nt 9378 to 8891) regions of HCV RNA showed that virions contained the entire sequence. This was in contrast to the report of Baumert *et al*., who reported that HCV-like particles produced in insect cells using a recombinant baculovirus containing cDNA of HCV structural proteins contained various shortened HCV RNAs[22]. Finally, we observed the expression of HCV proteins and virion-like structures using immunoelectron microscopy.

In this new culture system, cells were transfected with two plasmids. One contained the HCV genomic RNA-coding region between upstream T7 promoter and downstream T7 terminator, transcripts synthesized by bacteriophage T7 RNA polymerase would have a defined size. The other plasmid contained the open reading frame (ORF) of HCV polyprotein directly linked to a vaccinia late promoter. The doubly transfected cells were subsequently infected with vTF7-3 recombinant vaccinia viruses containing a T7 RNA polymerase gene under the control of a vaccinia promoter. Thus, T7 RNA polymerase was synthesized in the infected cells and in turn transcribed plasmid DNA encoding HCV genomic RNA. Meanwhile, vaccinia RNA polymerase transcribed DNA encoding HCV polyprotein. After polyprotein was processed, the resulting viral proteins packaged HCV genomic RNA and assembled it into virions, which were then released from cells via the secretory pathway. In the system, we took the advantage of the unique properties of vaccinia viruses. Vaccinia virus replicates entirely in cytoplasm and uses its own enzymes to replicate DNA and systhesize 5' capped and 3' polyadenylylated mRNA. Vaccinia DNA polymerase is able to replicate plasmid DNA in cytoplasm, increase the number of DNA copies, and transcribe cytoplasmic DNA that is linked to a vaccinia promoter. Meanwhile, the viral capping enzyme and poly(A) polymerase add a 5' cap and 3' poly(A) tail to the transcribes. The resulting mRNA can be translated by the host cell translation machinery. Beacuase HCV genomic RNA is synthesized by T7RNA polymerase of vaccinia while mRNA for the viral proteins is synthesized by vaccinia enzymes, RNA synthesis will not be restricted as RNA replication that is catalyzed by HCV enzymes.

For practical examination of this new system, we transfected BHK_{21} cells with pT7HCV and pVHCV respectively, or cotransfected the cells with pT7HCV plus pVHCV for comparison. After 5 d of culture, we detected approximately $3.24\pm0.25\times10^6$ copies of HCV RNA per mL of supernatant from cells transfected with pT7HCV and $3.60\pm0.18\times10^7$ copies of HCV RNA per mL from cells cotransfected with pT7HCV plus pVHCV. HCV RNA was not detected in cells transfected with pVHCV alone. These results suggest that cotransfection with pT7HCV plus pVHCV could increase virion production. Evidence from a previous study with *in vitro*-transcribed HCV RNA showed that although transfection of cells with genomic HCV RNA could result in the production of HCV virions, cotransfection with both genomic RNA and 5' capped RNA in which 5'UTR is deleted could increase virion production[13]. We hypothesize that it may be possible to further increase the production of viral particles by slight alterations of pT7HCV, including addition of mutations in the NS5a or NS3 regions (*e.g.*, serine to isoleucine at NS5 position 1179) to enhance replication[26-29], or addition of a hairpin-ribozyme cassette right after the end of the 3' untranslated region[30-32], which may also increase the titer of the resulting viral particles. However, the experimental benefits of these changes have yet to be determined.

Indeed, it also remains to be seen whether recombinant HCV virions identified in the present study are infectious. Evidence from a study with *in vitro*-transcribed full-length and subgenomic HCV RNA showed that recombinant viral particles were infectious and replication competent[13]. To determine whether the virions produced by our new method are infectious, future work may include purification of the particles by sucrose and CsCl gradient centrifugation according to Baumert *et al*.[22], to rule out interference by residual vaccinia viruses. Alternatively, we might generate a defective vaccinia virus that lacks D13L gene, which is essential for HCV production. Repression of D13L gene expression had no effect on viral replication and viral protein expression but could block formation of progeny virions[33].

In conclusion, the yield of recombinant HCV using our cell system increased 100- to 1000-fold compared to *in vitro*-transcribed HCV genomic RNA method or selective subgenomic HCV RNA molecule method. This culture system may enable us to provide academic and medical researchers with high quality HCV preparations as well as to generate attenuated HCV for vaccine development. Furthermore, it may be useful as a system for future drug screening and vaccine selection.

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