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Construction of RNAi Expressing Vector Targeting the Goat Prion Protein Gene (*PRNP*)

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Abstract: To explore the effect of RNA interference on the suppression of goat prion protein gene (*PRNP*), small interfering RNA (siRNA) expressing vectors pSilencer4.1-siRNA1 and a negative control pSilencer4.1-siRNA2 and the eukaryotic expressing vector pSG5-prion expressing parts of goat prion protein gene were designed and constructed. The recombinants were introduced into Human Embryonic Kidney (HEK) 293T cells mediated by GeneTran. RT-PCR was carried out to detect the expression of prion protein gene. The results showed that RNAi effects induced by pSilencer4.1-siRNA1 could successfully deplete the mRNA expression of goat prion protein gene in 293T cells 48 h after transfection with both vectors compared to the negative control. The findings demonstrated that pSilencer4.1-siRNA1 was constructed successfully to transfer antagonistic substance against goat prion protein gene.

Key words: RNA interference, siRNA, prion protein gene, goat, antagonistic, protein

INTRODUCTION

RNA interference (RNAi) is a promising antiviral strategy (Hu *et al*., 2002). Small interfering RNA (siRNA) can degrade mRNA which has a complementary sequence to the siRNA by RNAi (Tuschl *et al*., 1999). The siRNA has been widely used as an experimental tool to investigate the function of target genes because of its convenient, specific and potent gene silencing effect (Miyagishi *et al*., 2003; De Souza *et al*., 2006). Moreover, therapeutic application of siRNA targeting the gene of interest has been actively investigated. In addition to siRNA, DNA vectors that transcribe siRNA or short hairpin RNA (shRNA) are also available to induce RNAi. Vector-based approaches and siRNA share the same RNAi pathway but have different properties that affect the efficacy of RNAi-based therapy (Sui *et al*., 2002; Brummelkamp *et al*., 2002; Yu *et al*., 2002).

Prion diseases are a group of transmissible neurodegenerative disorders that include Creutzfeldt-Jakob Disease (CJD), Gerstmann-Straussler Syndrome (GSS), Kuru and Fatal Familial Insomnia (FFI) in humans as well as goat scrapie and bovine spongiform encephalopathy in animals (Aguzzi and Polymenidou, 2004). These diseases are caused by the conversion of the host cellular Prion Protein (PrP^{c}) into scrapie Prion Protein (PrP^{Sc}), a β-sheet-rich conformer that is infectious in the absence of nucleic acid (Prusiner, 1998). Researches (Bueler *et al*., 1993; Mallucci *et al*., 2002) have shown that the decline of expression level of endogenous PrP^{c} could prolong the incubation period of prion diseases. The decline of expression level of goat prion protein gene (*PRNP*) by RNAi pathway may be an effective method to prevent goat prion disease.

Two research groups (Brummelkamp *et al*., 2002; Sui *et al*., 2002) proposed a prospect method to control the gene expression and revealed that gene-specific suppression can be achieved by a DNA vector-based approach in mammalian cells. In this study, researhcers used the similar technology of DNA vector-based RNAi to inhibit the expression of PRNP, for the purpose of obtaining useful vectors and therapeutic approach to control goat prion diseases.

MATERIALS AND METHODS

Plasmid and cell: The siRNA expressing plasmid pSilencer 4.1-CMVneo was purchased from Ambion/Invitrogen Co., Ltd. The eukaryotic expressing plasmid pSG5 was purchased from Stratagene Co., Ltd. The competent cells of Escherichia coli DH5α were bought from Tiangen Biotech (Beijing, China) Co., Ltd. The Human Embryonic Kidney (HEK) 293T cells were bought from ATCC (American Type Culture Collection).

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Vector constructions

Design and synthesis of siRNAs: The target sequence of PRNP was searched via online software (http://www.ambion.com/techlib/misc/siRNA-finder.html). After homologue sequences blasting, researchers chose 5'-AAAACCTGGCGGAGGGGATGGAA-3' (nucleotides 84-106, GenBank Accession No. X74758.1) which was located at the coding sequences of PRNP as the target sites of PRNP. Based on the chosen sites, oligonucleotides named siRNA1 were designed according to the principle recommended by Ambion Online Software. Oligonucleotides named siRNA2 were designed as a negative control based on the other sites of PRNP. The specific restriction site, BamHI site was added to the 5'-end of sense and Hind III site was added to the 5'-end antisense of strand for insertion into the pSilencer 4.1-CMVneo vector. Chemically-synthesized siRNAs were purchased from Invitrogen (Shanghai, China). The sequences of the siRNAs are given in supplemental Table 1.

siRNAs annealing: The synthesized oligonucleotides were dissolved at a concentration of 100 μmol L^{-1}. Annealing of complementary oligonucleotides was done in 50 μL containing 5 μL 10×shDNA annealing buffer, 5 μL sense chain oligonucleotides (100 μmol L^{-1}), 5 μL antisense chain oligonucleotides (100 μmol L^{-1}) and 35 μL^{-1} ddH_2O for 5 min at 95°C, 5 min at 85°C, 5 min at 75°C and 5 min at 70°C. The annealed DNA fragments were diluted to 20 nmol L^{-1} for ligation.

Recombination and identification of expressing vectors: The siRNA expressing plasmid, pSilencer 4.1-CMVneo, was digested with BamH I and Hind III. Enzyme digestion was done in 50 μL containing 5 μL 10×buffer, 2 μL BamH I, 2 μL Hind III, 2 μg pSilencer 4.1-CMVneo and complementary ddH_2O for 3 h at 37°C. The products were electrophoresed on agarose gel and then were recovered using Agarose Gel DNA Purification kit Ver. 2.0 (Takara) according to manufacturer's instructions. Ligation reaction was performed in 16 μL containing 2 μL 10×T4 Ligation buffer, 1 μL annealed DNA fragments, 1 μL linearized pSilencer4.1-CMVneo vector, 0.5 μL T4 DNA ligase and 11.5 μL ddH_2O overnight at 4°C. The annealed DNA fragments were subcloned into the BamH I and Hind III sites of pSilencer 4.1-CMVneo to act as siRNA templates which will yield hairpin-like siRNAs (Fig. 1). The recombinant plasmids were named as pSilencer 4.1-siRNA1 and pSilencer4.1-siRNA2. According to the designation, the structure and transcribed yields of pSilencer4.1-siRNA1 and pSilencer4.1-siRNA2 are shown in Fig. 1.

The eukaryotic expressing plasmid, pSG5 was digested with EcoR I using similar methodology. The pSG5 vector recovered from agarose gel was treated with CIAP for preventing recircularization of the vector. Total DNA was extracted from goat blood samples using the DNAzol purification kit (Invitrogen) following manufacturer instructions. About 50 ng of genomic DNA was applied to PCR for amplification of target sequence of PRNP. PCR primers (Table 2, PrP) with EcoR I restriction site at 5'-end were designed from the chosen sequence of PRNP. Ligation reactions employed linearized pSG5 vectors and PCR products. The recombinant plasmids were named pSG5-prion. The structure and transcribed yields of pSG5-prion are shown in Fig. 2.

The competent cells of Escherichia coli DH5α were transfected with the recombinant plasmids. About 16 h after transfection, plasmids were extracted from the white clones. The recombinants designated as pSilencer4.1-siRNA1, pSilencer4.1-siRNA2 and pSG5-prion were identified with PCR initially and then verified by sequencing. The detecting primers for PCR identification were shown in Table 2.

Table 1: siRNA template oligonucleotides used in this study

siRNA	Sequence
siRNA1	
Sense	5'-GGATCCAACCUGGCGGAGGAUGGAATT'-3'
Antisense	5'-AAGCTTUUCCAUCCUCCGCCAGGUUTT-3'
siRNA2	
Sense	5'-GGATCCAAGCCACAUAGGCAGUUGGTT-3'
Antisense	5'-AAGCTTCCAACUGCCUAUGUGGCUUTT-3'

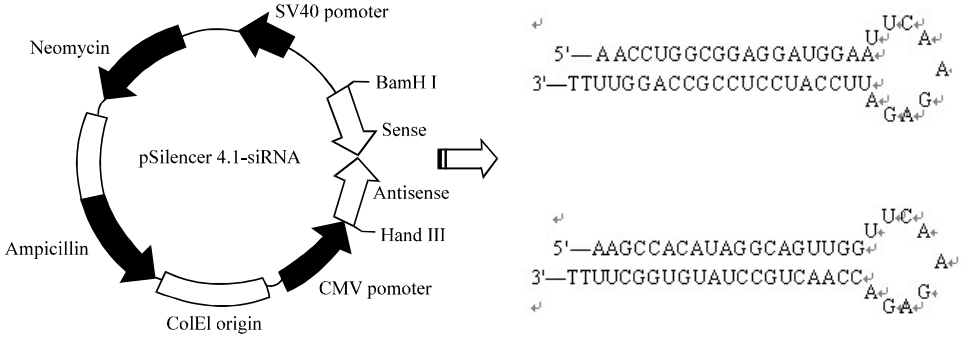

Fig. 1: Schematic diagram of structures and transcribed yields of pSilencer4.1-siRNA1 and pSilencer4.1-siRNA2 and hairpin-like siRNAs

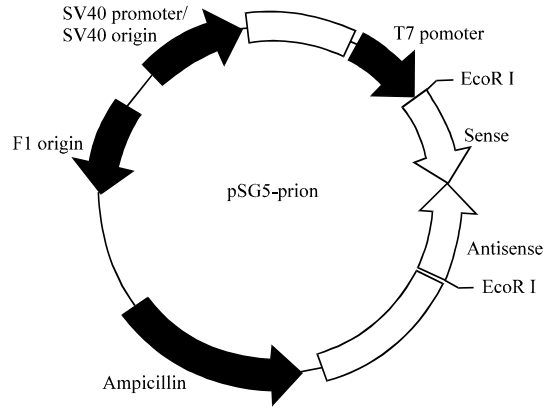

Fig. 2: Schematic diagram of structures and transcribed yields of pSG5-prion

Table 2: Primers used in this study

Primers designation	Sequences	Anticipate size (bp)
PrP		
Forward	5'-CCGAATTCAGGCGATTAAGTTGGGTA-3'	250
Reverse	5'-GCGAATTCCGGTAGGCGTGTACGGTG-3'	
Detecting primer for siRNAs vectors		
Forward	5'-AGGCGATTAAGTTGGGTA-3'	240
Reverse	5'-CGGTAGGCGTGTACGGTG-3'	
Detecting primer for pSG5-prion		
Forward	5'-CCAGGCGATTAAGTTGGGTA-3'	250
Reverse	5'-GCGGTAGGCGTGTACGGTG-3'	
Human beta-actin		
Forward	5'-TATGTGGGCGACGAGGCCCA-3'	300
Reverse	5'- AGTGGTACGGCCAGAGGCGT-3'	

Transfection and Reverse Transcriptase PCR (RT-PCR): The Human Embryonic Kidney (HEK) 293T cells were cultured in 6 well plates (1×10^5 cells per well) for 24 h before transfection in medium lacking penicillin and streptomycin. Cells were cotransfected with pSilencer4.1-siRNA1 and pSG5-prion or pSilencer4.1-siRNA2 and pSG5-prion at the ratio of 1:1 by using GeneTran (Invitrogen) according to manufacturer's guidelines. After 4 h, the transfection medium was changed to growth medium (2 mL medium per well) containing 0.5 mL DMEM and 20% FBS. Cells were harvested at 48 h after the cotransfection. The total RNA was isolated with Total RNA kit (Tiangen Biotech, China)) and reverse-transcribed to complementary DNA (cDNA) using a One Step RT-PCR kit (Takara) according to the manufacturer's instruction. cDNA was applied to PCR for analysis of expression of prion protein gene. PCR reactions were performed with PrP primers (Table 2) and human beta-Actin primers (Table 2) that could quantify the dosage of RNA in different samples.

RESULTS AND DISCUSSION

Identification of pSilencer4.1-siRNA1 and pSilencer4.1-siRNA2: PCR experiments were carried out for the identification of pSilencer4.1-siRNA1 and pSilencer4.1-siRNA2. As shown in Fig. 3, a fragment about 240 bp can be amplified from DNA of all identified cell clones. The following sequencing confirmed that sample 1 contained the sequence for siRNA1 and sample 2 contained the sequence for siRNA2 indicating that the designed DNA templates of siRNAs targeting PRNP were inserted into pSilencer 4.1-CMVneo successfully.

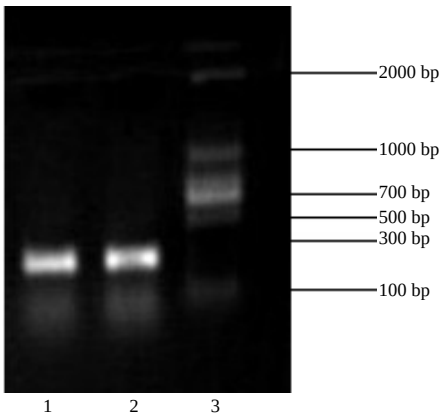

Fig. 3: Identification of pSilencer4.1-siRNA1 and pSilencer4.1-siRNA2 with PCR. Lane 1: pSilencer 4.1-siRNA1; Lane 2: pSilencer 4.1-siRNA2 and Lane 3: Marker

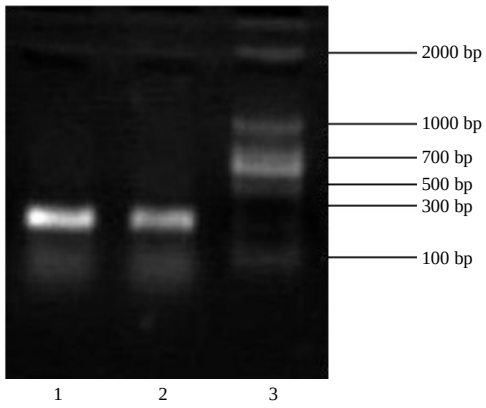

Fig. 4: Identification of pSG5-prion with PCR; Lane 1, 2: pSG5-prion and Lane 3: Marker

Identification of pSG5-prion: PCR was carried out for the identification of pSG5-prion. As shown in Fig. 4, a fragment about 250 bp can be amplified from DNA of the two identified cell clones. The following sequencing confirmed that these samples contained the target sequence indicating that the target sequence of PRNP was inserted into pSG5 successfully.

Suppression of the expression of prion protein gene in 293T cells: To confirm whether the expression of target

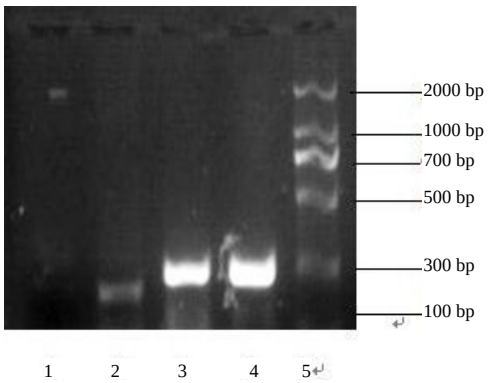

Fig. 5: Suppression of the expression levels of prion protein gene. Lane 1, 3: Cotransfected with pSilencer4.1-siRNA1 and pSG5-prion; Lane 2, 4: Cotransfected with pSilencer4.1-siRNA2 and pSG5-prion; Lane 1, 2: Using PrP primers; Lane 3, 4: Using human beta-actin primers; Lane 5: Marker

sequence of PRNP were suppressed by the recombinant RNAi vectors, RT-PCR was performed based on the total RNA isolated from 293T cells cotransfected with pSilencer4.1-siRNA1 and pSG5-prion, or with pSilencer4.1-siRNA2 and pSG5-prion. The figure of electrophoresis on agarose gel showed that the constructed pSilencer4.1-siRNA1 vector could effectively suppress the the target sequence of PRNP compared to pSilencer4.1-siRNA2, the negative control. PCR performed with human beta-Actin primers yielded 300 bp products (Fig. 5).

A DNA vector-based RNAi approach was applied in this study to suppress the expression of the target sequence of PRNP. The sequence of siRNA1 is homologous to the target sequence of PRNP so, RNAi effects induced by pSilencer4.1-siRNA1 could successfully deplete the mRNA expression of PRNP in 293T cells 48 h after transfection with both vectors compared to the negative control. The siRNA2 could not induce the RNAi effect so, target sequence of PRNP was expressed in 293T cells. The effective pSilencer4.1-siRNA1 will be the base of further study to breed transgenic goats which are resistant to prion diseases. Each year goat prion diseases cause great economic losses across the world. In order to reduce the economic loss and protect animal and human health, many countries plan to breed transgenic animals which are resistant to prion diseases.

The conformational conversion and subsequent disorders of PrP^{Sc} necessitate the presence of PrP^{c} (Bueler *et al*., 1993). A variety of functions have been proposed for mammalian PrP^{c} including involvement in cell death and survival, oxidative stress, immunomodulation, differentiation, metal ion trafficking (Pauly and Harris 1998), cell adhesion (Mange *et al*., 2002) and transmembrane signaling (Linden *et al*., 2008). But there is no evidence indicating that the deletion of *PrP* gene has deadly effect on mammalian physiology. Target sequence of PRNP was suppressed absolutely in this study. The effect of the pSilencer4.1-siRNA1 on expression of complete sequence of PRNP was not validated up to now. The results just provided strong evidence that the approach described above would be useful in suppressing the expression of PRNP.

CONCLUSION

This study shows that a recombined RNAi vector, pSilencer4.1-siRNA1 with stable suppression of the target sequence of PRNP was reported in this study. The findings provided new insight into preventing goat prion disease and a new method to provide breeding materials for producing therapeutic transgenic goats by somatic cell nuclear transfer.

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