·Basic Research·

Construction and identification of the eukaryotic expression vector carrying specific siRNA of LEDGF p52 gene

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Received: 2008-02-21 Accepted: 2008-10-08

Abstract

• AIM: To construct and identify LEDGFp52 eukaryotic expression vector for RNA interference.

• METHODS: Recombinants were designed and established by targeting gene LEDGFp52 and plasmid pGensil-1 based on LEDGFp52 cDNA sequences of Genomes. Two pairs of oligonucleotides were synthesized according to the Tuschl principle and inserted into plasmid pGenSil-l to generate siRNA eukaryotic expression vector. DH5α strains were transformed, plasmids were extracted, and recombinant vectors were identified by the restriction map and the sequence analysis. The cultured cells were transfected by the recombinant plasmid (pGensil-1-RNA.LEDGFp52-1). At 48 hours after transfection, the whole cell protein was extracted, and the protein level was detected using Western blotting with mouse anti-human LEDGFp52 monoclonal antibody.

• RESULTS: Recombinant plasmids completely concord with the designs by the restriction map and the sequence analysis, the protein level of LEDGFp52 was down-regulated at 48 hours after transfecting pGensil-1-LEDGFp52-1 expression vector into HeLa cells, the recombinant eukaryotic expression vectors were successfully constructed.

• CONCLUSION: siRNA recombinant can be successfully constructed by RNAi technique to inhibit the expression of LEDGFp52.

• KEYWORDS: LEDGFp52; RNA interference; proliferation; eukaryotic expression vector; vector construction; vector identification

Zhao HS, Wang Y. Construction and identification of the eukaryotic expression vector carrying specific siRNA of LEDGF p52 gene. *Int J Ophthalmol* 2008;1(4):324–327

INTRODUCTION

Lens epithelium derived growth factor p52 (LEDGFp52) protein was first identified through their interaction with transcriptional factors and splicing factors. It is encoded by exon 1-9 and contains 333 amino acids. It has strong interactions with the VP16 activation domain and with various components of the general transcriptional machinery, it is an active transcription activator[1]. The recent researches indicate LEDGF promoting growth and survival of many cell types and demonstrating potent ocular protective effect. LEDGF significantly and positively affects the survival of retinal photoreceptor cells [2], lens epithelial cells, cos7 (African Green Monkey SV40-transf'd kidney fibroblast cell line) cells, skin fibroblasts, and keratinocytes [3]. It also modestly stimulates the growth of lens epithelial cells, retinal pigmented epithelial cells, corneal epithelial cells, cos7 cells, fibroblasts, and keratinocytes [3]. Lens epithelium-derived growth factor can also increase survival and decrease DNA breakage of human RPE cells induced by oxidative stress[4]. In ophthalmic nervous activity, all the information processed by retina must be efferent through the axon of retinal ganglion cells (RGCs), so RGCs are the hub of information processing. Clinically, many ocular diseases can cause RGCs injury and ultimately lead to irreversible visual deprivation. Up to now, we still have no utility rescue for injured RGCs. Therefore, we designed and constructed LEDGFp52 eukaryotic expression vector for RNA interference, we aimed at providing an experimental basis for further investigation on the proper role of LEDGFp52 on the injured RGCs.

MATERIALS AND METHODS

Materials

Plasmid and bacteria strains *E.coli.* DH5α bacteria was provided by the Center Lab of Southwest Hospital, pGenSil-l plasmid was purchased from the Gensil Biomedical Company (Wuhan, China) and the proper diagram of the plasmid was shown as Figure 1 below.

Main reagents EcoRI, BamHI, HindⅢ and T4DNA ligase were purchased from TaKaRa, 100bp DNA Marker, Lipofectamine TM 2000, Plasmid extraction kit, Gel recovery

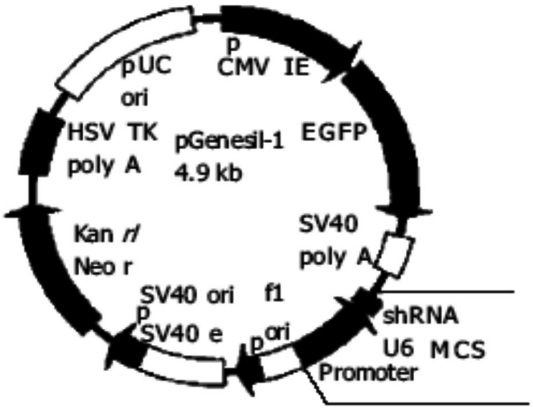

Figure 1 The map of pGenSil-1

kit, DNA fragment purification kit and LB nutrient medium were purchased from Invitrogen, LEDGFp52 mouse-anti-human monoclonal antibody was purchased from BD Biosciences, Goat-anti-mouse IgG and TMB were purchased from Tianwei (Beijing, China).

The specific sequence of siRNA.LEDGFp52 According to the LEDGFp52 nucleotide sequence provided by GenBank (AF098482.1 GI:4050033), based on Tuschl principle of design [5,6] on the internet of Ambion (design software to design two pairs of target sequences, on the basis of pGenSil-1 enzymatic site requirement, each had 19bp and BamH Ⅰ, Hind Ⅲ term recognition sequences existed. The primer were synthesized by Sangon Biocompany and diluted to 100pmol/L by distilled water. The total length was 60bp and the proper sequences as follows: LEDGFp52-1 sense 5'-GATCCCCGGAGATAGATAACAATCCATTCAAGAGA-TGGATTGTTATCTATCTCCTTTTTA-3';antisense:3'-GG-GCCTCTATCTATTGTTAGGTAAGTTCTCTACCTAAC-AATAGATAGAGGAAAAATTCGA-5',LEDGFp52-2sense: 5'-GATCCCCAGACGAAGTTCCTGATGGATTCA AGAG ATCCATCAGGAACTTCGTCTTTTTTA-3'; antisense: 3'-G GGTCTGCTTCAAGGACTACCTAAGTTCTCTAGGTA G TCCTTGAAGCAGAAAAATTCGA-5'.

Methods

The preparation and transfection of *E. coli.* DH5α competent cells Competent cells was prepared using Calcium chloride method, the plasmid was extracted according to the instruction of the kit, the DNA was selected and used in the downstream experiments which absorbance ratio between 1.8-2.0.

The construction of the eukaryotic expression vector of siRNA LEDGF p52 ① Primer annealing: 1μL sense and anti-sense strand oligonucleotides was aliquoted and slightly vortexed , before adding into 48μL 2× annealing buffer. The mix was incubated in 95℃ water bath for 4 minutes and in 75℃ water bath for 10 minutes. The mix was stored at 4 degree centigrade after natural cooling to room temperature. ②Vector linearizing: 1μg pGenSil-l plasmid, 10-buffer 2μL, BamH Ⅰ 1μL, Hind Ⅲ 1μL, ddH_2O 15μL were mixed, before incubating in 37℃ water bath for 2 hours. 1μL CIP was added, before incubating in 37℃ water bath for 1 hour. The enzymatic digestion product was analyzed by 10g/L agarose gel electrophoresis and the linearization property was recovered. ③ Linking and transforming: 2μL annealing primer was diluted to 10μL, 2μL diluted annealing primer were taken out and mixed with the recovered linearization property 1μL, 1-ligase buffer 1μL, T4 DNA ligase 1μL, ddH_2O 5μL, before linking in 16℃ water bath for overnight. And competent DH5α cells were transformed.④Recombinant screening and identifying: 200μL bacteria juice were taken out and spread on the anti-Kan plate, before incubating in 37℃ for 16 hours. The monoclone was picked up and the plasmids DNA was extracted and identified by BamHI and HindIII endonuclease digestion, the correct recombinants was collected and sent to Shangon Sequencing Department for sequence analysis. The confirming successful combinants were quickly amplified and the plasmids were extraceted and stored in -20℃. ⑤ Detecting the expression alteration of LEDGFp52 in HeLa cells is inhabited by pGenSil-1-siRNA. LEDGFp52-1. HeLa cells are lines of epithelium in human squamous carcinoma of the cervix, and there is certain level expression of LEDGFp52 in cell lines. pGensil-1-LEDGFp52-1 was introduced into HeLa cells according to the manual of Lipofectamine TM 2 000. The EGFP expression was observed in 24 hours and the cells were collected in 48 hours to detect the expression alteration of LEDGFp52 protein. ⑥ Westernblot analysis of the expression of LEDGFp52: The cells protein was extracted by quick method of preparation, SDS-PAGE electrophoresis was executed. The protein was transferred onto PVDF membrane and hybridized with mouse-anti-human LEDGFP52 monoclone antibody (1:1 000), incubated with goat-anti-mouse IgG (1:500), then, TMB substrate solution was used in color-alteration observing.

RESULTS

Identification of Recombinant Plasmid and Empty Vector by Endonuclease Digestion 15g/L agarose gel electrophoresis of the endonuclease digestion product of recombinant and empty vector indicated that circular plasmid had been dissected; the longer fragment was about 4.6kb and the shorter one was 410bp while the small fragment of empty vector was only 350bp (Figure 2).

Identification of the Recombinant by Sequencing The recombinant was sequenced via auto-gene sequencing instrument by Sangon Biocompany and the sequencing result was completely coincided with the designed sequence. The contained target gene sequence was nicety and the recombinant plasmid was successfully constructed (Figure 3).

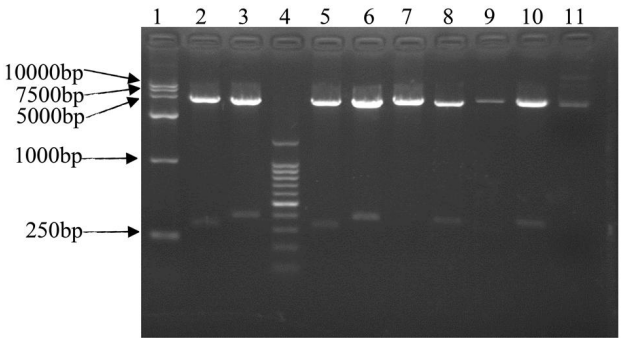

Figure 2 The restrictive endonuclease digestion map of the recombinant Line 1: DNA Marker VI (10 000, 7 500, 5 000, 2 500, 1 000, 250bp); Line 2, 5: pGenSil-1 empty vector digested by *EcoRI* and BamHI, released a small fragment which was 350bp; Line 3, 6, 8, 10: pGenSil-1-siRNA. LEDGFp52 recombinants digested by *EcoRI* and HindIII, released a fragment about4 10bp; Line 4: DNA Ladder 100 (1 500, 1 000, 900, 800, 700, 600, 500, 400, 300, 200, 100bp); Line 7: pGenSil- 1 circular plasmid which was 4900 bp; Line 9, 11: linearized product of pGenSil-1

Expression Inhibition of LEDGFp52 in HeLa Cells by pGensil–1–LEDGFp52–1 pGensil-1-LEDGFp52-1 successfully transfected HeLa cells, green fluorescence detected under phase contrast invert microscope in 24 hours (Figure 4). Westernblot detected the alteration of LEDGFp52 protein expression in 48 hours. Compared with control group and empty vector transfected group, the expression of LEDGFp52 protein in pGensil-1-LEDGFp52-1 transfected group showed down-regulation about 70% (Figure 5).

DISCUSSION

LEDGF promoted growth and survival of many cell types and demonstrated potent ocular protective effect, such as LEC, RPR, RPE [2-4]. The LEDGF gene encodes splice variants that may regulate gene transcription and mRNA splicing of a variety of proteins that are essential for cell growth and survival. Lens epithelium-derived growth factor (LEDGF) gene, which locates on human chromosome 9p21, generates two proteins by alternative splicing, viz., LEDGF-p75 and LEDGF-p52[7,8]. The expression of LEDGF can be detected in lens, retina, cornea and choriod. Bioinformatics suggests that LEDGFp52 may be much more active than we thought. Recent experimentation verified that lens injury could significantly promote RGCs survival and axonal regeneration both in vivo and in vitro, moreover, the regenerative axon could reach superior colliculus, supposed that injured lens released lens-derived neuroprotective substance slough the nature of the substance and the functional mechanism was unknown [9-11]. Therefore, researchers focused their insight on the macrophage and neurotrophic factors which had been verified to be beneficial to nerve regeneration by other investigations. These studies indicated that macrophage infiltration in retina could be

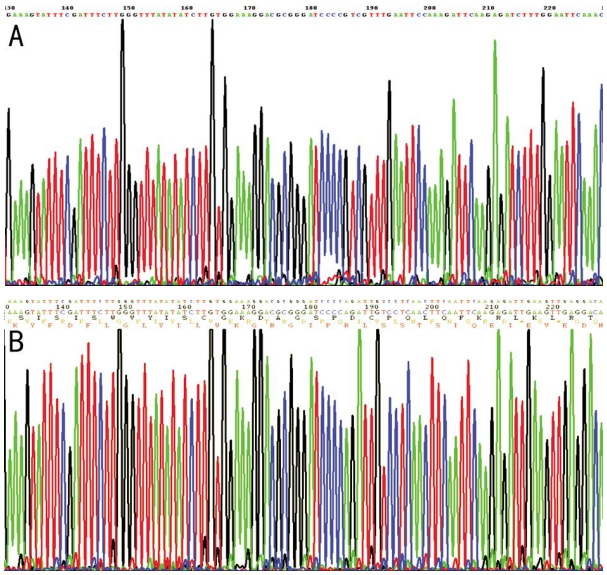

Figure 3 Partial sequence analysis of recombinant plasmid pGenSil –1 –LEDGFp52 pGensil-1-siRNA.LEDGFp52-1 (A); pGensil-1-siGNA.LEDGFp52-2(B)

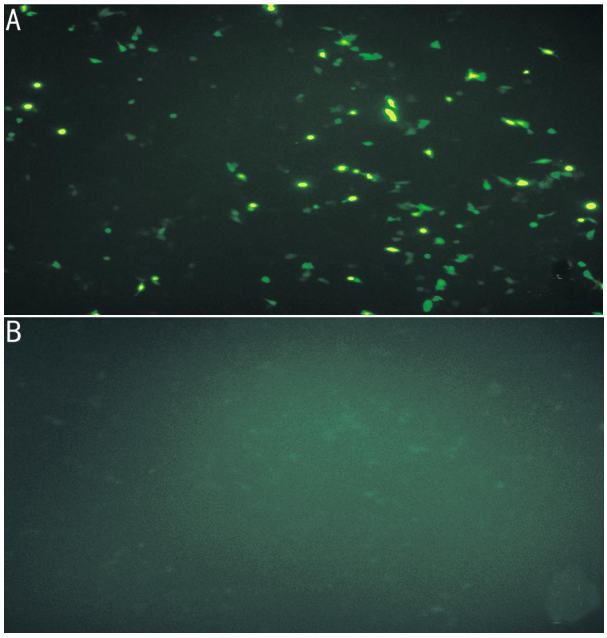

Figure 4 The EGFP appeared in 24 hours once the recom – binant pGenSil –1 –LEDGFp52 –1 transfected HeLa cells (A), but in control group didn´t appear EGFP expression at the same time (B)

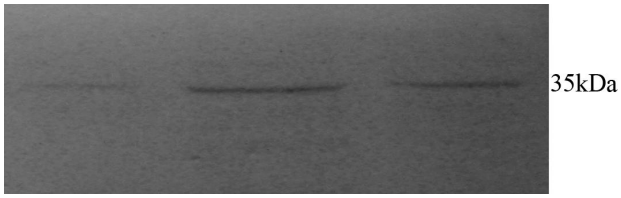

Figure 5 The alteration of LEDGFp52 protein expression in HeLa cell analyzed by Westernblot Left one was pGensil-1-LEDGFp52-1 transfected group. Middle one was control group. Right one was pGensil-1empty vector transfected group

detected after lens injury or zymosan injected in vitreous cavity, and activated macrophage could produce a polypeptide which promotes axon regeneration of RGCs. The molecular weight of this polypeptide was less than 30KD[12]. Macrophage infiltration usually peak in the 1st week after injury then descend in the 2nd week. However, the axonal regeneration could last to the 8th week or longer after lens injury. *In vitro*, coculture of lens and RGCs revealed that lens injury could significantly promote RGCs survival and axon outgrowth [13]. Thus, lens injury mediated RGCs protection and axon outgrowth cannot be merely attributed to the action of macrophage. In addition, there were no expression of the common neurotrophic factors, such as BDNF, NT-4, CNTF, NGFand bFGF [9,11] detected after lens injury using immunohistochemical technique and Westernblot. Furthermore, application of the receptor blocking agent for those factors did not affect RGCs survival in the case of lens injury [13]. All the indirect findings suggest that LEDGF may be a most possible candidate molecul which exert the neuroprotection after lens injury [14]. RNA interference as a method of gene expression inhibition, it has several merits, such as high efficiency, specificity, and stability as well as hereditability [15]. In this experiment, we had successfully constructed eukaryotic expression vector of siRNA specific for LEDGFp52, which could be used for the studying of its biological function on RGCs and which also provided the experimental evidence for further characterizing the lens-derived neuroprotective substance.

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