Brief Report

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Construction of recombinant adenoviral vector carrying *cyclinA2* gene

Yu Han[1], Liu Hong[2], Cuiping Zhong[1], Jianhua Qiu[1,*]

[1] *Department of Otolaryngology, Xijing Hospital, Fourth Military Medical University, Xi'an, Shaanxi, China;*
[2] *Xijing Hospital of Digestive Diseases, Fourth Military Medical University, Xi'an, Shaanxi, China.*

Summary **Cell cycle related molecules in mammalian cochleae could provide a new avenue to restore hearing loss caused by a variety of genetic and environmental insults. CyclinA2 is one of the most important regulators of cell cycle, but its role in the mammalian cochlea is still unknown. So, it is necessary to construct an adenovirus vector carrying *cyclinA2* gene for clarifying its function in the cochlea. In this study, the *cyclinA2* genes were cloned into the shuttle plasmid pDC316-mCMV-EGFP to construct pDC316-CyclinA2-mCMV-EGFP, which was co-transfected with the rescue plasmid pBHGloxΔE1,3Cre into 293 cells to obtain the recombinant adenovirus Ad.CyclinA2-EGFP. Then, the plasmid pDC316-CyclinA2-mCMV-EGFP and recombinant adenovirus Ad.CyclinA2-EGFP were identified by restriction enzymes and reverse transcription-polymerase chain reaction (RT-PCR). The recombinant adenovirus vector was purified by CsCl banding, and was titrated. Finally, the recombinant adenovirus vector carrying *cyclinA2* gene was constructed and confirmed by restriction enzyme analysis and RT-PCR. The titer of the recombinant adenovirus vectors reached 2.5×10^{-11} v.p/mL. Thus, we had successfully established the Ad.CyclinA2-EGFP vector, and it could express efficiently in various cells of cochlea. This study established the foundation for the further research of *cyclinA2* gene's function in the cochlea.**

Keywords: *cyclinA2*, adenovirus construction

1. Introduction

In the last decade, a series of gene therapy within the field of auditory neuroscience had undergone tremendous development. The inner ear offers several advantages for gene therapy: firstly, it is a well-compartmentalized receptacle isolated within the otic capsule, which is easily accessible through retroauricular injection and with lower risk of inoculating adjacent tissues. Secondly, it is composed of cochlear endolymph and perilymph that permit widespread diffusion of a locally introduced vector (*1,2*). Until now, varieties of viral vectors, including adenovirus, adeno-associated virus, retrovirus, and lentivirus, had been evaluated as delivery vehicles in gene therapy (*3-7*). Compared to the other viral vectors, adenovirus vectors hold a major advantage in that they were not dependent on cell replication and had their ability to transfect quiescent cells of the cochlea with high efficiency. Therefore, adenovirus had become among the most frequently used viral vectors in the inner ear, and it was very useful to construct adenovirus vectors for clarify functions of genes in the field of inner ear.

So far, several cell cycles related molecules had been determined to participate in the regulation of mammalian cochlea function, such as $p27^{Kip1}$ and Rb (*8-10*). These studies lead to the hypothesis that gentamicin-induced hair cells loss may be reversed by restarting cell cycle (*11*). CyclinA2 is one of the most important regulators of cell cycle, and it regulates two critical progression transitions: the G1/S transition into DNA synthesis and the G2/M entry into mitosis (*12*). Experimental data showed that cyclinA2 might promote the regeneration of cardiac muscle cells, which are considered as terminal cells, by cell cycle regulation (*13,14*). But it is not sure whether it provides insights into the regeneration of hair cells in

**Address correspondence to:*
Dr. Jianhua Qiu, Department of Otolaryngology, Xijing Hospital, Fourth Military Medical University, Xi'an, 710032, Shaanxi Province, China.
E-mail: jianhuaqi@126.com

mammalian. Therefore, it is very necessary to construct an adenovirus vector harboring *cyclinA2* gene for clarifying its function in the cochlea.

In the current study, the adenovirus vector carrying *cyclinA2* gene was constructed base on the AdMax vector systems. It would establish the foundation for further research of *cyclinA2* gene in the cochlea.

2. Materials and Methods

2.1. *Cells, enzymes, bacteria, plasmids and vectors*

Low-passage human embryonic kidney AD-293 cells and AdMax vector systems were obtained from Microbix Biosystems Corporation (Ontario, Canada). All cells were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal calf serum at 37°C in saturated humidified air with 5% CO_2. The cells were sub-cultured once every three days. Restriction enzymes were purchased from New England Biolabs (MA, USA) and used according to the manufacturer's instructions. *E. coli* DH-5α was purchased from GIBCO (CA, USA). Human *cyclinA2* cDNA clonewas purchased from OriGene Technologies (MD, USA).

2.2. *Reverse Transcription-PCR Analysis*

PCR was employed to amplify human *cyclinA2* gene from cDNA clone. According to the sequence of GeneBank, specific *cyclinA2* gene primers were designed and synthesized as following: F: 5'-ATTGC GGCCGCATGCCGGGCACCTCGAGGCATT-3', R: 5'-GCCGATATC TCACACACTTAGTGTCTCTG-3'. NotI and EcoRV sites were introduced into the sense and antisense primers respectively. PCR was performed in a total volume of 20 μL consisting of 0.4 μL each primer, 1.6 μL each dNTP, 2 μL 10× polymerase reaction buffer, 0.3 μL Pyrbest DNA polymerase and 1 μL DNA template. The PCR proceeded for 25 cycles of 94°C for denaturing, 55°C for annealing, and 72°C for extension. The PCR products were electrophoretically separated on a 1% agarose gel and were visualized by ultraviolet light. PCR products were purified from the agarose gel using DNA purification kit.

2.3. *Construction and identification of homologous recombinant adenoviral plasmid*

The shuttle vector pDC316-mCMV-EGFP and antisense fragment of the *cyclinA2* gene were restriction digested with NotI and EcoRV respectively. The digested products were purified and ligated with T4 DNA ligase, and then co-transformed into *E.coli* DH-5α cells. Thus, the fragment of the *cyclinA2* gene was cloned into the shuttle plasmid pDC316-mCMV-EGFP, and the homologous recombinant adenoviral plasmid was generated. The pDC316-CyclinA2-mCMV-EGFP was restriction digested with NotI and EcoRV to validate the successful construction.

2.4. *Generation and identification of the recombinant adenovirus Ad.cyclinA2-EGFP*

The monolayer of 293 cells was co-transfected with pDC316-CyclinA2-mCMV-EGFP and adenovirus DNA plasmid pBHGloxΔE1,3Cre using Lipofectamine 2000 (Invitrogen, NY, America) and incubated for 7 days at 37°C as described in the manual. The 293 cells were scraped off flasks with a rubber policeman, and lysed for three consecutive freezing/thawing cycles. The crude recombinant virusAd.CyclinA2-EGFP was collected from the supernatant by centrifugation. Virus was amplified once in 293 cells and subjected to one round of plaque purification. Viral suspensions in 3% sucrose were stored at −80°C until thawed for subsequent experiments. After three cycles of freezing/thawing, 5 μL of viral lysate were used for detection of the cyclinA2 gene in adenoviral particles with RT-PCR and its titer was determined.

3. Results and Discussion

We assumed that manipulating cell cycle related molecules in mammalian cochleae could provide a new avenue to restore hearing loss caused by a variety of genetic mutations and environmental insults (*11*). Thus, it is very important to construct adenoviral vector carrying *cyclinA2* gene for a better understanding of the roles of this critical factor in the cochlea, which would provide insights into protection and therapy of hearing impairment.

In the present study, we had successfully established the Ad.CyclinA2-EGFP vector base on the AdMax vector systems. First, we performed PCR amplification, and the PCR product of the plasmid was a strip of about 1289bp in 1% agarose gel electrophoresis (Figure 1). The expression of the *cyclinA2* gene was verified by gene sequence examination. Then, the shuttle vector

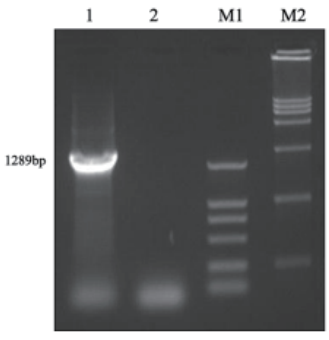

Figure 1. PCR identification of cyclinA2 gene. Lane 1: PCR products of *cyclinA2* gene. Lane 2: negative control. M1: DL200 Maker (2,000, 1,000, 750, 500, 250, 100 bp). M2: DL15000 Maker (15,000, 10,000, 7,500, 5,000, 2,500, 1,000 bp).

pDC316-CyclinA2-mCMV-EGFP was generated. As shown in Figure 2, the *cyclinA2* gene was released from the recombinant shuttle vector pDC316-CyclinA2-mCMV-EGFP by digesting with NotI and EcoRV. The 5.8 kb and 1.2 kb strips were detected by 1% agarose gel electrophoresis, representing the shuttle vector pDC316-mCMV-EGFP and reversal fragment of the cyclinA2 gene, respectively. Next, recombinants Ad.CyclinA2 -EGFP was generated in AD-293 cells. After recombinant adenoviral particles were transfected into AD-293 cells with Lipofectamine 2000 for seven days, the green fluorescence was monitored under fluorescence microscopy. As shown in Figure 3, compare with normal 293 cells, the appearance changes of 293 cells transfected with adenoviral particles were included losing their normal spindle, edema, grape-like, declining cell adhesion properties and plaque formation. And the recombinant adenovirus was prepared and purified by CsCl density gradient ultracentrifugation, and its titer was 2.5×10^{-11} v.p/mL.

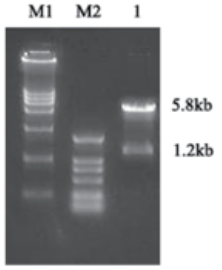

Figure 2. Digestion of pDC316-CyclinA2-CMV-EGFP with NotI and EcoRV. M1: DL15,000 (15,000, 10,000, 7,500, 5,000, 2,500, 1,000 bp). M2: DL2000 (2,000, 1,000, 750, 500, 250, 100 bp). Lane 1: pDC316- CyclinA2-CMV-EGFP.

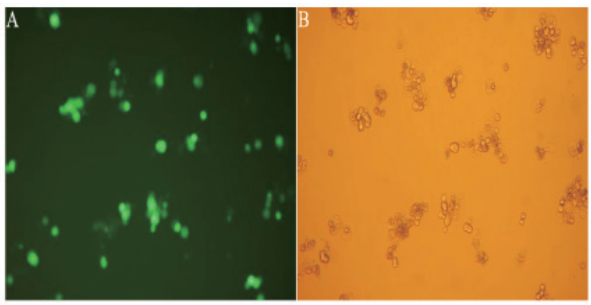

Figure 3. Cytopathic changes of the AD-293 cells transfected with adenoviral particles.

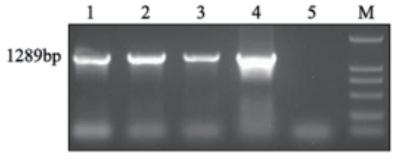

Figure 4. RT-PCR identification of the recombinant adenovirus. Lane 1: Ad.CyclinA2-EGFP stock solution. Lane 2: Ad.CyclinA2-EGFP stock solution diluted 10 times. Lane 3: Ad.CyclinA2-EGFP stock solution diluted 100 times. Lane 4: Positive control (pDC316-CyclinA2-mCMV-EGFP). Lane 5: Negative control. M: DL2000 Marker (2,000, 1,000, 750, 500, 250, 100 bp).

Finally, this vector was confirmed by restriction enzyme analysis and RT-PCR. Virus supernatant was collected by centrifugation of AD-293 cells after consecutive freezing/thawing cycles, and then was identified by RT-PCR amplification. As shown in Figure 4, a fragment of 1289 bp was obtained, indicating the correct generation of recombinant adenovirus.

With regard to gene therapy in the inner ear, adenovirus vector is one of the best viral vectors, which has ability to infect both dividing and non-dividing cells. In AdMax packaging system, the homologous recombination process was taken place in 293 cells, but not in bacteria. This system had lots of advantages, including easy operation, higher efficiency, and so on. Compared to AdEasy system, it only took 2 to 4 weeks to complete the recombination process with the greater than 98% success rate (*15*).

In conclusion, adenovirus vector harboring *cyclinA2* gene would be used to clarify its roles in the cochlea, including studies about hair cells regeneration. Our study established the foundation for the further research of *cyclinA2* gene's function in the cochlea.

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