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Immortalization of chicken embryonic liver-derived cell line by stable expression of hMRP18S-2 for serotype 4 fowl adenovirus propagation

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ABSTRACT

Inclusion body hepatitis and hydropericardium-hepatitis syndrome caused by serotype 4 fowl adenovirus (FAdV-4) have emerged in China since 2013. FAdV is usually propagated in primary chicken embryonic liver cells or embryo yolk sac. The aim of this work was to develop an immortalized CEL cell line by stable expression of human mitochondrial ribosomal protein 18S-2, named CEL-hMRP18S-2 cells, for the propagation of FAdV-4. The maximum cell density of CEL-hMRP18S-2 cells could reach 2.65×10^6 cells/ml in four-days culture. According to the mRNA levels of cell-cycle related genes in CEL-hMRP18S-2 cells tested by qRT-PCR, we speculated that the transformation of hMRP18S-2 into CEL cells caused the functional inactivation of p53 and the significant down-regulation of $p15^{INK4b}$ might cause the hyperphosphorylated form of Rb, releasing E2F-1 factor and enhancing the E2F-dependent transcription for cell cycle progression. It was suspected that the up-regulated c-Myc mRNA level at the initial period of immortalization might prompt transformed cells through the G0-G1 checkpoint. The normal CPE was observed in CEL-hMRP18S-2 cells infected by FAdV-4 and microcarrier suspension culture performed for FAdV-4 propagation with 9.0 $lgTCID_{50}$/ml suggested that CEL-hMRP18S-2 cells could be a useful continuous cell line for isolation of wild FAdV and production of FAdV-inactivated vaccine.

1. Introduction

Fowl adenoviruses (FAdVs) are non-enveloped and double-stranded DNA-viruses and belong to the genus aviadenovirus [1]. Based on restriction enzyme digest pattern and serum cross-neutralization test, FAdVs have been clustered into five species (A–E) with 12 serotypes (FAdV-1 to 8a and 8b to 11), infecting chickens and being responsible for inclusion body hepatitis (IBH), hydropericardium-hepatitis syndrome (HHS) and gizzard erosion and ulceration [2]. Severe FAdV cases with IBH and HHS in chicken flocks have been observed in China since 2013. And serotype 4 fowl adenovirus (FAdV-4), a member of the species Fowl adenovirus C, has been identified as a mainly epidemic agent of HHS in chicken flocks in China and other countries [3,4].

Fowl adenoviruses are usually grown in primary chicken embryonic liver (CEL) cells or primary chicken kidney (CEK) cells *in vitro* [5]. Embryonated eggs have also been reported as a sensitive medium for the isolation and propagation of FAdVs, by inoculating through chorioallantoic sac route for 10-12-day-old specific pathogen free (SPF) chicken embryos or through yolk sac route for 5-7-day-old SPF chicken embryos [6,7]. However, the preparation of these primary cells or SPF embryonated chicken eggs is a labor intensive and quality unsteady process, which might show a long-time lag and inconsistency in virus production from batch to batch and become a limiting factor during disease outbreak with an urgent need of vaccine. In this situation, continuously passaging cell line should be developed and served as a prior and homogenous cell substrate for virus propagation. Therefore, the purpose of this study was to develop and determine an immortalized chicken embryo liver cell line, which could support the growth of fowl adenovirus.

The general method of cell immortalization is to transform the primary cells with well-known immortalizing genes, such as over-expression of viral or cellular oncogenes, forced expression of human telomerase reverse transcriptase (hTERT), or a combination of both methods mentioned above [8]. Recently, it has been reported that human mitochondrial ribosomal protein S18-2 (hMRP18S-2), which is encoded by a cellular gene located on human chromosome 6p21.3

Abbreviations: FAdVs, fowl adenoviruses; hMRP18S-2, human mitochondrial ribosomal protein 18S-2; IBH, inclusion body hepatitis; HHS, hydropericardium-hepatitis syndrome; CEL cells, chicken embryonic liver cells; CEL-p cells, primary chicken embryonic liver cells

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[9,10], led to immortalization of primary rat embryonic fibroblasts and induced to express stem cell markers by binding to retinoblastoma (Rb) protein and advancing the cell cycle through G1 to S phase [11,12]. The derived cells lost cell contact inhibition and obtained the ability for anchorage-independent growth in soft agar with a higher cloning efficiency.

The present research is devoted to developing an immortalized chicken embryo liver cell line by transformation of hMRP18S-2 protein and characterizing the propagation ability of FAdV-4 in the immortalized cell line.

2. Materials and methods

2.1. Isolation and culture of chicken cells

Primary chicken embryo liver cells were isolated from 15-day-old SPF chicken embryos. Embryonic liver tissue was removed aseptically and rinsed twice gently with phosphate buffer saline (PBS, pH 7.2–7.4). The liver tissue was minced and treated with 0.25% (w/v) trypsin-EDTA solution at 37 °C for 30 min. Then the trypsinized cells were filtered through sterile 20 μm filter. The filtrate was added with Minimum Essential Medium (MEM, Gibco, 12492) containing 10% fetal bovine serum (FBS, Gibco, 10270) to stop the activity of residual trypsin and centrifuged at 800 × g for 5 min. The primary cell pellets were resuspended and cultured in MEM with 10% FBS, 100 units of penicillin/mL and 100 μg of streptomycin/mL, designated as CEL-p cell. DF-1 cells (ATCC® CRL-12203™) were cultured in DMEM (Gibco, 11965–084) with 10% newborn calf serum (Gibco, 16010–159) in T-flask at 5% CO_2 and 37 °C.

2.2. Viruses isolation

Liver samples of chickens with HHS were collected from flocks with HHS outbreaks in Jiangsu Province, China, and frozen at −80 °C. The sample homogenates were confirmed to be FAdV-4 positive by polymerase chain reaction (PCR) amplification of 766 bp fragment by PrimeStar® HS polymerase (Takara, R040Q) with primers as follows, forward primer: CAACTACATCGGGTTCAGGGATAACTTC, reverse primer: CCAGTTTCTGTGGTGGTTGAAGGGGTT. Reactions were performed according to the following protocol: 98 °C for 3 min, followed by 35 cycles of 98 °C for 10 s, 58 °C for 15 s, 72 °C for 15 s and final extension at 72 °C for 10 min. The PCR product was sequenced and blasted with reference FAdV strains. The extracts from the liver homogenates were inoculated through yolk sac route for 5-7-day-old SPF chicken eggs following standard embryo inoculation technique. The progeny FAdV-4 from infected chicken embryos were collected and stored as FAdV-4 working stocks at −80 °C for following studies.

2.3. Plasmid construction for expression of hMRP18S-2 and stable transfection of primary chicken embryo liver cells

The codon-optimized hMRP18S-2 cDNA with Kozak sequence was chemically synthesized by GenScript Biotechnology Company. The target DNA fragment was digested with *Kpn*I and *Not*I and then subcloned into expression vector pCAG which had been digested with the same restriction endonucleases to generate pCAG-hMRP18S-2.

On the day before transfection, CEL-p cells were prepared and inoculated in the 6-well plate in 2 ml MEM with 10% FBS at the initial cell density of 1×10^6 cells/ml. Before transfection, the culture media of CEL-p cells were replaced with opti-MEM (Gibco, 31985). CEL-p cells were transfected with pCAG-hMRP18S-2 plasmid mediated by linear polyethylenimine (PEI, Sigma-Aldrich, 764604) with the optimized procedure as previously described [13]. Two days after transfection, selection of the transfected cells was carried out by addition of 0.1 mg/ml G418 (Roche, 04727878001) for 2 weeks. G418-resistant cells were collected and split into 96-well plate to generate single cell clone in each well by serial dilutions as previously described [14]. And then screening pressure with 0.2 mg/ml G418 was performed for further 4 weeks. The fastest growing clone was selected for further experiments and designated as CEL-hMRP18S-2 cell. The selected cell clone could be cultured in MEM with 10% FBS and 0.1 mg/ml G418 and passaged every 4 days. Before cell counting, CEL-hMRP18S-2 cells were trypsinized and resuspended in the same volume of culture media. Viable cell density was monitored by a Fisher Scientific haemocytometer with the trypan blue dye-exclusion method. Additional observation and different tests were performed at different passages of CEL-hMRP18S-2 cells for more than 10 months.

2.4. Verification of hMRP18S-2 expression in CEL-hMRP18S-2 cells

For RT-PCR, total RNA from approximate 3×10^6 CEL-hMRP18S-2 cells and CEL-p cells were extracted with RNAeasy Mini Kit (Qiagen, 74104). RNA samples were reverse transcribed by SMART® MMLV reverse transcriptase (Clontech, 639522) to generate cDNA samples. The fragment of hMRP18S-2 coding sequence was amplified by PrimeStar® HS polymerase (Takara, R040Q) with the primers as follows, Forward: ATGGCCGCGTCCGTACTCAAC, Reverse: CTAGAGAGCACTCTGCGG GCC. Reactions were performed according to the following protocol: 98 °C for 3 min followed by 35 cycles of 98 °C for 10 s, 60 °C for 15 s, 72 °C for 20 s and final extension at 72 °C for 10 min.

For western blot, CEL-hMRP18S-2 cells and CEL-p cells were washed with PBS and lysed with commercial extraction kit (Beyotime, C3601) for protein extraction. Samples were separated with SDS-PAGE and subsequently transferred to nitrocellulose membrane. The transferred NC membrane was incubated with 1:100 diluted rabbit anti-human MRP18S-2 antibody (ABIN-971187) as the primary antibody and 1:5000 diluted HRP labeled goat anti-rabbit IgG (Santa Cruz Biotechnology, sc-2004) as the secondary antibody. The western blots were visualized using DAB HRP Color Development Kit (Beyotime, P0202) according to the instructions.

2.5. RNA extraction and quantitative reverse-transcription PCR

The RNA samples were prepared from CEL-p cells, DF-1 cells and CEL-hMRP18S-2 cells at the passage of 10, 30 and 60 using RNeasy Mini Kit (Qiagen, 74104) and treated with DNase I before reverse transcription. The cDNA was synthesized by using QuantiNova Reverse Transcription Kit (Qiagen, 205413) and quantitative Real-Time PCR assay was performed using QuantiFast SYBR Green PCR Kit (Qiagen, 204054) according to the manufacturer's instructions in the LightCycler 480 II (Roche, Germany). The sequences of primers used in this process were shown in Table 1. Chicken glycerealdehyde 3-phosphate dehydrogenase (chGAPDH) gene as loading control was used for the relative quantification. The fold change values for target genes compared with the samples of CEL-p cells were calculated by $2^{-\Delta\Delta Ct}$ method [15,16]. Each reaction was performed in triplicates and results were plotted as the average ± standard deviation of the mean.

2.6. Cell cycle analysis by flow cytometry

In brief, 2×10^6 CEL-p cells, DF-1 cells and CEL-hMRP18S-2 cells at the passage of 10, 30 and 60 were collected, washed with PBS (pH 7.2–7.4) and fixed with cold 70% (v/v) ethanol overnight at 4 °C. Cells were then washed in PBS (pH 7.2–7.4) and resuspended in RNase solution (250 μg/ml) for 30 min at 37 °C. Propidium iodide (Sigma, P4864) was added to a final concentration of 50 μg/ml and these preparations were incubated at room temperature for further 30 min. The stained cells were tested using FACS Calibur™ flow cytometer (Becton Dickinson, BD). Fluorescence data were obtained from 2×10^4 cells per sample and the percentage of cells in each phase of the cell cycle (G1, S and G2/M) was determined by CellQuest software (BD).