Construction and Identification of Eukaryotic Expression Vector of Vlgr1

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Abstract. To construct the Vlgr1 plasmid containing the functional domain, provide experimental material for the study of Vlgr1 - mediated signaling pathways and hydrolysis mechanisms. Methods: Primers were designed according to the gene sequences in the Gene bank, the DNA sequences of different domain regions were cloned and inserted into vector -pEGFP -N1 to construct Vlgr1--pEGFP -N1 expression plasmid, and then transfected into 293T cell line by double digestion, PCR and sequencing. The transfection was detected by fluorescence and Western Blot, the expression of Vlgr1 gene was observed. Results: It was confirmed that the plasmid was successfully expressed by gene sequencing, fluorescence method and Western Blot method. Conclusions: The Vlgr1 plasmid containing the functional domain was successfully constructed.

Introduction

G protein-coupled receptors (GPCRs) are collectively referred to as a class of membrane protein receptors. A variety of physiological processes in vivo have G protein-coupled receptor-mediated signal transduction systems [1], but we do not know much about the effect of G protein-coupled receptors on auditory aspects. Studies have shown that AChR and Adenosine receptors and other G protein-coupled receptors are expressed in the auditory system, in addition there are Vlgr1 and Celsr1 [2]and so on in the auditory system. Vlgr1 (Very large G protein-coupled receptor 1, Vlgr1) protein was encoded by vlgr1 gene,it is currently known as the largest membrane receptor, Belonging to the G protein coupled receptor subfamily, mainly expressed in the inner ear, retinal photoreceptor cells and nerve tissue [3]. Vlgr1 gene mutations can not only cause Usher syndrome, but also can cause hearing-related epilepsy[4], is the leading cause of deafness and blindness. However, the mechanism of adhesion G protein-coupled receptor Vlgr1 in hair cells and the pathogenesis of this gene mutation triggers USH2C are not clear. In this study, we constructed a Vlgr1 plasmid by manipulating the functional domain (including 7 helix, GPS, transmembrane and intracellular regions) of the vlgr1 gene, The effect of Vlgr1 - mediated signaling pathway and Vlgr1 on deafness was studied by using the plasmid as the experimental substance.

Materials and Methods

293T cell line was purchased from the American Type Culture Collection (ATCC) (Maryland, USA); PEGyl-N1 was purchased from Beijing Ji Bax Co., Ltd.; Nhe I HindIII was purchased from NEB; cell culture medium DMEM, fetal bovine serum, trypsin was purchased from Hyclone Corporation. Transfection reagent Lipofectamine 2000 was purchased from Invitrogen. Anti-beta-actin mouse monoclonal antibody, anti flag rabbit monoclonal antibody was purchased from sigma.

Construction the plasmid. The total RNA was extracted from the mouse ear tissue and reverse transcribed into cDNA. The cDNA product was then amplified by PCR to amplify the Vlgr1 gene fragment (1 to 498 amino acids, 3175-335 amino acids and 5618-628 amino acids). According to

the sequence of NCBI database Vlgr1 (NM_054053), three pairs of primers were designed. NheI restriction sites were added to the 5 'end of the first pair of upstream primers, the HindIII cleavage site was added to the 5 'end of the first, second, and third pair of primers, construct the flag-Vlgr1-myc-EGFP-pEGFP-N1 recombinant. The PCR primers for the Vlgr1 gene are shown in Table 1. The reaction conditions are as follows:Amplification conditions: 95 ℃ 30s, denaturation of 95 ℃ 5s, annealing of 60 ℃ 30s, extension of 72 ℃ 50s, 35 cycles and then extended 72 ℃ 2min, cooled to 4 ℃.

Table 1 Details of PCR Primers Employed in the Experiments

Primer name	Primer sequence(5'-3'')	Product size(bp)
1-Vlgr1-F	GGTGTTCGCCATGTCGGTGACCTCAGAACC	1494
1-Vlgr1-R	CCCAAGCTTTTCAGCGCCTCCTTGTATGGT	
2-Vlgr1-F	CAAGGAGGCGCTGAAAGACTAGGGGCCCAT	1086
2-Vlgr1-R	CAAGCTTTTCCAGGACGAAAGAGAAGG	
3.-Vlgr1-F	CTTTCGTCCTGGAACAAGCTGTCTGGGGC	1980
3-Vlgr1-F	GAGGTGGGTGTCAGCAATTTCCAGGACGAAAG	

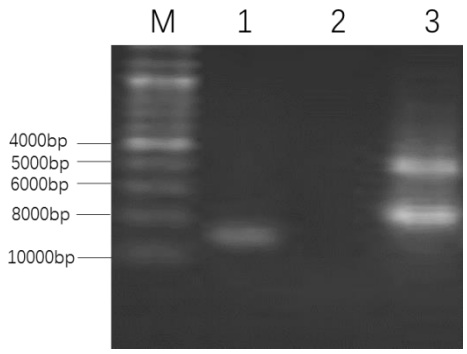
Construction and identification of recombinant subclone vectors. The A amplification fragments of the first pair of primers were ligated into the pEGFP-N1 vector by NheI, HindIII restriction site to form A-pEGFP-N1. The B amplification fragments of the second pair of primer were ligated into A-pEGFP-N1 by SLIC (ligation independent cloning, SLIC) to form A-B-pEGFP-N1 recombinants. The C amplification fragments of the third pair primers were linked to A-B-pEGFP-N1 by the SLIC method to form the A-B-C-pEGFP-N1 recombinant. The recombinant plasmid was transformed into Escherichia coli JM109, and the plasmid was extracted,sequencing was performed by preliminary identification using restriction enzyme digestion [5].

Transfection. The correct recombinant was transfected into 293T cells and incubated for 18-48 h in an incubator at 37 °C in a CO2 content of 5% and then observed under fluorescence microscopy to detect transfection levels [6].

Detection of protein expression in 293T cells by Western blot. Vlgr1 was transfected for 30 h, the total protein in 293T cells was extracted and subjected to SDS-PAGE electrophoresis, transfer film, closed, adding rabbit anti-Flag antibody (1: 1000),incubate overnight at 4℃, adding secondary antibody, ECL substrate developed, developed and fixed.

Results

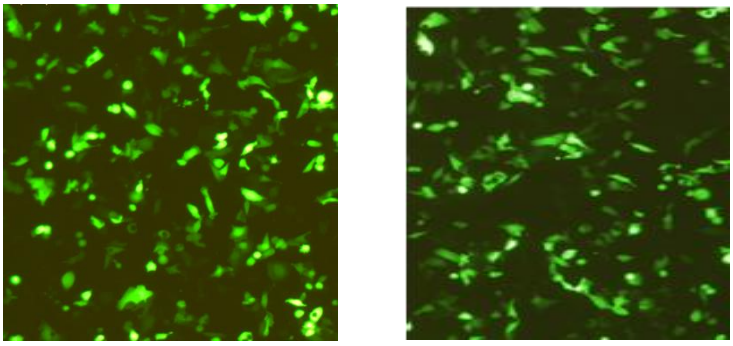
Identification of the recombinant Vlgr1--pEGFP -N1 by restriction enzyme digestion. By NheI, HindIII double digestion, about 4500bp fragment was cut out from Vlgr1--pEGFP -N1, indicating that the target gene fragment Vlgr1 has been inserted into the plasmid vector -pEGFP -N1, see Figure 1.



M.DNA Marker 10000; 1.Vlgr1-pEGFP-N1 plasmid; 2.Negative control; 3.NheI and HindIII enzyme digestion of Vlgr1-pEGFP-N1 plasmid

Figure 1 Identification of Vlgr1-pEGFP-1 plasmid by PCR and double restrictive enzyme digestion

Detection of the recombinant expression by fluorescence. After transfect 293T cells with vector and recombinant plasmid Vlgr1-pEGFP-N1, green fluorescence was observed under fluorescence microscope, indicating that transfection was successful and effective, see Figure 2



1.Vector ;2.Vlgr1-pEGFP-N1

Figure 2.Cell fluorescence after recombination plasmid

Western blot analysis of the expression. Inserted the flag tag on the Vlgr1 gene, Vlgr1 transfection was confirmed by flag antibody detection, the recombinant were successfully expressed in the cells, see Figure 3.

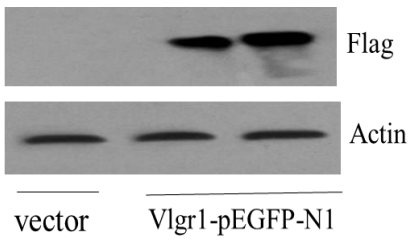


Figure 3 Western blot results

Discussion

VLGR1 is a member of the family of adherent receptors and is a central component of the complex pedicle junctions in the inner ear hair auditory cells. Knockout mice and mutant mouse models showed that loss of function of Vlgr1 will lead to abnormal cilia function and the disappearance of

hearing, it can be seen that Vlgr1 plays a very important role in the auditory signal transduction and the development process of cochlear formation. Vlgr1 is expressed in many tissues, including the liver, lung, brain, cochlea and retina[7]. In the cochlea, Vlgr1 is mainly expressed in hair cells, is the main component of the composition of the ankle[8]. Studies have shown that mice lacking Vlgr1, Usherin, Vezatin and Whirlindin can not be located in the basal part of the cilia. Studies have shown that Vlgr1 is also expressed in banded synapses at the bottom of hair cells[9,10]. Studies have shown that mutant VLGR1 can lead to Usher Syndrome, which includes congenital hearing loss and progressive retinitis. However, the molecular mechanism of Vlgr1-mediated intracellular signal transduction and the mechanism leading to Usher Syndrome are still poorly understood. In this experiment, we successfully constructed the Vlgr1 plasmid recombinants. Help us to further study the mechanism of Vlgr1 in the human body, as well as its signal transduction pathways and ways, but also help us to find The mechanism of Vlgr1 in auditory function.

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