論文の内容の要旨

論文題目　**Development of technologies for the applications of RNA interference**

(RNA 干渉の応用に向けた技術の開発)

氏名　カシム　ヴィヴィ

Introduction

RNA interference (RNAi) is now well-known as a post-transcriptional gene silencing mechanism induced by double-stranded RNAs (dsRNAs). It was first discovered in 1998 by Fire *et al.* in *Caenorhabditis elegans*, and has been found in evolutionally diverse organisms, such as plants, flies and protozoan. In RNAi, long dsRNAs are cleaved into small-interfering RNAs (siRNAs) of 21-23 nucleotides (nts) in length by Dicer, a member of the RNAse III family. These siRNAs are then incorporated into RNA-Induced Silencing Complexes (RISCs), which then bind to the target messenger RNA (mRNA) having sequences complementary to the incorporated siRNA, leading to the degradation of the target mRNA.

However, in the case of mammalian cells, it was known that long dsRNAs trigger interferon response, a major immune reaction, resulting in inhibition of translational step of target genes, induction of apoptosis, and nonspecific cleavage of single-stranded RNAs by RNase L. Initially, these were hurdles for applying RNAi-mediated gene silencing in mammalian cells. A key finding to avoid these problems was reported by Tuschl *et al.* in 2001, i.e. a 21-23 nt chemically synthesized siRNAs with 3' overhangs of 2 or 3 nt could bypass the interferon pathway and induced RNAi in mammalian cells.

Compared to other gene-silencing technologies that have been used so far, such as antisense RNAs and dominant-negative molecules, inhibition of function of a gene of interest using RNAi has several advantages. First, a specific sequence can be designed that possesses a strong suppressive effect. Second, vector-mediated systems have been developed to induce RNAi in mammalian cells, allowing us to achieve high cost-performance. Thus, RNAi provides promising experimental and therapeutic tools today. There are, however, still rooms to improve the technology. In this thesis, I describe novel methods to control siRNA

expression and to select efficient siRNA target sites.

Control of siRNA expression using the Cre-*loxP* recombination system

For practical use of siRNA, it is necessary to develop a method to control the expression of siRNAs. To express siRNAs, promoters recognized by RNA polymerase III (pol III) such as U6 and H1 promoters have been used. They are strong promoters that express short RNAs. In spite of the merits of pol III that achieves high level expression and adds no extra sequences to its transcripts, these promoters are activated constitutively and ubiquitously. Furthermore, knocking-down genes essential for survival may lead to fatal results, which limits the use of this system. Thus, it is desirable to develop a system to express siRNA timely in a tissue specific manner.

For this reason, I first tried to develop a controllable siRNA expression vector that can be switched-on by the action of Cre recombinase proteins. A major siRNA expression vector consists of sense - loop - antisense sequences under the control of U6 promoter, which gives rise to hairpin-type siRNAs. To develop a Cre recombinase-controllable siRNA expression vector, instead of the canonical loop sequence, I inserted the sequence "loxP - a stretch of 7 thymine residues - 809 bp linker fragment - loxP" between the sense and antisense sequences. In its original form, there are 4 consecutive thymine residues that act as a transcriptional terminator of pol III. Therefore, transcription from this vector is supposed to stop right after the sense sequence, resulting in

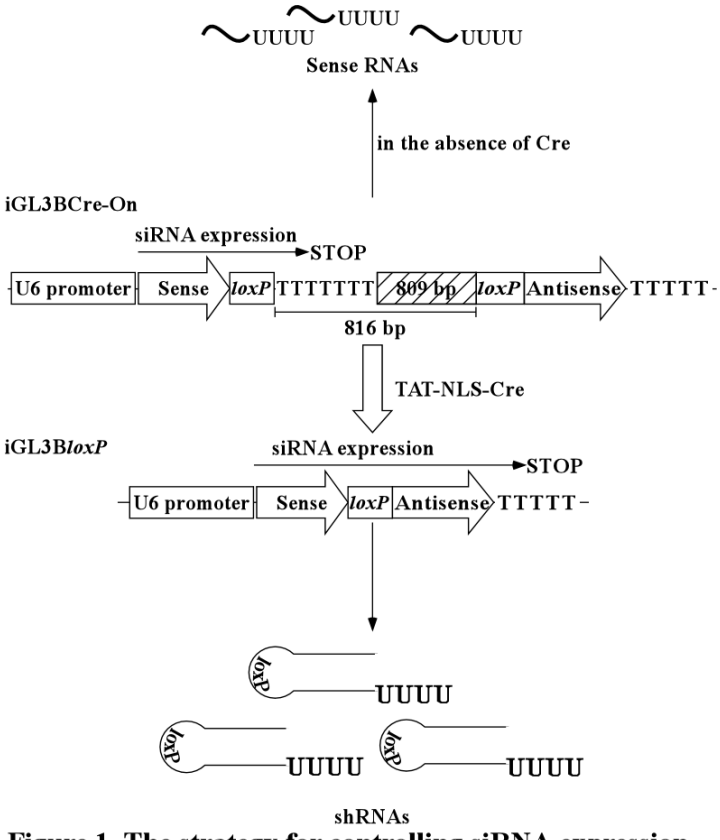

Figure 1. The strategy for controlling siRNA expression vector using exogenously provided Cre recombinase.

transcription of only the sense RNAs which have no suppressive activity. In the presence of Cre recombinase, recombination occurs between the two *loxP* sites, leaving only one *loxP* site between the sense and antisense sequences. The resultant DNA is transcribed to produce hairpin-type siRNAs, with loop of a *loxP* sequence.

To test the feasibility of this system, I constructed a Cre recombinase-controllable siRNA expression vector targeted against the firefly luciferase, termed iGL3BCre-On vector, and cotransfected it together with firefly and *Renilla* luciferase reporter vectors. Cre recombinase was provided exogenously by adding recombinant TAT-NLS-Cre recombinase protein (TAT-NLS-Cre) 4 hours after transfection. The result demonstrated that only in the presence of TAT-NLS-Cre siRNA expression was switched-on and, consequently, firefly luciferase activity was suppressed. Moreover, the suppressive effect was dose-dependent on TAT-NLS-Cre.

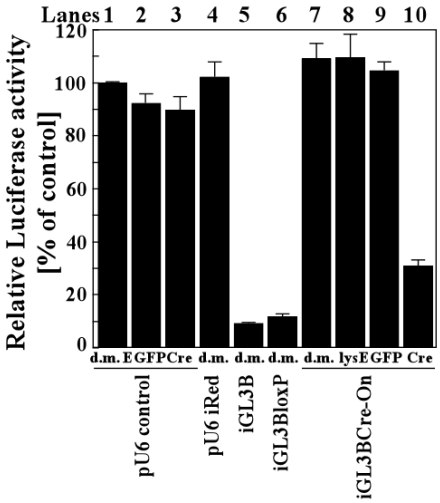

Figure 2. Cre-On siRNA expression vector was switched-on only in the presence of Cre recombinase (lane 10). Lanes 1-3, control vector with the addition of either dialysis medium, TAT-NLS-EGFP or TAT-NLS-Cre; lane 4, siRNA expression vector against DsRed with the addition of dialysis medium; lane 5, iGL3B with the addition of dialysis medium; lane 6, iGL3BloxP with the addition of dialysis medium; lanes 7-10, iGL3BCre-On with the addition of either dialysis medium, residual *E. coli* lysate, TAT-NLS-EGFP or TAT-NLS-Cre.

Therefore, the expression of siRNA could be controlled by using the Cre-*loxP* recombination system. Cre recombinase can be provided endogenously by either establishing Cre recombinase stable cell-lines or using Cre recombinase transgenic mice. Although endogenous expression of Cre recombinase has an advantage in maintaining long-term recombination activity, a suitable expressing system has to be selected and appropriate cells or mice have to be established for each experimental setting. Alternatively, Cre recombinase protein can be exogenously added to cells of interest. As TAT fusion proteins are taken up by cells, recombinant TAT-NLS-Cre recombinase proteins can be used to deliver the Cre recombinase to the target cells. The results demonstrated here show that TAT-NLS-Cre is able to switch on the expression of siRNA from the expression vector.

Screening of siRNA target sequences by using fragmentized DNA

The second problem I tried to solve was to select the siRNA target sequences with high RNAi activity. As described above, unlike in *C. elegans*, long dsRNAs can not be used to induce RNAi in mammalian cells due to the undesirable interferon responses. To avoid interferon responses, RNAi should be induced in mammalian cells by using siRNAs. As suppression efficacy depends very much on the target sequences, choosing an appropriate target sequence is the key for this technology. Although several algorithms and computer programs for target sequence predictions have been developed, these systems have a disadvantage. A substantial fraction of high efficiency target sequences might be overlooked, as the factors influencing the RNAi activity are complex and not wholly understood yet. For example, the length of siRNA target sequences should usually be pre-set for algorithm-based predictions, yet it has been reported that, in some cases, 25-27-nt siRNAs could give higher suppressive effects compared to 21-nt siRNAs.

Another issue to be considered for selecting RNAi target sequences is cytotoxicity that might be induced by particular dsRNAs. Recently it was reported that dsRNAs with a particular sequence, regardless of their length, could induce interferon dependent- and/or independent-cytotoxicity. As the mechanisms of these cytotoxic effects remain to be understood, the current programs and predictions can not exclude cytotoxic sequences.

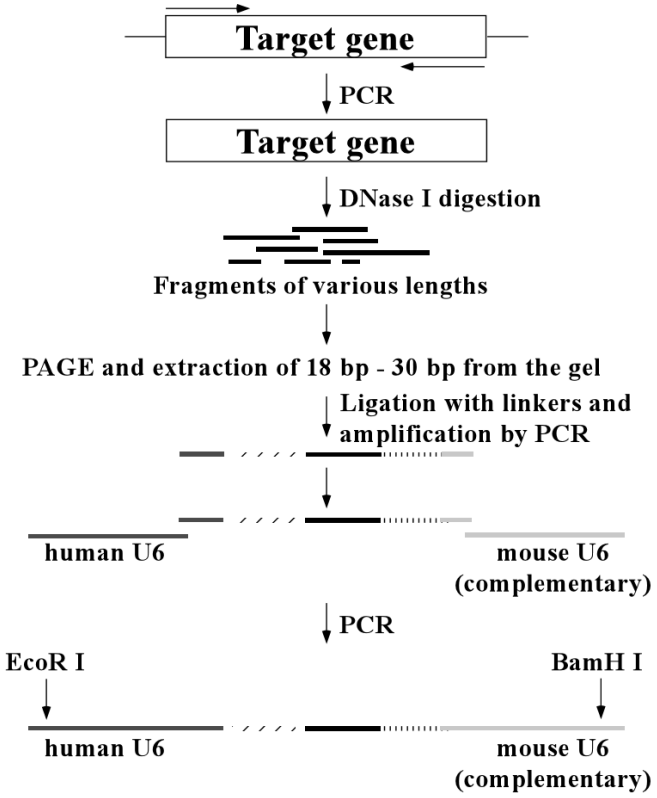

Figure 3. Strategy for constructing parallel-type siRNA expression libraries with siRNA target sequences originated from fragmentized target genes.

To solve these problems, I developed an experimental approach for determining siRNA target sequences that exhibit high RNAi activity but lack cytotoxic activity. In this system, target genes were fragmentized by using DNase I, and fragments

with the length between 18-30 bp were collected. After ligation with linkers and PCR amplification, fragments were further amplified by PCR again together with the human U6 promoter and the mouse U6 promoter. PCR products were then inserted into a backbone plasmid vector. The resulting "parallel-type" siRNA expression vectors contained the human U6 promoter, a target gene-derived fragment coding for 18-30 bp siRNA, and the mouse U6 promoter (whose direction is inverse to that of human U6 promoter) consecutively.

I generated parallel-type siRNA expression vector pools for two reporter genes, i.e DsRed and GFP. To simplify the screening process, I assayed groups of five or more parallel-type siRNA expression vectors. The groups were cotransfected together with the reporter genes, and the suppressive activity of each group was determined. Only those groups with more than 50% suppression of the target genes were selected, and individual siRNA expression vector from those groups was further assayed.

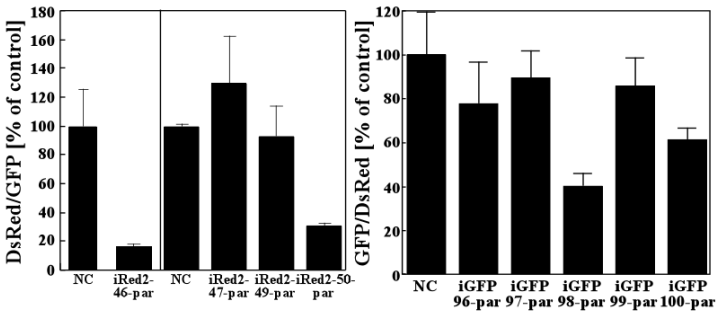

Figure 4. High suppressive activity siRNA target sites against DsRed (left panel) and GFP (right panel) were obtained by screening the parallel-type siRNA expression vectors library.

By using this method, I could efficiently screen more than 110 siRNA expression vectors for each reporter genes, and selected target sequences that have high RNAi activity. Furthermore, I also found that some clones exhibited cytotoxicity regardless of their lengths. Thus, this method enables us to find target sequences that produce high RNAi activity, and at the same time, avoid the cytotoxic sequences.

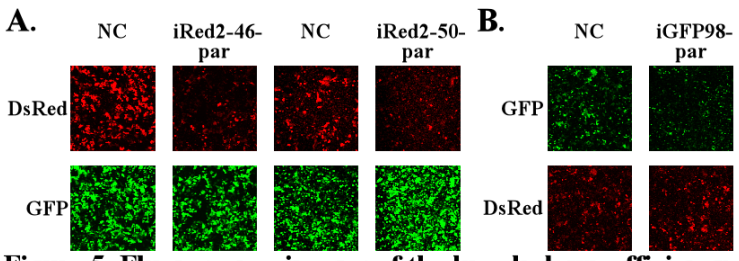

Figure 5. Fluorescence images of the knock-down efficiency against DsRed (A) and GFP (B) as shown in Figure 4.

Conclusions and Future Perspectives

In this thesis, I developed methods for controlling siRNA expression vectors and for selecting highly active

siRNA target sequences, which were two major issues that remained to be overcome for application of RNAi technology.

Controlling siRNA expression vectors using the Cre-*lox*P recombination system enables the spatio-temporal control of gene expression, making it possible to apply RNAi in various experimental systems. This also allows us to knock-down essential genes for life. Furthermore, besides using exogenously delivered Cre recombinase, delivery can be achieved by establishment of stable Cre recombinase-expressing cell lines or transgenic animals. The choice of delivery systems depends on the needs of each experiment.

Screening siRNA target sequences using parallel-type siRNA expression libraries generated from fragmentized target gene enables to select siRNA target sequences with desired RNAi activity, and the method simultaneously avoids cytotoxic sequences. Alternatively, this method could also be used to obtain cytotoxic sequences, and this might provide a source for elucidating the nature and mechanisms of cytotoxic short dsRNAs.

Collectively, I believe that these procedures I have established allow us to use highly efficient and specific siRNAs for accurate and precise control of gene expression, contributing to the further application of the RNAi technologies in both research and therapeutic fields.