Construction and Identification of Recombinant HeLa Cells with Human Cytomegalovirus Tegument Protein UL23 Expression

ABSTRACT

Aim: Human cytomegalovirus (HCMV), a member of the human herpesvirus family, is a common opportunistic virus causing severe ailments and deaths in people with immature or compromised immune systems. The function of UL23, a tegument protein encoded by HCMV, is poorly understood yet. We established a HeLa cell line with stable and effective expression of HCMV pUL23 to study the situation of viral proteins in host cells in vitro.

Material and method: To generate recombinant lentiviral particles, DNAs of constructs pCDH-UL23-FLAG, psPAX2, and pMD2.G were co-transfected into Lenti-X 293T cells. The stably pUL23-expressing HeLa cells resistant to puromycin were obtained after recombinant lentivirus infection. The UL23 gene was successfully amplified through RT PCR in DNA extraction from the cell line and the expression of pUL23 was identified through Western blotting.

Results and discussion: The results verified that a HeLa cell line stably expressing pUL23 was established with a lentivirus expression system.

Conclusion: The HeLa cell line with stable and effective expression of HCMV pUL23 paves the way for future study on biological functions and mechanism of protein UL23.

KEYWORDS pUL23, human Cytomegalovirus (HCMV), lentiviral packaging

INTRODUCTION

Human cytomegalovirus (HCMV) belongs to the β-herpesvirus and has a high infection rate in the human population. Even in the developed country the infection rate of HCMV can achieve 60%, while almost 100% infection rate in developing countries. HCMV can be long latent, it is pathogenic to immunocompromised individuals (children, organ transplantation patients, AIDS patients, etc.), and can even cause death. HCMV expresses viral proteins to modulate the host immune responses at every step of its life cycle, which play a crucial role in viral pathogenesis. At present, little is known about HCMV, and there are few reports on pUL23. UL23 is a cortical protein encoded by the HCMV UL23 gene, which accumulates in the nuclear periphery of the cytoplasm and belongs to the US22 gene family. However, UL23 is dispensable for viral replication in cultured cells and little is known about its functions. Dunn et al. reported that after the HCMV strain Towne lacked pUL23, the rate of virus reproduction on human foreskin fibroblasts (HFF) was not suppressed, it showed accelerated proliferation, indicating that pUL23 might play an important role in regulating its own growth. In this study, we used a lentivirus expression system to establish a cell model with expression of UL23 gene, aiming to lay a foundation for the study of pUL23 functions.

MATERIALS AND METHODS

Cells, viruses, and plasmids

Plasmids pCDNA3.1–UL23-3 × Flag, pCDH-EGFP, pCDH, psPAX2, and pMD2.G; cell lines of LentiX293T and HeLa, as well as strain Stbl3 were all preserved in our laboratory.

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**Reagents**

DMEM high-glucose medium was purchased from Gibco. Protein Marker, transfection reagent, Trizol, and restriction endonuclease were purchased from Thermo. T4 DNA ligase, DNA Marker, and reverse transcription kit were purchased from Takara. Plasmid extraction kit and gel extraction kit are purchased from Omega. Pfu DNA polymerase was purchased from Beijing TransGen Biotech Co., Ltd. Puromycin (Puro) was purchased from DingGuo Biotechnology Co., Ltd. IP lysis buffer was purchased from Beyotime. Mouse anti-β-actin, mouse anti-FLAG and goat-anti-mouse IgG were purchased from Proteintech. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), western blot testing reagents, LB medium. The primers were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

**Culture of LentiX293T and HeLa**

LentiX293T and HeLa were cultured in a humidified incubator (5% CO₂, 37°C). To reduce the background expression, unless otherwise specified, the culture media used in the experiments was high-glucose media containing streptomycin (100 U/mL), penicillin (100 U/mL), and 10% FBS.

**Amplification of target gene**

The primers were designed according to the sequences of UL23-3×Flag, see Table 1. The fragments UL23-3×Flag was amplified with the plasmid pCDNA3.1-UL23-3×Flag as template. PCR conditions were pre-denaturation at 94°C for 5 min; denaturation at 94°C for 30 s, annealing with TM value of 52°C for 30 s, extension at 72°C for 2 min, total 35 cycles; extension at 72°C for 10 min. PCR products were identified by 2% agarose gel electrophoresis.

**Construction and identification of the recombinant vector**

The pCDH vector and the PCR product were digested with restriction enzymes BamHI and NheI, and transformed to Stbl3 competent cells after ligated at 16°C overnight. Select 4 clones, inoculate in 4 ml LB tubes containing ampicillin, and incubated in an incubator at 37°C with shaking at 200 rpm overnight. The plasmids were identified by double digestion, and the positive clones were sequenced.

**Packaging and infection of lentivirus**

On the first day, 6 × 10⁶ Lenti×293T cells were seeded in a cell culture dish, the complete medium was 10 ml, and the cells were incubated at 37°C with 5% CO₂ according to the method reported by Tiscornia et al. The next day, when the cell density reached about 80%, shuttle plasmid (20 μg), pSAX2 (15 μg), and pMD2.G (5 μg) were transfected into the cells with the steps in accordance with the instructions of Lipofectamine 2000. On the third day, 10 ml complete medium was used for exchange at 16 h after transfection. On the fourth day, the supernatant containing virus was collected and centrifuged at 1500 rpm for 4 min at 48 h after transfection. The cell debris was removed by filtering through a 0.45 μm low protein binding filter (to reduce virus loss). At 30 min before infection, the target cells used to be infected by lentivirus were added with polybrene to a final concentration of 8 μg/ml for pre-adaptation, and the supernatant was also added with polybrene to a final concentration of 8 μg/ml, and then used to replace the culture medium of the target cells. At 24 h after infection, the infection fluid was replaced by fresh medium. At 72 h after infection, puro was added.

**RT-PCR**

The total RNA was extracted according to the instructions of Trizol reagent. Then, 500 ng of total RNA was added to the 10 μL reverse transcription system. CDNA was obtained by reverse transcription according to the instructions of the reverse transcription kit. UL23 gene was amplified with 1 μL of cDNA as template, and reverse-transcription product PCR using the forward and reverse primers of UL23 gene (Table 1). PCR conditions were pre-denaturation at 94°C for 5 min, denaturation at 94°C for 30 s, annealing with TM value of 58°C for 30 s, extension at 72°C for 2 min, total 35 cycles; extension at 72°C for 10 min. PCR products were identified by 1.5% agarose gel electrophoresis.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’ to 3’)</th>
<th>Purpose</th>
</tr>
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<tbody>
<tr>
<td>Primer 1</td>
<td>CCGGATCTCTTTATACCTGCTCCG</td>
<td>Amplification and cloning of UL23 in pCDH vector</td>
</tr>
<tr>
<td>Primer 2</td>
<td>GCGGATCCACGCTCTGACTCTAGAT</td>
<td>Amplification and cloning of UL23 in pCDH vector</td>
</tr>
<tr>
<td>UL23-RT-PCR-F</td>
<td>CCCAGGCTTGCCACCATGTCGGTAATCAAGGACTGTT</td>
<td>Strand specific primers used for RT-PCR of UL23 expression</td>
</tr>
<tr>
<td>UL23-RT-PCR-R</td>
<td>CCGGATCTCTACGGCTGCTGCTAAAAAGT</td>
<td>Strand specific primers used for RT-PCR of UL23 expression</td>
</tr>
</tbody>
</table>
Western blotting

The cells were washed with PBS 3–4 times. To each well, IP lysis buffer was added for cell disruption which was performed on ice for 15 min, then extract the total protein. The cell lysate was collected and 5×SDS gel loading buffer was added. After protein quantification, an appropriate amount of protein was subjected to 12% SDS-PAGE. According to the corresponding molecular weight of the protein, the protein on the separating gel was transferred to the nitrocellulose membrane. The membrane was sealed with 5% skim milk at room temperature for 1 h, then, respectively incubated with mouse anti-flag monoclonal antibody (diluted at 1:2000), shaking at 4°C overnight, washed with TBST buffer for three times, with 20 min each time. Then, the membrane was incubated with goat anti-mouse IgG antibody (diluted at 1:5000). After shaking at 4°C for 1 h, then TBST buffer washed for three times, with 20 min each time. Finally, gel infrared imaging was performed.

RESULTS

Construction of the plasmid for induced expression

The UL23 fragments (Fig. 1A) were obtained by PCR with pcDNA3.1-UL23-3×Flag as template, and the bands were at 852 bp which size was consistent with the expectation. The UL23 fragment was separately ligated with the pCDH vector, and identified by digestion with BamHI and EcoRI (Fig. 1B), and pCDH-UL23-3×Flag has two clones that could generate bands at the corresponding sites. Sequencing results were compared with those genes on GenBank. The results showed that both the inserted sequence and the read-through box were correct and the plasmid was constructed successfully.

Establishment of the cell line with stable expression of exogenous protein

Enhanced green fluorescent protein (EGFP) could be sensitively captured by fluorescence microscope, and could quickly reflect the expression in single cells. Therefore, the recombinant lentiviral vector pCDH-EGFP carrying the reporter gene EGFP was used as a positive control, which could indirectly reflect the real-time expression of the target protein UL23. Under the fluorescence microscope, the HeLa cells in the positive control group can show green fluorescence (Fig. 2), indicating that the induction system could express the exogenous protein normally.

RT-PCR detection of UL23 expression in HeLa cells

Using the HeLa UL23 cellular cDNA as a template, an 852 bp gene fragment was amplified. RT-PCR could detect the mRNA of UL23, but the control cells did not amplify the target band (Fig. 3).

Western blotting detection of pUL23 in HeLa cells expression

Western blotting results showed that the overexpression of UL23 in the recombinant cell line was observed when

![Fig. 1](image1.png) Identification of recombinant plasmids by agarose gel electrophoresis (A) PCR product of the UL23 gene; (B) Identification of a recombinant lentiviral vector by double restriction endonuclease digestion using BamHI and NheI.

![Fig. 2](image2.png) Overexpression of UL23 recombinant HeLa cell line Screening the induced expression of EGFP in positive-control HeLa by fluorescence microscopy (×100 magnification for all images).
Fig. 4 Detection of UL23 encoding protein by Western-blotting. 1: HeLa 2: HeLa-EGFP 3: HeLa-UL23-3xFlag.

the expression of the internal control protein β-actin was almost identical (Fig. 4).

CONCLUSION

Lentivirus vector are gene therapy vectors developed on the basis of HIV-1 (human immunodeficiency Type I virus), and it has many researches in gene therapy. The distinguishing feature of the generic retrovirus vector is that it has the ability to infect many different cells, no matter split cells or nonsplitting cells. The lentivirus can insert the exogenous gene into the cell genome to achieve stable expression of the target gene. At the same time, lentivirus is highly secure and do not induce any effective cellular immune responses. Viral packaging is a complicated process, which could involve the interaction between the viral vector and the packing cells. Therefore, the density of LentiX293T packaging cells is an important limiting factor. By making full use of the 3D space formed by the medium, a large number of cells can be increased within a certain range, thus, increasing the chance of binding with foreign DNA, increasing the number of plasmids entering the cells, and obtaining higher virus titer. In this study, we successfully established a HeLa cell line with low background and efficiently induced the expression of the HCMV protein UL23, which laid the foundation for the study of the functions of pUL23.

ACKNOWLEDGMENTS

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