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Construction and identification of Fas-targeting siRNA-expressing plasmid*

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Abstract: Objective: To study the therapeutic potential of Fas inhibition in different diseases, a Fas-targeting siRNA (small interfering)-expressing plasmid was constructed. Methods: The U6 promoter cassette and siFas (small interfering RNA that inhibit Fas expression) template sequence were obtained by PCR method. They were cloned into modified pcDNA3.1. The resultant plasmid pU6-siFas was transfected into P815 cells with lipofectin2000 and selected under G-418-containing culture medium. Fas inhibition in stably transfected cells was detected by immunocytochemistry. Results: The plasmid pU6-siFas efficiently reduced the expression of Fas and conferred G-418 resistance in P815 cells. Conclusion: The successful construction of the siRNA expressing plasmid will facilitate the application of RNA interference technique and lay the foundation for further study of Fas inhibition in the treatment of different diseases such as aplastic anemia and acute liver failure.

Key words: RNA interference, Fas protein, Plasmid, Vector construction
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INTRODUCTION

Fas antigen is a receptor that crosslinks with its ligand (FasL) or antibody initiating a signal transduction cascade that leads to apoptosis. Increased Fas expression correlated with various kinds of diseases such as aplastic anemia (Ismail *et al*., 2001) and acute liver failure (Galle *et al*., 1995). Inhibiting Fas expression is an important treatment option for these diseases (Killick *et al*., 2000; Song *et al*., 2003).

RNA interference method is now becoming a powerful tool to specifically inhibit target gene expression in mammalian cells. And its potential use as gene therapy in clinical settings has been demonstrated (Caplen, 2004). Here we reported the design and construction of a small interfering RNA (siRNA)-expressing plasmid that can inhibit Fas expression efficiently in mammalian cells, which lays the foundation for further therapeutic study of Fas inhibition in different diseases.

MATERIALS AND METHODS

PCR cloning of U6+27 promoter cassette

As reported in Liu *et al.*(2003), briefly, mouse genome DNA was prepared from the liver tissue to be used as template in PCR. The following primers were used to clone the mouse U6+27 promoter cassette. Forward primer: 5'-CGGGATCCGATCCGACG CCGCCATCTCTAG-3'; backward primer: 5'-CGGT CGACTAGTATATGTGCTGCCGAAGCG-3'. They have BamHI site at 5' end and SalI site at 3' end. The PCR reaction started with 5 min at 95 °C for template pre-denaturation. This was followed by 30 cycles

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with each cycle consisting of 60 s at 95 °C, 60 s at 62 °C and 80 s at 72 °C, finally, 10 min at 72 °C for elongation. PCR products were cloned into pGEM-T easy plasmid for sequencing.

Generation of DNA sequence for transcription of 21 bp hairpins RNA of Fas (shFas)

The sequence (413−425) 5′-AAGTGCAAGTG CAAACCAGACTT-3′ from the mouse Fas complete cds (GenBank accession number M83649) was chosen as siRNA target site (Song *et al*., 2003) and the design of the corresponding DNA sequence was according to this site. The DNA sequence was generated by PCR method using the following primers: forward primer: 5′-GCGTCGACGTGCAAGTGCAAACC AGACTTCAAGAGAGTCTG-3′; backward primer: 5′-CCAAGCTTCTCGAGAAAAAAGTGCAAGTG CAAACCAGACTCTCTTG-3′. They have SalI site at the 5′ end, and XhoI and HindIII sites at the 3′ end. The sequences underlined were the regions where the two primers were complementary so that they could be elongated in PCR reaction by using each other as template. The PCR mixture includes 10% Taq 10x buffer, 500×10^{-12} mol forward and backward primers, 0.2 μmol/L of dATP, dCTP, dGTP and dTTP, and 3 units Taq DNA polymerase. The final volume was brought to 100 μl. The reaction started with 5 min at 95 °C for template pre-denaturation. Then the mixture was cooled down naturally to room temperature. This was followed by 20 cycles with each cycle consisting of 60 s at 95 °C, 60 s at 62 °C and 60 s at 72 °C, finally, 5 min at 72 °C for elongation. The PCR products were cloned into pGEM-T easy plasmid and sequenced to confirm its validity.

Construction of siFas-expressing plasmid vector

We constructed the siFas-expressing plasmid vector using pcDNA3.1 as the vector backbone. The schematic presentation of vector construction process was shown in Fig.1. Briefly, the U6+27 promoter cassette and shFas hairpin template were serially ligated into the BamHI, SalI and HindIII-digested pUC19 to obtain pUC19/U6-siFas. The pUC19/U6-siFas was then digested with BamHI and XhoI and the U6-siFAS fragment was sub-cloned into BamHI and XhoI-digested pcDNA3.1 (Invitrogen) to obtain pcDNA3.1/U6-siFas. To ensure the full activity of U6 promoter, the pcDNA3.1/U6-siFas was double-digested by BglII and BamHI to eliminate the CMV (cytomegalovirus) promoter sequence. Then the large vector fragment was re-ligated together by T4 DNA ligase. The resultant plasmid was named pU6-siFas.

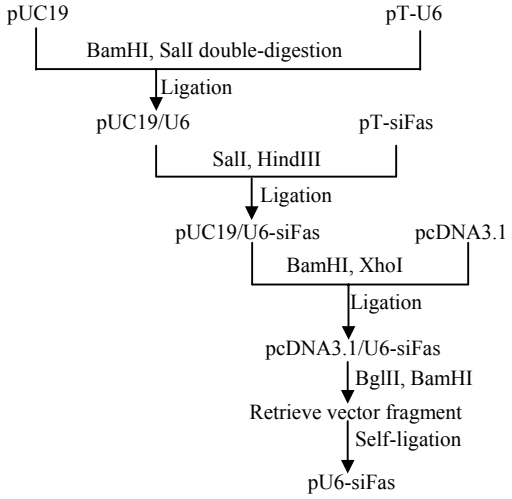


Fig.1 Schematic presentation of the construction process of plasmid pU6-siFas

Plasmid transfection of P815 cells

P815, a mouse mastocytoma cell line expressing certain level of Fas, was kindly provided by the Department of Immunology, the Fourth Military Medical University. These cells, which grew adhered together and proliferated vigorously, were propagated in DMEM (dulbecco's modified medium) complete culture medium with 10% heat-inactivated fetal bovine serum. To determine whether the constructed plasmid can inhibit Fas expression in mammalian cells, the P815 cells were transfected with pU6-siFas and empty pcDNA3.1 as control with lipofectin2000 reagents (Invitrogen) as described by the manufacture. Briefly, P815 cells had 85% confluence in 24 well culture vessel at the time of transfection. One μg plasmid and 3 μl lipofectin2000 were mixed together and added to cells in the culture medium. The cells were introduced at 1:10 dilution into fresh growth medium 24 h after transfection. Forty-eight hours later, G-418 was added into the medium at a final concentration of 700 μg/ml. After two-week's selection, clones were picked individually and expanded. The expression of Fas in individual clones was detected by immunocytochemistry.

Immunocytochemistry

The expression of Fas was detected by Avidin-Biotin Complex (ABC) Method. Briefly, cells smear on slides was dried naturally at room temperature. Then the slides were fixed with iced acetone for 15 min followed by incubation with 3% H_2O_2 in methanol at 37 °C for 30 min to quench endogenous peroxidase activity. After blocking in goat serum in phosphate-buffered saline at 37 °C for 30 min, the slides were incubated with rabbit anti-Fas polyclonal antibody (Boster Biotech, Wuhan, China) at 4 °C overnight, followed by incubation with Biotinylated anti-Rabbit IgG (Zhong Shan Biotech, Beijing, China) at 37 °C for 60 min. Then drops of ABC complex were added and the slides were incubated at 37 °C for 30 min, followed by incubation in fresh DAB (diaminobenzidin) solution. The reaction was stopped by washing in running water when uniform brown color first become visible on the slides with non-transfected P815 cells.

RESULTS

The U6+27 promoter cassette and shFas DNA template were cloned by PCR method

As shown in Fig.2, the PCR product of U6 promoter was 300 bp to 400 bp, corresponding to the size of U6+27 sequence reported in GenBank. The PCR product of shFas DNA template was less than 100 bp, corresponding to the size of the sequence we designed (about 75 bp). Further sequencing confirmed their validity and that no nucleotide mutation existed.

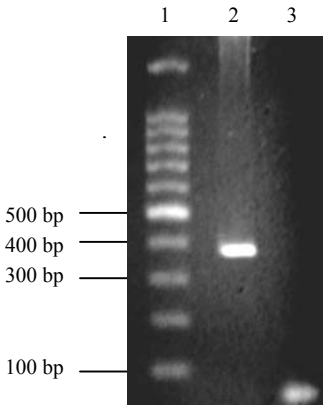


Fig.2 Agarose gel electrephoresis of PCR products
1. 100 bp DNA marker; 2. U6+27 promoter PCR product; 3. siFas DNA template PCR product

siFas-expressing plasmid pU6-siFas was constructed

The plasmid structure is shown in Fig.3. There was one BglII site at the 13th nucleotide sequence in the original pcDNA3.1. BglII, BamHI double-digestion and re-ligation successfully eliminated the CMV promoter and kept the other essential elements in the original pcDNA3.1. Thus the resultant plasmid pU6-siFas had the following features: SV40 origin for episomal replication and simple vector rescue in cell lines expressing the large T antigens such as COS-1 and COS-7 cells; ampicillin resistance gene and pUC origin for selection and maintenance in *Escherichia coli*; neomycin resistance gene for selection under G-418. The elimination of CMV promoter in the original pcDNA3.1 may reduce the promoter interference (Emerman and Temin, 1984) and allow more efficient transcription of siRNA. The size of the final plasmid was about 4.9 kb.

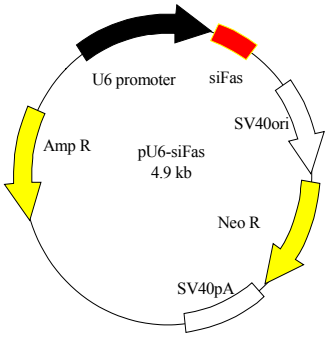
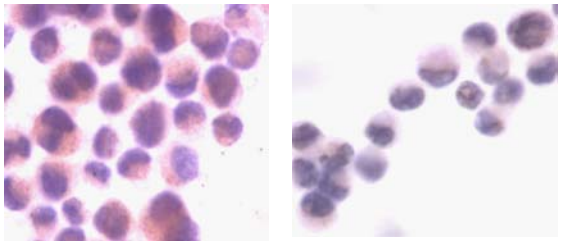


Fig.3 The structure of pU6-siFas

pU6-siFas can efficiently inhibit Fas expression in P815 cells

Plasmids were transfected into P815 cells with lipofectin2000. G-418 was used to select the stably transfected cell clones. As shown in Fig.4, the P815



(a) (b)

Fig.4 Immumohistochemistry detection of Fas expression in P815 cells (a) Fas expression in pcDNA3.1 transfected P815 cells; (b) Fas expression is significantly reduced in pU6-siFas transfected P815 cells

(data not shown) as well as pcDNA3.1-transfected P815 cells express Fas (Fig.4a) with the Fas expression level being significantly reduced in pU6-siFAS transfected P815 cells (Fig.4b). These results demonstrated that the plasmid pU6-siFas efficiently reduced the expression of Fas and conferred G-418 resistance to P815 cells.

DISCUSSION

Fas-mediated apoptosis is involved in the pathophysiology of various kinds of diseases such as acute liver failure (Galle *et al*., 1995) and bone marrow failure (Ismail *et al*., 2001). It was shown that siRNA targeting Fas can protect mouse from fulminant hepatitis (Song *et al*., 2003). Chronic aplastic anaemia (CAA) is a syndrome involving increased apoptosis in stem cells. It is reported that CD34+ cells in CAA patients often have upregulated Fas antigen expression (Ismail *et al*., 2001; Maciejewski *et al*., 1995), which is associated with increased stem cell apoptosis. We suppose that inhibiting Fas expression on CAA BM CD34(+) cells may be effective for the treatment of bone marrow failure.

Several approaches can be applied for specific inhibition of target gene expression at mRNA level such as antisense, riboenzyme, DNAzymes, RNA interference (RNAi) or function at protein level such as small molecule chemicals and peptides. We used RNAi method, because the design of siRNAs is much simpler and the inhibition of gene expression is both specific and efficient.

siRNA can be chemically synthesized in vitro or transcribed by vectors in vivo. In vitro synthesis is fast and may be clinically effective, but is also very expensive and RNA is unstable. Besides, the inhibition of gene expression is transient. Compared to in vitro synthesis, in vivo transcription by siRNA expressing vector is cheaper and easier to handle. And stably transduced cell lines can be obtained if the siRNA-expressing vectors harbor a selectable marker. So we constructed the pU6-siFas plasmid which contains RNA polymerase III promoters U6+27 (Paul and Good, 2002) and head-to-head DNA template for transcription of small hairpin RNA (shRNA) that is later processed into siRNA in cytoplasm by Dicer enzyme. The neomycin resistance gene confers G-418 resistance and thus stably transfected cell clones can be selected in G-418-containing culture medium.

Generally, viral vectors possess higher transduction efficiency than plasmid. Still, many investigators and most pharmacy companies prefer the use of plasmids as potential gene therapy agents, because plasmid meets many of the prerequisites for a clinically viable treatment: ease of manufacturing, low toxicity and immunologically innocuous (to allow repeated administrations, and insensitivity to pre-existing immunity). Besides, the possibility that plasmid generate effects on gene expression independent of the expressed siRNA may be lower than that of viral vectors (Caplen, 2004). Although the transfection efficiency of plasmids is currently still unsatisfactory, it may be improved or optimized by developing new delivery methods such as new classes of cationic lipids (Hirko *et al*., 2003) and nanoparticles (Kaul and Amiji, 2004).

RNAi has become a powerful way to specifically silence target gene expression and its potential clinical application is now being actively investigated. Although RNAi is facing the same problems with other gene-therapies such as drug delivering, some initial studies have shown encouraging results. For example, it has been demonstrated that high-pressure tail-vein injections of synthetic siRNAs or shRNAs expressed from vectors can induce sequence-specific silencing in tissues (principally the liver) of adult mice (Song *et al*., 2003; Xia *et al*., 2002; Lewis *et al*., 2002; McCaffrey *et al*., 2002).

In conclusion, we designed a Fas siRNA-expressing plasmid and tested its efficiency in the P815 cell line. This work laid the foundation for further therapeutic study of Fas inhibition in different diseases such as aplastic anemia and acute liver failure.

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