Construction and Identification of Retro RNAi System of the Specific Silencing CD46*

WANG Mei-juan, GAO Mei-hua△, ZHANG Bei, XIE Xi-he , WANG Bing

(Department of Immunology, Medical College of Qingdao University，Qingdao 266071, China)

ABSTRACT Objective: To construct recombinant retrovirus encoding shRNA targeted human CD46 and identify its function. Methods: shRNA was synthesized, annealed and inserted into pSUPER plasmids digested with *Bgl*II and *Hind*III to construct the recombinant pSUPER RNAi plasmids(pSUPER-CD46). Recombinant pSUPER-CD46 plasmid was identified by enzyme digestion and sequencing analysis. The plasmids were transfected into Jurkat cells in vitro and the inhibitory efficiency of target genes expression was observed with RT- PCR and Western blot. Recombinant pSUPER-CD46 vector was identified correct by enzyme digestion and sequencing analysis. Result: The siRNA eukaryotic expression vectors constructed targeting on CD46 could reduce the expressions of target genes and it might be able to use for the exploration of new anti-fibrosis drugs genetically. Conclusion: The eukaryotic expression vectors were constructed successfully. Future work could investigate the relationship between CD59 and CD46 in T cell signal transduction.

Key words: CD59; ShRNA; CD46; Retroviral vector

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Introduction

CD46 and CD59 (cell surface complement regulatory proteins, CReg), are widely expressed in human lymphoid and non-lymphoid cells. CReg proteins are up-regulated on certain activated leucocyte subsets which indicate that the expression would be increased following immune responses in vivo[1].

CD59 is a widely expressed GPI-anchored glycoprotein regulating complement by binding to C8 and C9 and inhibiting formation of the C5b-9 membrane attack complex [2]. Korty showed that cross-linking of CD59 with antibodies can activate human T lymphocytes[3]. The ability of CD59 to transduce signals in human T cells is mediated by Lck[4]. Decker also demonstrated that CD59could transduce signals indepe ndently of CD3/TCR by showing that CD59 cross-linking activates PKC and ERK2 in Jurkat T cells defective in expression of CD3/TCR. In the presence of PMA, CD59 cross-linking on CD3-de fi cient Jurkat T cells also lead to the increasing expression of CD25 (IL-2 receptorα chain)[5].

The transmembrane protein CD46 is a complement regulatory protein ubiquitously expressed that acts as a cofactor for the cleavage of the C3b and C4b complement products by factor I and therefore protects cells from lysis by autologous complement [6]. CD46 also transduces intracellular signals, which leads to intracellular calcium increasing[7], and tyrosine phosphorylation of the intracytoplasmic region CD46-Cyt2 has been described [8]. CD46 stimulation in human PBL leads to the Tyr phosphorylation of two adapter proteins, CBL and LAT, both involved in T cell activation (CD46 also acts as a co-stimulator, and promotes T cell proliferation upon its cross-linking with the TCR-CD3[9]. Similar results were reported by Fernandez , who showed that Crry, the CD46 homologue in mice, co stimulated murine T cells[10]. Zaffran showed that CD46/CD3 co-stimulation induced a synergistic activation of ERK 1/2 MAPKs, Vav and Rac. The aims of the present study were to construct recombinant retrovirus encoding shRNA targeted human CD46 and identify its function,and prepared for future work[11].

1 Materials and Methods

1.1 Bacteria, plasmids and reagents

The pSUPER retro neo+gfp vector, Jurkat T-lymphocytes cell culture and coli JM109 were conserved in our laboratory. The restriction enzymes were purchased from Fermentas, E.Z.N.A. Gel Extraction kit was from OMEGA;RPMI 1640 was purchased from GIBCO (Gaithersburg, MD, USA); DNA Ligation Kit from Takara; The packaging cell line PA317 was obtained from CELL bank of Shanghai and cultured in dulbecco's minimum essential medium (DMEM, Hyclone containing 10% fetal bovine serum (FBS, PAA, Germany >. Lipofectamine 2000 (Invitrogen). Other chemicals and reagents available were from local commercial sources.

1.2 Methods

1.2.1 Designing and synthesis specific oligonucleotides of CD46 According to the advice of Ambion (http://www.ambion.com/techlib/misc/siRNA-design. html) and the software of RNAstructure5.2 on RNAi target sequences, 4 pairs of 19bp specific sequences were selected from human CD46 mRNA (GenBank, Accession: X59408,X59405, AK222822,BC030594), designing 2 pairs of 60nt oligonucleotides differently and a pair of negative control sequence. Each strand includes the sequence forming enzy-

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Author: Wang Mei-juan, female, Current Master Student.

Tel:15006489773; E-mail:huanhuaweiyu@163.com

△Corresponding Author: GAO Mei-hua, female, professor, doctoral suervisor. Tel: 0532-83780021; E-mail: Meihuagao@yahoo.com.cn

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me cut site at the two endpoints, 5 thymines as termination signal and 2 inverse complementary 19 nt CD46 specific sequences separated by 9nt district forming hairpin structure. Each pair of 60nt oligonucleotides formed double-stranded DNA with *Bgl*II and *Hind* III at the endpoint (Tab1).The oligonucleotide sequences were synthetized by Sangon (Shanghai, China). Bold letters indicate CD46-targeting sequences, including sense strand and complementary antisense strand, forming a hairpin structure after transcript.

Table 1 Oligonucleotides used in RNAi

No	Sequence	Location
T1T2	5'GATCCCCTTCAGTGTGGAGTCGTGCTTTCAAGAGAAGCACGACTCCACACTGAATTTTTA3′	782nt-800nt
	5'AGCTTAAAAATTCAGTGTGGAGTCGTGCTTCTCTTGAAAGCACGACTCCACACTGAAGGG3′	
	5'GATCCCCCATTTGAAGCTATGGAGCTTTCAAGAGAAGCTCCATAGCTTCAAATGTTTTTA3	
C	5'AGCTTAAAAACATTTGAAGCTATGGAGCTTCTCTTGAAAGCTCCATAGCTTCAAATGGGG3′	262nt-280nt
	5'GATCCCCTCGGGATGGTTATTGGCTCTTCAAGAGAGAGCCAATAACCATCCCGATTTTTA3′	
	5'AGCTTAAAAATCGGGATGGTTATTGGCTCTCTCTTGAAGAGCCAATAACCATCCCGAGGG3′	

1.2.2 Construction of recombinant plasmids CD46−Psuper Annealing oligonucleotides 1 OD oligos was dissolved in 11 μl deionized H_2O which was about 33μg/μl, then 1μl was taken from each oligo (forward and reverse), and 48μl annealing buffer (TE, PH8.0) was added. The annealed oligos were incubated at 94℃ for 4 min, 80℃ for 4min, 70℃ for 10min , 37℃for 20min, then slowly cooled down to room temperature. pSUPER plasmids Primary plasmids were digested with *Bgl*II and *Hind*III. The final products were recovered by agarose gel electrophoresis and inserted into digested pSUPER plasmids. The oligos were inserted into the digested pSUPER plasmids at the molar ratio of 7:1. The directional cloning was accomplished with the aid of T4 DNA ligase. A negative control reaction was performed with no insert. 4) Transformation of the ligation products. The recombinant plasmids were transformed into competent cells of host strain, JM109. The positive clones were identified. The colonies were randomly picked up and cultured in an ampicillinum broth. Then several colonies were chosen until a positive clone was located by digesting with *Eco*RI and *Hind* III primarily. Then, sequencing is carried out for further identification. The point region were amplified by PCR using plasmids as templates, with the following primers: pSUPER-a: 5'-CCTTTATCCAGCCCTCACTC-3'pSUPER-s:5'-AGACTGCCTTGGGAAAAGCG-3'Finally, the insert sequences were sequenced by Shanghai Sangon Biological Engineering Technology & Service Co.

1.2.3 Cell culture and transfection The packaging cell line PA317 was cultured in DMEM medium with 10% fetal bovine serum (FBS) and 100U/mLpenicillin/100mg/mL streptomycin at 37℃ in a humidified incubator with 5% CO_2 for 24h. Cells were plated in a 24-well plate at the density of 2×10^8/L. With the cell confluence reaching 80%, transient transfection were carried out according to the ratio of 1:1.25 for preparing the DNA-Lipofectarnine 2000 complex in a total volume of 500μl DMEM, which was mixed gently and incubated for 20 min at room temperature. During the incubation at 37℃ in a humidified incubator with 5% CO_2, 500μl fresh growth medium was added to cells. 4h later, 1.5 ml DMEM medium containing 10% fetal bovine serum was added. The siRNAs were incubated with the cells for 24 h. Forty-eight hours post-transfection, the supernatant was collected, filtered through a 0.45μm msyringe filter, and infected the Jurkurt cell. Jurkat cells were cultured in RPMI 1640 with 10% fetal bovine serum, 100U/ml penicillin and 100 g/ml streptomycin in a humid wet incubator (37℃, 5% CO_2). After adjusting the cell density to 5×10^5/mL, they were inoculated in 24-well plates. Next day, the cells were incubated with 10^4 cfu (colony forming units) per well recombinant retrovirus containing polybrene (8μg/mL final concentration) at 37℃ for 2 h, then the medium was replaced. 12 h later, the medium was removed and replaced with fresh medium containing G418(800μg/mL, Amresco). This was repeated 3 times at 3-day intervals. On day 14, colonies of G418-resistant monoclonal cells were screened for stable expression of siRNAs. The efficacy of the two target sequences was evaluated and the best chosen for sub-sequent experiments.

1.2.4 RNA Extraction and RT−PCR Analysis Total RNA was extracted by using Trizol reagent according to the manufacturer's protocol. The expression of CD46 was detected by RT-PCR one step assay provided by Promega Company. DNA primer sequences were designed as follows: for human CD46, sense 5'AgCACTGGATGCTTTGTGAGT3'and antisense 5'GAGTCACCAGCAGAAGAA3', 5'ATATTCAGCTCCACCATCTGCT-3' with a 267 bp product in length,for glyceraldehydes-3-phosphate dehydrogenase (GAPDH), sense 5'CGTGGAAGGACTCATGACCA3' and antisense 5'TCCAGGGGTCTTACTCCTTG3', with a 509 bp product in length. The amplification conditions for both CD46 and GAPDH were as follows: initial denaturation at 94℃ for 2 minutes, 40 cycles of 94℃ for 30 seconds, 47℃ for 1 minute, 68℃ for 1m-

inutes and extended incubation at 68 ℃ for 7 minutes. Aliquots of PCR product were electrophoresed in 1 % agarose gels.

2 Result

2.1 pSUPER retro neo+gfp vector digested with *Bgl* Ⅱ and *Hind*Ⅲ restriction enzymes

The empty plasmid was digested by *Bgl* Ⅱ (2424 site) and *Hind* Ⅲ (1441 site) restriction enzyme, the removed fragment of the empty plasmid was (2424-1441)983bp, the left fragment of the empty plasmid was(8371-983)7388bp(Fig.1).

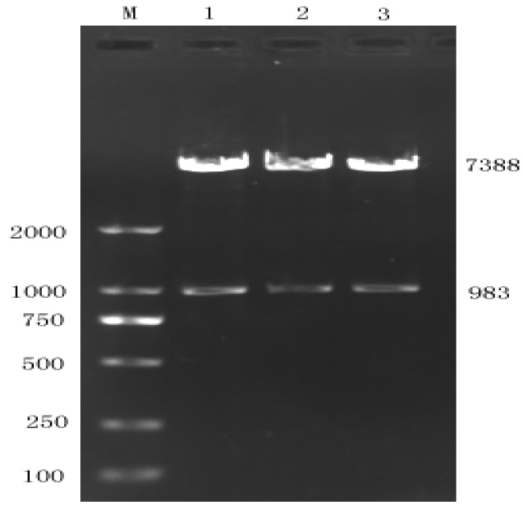

Fig.1 Linearize the pSUPER retro neo+gfp vector with Bgl Ⅱ and HindⅢ restriction enzymes

2.2 PCR Identification of the recombinants

Aliquots of PCR product were electrophoresed in 1 % agarose gels. The positive clone was 397 bp, and the negative clone was 1,318 bp (397 - 62 + 983) (Fig.2).

2.3 Double Enzyme digestion of the recombinants

The empty and recombinant plasmid was digested by *EcoR* I (2645 site) and *Hind* Ⅲ (1441 site) restriction enzyme, the removed fragment of the empty plasmid was 1,204 bp (2645 - 1441), the fragment of the recombinant plasmid was 281 bp (1204 - 983 + 62) (Fig.3).

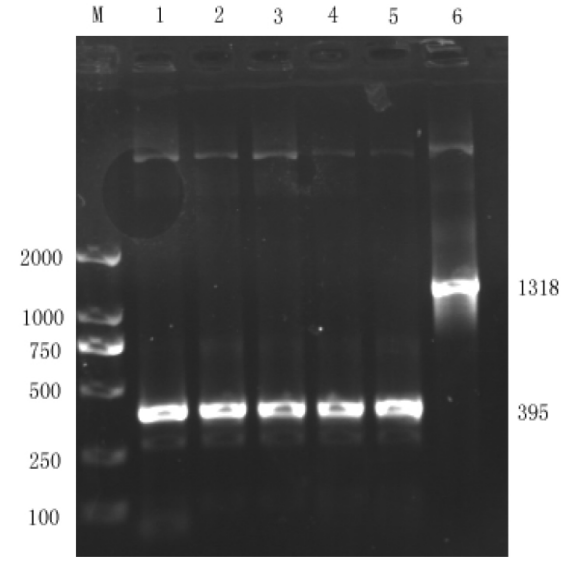

Fig.2 PCR Identification of the recombinants:M. DL2000 DNA marker; ①～②：pSUPER-siCD46-T1③pSUPER-siCD46-T2；④～⑤pSUPER-siCD46-C；⑥pSUPER；pSUPER-siCD46 was about 395bp; pSUPER was about 1318bp

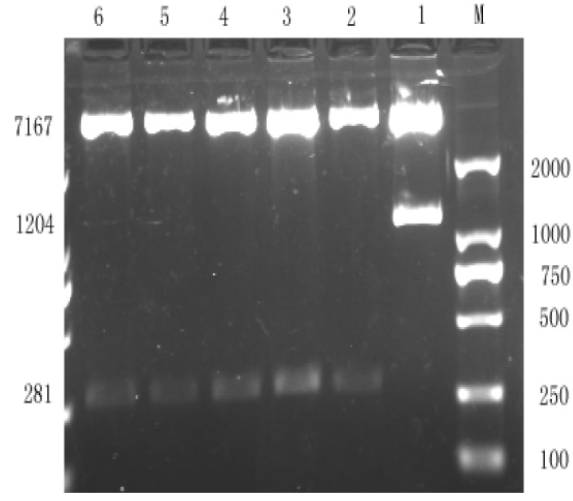

Fig.3 Double Enzyme digestion of the recombinants:M. DL2000 DNA marker; ①: pSUPER; ②~③: pSUPER-siCD46- C; ④pSUPER siCD46-T2; ⑤～⑥pSUPER siCD46-T1,digested by *EcoR* I and *Hind*Ⅲ, pSUPER-siCD46was about 281bp;pSUPER was about 1204bp

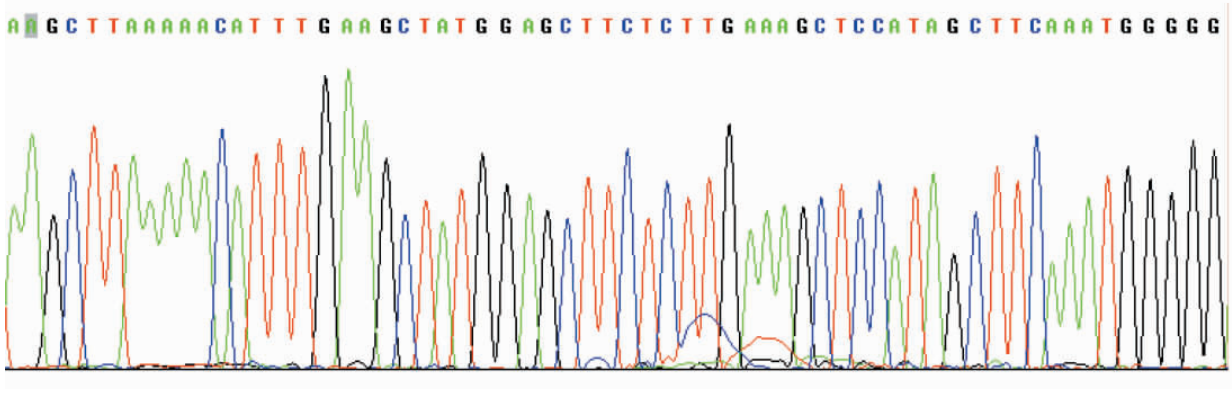

Fig.4 Sequence of the recombinants pSUPER-siCD46-T2

2.4 Sequence identity of the recombinants

The recombinant vector was identified by DNA sequencing. And the sequence demonstrated that 60bp has inserted into the pSUPER vector.

2.5 Expression of CD46 protein and inhibition by siRNA

Jurkat cells transfected with pSUPER retro neo+gfp recombinantplasmid with each siRNAs were observed by fluorescencemicroscope at 36-48 h post-transfection (Fig.5). The efficiency of transfection was confirmed by the highly expressed reporter gene GFP protein. There was obvious reduction effect induced by all CD46 specific siRNAs except the scrambled one (Fig.5).

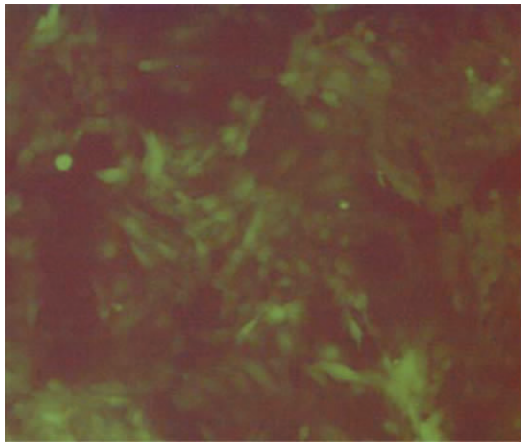

Fig.5 Fluorescence microscope images of PA317after transfected. 200× ;

2.6 RT-PCR identification of CD46 mRNA

The expression of CD46 mRNA in group Ⅲ was significantly lower than that of group Ⅰ and group Ⅱ ($P<0.05$) (Figure 6). CD55 mRNA level in Ⅳ group decreased significantly compared with group Ⅰ and Ⅱ ($P<0.05$) (Figure 3B), while there was no statistical significance between group Ⅰ and Ⅱ.

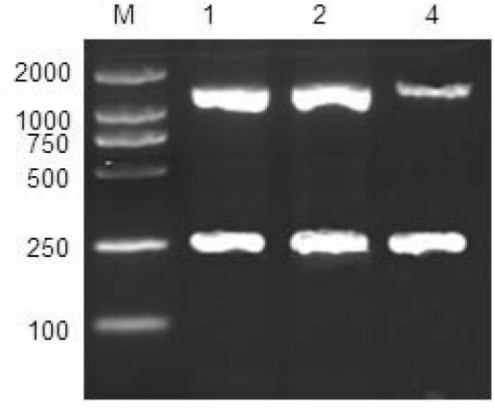

Fig.6 CD46 mRNA level were detected in stable transfected Jurkat cells by RT-PCR:M: DL 2000DNA marker ;1: Untransfected Jurkat cells; 2: Jurkat cells transfected with pSUPER; 4: Jurkat cells transfected with pSUPER-siCD46

3 Discussion

CD59 is an 18-20-kDa GPI-anchored glycoprotein. Because of its method of cell surface anchoring, it does not span the plasma membrane. Despite of this, it had shown that antibody-mediated cross-linking of CD59 on the surface of human hematopoietic cells resulted in a rapid and robust increase in the level of tyrosine phosphorylation of several intracellular proteins. Studies had shown that antibody cross-linking of CD59 induced a series of intracellular signaling events including the activation of protein-tyrosine kinases (PTK). Korty showed that cross-linking of CD59 with antibodies can activate human T lymphocytes [3]. The ability of CD59 to transduce signals in human T cells is mediated by Lck [4]. Human T-cell activation by CD59 cross-linking leads to activation of Lck and ZAP-70 PTKs that stimulate the TCR/ZAP-70 signaling cascade[5]. Lck and Fyn initiate TCR signaling pathways by phosphorylation of CD3 immunoreceptor tyrosine-based activation motifs (ITAMs) leading to recruitment and activation of ZAP-70 and Syc PTKs that activate downstream signaling pathways[12]. Deckert also demonstrated that CD59 can transduce signals independently of CD3/TCR by showing that CD59 cross-linking activates PKC and ERK2 in Jurkat T cells defective in expression of CD3/TCR. In the presence of PMA, CD59 cross-linking on CD3-deficient Jurkat T cells also leads to enhanced expression of CD25 (IL-2 receptorα chain)[5]

Two reports had described the presence of an 80-kDa glycoprotein present in immunoprecipitations of the activated complement-binding proteins CD55 and CD59, a possible transmembrane link of the intracellular kinases to the extracellular receptor [4]. The association of a transmembrane component with a GPI-linked receptor had been not uncommon. The mechanism by which GPI-linked CD59 sent messages was unknown. It is possible that CD59 needs a transmembrane panning co-receptor to send signals.

CD46 is a type I membrane glycoprotein expressed by all human nucleated cells. CD46 and CD59,are expressed at higher levels on recently activated/memory lymphocytes (CD45RO+) than naıve lymphocytes (CD45RO-) could indicate that the changes in CReg protein expression observed in vitro occur also followinglymphocyte activation in vivo. CReg proteins are up-regulated on certain activated leucocyte subsets indicate that levels would be increased following immune responses in vivo [1]. However, CD46 is also capable of transducing signals into several cell types, including macrophages, dendritic cells, B and T cells. The cytoplasmic domain of CD46 encodes putative signals for the protein kinases PKC, CK2 and Src [13]. Wang found that cross-linking CD46 on Jurkat T lymphoma cells led to tyrosine phosphorylation in the CD46 intracellular tail. CD46 phosphorylation was dependent on the activity of the Src-PTK Lck[14]. Analyzing the ability of CD46 to transduce signals, Astier showed that cross-linking of CD46 on human T cells induced phosphorylation of two adaptor proteins, p120CBL and the linker for activation of T cells, LAT, known to regulate T cell receptor (TCR) signaling. Efficient T-cell activation includes TCR engagement by antigen and co-stimulation by accessory rece-

ptors, such as CD28, CD4, CD8 and CD2 [15]. CD46 also acts as a co-stimulator, and promotes T cell proliferation upon its cross-linking with the TCR-CD3. Similar results were reported by Fernandez C. they showed that Crry, the CD46 homologue in mice, costimulates murineT cells[10]. Zaffran showed that CD46/CD3 co-stimulation induced a synergistic activation of ERK 1/2 MAPKs, Vav and Rac [11]. The costimulatory effect of CD46 is accompanied by morphological changes in human T cells which become adherent and reorganize their actin filaments in a lamellipodia-like structures and in thin protrusions. These changes are probably mediated by Vav and are functionally important.

This study constructed recombinant retrovirus encoding shRNA targeted human CD46 and identify its function. And the eukaryotic expression vectors were constructed successfully. This work is very useful to our further research.In the future work we could investigate the function change when lower the expression level of CD46 and /or CD59 by RNAi,then identify the relationship between CD59 and CD46 in T cell signal transduction.

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人 CD46 RNAi 逆转录病毒载体系统的构建与鉴定*

王美娟 高美华△ 张 蓓 解西河 王 冰

(青岛大学医学院免疫学教研室 山东 青岛 266071)

摘要 目的:构建并筛选携带针对 CD46 基因的 pSUPER retro RNAi 逆转录病毒载体,从而特异、有效地抑制人 CD46mRNA 水平。方法:利用 DNA 重组技术,将 2 条 60nt 能转录产生靶向 CD46 小发夹 RNA (shRNA) 的寡核苷酸序列,定向克隆入逆转录病毒载体 pSUPER retro,并转化大肠杆菌 JM109。结果:重组载体经 PCR 及限制性内切酶酶切鉴定初步成功后送测序,结果表明序列正确。结论:特异性沉默 CD46 基因的 pSUPER retro RNAi 逆转录病毒载体构建成功,为后续转染 Jurkat 细胞,研究 CD46 在 T 细胞的信号转导中的作用奠定了基础,对进一步研究 T 细胞相关疾病及开展细胞免疫缺陷的治疗方面提供了新的思路与方向。

关键词:CD59;CD46;ShRNA;逆转录载体

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作者简介:王美娟(1983-),女,硕士生,Tel:15006489773,E-mail:huanhuaweiyu@163.com

△通讯作者:高美华(1953-),女,教授,博士生导师,Tel:0532-83780021,E-mail: Meihuagao@yahoo.com.cn

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