Construction of Retrovirus Vector with Ubc9 Gene and Recombinant Virus Producing Cell Line*

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ABSTRACT Objective: To establish a recombinant retrovirus vector containing Ubc9 gene and to establish a stable virus packaging cell line expressing Ubc9 effectively and stably. Methods: Ubc9 gene was amplified from the plasmid pcmv6-xl6-ubc9 by PCR technique and subcloned to the retroviral vector pMSCVneo to construct the recombined retrovirus vector. The recombinant plasmid was transfected into packaging cell line PT67 with PolyJetTM and the efficient virus-producing cell line PT67-Ubc9 was screened out following G418 selection and collected virus infected NIH/3T3 cells. Results: The recombinant retroviral vector pMSCV-Ubc9 was identified by restrictive analysis and DNA sequencing. A stable virus producing cell line was selected and the retrovirus was effectively transfected into NIH3T3 cells. Conclusion: The recombinant retroviral vector pMSCV-Ubc9 was constructed successfully. A stable viral producing cell line PT67-Ubc9 was selected and established.

Key words: Ubc9 gene; Retroviral Vector pMSCVneo; PT67

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Introduction

Several covalent modi?cation of proteins, such as phosphorylation, acetylation, and methylation, play important role in biology function of protein. Recent years some ubiquitin-like proteins were found, they have remarkably similar secondary structure with ubiquitin and they covalently conjugate to other proteins in the similar multistep process like ubiquitination [1,2,3]. The small ubiquitin-like protein modifiers (SUMO) have emerged as the key members. Unlike ubiquitylation which primarily targets the substrate for degradation, sumoylation participates in numerous biological processes, including nucleocytoplasmic translocalization, transcriptional regulation, DNA repair and replication, as well as mitotic and meiotic chromosome behavior [4]. Now, more than 100 SUMO target proteins have been reported, such as RanGAP1, PML, Sp100, p53 and c-Jun [5]. SUMO conjugation requires an E1-activating enzyme (Aos1/Uba2) , an E2-conjugating enzyme (Ubc9) and a variety of E3 ligases (RanBP2, the PIAS proteins, and the polycomb group protein Pc2). As the unique conjugating enzyme, Ubc9 is critical for sumoylation and it regulates various processes. In mammalian cells, Ubc9 protein down-regulation was associated with the defects in cytokinesis and furthermore increases the number of apoptotic cells [6]. In lower eukaryotes, Ubc9 was an important protein for normal mitosis and cell cycle progression. Cell cycle arrest and abortive mitosis occurred if its absence [7]. There were accumulating reports indicating that ligase enzyme Ubc9 was found overexpressed in several malignancies, such as lung adenocarcinoma, ovarian carcinoma , and melanoma[8-10].

In order to further study the role of Ubc9 in cells. The construction retrovirus vector with Ubc9 gene and establishment of recombinant virus cell line producing Ubc9 gene were needed.

1 Materials and methods

1.1 Materials

T4 DNA ligase, restriction endonucleases were purchased from TAKARA；DNA gel extract kit, Taq enzyme, PCR purified kit, low-dose plasmid extraction kit were from Bioteke Corporation; DMEM/HG culture medium and fetal bovine serum were from Gibico Company; PolyJet™ were purchased from SignaGen Laboratories；Plasmid (pMSCVneo pcmv6-xl6-ubc9) was provided by Professor Xu Xiang (Third Military Medical University, China)；NIH3T3 cells were from Clontech. bacillus coli DH5a and PT 67 were stored by our laboratory; Other reagents from Sigma. DNA sequencing was completed by Invitrogen Corporation.

1.1.1 Amplication and cloning of Ubc9 gene

Ubc9 gene was obtained by PCR amplication from the plasmid pcmv6-xl6-ubc9. The PCR primers were designed containing the restriction site of *EcoR* Ⅰ and *Xho* Ⅰ. Upstream primer: 5'cggaattcatgtcggggatcgccctcag 3'Downstream primer: 5'tccgctcgagttatgagggcgcaaacttc 3'. PCR was conducted in 50μl reaction solution. The reaction mixture contained 25μl Taq MasterMix2*, 5μl Template, 2 μl Forward primer (10μM), 2 μl Reverse primer (10μM) , and 16 μl RNase-Free water. PCR cycles were conducted with preamplification denaturation at 94℃ for 2 minutes, fol-

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lowed by 30 cycles of denaturation at 94℃ for 30 seconds, annealing at 60℃for 30 seconds, and extension at 72℃ for 30 seconds. The final step of extension at 72℃ for 5 minutes. The PCR products were analyzed by electrophoresis on 2% agarose gel and reclaim. Store at -20℃.

1.1.2 Construction of recombination retroviral vector pM-SCV-Ubc9

Plasmid (pMSCVneo) and Ubc9 gene were digested by the restriction enzymes *EcoR* Ⅰ and *Xho* Ⅰ. The reaction mixture contained 3μl 1× buffer H, 1μl *EcoR* Ⅰ, 1μl *Xho* Ⅰ, 15μl Plasmid, 10μl ddH_2O. The mixture was kept at 37℃ for 2 hours, then incubated at 65℃ for 20 minutes. The size of pMSCVneo was checked by electrophoresis on 1% agarose gel and reclaim. T4 DNA ligase was used to join them at 16 ℃ for 12 hours, then the ligation mixture was transferred into frozen competent DH5α. Five clones were respectively selected and identified by restrictive analysis and DNA sequencing . At the same time, the empty vector as a contrast.

1.2.3 The virus vector package in PT67 cell lines

A total of 105 PT67cells were seeded onto 60 mm culture dishes and allowed to grow to 50 %--70 % confluence in DMEM with 10 % fetal calf serum (complete culture medium). The PT67 cells was transfected by liposome with recombination retroviral vector pMSCV-Ubc9 for 12hours (following the PolyJetTM introduction), then the transfected complex containing medium gently were removed and the fresh complete culture medium were refilled . 72 hours after transfection, complete culture medium containing 400 μg/ ml G418 was added into PT67cells until positive clones were obtained. The positive clone was digested by 0.25 % Trypsin-EDTA and moved to 24 wells culture plate, then expanded culture in 100 ml flasks. When PT67cells growed to 80 % confluence, the supernatant was collected and aggregated virus by centrifugation (1000rpm) for 5 min at 4℃. The virus pellet was collected by centrifugation at 25000rpm for 90 min at℃. Resuspend the virus by 0.5-1% TNE, and incubated overnight at 4℃. The suspension stored at - 80℃. No cryoprotectant is required.

1.2.4 Target cell (NIH/3T3 cells) infection

The NIH/3T3 cells 12-18 hours before infection were cultured at a cell density of 2×10^5 per 60-mm plate. For infection, the collected medium with virus from packaging cells was diluted at least 2-fold with fresh medium, then added into NIH/3T3 cells. In order to enhance the infection efficiency, polybrene was add with the final concentration of 8μg/ml. Then replace the medium with fresh virus-containing medium 24 hours later. 48 hours after the infection, complete culture medium containing 400 μg/ ml G418 was added in NIH/3T3cells to obtaine the positive cells.

1.2.5 The expression of Ubc9 in the infected NIH/3T3 cells by Western blot analysis

The total protein was isolated from positive cells and the expression of Ubc9 was detected by Western blotting. The protein was separated in gel, then transferred into PVDF membrane. The membrane was blocked with 5% fat-free milk for 30 mins, then incubated with the Rabbit -anti- Ubc9 at 1:1000 dilution at 4℃ overnight. After wash in TBS on a shaker for 30mins the secondary Goat anti-Rabbit serum maked with HRP was added at 1∶10000. Development of the color with ECL after completely washing and the picture was taken. At the same time, NIH/3T3 and empty vector (pMSCVneo) - NIH/3T3 were used as a contrast.

2 Results and analysis

2.1 Amplification Ubc9 gene

The size of ubc9 gene amplified by PCR from Plasmid pcmv6-xl6-ubc9 was 477pb checked by electrophoresis on 2% agarose gel , which showed that the ubc9 gene was successfully obtained (Fig.1).

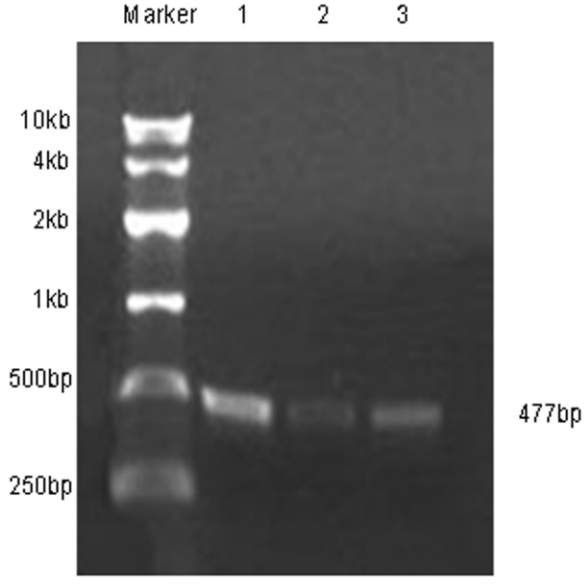

Fig.1 Amplification Ubc9 gene 1)DNA Maker 2,3,4)Amplification Ubc9 gene

2.2 Identification of recombination retroviral vector pM-SCV-Ubc9 by restrictive analysis and DNA sequencing

The recombination retroviral vector DNA contained Ubc9 gene were detected by the endonuclease (*EcoR* Ⅰ, *Xho* Ⅰ) digestion on the 2% Agarose gel electrophoresis of product of recombinant and empty vector.(Fig.2); The sequence data revealed its sequence contain Ubc9 gene and all base pairs were nicety and the recombinant plasmid was successfully constructed.

2.3 Western blotting detection the expression of Ubc9 gene in target cell

Through the Western blotting detection, the Ubc9 protein was high expression in pMSCV- Ubc9- NIH/3T3. At the same time, use NIH/3T3 and empty vector (pMSCVneo) -NIH/3T3 as a contrast. (Fig.3)

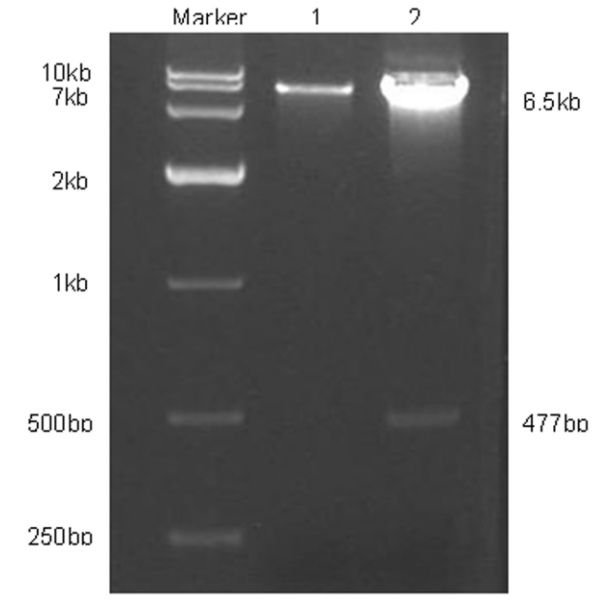

Fig.2 Digestion of pMSCV-Ubc9 :1) DNA Maker 2) pMSCVneo 3) endonuclease (EcoR Ⅰ.Xho Ⅰ) digestion pMSCV-Ubc9

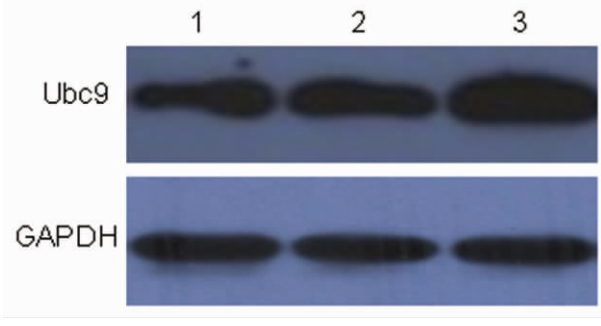

Fig.3 The expression of Ubc9 gene in NIH/3T3 cell 1) NIH/3T3 cells 2) pMSCVneo- NIH/3T3 cells 3) pMSCV- Ubc9-NIH/3T3 cells

3 Discussion

As the SUMO E2 enzyme, Ubc9 recognizes and binds to a SUMO consensus sequence, ψ KX(D/E), where ψ is a hydrophobic amino acid and X is any amino acid, present in most target proteins [11]. The Ubc9-SUMO complex can directly interact with specific substrates, and this interaction leads to the formation of an isopeptide bond between SUMO and a lysine residue on its target[12,13]. So sumoylation is dependent upon the expression of Ubc9. Recent years some experiments show that Ubc9 is essential for cell viability, but it is notable that depletion of Ubc9 in chicken cells was associated with increased numbers of binucleate cells and knockout of Ubc9 in mouse led to aberrant nuclear morphologies and defects in chromosome segregation [14,15]. In Drosophila, Ubc9 loss-of-function studies similarly showed mitotic defects in hemopoietic tissues [16]. Ubc9 yeast mutants were more sensitive to DNA damaging agents[17,18]. Recent reports indicated that Ubc9 had high expression in human premalignant conditions in response to low-grade, longterm genotoxic stress, implying that upregulation of sumoylation may be an adaptive process to genotoxic stress[19]. There also had been reported that Ubc9 was over-expressed in certain tumors, so ubc9 was proposed as a potential clinical marker for some specific human abnormality diagnosis [4]. Some experiment revealed that the Ubc9-SUMO system might participate in the proliferation and differentiation of neuronal cells in the developing brain and in neuronal plasticity in the adult brain[20]. So, it is very necessary to establish a recombinant retrovirus vector containing Ubc9 gene.

This article used retrovirus vector because it had acceptance of big fragment insert and produces high and stable gene transfer efficiency. The Murine Stem Cell Virus (MSCV) vectors were derived from the Murine Embryonic Stem Cell Virus (MESV) and the LN retroviral vectors. pMSCVneo contains E.coli Ampr gene for propagation and antibiotic selection in bacteria. It also has the neomycin resistance gene which can select antibiotic cell. The RetroPack PT67 Cell Line was derived from a mouse fibroblast (NIH 3T3) cell line designed for stably producing high-titer retrovirus. PT67 cells package virus with a polytropic envelope, that recognizes receptors on mouse, rat, human, cat, dog, and monkey cells. Thus, virus packaged by RetroPack PT67 has a broad mammalian host range.

Over the past ten years after its discovery, the small ubiquitin-like protein modifier had become a key regulator of proteins. The studies indicated that sumoylation has participated in a wider cell biological processes. Although new SUMO targets were identified rapidly, many fundamental questions remain unanswered. This study successfully constructed a retroviral vector carrying the Ubc9 gene for future studies.

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Pmscv-Ubc9 逆转录病毒表达载体的构建及产毒细胞系的建立 *

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摘要 **目的** 构建含 Ubc9 的逆转录病毒表达载体，筛选建立携带该基因的高滴度产毒细胞系，深入研究 SUMO 化修饰的作用。**方法** 聚合酶链反应(PCR)扩增获取目的基因 Ubc9，定向插入逆转录病毒表达载体 pMSCVneo，形成重组质粒 pMSCV-Ubc9，脂质体法将 pMSCV-Ubc9 转染逆转录病毒包装细胞 PT67；G418 筛选产毒细胞克隆，扩大培养产毒细胞克隆，收获病毒感染 NIH3T3 细胞。**结果** 限制性酶切和测序鉴定证实 Ubc9 正确插入逆转录病毒表达载体。G418 筛选获得稳定产毒的抗性细胞克隆，收获病毒能有效感染 NIH3T3 细胞。**结论** 携带 Ubc9 基因的重组逆转录病毒表达载体 pMSCV-Ubc9 构建成功，转染 PT67 细胞后包装出重组逆转录病毒，进而筛选获得了能转录表达 Ubc9 的产毒细胞系 PT67-Ubc9。

关键词 Ubc9 基因；逆转录病毒载体 pMSCVneo；PT67 细胞

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