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Enhancement of RNA Interference Effect in P19 EC Cells by an RNA-dependent RNA Polymerase

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ABSTRACT

Background: RNA interference (RNAi) is a phenomenon uses double-stranded RNA (dsRNA) to specifically inhibit gene expression. The non-specific silencing caused by interferon response to dsRNA in mammalian cells limits the potential of utilizing RNAi to study gene function. Duplexes of 21-nucleotide short interfering dsRNA (siRNA) inhibit gene expression by RNAi. In some organisms, siRNA can also function as a primer converting mRNA into dsRNA that are further cleaved to produce more siRNA. This activity involves the enzyme RNA-dependent RNA polymerase (RdRP). There are no known RdRP involved in RNAi in mammals. By using an RdRP from *Caenorhabditis elegance* named *ego-1,* investigators intend to enhance RNAi effect in mammalian cells. The aims of this project were: 1) to investigate the efficiency of siRNA to enhanced green fluorescent protein (eGFP) gene silencing and 2) to enhance the RNAi effect. **Methods:** We used a vector-based siRNA to target eGFP. Also we used a vector expressing *ego-1* to test for a possible amplification effect of RNAi. The expression of eGFP in the cells was detected by using fluorescent microscopy, flowcytometry and Western-blotting. **Results:** Transfection of the plasmid into P19 cells significantly decreased eGFP fluorescence. In addition, eGFP protein was reduced. Preliminary data suggested that the presence of *ego-1* enhanced the RNAi effect. **Conclusion:** The results indicated that use of hairpin siRNA expression vectors for RNAi is a promising method to inhibition of gene expression in mammalian cells. Also, introducing RdRP enzyme to mammalian cells might amplify the RNAi effect in the cells. *Iran. Biomed. J. 13 (1): 19-25, 2008*

Keywords: Gene silencing, RNA interference (RNAi), RNA-dependent RNA polymerase (RdRP), Double-stranded RNA (dsRNA)

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INTRODUCTION

In 1998, Fire and colleagues [1] found that the injection of double-stranded RNA (dsRNA) into *Caenorhabditis elegans* led to an efficient sequence-specific gene silencing, which is referred to as RNA interference (RNAi). RNAi has been linked to many previously described silencing phenomena such as post-transcriptional gene silencing in plants [2]. It is also evident in unicellular organism, metazoans, such as *Drosophila,* and lately mammals [1, 3-6]. Based on the current research on plants and *C. elegans,* the biological role of RNAi is suggested to be involved in the defence against endogenous parasitic or exogenous pathogenic nucleic acids [7].

The effector molecules in the mechanism are 21-23 nucleotide short RNAs, which called short interfering dsRNA (siRNA) [8]. Indeed, Dicer, an RNase III family member, cleaves long dsRNA to produce siRNA. Introduction of chemically synthesized dsRNA to the cells leads to degradation of homologous RNA. This finding provided a new tool for studying gene function in various organisms [9].

However, the applicability of this approach is limited in mammals, because the introduction of dsRNA longer than 30 nucleotides induces a sequence non-specific IFN response [3]. Two enzymes, protein kinase PKR and 2'-5'-oligoadenylate synthetase, will be activated by long dsRNA, cause non-specific degradation of mRNA, the stall of translation and finally cell death [10, 11]. To avoid this IFN response, Elbashir and colleagues [3] introduced chemically synthesized siRNA into mammalian cells. It has been shown that the

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synthetic siRNA were functional *in vivo* and could induce loss of luciferase gene reporter activity comparable to long dsRNA [3, 6, 12, 13]. However, the limitation of this method is that the transfected synthetic siRNA works for only a few days in mammalian cells. Despite the early stage of RNAi studies in mammals, here, we investigated the siRNA method for gene silencing in P19 line of murine embryonic carcinoma stem cell [14], by using the enhanced green fluorescent protein (eGFP) gene as a target. To overcome the limitation of using siRNA discussed above, we used a vector, based on suppression of endogenous RNA (pSUPER) system that directs the synthesis of siRNA-like transcripts.

The current studies based on evidences from *C. elegance* and *Drosophila* suggest that the siRNA can also function as a primer converting mRNA into dsRNA that are further cleaved by Dicer to produce more siRNA. This activity had been shown to involve the enzyme, RNA-dependent RNA polymerase (RdRP), and is believed to be the reason behind the amplification effect seen in systemic silencing [15, 16]. Mammals seem do not have any homolog enzyme of RdRP. Therefore, we intended to enhance the RNAi by introducing an RdRP from *C. elegance* named *ego-1* to test for a possible amplification effect.

MATERIALS AND METHODS

Culture and transfection of cells. P19 EC cell line of murine embryonal carcinoma stem cells (1.5×10^6 P19 cells/60 mm tissue culture dish) was cultured under conditions previously described [17]. Cells were split 24 h before the transfection. These cells were transfected using the calcium phosphate co-precipitation method [18]. The cells were cotransfected with plasmids expressing *Pgk-1* gene (pB17), eGFP (pML8) together with either a plasmid expressing specific siRNA for eGFP (pJC2), or pSUPER (control). For pJC2, the following sequence of eGFP was selected: 5'-GCTGACCCTGAAGTTCATCT-3'. To enhance the RNAi effect, we used pKJ283 plasmid, contains *ego-1* gene. In all transfection, equal ratio of each plasmids DNA was used (all plasmids kindly provided by Dr. McBurney's Laboratory, Ottawa Regional Cancer Center, Ottawa, Canada), and a total of 5 µg DNA was transfected using 60 mm tissue culture dishes. For stabling cell population, the selection was done with 200 µg of *puromycin* per ml.

Fluorescence microscopy. Fluorescent intensity of GFP-positive cells was visualized by fluorescence microscopy at 8 days post-transfection, in living P19 cells. For this, P19 cells (1.5×10^6 P19 cells/60 mm tissue culture dish) were plated and transfected with 5 µg of each plasmid, DNA by the calcium phosphate method. GFP expression was clearly visualized under viable conditions. Fluorescence micrographs were taken 8 days after transfection.

Flow cytometry. For flow cytometry, P19 EC cells were washed with PBS. Single cell suspensions were prepared and the cells were directly analyzed for GFP expression. Detector settings were adjusted with untransfected cells. Determination of fluorescent intensity was carried out on FACS (LSR, Becton Dickenson, USA) with 10,000 events captured per sample. The cell gated for positive GFP signals. The acquisition and analysis of the FACS data were performed with CELLQUEST software (Becton Dickenson).

Western-blotting. For Western-blotting, transfected cells, grown in 24-well plates, were trypsinized and harvested in SDS sample buffer. The protein concentration (1.5-2.5 mg/ml) of the resulting total extracts was determined according to the Bradford using bovine serum albumin as standard (DC Protein Assay; Bio-Rad, Canada). Equal amounts of total protein (25 µg) were separated on 10% polyacrylamide gels and transferred to nitrocellulose. Membranes were blocked with 5% milk in PBS and then incubated with antibody against eGFP (rabbit anti-GFP antibody, dilution 1/2000, Sigma, cat # G1544, USA). Secondary antibody (dilution 1/10,000) detection was performed using the goat anti-rabbit horseradish peroxidase (HRP)-conjugated (Santa Cruz, USA). Finally, the membranes were exposed to X-ray film (Kodak MR-1) for various times (between 30 s and 5 min).

RESULTS

RNAi effect in embryonal carcinoma cells. We used pSUPER based vector (pJC2) to determine whether an RNAi model was feasible in the P19 EC cells. The eGFP gene was selected as the target because its activity is easily detectable. Based on pSUPER system, pJC2 was used to produce a siRNA bearing a 20 nucleotide sequence from eGFP (Fig. 1). Initially, we tested the effect of pJC2 on

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expression of eGFP and *puromycin.* Following the transient experiments, we attempted to investigate whether the siRNA could be stably expressed in EC cells. So, pB17 was included in all transfections. The cells were transfected with pB17, pML8 and either pJC2 or pSUPER (control). Then, the cells were subjected to *puromycin* selection 48 h post-transfection and were grown for 8 days before analysis. Single-cell suspensions were created from the transfected EC cells and subjected to FACS to determine the eGFP intensity of cells. FACS analysis of pooled stable population indicated that 93.48% of the cells in the control samples were eGFP-positive (Fig. 2A), while only 47.07% remained positive in the pJC2 samples (Fig. 2B).

Enhancement of RNAi by ego-1. In an attempt to enhance the RNAi effect observed in P19 EC cells, we performed experiments which the pKJ283 (Fig. 3), the *ego-1* construct, was introduced in combination with pJC2. FACS analysis indicated that, the percentage of the eGFP-positive cells decreased to 16.06% with pKJ283 (Fig. 4). To

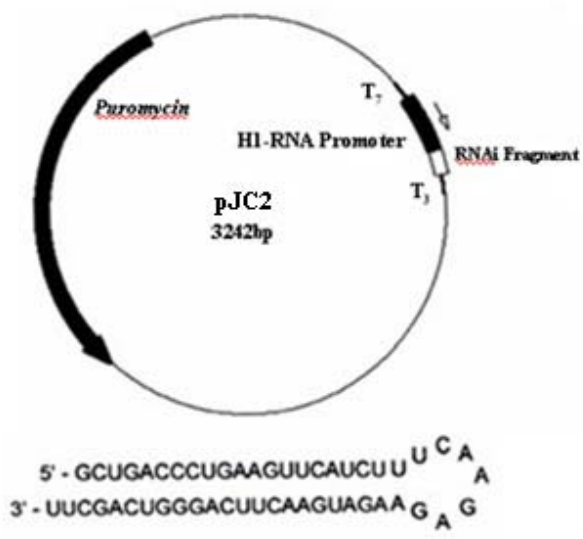


Fig. 1. Schematic drawing of pJC2 vector and its predicted transcript. The H1-RNA promoter is used to derive the small interfering RNA which contains the same 20 nucleotide sequence in sense and anti-sense format from eGFP. The predicted transcript forms a hairpin with the complementary pairing of the two 20 nucleotide sequences separated by a short loop region.

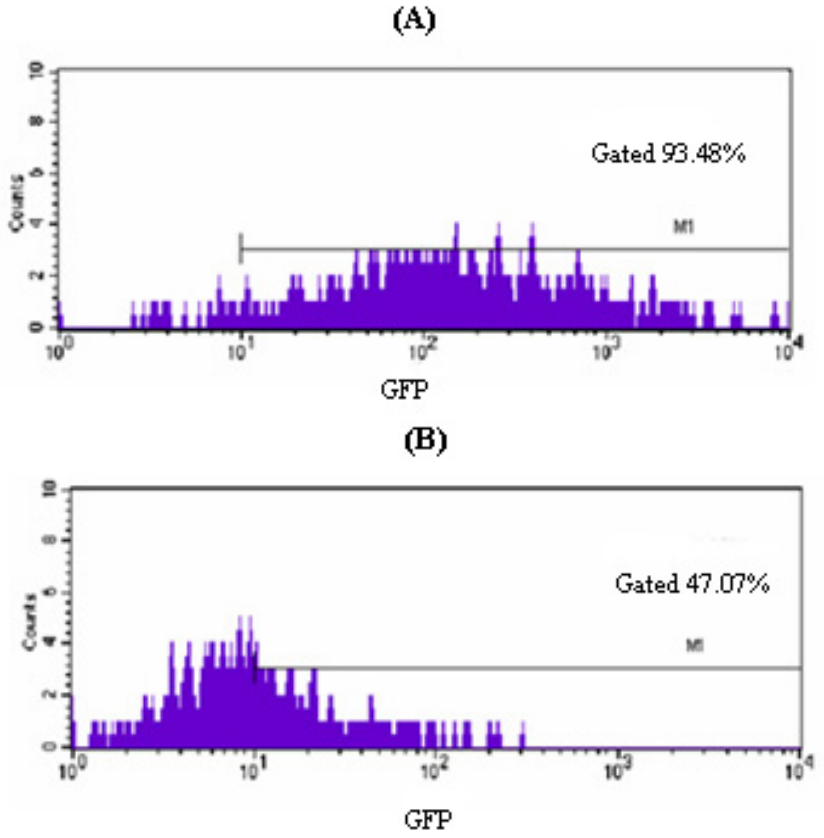


Fig. 2. Stable RNA interference effect with pJC2 in EC cells. FACS analysis of EC cells 8 days after initial *puromycin* selection at 48 h after transfection. The cells were transfected with pB17, pML8 and either pSUPER (control) **(A)**, or pJC2 **(B)**. B shows effect of pJC2 in stable on the number of positive GFP cells. The peak patterns represent the fluorescent profile of the cells. The bar indicates the gated region for positive GFP cells. The number reflects the percentage of the positive cells.

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DISCUSSION

The use of siRNA to inhibit gene expression in mammalian cells is a promising new approach for analysis of gene function [3]. Here, we have demonstrated that siRNA can be synthesized by *in vitro* transcription providing economical alternatives to chemical synthesis of siRNA. We also found that hairpin siRNA can inhibit gene expression in mammalian cells. The technique of RNAi has been widely used in *C. elegans* and plants to inhibit gene expression due to its highly sequence specific silencing and powerful systemic spread of the silencing effect [1]. Recently, the successful discovery of using siRNA to induce RNAi in mammalian cells has opened new possibilities with this technique [3, 19]. Here, utilizing the hairpin siRNA, we demonstrated that it is possible to produce RNAi effect in P19 EC cells by using the eGFP gene as a target. The sequence specific silencing manner of RNAi was also confirmed since only the targeted gene was silenced by the siRNA.

The RNAi response to dsRNA can be triggered effectively in undifferentiated embryonic cells. The observation is in line with previous findings that these cells are deficient in some of the dsRNA- and IFN-activated enzymes and that induction of IFN genes by dsRNA or viral infection in these cells is impaired [20].

However, application of RNAi in response to long dsRNA in cultured mammalian cells has been generally unsuccessful, most likely because of the known, non-specific effects of dsRNA longer than 30 nucleotide on gene expression and cell growth, mediated by the dsRNA-dependent protein kinase

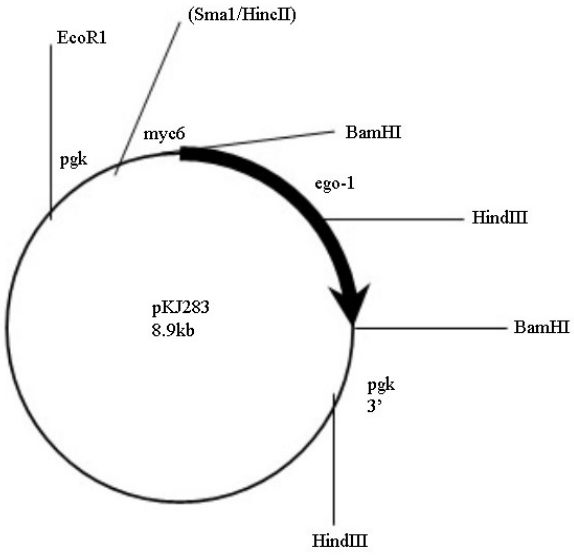


Fig. 3. Schematic drawing of pKJ283 expressing *ego-1*.

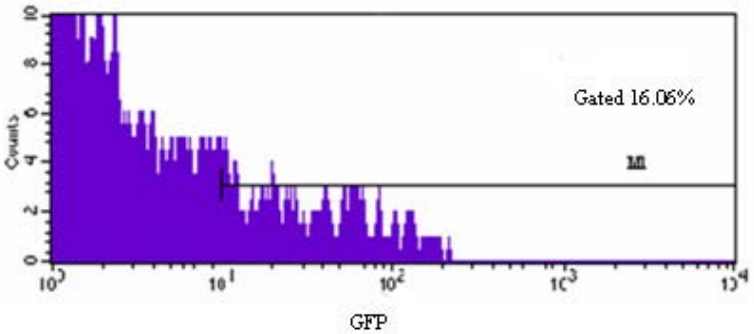


Fig. 4. Stable RNA interference effect with pKJ283 in EC cells. FACS analysis of EC cells 8 days after initial *puromycin* selection at 48 h after transfection. The cells were transfected with pB17, pML8, pJC2 and pKJ283. The diagram shows effect of pKJ283 in stable on the number of positive GFP cells in comparison to the results without pKJ283 (Fig. 2B). The peak patterns represent the fluorescent profile of the cells. The bar indicates the gated region for positive GFP cells. The number reflects the percentage of the positive cells.

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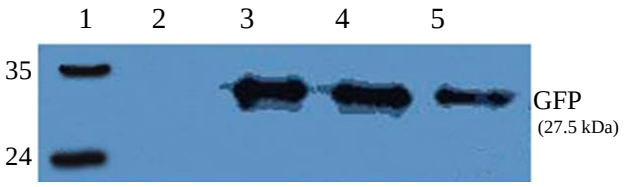


Fig. 5. Western-blot analysis of eGFP protein (MW, 27.5 kDa) expression in EC cells. Lane 1**,** marker; lane 2**,** Un-transfected P19 cells; lane 3, transfected P19 cells with: pB17, pML8, pSUPER; lane 4, transfected P19 cells with: pB17, pML8, pJC2; lane 5, transfected P19 cells with: pB17, pML8, pJC2, pKJ283. GFP was detected with anti-GFP antibody. In agreement with the FACS analysis results, inhibition of eGFP expression was more efficient in pKJ283-transfected cells.

PKR and RNase L, both acting as effectors of the IFN response [20]. These inhibitory effects have impeded studies of RNAi in mammals. Recent work by Elbashir and colleagues [3] has shown that siRNA [21- to 22-nucleotides short interfering RNAs] could induce silencing in numerous mammalian cell lines, presumably by entering the RNAi pathway. This non-specific effect can be circumvented by the use of synthetic siRNA, which can mediate strong and specific suppression of gene expression [3]. However, this reduction in gene expression is transient, which severely restricts its applications. To overcome this limitation, we used a mammalian expression pSUPER based on vector, named pJC2, that directs the synthesis of siRNA-like transcripts in mammalian cells [19]. siRNA expression mediated by this vector causes efficient and specific down-regulation of gene expression, resulting in functional inactivation of the targeted genes. Using this vector, stable expression of siRNA mediates persistent suppression of gene expression, allowing the analysis of loss-of-function phenotypes that develop over longer periods of time. Therefore, the pSUPER system constitutes a new and powerful system to analyze gene function in a variety of mammalian cell types [19]. The pJC2 vector contains a polymerase-III H1-RNA promoter to make the siRNA transcripts in a form of a hairpin ensuring the dsRNA formation [19, 21, 22]. For this plasmid, there was a gene-specific insertion such that it specifies a 20-nucleotide sequence derived from the target transcript, separated by a short spacer from the reverse complement of the same 20-nucleotide sequence. The resulting transcript is predicted to fold back on itself to form a 20-base pair stem-loop structure. The pB17 contains a fragment from the murine *Pgk-1* gene that greatly increases the expression of integrated genes from transfection [23]. So, pB17 was included in all transfections.

These hairpins siRNA were capable of suppressing eGFP gene expression, specifically. Apparently, the stable RNAi effect in EC cell was not a complete inhibition like in *C. elegans* [1]. On the other hand, looking at individual stable clones where high inhibition was evident, there appeared to be complete abolishment of the gene expression. Probably, these clones uniformly integrated a low copy number of reporter gene. Since the ratio of the transfected DNA was the same, we expected an equal number of siRNA construct being integrated into the genome. This suggests that the RNAi with lower copy number of its targeted gene was more effective in the EC cells.

The results showed that, RdRP did induce a change in RNAi in the cells. The inhibition observed with the enzyme was significantly different from the ones without. This suggests a possible enhancement of RNAi by the amplification of the siRNA with RdRP in EC cells. Nevertheless, this is still a preliminary evidence of the effect of RdRP. The effects of RNAi are potent and systemic in nature [24]. This has led to a proposed mechanism in which RdRP play a role in both triggering and amplifying the silencing effect [24]. It is speculated to play a role in the amplification of the dsRNA signal, allowing its spread throughout the organism [25-28]. RdRP proteins have been identified in a variety of organisms that exhibit RNAi-like phenomena. There are three types of the enzyme, including *ego-1*, *rrf-1*, and *rrf-3* in *C. elegans* [16]. Two recent reports specified a function for an RdRP activity in RNAi in *C. elegans* [16] and *Drosophila* [15] such that the siRNA prime an RdRp for RNA synthesis to produce more dsRNA, thereby amplifying the response. Indeed, in plants and *C. elegans*, two distinct populations of small RNAs have been proposed to participate in RNAi: "Primary siRNA" (derived from Dicer nuclease-mediated cleavage of the original trigger) and "secondary siRNA" (additional small RNAs whose synthesis requires RdRP) [29].

There are, however, no known homologs of RdRPs involved in RNAi in coelomates whose genomes have been sequenced completely (*Drosophila*, human, and mouse).

Considering the important role of the PdRP in RNAi in plants and *C. elegance* [15, 16], it might explain the less effective RNAi observed in mammalian cells since they do not have this enzyme. The introduction of possible RdRP became an appealing strategy for a possible amplification effect of the RNAi in the EC cells.

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The results indicated that the plasmid vector-based siRNA system is a promising method to inhibit gene expression in mammalian cells. The ability to create permanent cell lines with a desired loss-of-function phenotype extends the utility of RNAi as a method for probing gene function in mammalian cells. This capability enables the production of large numbers of silenced cells for biochemical analysis, studies of gene function and therapeutic gene silencing.

The mammalian RNAi pathway likely differs from that of *C. elegans*, plants, and fungi that all employ RdRPs to amplify the RNAi response. An RdRP, however, could have a role in mammalian RNAi to generate trigger dsRNA. The present study showed that *ego-1,* an RdRP could enhance RNAi activity over conventional siRNA duplexes in cultured mammalian cells.

The ability to inhibit gene expression with hairpin siRNA synthesized in mammalian cells is likely to have broad application. In particular, this approach should facilitate studies of gene function in transfectable cell lines, studies in intact animals, and gene therapy.

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