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Construction and expression of lentiviral vectors encoding recombinant mouse CREBZF in NIH 3T3 cells

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ABSTRACT

CREBZF, also known as Zhangfei or SMILE, is a member of the CREB/ATF protein family. CREBZF has mainly been considered as a basic region-leucine zipper transcription factor that functions in coordination with other transcription factors and plays a role in latent HSV-1 infection, apoptosis and the mammalian endoplasmic reticulum stress and unfolded protein response. In this study, we constructed recombinant lentiviral vectors for CREBZF short hairpin RNA (shRNA) expression and over-expression to improve understanding of the mechanisms regulating CREBZF. The CREBZF ORF sequence was cloned into the lentiviral shuttle plasmid pCD513B-1, and various shRNA oligonucleotides and one negative control (shN) were cloned into the pCD513B-U6 expression vector. The recombinant lentivirus was packaged and transduced into NIH 3T3 cells. CREBZF mRNA and protein expression were examined using real-time reverse transcription-polymerase chain reaction (RT-qPCR) and western blotting, respectively. The over-expression vector and the most effective shRNA vector significantly affected the expression of CREBZF mRNA and protein. Both of the CREBZF recombinant lentiviral vectors were successfully constructed. The over-expression vector significantly increased the expression of exogenous CREBZF and inhibited the growth of NIH 3T3 cells compared to controls. The most effective shRNA lentiviral vector, pCD513B-U6-CREBZF-shRNA-3, was transformed, leading to significant knockdown of the CREBZF gene. We conclude that CREBZF the recombinant lentiviral vectors are promising tools for regulating the expression of CREBZF in NIH 3T3 cells.

1. Introduction

Viral vectors are highly efficient vehicles for transforming exogenous genes and have a promising future in clinical applications. Lentiviral vectors have a number of advantages for research, such as the ability to carry relatively larger exogenous gene fragments, effective control of delivery and integration, stable transduction of exogenous genes into actively dividing as well as resting and differentiated cells, the ability to be condensed into high titers and the ability to express genes long-term *in vivo* without causing an immune response (Blesch, 2004). Such characteristics make lentiviral vectors one of the preferred delivery systems for

Abbreviations: ATF, activating transcription factor; b-ZIP, basic region-leucine zipper; CREB, cAMP response element-binding protein; CREBH, cAMP responsive element-binding protein H; dsRNA, double-stranded RNA; ERRα, estrogen receptor-related receptor; HCF-1, related host cell factor 1; HIV, human immunodeficiency virus; HSV-1, herpes simplex virus-1; NRs, nuclear receptors; RNAi, RNA interference; RT-qPCR, real-time reverse transcription-polymerase chain reaction; RT-PCR, reverse transcription polymerase chain reaction; shRNA, short hairpin RNA; SHP, small heterodimer partner; SMILE, SHP-interacting leucine zipper protein; trkA, tropomyosin-related kinase A; UPR, unfolded protein response.

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exogenous genes transfer. The third generation human immunodeficiency virus (HIV)-derived lentiviral vectors contain four separate plasmids for the production of transducing particles. Compared to the first- and second-generation vectors, the third generation is safer and yields higher titers (Dull et al., 1998). Major applications of the third generation have been in generating transgenic animals, studying gene functions, modeling neurological diseases and developing therapeutic approaches and hepatocyte transplantation (An et al., 2014; Chandrashekran et al., 2014; Li et al., 2012; Nguyen et al., 2009).

RNA interference (RNAi) is a post-transcriptional gene silencing mechanism in which double-stranded RNA (dsRNA) effector molecules trigger the degradation of complementary mRNA transcripts, effectively controlling the expression of desired genes. RNAi has been widely used as a rapid reverse genetics approach to analyze gene function as well as to ablate specific genes for therapeutic purpose, such as cancer and viral infections (Ohrt and Schwille, 2008). The exquisite sequence specificity and high potency make RNAi an attractive gene silencing approach (Hannon and Rossi, 2004).

CREBZF, which is also called Zhangfei and SHP-interacting leucine zipper protein (SMILE), is a member of the cAMP response element-binding protein (CREB)/activating transcription factor (ATF) family of basic region-leucine zipper (b-ZIP) transcription factors identified by their interaction with herpes simplex virus-1 (HSV-1) related host cell factor 1 (HCF-1) (Lu and Misra, 2000). As a bZIP transcription factor, CREBZF can heterodimerize with other transcription factors as a co-regulator, binding to target promoters and regulating downstream genes, which might include VP16 (Vmw65 or αTIF) (Akhova et al., 2005), Luman (LZIP or CREB3) (Misra et al., 2005), activating transcription factor 4 (ATF4 or CREB2) (Hogan et al., 2006), small heterodimer partner (SHP) (Xie et al., 2008), tropomyosin-related kinase A (trkA) (Valderrama et al., 2008), nuclear receptors (NRs) (Xie et al., 2009a, 2009b), estrogen receptor-related receptor (ERRα) (Xie et al., 2009a, 2009b), cAMP responsive element-binding protein H (CREBH) (Misra et al., 2011), Smad 8 (Lee et al., 2012a, 2012b) and p53 (López-Mateo et al., 2012).

Currently, the precise physiological functions of CREBZF remain unclear. CREBZF has been identified as a co-repressor of VP16, inhibiting the replication of HSV (Akhova et al., 2005). However, CREBZF represses GR-, CAR- and HNF4a-mediated target gene expression (Xie et al., 2009a, 2009b), represses CREBH-mediated transactivation of target genes (Misra et al., 2011), impairs glucose-stimulated insulin secretion as well as insulin gene expression and inhibits the expression of beta-cell enriched transcription factors in INS-1 cells (Lee et al., 2012a, 2012b), suppress the expression of Brn3a in Vero cells (Bodnarchuk et al., 2012), suppresses the unfolded protein response (UPR) and inhibits the growth of osteosarcoma cells (Bergeron et al., 2013). However, CREBZF also increases the transactivation of ERs in ER-positive MCF-7 and T47D cells (Xie et al., 2008) and increases ERRα activity in HEK 293T and HepG2 cells (Xie et al., 2009a, 2009b). In this study, we used a third generation lentiviral packaging system and constructed a recombinant lentiviral vector encoding the mouse CREBZF gene and a shRNA that targets CREBZF. We screened for an effective RNAi segment against the CREBZF CDS sequences and generated a new tool for identifying the functions of CREBZF.

2. Materials and methods

2.1. Cell culture

HEK 293T cells (human embryonic kidney cells expressing the large simian virus 40 T-antigen) and NIH 3T3 cells (mouse embryonic fibroblast NIH 3T3 cells) were cultured in high glucose DMEM with 10% fetal bovine serum (FBS; Invitrogen, Inc., Carlsbad, CA, USA) and 100 IU/ml penicillin and 100 μg/ml streptomycin solution in a humidified incubator at 37 °C with 5% CO_2.

2.2. Construction and identification of the CREBZF over-expression lentiviral vector

Primers were designed using the CREBZF sequence in GenBank (GenBank accession number: NM_145151.3). The forward primer was 5′-GCTCTAGATCGGAGTGGCTGCGGC CTAC-3′, and the reverse primer was 5′-CGGGATCCGCGGG AACGACTGCTCTGTAAC-3′. They both contained *Xba* I and *BamH* I restriction enzymes sites. Mouse ovary genomic DNA was used as the template, and reverse transcription polymerase chain reaction (RT-PCR) was used to clone the CREBZF gene using the following reaction conditions: 95 °C pre-denaturation for 5 min; 98 °C denaturation for 10 s, 51 °C annealing for 5 s, 72 °C extension for 1.5 min for 30 cycles; 72 °C extension for 10 min. The RT-PCR products were resolved with 1% agarose gel electrophoresis, and the PCR products were purified, sequenced and cloned into the shuttle plasmid pCD513B-1 to construct the pCD513B-CREBZF lentiviral over-expression vector.

2.3. Construction and identification of CREBZF shRNA lentiviral vectors

To identify effective shRNA targets in the CREBZF cDNA coding sequence (GenBank accession number: NM_145151.3), the following criteria were used: (1) the sequence should begin with a G dinucleotide; (2) the sequence should be 19 nucleotides long; (3) the G/C content should be <50%; (4) the sequence should not be homologous to other coding sequences by BLAST. According to these criteria, three pairs of cDNA oligonucleotides targeting the mouse CREBZF mRNA were designed using online software (https://rnaidesigner.invitrogen.com/rnaiexpress/). One negative control shRNA containing a scrambled sequence was also designed. The three RNAi candidate target sequences and the negative control are shown in Table 1. Sense and antisense primers containing the sense siRNA sequence, a 9 bp loop sequence, the antisense siRNA sequence, and an RNA polymerase III terminator sequence were designed to contain *EcoR* I and *BamH* I restriction sites at the 5′ and 3′ ends, respectively. After pCD513B-U6 was linearized with *EcoR* I and *BamH* I, the primers were annealed and inserted into pCD513B-U6 downstream of the U6 RNA polymerase III promoter. The resulting plasmids containing the shRNA sequences or the negative control were named pCD513B-U6-CREBZF-shRNA-1, -2, -3 and pCD513B-U6-N-shRNA, respectively. The recombinant