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Research Paper

Construction and identification of recombinant lentivirus–mediated gene transfer system for rat transducer of regulated CREB activity 1☆

Ying Shi, Shigang Cheng, Xu Chen, Chuanguo Xiao*

Department of Urology, Union Hospital Affiliated to Tongji Medical College, Huazhong University of Science & Technology, Wuhan 430022, Hubei Province, China

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Abstract

Objective: To construct a recombinant lentivirus vector which carries SD rat transducer of regulated CREB activity-1(TORC1) gene and examine its ability to express the TORC1 gene *in vitro*. **Methods:** The coding sequence of SD rat TORC1 gene was amplified using PCR and cloned into pGC-FU vector. 293T cells were transfected using Lipofectamine 2000 and packaged for the recombinant lentivirus particles. When the cloned sequence was identified to be right, the recombinant lentivirus particles were amplified in a large quantity. The titer of virus was determined by real-time PCR and the level of TORC1 expression was examined by Western blot. **Results:** The recombinant lentivirus vector carrying TORC1 was constructed successfully and could express TORC1 at a high level in 293T cells *in vitro*, and the titer determined by real-time PCR was 2×10^8 TU/ml. **Conclusion:** The recombinant lentivirus vector could express TORC1 gene at a high level, and was very helpful in the study of exploring the effect of TORC1 on spinal cord injury.

Key words: TORC1; lentivirus vector; spinal cord injury

INTRODUCTION

Spinal cord injury(SCI) often causes patients serious and permanent functional disabilities due to irreversible neurological trauma. However, to date there is no therapeutic strategy that can significantly promote regeneration of damaged axons of neurons, and therefore numerous researchers focus on this. Recent studies *in vitro* and *in vivo*[1-3] indicate that therapeutic gene delivery by virus vector is a promising approach for SCI. Compared with adenovirus and retrovirus, lentivirus is characterized by slight immune responses and long and stable expression, which make it a powerful tool for research and therapy of SCI[4,5].

cAMP response element binding protein(CREB) is one of the bZIP superfamily of transcript factors, and a key component of numerous and diverse biological processes ranging from development to plasticity to disease in the central nervous system(CNS)[6] as well. Recently Zhou Y *et al.*[7] and Kovacs KA *et al.*[8] reported that transducer of regulated CREB activity-1 (TORC1) played a critical role in the activation of CREB, which indicated that TORC1 might be a new therapeutic target in CNS.

In this study, we intended to construct a recombinant lentivirus vector carrying rat TORC1 with high titer and examine the expression level of TORC1 *in vitro* after 293T cell was transfected. These findings would lay the first stone for the future exploit of TORC1 in the treatment of SCI.

MATERIALS AND METHODS

Plasmids and reagents

Lentivirus packaging system including plasmid pGC-FU, pHelper 1.0, pHelper 2.0 and plasmid TORC1 were purchased from Shanghai GeneChem Co. China. 293T and *E.coli* DH5α were from our lab. *Age*Ⅰ and *EcoR*Ⅰ were from NEB Inc, USA. In-Fusion kit was from BD

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*Corresponding author

E-mail address: xiaocg@mails.tjmu.edu.cn

Co. USA. RNA PCR kit, Agarose Gel DNA Purification kit and SYBR Master Mixture were from Takara Co. Japan. Endo-free Plasmid Mini Kit was from Qiagen Co. USA. Trizol reagent and Lipofectamine 2000 were purchased from Invitrogen Co. USA. Anti-flag antibody and Goat anti-mouse IgG coupled to HRP were from Sigma Co. USA and Santa-Cruz Co. USA, respectively. RIPA lysis buffer and ECL were from Beyotime Co. China.

Construction and identification of recombinant TORC1 lentivirus vector

To obtain the coding region of TORC1, primers were designed using plasmid TORC1 as template: TORC1-Age I: 5'-CAGGATCCCCGGGTACCGGTCGCCACCATGGCGACTTCGAACAATC-3'-TORC1-EcoRI:5'-ATAAGCTTGATATCGAATTCTACTTGTCATCGTCATCCTTGTAGTCCAGGCGGTCCATTCGGAAG-3', sequences underlined were restriction sites of *Age*Ⅰ and *EcoR*Ⅰ respectively, and the sequence in pane coded flag tag. The cycle parameters were as follows: 94℃ for 30 s, then 30 cycles of 94℃ for 30 s, 55℃ for 30 s, 72℃ for 4 min, followed by a final extension of 72℃ for 10 min. The PCR product was 1 963 bp in length.

Plasmid pGC-FU and the PCR product were digested with *Age*Ⅰ and *EcoR* Ⅰ respectively, and then purified using Agarose Gel DNA Purification kit. The two recovered products were ligated using In-Fusion kit, and the ligated product was used to transfect *E.coli* DH5α. Individual growing clones were picked out and mixed with LB broth containing AMP. 1 μl of each mixture was taken to be PCR template and the rest was incubated at 37℃ for 12 h. To confirm the ligation was correct, primers were designed as follows: TORC1-SEQF: 5'-AGCTGGAGCAGTTCAACATG-3', pGC-FU-SEQR:5'-CGTCGCCGTCCAGCTCGACCAG-3'. The cycle parameters were:94℃ for 30 s, then 30 cycles of 94℃ for 30 s, 60℃ for 30 s, 72℃ for 30 s, followed by a final extension of 72℃ for 6 min, and the product was 514 bp in length. The clones whose PCR results were positive were subject to DNA sequencing.

Packaging, concentration and titration of recombinant TORC1 lentivirus vector

Lentivirus packaging system included recombinant pGC-FU vector, pHelper 1.0 and pHelper 2.0. DNA of the three plasmids was extracted using Endo-free Plasmid Mini Kit and then used to transfect 293T cells using Lipofectamine 2000 according to the manufacturer's instructions. 48 h later, the supernatant was collected and centrifuged using filtrator at 4 000 g, 4℃ for 15 min, and then the collection was concentrated again by centrifugation at 1 000 g, 4℃ for 10 min. The final concentration was the desired virus.

The titer of recombinant lentivirus was determined by real-time PCR. The viral concentration mentioned above was served as stock solution, and a 10-fold dilution series was performed until dilution fold reached 10^{-5}. The six samples were used to transfect 1×10^5 293T cells respectively. 4 d later, the total cellular RNA of each group was extracted using Trizol according to the manufacturer's instructions. 20 μl cDNA was obtained after reverse transcription and 1 μl cDNA was taken to perform real-time PCR using SYBR Master Mixture. Primers for TORC1 were: sense, 5'-CCCAAGTCCTGCGAAGTC-3',antisense, 5'-GGAAGTGGATGGTGCTGAG-3'. Primers for GAPDH were:sense,5'-GTGGACATCCGCAAAGAC-3'. antisense, 5'-AAAGGGTGAACGCAACTA-3'.

Detection of TORC1 expressed by recombinant lentivirus *in vitro*

293T cells transfected by recombinant TORC1 lentivirus were washed with PBS, collected and lysed in RIPA lysis buffer 48 h later. Purified lysate(40 μg) of each sample was electrophoresed on 10% SDS-PAGE and then transferred to nitrocellulose filters. The filters were blocked in TBST containing 5% milk for 1 h and then incubated overnight at 4℃ with 1:4 000 diluted mouse anti-flag antibody. Goat anti-mouse IgG coupled with HRP(1:8 000) were used as the secondary antibody and bands were visualized by ECL.

Statistical analysis

The results of real-time PCR were analyzed by the self-contained software of iQ5(Bio-rad Co. USA).

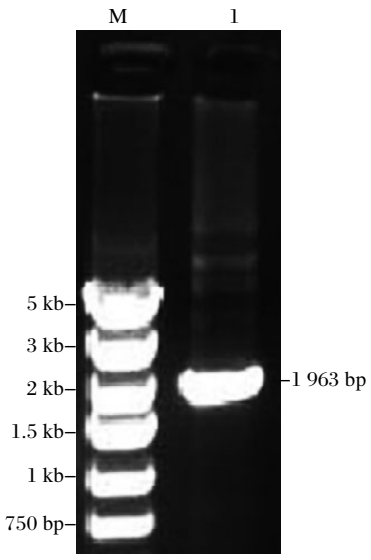
RESULTS

Construction, identification and sequencing of recombinant TORC1 lentivirus vector

The coding region of TORC1 was amplified using plasmid TORC1 as template and TORC1-AgeI and TORC1- EcoRI as primer, and the PCR product was identified by the electrophoresis on 2% agarose gel(***Fig. 1***). Plasmid pGC-FU was linearized after the digestion with Age I and EcoRI. The PCR product mentioned above was digested with Age I and EcoR I as well, and then cloned into plasmid pGC-FU. The positive clones were identified by PCR(***Fig. 2***) and the result of DNA sequencing further confirmed that the inserted fragment was consistent with our target sequence.

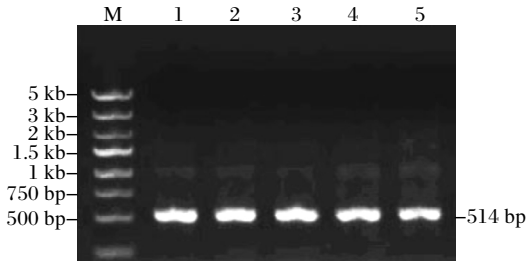
Proliferation and titration of recombinant TORC1 lentivirus vector

293T cells were co-transfected by the lentivirus packaging system, and the supernatant was collected 48 h later. The stock solution of recombinant virus was



M:Marker, Lane 1:TORC1

Fig. 1 The coding region of TORC1 amplified by PCR



M:Marker; Lane 1, 2, 3, 4, 5 were all positive clones

Fig. 2 Identification of positive clones

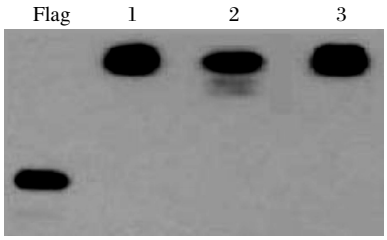
obtained by two-step centrifugation of the supernatant at 4℃. A 10-fold dilution series of 6 experimental groups and the control were subject to real-time PCR, and analysis of cycle threshold(Ct) was represented(***Table 1***). Usually it was considered to have statistical significant difference when the difference of Ct between experimental group and control was more than 2. The results showed that the difference of Ct between the group of 10^{-4} dilution fold and control was more than 2, and therefore it was considered that there were virus particles in this experimental group. On the assumption that one virus particle existed in that group, the titer of the stock solution was namely 1×10^7 TU/ml. Since only 1 μl cDNA of each group was used to perform real-time PCR, the results just demonstrated one-twentieth of the sum and the final titer of recombinant virus was 2×10^8 TU/ml.

The expression level of TORC1 *in vitro*

Flag was one kind of protein tag which infused with target protein. We treated the protein sample obtain from 293T cells transfected with the recombinant TORC1 lentivirus vector using anti-flag antibody, and observed bands of 80~90 ku(***Fig. 3***), which matched with the protein weight of TORC1-flag infusion protein. The result showed that the recombinant virus could express TORC1 *in vitro* as expected.

Table 1 **The analysis of Ct of experimental groups and the control**

Group	Ct of Actin	Ct of TORC1	The mean Ct of TORC1
1 μl	14.1	24.65	24.71
		24.77	
10^{-1} μl	13.06	26.57	26.43
		26.29	
10^{-2} μl	13.59	29.01	29.015
		29.02	
10^{-3} μl	13.53	31.67	33.187
		32.07	
10^{-4} μl	13.88	32.85	33.33
		33.81	
10^{-5} μl	14.17	32.62	33.635
		34.65	
Control	14.67	41.38	41.38
		N/A	



Lane 1, 2, 3 were protein samples from 293T cells transfected with different positive clones.

Fig. 3 Detection of TORC1-flag fusion protein

DISCUSSION

Recombinant virus vectors carrying therapeutic genes are attractive options for the treatment of diseases and trauma of CNS. To date, lentivirus is considered as an excellent choice due to its characteristic nature: it can deliver 8 kb of sequence and mediate gene transfer into any neuronal cell type. The expression of target genes is sustained, and normal cellular function *in vitro* and *in vivo* are not compromised[4]. The delivery of BDNF[9], NT-3[10], RARβ2[11] using lentivirus and Schwann cells, olfactory ensheathing cells and neural progenitor cells[12] modified by lentivirus carrying therapeutic genes[13,14] have been proven to be useful in the promotion of axonal regeneration *in vitro* and *in vitro*. The increasing understanding of pathogenetic mechanisms of axonal damages involved in SCI is leading to more effective therapeutic or preventive interventions[15].

CREB as a mediator of cAMP-inducible transcription, plays a critical important role in growth, survival and synaptic function of neurons. CREB target genes include genes that control neurotransmission, cell structure, signal transduction, transcription and

metabolism, which are more than 100 in sum[6,16]. Phosphorylation of CREB at Ser133 induced by Ca^{2+} or cAMP was once regarded as the starting point of CREB activation and transcription of downstream genes[16,17]. However, some reports challenged the hypothesis and believed that there would be other agents involved in the progress of CREB activation[4,16-18]. In 2003, Conkright MD *et al.*[19] and Iourgenko V *et al.*[20] reported that TORCs could dramatically increase CREB transcriptional activity independently of CREB Ser133 phosphorylation. Further research demonstrated that TORC1 was required for CREB-target gene transcription in neurons by nuclear translocation[7,8]. Based on the importance of CREB-mediated transcription in CNS and the function of TORC1 in CREB activation, we believe that TORC1 would be a promising therapeutic target for SCI.

In this study, we succeeded in constructing recombinant TORC1 lentivirus and the titer reached 2×10^8 TU/ml. The result of Western blot demonstrated that the recombinant virus vector was capable of expressing TORC1 efficiently *in vitro* as well, which is critical for the next research of neurons and animal models. In theory lentivirus can deliver 8 kb of axogenic sequence, but it is not the case based on our experience. First we tried to keep the green fluorescent protein(GFP) gene of pGC-FU vector along with the TORC1 gene, which meant the length of transcript region was more than 2 kb. However, the titer of obtained recombinant vector was not stable and could not meet with our expectation. Finally we ruled out GFP gene and obtained recombinant lentivirus with desired titer. Besides, it is important to determine when(after SCI occurrence) the recombinant TORC1 lentivirus will be most effective. There are still many problems we need to overcome.

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