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Full Length Research Paper

Silencing effect of shRNA expression vectors with stem length of 21, 27 or 29 bp

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In this study, shRNA vectors having different stem length were constructed and their silencing effect was tested in mouse embryonic fibroblast and *in vivo*. Interfering RNAs were designed with stems of 21, 27, and 29 bp. The enhanced green fluorescent protein gene was used as target gene. The synthesized single strands were annealed and cloned into psiSTRIKE. Then, the recombinant plasmids were transfected into mouse embryonic fibroblast with lipofection and injected into leg muscle of mouse. The mRNA expression level of the green fluorescent protein gene was checked by real-time quantitative polymerase chain reaction (RT-PCR). The silencing effect of the 29 bp shRNA vector was stronger than that of the 21 and 27 bp in cell, but there was no significant difference among them when injected in muscle. The findings in this study are of interest for selecting the best hairpins for mouse individuals.

Key words: Gene silenging, shRNA, enhanced green fluorescent protein, mouse embryonic fibroblast.

INTRODUCTION

RNA-mediated gene silencing is an ancient and conserved mechanism that recognizes double-stranded RNA (dsRNA) as a signal to trigger sequence-specific degradation of homologous mRNA. It extensively resides in all kinds of animal and plant (Hannon, 2002; Hutvagner and Zamore, 2002). There are two techniques usually used in RNA interference in mammalian cells. One relies on the transfection of cells with a small interfering RNA (siRNA) duplex, a 19 bp synthetic double-stranded RNA (dsRNA) bearing two overhanging bases at 3′ end (Elbashir et al., 2001). The other makes use of small hairpin RNA (shRNA) transcribed from expression plasmids or viral vectors in mammalian cells (Brummelkamp et al., 2002; Hasuwa et al., 2002; Miyagishi and Taira, 2002; Sui et al., 2002; Yu et al., 2002). The former can only induce short-term interference effect, while the latter can get a stable and long-lasting gene silencing effect.

To be effective, the shRNAs are designed to follow the rules predicated by the specifics of the cellular machinery and are presumably processed similar to the microRNA maturation pathways. The basic structure of an ordinary shRNA consists of paired antisense and sense strands connected by a loop of unpaired nucleotides. A duplex stem of 19 to 29 bp, either fully paired or with miRNA-style internal mismatches, is commonly used in vector expressed shRNAs (Zeng et al., 2002; Cai et al., 2004; Silva et al., 2005; Stegmeier et al., 2005; Chung et al., 2006; Boudreau et al., 2008). The structure of shRNAs has been found to affect the silencing ability. Moreover, in the case of synthetic shRNAs, the lengths of both stems and loops can affect efficacy (Li et al., 2007; Vlassov et al., 2007). shRNAs assimilate into the endogenous pathway and in doing so, are significantly more efficient than siRNAs. Today, induction of gene silencing using shRNA vector is widely used for many applications. For example,

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Abbreviations: dsRNA, Double-stranded RNA; **siRNA,** small interfering RNA; **dsRNA,** double-stranded RNA; **shRNA,** small hairpin RNA; ***egfp*,** enhanced green fluorescent protein gene; **MEF,** mouse embryonic fibroblasts; **BLAST,** Basic Local Alignment Search Tool; **DMEM,** Dulbecco's modified Eagle's minimal essential medium; **FBS,** fetal bovine serum.

large libraries of DNA vector-based RNAi reagents are being used for various loss-of-function screens, cell lines or transgenic animals that express shRNA are being created, therapeutic approaches based on shRNA expression vector are being investigated (Xia et al., 2002; Carmell et al., 2003; Berns et al., 2004; Xia et al., 2004).

Despite prior results showing excellent specificity in RNAi, recently many studies have shown that there are multiple specific and non-specific off-target effects in siRNA or shRNA mediated gene silencing (Alan et al., 2003; Fedorov et al., 2006; Grimm et al., 2006; Candace et al., 2008). Specific off-target effects are mediated by partial sequence complementarity of the RNAi construct to mRNAs other than the intended target. Non-specific off-target effects include a wide variety of immune and toxicity related effects that are intrinsic to the RNAi construct itself or its delivery vehicle (Rao et al., 2009). Through choosing suitable target sequence, specific off-target effects can be avoided. Non-specific off-target effects are concerned with the length of mRNA transcribed by shRNA vector, whichshows that longer sequences induce unintended effects more easily.

Traditionally, 21 bp siRNA or shRNA was used extensively in mammalian to inhibit expression of specific genes for experimental and therapeutic purposes. Recently, Kim et al. (2005) found that 27 bp siRNA are substrates of the dicer endonuclease, directly linking the production of siRNAs to incorporate in the RNA-induced silencing complex. Meanwhile, Siolas et al. (2005) reported that chemically synthesized shRNAs with 29 bp stem can be cleaved into 22 bp small RNAs by dicer. Both results hint that 27 or 29 bp siRNA may be more potent inducers in RNA interference than conventional 21 bp. Here, we constructed a series of shRNA expression vectors targeting to enhanced green fluorescent protein gene (*egfp*) with stem length 21, 27, 29 bp, respectively. The silencing effects were evaluated by real-time quantitative reverse transritase polymerase chain reaction (RT-PCR) in transfected mouse embryonic fibroblasts (MEF) or intramuscular injected mice legs.

MATERIALS AND METHODS

Plasmid construction

shRNAs having different stem length were designed targeting *egfp* (GenBank accession no. 1377914) by the online design process (http://www.promega.com/siRNADesigner), with unanimous loop sequence (TTCAAGAGA). The designed shRNA sequence was analyzed by Basic Local Alignment Search Tool (BLAST) search to ensure that they did not have significant sequence homology with other genes in mouse genome. Three fragments were designed for every stem length and the most effective were screened by preliminary experiments (Figure 1).

All plasmid constructions were carried out using standard cloning procedures. Oligonucleotides were purchased from Invitrogen Corporation, China. The complementary oligonucleotides were annealed (annealing buffer: 100 mM potassium acetate, 30 mM HEPES- KOH pH 7.4, 2 mM Mg-acetate) and ligated into vector psiSTRIKE. Positive clones were identified by restriction digestion and confirmed by sequencing. According to different stem length, they were named EGFP-21siRNA, EGFP-27siRNA and EGFP-29siRNA, respectively.

Cell culture, transfection and intramuscular injection

MEFs were isolated from 13.5 day old fetuses from *egfp* transgenic mouse and cultured in Dulbecco's modified Eagle's minimal essential medium (DMEM) (Gibco, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco, USA). Twenty-four hours before transfection, 1×10^5 cells were seeded in each well of 24-well plate (Costar, USA) and cultured in medium without antibiotics. After achieving greater than 60% confluence, 0.6 µg of each shRNA expression construct was used for transfection in each well. Lipofectamine 2000 reagent (Invitrogen, USA) was used as the transfection reagent in a ratio of 1 µg of DNA: 1.5 µl lipofectamine according to the manufacturer's instructions. Non- transfected and scrambled shRNA transfected cells were used as controls. The lipofectamine/DNA complexes were removed 6 h after transfection and fresh medium was added to the cells. To produce stably transfected cells, 500 µg/ml Geneticin (G 418) was added to the medium (DMEM + 15% FBS) 48 h after transfection to select for clones containing the insert. The cells were left in selective medium for 15 days after which they were trypsinized and cultured in selective medium for propagation.

egfp transgenic mice of 13 days after weaning were used in the *in vivo* experiments. Plasmid DNA was delivered into mice using the intramuscular injection method. Plasmid DNA of 50 µg was diluted in 0.5 ml DMEM and rapidly injected into the hind-leg muscle of the mice. Three days later, the mice were sacrificed and their leg muscle around the injected dot was peeled and assayed for mRNA expression by real-time quantitative real time-polymerase chain reaction (RT-PCR) amplification.

RNA isolation and reverse transcription

Total RNA from the MEFs and leg muscle were extracted using the TRIzol reagent (Invitrogen, USA) according to manufacturer's instructions. Concentration of each RNA sample was measured using UV-1700 spectrophotometer. Only the RNA samples with 260/280 ratio between 1.9 and 2.1 and 260/230 ratio greater than 2.0 were used for the analysis. The integrity of RNA samples was also confirmed by agarose gel electrophoresis. First-strand cDNA was synthesized by reverse transcribing 300 ng of total RNA in 20 µl final reaction volume by transcriptor reverse transcriptase (Roche Applied Science, Germany).

Primer design and real-time quantitative PCR

To facilitate the real-time PCR analysis of the investigated gene and internal control under same reaction conditions, primers and probes were designed using Primer express 2.0 software (Applied Biosystems, USA) under default parameters. The primer and probe sequences are given in Table 1. The PCR mixture contained 1 µl diluted cDNA, 10 µl 2.5×RealMasterMix (Probe) (Tiangen, China), 1.25 µl 20×Probe Enhancer solution, 200 nM probe and 900 nM of each gene-specific primer in a final volume of 25 µl. PCRs with no template were also performed for each primer pair as blank controls. The real-time PCR was performed by Bio-Rad iQ5 system (Bio-Rad Laboratories, USA) under the following conditions: 2 min at 95°C, 40 cycles of 20 s at 95°C and 40 s at 57°C and 30 s at 68°C in 96-well optical reaction plates. Data were analyzed using the $2^{-\triangle\triangle CT}$ method after 40 cycles.

21 bp (417-437)	ACCGCACAAGCTGGAGTACAACTATTCAAGAGATAGTTGTACTCCAGCTTGTGCTTTTTC TGCAGAAAAAGCACAAGCTGGAGTACAACTATCTCTTGAATAGTTGTACTCCAGCTTGTG
27 bp (489-515)	ACCGGTGAACTTCAAGATCCGCCACAACATTTCAAGAGAATGTTGTGGCGGATCTTGAAGTTCACCTTTTTC TGCAGAAAAAGGTGAACTTCAAGATCCGCCACAACATTCTCTTGAAATGTTGTGGCGGATCTTGAAGTTCAC
29 bp (429-457)	ACCGTACAACTACAACAGCCACAACGTCTATATTCAAGAGATATAGACGTTGTGGCTGTTGTAGTTGTACTTTTTC TGCAGAAAAAGTACAACTACAACAGCCACAACGTCTATATCTCTTGAATATAGACGTTGTGGCTGTTGTAGTTGTA
Scramble sequence	ACCGCCGTACTAGAGCAATAAACGTTCAAGAGACGTTTATTGCTCTAGTACGGCTTTTTC TGCAGAAAAAGCCGTACTAGAGCAATAAACGTCTCTTGAACGTTTATTGCTCTAGTACGG

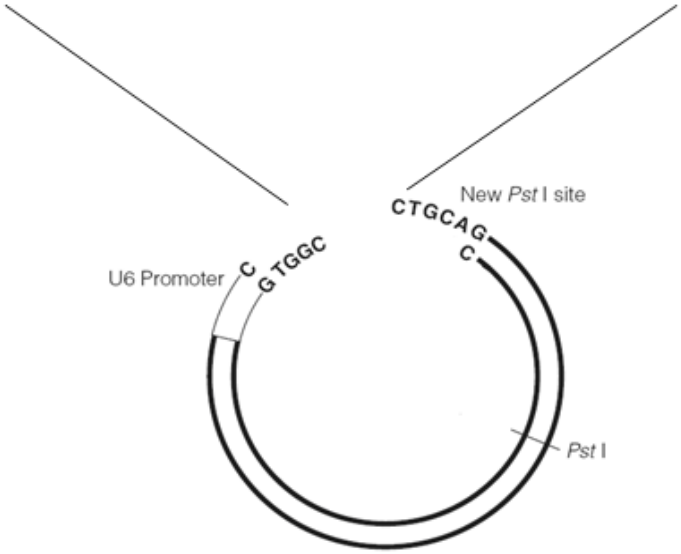

Figure 1. Schematic of shRNA vectors embracing different stem length. All of the sequences have a direction of 5′-3′. The numbers represent the site that the sequences locate in the *egfp*. Designed shRNA can be ligated into psiSTRIKE vector and form a new *Pst*I site which will help to identify the recombinant.

Table 1. The primer and probe sequences used for real-time PCR analysis.

Gene name	Primer sequence*	Amplicon length (bp)
EGFP	^{397}5'- GAGGAC GGCAACATCCTGG-3' ^{486}5'- GATGCCGTTCTTCTGCTTGTC -3' ^{418}5'-CACAAGCTGGAGTACAACTACAACAGCCA-3' (Probe)	90
GAPDH	^{221}5'-AGTCAAGGCC GAGAATGGGA-3' ^{328}5'-ACATACTCAGCACCGGCCTCA-3' ^{258}5'-AAGCCCATCACCATCTTCCAGGAGC-3'(Probe)	108

Primer (upper two lines) and probe (lower line) sequences along with their position (numbers given as superscript on the left) on full-length cDNA sequence.

RESULTS

Identification of recombinant plasmids

Recombinant plasmids were extracted from the bacteria and then digested by *Pst*I enzyme. Correct recombinant plasmids would be cut into two fragments (3655 and 962 to 970 bp) (Figure 2). Positive samples identified were sent to Invitrogen Company to sequence. The results of sequencing were analyzed with ClustalX software.

Microscopy of the MEFs after transfection

The growing MEFs isolated from *egfp* transgenic mice were spindle and polygon shaped and emitted intense

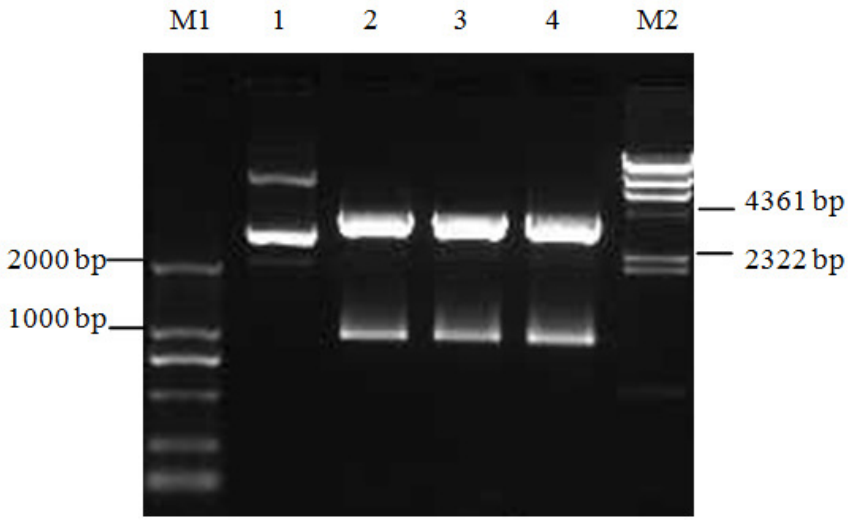

Figure 2. Recombinant plasmids digested by *Pst*I. M1, DL 2000 marker; 1, positive control; 2 to 4, positive plasmids; M2, HindIII maker.

fluorescence (Figure 3a and b). The fluorescence of the cells began to weaken at 24 h and attained maximum silencing effect at 48 h after transfection of the shRNA vectors. Some cells began to die at 3rd day and monoclone was acquired at 15th day after exposure to G-418. As shown in Figure 3, silencing effect was observed in every transfected group (Figures 3c to h) and was not shown in negative group (Figures 3i and j).

Real-time quantitative RT-PCR

The MEFs were collected at 24, 48, and 72 h after transfection, respectively. Real-time PCR and TaqMan detection were performed on the corresponding cDNA synthesized from each sample. Two biological replicates for each sample were used for real-time PCR analysis and three technical replicates were analyzed for each biological replicate. The data were analyzed using the $2^{-\Delta\Delta Ct}$ method (Kenneth and Thomas, 2001), where $^{\Delta\Delta}C_T$= ($C_{T,\ Target}$ - $C_{T,\ Gapdh}$) Time x - ($C_{T,\ Target}$ - $C_{T,\ Gapdh}$) Time 0 (Tables 2 to 4). As shown in Figure 4, all of the shRNA vectors with different stem length inhibited the expression of EGFP gene effectively. EGFP-21siRNA, EGFP-27siRNA and EGFP- 29siRNA made the gene expression level reduced to 45, 32, 25%, respectively. In comparison, EGFP-29siRNA was the most effective one. The silencing effect reached peak at 24 h after transfection by EGFP-21siRNA and EGFP-27siRNA and at 48 h by EGFP-29siRNA.

To test the *in vivo* silencing effects, we diluted the shRNA vectors with DMEM and then, injected them into leg muscle of mice. The EGFP mRNA degradation induced by shRNA was monitored by real-time quantitative RT-PCR. EGFP-21siRNA, EGFP-27siRNA and EGFP-29 siRNA made the expression of target gene down to 8.9, 9.7, 9%, respectively (Figure 5). There were no obvious difference among *in vivo* silencing effects of shRNAs with stem length of 21, 27 and 29 bp.

DISCUSSION

In the present study, shRNA expression vectors having 21, 27 or 29 bp stem length were constructed and then transfected into mouse embryonic fibroblasts with lipofection and injected into leg muscle of mouse. The mRNA levels of target and reference gene were quantified by real-time quantitative RT-PCR to find the difference of their silencing effect. The enhanced green fluorescent protein gene was used as target gene, so that the gene silencing could be observed easily by microscopy.

Once delivered into mammalian cells, siRNA will immediately demonstrate gene silencing effect (Persengiev et al., 2004). We observed fluorescein failing on MEFs under microscope at 48 h after shRNA expression vectors were transfected into them. The mRNA levels of *egfp* in the first three days after transfection in MEFs were quantified by real-time quantitative RT-PCR. The results showed that, the gene silencing induced by 21 bp shRNA vector emerged earlier than that of 27 and 29 bp, which may relate to their mechanism of action. Our data showed that shRNA vectors with different stem length could effectively silence the target gene when transfered into MEFs (Tables 2 to 4). The silencing effect of EGFP-27siRNA and EGFP-29siRNA were more potent than that of EGFP-21siRNA in MEFs, consistent with the previous studies (Kim et al., 2005; Siolas et al., 2005), which showed that it is practicable to adopt 27 and 29 bp shRNA vectors in the study of gene silencing in

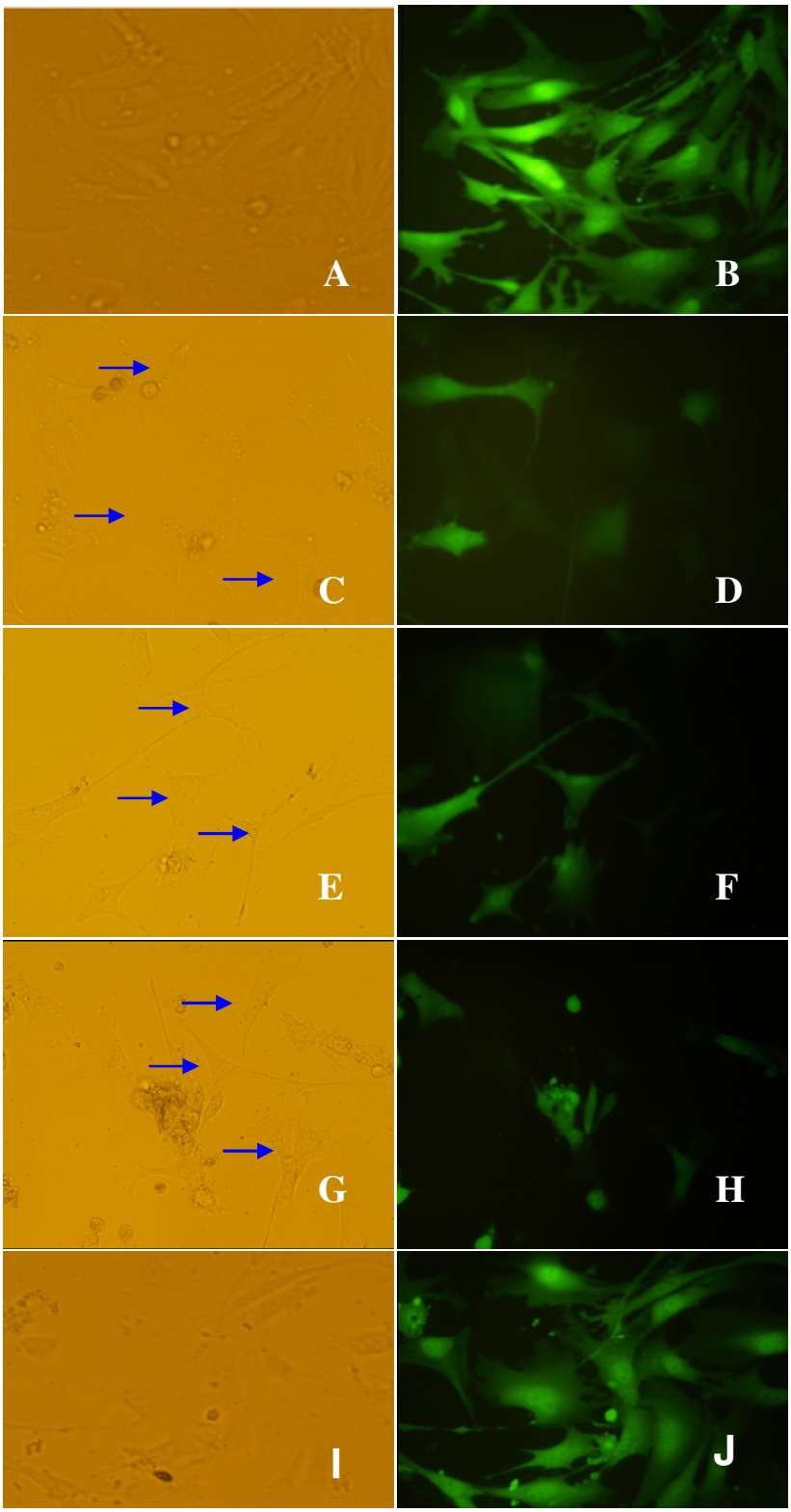

Figure 3. Microscopy of mouse embryonic fibroblast. Arrows indicated the cells visible in white light but nearly unvisible under ultraviolet. (A, B), MEF separately exposed under white light and UV; (C,D), MEF transfected by EGFP-21 siRNA exposed separately under white light and UV; (E,F), MEF transfected by EGFP-27 siRNA exposed separately under white light and UV; (G,H), MEF transfected by EGFP-29 siRNA exposed separately under white light and UV; (I,J), MEF transfected by scramble sequence exposed separately under white light and UV.

Table 2. The result analyzed by $^{\Delta\Delta}$CT method at 24 h after transfection.

Group	*egfp* av.ct	*gapdh* cv.ct	$^{\Delta}$ct	$^{\Delta\Delta}$ct	$2^{-\Delta\Delta}$ct
Positive control	24.17138	24.67054	-0.49916	0	1
EGFP-21siRNA	23.54118	22.88129	0.65989	1.15905	0.45
EGFP-27siRNA	21.88658	21.61149	0.27509	0.77425	0.58
EGFP-29siRNA	22.50081	21.49481	1.0060	1.50516	0.35
Scramble sequence	23.57426	24.08869	-0.51443	-0.01527	1.01

Table 3. The result analyzed by $^{\Delta\Delta}$CT method at 48 h after transfection.

Group	*egfp* av.ct	*gapdh* cv.ct	$^{\Delta}$ct	$^{\Delta\Delta}$ct	$2^{-\Delta\Delta}$ct
Positive control	21.87661	22.18688	-0.31027	0	1
EGFP-21siRNA	23.87798	23.42545	0.45253	0.7628	0.58
EGFP-27siRNA	22.98277	21.64324	1.33953	1.6498	0.32
EGFP-29siRNA	23.93882	22.27868	1.66014	1.97041	0.25
Scramble sequence	23.74599	23.33625	-0.09026	0.22001	0.86

Table 4. The result analyzed by $^{\Delta\Delta}$CT method at 72 h after transfection.

Group	*egfp* av.ct	*gapdh* cv.ct	$^{\Delta}$ct	$^{\Delta\Delta}$ct	$2^{-\Delta\Delta}$ct
Positive control	24.15574	24.55848	-0.40274	0	1
EGFP-21siRNA	24.21644	24.06912	0.14732	0.55006	0.68
EGFP-27siRNA	23.72680	23.29399	0.43281	0.83555	0.56
EGFP-29siRNA	24.61650	24.43256	0.18394	0.58668	0.67
Scramble sequence	23.96651	24.40233	-0.43582	-0.03308	1.02

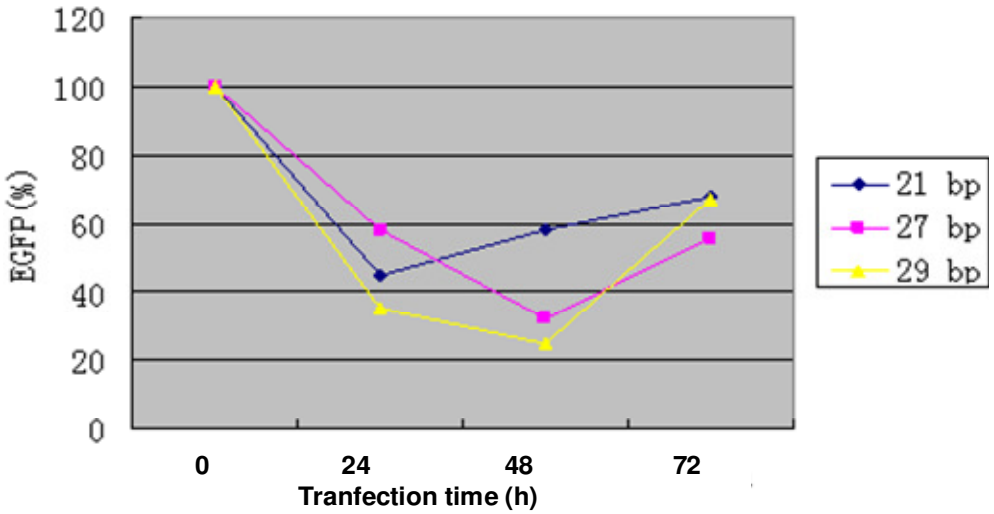

Figure 4. The silencing effectiveness in MEFs induced by shRNA vectors at different time point. Blue diamonds, EGFP-21siRNA; pink squares, EGFP-27siRNA; yellow triangles, EGFP-29siRNA.

mammalian cells. A stable cell line was obtained after 15 days of screening and selection by G 418, which indicated the interference vector had integrated into the genome and expressed.

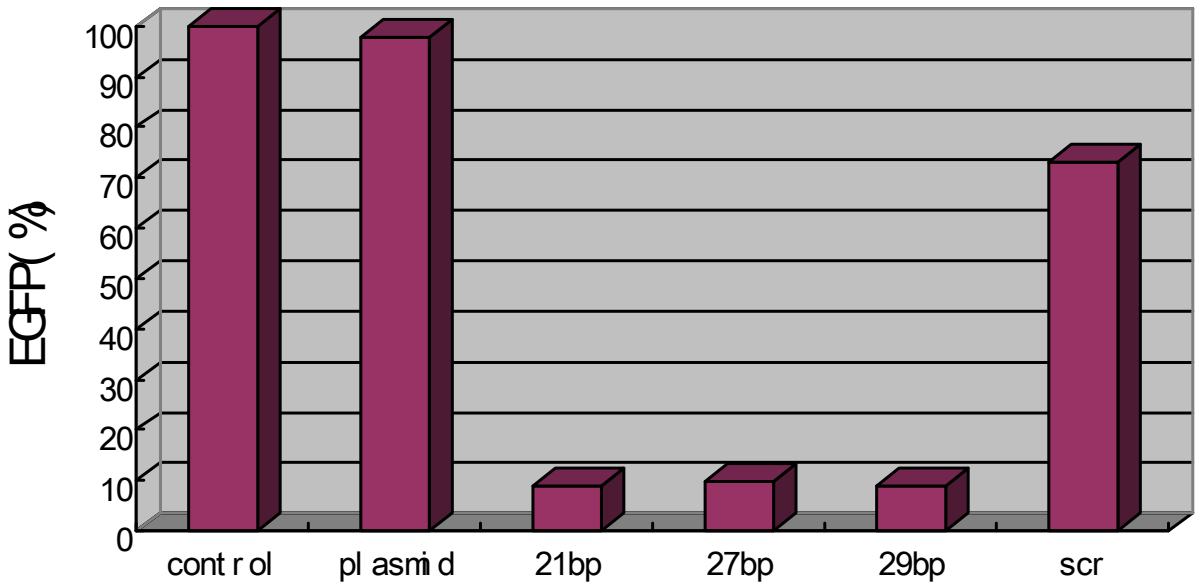

Figure 5. The silencing effect in individual induced by different stem shRNA vector at 72 h after injection. The amount of EGFP mRNA was determined using real-time RT-PCR and normalized to GAPDH mRNA. Injection with each construct was done in triplicate. Control, non-injected muscle; plasmid, psiSTRIKE vector; 21 bp, EGFP-21siRNA; 27 bp, EGFP-21siRNA; 29 bp, EGFP-21siRNA; scr, scrambled shRNA control.

The expression of *egfp* nearly disappeared 72 h after injection of shRNA vectors into leg muscle of mice. This *in vivo* silencing effect was obviously stronger than that induced by cell transfection. This may be due to the difference of siRNA delivery and expression regulation between *in vitro* and *in vivo*. Kim et al. (2005) show synthetic 25 and 27 bp siRNAs need less dosage than that of 21 bp, when leading to the maximum silencing effect. In this experiment, we found there was not obvious difference of silencing effect among different shRNA vectors, which should ascribe to excessive dosage of the shRNA vectors into mouse muscle. Next, we will do experiments to determine the suitable dosage in attaining maximum silencing effect.

Unexpectedly, we found the negative control also inhibited the target gene expression by 27%, which make it imperative to fully evaluate the adverse effect of *in vivo* RNA interference. Some researchers show that siRNA (<30 bp) may induce interferon response too (Alan et al., 2003; Sledz et al., 2003), which was traditionally thought to be impossible. Enough siRNAs could bind to nuclear-cytoplasmic transport protein (exportin-5) and saturate its pathways (Grimm et al., 2006) and eventually influence the effects of endogenous miRNAs. Therefore, in addition to gene silencing, there is also a complex network of adverse effect of RNA interference which needs further in-depth study.

Conclusions

In this study, it was reviewed that shRNA vectors of 27 and 29 bp stem length could induce a more potent silencing effect than that of 21 bp in MEFs. It can be predicted that in the near future, shRNA vectors of 27 or 29 bp are bound to be the best choice in gene function study and disease treatment. The birth of the first transgenic RNAi mouse showed that RNA interference could be applied in the production of transgenic animal (Hasuwa et al., 2002). Furthermore, we will produce transgenic RNAi mouse by microinjection in order to acquire long-term and stable interference in individual.

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