Interference of EGFP RNA in Human NT-2/D1 Cell Lines Using Human U6 Promoter-based siRNA PCR Products

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Abstract RNA interference (RNAi), a process of sequence-specific gene suppression, has been known as a natural gene regulatory mechanism in a wide range of lower organisms. Recently, we have reported that a transfection of human U6 promoter (hU6) driven hairpin small-interference RNA (siRNA) plasmid specifically knocks down the target gene by post-transcriptional gene silencing in mammalian cells. Here we report that transfection of polymerase chain reaction (PCR) products, containing human U6 promoter with hairpin siRNA, knocks down the target gene expression in human teratocarcinoma NT-2/D1 cells. Moreover, we showed 3′ end termination sequence, 5 Ts, is not critical elements for knocking down in PCR-based siRNA system. Therefore, the PCR-based siRNA system is a promising tool not only for the screening but also to temporally regulate gene expression in the human progenitor cells.

Keywords: gene silencing, small interference RNA, PCR, human teratocarcinoma NT-2/D1 cells

RNAi is a rapidly developing technology for repressing specific gene expression in various biological systems including plants [1], *Caenorhabditis elegans* [2], Drosophila [3,4] and mammalians [5] which can be applied to reverse genetics, gene therapy, and functional genomics. RNAi is the mechanism of sequence-specific post-transcriptional gene knock-down. When double-stranded RNAs (dsRNAs) are introduced into an organism, sequence-specific destruction of endogenous target messenger RNAs (mRNAs) occurs. This process involves the cleavage of long dsRNA into active 21~23 nt length small interference RNA (siRNA) by Dicer [6] or another RNase III-like enzyme [7]. These siRNAs are incorporated into RNAi-induced silencing complex [8] and then are used as a guide to degrade the corresponding mRNA. Even though siRNA has a powerful knock-down effect to specific target gene, still there was an obstacle which prevents application of siRNA because of non-specific reduction of mRNA by interferon response [9]. To overcome this non-specificity, direct transfection method of siRNA, which does not trigger interferon responses, namely activation of dsRNA-dependent protein kinase or 2′,5′-oligoadenylate synthetase has been introduced [10].

Transfection of chemically synthesized [11] or *in vitro* transcribed [12] siRNA suppressed gene expressions in a variety of mammalian cell lines including human embryonic kidney (HEK 293) and HeLa cells without any side effect caused by activation of anti-viral host defense mechanisms. On the other hand, transfection of siRNA reduces gene expression for only a short period compared to transfection of dsRNA. To overcome this limitation, we transfected a human U6 promoter driven plasmid vector, which can stably express siRNA in host cells [13]. The siRNAs originated from a H1 promoter driven plasmid vectors was also reported to be able to effectively suppress target gene expression [14]. These promoters can transcribe small RNAs by RNA polymerase III (RNA-pol III), and this transcription is terminated when RNA-pol III meets 4~5 Ts, which is located at the 3′ end of the gene construct, making it possible to directly produce siRNAs [14]. Recently retroviral and lentiviral vector based siRNA were also introduced to increase transfection efficiency and stable integration into the chromosome of host cell [16,17].

However the most critical step in designing siRNA, is to find an effective siRNA sequence for the target genes. Although there are some principles to identify the effective siRNA site in the target gene, in general, such a selection needs to be done by empirical way. Although vector-based siRNA appears to be the most promising technology for a long-lasting suppression of gene expression, it may not be suitable for screening of the effective siRNA sequence. In the present study, we established PCR-based siRNA system using human U6 promoter (hU6) in a multipotent embryonal human tetratocarcinoma cell line (NT-2/D1) [18].

NT-2/D1 cells have been shown to differentiate into neurons and astrocytes by treatment with retinoic acid [19]. NT-2/D1 derived neurons (NT-2/N) have been studied as experimental models for elucidating the mechanisms of various neurodegenerative diseases [20]. NT-2/D1 derived astrocytes have been used as models for

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glutamate transport dysfunction in various neurodegenerative diseases [21]. Thus, successful gene-knockdown in NT-2/D1 by PCR-based human U6 promoter driven siRNA would be a good tool to investigate mechanisms of neural cell functions as well as to screen effective siRNA sequence for target genes expressed in these human cells.

Fig. 1 shows a scheme for a production of PCR based siRNA. In the first round PCR, hU6 promoter (GenBank accession No. M14486, gene sequence 64-355) and 27 nt of U6 5′-coding sequence was amplified with a primer set (U6-F: 5′-TCTTTGGAATTCAAGGTCGGGCAGGAAGAGGGCCTA-3′, U6-R: 5′-CGCGGATCCTAGTATATGTGCTGCCGAAGC-3′) using plasmid pUC-hU6 [13] as a template. Simultaneously hairpin-siRNA for enhanced green fluorescent protein (EGFP) gene consists of 21 nt sense strand of siRNA, 9 nt spacer and 21 nt antisense strand of siRNA was generated by PCR using another primer set (siEGFP-F: 5′-CGC GGA TCC GGC GAT GCC ACC TAC GGC AAG CTC GAG ATC-3′, siEGFP-R: 5′-GCT CTA GAG GCG ATG CCA CCT ACG GCA AGG ATC TCG AGC T-3′). After 100-times dilution, these two the first round PCR products were mixed and used as template for the second round PCR reaction. Since 5′ end of siEGFP-F primer contains complementary sequence to 3′ end of hU6 promoter region, the second round PCR using U6-F primer and siEGFP-R primer produced hU6-siEGFP. The first and second round PCR reaction were performed with Eppendorf HotMaster Taq polymerase (Eppendorf, Westbury, NY, USA) under the condition of pre-incubation at 95°C for 5 min; 35 cycles of 95°C for 40 sec, 55°C for 30 sec and 72°C for 30 sec; and post-extension at 72°C for 5 min. To produce PCR fragment containing hU6 promoter with a random sequence for testing hU6 promoter activity in the cells, we used a primer set U6-F and U6-R1 (5′-AAA AAT TCT AGA TGT AAA AAT AGT GTT GTG TGC CTA GGA TAT GTG CTG CCG AAG CGA GCA C-3′) or U6-R2 (5′-TAG TGT TGT GTG CCT AGG ATA TGT GCT GCC GAA GCG AGC AC-3′) using plasmid pUC-hU6 as a template.

NT-2/D1 cells were cultured as described previously [18]. Briefly, 1×10^6 cells were seeded in a 6 well culture plate in Dulbecco's Eagle medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% fetal bovine serum (Novatech, Grand Island, NE, USA), 2 mM glutamine (Invitrogen) and 0.4% penicillin-streptomycin (Invitrogen). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C. Transient transfection of mammalian EGFP expressing vector (pEGFP-C1, BD Bioscience, Palo Alto, CA, USA) and/or hU6-siEGFP was performed by using Lipofectamine™ 2000 (Invitrogen) on subconfluent cells in a 6-well culture plate with the ratio of 1:3 (DNA:Lipofectamine reagents, respectively). The gene silencing effect of hU6-siEGFP transfection in NT-2/D1 cells co-transfected with pEGFP-C1. Transfection efficiency was normalized with 0.5 μg of pEGFP-C1 (Clontech, Palo Alto, CA, USA). The expression of EGFP protein was directly observed by a fluorescence microscope (Leica, Bannockburn, IL, USA) and EGFP gene expression was assessed by RT-PCR.

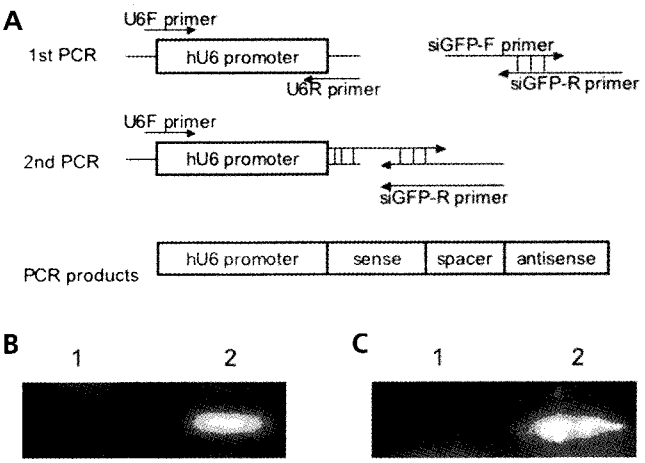

Fig. 1. Construction of the human U6 (hU6) promoter-driven PCR products for siRNA and measurement of hU6 promoter activity in NT2/D1 cells. A schematic diagram for construction of hU6 promoter PCR-based siRNA is showed in (A). In the first round PCR, hU6 promoter and 27 nt of U6 5′-coding sequence was amplified using a primer set (U6F and U6R) and a plasmid pUC-hU6 as a template. Simultaneously hairpin-siRNA for enhanced green fluorescent protein (EGFP) gene consists of 21 nt sense and antisense strand of siRNA, 9 nt spacer and with/without 5 Ts, was generated by PCR using a primer set (siEGFP-F and siEGFP-R). In the second round PCR reaction using a primer set (U6-F and siEGFP-R) and the first PCR products as templates produced hU6-siEGFP, since 5′ end of siEGFP-F primer contains complementary sequence to 3′ end of hU6 promoter region. To add 5 Ts at the 3′ end of hU6-siEGFP, we added 5 As to the 3′ end of siEGFP-R primer. Activity of hU6 promoter in the NT2/D1 cells was accessed by RT-PCR using total RNA from the cells without (lane 1) or with (lane 2) transfection of hU6-random PCR products, which contain hU6 promoter and random sequence. Human U6 promoter showed high activity in the NT2/D1 cells either transfected without (B) or with (C) 5 Ts in the 3′ end of the hU6-random PCR products.

After 48 h of transfection, total RNA was extracted from NT-2/D1 cells with RNeasy® Protect Mini Kit (Qiagen, Valencia, CA, USA), according to the manufacturer's protocol. One μg of the RNA was reverse-transcribed and amplified using the SuperScript™ ONE-STEP™ RT-PCR system (Invitrogen). To assess hU6 promoter activity, we transfected the cells with PCR fragments (hU6-random) containing hU6 promoter, 27 nt of U6 5′-coding sequence and a random sequence instead of siEGFP. Then transcription of 27 nt of U6 5′-coding sequence and the random sequence was detected by RT-PCR with a primer set (sense: 5′-GTGCTCG-CTTCGGCAGCACAT-3′, antisense: 5′-AATAGTGTT-GTGTGCCTAGGA-3′ or 5′-TAG TGT TGT GTG CCT AGG ATA-3′). The EGFP gene expression was analyzed by RT-PCR with an EGFP-specific primer set (sense 5′-CAAGGACGACGGCAACTACAAGACC-3′, antisense 5′-GCGGACTGGGTGCTCAGGTAGTGGT-3′). As an internal control, β-actin was amplified by RT-PCR with a

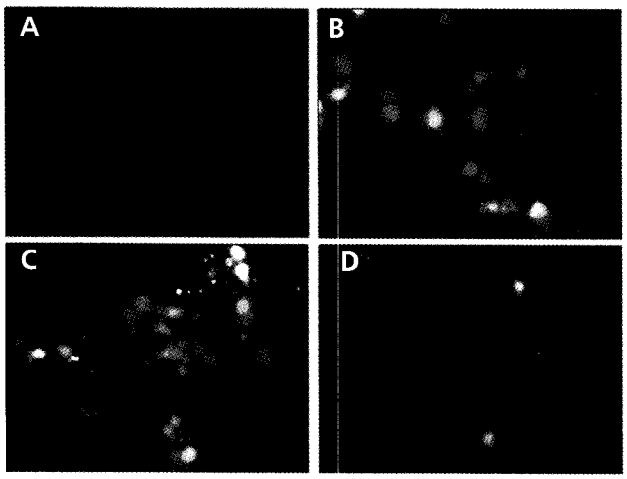

Fig. 2. Typical fluorescent microscopic pictures of NT2/D1 cells. (A) NT2/D1 cells transfected with 300 ng of pcDNA 3.1 as a negative control for fluorescent signal. (B) NT2/D1 cells with 300 ng of EGFP expression vector (pEGFP-C1) as a positive control. (C) NT2/D1 cells cotransfected with 300 ng of pEGFP-C1 and 300 ng of hU6-random PCR products as a negative control for siRNA. (D) NT2/D1 cells cotransfected with 300 ng of pEGFP-C1 and 300 ng of hU6-siGFP PCR products.

β-actin specific primer set (sense: 5′-GACAGGATGCAGAAGGAGAT-3′, antisense: 5′-TTGCTGATCCACATCTGCTG-3′).

We found that NT-2/D1 cells transfected with hU6-random showed high transcription level of 27 nt of hU6 5′-coding sequence and the random sequence by RT-PCR, while mock transfected NT-2/D1 cells didn't show any expression of this gene piece (Fig. 1). Transfection of NT-2/D1 cells with 5 Ts in the 3′ end of the hU6-random PCR products made no difference in the expression level compared to NT-2/D1 cells transfected with hU6-random PCR without 5 Ts in the 3′ end. This result indicates that the hU6 promoter has an activity of transcription of small gene sequence in NT-2/D1 cells and 5 Ts in the 3′ end is not require for the hU6 promoter activity.

NT-2/D1 cells transfected with pEGFP-C1 expressed EGFP protein (Fig. 2B) and genes (Fig. 3, lane 2), with the gene expression ratio between EGFP and β-actin (EGFP/β-actin) = 1.10 ± 0.0181 (mean ± S.D., n = 3), while cells transfected with control vector, pcDNA 3.1 (BD Bioscience), did not show fluorescent signals (Fig. 2A) or a EGFP band on the gel (Fig. 3, lane 1, EGFP/β-actin = 0). Moreover, EGFP expression was not suppressed by nonspecific siRNA (Fig. 2C). Co-transfection of hU6-siEGFP suppressed EGFP protein expression of NT-2/D1 cells transfected with pEGFP-C1 (Fig. 2D). EGFP gene expression of NT-2/D1 cells transfected with pEGFP-C1 was dose-dependently suppressed by hU6-siEGFP co-transfection (Fig. 3, lane 3~6, EGFP/β-actin = 0.84 ± 0.091, 0.63 ± 0.038, 0.17 ± 0.012, and 0 ± 0 respectively). These results show that siRNA PCR products, which contain hU6 promoter and hairpin structure with 9-mer spacer without a 5 Us 3′ terminator sequences can be applicable for specific gene silencing of target gene in NT-2/D1 cells.

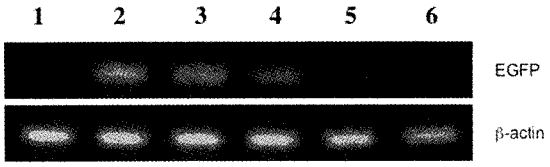

Fig. 3. Effects of hU6-siGFP PCR products on EGFP gene expression level in NT2/D1 cells were analyzed by RT-PCR and gel electrophoresis. Lane 1 shows a negative control from NT2 cells transfected with 1.5 μg of pcDNA 3.1. Lane 2 shows a positive control from NT2/D1 cells transfected with 1.5 μg of pEGFP-C1 alone. Lane 3 shows a negative control for siRNA from NT2 cells cotransfected with 1.5 μg of pEGFP-C1 and 1.5 μg of hU6-random PCR products (lane3). Lane 4, 5, and 6 show EGFP expression in NT2/D1 cells cotransfected with 1.5 μg of pEGFP-C1 and 1.5, 3, and 5 μg of hU6-siGFP PCR products, respectively.

In a previous study, we constructed hU6 promoter driven EGFP siRNA plasmid based on pUC19 (Invitrogen) backbone. Although the vector based RNAi is useful for stable gene silencing, it is cumbersome for screening of target site of siRNA because of the cloning process, which involve bacterial culture or ligation process. Although several reports, which show similar RNAi construction strategy with ours, were documented [22,23], in the current study, we are reporting PCR fragments based RNAi system which is useful for gene silencing in neural progenitor cell lines. Using this technology, we will be able to prepare significant number of RNAi expression gene constructs to test siRNA sequence targeting various genes in a day.

First, we tested whether hU6 promoter in the PCR fragment is able to transcribe small fragment of RNA in the mammalian cells. Since we were able to detect gene transcript of random gene sequence we inserted after hU6 promoter in NT-2/D1 cell received gene transfection of hU6-random PCR fragment, hU6 promoter is active in the mammalian cells. We also found omission of 5 Ts in the 3′ end, as an ending sequence for hU6 promoter did not alter the expression of siRNA. Thus, we are able to use this PCR based expression system with out addition of 5 Ts in the 3′ end. We further investigate knock down of the exogenous EGFP gene and protein expressions by the PCR based siRNA construct without 5 Ts in the 3′ end. PCR based EGFP targeting RNAi was able to not only suppress protein expression but also dose dependently suppress EGFP expression induced by transfection of pEGFP-C1. Although further investigation may be necessary, since transfection with hU6-random PCR product did not alter EGFP expression and hU6-EGFP PCR product did not change β-actin gene expression level, transfection with hU6-EGFP PCR product may selectively suppress EGFP expression.

To date, though mechanism of RNAi in mammalian cells is not clear, many useful methodologies have been developed for synthesizing siRNA. However, target site

screening for specific gene knock-down still has been executed by empirical trials using several candidate siRNAs. In this study, we established fast and convenient method to generate siRNAs using PCR. When we generate hU6-siEGFP PCR products which containing hU6 promoter and small hairpin loop without 5 Ts in the 3′ end, siRNAs from PCR products successfully suppressed exogenous target gene expression in NT-2/D1 cells. Since two step PCR method ensure the production of target sequence containing hU6 promoter and specificity of siRNA was adequate, this rapid and convenient gene silencing method can be also applied to gene function study in mammalian cells.

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