SEQUENCE SPECIFIC INHIBITION OF HUMAN CHEMOKINE GENE, CXCL7, BY SMALL INTERFERING RNAS IN LEWIS LUNG CARCINOMA CELLS

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DİLAY KIZIŞAR

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submitted by **DİLAY KIZIŞAR** in partial fulfillment of the requirements for the degree of **Master of Science in Chemistry Department, Middle East Technical University** by,

Prof. Dr. Canan ÖZGEN _____
Dean, Graduate School of **Natural and Applied Sciences**

Prof. Dr. İlker ÖZKAN _____
Head of the Department, **Chemistry**

Prof. Dr. Mahinur S. AKKAYA _____
Supervisor, **Chemistry Dept., METU**

Examining Committee Members

Prof. Dr.İnci GÖKMEN _____
Chemistry Dept., METU

Prof. Dr. Mahinur S. AKKAYA _____
Chemistry Dept., METU

Assist. Prof. Dr. Salih ÖZÇUBUKÇU _____
Chemistry Dept., METU

Assist. Prof. Dr. İrem EREL _____
Chemistry Dept., METU

Assoc. Prof. Dr. Ayşe Begüm TEKİNAY _____
Institute of Materials Science and Nanotechnology,
Bilkent University

Date: 19/09/2013

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Name, Last name: Dilay KIZIŞAR

Signature:

ABSTRACT

SEQUENCE SPECIFIC INHIBITION OF HUMAN CHEMOKINE GENE, CXCL7, BY SMALL INTERFERING RNAS IN LEWIS LUNG CARCINOMA CELLS

Dilay KIZIŞAR
M.Sc., Department of Chemistry
Supervisor: Prof. Dr. Mahinur S. AKKAYA

September 2013, 50 pages

In this study, we aimed to design small interfering RNAs to silence the expression of a gene that is a member of chemokines playing crucial roles on cancer. Small interfering RNAs (siRNAs) are generated by the cleavage of 20-25nt in length dsRNAs by Dicer, which is a member of ribonuclease III family of nucleases, which mediate RNAi-induced specific mRNA degradation and they are known to be an important part of the gene regulation mechanism in all eukaryotes. Recently, siRNAs are being utilized as promising treatment approaches. In this study, we targeted a gene involved in lung cancer CXCL7 via designing potent siRNA sequences and carried out optimization for efficient transfection. The levels of silencing in mouse cell culture were determined by the use of Flow Cytometry.

Keywords: RNAi, siRNA, Chemokine, CXCL7, Lung Cancer

ÖZ

İNSAN CXCL7 GENİNİN DENEYSEL AKCİĞER KANSERİ MODELİNDE SEKANS SPESİFİK SİRNA YÖNTEMİ İLE İNHİBİSYONU

Dilay KIZIŞAR
Yüksek Lisans, Kimya Bölümü
TezYürütücüsü: Prof. Dr. Mahinur S. AKKAYA

Eylül 2013, 50 sayfa

siRNA'lar (küçük, hücresel süreçlere etki eden ribonükleik asit) 20-25 nükleotit boyundaki çift sarmallı ribonükleik asitlerin Dicer adı verilen ribonükleaz III enzimi tarafından kesilmesiyle oluşmakta olup, bu süreç RNA interferans tarafından etkilenen mesajcı RNA degradasyonunu spesifik olarak başlatmaktadır. siRNA'ların tüm ökaryotlarda gen regülasyon mekanizmasının önemli bir parçası olduğu bilinmektedir. Son yıllarda, bu metot gelecek vaadeden bir tedavi yöntemi olarak yoğun ilgi görmekte ve bu alanda birçok çalışma gerçekleştirilmektedir. Bu çalışmada, akciğer kanserinde görülen CXCL7 genini susturmak için siRNA sekansları tasarlanmış ve transfeksiyon optimizasyonları yapılmıştır. Susturma düzeyleri fare kanser hücrelerinde Akım Sitometrisi cihazı kullanılarak hesaplanmıştır.

Anahtar Kelimeler: RNA interferans, Kemokin, siRNA, CXCL7, Akciğer Kanseri

To my parents,

To my beloved brothers, Murat and Kayhan

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TABLE OF CONTENTS

LIST OF TABLES

TABLES

LIST OF FIGURES

FIGURES

LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
BLAST	Basic Local Alignment Search Tool
bp	base pairs
cDNA	complementary DNA
DNA	Deoxyribonucleic acid
eGFP	enhanced Green Fluorescent Protein
FACS	Fluorescence-activated cell sorting
FBS	Fetal Bovine Serum
GFP	Green Fluorescent Protein
IRES	Internal Ribosomal Entry Site
LLC	Lewis Lung Carcinoma
mRNA	messenger RNA
nt	nucleotide
Opti-MEM	Opti-MEM I Reduced serum media
ORF	Open Reading Frame
qRT-PCR	quantitative Real Time-PCR
PCR	Polymerase Chain Reaction
PKR	Protein Kinase R
RISC	RNA-Induced Silencing Complex
RNA	Ribonucleic Acid
RNase	Ribonuclease
RNAi	RNA interference
RPMI	Roswell Park Memorial Institute

siRNA	small interfering RNA
siRNP	siRNA-Protein complex
si236	siRNA targeting the mRNA of CXCL7 at position 236
si391	siRNA targeting the mRNA of CXCL7 at position 391
siUTR919	siRNA targeting the mRNA of CXCL7 at position 919
siGFP	siRNA targeting the eGFP gene
siNEG	Negative control siRNA
UTR	Untranslated Region

CHAPTER 1

INTRODUCTION

1.1 RNA Interference

RNA interference (RNAi) is an evolutionarily conserved mechanism in living organisms by which 21-22 nucleotide double stranded RNAs mediate sequence specific, post-transcriptional gene silencing.[1,2] This process allows to control the expression of genes of interest and to create new agents for treatment of various genetically regulated diseases.

RNAi has been used as a promising tool not only to understand the cellular mechanism of interest of the genes and gene products but also to discover potent agents for therapeutic applications.

1.1.1 The history of RNAi discovery

The revolutionary discovery of RNAi was awarded a Nobel Prize to A. Fire and C. Mello with their seminal work demonstrating in 1998 that dsRNA molecules induce efficient gene silencing in the nematode *Caenorhabditis elegans*.[3] These results were immediately validated by the demonstration of RNAi in *Drosophila melanogaster*.[4] However, the full potential of RNAi mechanism only became clear when Tuschl and his colleagues revealed that 21-bp RNA duplexes with symmetric 2-nt 3' overhangs, termed small interfering RNAs (siRNAs), could reliably induce RNAi in a range of mammalian cells.[5] Soon afterward, it became definite that many different human cell types had this evolutionarily conserved mechanism, RNAi, which could be harnessed to silence any gene of interest.

The knowledge that small double stranded RNAs can affect gene expression has had a tremendous impact on research and RNAi has expeditiously become one of the most promising new approaches for treatment of many diseases.[6]

1.1.2 The mechanism of RNAi

The mechanism of RNAi starts with the cleavage of dsRNAs by a cellular enzyme called Dicer (Figure 1.1). It is a member of the RNase III family of nucleases that specifically

cleave double-stranded RNAs to generate siRNAs in an ATP-dependent manner and is evolutionarily conserved in worms, flies, plants, fungi and mammals.[7]

After Dicer cleavage, RNA Induced Silencing Complex (RISC), which is a multi-protein complex as the machinery of RNAi, recognizes processed 21-nt RNA molecules, called small interfering RNAs (siRNAs).[4,8] The selection of the siRNA strand to be incorporated to RISC is governed by the thermodynamic features of the siRNA duplex.[9,10] The lower stability of the 5' end of the guide strand has been suggested to be crucial for the incorporation of the strand into the RISC complex.[11]

The result of the incorporation of siRNA into RISC multi-protein complex leads the formation of siRNA-protein complex, siRNP.[12] Although this process is independent from ATP, the unwinding of siRNA strands requires ATP. Once unwound, only the antisense strand guides RISC to messenger RNA that has a complementary sequence, which results in the endonucleolytic cleavage of the target mRNA.[4,13,5]

The resulting degraded mRNA cannot be translated and the production of its encoded protein is blocked.

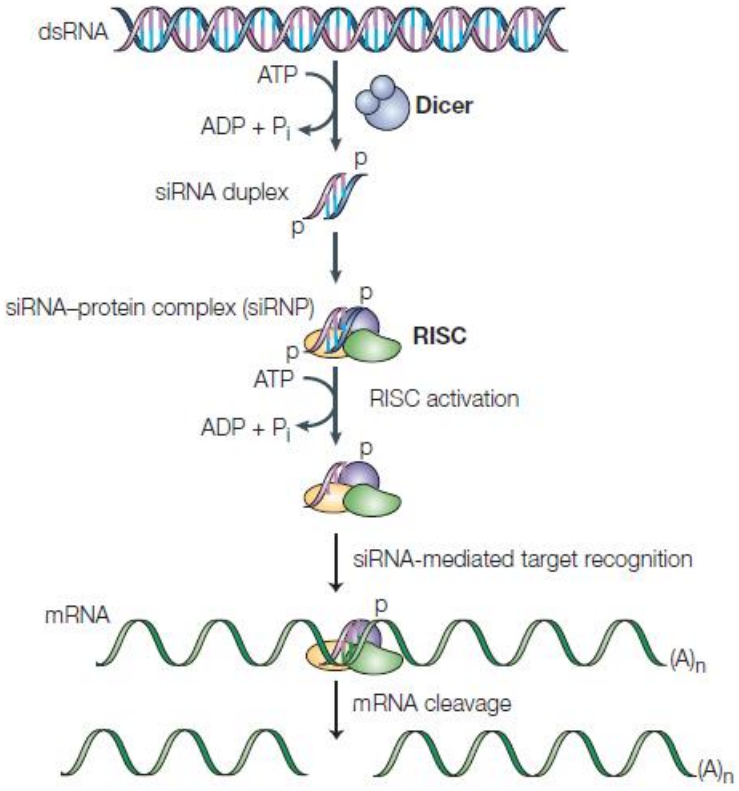


Figure 1.1 The mechanism of RNAi (adapted from[12]).

However, RNAi mechanism differs by the involvement of the protein Ago2, which acts as the catalytic engine of the process, of the RISC multi-protein complex in mammalian systems as illustrated in Figure 1.2.[14]

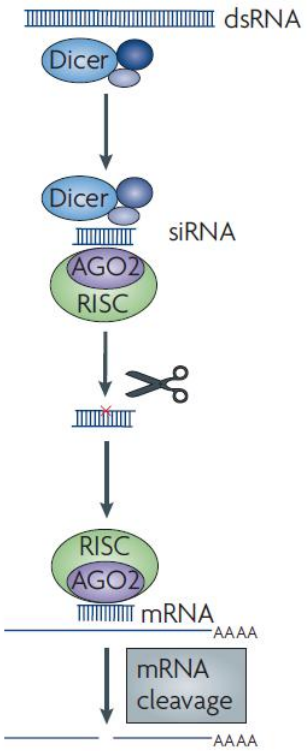


Figure 1.2 RNAi mechanism in mammalian systems (adapted from[14]).

1.2 Small interfering RNA

Since the underlying mechanism of how RNAi works was understood, siRNAs have become promising tools for exploring gene functions. siRNAs are defined as 21 nt long dsRNA molecules containing dinucleotides 3' overhangs and 5' phosphates, processed through the cleavage of dsRNAs by the Dicer cellular enzyme.[7]

However, as the great potential of RNAi for therapeutic applications have appeared, synthetic siRNAs are being utilized as the mediators of RNAi to silence the gene of interest.

Chemically synthesized siRNAs do not bear 5′-phosphate groups crucial for incorporation into RISC; however, cellular kinases correct this efficiently by phosphorylating at 5'-OH

ends, making it unnecessary to enzymatically phosphorylate synthetic siRNAs before using them.[5,15]

1.2.1 The structure of siRNAs

siRNAs are 19 nucleotide-long double stranded RNA duplexes with two nucleotide 3'overhangs and 5'phosphates.[7] The strands of siRNA duplexes are classified as sense or passenger strand and antisense or guide strand, in which the antisense strand was recognized by the RISC and the sense strand was not used and degraded. The structural features of siRNA are emphasized in Figure 1.3.

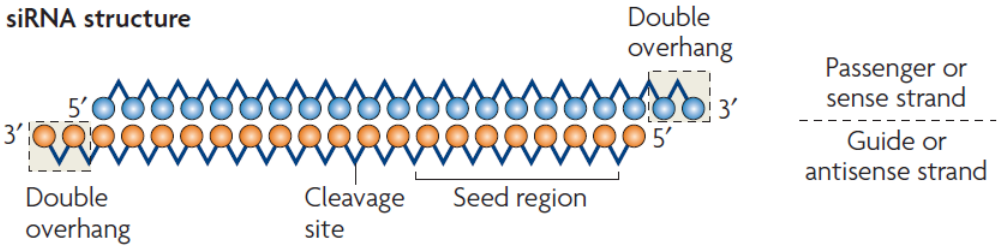


Figure 1.3 A schematic illustration of the important features of siRNA structure (double overhangs, mRNA cleavage site and seed region, adapted from[14]).

The siRNAs may be effective in mammalian systems, where long dsRNAs cannot be used since they cause activation of the dsRNA-dependent protein kinase (PKR) response.[16]

Activated RISC as a result of anti-sense strand loading preferentially cleaves target mRNA between nucleotides 10 and 11 relative to the 5′ end of the complementary targeting strand.[17,18]

Moreover, siRNA sequences containing internal repeats more than three bases or palindromes may form internal fold-back structures. These hairpin-like structures may exist in equilibrium with the duplex siRNA and may result in reduction of the effective concentration and functionality of siRNA.[19]

1.2.2 Thermodynamic profile

Several studies revealed the importance of siRNA thermodynamic profile for the incorporation of antisense strand into the RISC multi-protein complex. The siRNA strand, which is less stable at 5´end, is preferably loaded into the RISC, so the low stability of seed region of the antisense strand correlates with siRNA efficiency. siRNAs with more A or U at the seed region appear to be more efficient because of the fact that less hydrogen bonding between A and U than between G and C causes lower stability.[9,10] Figure 1.4 demonstrates the thermodynamic relations in RNAi mechanism.

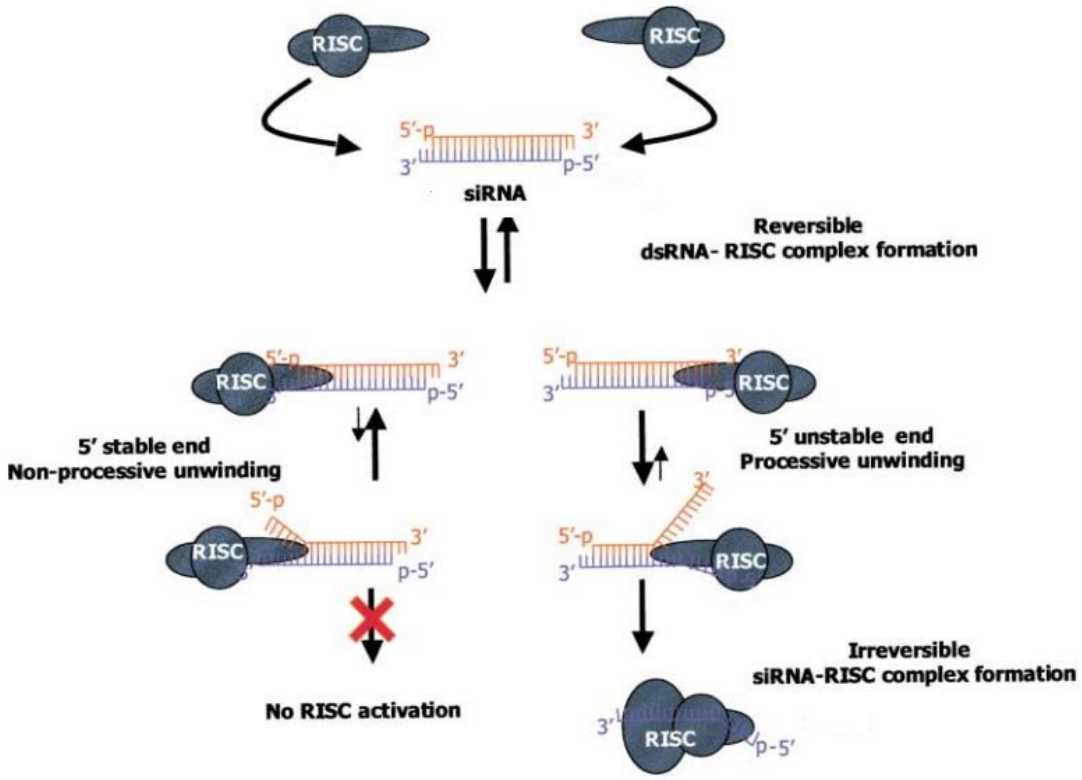


Figure 1.4 A proposed model for the RISC activation based on the thermodynamic characteristics within an siRNA (adapted from[9]).

1.2.3 GC content

The GC content of the most potent siRNAs usually varies between 36 – 52 % in functional siRNAs as shown in Figure 1.5.[17] siRNA duplexes with a GC content higher than 52% may result in poor dissociation of the strands, while siRNAs with a GC content lower than 30% may interact less well with the mRNA recognition site.[20]

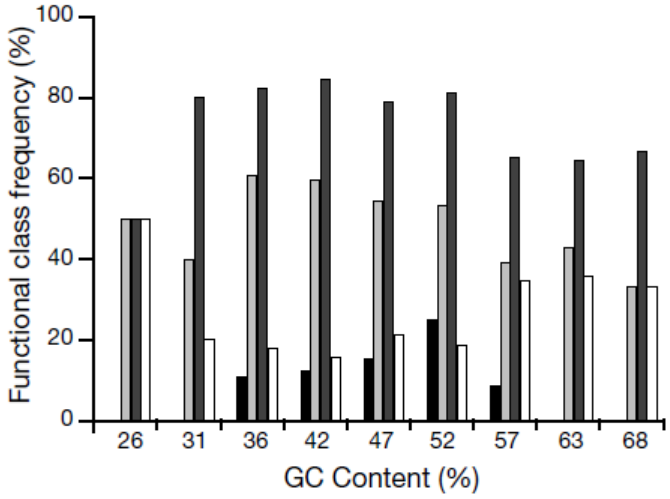


Figure 1.5 Functional silencing classes sorted by G/C content (between 26% and 68%, adapted from[17]).

Although long double-stranded RNAs provoke an interferon response, short interfering RNAs (siRNAs) have been reported to specifically inhibit gene expression in many different mammalian cell lines.[21,22,23,24] Also, it has been shown that even single nucleotide mismatches between the antisense strand of the siRNA and target mRNA can abrogate RNA interference.[18]

1.2.4 Low seed frequency

siRNA duplexes may cause off-target effects which lead to unintended alteration of non-target gene expression. It can be prevented by avoiding siRNA sequences that have complementarities to other genes even if they seem to be potent for silencing of the gene of interest. Furthermore, off-targeting is dependent on the concentration and mediated by either the passenger strand or the guide strand of siRNA.[25]

Khvorova et al. (2008) revealed that siRNA duplexes with low seed complement frequencies induced fewer off-target effects and false positive phenotypes than duplexes with medium or high seed complement frequencies (Figure 1.6).[26]

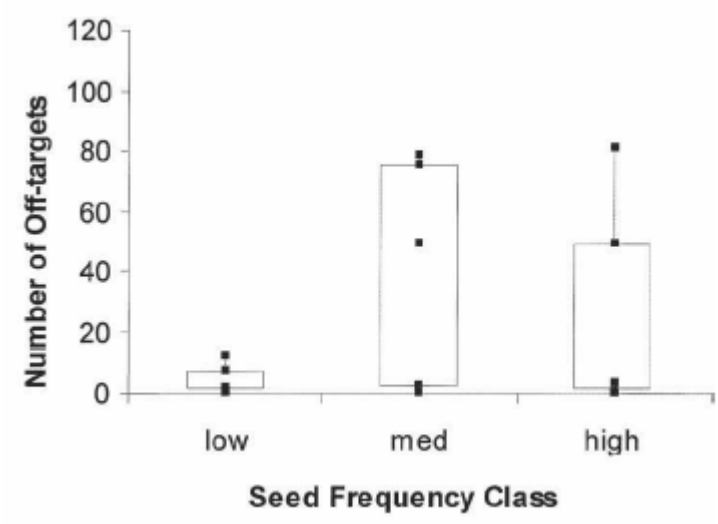


Figure 1.6 The relationship between seed complement frequencies and off-targets[26].

1.2.5 Point-specific base preferences

Various reports analyzing the sequence features of efficient siRNAs have shown preferences for certain nucleotides at specific positions within the siRNA.[17,27,28,29] Table 1.1 summarizes those preferences of nucleotides at specific positions within the siRNA sequence.

Table 1.1 Nucleotide preferences at specific points established in the literature.

Citation	Position	1	3	6	10	11	13	16	19
[17]	Preferred		A		U		A/C/U		A/U
	Not preferred						G		G/C
[28]	Preferred	G/C							A/U
	Not preferred	A/U							G/C
[29]	Preferred					C/G	A	G	U
	Not preferred			C		A/U			G
[30]	Preferred	G/C			A/U				A/U

1.3 Chemokines and cancer

Chemokines are a family of chemotactic cytokines whose primary function is the regulation of cell trafficking. Many cancers have a complex network of chemokines that affect tumor growth, cell survival and migration, metastatic spread and angiogenesis.[30]

The pivotal roles of chemokines and their receptors in the tumor microenvironment have been assertively established and reviewed by different reports.[30,31,32]

1.3.1 The structural features and classification of chemokines

Chemokines adopt a characteristic fold that consists of an N-terminal unstructured domain which is critical for signaling, a three-stranded β-sheet connected by loops and turns, and a C-terminal helix (Figure1.7).

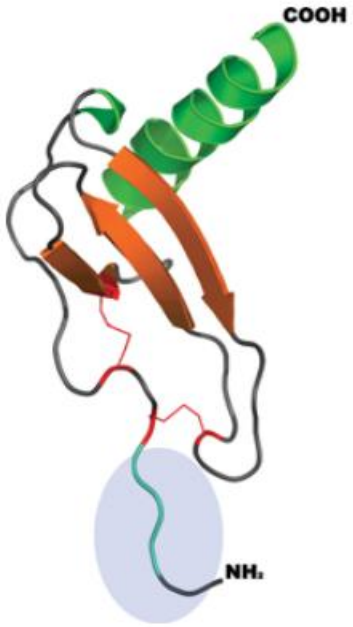


Figure 1.7 The structural features of chemokines (adapted from[33]).

The protein family of chemokines are classified into four subfamilies -CXC, CC, C and CX3C- depending on the arrangement of the first two cysteins that are adjacent to the N-terminus of the molecule.[34]

1.3.2 ELR+ Chemokines in cancer

The CXC chemokines are potent promoters of angiogenesis in the chemokine family.[35] They are strong inducers of chemotaxy which arises from the proleolytic NH_2-terminal cleavage of ELR motif (Glu-Leu-Arg) by attracting neutrophils. Neutrophils play a crucial role on the tumor microenvironment by secreting chemotactic substances. However, it has been shown that the tumor cells themselves mediate neutrophil recruitment to sites of tumorigenesis by secreting CXC chemokines.[36]

1.3.3 CXCL7 chemokine as a potential target for cancer research

CXCL7 is one member of precursor plasma chemokines that gain receptor affinity by being proleolytically processed.[37]

Recently, overexpression of CXCL7 chemokine was revealed to be associated with advanced tumor stage in an animal model.[38]

However, unlike the other members of ELR+ CXC chemokines, the function of CXCL7 chemokine in cancer has not been investigated yet.

1.4. siRNA treatment in cancer

In cancer research, the process of tumorigenesis has been recognized as the result of the overexpression of oncogenes, growth factors and mutant tumor suppressors, even if the underexpression of other proteins may influence the process.[39]

Recently, it has been reported in various studies that chemokines are involved in many aspects of carcinogenesis like tumor growth, angiogenesis and metastasis developments.[30,37,40]

RNAi can be employed by designing efficient siRNAs as the mediators, to study the underlying mechanisms in cancer.

For instance, Shim et al. (2005) achieved a remarkable RNAi silencing which resulted in the inhibition of breast cancer metastasis and demonstrated that a combination of two siRNAs targeting CXCR4 gene, which is a member of chemokine family, induced a higher level of silencing than each siRNA alone.[41]

Recently, Davis et al. (2010) conducted the first clinical trial of siRNA treatment in humans by using a targeted nanoparticle delivery system to treat patients with solid cancer, who were refractory to standard therapies.[42]

1.4.1 siRNA delivery by transfection

siRNAs have a strong negative charge due to the negative phosphate charges on the siRNA backbone and by that means, they cannot readily pass through biological membranes to be taken up by cells.[43]

Moreover, siRNAs have a small size due to their shortness in length, in comparison with other oligonucleotides provoking gene silencing, which makes them prone to rapid degradation in physiological conditions. Therefore, they are required to be employed by encapsulating them with a delivery agent, to overcome the challenges in their proper uptake by the cells.

The most widely used transfection method is the use of cationic lipid-based reagents as nucleic acid carriers. They are also being utilized as the most common method for siRNA transfections. These delivery agents bind to the negatively charged DNA or RNA molecules and together form positively charged lipid–nucleic acid complexes.

1.5 Assessment of gene silencing

The silencing of gene expression can be detected on either the mRNA or the protein level. There are various techniques to assess the silencing triggered by siRNA, such as qRT-PCR for detection on the mRNA level and Western Blotting for detection on the protein level. Moreover, the use of bicistronic vectors, which contain cDNA sequences of the protein of interest and a reporter fluorescent protein, allows for the assessment of gene silencing by measuring the fluorescence intensity. If the expression of the protein of interest is blocked, the expression of the fluorescence protein will also be blocked and this permits the use of the level of the fluorescence protein as a marker of the gene silencing.

1.6 Aim of the study

In this study, we aimed to design potent siRNA sequences for the inhibition of human chemokine gene, CXCL7, which is a member of chemokines known to play significant roles in cancer. The selected siRNA candidates were transfected by a conventional cationic lipid formulation and the assessment of silencing was carried out in mammalian cell culture and analyzed by Flow-Cytometry. Since the function of the CXCL7 gene in tumorigenesis has not been fully understood, the siRNAs used in this thesis can be utilized to examine the phenotypic changes caused by the silencing of the gene in cancer cells.

CHAPTER 2

MATERIALS AND METHODS

2.1 Rational siRNA design

The success of gene silencing is highly dependent on the choice of siRNA sequence targeting the gene of interest. There are several features that should be taken into consideration while designing siRNA sequences.

Initial explorations to predict which regions to target within a long mRNA sequence focused on determining similarities among experimentally validated siRNAs. These reports as mentioned in Table 1.1 established a set of rules for designing potent siRNA sequences.

Among them, Reynolds et al. (2004) indicated the most important characteristics of a siRNA sequence by performing statistical analysis of 180 siRNAs targeting the mRNA of two genes.[17] Their comprehensive analysis gave insights on selecting of efficient siRNAs and introduced general criteria for successive RNAi applications as defined in Table 2.1.

Table 2.1 General criteria for rational design of functional siRNAs (adapted from[17]).

Criterion	Effect	Score
I. 30-52 % G/C content	Positive	+ 1
II. At least 3 A/U bases at positions 15-19	Positive	+ 1 each
III. Presence of a 'U' at position 10	Positive	+ 1
IV. Presence of an 'A' at position 19	Positive	+1
V. Presence of an 'A' at position 3	Positive	+ 1
VI. Absence of internal repeats more than 3 bases	Positive	+ 1
VII. A base other than G or C at position 19	Negative	- 1
VIII. A base other than G at position 13	Negative	- 1

In addition, there have been different reports that analyzed siRNA sequences and established some rules concerning position-specific preferences (Table 1.1). However, there are some inconsistencies for nucleotide preferences of each position of siRNA. Although there are common preferred nucleotides at positions 1 and 19 in some reports, a few consistencies can be observed at other positions in individual studies. Thereby, if these experimental results were utilized to select siRNA sequences, many sequences might be selected as candidates to achieve RNAi silencing.

Moreover, the efficient siRNAs can be found with a high success rate among designable siRNAs in the case of *Drosophila Melanogaster* as well as other lower eukaryotes; however, about 80% of theoretically potent siRNAs would not be highly functional in the case of mammalian systems, as stated in the review of Ui-Tei et al.(2006).[44]

Before getting into rational design, siRNAs candidates should be selected as a short list by a computer-based algorithm in order to select efficient siRNA sequences that have a full complementarity to the mRNA of the gene of interest.

2.1.1 Bioinformatics for siRNA design

There is a wide variety of bioinformatics tools to search for potent siRNA sequences that are entirely complementary to the mRNA of the gene of interest.

For designing of the siRNAs, review papers on the effectiveness of various available algorithms were firmly studied. In addition, published papers on some of the most highly ranked algorithms were examined. The algorithms reported to be the best in predicting the effectiveness of siRNAs, which were tested and confirmed experimentally, were selected for use in this study.[45,46,47]

The mRNA sequence of CXCL7 in FASTA format was extracted from National Center for Biotechnology Information (NCBI, http://www.ncbi.nlm.nih.gov/) and all the other information identifying CXCL7 gene, including accession number and gene id, were used for operating the computational design tools and illustrated in Table 2.2. The official symbol of CXCL7 was provided by Mouse Genome Informatics (MGI) as Ppbp and its corresponding name is pro-platelet basic protein as the official full name.

Table 2.2 The genetic information of CXCL7 provided by NCBI.

Gene ID	57349
Accession Number	NM_023785
Gene Type	Protein coding
Organism	Mus Musculus
mRNA sequence in FASTA format	>gi|141802684|ref|NM_023785.2| Mus musculus pro-platelet basic protein (Ppbp), mRNA AACATAGCAGCAGTGTTCTGGGCAGCTCAGCCTCACGTTGTTCCCT CCTGGCTCTGGGGACAGTTCTAGCTCACTGCTCTTCATTATGGGCT TCAGACTCAGACCTACATCGTCCTGCACCAGGGCCTGCCCACTTCA TAACCTCCAGATCTTGCTGCTGCTGGGCCTGATCCTTGTTGCGCTG GCTCCCCTTACAGCTGGAAAATCTGATGGCATGGACCCATATATCG AACTGCGCTGCAGATGTACGAATACCATCTCTGGAATCCCATTCAA TTCTATCTCCCTTGTGAATGTGTACAGGCCAGGAGTTCACTGTGCT GATGTGGAAGTGATAGCCACACTGAAGAATGGACAAAAAACGTGCC TGGACCCAAATGCCCCTGGCGTCAAGAGAATCGTCATGAAAATCTT GGAAGGTTACTGACCAGCTGCTTCATCTGTGCCAAACCATTTTCCT GCTCAGGAAGGGTTGAGGGTTTGAAATCTGGACTTGGTAGAATTAT TTTTCCACTTTGAAATTGGGATGTTCTCTCGTTCTGTCTCAGAAAT CACACTGTATTCTGAGAAGGCATGATAAAGAATGTCACAACAAACA AACAAACAATGATGAAGACGGGCAAGCCAAGTTGCAACCTGCAATT TATGGTCTATTTCTGCAGTTCTTGGCTAAGCATTCTGACATGTCCT GCATTTCTCTATTTGAGCTTTTCTTTTCAAATGTACTATGTCAAGT TTTCATTCTGTGTTTTTAATAATTATTTCTTTTAGTTTTGAGATTA TAATTACATCCCTTCCCCTTTCATTTTTCTCCTTCTGAACCCTTCT ATATACCCATCCTTGCTCTCTTCAAATCCATGGCTGCTTTTTTTTC CCATTGAGCATTGTTATATACATATATAATGATAAATTTCGTAGTG GTCAATTGCAACGGAAATCGCCTGGAGTCAAGCAGTTTTCGCTCTG GTCATGACCATGTTTTTCCAATTTCTTATCCCTAGAATGTCTTGTT TCCAAGCAACCTTTGCAACATTTTACATATGTTTACTTGAAACTGA ACACAATAAAATCATAGAAAGTTAAAAAAAAAAAAAAAAAAAAAA AAA

A short list of potent siRNAs was created based on the best siRNAs predicted by the algorithms. Each algorithm ranked the siRNAs using various ranking systems including a numerical and a star system. The potential short-listed siRNAs were tested using NCBI's program BLAST (Basic Local Alignment Search Tool) to ensure specificity that they were only complementary to their target mRNAs to avoid probable off-targets.

Subsequently, the potential siRNA sequences were checked for immunostimulatory and cytotoxicity effects. For instance, a 4 base-pair motif "UGGC" in the RISC-loading strand of the siRNA is known to cause immunostimulatory effects[26]; whereas, the 5'-GUCCUUCAA-3' motif in either strand has been found to cause cellular toxicity.[48]Moreover, siRNA containing 5'-UGUGU-3' motif should be avoided for not to activate innate immune response as identified by MacLachlan et al. (2005).[49]

Afterwards, siRNA candidates were eliminated by assigning scores in accordance with the general criteria regarding functionality of siRNA proposed by Reynolds et al.(2004) as described in Table 2.1. In addition, the point-specific preferences shown in Table 1.1 were taken into consideration since they have significant importance of being experimentally validated.

As a result, three candidate siRNAs targeting the CXCL7 gene were designed by using the shortlist created by siDESIGN Center (Dharmacon Research, Inc., Lafayette, CO, http://www.dharmacon.com/sidesign/).

2.1.2 The sequences of selected siRNA candidates

In order to achieve silencing of CXCL7 gene, three candidate siRNAs were selected within the mRNA target. Two siRNAs were selected among the Open Reading Frame (ORF) region and one siRNA candidate was selected from the 3'-UTR (Untranslated Region) of the mRNA of CXCL7 gene.

The selected siRNA candidates were named as si236, si391 and siUTR919, indicating the positions where they target in the mRNA sequence based on the first paired nucleotide as pointed out in Table 2.3.

The sequences of siRNAs against CXCL7 gene were highlighted (in green) in Table 2.3 which indicates which positions exactly were targeted against its corresponding mRNA.

Table 2.3 The targeting positions of selected siRNAs in the mRNA sequence of CXCL7.

```
   1 AACATAGCAGCAGTGTTCTGGGCAGCTCAGCCTCACGTTGTTCCCTCCTGGCTCTGGGGA

  61 CAGTTCTAGCTCACTGCTCTTCATTATGGGCTTCAGACTCAGACCTACATCGTCCTGCAC

 121 CAGGGCCTGCCCACTTCATAACCTCCAGATCTTGCTGCTGCTGGGCCTGATCCTTGTTGC

 181 GCTGGCTCCCCTTACAGCTGGAAAATCTGATGGCATGGACCCATATATCGAACTGCGCTG

 241 CAGATGTACGAATACCATCTCTGGAATCCCATTCAATTCTATCTCCCTTGTGAATGTGTA
          <si236>
 301 CAGGCCAGGAGTTCACTGTGCTGATGTGGAAGTGATAGCCACACTGAAGAATGGACAAAA

 361 AACGTGCCTGGACCCAAATGCCCCTGGCGTCAAGAGAATCGTCATGAAAATCTTGGAAGG
                                    <si391>
 421 TTACTGACCAGCTGCTTCATCTGTGCCAAACCATTTTCCTGCTCAGGAAGGGTTGAGGGT

 481 TTGAAATCTGGACTTGGTAGAATTATTTTTCCACTTTGAAATTGGGATGTTCTCTCGTTC

 541 TGTCTCAGAAATCACACTGTATTCTGAGAAGGCATGATAAAGAATGTCACAACAAACAAA

 601 CAAACAATGATGAAGACGGGCAAGCCAAGTTGCAACCTGCAATTTATGGTCTATTTCTGC

 661 AGTTCTTGGCTAAGCATTCTGACATGTCCTGCATTTCTCTATTTGAGCTTTTCTTTTCAA

 721 ATGTACTATGTCAAGTTTTCATTCTGTGTTTTTAATAATTATTTCTTTTAGTTTTGAGAT

 781 TATAATTACATCCCTTCCCCTTTCATTTTTCTCCTTCTGAACCCTTCTATATACCCATCC

 841 TTGCTCTCTTCAAATCCATGGCTGCTTTTTTTTCCCATTGAGCATTGTTATATACATATA

 901 TAATGATAAATTTCGTAGTGGTCAATTGCAACGGAAATCGCCTGGAGTCAAGCAGTTTTC
                    <siUTR919>
 961 GCTCTGGTCATGACCATGTTTTTCCAATTTCTTATCCCTAGAATGTCTTGTTTCCAAGCA

1021 ACCTTTGCAACATTTTACATATGTTTACTTGAAACTGAACACAATAAAATCATAGAAAGT

1081 TAAAAAAAAAAAAAAAAAAAAAAAAA
```

The sequences of sense strands of each candidate siRNA were scored based on the characteristics of each one, according to the design criteria in Table 2.1 and both the sequences and their scoring are demonstrated in Table 2.4.

Table 2.4 The sequences of three candidate siRNAs against CXCL7 and the scoring.

siRNA	Sense Strand Sequence	Score*
si236	5'-CGCUGCAGAUGUACGAAUA-3'	8
si391	5'-CAAGAGAAUCGUCAUGAAA-3'	8
siUTR919	5'-UCGUAGUGGUCAAUUGCAA-3'	7

* The scores higher than 6 are expected to be efficient as stated by Reynolds et al.(2004).[17]

To confirm the silencing by siRNA treatment, a positive control and a negative control were included in an experimental set.

The sequence of the positive control siRNA duplex was proven to be efficient and used as a control by various studies.[50,51,52] The sequence of siRNA targeting eGFP was also confirmed by checking its full complementarity to eGFP sequence of pIRES2-eGFP vector using nucleotide BLAST alignment tool (blastn).

Negative control siRNA was designed by scrambling the sequence of one of the siRNA candidates. It has exact amount of bases with the siRNA but differed in the order of the nucleotides. Nucleotide BLAST alignment (blastn) was conducted for the negative control siRNA sequence in comparison with any sequence covering human and mouse genomes.

The positive and negative controls were named as siGFP and siNEG correspondingly. The sequences of siRNA duplexes, including the controls, are presented in Table 2.5.

Table 2.5 The sequences of sense strands of three siRNA candidates targeting CXCL7 gene and control siRNAs.

siRNAs	Sequence
ORF region-position 236 (CXCL7)	5'-CGCUGCAGAUGUACGAAUA*dTdT*-3' 3'-*dTdT*GCGACGUCUACAUGCUUAU-5'
ORF region-position 391 (CXCL7)	5'-CAAGAGAAUCGUCAUGAAAd*TdT*-3' 3'-*dTdT*GUUCUCUUAGCAGUACUUU-5'
3'UTR region- position 919 (CXCL7)	5'-UCGUAGUGGUCAAUUGCAA*dTdT*-3' 3'-*dTdT*AGCAUCACCAGUUAACGUU-5'
Positive control siRNA (eGFP)	5'-CAAGCUGACCCUGAAGUUC*dTdT*-3' 3'-*dTdT*GUUCGACUGGGACUUCAAG-5'
Negative control siRNA (non-targeting any gene in human, mouse, rat.)	5'-AUAAUACCGGUCGAAGGCU*dTdT*-3' 3'-*dTdT*UAUUAUGGCCAGCUUCCGA-5'

All siRNAs used in this thesis were chemically synthesized by Dharmacon Inc.(Lafayette, CO) and received as desalted, pre-annealed duplexes in standard format. Each siRNA including control ones were suspended in RNase-free water to give 100 μM stock solutions of each siRNA duplex. For cell culture experiments, aliquots of 10 μM siRNA duplexes were prepared using main stock solution(100 μM) to avoid repeated freeze-thaw cycles.

2.2 Cell culture

Lewis Lung Carcinoma, abbreviated as LLC, is a mouse cell line which is widely used as a model for metastases and suitable for studying the functions of therapeutic agents in cancer.

To examine the silencing ability of siRNAs targeting CXCL7 gene, we used a mouse cell line stably expressed pIRES2-CXCL7-eGFP plasmid, which was constructed by Unver[53] at Hacettepe University, Basic Oncology. This cell line was a gift from colleagues studying over-expression of CXCL7 gene in lung cancer. They used a mammalian expression vector, pIRES2-eGFP (BD Biosciences Clontech) which is designed for the efficient selection by

Flow-Cytometry of transfected mammalian cells expressing eGFP and the protein of interest, to construct the CXCL7 gene. pIRES2-eGFP vector contains a multiple cloning site (MCS) for construction of the gene of interest, internal ribosome entry site (IRES) and enhanced green fluorescent protein (eGFP) within the sequence (Figure 2.1). IRES region permits both the gene of interest and the eGFP gene to be translated from a single bicistronic mRNA and enables the utilization of eGFP as a reporter of gene expression. The insert DNA for coding mouse CXCL7 gene was cloned into MCS and the resulting plasmid was transfected into LLC cells.

In this study, we named this modified LLC cell line as pIRES2-CXCL7-eGFP/LLC cells, since they had been selected to stably express pIRES2-CXCL7-eGFP vector.

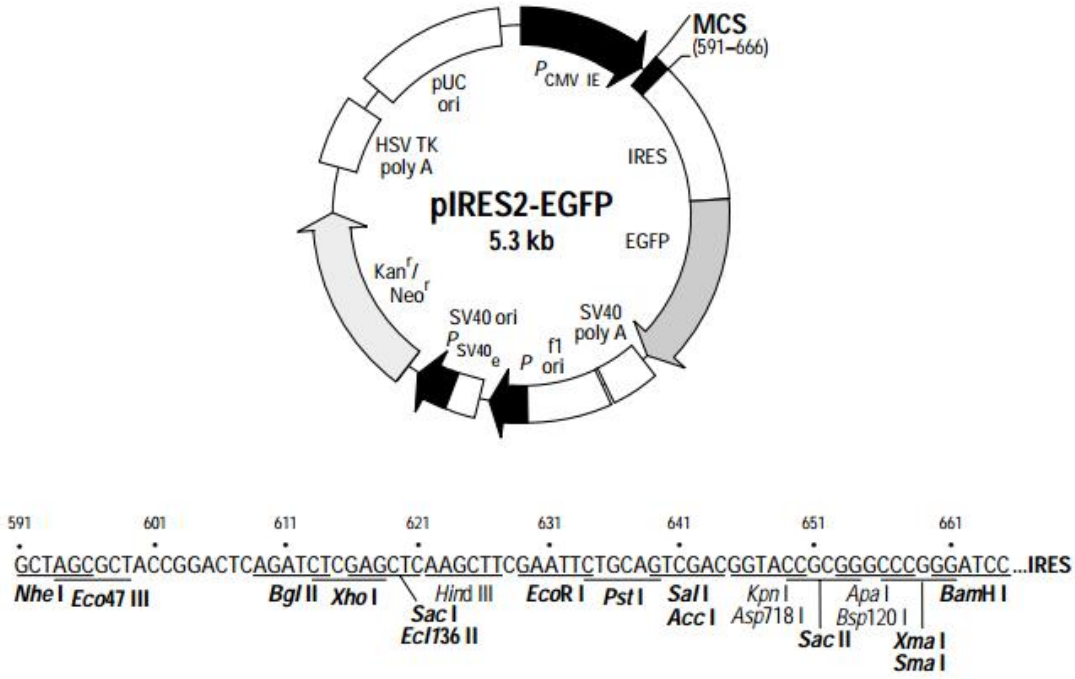


Figure 2.1 The restriction map and multiple cloning site of pIRES2-eGFP vector.

2.2.1 Cell passage and maintenance

Mammalian cells are prone to gradually change in culture conditions. It is difficult to consistently maintain them in perfect health. Cell passage is the technique to keep cells

healthy by exchanging the growth medium by transferring a known amount of cells to a new sterile culture flask for growth in a CO_2 incubator.

In this study, LLC cell cultures were maintained in full RPMI medium (RPMI medium supplemented with 2mM L-Glutamine and containing 1.0% Penicillin/Streptomycin and 10.0 % Fetal Bovine Serum (FBS)) in a humidified atmosphere consisting of 5% CO_2 and 95% airat 37ºC. ThepIRES2-CXCL7-eGFP/LLC cells were cultured in full RPMI medium containing neomycin as a selective antibiotic in a 450 µg/mL concentration.

Cell passages were performed twice a week by changing the growth medium in sterile T_{25} cell culture flasks. After aspiration of the old medium, 5 mL of full RPMI medium was added into the cells in each passage. The detachment of the cells from the cell culture flask was carried out by tapping the flask against the palm of the hand.

2.2.2 Cell counting by hemacytometer

A hemacytometer is used for counting the cells. Fuchs-Rosenthal hemacytometer was used to count LLC cells for determining cell concentration. It is laser-etched to have grids consisting of 16 one square millimeter areas orientated by triple lines, and each such area subdivided into 16 squares as represented in Figure 2.2. A cover slip is placed over the counting surface and the cell suspensions are filled in the hemacytometer by capillary action.

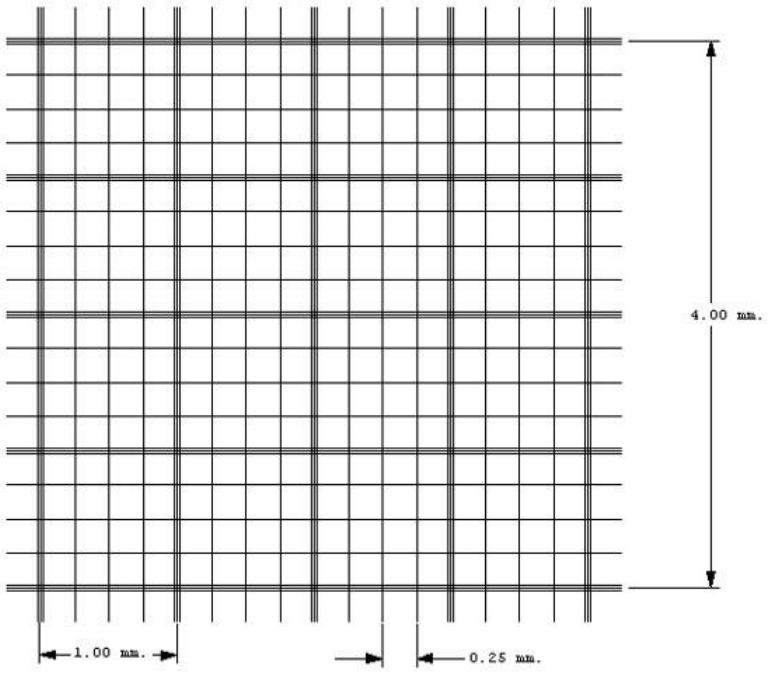


Figure 2.2 The Fuchs-Rosenthal Ruling.

When the culture flask containing modified LLC cells reached a confluency of about 75-80%, the flask was taken from the CO_2 incubator and put into a sterile cell culture hood. First, the supernatant was removed without disrupting the cells with a 5 mL sterile tube. Then, the fresh full RPMI medium was added into the flask. After the cells were detached from the surface of the culture flask by tapping, the cells were carefully suspended by pipetting with a sterile pasteur pipette to make them uniformly distributed to count the cells easily. For cell counting, 10 µL of cells were mixed with 10 µL of 0.4% tryphan blue solution and loaded into the chamber.

The tryphan blue staining is also a common method for identifying dead cells. Only the intact live cells resist penetration of dye across the membrane, so dead cells with compromised membranes become stained and blue.

The live cells were counted under a phase contrast microscope (Olympus) and the concentrations of the cells were calculated as in the followings;

Cells per mL = Average count per square x dilution factor x 5,000, where the dilution factor is ½.

The appropriate number of cells were transferred into a sterile tube and washed with full RPMI without neomycin by centrifugation to get rid of the presence of antibiotic during transfections. After washing, the cells were resuspended in full RPMI medium and distributed into the wells of a cell culture multi-well plate.

2.3 siRNA delivery by lipofection

An important factor for efficient gene silencing by utilizing RNAi is the type of delivery. A popular method for efficient siRNA delivery is the use of cationic lipids, which possess a positively charged head and a hydrophobic tail. When combined with nucleic acids, the cationic heads of the lipid interact with the phosphate backbone, which result in formation of lipoplexes (siRNA-lipid complexes). These complexes are then easily transported by endocytosis through the negatively charged cell surface.[54]

Efficient gene silencing in siRNA-mediated RNA interference (RNAi) experiments requires proper uptake of siRNA into the cells of interest. For in vitro experiments, transfection is an easy and rapid method of siRNA delivery.

In this study, Lipofectamine RNAiMax reagent (Life Technologies, Inc., Cat No: 13778-100) was used for siRNA transfection. It is a proprietary transfection agent that is particularly improved for siRNA applications with high efficiency and minimal cytotoxicity in many cell types by Invitrogen Life Science Technologies.

Also, Opti-MEM I Reduced serum medium (Life Technologies, Inc., Cat no: 31985-062) was utilized for the dilutions of RNAi duplexes and Lipofectamine RNAiMax reagent before generating siRNA-lipid complexes, as recommended by the provider of the transfection agent, Invitrogen Life Science Technologies.

2.3.1 Conventional transfection method

This method was classified as conventional because it requires seeding the plates one day before transfection. Particularly in siRNA transfections, the cells are recommended to be seeded in a concentration that each well has a confluency of 30-40% the next day. The below picture was taken one day after seeding the CXCL7-expressing LLC cells onto multi-well plates and the confluency can be seen to set between 30-40% (Figure 2.3).

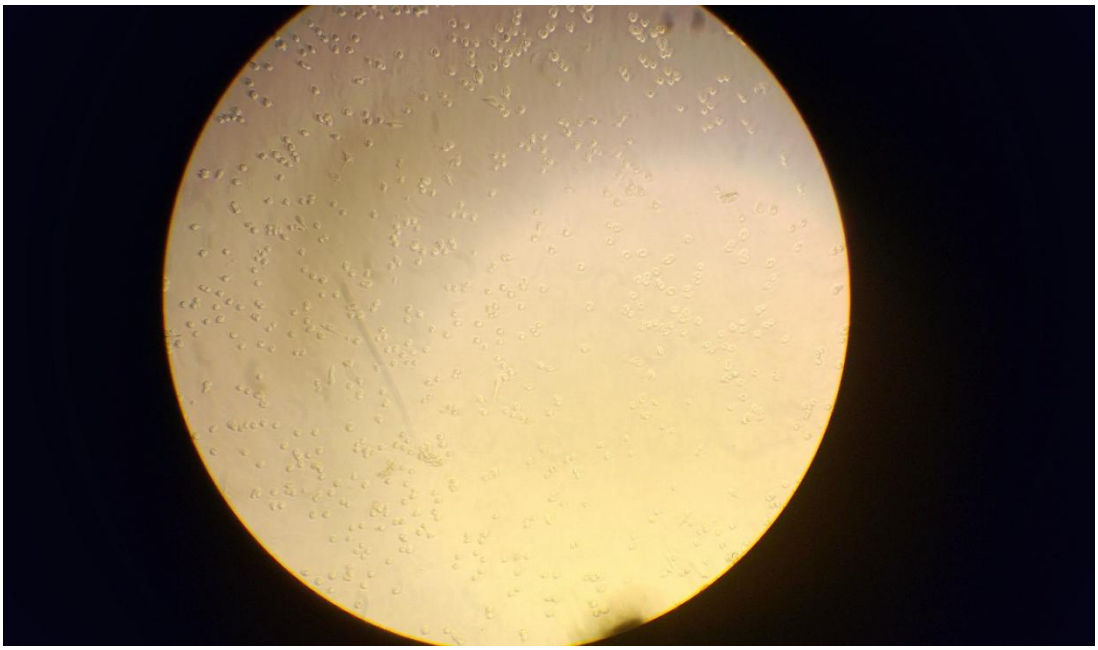


Figure 2.3 The representative picture of LLC cells (taken by an inverted microscope (Olympus, CH40)).

An experimental set for analyzing the silencing of CXCL7 gene was illustrated in Table2.6 in which conventional transfections were carried out in 48-well cell culture plates. Each set contains a positive control known to provide high knockdown of its target gene (eGFP) and a negative control to assess that the silencing of the gene is sequence-specific. A mock-

transfection (reagent control) was performed in each experimental set which contained no siRNA. This control was included to determine any non-specific effects that may be caused by the transfection mix or the process.

Table 2.6 The experimental set-up for silencing of CXCL7 by siRNA transfection.

Experimental Set	siRNA (10μM stock)	Lipofectamine RNAiMAX
Untransfected	None	None
siGFP (Positive control)	7.5 μL	0.75 μL
siNEG (Negative control)	7.5 μL	0.75 μL
si236	7.5 μL	0.75 μL
si391	7.5 μL	0.75 μL
siUTR919	7.5 μL	0.75 μL
Mock (Reagent Control)	None	0.75 μL

Each well was transfected with 0.75 μL Lipofectamine RNAimax reagent and 7.5 pmol of siRNA (25nM final concentration).

Master mix for lipofectamine dilution:

For dilution of transfection reagent, a master mix was prepared for an experimental set of 6 reactions in a single tube. 194 μL of Opti-MEM medium was added into an RNAse-free microfuge tube and 6.0 μL of Lipofectamine RNAimax reagent was introduced by pipetting gently and waited for 5 min.

Dilution of siRNA:

Opti-MEM medium of 48.5 μL was mixed gently with 1.5 μL of each siRNA (10 μM), half of which was mixed with 25 μL of lipofectamine master mix and incubated for 10 min at room temperature.

Each siRNA-reagent complex (50 μL) was added drop-wise onto wells containing 250 μL volume of cells (2.0 x 10^4 cells/well) in a 48-well plate. The plate was observed on an inverted microscope (Olympus, CH40) to confirm that the cells were healthy and then, incubated for 48h in a CO_2 incubator for further being analyzed by Flow-Cytometry.

The exact protocol was performed in triplicates at separate times.

2.3.2 Reverse transfection method

In conventional transfection, siRNA and lipid-based transfection reagent are complexed first and then added onto pre-plated cells. However, in reverse transfection, all three components including the cells are added into the wells at exactly the same time.

Each siRNA and Lipofectamine RNAimax reagent was diluted in 50 μL of Opti-MEM medium separately. They were mixed gently inside each well and allowed to form siRNA-reagent complexes for 10 min.

Meanwhile, the cells were counted by using a hemacytometer to adjust the concentration as 5.0×10^4 cells/well. Each well containing the siRNA-reagent complexes (100 μL) including a mock control was filled with 500 μL of cells in a 24-well plate. The final concentration of siRNA was 25 nM. The cells were allowed to incubate at 37ºC for 48h in a CO_2 incubator.

This method was also performed in triplicates at separate times.

2.4 Flow-Cytometry

Flow-Cytometry enables to isolate populations of cells that express fluorescent proteins by using laser technology. In this study, we utilized it for the determination of the level of eGFP expression.

Since flow cytometry operates at the single-cell level, the efficacy of siRNA-mediated silencing can be determined independently from variabilities in transfection efficiency.

The use of bicistronic vectors enables to measure the knockdown of a target gene by using flow cytometry.[52] Since the siRNA will degrade the entire mRNA including the target gene and eGFP, the level of eGFP expression is expected to reflect the efficiency of siRNA and the inhibition of the gene of interest.

Flow-Cytometry (FACSAria II, BD Biosciences) was used to analyze the cells after siRNA transfections and FACsDiva software 6.0 was used to interpret the flow cytometric data of the silencing experiments by using the instrument parameters employed by Unver[53].

2.4.1 The working principle of Flow-Cytometry

Flow cytometers works with a suspension of single, unclumped cells. The cells in suspension are introduced to the system via a sample probe. After the sample is dispensed into a cuvette flow cell, the isotonic sheath fluid creates the fluid stream by hydrodynamically focusing the cells so that they can pass through one cell at a time.

As each single cell exits the flow cell, it becomes exposed to laser beams. While the cell is in contact with the laser, intrinsic and extrinsic features are recorded through a series of dichroic mirrors and photo multiplier tubes. Forward scatter of light determines cell size while side scatter of light determines granularity. Finally, the data are converted to an electronic signal, which can be analyzed for multiple purposes by the software of the system.[55]

2.4.2 The enhanced Green Fluorescent Protein (eGFP)

The accessibility of fluorescent proteins provides the opportunity for real-time determination of gene expression and protein localization.

The green fluorescent protein (GFP) extracted from the jellyfish *Aequorea victoria* has been widely utilized as an important marker for gene expression. However, Zhang and his colleagues constructed a GFP variant which makes the protein 35 times brighter and optimized for higher expressions in mammalian cells.[56] Since then, eGFP has been used as a reporter gene especially in mammalian applications.

In this work, the levels of eGFP expression were measured by Flow-Cytometry and employed as a marker for the level of CXCL7 expression.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 The determination of siRNA silencing analyzed by Flow-Cytometry

The levels of eGFP expression of pIRES2-CXCL7-eGFP/LLC cells after siRNA transfections were normalized to the level of LLC cells to avoid the background noise raised by self-autofluorescence of non-recombinant cells. Figure 3.1 represents the autofluorescence of LLC cells and for further analysis, the P3 area is focused to measure the level of eGFP expression of recombinant cells.

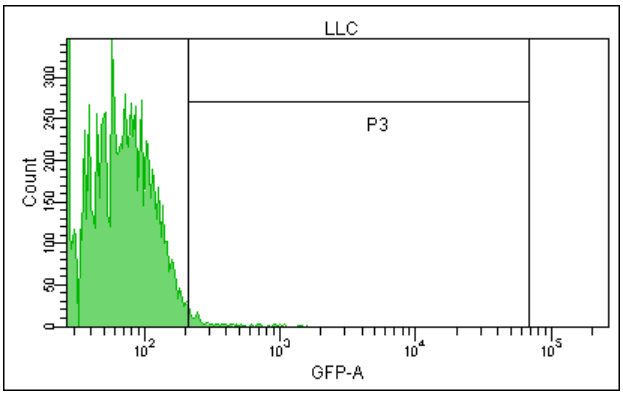
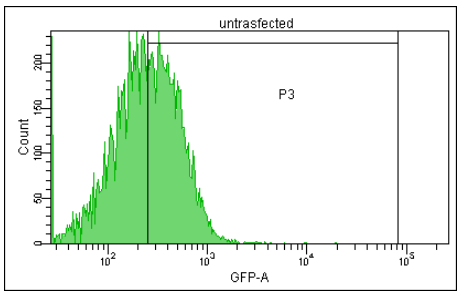


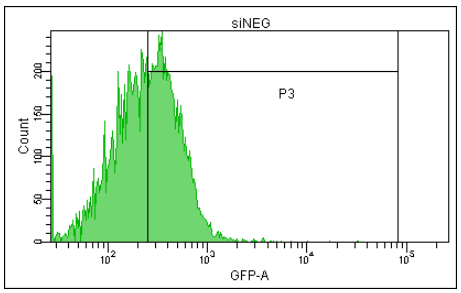
Figure 3.1 The eGFP expression of LLC cells by Flow-Cytometry.

3.2 The optimization of siRNA concentration on positive control siRNA

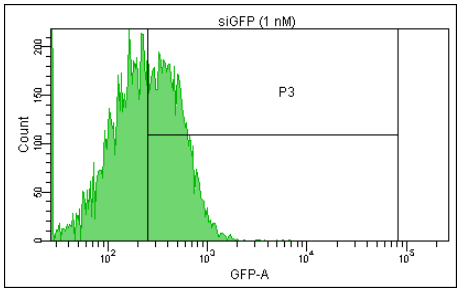
Positive control siRNA, namely siGFP, was used for transfection to optimize the concentration of siRNAs. pIRES2-CXCL7-eGFP/LLC cells were transfected with siGFP at various concentrations. Figure 3.2 shows the levels of eGFP expressions after siGFP transfection and it can be observed that 25 nM is the optimum concentration of siRNA for transfection of recombinant LLC cells. The untransfected cells were used as a reference point and the negative control siRNA did not result in a decrease in the fluorescence level in the eGFP expressing cells as expected.

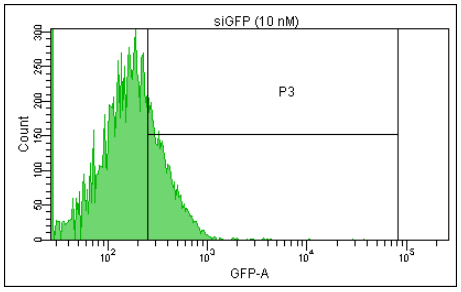
a.

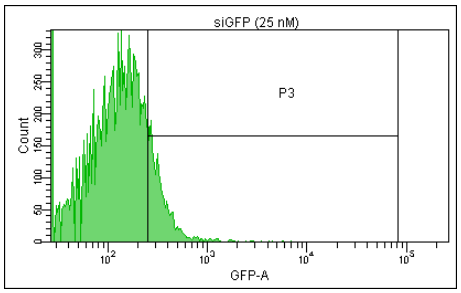




b.







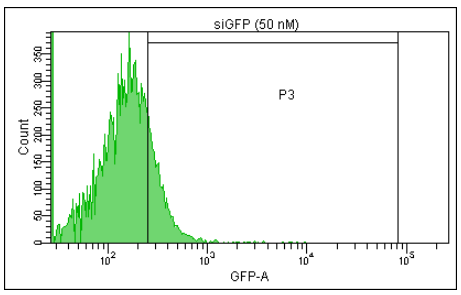


Figure 3.2 The expressions of eGFP of pIRES2-CXCL7-eGFP/LLC cells after siRNA transfections; a) untransfected cells and negative control siRNA-transfected cells b) transfected with siGFP at concentrations of 1 nM, 10 nM, 25 nM and 50 nM.

3.3 The determination of CXCL7 silencing after siRNA transfections

3.3.1 The results of conventional transfection method

Figure 3.3 gathers all the data of an experimental set of siRNA transfection by conventional method as the expression level of eGFP. The results of the other two experimental sets are presented in Appendix A.

Control samples for detecting, the transfection agent alone (mock control) or a nonspecific siRNA (negative control) demonstrated the same fluorescence profile with the untransfected cells allowing comparison of the silencing levels.

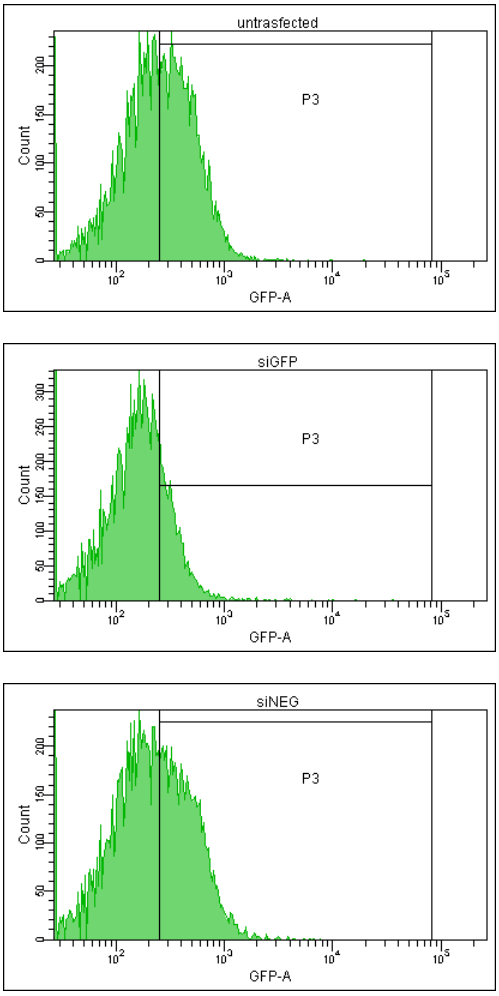


Figure 3.3 The results of an experimental set as the level of eGFP expression after conventional siRNA transfections.

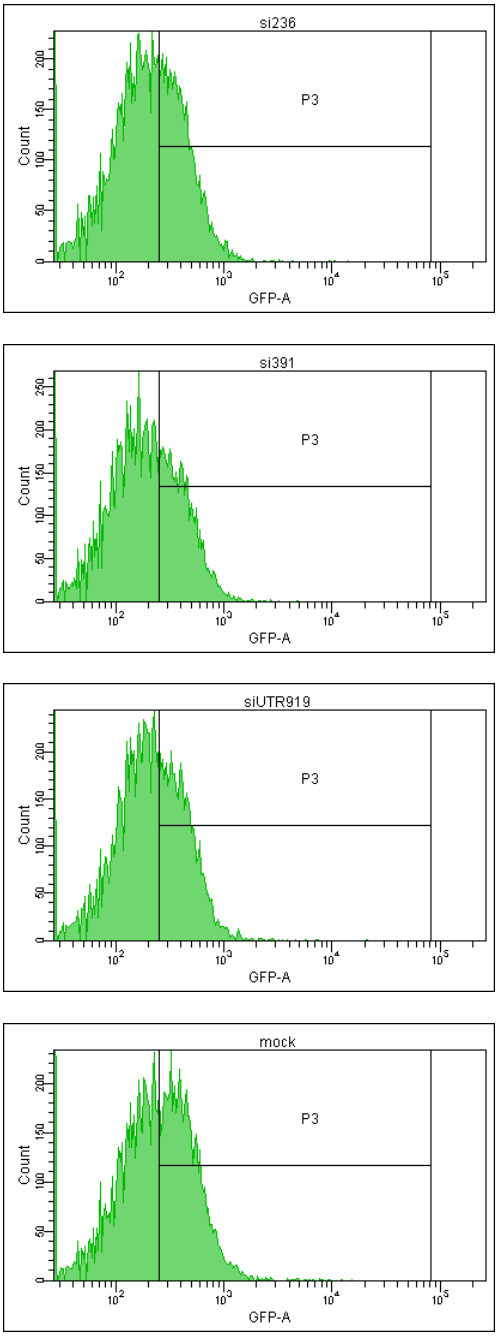


Figure 3.3 (continued)

The flow cytometric results demontrated that positive control siRNA (siGFP) decreased the level of eGFP by 54% (mean value) compared to untransfected cells, whereas si236 and si391 gave significant reductionsof 20% and 27% correspondingly (Figure 3.4).

Samples	**% Silencing**				
	Set 1	Set 2	Set 3	Average	Std-Dev
Untransfected	-	-	-	-	-
siGFP	55.29	55.34	51.42	54.01667	2.248918
siNEG	-0.4	-3.56	2.4	-0.52	2.981812
si236	20.36	18.45	20.71	19.84	1.216429
si391	27.94	20.06	28.93	25.64333	4.86058
siUTR919	17.76	6.8	15	13.18667	5.700573
mock	3.39	0	-2.14	0.416667	2.788446

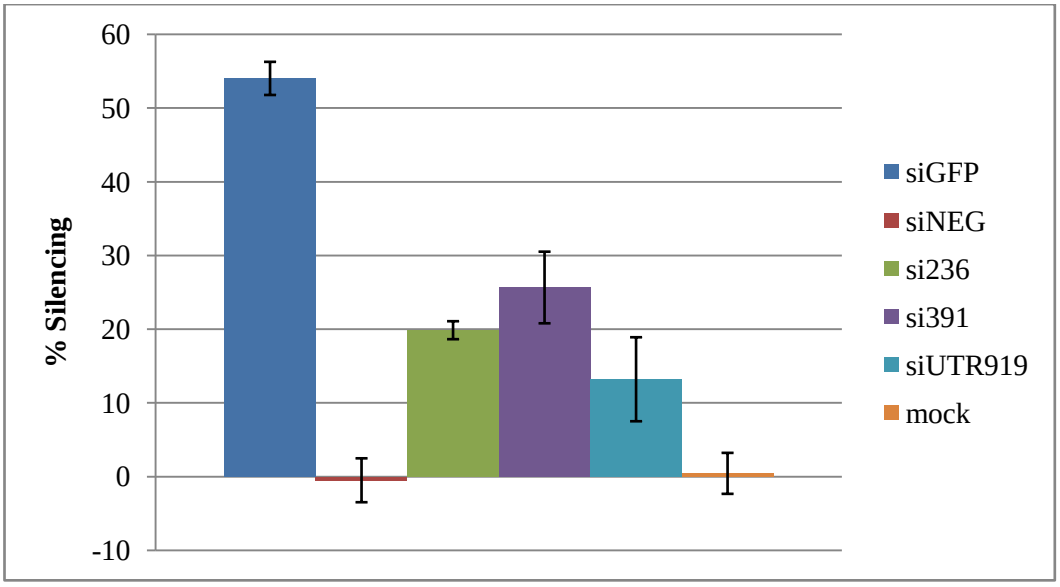


Figure 3.4 The percentages of relative silencing of siRNAs and controls compared to untransfected cells based on the data of three different experimental sets by conventional transfection method. The % silencing values are presented above the figure.

3.3.2 The results of reverse transfection method

All the measurements of eGFP expression as a representative experimental set of three repeats of siRNA transfections by reverse transfection method are represented in Figure 3.5.

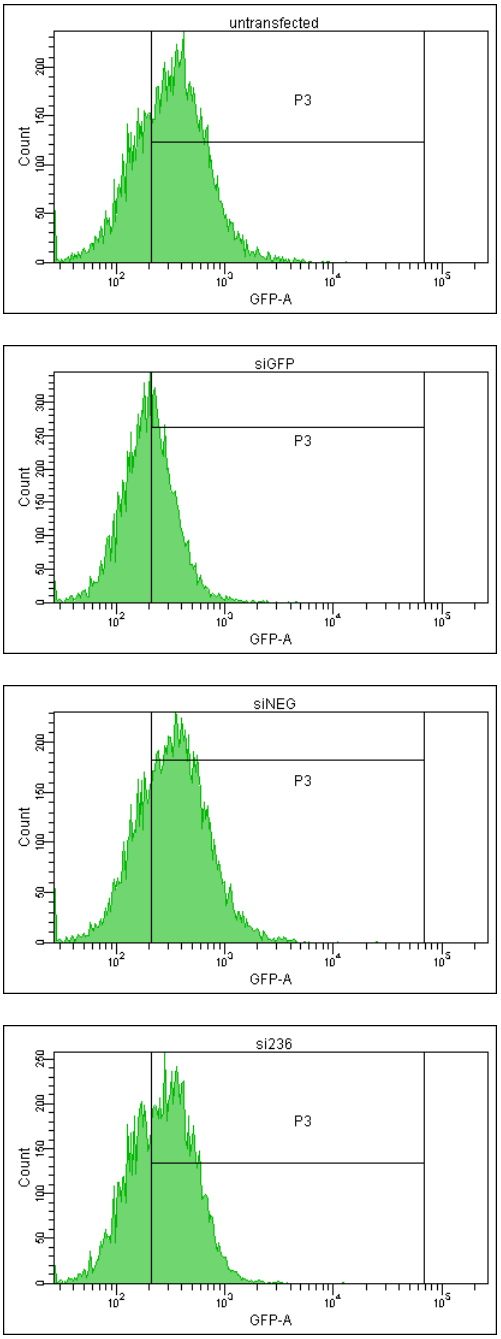


Figure 3.5 The results of an experimental set as the level of eGFP expression after siRNA reverse transfections.

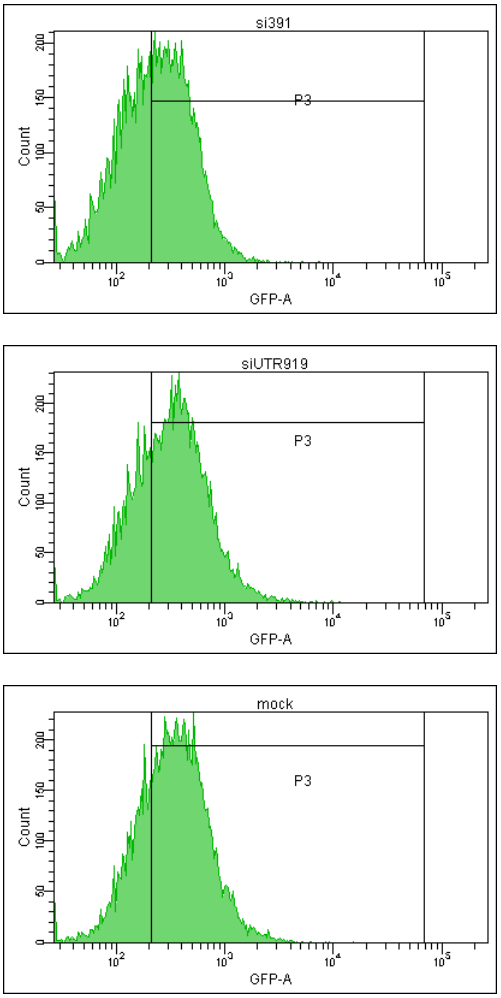


Figure 3.5 (continued)

The percentages of relative silencing induced by each transfection were calculated by comparing them to the result of untransfected cell population as shown in Figure 3.6. The negative and mock control results did not induce any silencing effects as expected.

The positive control siRNA decreased the eGFP expression by 36% (mean value) while siRNA candidates for targeting CXCL7 achieved 11-17% decrease in eGFP level compared to untransfected cells.

The experimental sets of reverse transfections are included in Appendix B.

Samples	% Silencing				
	Set 1	Set 2	Set 1	Average	Std-De
Untransfected	-	-	-	-	-
siGFP	35.05	39.79	32.03	35.62333	3.911641
siNEG	-3.29	-0.14	3.92	0.163333	3.614559
si236	9.44	11.18	11.44	10.68667	1.087443
si391	20.17	12.52	17.65	16.78	3.8985
siUTR919	1.43	0.6	5.55	2.526667	2.650968
mock	-4.29	-1.19	-3.59	-3.02333	1.625833

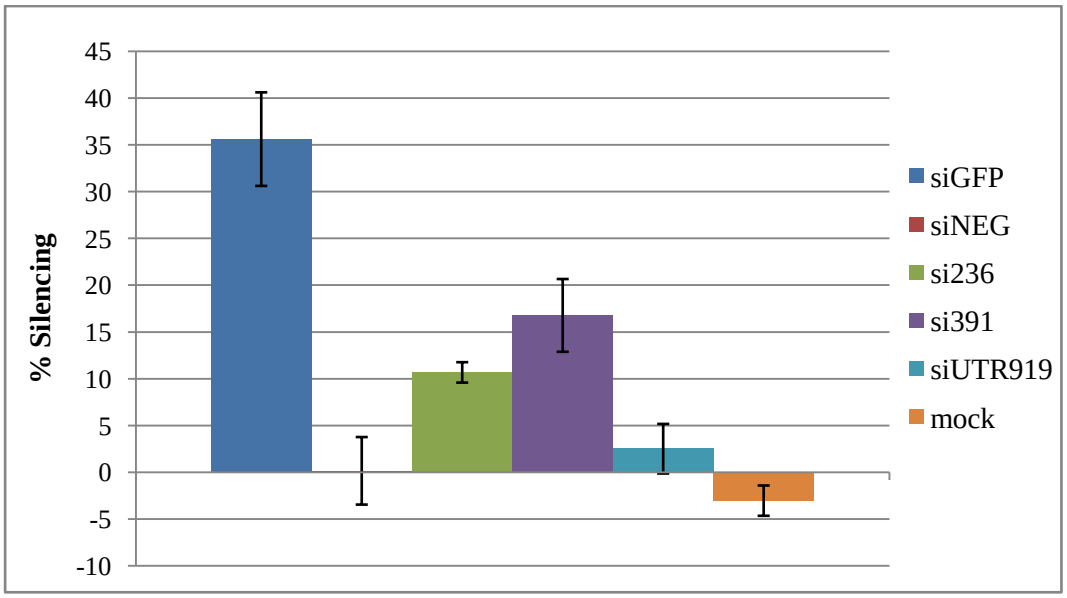


Figure 3.6 The percentages of relative silencing of siRNAs and controls compared to untransfected cells based on the data of three different experimental sets by reverse transfection method. The values are presented above the figure.

The effect of the transfection reagent which can be observed in the result of mock transfection in Figure 3.6 can be interpreted as a false-negative contribution to the results of siRNA transfections against CXCL gene by reverse transfection method.

3.3 The effect of transfection on cell granularity and viability

The changes in cell size, granularity and viability were recorded by FACsDiva software. Side scatter (SSC) parameter is the record of the amount of light that scatters perpendicular to the laser beam within a cell population, whereas Forward scatter (FSC) parameter is the

record of the amount of the light that scatters axial to the laser beam within a cell population.[55] P2 area encompasses the viable cells that are in the range of SSC (vertical axis which indicates the granularity of the cells) and FSC (horizontal axis which indicates the size of the cells) values in the defined area (Figure 3.7). As shown in Figure 3.7, neither the siRNA nor the transfection reagent alone (mock-transfected) did affect the cell granularity and size of the treated populations compared to untransfected population.

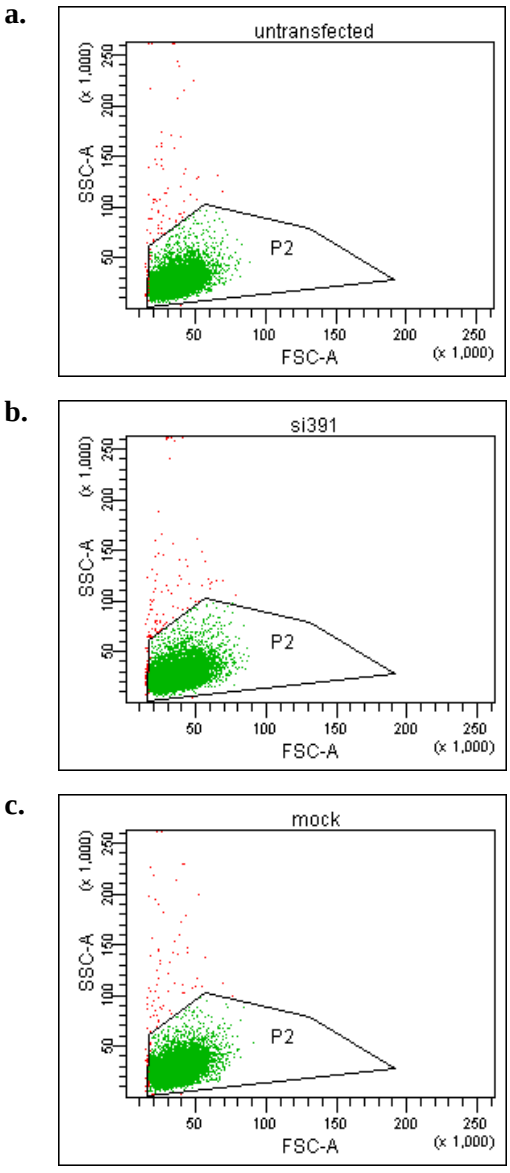


Figure 3.7 Forward scatter and side scatter properties of the cells; a) untransfected, b) siRNA-transfected, c) mock-control transfected.

3.4 The importance of targeting region of the mRNA and experimental validation of functional siRNAs

Designing of three siRNAs targeting the mRNA of CXCL7 gene, one of the candidates was selected from the 3'UTR region and two of them were complementary to ORF region. The flow cytometry results indicated that siRNAs targeting ORF region (si236 and si391) significantly inhibited the expression of CXCL7 while siRNA targeting 3'UTR region (siUTR919) was not effective.

However, siRNA assigned with the highest score by bioinformatic tools (si236) was not as efficient as si391. Both siRNAs (si236 and si391) were assigned with the same score according to general criteria established in the literature (Table 2.1) which si391 showed relatively better silencing experimentally.

Experimental confirmation of the siRNAs were valuable to not fully relying on package programs for siRNA design since as highlighted in the Table 2.1, Reynolds' parameters are proven to be extremely important and they should always be considered and included for designing efficient siRNAs.

Furthermore, a pool of si236 and si391 (named as siORFs) were transfected to recombinant LLC cells in parallel to experimental sets of conventional transfections and resulted in a silencing level in between the levels of the two siRNAs as shown in Figure 3.8. Unlike the study of Shim et al. (2005), a combination of two potent siRNAs did not increase the level of silencing higher than each siRNA alone.

The experimental sets of conventional transfections, including siORFs, are included in Appendix A.

Samples	% Silencing				
	Set 1	Set 2	Set 3	Average	Std-Dev
Untransfected	-	-	-	-	-
si236	20.36	18.45	20.71	19.84	1.216429
siORFs	25.75	19.09	19.64	21.49333	3.696625
si391	27.94	20.06	28.93	25.64333	4.86058

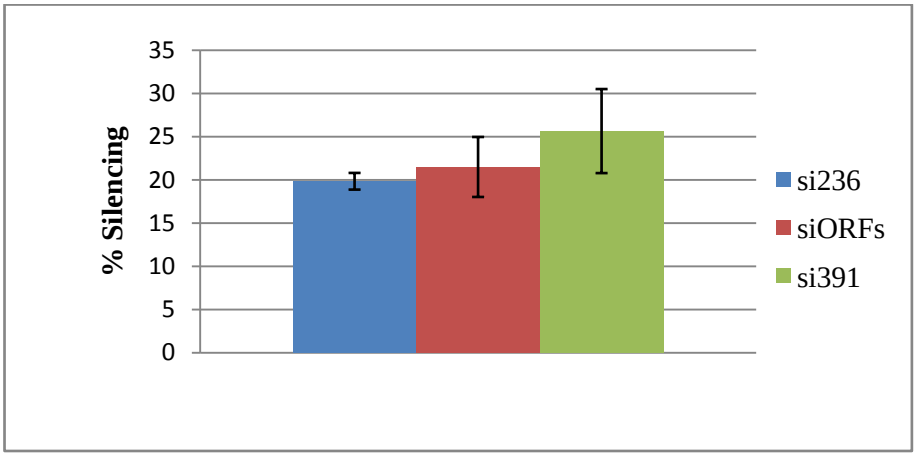


Figure 3.8 The comparison of the effectiveness of siRNAs, si236 and si391, and siORFs as the pool of the two siRNAs. The values are presented above the figure.

3.5 The comparison of transfection methods on silencing of CXCL7

In siRNA applications, the choice of the transfection method depends on the nature of the cell. We applied both conventional transfection and reverse-transfection methods in modified LLC cells overexpressing CXCL7 gene.

In this study, the conventional transfection method gave better results of silencing compared to reverse transfection method performed in CXCL7 expressing LLC cells.

However, the half-life of wild-type GFP in mammalian cells is about 26h[57], which means the fluorescence of the protein can be observed within the cells even if the mRNA was degraded. In this study, we utilized eGFP protein which is a GFP variant with higher fluorescence and stability.Since we analyzed for the fluorescence intensities of the cell populations at 48h after transfections, the long half-life of eGFP might have affected the results of silencing levels; showing lower silencing than that of the real potency.

Also, the high passage number of the recombinant cells used in this study may have negatively influenced the transfection efficiency of siRNAs.

CHAPTER 4

CONCLUSION

In this study, siRNA sequences were designed against CXCL7 gene as a potential target for understanding how the tumorigenesis will be affected in the absence of its corresponding protein. Various bioinformatics tools which have been specifically designed for selection of potent siRNAs were utilized. Three candidate siRNAs were chosen to be assessed with experimental validation.

A modified mouse cell line, abbreviated as pIRES2-CXCL7-eGFP/LLC, was used for silencing experiments. LLC cell line was also cultured to avoid accounting for the autofluorescence coming from the fluorescent molecules already present in the cells. All siRNAs were transfected into the cells by using a conventional cationic lipid formulation which enables the uptake of siRNAs by endocytosis through negatively charged cell surface.

The silencing levels were measured by Flow Cytometry as the level of eGFP expression. We observed a significant reduction in the level of eGFP expression as a reporter of CXCL7 silencing when treated with targeting siRNAs. Our results suggest that two of three siRNA sequences, si391 and si236, can be employed as potent inducers of CXCL7 silencing to study the functionality of this chemokine in cancer cells.

The transfection of the LLC cells overexpressing CXCL7 gene with a pool of si236 and si391 showed a reduction value in between the values of the transfections of each siRNA alone.

In conclusion, efficient siRNAs targeting CXCL7 chemokine have been successfully delivered into LLC cells and silenced the level of expression in a sequence specific manner.

Further studies are needed to confirm and examine the phenotypic effects resulted by the silencing of this chemokine and to determine whether siRNAs designed in this study may serve as a therapeutic agent for cancer treatment.

REFERENCES

(1) Sharp, P. A. *Genes Dev.* **2001**, *15*, 485–490.

(2) Hannon, G. J. *Nature* **2002**, *418*, 244–251.

(3) Fire, A.; Xu, S.; Montgomery, M. K.; Kostas, S. A.; Driver, S. E.; Mello, C. *Nature* **1998**, *391*, 806–811.

(4) Hammond, S. M.; Bernstein, E.; Beach, D.; Hannon, G. J. *Nature* **2000**, *404*, 293–296.

(5) Elbashir, S. M.; Harborth, J.; Lendeckel, W.; Yalcin, A.; Weber, K.; Tuschl, T. *Nature* **2001**, *411*, 494–498.

(6) Castanotto, D.; Rossi, J. J. *Nature* **2009**, *457*, 426–433.

(7) Bernstein, E.; Caudy, A. A.; Hammond, S. M.; Hannon, G. J. *Nature* **2001**, *409*, 363–366.

(8) Zamore, P. D.; Tuschl, T.; Sharp, P. A.; Bartel, D. P. *Cell* **2000**, *101*, 25–33.

(9) Khvorova, A.; Reynolds, A.; Jayasena, S. D. *Cell* **2003**, *115*, 209–216.

(10) Schwarz, D. S.; Du, T.; Xu, Z.; Aronin, N.; Zamore, P. D. *Cell* **2003**, *115*, 199–208.

(11) Tomari, Y.; Matranga, C.; Haley, B.; Martinez, N.; Zamore, P. D. *Science (80-.).* **2004**, *306*, 1377–1380.

(12) Dykxhoorn, D. M.; Novina, C. D.; Sharp, P. A. *Nat. Rev. Mol. Cell Biol.* **2003**, *4*, 457–467.

(13) Nykänen, A.; Haley, B.; Zamore, P. D. *Cell* **2001**, *107*, 309–321.

(14) De Fougerolles, A.; Vornlocher, H.-P.; Maraganore, J.; Lieberman, J. *Nat. Rev. Drug Discov.* **2007**, *6*, 443–453.

(15) Weitzer, S.; Martinez, J. *Nature* **2007**, *447*, 222–226.

(16) Elbashir, S. M.; Lendeckel, W.; Tuschl, T. *Genes Dev.* **2001**, *15*, 188–200.

(17) Reynolds, A.; Leake, D.; Boese, Q.; Scaringe, S.; Marshall, W. S.; Khvorova, A. *Nat. Biotechnol.* **2004**, *22*, 326–330.

(18) Elbashir, S. M.; Martinez, J.; Patkaniowska, A.; Lendeckel, W.; Tuschl, T. *EMBO J.* **2001**, *20*, 6877–6888.

(19) Kirchner, R.; Vogtherr, M.; Limmer, S.; Sprinzl, M. *Antisense Nucleic Acid Drug Dev.* **1998**, *8*, 507–516.

(20) Dykxhoorn, D. M.; Lieberman, J. *Annu. Rev. Biomed. Eng.* **2006**, *8*, 377–402.

(21) Tuschl, T. In *RNAi: A Guide to Gene Silencing*; 2002; pp. 265–296.

(22) Sandy, P.; Ventura, A.; Jacks, T. *Biotechniques* **2005**, *39*, 215–224.

(23) Chang, K.; Marran, K.; Valentine, A.; Hannon, G. J. *Cold Spring Harb. Protoc.* **2012**, *2012*, 957–961.

(24) Semizarov, D.; Frost, L.; Sarthy, A.; Kroeger, P.; Halbert, D. N.; Fesik, S. W. *Proc. Natl. Acad. Sci.* **2003**, *100*, 6347–6352.

(25) Fedorov, Y.; Anderson, E. M.; Birmingham, A.; Reynolds, A.; Karpilow, J. O. N.; Robinson, K.; Leake, D.; Marshall, W. S.; Khvorova, A. *RNA* **2006**, *12*, 1188–1196.

(26) Anderson, E. M.; Birmingham, A.; Baskerville, S.; Reynolds, A.; Maksimova, E.; Leake, D.; Fedorov, Y.; Karpilow, J.; Khvorova, A. *RNA* **2008**, *14*, 853–861.

(27) Ui-Tei, K.; Naito, Y.; Takahashi, F.; Haraguchi, T.; Ohki-Hamazaki, H.; Juni, A.; Ueda, R.; Saigo, K. *Nucleic Acids Res.* **2004**, *32*, 936–948.

(28) Hsieh, A. C.; Bo, R.; Manola, J.; Vazquez, F.; Bare, O.; Khvorova, A.; Scaringe, S.; Sellers, W. R. *Nucleic Acids Res.* **2004**, *32*, 893–901.

(29) Jagla, B.; Aulner, N.; Kelly, P. D.; Song, D. A.; Volchuk, A.; Zatorski, A.; Shum, D.; Mayer, T.; De Angelis, D. A.; Ouerfelli, O.; Rutishauser, U.; Rothman, J. E. *RNA* **2005**, *11*, 864–872.

(30) Balkwill, F. *Nat. Rev. Cancer* **2004**, *4*, 540–550.

(31) Zlotnik, A. *Int. J. Cancer* **2006**, *119*, 2026–2029.

(32) Mantovani, A.; Savino, B.; Locati, M.; Zammataro, L.; Allavena, P.; Bonecchi, R. *Cytokine Growth Factor Rev.* **2009**, *21*, 27–39.

(33) O'Hayre, M.; Salanga, C. L.; Handel, T. M.; Allen, S. J. *Biochem. J.* **2008**, *409*, 635–649.

(34) Ruffini, P. A.; Morandi, P.; Cabioglu, N.; Altundag, K.; Cristofanilli, M. *Cancer* **2007**, *109*, 2392–2404.

(35) Belperio, J. A.; Keane, M. P.; Arenberg, D. A.; Addison, C. L.; Ehlert, J. E.; Burdick, M. D.; Strieter, R. M. *J. Leukoc. Biol.* **2000**, *68*, 1–8.

(36) Gregory, A. D.; Houghton, A. M. *Cancer Res.* **2011**, *71*, 2411–2416.

(37) Van Damme, J.; Struyf, S.; Opdenakker, G. *Sem. Cancer Biol.* **2004**, *14*, 201–208.

(38) Chiang, H.; Wang, Y.; Chou, C.; Liao, A. T.; Chu, R.; Lin, C. *BMC Vet. Res.* **2012**, *8*, 216.

(39) Xie, F. Y.; Woodle, M. C.; Lu, P. Y. *Drug Discov. Today* **2006**, *11*, 67–73.

(40) Müller, A.; Homey, B.; Soto, H.; Ge, N.; Catron, D.; Buchanan, M. E.; McClanahan, T.; Murphy, E.; Yuan, W.; Wagner, S. N.; Barrera, J. L.; Mohar, A.; Verástegui, E.; Zlotnik, A. *Nature* **2001**, *410*, 50–56.

(41) Liang, Z.; Yoon, Y.; Votaw, J.; Goodman, M. M.; Williams, L. *Cancer Res.* **2005**, *65*, 967–971.

(42) Davis, M. E.; Zuckerman, J. E.; Choi, C. H. J.; Seligson, D.; Tolcher, A.; Alabi, C. A.; Yen, Y.; Heidel, J. D.; Ribas, A. *Nature* **2010**, *464*, 1067–1070.

(43) Aagaard, L.; Rossi, J. J. *Adv. Drug Deliv. Rev.* **2007**, *59(2-3)*, 75–86.

(44) Ui-Tei, K.; Naito, Y.; Saigo, K. *J. Biomed. Biotech.* **2006**, *2006*, 65052.

(45) Ding, Y.; Chan, C. Y.; Lawrence, C. E. *Nucleic Acids Res.* **2004**, *32*, 135–141.

(46) Chalk, A. M.; Wahlestedt, C.; Sonnhammer, E. L. L. *Biochem. Biophys. Res. Commun.* **2004**, *319*, 264–274.

(47) Lu, Z. J.; Mathews, D. H. *Nucleic Acids Res.* **2008**, *36*, 640–647.

(48) Hornung, V.; Guenthner-Biller, M.; Bourquin, C.; Ablasser, A.; Schlee, M.; Uematsu, S.; Noronha, A.; Manoharan, M.; Akira, S.; de Fougerolles, A.; Endres, S.; Hartmann, G. *Nat. Med.* **2005**, *11*, 263–270.

(49) Judge, A. D.; Sood, V.; Shaw, J. R.; Fang, D.; McClintock, K.; MacLachlan, I. *Nat. Biotechnol.* **2005**, *23*, 457–462.

(50) Wong, S. Y.; Haack, H.; Kissil, J. L.; Barry, M.; Bronson, R. T.; Shen, S. S.; Whittaker, C. A.; Crowley, D.; Hynes, R. O. *Proc. Natl. Acad. Sci.* **2007**, *104*, 12784–12789.

(51) Berezhnoy, A.; Brenneman, R.; Bajgelman, M.; Seales, D.; Gilboa, E. *Mol.Ther. Nucleic Acids* **2012**, *1*, e51.

(52) Kamio, N.; Hirai, H.; Ashihara, E.; Tenen, D. G.; Maekawa, T.; Imanishi, J. *Biochem. Biophys. Res. Commun.* **2010**, *393*, 498–503.

(53) Unver, N. Ph.D. Thesis, Hacettepe University, Ankara, 2012.

(54) Elouahabi, A.; Ruysschaert, J.-M. *Mol. Ther.* **2005**, *11*, 336–347.

(55) BD Biosciences., *BD FACSAria II User's Guide Revision A*, 2009, http://www.bdbiosciences.com/.

(56) Zhang, G.; Gurtu, V.; Kain, S. R. *Biochem. Biophys. Res. Commun.* **1996**, *227*, 707–711.

(57) Corish, P.; Tyler-Smith, C. *Protein Eng.* **1999**, *12*, 1035–40.

APPENDICES

Appendix A: The results of conventional method siRNA transfections

Set 2:

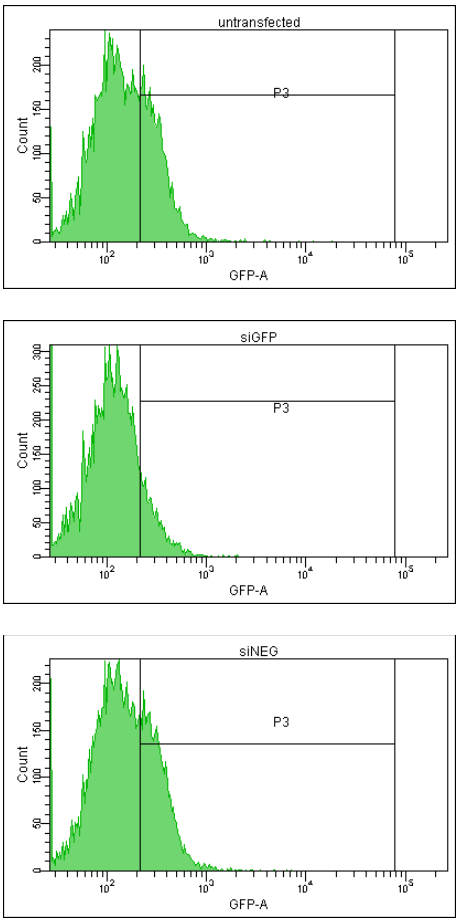
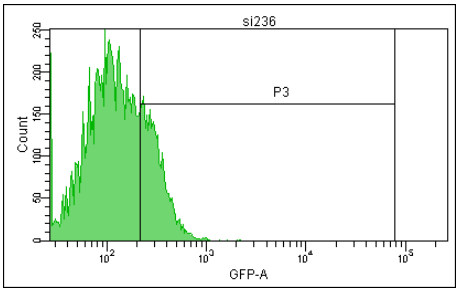
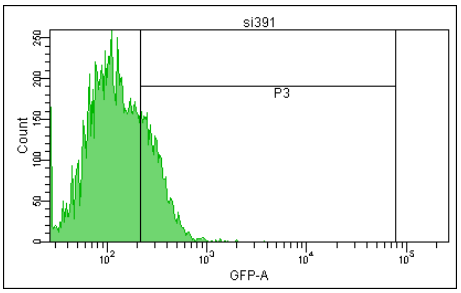
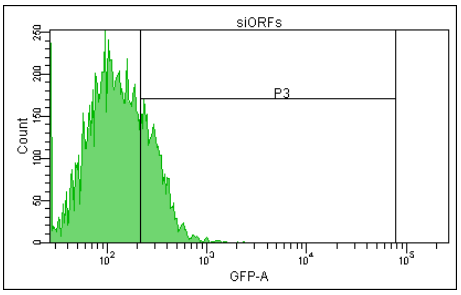
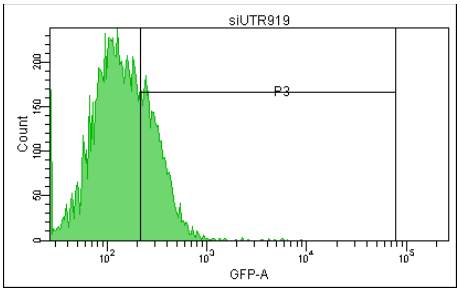


Figure A.1 The results of levels of eGFP expression after conventional transfections of the experimental set 2.









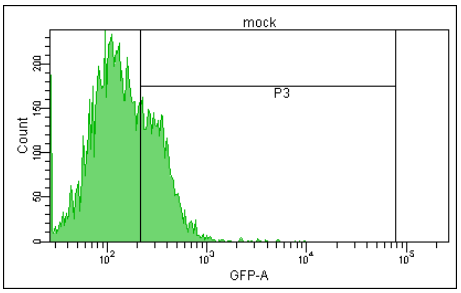


Figure A.1 (continued)

Set 3:

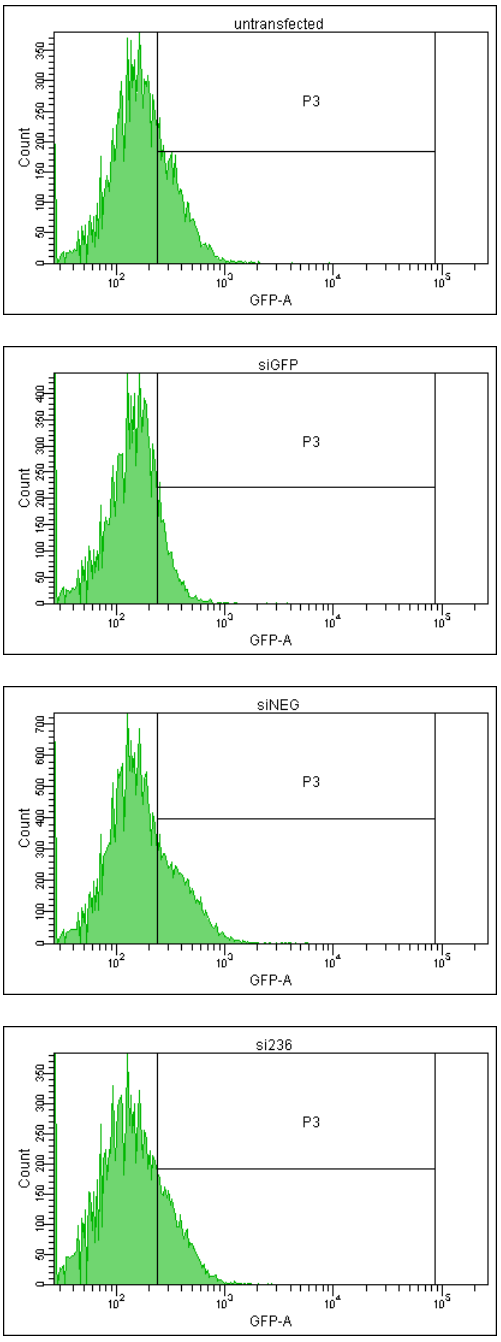


Figure A.2 The results of levels of eGFP expressions after conventional transfections of the experimental set 3.

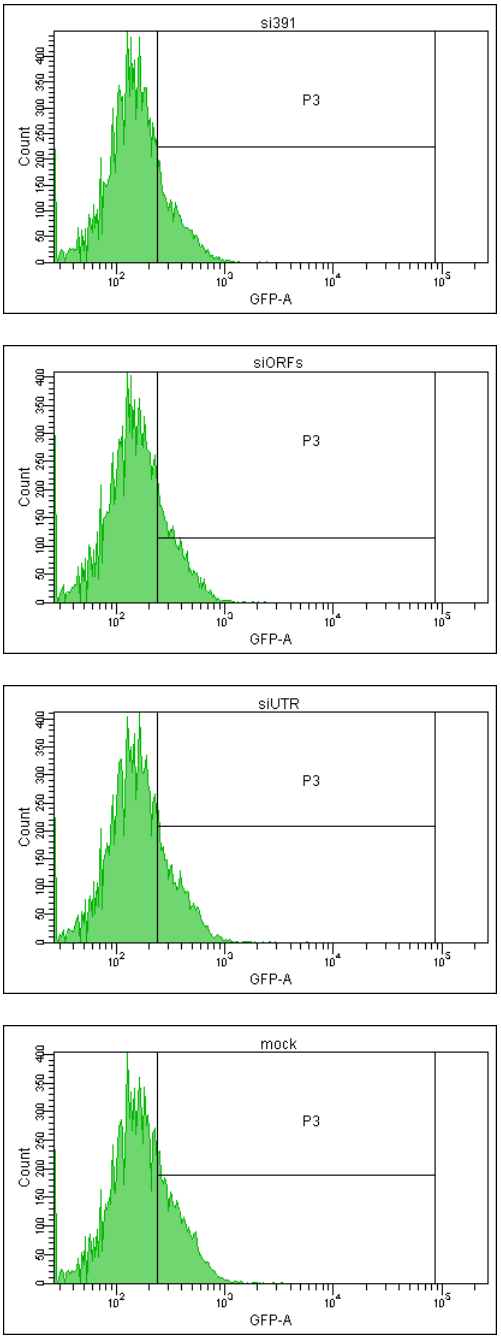


Figure A.2 (continued)

Appendix B: The results of reverse method siRNA transfections

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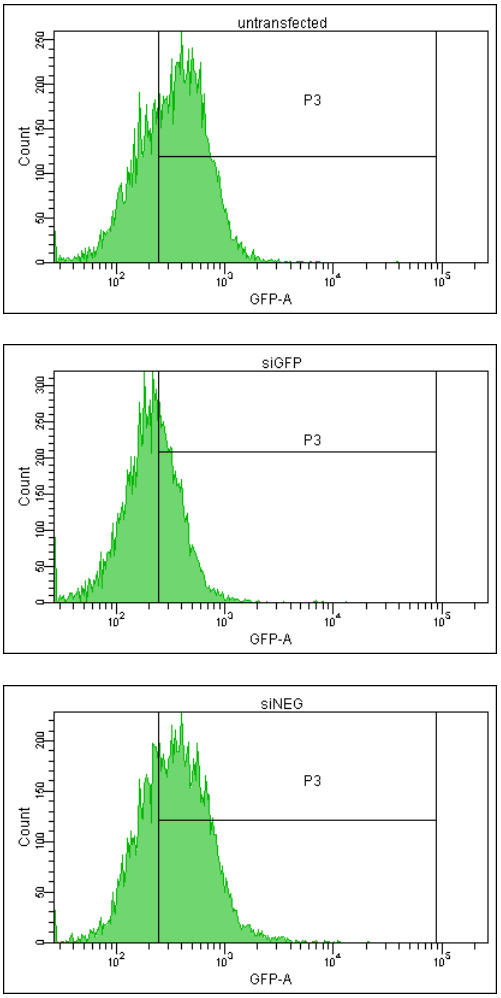
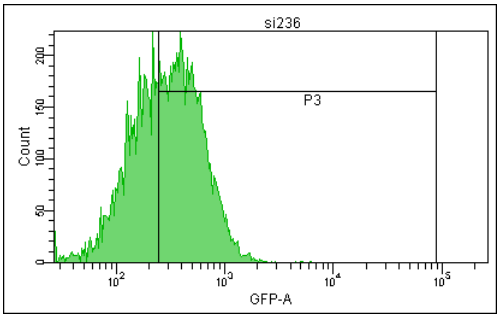
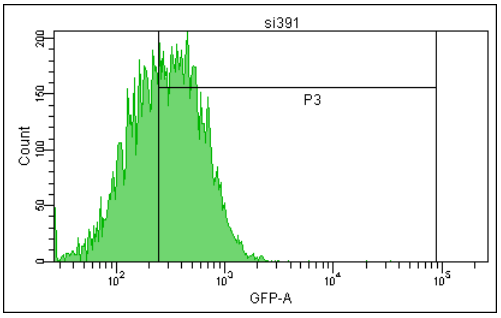
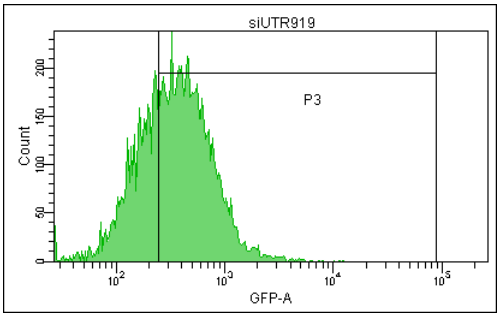


Figure B.1 The results of levels of eGFP expressions after reverse transfections of the experimental set 2.







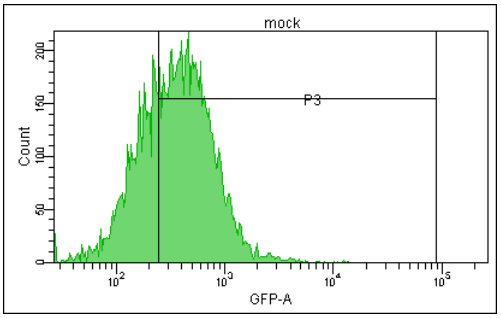


Figure B.1 (continued)

Set 3:

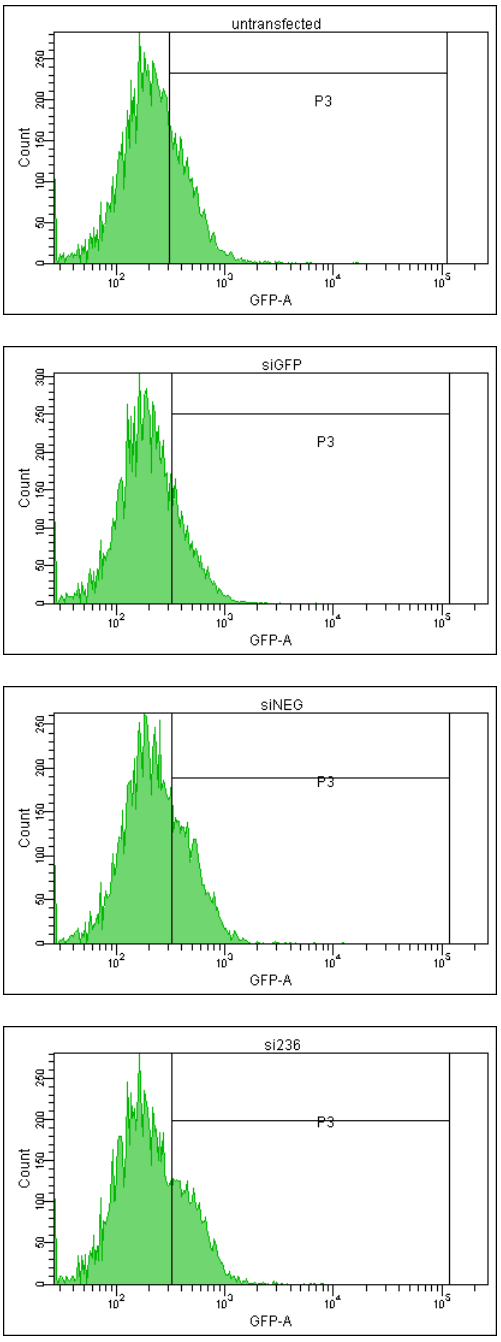


Figure B.2 The results of levels of eGFP expressions after reverse transfections of the experimental set 3.

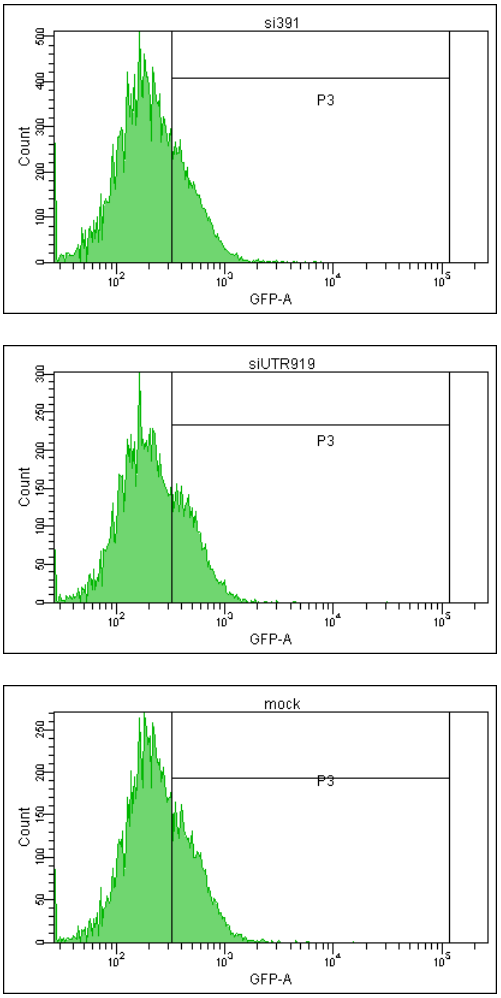


Figure B.2 (continued)