การยับยั้งการแสดงออกของยีนดีเอสซีอาร์วันโดยใช้เอสไออาร์เอ็นเอ ในเซลล์ลิมโฟบลาสต์จากผู้ป่วยโรคดาวน์ซินโดรม

นางสาวพรรณธิภา ตรีจันทร์ทอง

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
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DSCR1 GENE SILENCING BY siRNA IN LYMPHOBLAST CELLS FROM PATIENT WITH DOWN SYNDROME

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Thesis Title	*DSCR1* gene silencing by siRNA in lymphoblast cells from patient with Down syndrome.
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พรรณธิภา ตรีจันทร์ทอง : การยับยั้งการทำงานของยีนดีเอสซีอาร์วันโดยใช้เอสไออาร์เอ็นเอในเซลล์ลิมโฟบลาสต์จากผู้ป่วยดาวน์ซินโดรม (*DSCR1* GENE SILENCING BY siRNA IN LYMPHOBLAST CELLS FROM PATIENT WITH DOWN SYNDROME) อาจารย์ที่ปรึกษา: รศ.ดร. วรวุฒิ จุฬาลักษณานุกูล, อาจารย์ที่ปรึกษาร่วม : นพ.วีรยุทธ ประพันธ์พจน์, จำนวน106 หน้า

ยีน *DSCR1 (Down Syndrome Critical Region 1 gene)* เป็นยีนที่มีส่วนเกี่ยวข้องกับขบวนการเรียนรู้และการจดจำในมนุษย์โดยในสมองของทารกผู้ป่วยดาวน์ซินโดรมพบการแสดงออกของยีน *DSCR1* ที่มากเกินปกติ (over-expression) ส่งผลทำให้ปริมาณโปรตีน calcipressin ซึ่งเป็นผลิตภัณฑ์จากยีน *DSCR1* เพิ่มขึ้นตามไปด้วยและสามารถยับยั้งการทำงานของโปรตีนที่มีส่วนสำคัญในขบวนการการเรียนรู้และการจดจำที่เรียกว่า calcineurin ได้ ดังนั้นหากสามารถยับยั้งการทำงานของ *DSCR1* ก็อาจส่งผลให้ผู้ป่วยมีการเรียนรู้และจดจำที่เป็นปกติได้. เทคนิค RNAi (RNA interfering technology) เป็นเทคนิคที่เลียนแบบกระบวนการยับยั้งการทำงานของยีนที่เกิดขึ้นเองตามธรรมชาติ (biogenesis pathway) ปัจจุบันจึงได้ถูกนำมาใช้ในการยับยั้งการทำงานของยีนต่างๆ โดยกุญแจสำคัญของกระบวนการนี้คือ siRNA (small interference RNA) ซึ่งมีลำดับเบสที่ complementary กับ mRNA ของยีนเป้าหมาย โดยจะเกิดการจับและทำลาย mRNA เป้าหมาย ส่งผลให้ยีนไม่สามารถทำหน้าที่ต่อไปได้ วัตถุประสงค์ของงานวิจัยในครั้งนี้ เพื่อศึกษาถึงผลของ siRNA ต่อการยับยั้งการทำงานของยีน *DSCR1* ที่มีมากเกินปกติในเซลล์ลิมโฟบลาสต์ของผู้ป่วย โดยศึกษาการแสดงออกของยีน *DSCR1* ในเซลล์ลิมโฟบลาสต์จากคนปกติและผู้ป่วย ทั้งในระดับmRNAและโปรตีน โดยวิธี real time PCR และ western blot ตามลำดับ และทำการเปรียบเทียบการแสดงออกของยีน คู่กับเซลล์ที่ถูก treat ด้วย siRNA ในความเข้มข้นที่แตกต่างกัน 3 ระดับ (0.4 ,0.8 และ 1 μg) พบว่า ในคนปกติ siRNA ทุกระดับ (t test, Pr=0.7878, 0.7099 และ 0.4103) ไม่มีผลต่อการแสดงออกของ mRNA จากยีน *DSCR1* ในวันที่ 14 หลังการ treat ด้วย siRNA คาดว่าอาจจะเป็นสืบเนื่องจากระยะเวลาที่นานเกินไปในการเพาะเลี้ยงเซลล์ให้เพียงพอต่อการสกัด mRNA ซึ่งเมื่อติดตามอัตราการรับ siRNA สู่เซลล์ลิมโฟบลาสต์ โดยการติดยีนเรืองแสง*DsRed2* ซึ่งจะให้โปรตีนเรืองแสงสีแดง (Red Fluorescent Protein) เข้ากับ vector ของ siRNA พบว่า siRNA จะอยู่ภายในเซลล์ได้ 8 วัน จากเหตุผลนี้ เราจึงได้ทำการทดลองเพิ่มบางส่วน โดยการลดปริมาณตัวอย่างลง เพื่อให้เพียงพอต่อการสกัด mRNA และ treat ด้วย siRNAที่ความเข้มข้น 0.8 μg ซึ่งเป็นความเข้มข้นที่เหมาะสมที่สุด พบว่า ไม่มีความแตกต่างกันในระดับของ mRNA (t test, Pr=0.3431) นอกจากนี้ เราได้วัดอัตราการรับ siRNA เปรียบเทียบกันระหว่างลิมโฟบลาสต์ และ ไฟโบบลาสต์ พบว่าอัตราการรับ ไม่แตกต่างกันถึงแม้ว่าจะเป็นเซลล์คนละชนิดกัน และมาจากเนื้อเยื่อที่แตกต่างกัน.

ภาควิชา พฤกษศาสตร์ ลายมือชื่อนิสิต .....

สาขาวิชา พันธุศาสตร์ ลายมือชื่ออาจารย์ที่ปรึกษา .....

ปีการศึกษา 2550 ลายมืออาจารย์ที่ปรึกษาร่วม .....

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KEY WORDS : *DSCR1* / siRNA / *DsRed2*

PANTIPA TRICHANTONG : *DSCR1* GENE SILENCING BY siRNA IN LYMPHOBLAST CELLS FROM PATIENT WITH DOWN SYNDROME. THESIS ADVISOR: ASSOC. PROF. Dr. WARAWUT CHULALAKSANANUKUL, THESIS CO-ADVISOR: VERAYUTH PRAPANPOJ MD; 106 pp.

DSCR1 (Down Syndrome Critical Region 1) gene directly affect to the learning and memory process in human expecially in fetal Down Syndrome (DS) brain. *DSCR1* belongs to a highly conserved calcineurin inhibitor family called calcipressin. *DSCR1* can bind to and inhibit calcineurin, a protein important for learning and memory. RNAi technique which is a naturally occurring cellular mechanism that induces post transcriptional gene silencing .Small interfering RNA (siRNA) molecules are the key intermediaries in post transcriptional gene silencing which when exogenously administered can inhibit the expression of any given target gene. Since *DSCR1* is overexpressed in DS fetal brain, it is possible that normalizing *DSCR1* expression may restore normal brain function in DS individual. The goal of this study is to inhibit *DSCR1* gene in lymphoblast cells by siRNA. In this research we studied *DSCR1* gene expression level in both mRNA and protein by real time PCR and western blot consequently.The comparison result of mRNA level between untreated and treated with 3 concentrations of siRNAs (0.4 ,0.8 and 1 μg) in control and case samples indicated that siRNAs did not affect to mRNA level of *DSCR1* gene in both of samples at 14th day (Pr=0.7878, 0.7099 and 0.4103) From above result it might be the long period of post-transfection. The *DsRed2* gene was cloned into siRNA plasmid vector to indicate the transfection efficiency. RFP signal was shown that siRNAs were effectively within 8 days of post transfection .From this result , I decide to make an additional experiment by decrease time period and sample quantity, harvest lymphoblast cell lines 5 samples from normal and measure mRNA at 5th day of post-transfection at appropriate siRNA concentration (0.8 μg) for test *DSCR1* suppression . After changing the time, I found siRNA cannot knockdown *DSCR1* gene (t test, Pr=0.3431), Moreover we tested transfection efficiency in both of fibroblast and lymphoblast cells from same case sample. We demonstrate that the percentage of transfection efficiency and period of signal in both of cell types are not different.

Department of Botany Student's signature.....

Field of study Genetics Advisor's signature.....

Academic year 2007 Co-advisor's signature.....

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LIST OF ABBREVIATIONS

DS	=	Down syndrome
DSCR1	=	Down syndrome critical region 1
RNAi	=	RNA interference
siRNA	=	small interfering RNA
shRNA	=	short hairpin RNA
RT-PCR	=	Reverse transcriptase polymerase chain reaction

CHAPTER I

INTRODUCTION

Background and rationale

Down syndrome (DS) is the most common chromosomal abnormality found in humans. It occurs at a rate of 1 in 700 live births and is much more common than other genetic abnormalities. It is also the most common cause of intellectual disability in the community. Individuals with DS have abnormalities in every organ system of the body, including brain defects, heart defects, leukaemia, gastrointestinal anomalies, bone abnormalities, facial dismorphology, eye problems and immune defects which make them prone to infections. In addition, the most of DS develop Alzheimer's disease by the age of 35. Notably, many of the problems experienced by people with DS also occur in the rest of the population albeit at a lower frequency and later in life. Therefore, DS is very useful model for common disorders and what we learn from DS is applicable to the broader community. Most individuals (95%) with trisomy 21 have 3 free copies of human chromosome 21 (HC 21) instead of the normal two; full or partial (*1*). In about 5% of patients, 1 copy is translocated to another acrocentric chromosome, most often chromosome 14 or 21 *(2, 3)*. In 2 to 4% of cases with free trisomy 21 there is recognizable mosaicism for a trisomic and a normal cell line (*4*). DS is caused by increases in the amounts of a number of normal gene products. The identification of genes on HC21 and the elucidation of the function of the proteins encoded by these genes have been a major challenge for the human genome project and for research in DS (*5*). Among the genes located in the DS region is the DS critical region 1 (*DSCR1*) gene.

The *DSCR1* gene consist of four exons, (exons1-4) can be alternatively spiced to produce number of different mRNA isoforms (isoform A, B and C) . *DSCR1*, located in the 21q22.1-q22.2 region, is implicated in both the heart defects and neurological deficits observed in individuals with DS because it regulates a cellular pathway critical for heart and brain development and function. Therefore, the overexpression of *DSCR1* gene may be involved in pathogenic abnormalities of mental retardation and/or heart defects in patients with DS. The DSCR1 belongs to a new family of genes that bind and inhibit calcineurin (*6, 7*). The name calcipressin1 has been suggested for the calcineurin inhibitory DSCR1 protein product.

Calcineurin is a calcium/calmodulin-activated serine/threonine phosphatase (PP2B) that is an important enzyme in Ca^{2+}-dependent eukaryotic signal transduction pathways (*8*).

The *DSCR1*-binding region in calcineurin A is located in the linker region between the calcineurin A catalytic domain and the calcineurin B-binding domain, outside of other functional domains previously defined in calcineurin A. *DSCR1* induction during adaptation to oxidative stress is also a calcium-dependent process (*9,10*). Calcineurin plays important roles in immune stimulation, and calcineurin-dependent signal transduction pathway have been extensively characterized during T cell activation (*11*). Morever, calcineurin plays a critical role in cellular responses to various extracellular signals and environmental stresses and is important in the regulation of apoptosis (*12*), memory and learning processes, skeletal and cardiac muscle growth and differentiation.

For my research, I would like to present the study of *DSCR1* gene silencing by using RNA interference (RNAi) technique is a naturally occurring cellular mechanism that induces post-transcriptional gene silencing in diverse cell types and has attracted much attention as a potential therapeutic strategy. Small interfering RNAs (siRNA) are short duplexes of RNA approximately 20-25 nucleotides that are the key intermediaries in this process. Since its discovery in the late 1990s, RNAi/siRNA technology has broad application in target validation, probing gene function (transgenomics), and may serve as a potential therapeutic strategy for the inhibition of disease-associated genes (*13*).

This technique was first developed in *Caenorhabditis elegans*, and was rapidly applied to a wide range of organisms. Methods for expressing siRNAs in cells in culture and in vivo using viral vectors, and for transfecting cells with synthetic siRNAs, have been developed and are being used to establish the functions of specific proteins in various cell types and organisms.

From all above informations, I decide to study about the gene silencing in *DSCR1* gene, located on chromosome 21, is overexpressed in the brain of DS fetus and encodes an inhibitor of calcineurin which regulated learning and memory to address its role in mental retardation associated with DS. By the siRNA technique has board application in this experiment. The overexpression of *DSCR1* has been shown to effect learning and memory on *Drosophila* (*14*). Thus, it's possible that overexpress of *DSCR1* in human may be one of the important factors contribution to abnormalities found in DS individual. Many knowledge obtained from this study, It may serve as a potential therapeutic strategy for the inhibition of *DSCR1* gene in DS patient compare with normal human and I expected this research is an extremely valuable information for DS patient treatment in the future.

Research questions

1. Can siRNA decrease *DSCR1* gene expression level in lymphoblast cells?
2. What is the appropriate concentration of siRNA for decreasing *DSCR1* gene expression level in lymphoblast cells?

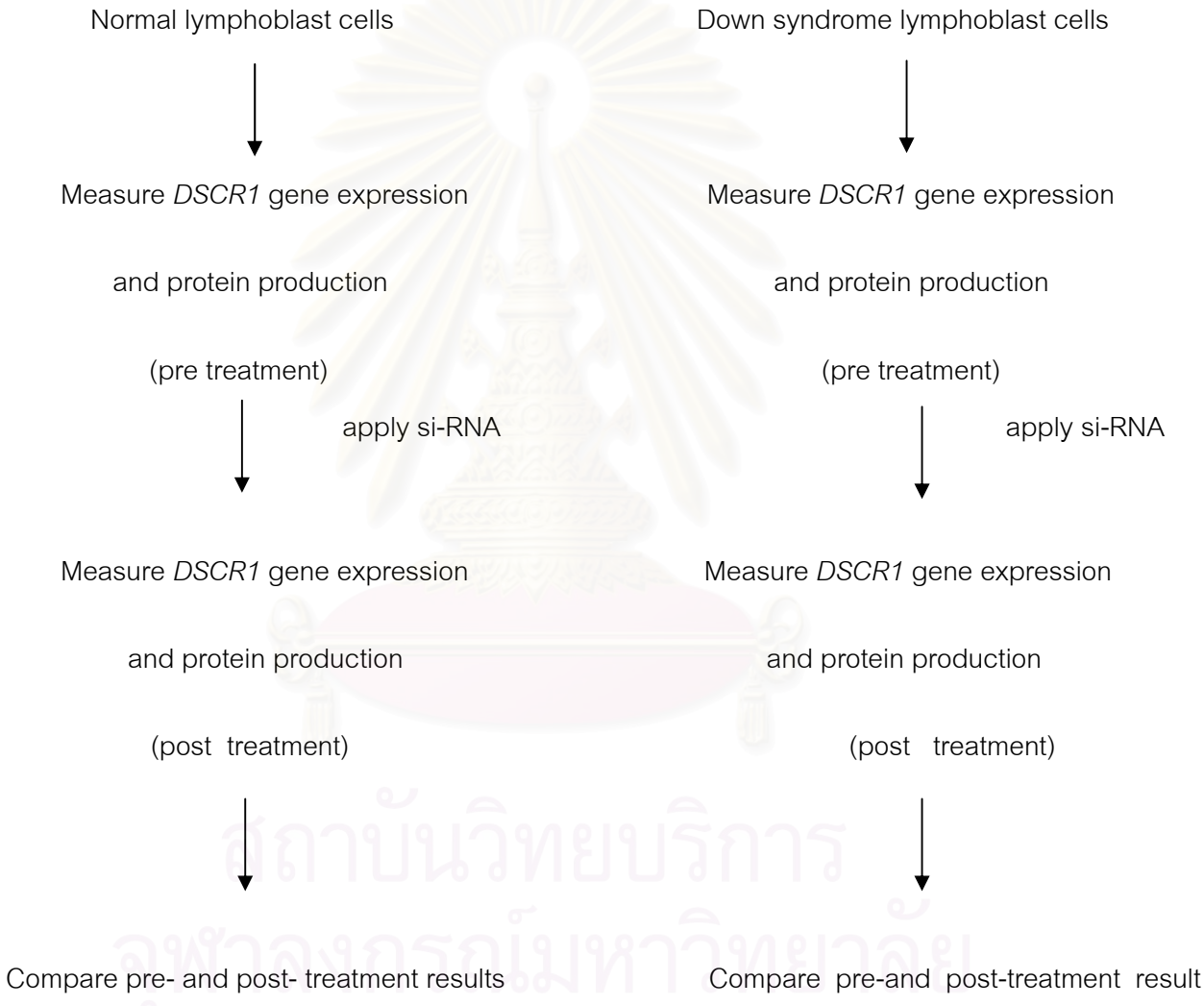
Objectives

1. To study the *DSCR1* gene silencing by siRNA in lymphoblast cells from patient with Down syndrome.
2. To determine the appropriate concentration of siRNA to silence *DSCR1* gene in lymphoblast cells from patient with Down syndrome.

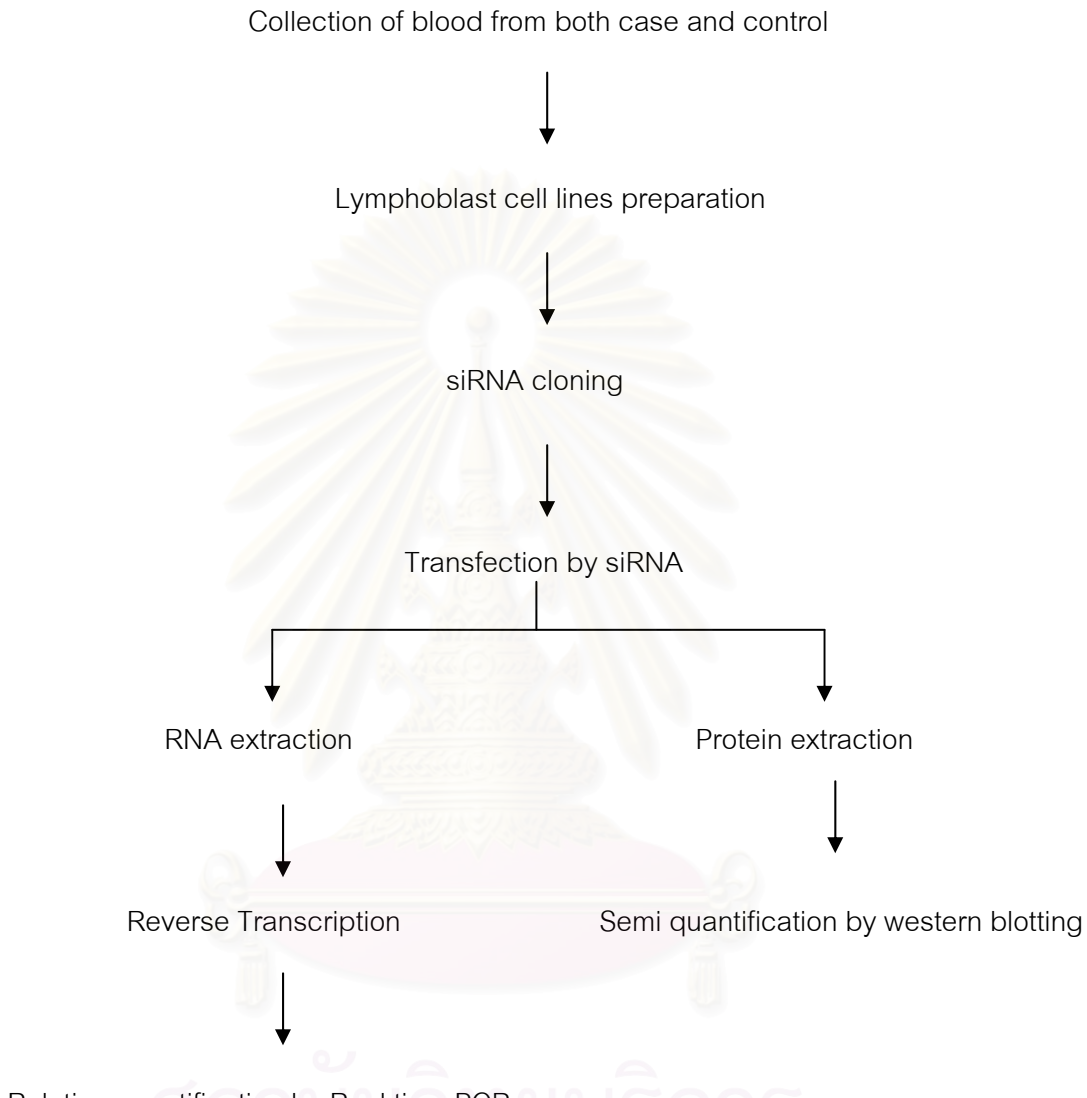
Hypothesis

1. *DSCR1* gene expression level is decreased by si-RNA.

Conceptual framework



Methodologies



Expected Benefit

This study will give extremely valuable information about the using of siRNA technique for silence the *DSCR1* gene which is overexpressed in the brain of Down Syndrome fetus and encoded an inhibitor of calcineurin. It is the leading genetic cause of mental retardation and many clinical abnormalities. Moreover, my expectation is to help the Down Syndrome treatment potentially to revolutionise future healthcare.

Research Methodology

1. Collection of blood samples from Down syndrome patients and normal human (control)
2. Preparation of lymphoblast cells lines
3. Cloning siRNA
4. siRNA transfection into lymphoblast cells by lipofectamine 2000
5. Find the uptake rate of siRNA by using RFP reporter
6. RNA extraction
7. Two step RT-PCR
8. Agarose electrophoresis
9. Relative quantification real time PCR
10. Data analysis
11. Protein extraction
12. Semi quantification by western blotting.
13. Data analysis
14. Conclusion

CHAPTER II

REVIEW OF THE RELATED LITERATURES

1. Down syndrome

A. History of Down syndrome

English physician John Langdon Down first characterized Down syndrome as a distinct form of mental retardation in 1862, and in a more widely published report in 1866 entitled Observations on an ethnic classification of idiot (*15*). Due to his perception that children with Down syndrome shared physical facial similarities (epicanthal folds) with those of Blumenbach's Mongolian race, Down used terms such as mongolism and Mongolian idiocy (*16*). Idiocy was a medical term used at that time to refer to a severe degreeof intellectual impairment. Down wrote that mongolism represented retrogression, the appearance of Mongoloid traits in the children of allegedly more advanced Caucasian parents. By the 20th century, Mongolian idiocyhad become the most recognizable form of mental retardation. Most individuals with Down syndrome were institutionalized, few of the associated medical problems were treated, and most died in infancy or early adult life. With the rise of the eugenics movement, 33 of the (then) 48 U.S. states and several countries began programs of involuntary sterilization of individuals with Down syndrome and comparable degrees of disability. The ultimate expression of this type of public policy was the German euthanasia program begun in 1940. Court challenges and public revulsion led to discontinuation or repeal of such programs during the decades after World War II.

Until the middle of the 20th century, the cause of Down syndrome remained unknown. However, the presence in all races, the association with older maternal age, and the rarity of recurrence had been noticed. Standard medical texts assumed it was caused by a combination of inheritable factors which had not been identified. Other theories focused on injuries sustained during birth *(17).*

With the discovery of karyotype techniques in the 1950s, it became possible to identify abnormalities of chromosomal number or shape. In 1959, Professor Jérôme Lejeune discovered that Down syndrome resulted from an extra chromosome. The extra chromosome was subsequently labeled as the 21st, and the condition as trisomy 21.

In 1961,nineteen geneticists wrote to the editor of The Lancet suggesting that Mongolian idiocy had misleading connotations, had become an embarrassing term, and should be changed (*18,19,20*). The Lancet supported Down's Syndrome. The World Health Organization (WHO) officially dropped references to mongolism in 1965 after a request by the Mongolian delegate (*21, 22*). However, almost 40 years later, the term mongolism still appears in leading medical texts such as Review of Medical Physiology, 22nd Edition, 2005, by Professor William Ganong and General and Systematic Pathology, 4th Edition, 2004, edited by Professor Sir James Underwood.

In 1975, the United States National Institutes of Health convened a conference to standardize the nomenclature of malformations. They recommended eliminating the possessive form the possessive use of an eponym should be discontinued, since the author neither had nor owned the disorder. Although both the possessive and non-possessive forms are used in the general population, Down syndrome is the accepted term among professionals in the USA, Canada and other countries, Down's syndrome is still used in the United Kingdom and other areas (*23, 24*).

B. Incidence

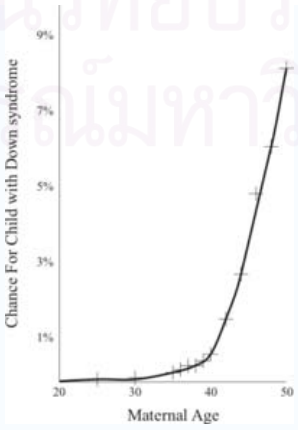


Figure 1 Incidence of Down syndrome

The incidence of Down syndrome is estimated at 1 per 800 to 1 per 1000 births.In 2006, the Center for Disease Control estimated the rate as 1 per 733 live births in the United States 5429 new cases per year.Approximately 95% of these are trisomy 21. Down syndrome occurs in all ethnic groups and among all economic classes.

Maternal age influences the risk of conceiving a baby with Down syndrome.At maternal age 20 to 24, the risk is 1/1490; at age 40 the risk is 1/60, and at age 49 the risk is 1/11(25). Although the risk increases with maternal age, 80% of children with Down syndrome are born to women under the age of 35, reflecting the overall fertility of that age group. Other than maternal age, no other risk factors are known. There does not appear to be a paternal age effect.

C. Clinical feature

Down syndrome, a particular combination of phenotypic features that includes mental retardation and characteristic facies, is caused by trisomy 21 (*26*), one of the most common chromosomal abnormalities in liveborn children.

Individuals with Down syndrome often have specific major congenital malformations such as those of the heart (30-40% in some studies), particularly the atrioventricular canal, and of the gastrointestinal tract, such as duodenal stenosis or atresia, imperforate anus, and Hirschsprung disease. Some of these clinical features have been incorporated into the preliminary phenotypic maps of chromosome 21 (*27,28,29)* .

Leukemia (both ALL and AML) and leukemoid reactions show increased incidence in Down syndrome (30, 31). Estimates of the relative risk have ranged from 10 to 20 times higher than the normal population; in particular, acute megakaryocytic leukemia occurs 200 to 400 times more frequently in the Down syndrome than in the chromosomally normal population (*32*). Transient leukemoid reactions have been reported repeatedly in the neonatal period, and this phenotype has been tentatively mapped to the proximal long arm of chromosome 21 (*33*). Ninety percent of all Down syndrome patients have a significant hearing loss, usually of the conductive type (34).

For additional defects and phenotypic characteristics (*35)*. Patients with Down syndrome develop the neuropathologic hallmarks of Alzheimer disease at a much earlier age than individuals with Alzheimer disease without trisomy 21 (*36*). Characteristic senile plaques and neurofibrillary tangles are present in the brain of all individuals with Down syndrome over the age of 40 years (*37*). The triplication of the amyloid precursor protein gene may be the cause of this phenomenon. Several mutations in the *APP* gene have been described in families with early-onset Alzheimer disease without trisomy 21.

In a retrospective study of high-altitude pulmonary edema performed at Children's Hospital in Denver, identified 6 of 52 patients as having Down syndrome. Five of the 6 children had preexisting illnesses including chronic pulmonary hypertension, existing cardiac defects with left-to-right shunt, or previous defects of left-to-right shunt that had been repaired. One child had Eisenmenger syndrome (*38*) suggested caution in traveling to even moderate altitudes with children with Down syndrome.

To determine whether newborns with DS have decreased blood T4 concentrations at the time of the neonatal screening (*39*) conducted an observational study in a large and representative cohort of Dutch children with DS born in 1996 and 1997. Results of congenital hypothyroidism (CH) screening measuring T4, TSH, and T4-binding globulin concentrations were analyzed in comparison with clinical information obtained by interviewing the parents and data from the general newborn population and a large control group. The mean T4 concentration of the 284 studied children with DS was significantly decreased. The individual T4 concentrations were normally distributed but shifted to lower concentrations. Mean TSH and T4-binding globulin concentrations were significantly increased and normal, respectively. The decreased T4 concentration, left-shifted normal distribution, and mildly elevated TSH concentrations pointed to a mild hypothyroid state in newborns with DS and supported the existence of a DS-specific thyroid (regulation) disorder.

D. Treatment

Despite continued hope, no significant medical treatments for mental retardation associated with Down syndrome have been forthcoming. However, the dramatic improvements in medical care described here have greatly improved the quality of life and increased life expectancy.

E. Karyotype

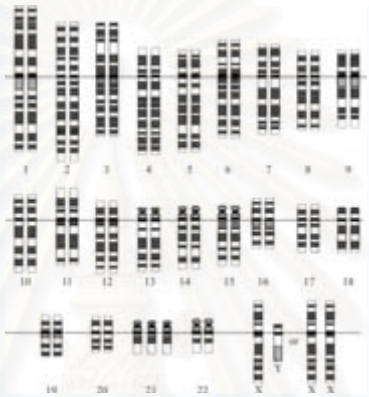


Figure 2 Karyotype of Down syndrome

Karyotype for trisomy Down syndrome. Notice the three copies of chromosome 21 Down syndrome is a chromosomal abnormality characterized by the presence of an extra copy of genetic material on the 21st chromosome, either in whole (trisomy 21) or part (such as due to translocations). In general, this leads to an overexpression of the genes (*40, 41*). Understanding the genes involved may help to target medical treatment to individuals with Down syndrome. It is estimated that chromosome 21 contains 200 to 250 genes (*42*). Recent research has identified a region of the chromosome that contains the main genes responsible for the pathogenesis of Down syndrome, located proximal to 21q22.3. The search for major genes involved in Down syndrome characteristics is normally in the region 21q21–21q22.3.

The effects of the extra copy vary greatly among individuals, depending on the extent of the extra copy, genetic background, environmental factors, and random

chance. Down syndrome occurs in all human populations, and analogous effects have been found in other species such as chimpanzees (*43*) and mice.

F. Cytogenetics

Trisomy 21 (47, XX, +21) is caused by a meiotic nondisjunction event. With nondisjunction, a gamete (*i.e.*, a sperm or egg cell) is produced with an extra copy of chromosome 21; the gamete thus has 24 chromosomes. When combined with a normal gamete from the other parent, the embryo now has 47 chromosomes, with three copies of chromosome 21. Trisomy 21 is the cause of approximately 95% of observed Down syndromes, with 88% coming from nondisjunction in the maternal gamete and 8% coming from nondisjunction in the paternal gamete.

-Mosaicism

Trisomy 21 is caused prior to conception, and all cells in the body are affected. However, when some of the cells in the body are normal and other cells have trisomy 21, It is called Mosaic Down syndrome (46, XX/47, XX, +21).This can occur in one of two ways. A nondisjunction event during an early cell division in a normal embryo leads to a fraction of the cells with trisomy 21 or a Down syndrome embryo undergoes nondisjunction and some of the cells in the embryo revert back to the normal chromosomal arrangement.There is considerable variability in the fraction of trisomy 21, both as a whole and among tissues. This is the cause of 1–2% of the observed Down syndromes.

- Robertsonian translocation

The extra chromosome 21 material that causes Down syndrome may be due to a Robertsonian translocation.In this case, the long arm of chromosome 21 is attached to another chromosome,often chromosome 14 (45, XX, t(14;21q)) or itself (called an isochromosome, 45, XX, t (21q;21q)). Normal disjunctions leading to gametes have a significant chance of creating a gamete with an extra chromosome 21. Translocation Down syndrome is often referred to as familial Down syndrome. It is the cause of 2-3%

of observed cases of Down syndrome. It does not show the maternal age effect, and is just as likely to have come from fathers as mothers.

- Duplication of a portion of chromosome 21

Rarely, a region of chromosome 21 will undergo a duplication event. This will lead to extra copies of some, but not all, of the genes on chromosome 21 (46, XX, dup(21q))(*44*). If the duplicated region has genes that are responsible for Down syndrome physical and mental characteristics, such individuals will show those characteristics. This cause is very rare and no rate estimates are available.

G. Mapping

-Down Syndrome Critical Region

Mapping of the chromosomal region that, if triplicated, results in the phenotypic characteristics of Down syndrome has been facilitated by the use of DNA samples from individuals who have partial trisomy 21 with or without features of the Down syndrome phenotype (*45, 46, 47*). Although detailed analysis of these DNAs is still under way, an area of approximately 5 Mb between loci D21S58 and D21S42 has been identified that is associated with mental retardation and most of the facial features of the syndrome. In particular,a subregion that includes D21S55 and MX1, the latter being located in band 21q22.3, has been associated with mental retardation and several morphologic features, including oblique eye fissure, epicanthus,flat nasal bridge, protruding tongue, short broad hands, clinodactyly of the fifth finger, gap between first and second toes, hypotonia, short stature, Brushfield spots, and characteristic dermatoglyphics (*48*). Additional phenotypic characteristics may map outside the minimum critical region (symbolized DCR). Material from other rare patients who have features of Down syndrome but no visible chromosomal abnormality may help to narrow down the critical region.In several such studies, however,no triplicated region has been identified (*49,50*). It is possible that these patients do not have any chromosome 21 abnormality and their phenotype is a phenocopy of Down syndrome.

By analysis of a 3-generation Japanese family containing 4 Down syndrome individuals with partial trisomy 21 (*51, 52*) defined a 1.6-Mb region between LA68 and ERG in 21q22 as theDown syndrome critical region. They constructed a contig map covering more than 95% of this 1.6-Mb region.

-*Genes within Down Syndrome Critical Region*

A gene (*DSCR1*) from the Down syndrome critical region that is highly expressed in brain and heart,and suggested it as a candidate for involvement in the pathogenesis of DS, in particular mental retardation and/or cardiac defects (*51*).

Identified *DSCR2* within the Down syndrome critical region 2 between DNA marker D21S55 and MX1 (52).

Identified *DSCR3* within the Down syndrome critical region (*53*).

Identified *DSCR4* as 2 ESTs that map to the 1.6-Mb Down syndrome critical region. *DSCR4* is predominantly expressed in placenta (*54*).

Using indexing-based differential display PCR on neuronal precursor cells to study gene expression in Down syndrome (*55*), found that genes regulated by the *REST* transcription factor were selectively repressed.One of these genes, *SCG10*, which encodes a neuron-specific growth-associated protein, was almost undetectable. The REST factor itself was also downregulated by 49% compared to controls. In cell culture, the Down syndrome cells showed a reduction of neurogenesis, as well as decreased neurite length and abnormal changes in neuron morphology. The authors noted that REST-regulated genes play an important part in brain development, plasticity, and synapse formation, and they suggested a link between dysregulation of *REST* and some of the neurologic deficits seen in Down syndrome.

2. Down syndrome critical region 1gene (*DSCR1* gene)

A.*DSCR1* gene structure and function

The study of patients with partial trisomy 21 has defined an area of approximately 3 Mb at chromosomal region 21q22 as the minimal candidate region for the Down syndrome phenotype.Using a novel exon cloning strategy (*56)*, identified several putative exons from region 21q22.1-q22.2. One exon was used to isolate fetal brain cDNAs corresponding to a gene that the authors designated *DSCR1*.The predicted 171-amino acid protein contains 2 proline-rich regions, a putative DNA-binding domain, and an acidic region. Northern blot analysis revealed that the 2.2-kb *DSCR1* transcript is expressed at the highest levels in fetal brain and adult heart and at lower levels in various other tissues. An additional 2-kb mRNA was detected in fetal and adult liver. Increased expression in the brains of young rats compared with adults suggested to that *DSCR1* plays a role during central nervous system development.

-Gene function

Demonstrated that *DSCR1* protein is overexpressed in the brain of Down syndrome fetuses, and interacts physically and functionally with calcineurin A , the catalytic subunit of the Ca(2+)/ calmodulin-dependent protein phosphatase PP2B (57). The *DSCR1*-binding region in calcineurin A is located in the linker region between the calcineurin A catalytic domain and the calcineurin B-binding domain, outside of other functional domains previously defined in calcineurin A. *DSCR1* belongs to a family of evolutionarily conserved proteins with 3 members in humans: *DSCR1, ZAKI4* and *DSCR1L2*. Overexpression of *DSCR1* and *ZAKI4* inhibited calcineurin-dependent gene transcription through the inhibition of NFAT translocation to the nucleus. The authors hypothesized that members of this family of human proteins are endogenous regulators of calcineurin-mediated signaling pathways and may be involved in many physiologic processes.

In mice and humans, *MCIP1* is expressed primarily in cardiac and skeletal muscles (*58*), and transcription of the *MCIP1* gene, but not of the *MCIP2* gene, is

potently stimulated by activated calcineurin, thereby establishing a negative feedback mechanism that presumably serves to protect cells from otherwise deleterious consequences of unrestrained calcineurin activity (*59*), identified an alternative calcineurin-responsive promoter 5-prime of exon 4 in the *MCIP1* gene.

Referred to the proteins encoded by the *MCIP* genes as calcipressins (*60,61*). Functional analysis showed that when expressed in yeast, *DSCR1* and *ZAKI4* inhibited calcineurin function. The authors proposed that increased expression of *DSCR1* in trisomy-21 individuals may contribute to the neurologic, cardiac, or immunologic defects of Down syndrome.

Showing significant expression of *DSCR1* in brain, spinal cord, kidney, liver, mammary gland, skeletal muscle, and heart. Within the brain, *DSCR1* was predominantly expressed in neurons of the cerebral cortex, hippocampus, substantia nigra, thalamus, and medulla oblongata without regard to age in humans or rats (*62, 63*). Postmortem studies of 8 patients with Alzheimer disease and 8 controls showed that *DSCR1* expression in the cerebral cortex and hippocampus of AD patients was approximately double that of controls, and moreover, that *DSCR1* levels in brains with extensive neurofibrillary tangles were 3 times higher than in controls. Previous studies had shown that decreased calcineurin phosphatase activity allowed accumulation of hyperphosphorylated tau protein and cytoskeletal changes in the brain similar to those observed in AD (*64*). Since *DSCR1* inhibits calcineurin activity, (*65)* suggested that increased DSCR1 levels may cause accumulation of hyperphosphorylated tau protein and production of neurofibrillary tangles, thereby promoting the development of AD. Cell studies showed that toxic levels of aggregated amyloid beta 1-42 peptide directly stimulated *DSCR1* expression, perhaps as a protective mechanism against calcineurin-induced apoptosis.The authors suggested that while DSCR1 overexpression may initially be protective, chronic overexpression may eventually lead to the formation of neurofibrillary tangles associated with AD.

Demonstrated that *DSCR1* was induced in human endothelial cells in response to VEGF, TNFA, and calcium mobilization, and this upregulation was inhibited by

inhibitors of the calcineurin -NFAT signaling pathway, as well as by PKC inhibition and a calcium chelator (*66,67,68,69*) .Hypothesized that upregulation of *DSCR1* in endothelial cells may act as an endogenous feedback inhibitor of angiogenesis by regulating the calcineurin-NFAT signaling pathway.

Reported that 2 genes, DSCR1 and DYRK1A, that lie within the Down syndrome critical region of human chromosome 21 act synergistically to prevent nuclear occupancy of NFATc transcription factors, which are regulators of vertebrate development (*70*). Using mathematical modeling to predict that autoregulation within the pathway accentuates the effects of trisomy of *DSCR1* and *DYRK1A*, leading to failure to activate NFATc target genes under specific conditions (*71*).The authors' observations of calcineurin- and Nfatc-deficient mice, Dscr1- and Dyrk1a-overexpressing mice, mouse models of Down syndrome, and human trisomy 21 are consistent with these predictions. Suggested that the 1.5-fold increase in dosage of DSCR1 and DYRK1A cooperatively destabilizes a regulatory circuit, leading to reduced NFATc activity and many of the features of Down syndrome concluded that more generally, their observations suggest that the destabilization of regulatory circuits can underlie human disease (*72*).

-Gene structure

DSCR1 spans nearly 45 kb and contains 7 exons, 4 of which are alternative first exons. They found tissue-specific expression patterns for the alternative transcripts (73).

B. Mapping

By analysis of somatic cell hybrids and *YACs*, confirmed that the *DSCR1* gene is located at 21q22.1-q22.2. mapped the mouse *DSCR1* gene to chromosome 16 (*74,75*).

3. RNAi/siRNA technology

RNA interference (RNAi) is a naturally occurring cellular mechanism that induces post-transcriptional gene silencing in diverse cell types and has attracted much attention as a potential therapeutic strategy (*76*). Small interfering RNAs (siRNA) are short duplexes of RNA (21–23nt) that are the key intermediaries in this process. Since its discovery in the late 1990s (*77*), RNAi/siRNA technology has made a tremendous impact in molecular biology and has the potential to revolutionise future healthcare. The siRNA/RNAi technology has broad applications in target validation, probing gene function (transgenomics), and may serve as a potential therapeutic strategy for the inhibition of disease-associated genes. Indeed, many now consider RNAi/siRNA technology as the simplest, most effective gene silencing tool that has largely superceded its predecessors such as antisense oligonucleotides, ribozymes and DNAzymes (*78*).

A. Emergence of RNAi for posttranscriptional gene silencing

The technology of siRNA was borne out of work to elucidate the process of RNAi and post-transcriptional gene silencing initially pioneered in plants (*79*). In the early 1990s it was known that the introduction of transgenes into plants led to inhibition of the endogenous gene and the transgene itself a phenomenon termed as co-supression. It was also known that gene silencing occurred post-transcriptionally as transcripts from both genes were produced but were then degraded rapidly in the cytosol; hence, the term post-transcriptional gene silencing. The subsequent identification of small (21–23nt) fragments of double stranded (ds) RNA in plants strongly implied the involvement of dsRNA in this process (*80*).This led to the pioneering finding in the nematode, *Caenorhabditis elegans*, that long term gene silencing could indeed be achieved through the injection of dsRNA (*81*). Soon after, similar findings were described in other organisms: fruit flies, frogs(*82*), mice (*83*) and now human cells (*84*) implying that the natural RNAi process is conserved amongst a variety of invertebrate and vertebrate species.

RNAi operates by cleaving dsRNA with Dicer, an RNAse III nuclease, to form siRNA, the siRNA then causes significant down-regulation of homologous messenger RNA. The mechanism of action (covered in more detail below) is thought to differ between organisms, thus research is currently elucidating common pathways and key molecules in the process, which among other putative roles is commonly believed to be an antiviral defence mechanism (*85*). For example, the introduction of long dsRNA (greater than 30nts in length) into mammalian cells leads to a global inhibition of gene expression via a protein kinase dependent-mediated interferon response. However, siRNAs (less than 30nts) do not elicit this toxic immune response and cellular administration of these molecules can yield potent gene silencing. Thus, administration of siRNAs to cells offers a convenient way of activating the intracellular RNAi machinery against a specific gene of interest. The power of this application was only realized after Tuschl's laboratory showed that exogenously applied siRNAs can be used in cultured mammalian cells for targeted inhibition of gene expression (*86*). Their discovery lead to an explosion of activity in this area and currently it is one of the fastest developing fields in the academic and commercial sectors.

Although RNAi represents one of the most powerful biological tools ever to be introduced, recent literature reports have also highlighted several challenges facing this technology including issues relating to appropriate design, effective delivery, toxicity and specificity of action. Some of these issues appear similar to those encountered by conventional anti-mRNA approaches such as antisense oligonucleotides, ribozymes and deoxyribozymes (*87*). In this article, we will focus on reviewing the recent studies aimed at improving the design and exogenous (non-viral vector-mediated) delivery of siRNAs. The endogenous expression of dsRNA or short hairpinRNAs using viral vectors for cellular gene silencing is not covered and the reader is referred to other texts (*88*).

B. Mechanism of RNAi action

RNAi can be mediated by exogenous delivery of siRNA or micro RNA (miRNA), the latter is not discussed in detail here. The siRNAs are a class of molecules usually

comprising of 21–23 nucleotides (nts). duplexes characteristically housing two nucleotide overhangs at each 30 terminus . Fig. 3 schematically presents the anatomy of an exogenous synthetic siRNA duplex. Inside cells, these duplexes are recognized by the RNA-induced silencing complex (RISC) present in the cytosol (*89*), whereby the antisense RNA strand is then guided by this complex to the complementary sequence in target mRNA. The RISC complex then, by mechanisms that are not fully understood, initiates its hydrolysis and thus, prevents or “silences” gene expression (summarised in Fig. 4). Work is ongoing to unravel the detailed mechanism of RNAi, for instance a cytosolic siRNase enzyme was recently identified and implicated in the regulation of siRNA/RNAi through degradation of dsRNA and siRNA (*90*).

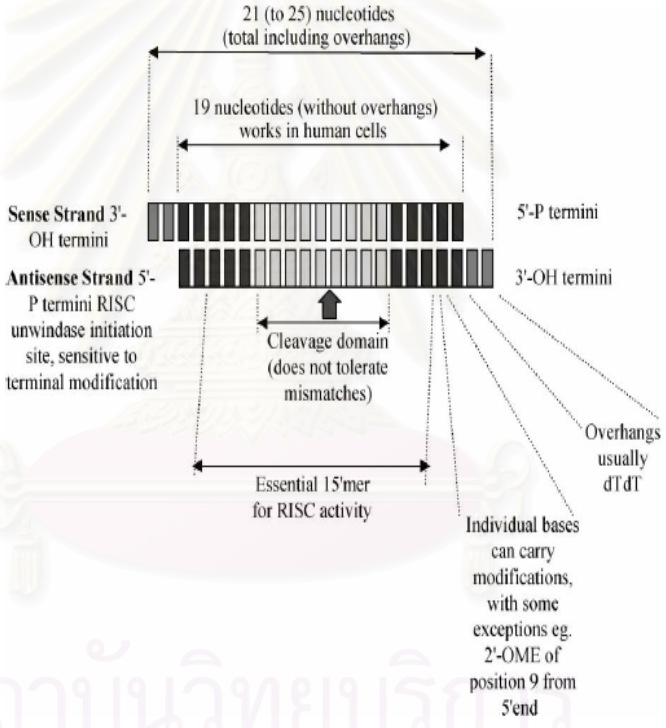


Figure 3. Anatomy of a synthetic siRNA duplex.

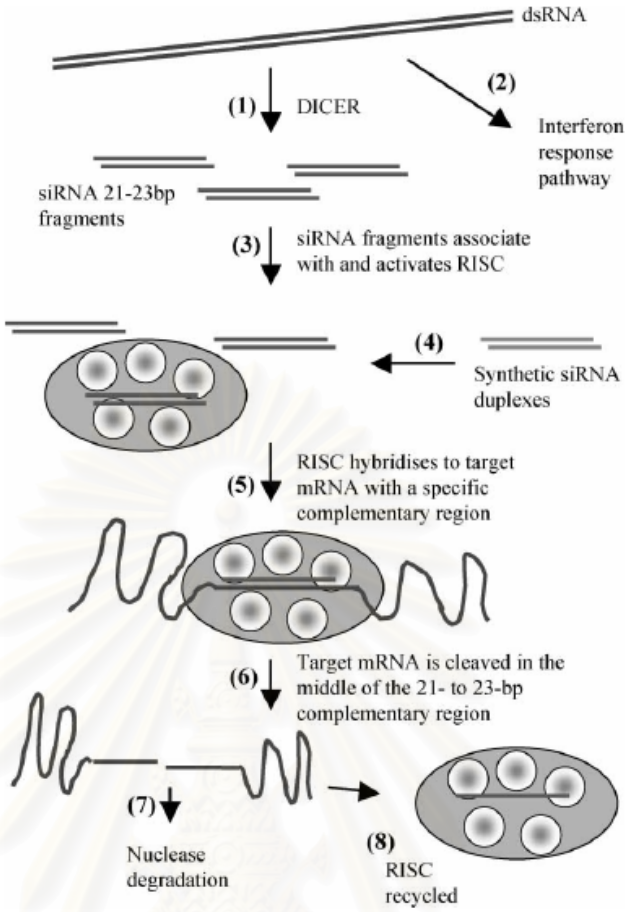


Figure 4. The RNA i/ siRNA pathway for gene silencing.

C. RNA-Induced Silencing Complex (RISC)

The RISC is a multi-component magnesium dependent nuclease that utilizes siRNA as a guide to target specific mRNA degradation (*89*). The exact composition of RISC remains unclear, although gradually its components are being verified. For instance, the components of RISC identified to date in *Drosophila* include Argonaute2 (AGO2) (*90*), fragile X protein (dFXR), Vasa intronic gene protein (VIG) (*91*) Tudor SN (staphylococcal nuclease) and siRNA (*92*). In human cells, however, much less has been determined, with the only identified components being single stranded siRNA, the Argonaute proteins eIF2C1 and eIF2C2 in HeLa cells (*93*) and Tudor SN (*94*). The putative RISC components and interactions are schematically summarised in Fig. 5. It has been demonstrated that an ATP dependent helicase unwinding of double stranded

siRNA is required before formation of the RISC complex (*95*). The unwindase action is proposed to be a DEAD box helicase p68 (*96*). The antisense strand of this unwound siRNA duplex directs the sequence-specific cleavage of the mRNA.

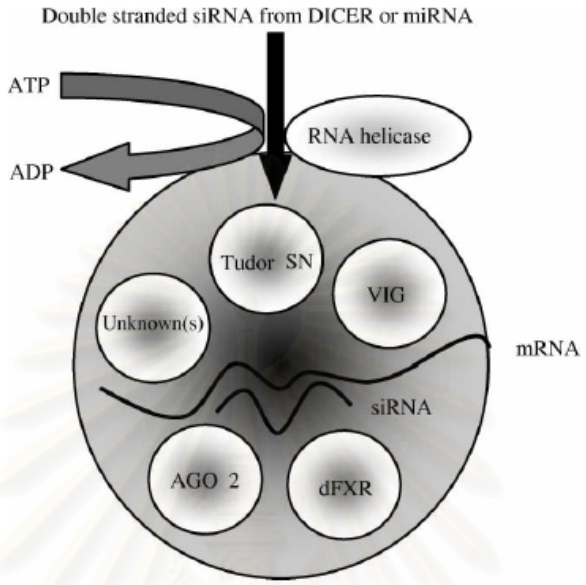


Figure 5. Current model for RISC complex in *Drosophila*.

It is clear that a number of key questions remain to be answered about the RISC complex. What is the total composition of the RISC complex? What is the interaction between the known components? Does RISC form as a response to the presence of siRNA or is it a preformed complex into which siRNA is incorporated? Is RISC localised to one area of the cell? Incrementally, our understanding of RISC is improving, and elucidating the answer to these and other questions that will enable a greater understanding of the mechanisms involved and a better exploitation of such knowledge both in terms of the cytosolic targeting of siRNA delivery vehicles and in the design of RISC favoured siRNAs.

RNAi in vertebrate cells relies on a cellular post-transcriptional gene regulatory mechanism that uses endogenously encoded, ~22- nucleotide (nt) , single-stranded RNAs to guide a ribonucleoprotein complex, the RNA inducd silencing complex (RISC), to target mRNAs. At least 300 of these microRNAs (miRNAs) are found in the human genome; they are believed to have a key role in regulation vertebrate differentiation and

development. miRNAs are initially transcribed by RNA polymerase II (Pol II) as part of a long primary miRNA (pri-miRNA) precursor (Fig 6). Mature miRNAs form part of one arm of an ~85-nt RNA stem-loop in the pri-miRNA. The first step in miRNA processing is mediated by the RNase III enzyme Drosha, which cleaves the stem ~22-nt away from the terminal loop to generate an ~65-nt pre-miRNA hairpin intermediate. Drosha cleavage defines one end of the mature miRNA and leaves a characteristic 2-nt 3' overhang. The pre-miRNA is transported to the cytoplasm by the nuclear export factor Exportin-5, where it interacts with a second RNase III enzyme called Dicer. Dicer binds the 2-nt 3' overhang found at the base of the pre-miRNA hairpin and cleaves ~22 nt away from the base, removing the terminal loop and leaving another 2-nt 3' overhang. The resultant duplex intermediate interacts with RISC components, including Argonaute-2, which selectively incorporate the RNA strand whose 5' end is less tightly base-paired. Once programmed, RISC can downregulate the expression of homologous mRNAs.

The miRNA biogenesis pathway includes three distinct RNA intermediates: the initial pri-miRNA transcript, the pre-miRNA hairpin and the miRNA duplex. All can be used as entry points to allow programming of RISC with artificial miRNAs called small interfering RNAs (siRNAs). The first approach to vertebrate RNAi to be described used transfection of synthetic siRNA duplexes, identical in structure to miRNA duplex intermediates, into cultured cells. This approach efficiently downregulates target mRNAs but has the disadvantage of being both transient and expensive. A second RNAi approach uses short hairpin RNAs (shRNAs) transcribed under the control of RNA polymerase III (Pol III). shRNAs are similar to pre-miRNA hairpins, and shRNA expression vectors can be used to establish stable RNAi responses in culture or in transgenic mice. The problem with the shRNA approach is essentially Pol III promoters, unlike Pol II promoters, do not lend themselves to regulation. A third approach to the induction of a specific RNAi response in mammalian cells relies on the transcription, by Pol II or Pol III, of artificial pri-miRNA transcripts that undergo the same processing steps as natural pri-miRNAs but result in the production of siRNA. Silva *et al.* (Silva.J.M.,2005) and Dickens *et al.* (*97*) show that such artificial pri-miRNAs not only induce an

exceptionally potent RNAi reponse but also allow regulated RNAi when using Pol II-dependent promoters to drive pri-miRNA expression.

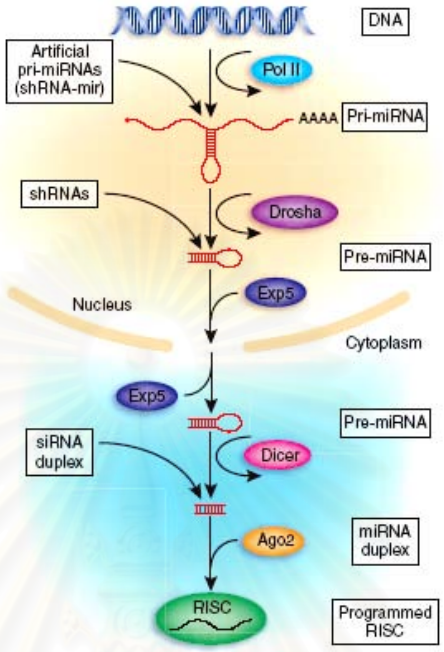


Figure 6. The miRNA biogenesis pathway in vertebrate cells.

D. siRNA Construction

In vivo expression of siRNAs can be effectively achieved by generating DNA cassettes that contain a U6 promoter, siRNA template and transcription termination sequences and then transfecting these cassettes into eukaryotics cells. In cells, RNA molecules, and U6 promoters have been used successfully to generate siRNAs in mammalian cells. Several approaches for RNA interference studies use this model. In one approach, individual promoters direct the transcription of sense and anti-sense strands of the siRNAs.The DNA units are generated by PCR and can be directly transfected into the cells or subcloned into appropriated vectors prior to transfection (*98*).

The siLentGene™-2 U6 Hairpin Cloning Systems use an approach in which siRNAs are expressed as fold-back stem-loop structures and are transcribed from the U6 promoter (*99*). A DNA cassette containing a U6 promoter,a hairpin siRNA target

sequence and the transcription termination sequence are generated by a single PCR amplification. The resulting PCR product can be directly transfected into human cells for rapid screening of optimal target sequences or may be subcloned into the provided dephosphorylated psiLentGene™-Basic Vector for transient suppression of the gene of interest. The systems also provide vectors with antibiotic resistance markers, neomycin, for long-term stable RNAi analysis.

- *Hairpin DNA Cassette*

To generate a DNA cassette containing hairpin structures, needed 1) A U6 cassette template containing the human U6 promoter; 2) An upstream primer that is complementary to the 5´ end of the U6 promoter region, and 3) A downstream primer that is complementary to the 3´ end of the promoter. The downstream primer contains the hairpin siRNA target sequence and a region that is complementary to the 3´ end of the U6 promoter.

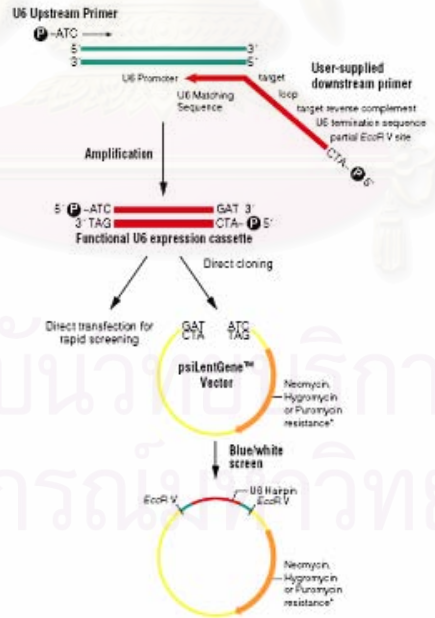


Figure7. Overview of the siLentGene™-2 U6 Hairpin Cloning Systems procedure.

E. Evolution

Based on parsimony-based phylogenetic analysis, the most recent common ancestor of all eukaryotes most likely already possessed an early RNA interference pathway; the absence of the pathway in certain eukaryotes is thought to be a derived characteristic. The ancestral RNAi system probably contained at least one dicer-like protein, one argonaute, one PIWI protein, and an RNA dependent RNA polymerase that may have also played other cellular roles(100). A large-scale comparative genomics study likewise indicates that the eukaryotic crown group already possessed these components, which may then have had closer functional associations with generalized RNA degradation systems such as the exosome. This study also suggests that the RNA-binding argonaute protein family, which is shared among eukaryotes, most archaea, and at least some bacteria (such as *Aquifex aeolicus*), is homologous to and originally evolved from components of the translation initiation system.

The ancestral function of the RNAi system is generally agreed to have been immune defense against exogenous genetic elements such as transposons and viral genomes (*101*). Related functions such as histone modification may have already been present in the ancestor of modern eukaryotes, although other functions such as regulation of development by miRNA are thought to have evolved later (*102*).

RNA interference genes, as components of the antiviral innate immune system in many eukaryotes, are involved in an evolutionary arms race with viral genes. Some viruses have evolved mechanisms for suppressing the RNAi response in their host cells, an effect that has been noted particularly for plant viruses. Studies of evolutionary rates in *Drosophila* have shown that genes in the RNAi pathway are subject to strong directional selection and are among the fastest-evolving genes in the *Drosophila* genome (*103*).

- Gene knockdown

The RNA interference pathway is often exploited in experimental biology to study the function of genes in cell culture and *in vivo* in model organisms. Double-stranded

RNA is synthesized with a sequence complementary to a gene of interest and introduced into a cell or organism, where it is recognized as exogenous genetic material and activates the RNAi pathway. Using this mechanism, researchers can cause a drastic decrease in the expression of a targeted gene. Studying the effects of this decrease can show the physiological role of the gene product. Since RNAi may not totally abolish expression of the gene, this technique is sometimes referred as a knockdown, to distinguish it from "knockout" procedures in which expression of a gene is entirely eliminated.

Extensive efforts in computational biology have been directed toward the design of successful dsRNA reagents that maximize gene knockdown but minimize off-target effects. Off-target effects arise when an introduced RNA has a base sequence that can pair with and thus reduce the expression of multiple genes at a time. Such problems occur more frequently when the dsRNA contains repetitive sequences. It has been estimated from studying the genomes of *H. sapiens*, *C. elegans*, and *S. pombe* that about 10% of possible siRNAs will have substantial off-target effects. A multitude of software tools have been developed implementing algorithms for the design of general(*104*), mammal-specific(*105*) and virus-specific(*106*) siRNAs that are automatically checked for possible cross-reactivity.

Depending on the organism and experimental system, the exogenous RNA may be a long strand designed to be cleaved by dicer, or short RNAs designed to serve as siRNA substrates. In most mammalian cells, shorter RNAs are used because long double-stranded RNA molecules induce the mammalian interferon response, a form of innate immunity that reacts nonspecifically to foreign genetic material (*107*). Mouse oocytes and cells from early mouse embryos lack this reaction to exogenous dsRNA and are therefore a common model system for studying gene-knockdown effects in mammals (*108*). Specialized laboratory techniques have also been developed to improve the utility of RNAi in mammalian systems by avoiding the direct introduction of siRNA, for example, by stable transfection with a plasmid encoding the appropriate sequence from which siRNAs can be transcribed, (*109*) or by more elaborate lentiviral

vector systems allowing the inducible activation or deactivation of transcription, known as conditional RNAi (*110*) .

-*Functional genomics*

Most functional genomics applications of RNAi in animals have used *C. elegans* (*111*) and *D. melanogaster*, (*112*) as these are the common model organisms in which RNAi is most effective. *C. elegans* is particularly useful for RNAi research for two reasons: firstly, the effects of the gene silencing are generally heritable, and secondly because delivery of the dsRNA is extremely simple.Through a mechanism whose details are poorly understood, bacteria such as *E. coli* that carry the desired dsRNA can be fed to the worms and will transfer their RNA payload to the worm via the intestinal tract. This delivery by feeding is just as effective at inducing gene silencing as more costly and time-consuming delivery methods, such as soaking the worms in dsRNA solution and injecting dsRNA into the gonads. Although delivery is more difficult in most other organisms, efforts are also underway to undertake large-scale genomic screening applications in cell culture with mammalian cells (*113*).

Approaches to the design of genome-wide RNAi libraries can require more sophistication than the design of a single siRNA for a defined set of experimental conditions. Artificial neural networks are frequently used to design siRNA libraries (*114*) and to predict their likely efficiency at gene knockdown. Mass genomic screening is widely seen as a promising method for genome annotation and has triggered the development of high-throughput screening methods based on microarrays. However, the utility of these screens and the ability of techniques developed on model organisms to generalize to even closely-related species has been questioned, for example from *C. elegans* to related parasitic nematodes.

Functional genomics using RNAi is a particularly attractive technique for genomic mapping and annotation in plants because many plants are polyploid, which presents substantial challenges for more traditional genetic engineering methods. For example, RNAi has been successfully used for functional genomics studies in the

hexaploid wheat *Triticum aestivum*, as well as more common plant model systems *Arabidopsis thaliana* and *Zea mays*.

F. Technological applications

-Medicine

It may be possible to exploit RNA interference in therapy. Although it is difficult to introduce long dsRNA strands into mammalian cells due to the interferon response, the use of short interfering RNA mimics has been more successful. The first applications to reach clinical trials were in the treatment of macular degeneration and respiratory syncytial virus. Developed by siRNA Therapeutics and Alnylam Pharmaceuticals respectively. RNAi has also been shown effective in the reversal of induced liver failure in mouse models.

Other proposed clinical uses center on antiviral therapies, including the inhibition of viral gene expression in cancerous cells, the silencing of hepatitis A and hepatitis B genes,silencing of influenza gene expression, and inhibition of measles viral replication. Potential treatments for neurodegenerative diseases have also been proposed, with particular attention being paid to the polyglutamine diseases such as Huntington's disease. RNA interference is also often seen as a promising way to treat cancer by silencing genes differentially upregulated in tumor cells or genes involved in cell division. A key area of research in the use of RNAi for clinical applications is the development of a safe delivery method, which to date has involved mainly viral vector systems similar to those suggested for gene therapy.

Despite the proliferation of promising cell culture studies for RNAi-based drugs, some concern has been raised regarding the safety of RNA interference, especially the potential for off-target effects in which a gene with a coincidentally similar sequence to the targeted gene is also repressed. A computational genomics study estimated that the error rate of off-target interactions is about 10%. One major study of liver disease in mice led to high death rates in the experimental animals, suggested by researchers to be the result of oversaturation of the dsRNA pathway.

When *DSCR1* is overexpress in DS fetal brain, it's possible that normalizing *DSCR1* expression may restore normal brain function in DS individual. In this study, the RNAi/siRNA technology is used for *DSCR1* gene silencing in lymphoblast cell. The si-RNA induces post-transcriptional gene silencing. The results from this study may serve as a potential therapeutic strategy for Down syndrome in future.

-Biotechnology

RNA interference has been used for applications in biotechnology, particularly in the engineering of food plants that produce lower levels of natural plant toxins. Such techniques take advantage of the stable and heritable RNAi phenotype in plant stocks. For example, cotton seeds are rich in dietary protein but naturally contain the toxic terpenoid product gossypol, making them unsuitable for human consumption. RNAi has been used to produce cotton stocks whose seeds contain reduced levels of delta-cadinene synthase, a key enzyme in gossypol production, without affecting the enzyme's production in other parts of the plant, where gossypol is important in preventing damage from plant pests. Similar efforts have been directed toward the reduction of the cyanogenic natural product linamarin in cassava plants.

Although no plant products that use RNAi-based genetic engineering have yet passed the experimental stage, development efforts have successfully reduced the levels of allergens in tomato plants and decreased the precursors of likely carcinogens in tobacco plants. Other plant traits that have been engineered in the laboratory include the production of non-narcotic natural products by the opium poppy,resistance to common plant viruses,and fortification of plants such as tomatoes with dietary antioxidants. Previous commercial products, including the Flavr Savr tomato and two cultivars of ringspot-resistant papaya, were originally developed using antisense technology but likely exploited the RNAi pathway.

4. Relative quantification real time PCR

The two most commonly used methods to analyze data from real-time, quantitative PCR experiment are absolute quantification and relative quantification.

Absolute quantification determines the input copy number, usually by relating the PCR signal to a standard curve. Relative quantification relates the PCR signal of the target transcript in a case to that of another sample such as an unrelated control. The $2^{-\Delta\Delta_{Ct}}$ method is a convenient way to analyze the relative changes in gene expression for realtime quantitative PCR experiments (*115*) .

First of all, the Δ c_t value for each sample is determined by calculating the differences between c_t value of the target gene and c_t value of the endogenous reference gene. This is determined for each unknown sample as well as for the calibrator sample.

Δc_t (sample) = c_t target gene - c_t reference gene

Δc_t (calibrator) = c_t target gene - c_t reference gene

Next, the $\Delta\Delta c_t$ value for each sample is determined by subtracting the Δc_t value of the calibrator from the Δc_t value of the sample.

$\Delta\Delta c_t = \Delta c_t$ (sample) - Δc_t (calibrator)

If the PCR efficiencies of the target gene and endogenous reference gene are comparable, the normalized level of target gene expression is calculated by using the formula normalized target gene expression level in sample = $2^{-\Delta\Delta_{Ct}}$

CHAPTER III

MATERIALS AND METHODS

Research instruments

1. Pipette tip : 10 µl, 200 µl, 1000 µl (Axygen)
2. Fiter-tip : 10 µl, 100 µl,200 µl, 1000 µl (Axygen)
3. Microcentrifuge tube : 0.2 ml, 0.5 ml, 1.5 ml (Axygen)
4. Polypropylene tube : 15 ml (Sterilin)
5. Beaker : 50 ml, 100 ml, 500 ml, 1000 ml (Pyrex)
6. Flask : 100 ml, 200 ml (Pyrex)
7. Bottle : 100 ml, 500 ml, 1000 ml (Pyrex)
8. Cylinder : 50 ml, 100 ml, 500 ml (Pyrex)
9. Glass pipette : 5 ml, 10 ml (Pyrex)
10. Pipette rack (Axygen)
11. Cell line culture flask (Corning)
12. Stirring-magnetic bar
13. Stirring hot plate (Schott)
14. Fume hood (Captair)
15. Micropipette : 10 µl, 20 µl, 100 µl, 200 µl, 1000 µl (Gilson)

16. Pipette boy (Brand)
17. Waterbath (Memmert)
18. Centrifuge (Haraeus)
19. Vortex (Scientific industries)
20. Microcentrifuge (Hettich)
21. Electrophoretic chamber (Amersham)
22. Electrophoretic power supply (Amersham)
23. Parafilm
24. Spectrophotometer (Thermo specific)
25. Cuvette (Starna)
26. Gel doc (Syngene)
27. Thermal cycler (MJ research)
28. Refrigerator (Sanyo)
29. Freezer : -20 ºC (Sanyo)
30. Freezer: -80 ºC (Thermo Forma)
31. Freezer: -196 ºC (Forma Scientific)
32. Comb (Amersham)
33. Gel doc software (Syngene)
34. Safety cabinet (Nuaire)
35. Incubator (Forma Scientific)

36. Realtime-PCR machine (MJ research)

37. Microcentrifuge tube for realtime PCR machine (Bio-Rad)

38. Microcentrifuge for realtime PCR machine (Bio-Rad)

39. Cryogenic vial (Corning)

40. Beacon designer software for TaqMan assay

41. Biotools software (Chang Bioscience)

42. MJ Opticon Monitor Analysis software version 3.1 (Bio-Rad)

43. 24 well pate

44. Semi-dry blotter (Weltech)

45. Gel gradient former

46. Film cassette

47. Medical X-ray film (Kodak)

Reagents

A. General reagents

1. Double distilled water
2. Absolute ethanol (Merck)
3. Phenol/chloroform (USB)
4. Sucrose (USB)
5. Tris base (USB)
6. EDTA (Fluka)

7. Hydrochloric acid (Merck)

8. Sodium chloride (Merck)

9. Ammonium acetate (USB)

10. Ethidium Bromide (Sigma)

11. Sodium hydroxide (AnalaR)

12. Sodium dodecyl sulfate (USB)

13. Proteinase K

14. Triton X-100 (USB)

15. 100 base pair ladder (Sigma)

16. 100% DMSO

17. RPMI (Biological Industries)

18. B-mercapto-ethanol

B. Reagents for PCR

1. 10X PCR buffer (Qiagen)

2. Magnesium chloride (Qiagen)

3. Deoxynucleotide triphosphates (dNTP) (Qiagen)

4. Oligonucleotide primers (Proligo)

5. HotstarTaq DNA polymerase (Qiagen)

C. Reagents for Reverse transcription

1. RNA blood mini kit (Qiagen)
2. Improm-II™ reverse transcription system (Promega)
3. RNase-free DNase set (Qiagen)
4. DEPC-treated water (USB)

D. Reagent for quantitative realtime-PCR

1. QuantiTect Probe PCR Kit (Qiagen)
2. Oligonucleotide primer (Proligo)
3. TaqMan probes (Operon)

E. Reagent for cloning and transfection

1. siLentGene-2 U6 Hairpin Cloning Systems (Human) Kit (Promega)
2. DNA purification Kit (Qiagen)
3. LB plate
4. LB media
5. *E.coli* strain JM109
6. SOC media
7. X-gal
8. IPTG
9. DsRed II plasmid (Takara Bio)
10. Oligonucleotide primer (Proligo)

11. Restriction enzyme EcoRV (Biolab)

12. Restriction enzyme NheI (Biolab)

13. Restriction enzyme XhoI (Biolab)

14. Amplicilin

15. G-418 (Sigma)

16. Lipofectamine 2000 (Invitrogen)

17. Qiaquick® gel extraction kit (Qiagen)

F. Reagent for Western Blotting

1. Tween20

2. PIPES (Sigma)

3. Proteinase inhibitor

4. TEMED

5. APS

6. Goat Anti-Calcipressin 1 / DSCR1 Antibody (Santacruz)

7. Donkey anti-goat antibody (Santacruz)

Procedure

1. Cell lines and cell culture

The peripheral blood samples of cases, who were already characterized from Rajanukul Insitute.

The 5 normal control samples and 10 case samples were already collected and processed as lymphoblast cell line.

RPMI media + 20% Fetal brovine serum and placed in 5% CO_2, 37°C incubator with humidity. Lymphoblast cells were grown in 7 days .

2. siRNA designing and cloning

A. siRNA primer design & amplification

siRNA is cloned by siLentGene-2 U6 Hairpin Cloning Systems-Neomycin (Human) Kit (Promega). siRNA downstream primer is designed by Promega siRNA designer program and prevented off-target by Blast program. The downstream primer contains the following: 5' phosphate group, partial EcoR V sequence to generate an EcoR V site when the amplified cassette is ligated into one of the psiLentGene vectors, U6 terminator sequence to allow the U6 polymerase to terminate with the 3' overhangs that are required for successful siRNAs, target sequence reverse complement-loop sequence-target sequence to create fold-back stem-loop structures and U6 cassette matching sequence to allow the down-stream primers to bind to the U6 cassette during amplification.

1. Down stream primer is designed and used for generate siRNA as follow;

EcoRV Half site	U6 Terminator	Target	Loop	Target reverse complement	U6Cassette Matching Sequence
5'-ATC	TAAAAA	GAGGACGCA TTCCAAATCA	AGAGAACTT	TGATTTGGAA TGCGTCCTC	GGTGTTTCGTCC TTTCCACAAGA

Table1 : siRNA sequence as down stream primer construct.

2. Prepare reaction mix as follow ;

Table 2 : Component for siRNA amplification

No.	PCR Reagent	Stock concentration	Final concentration
1	Nuclease-Free Water	variable	-
2	siLentGene High Fidelity PCR Master Mix (Promega)	2 X	1 X
3	siLentGene U6 Cloning Upstream Primer (Promega)	20 µM	0.4 µM
4	siRNA downstream primer (Proligo)	20 µM	0.4 µM
5	siLentGene U6 Cassette DNA template (Promega)	1ng/µl	0.02ng

Table 3 : PCR condition

Step	Temperature and incubation time
1. Initial denaturation	95°C/ 15 min
2. 30 cycles of Denaturation Annealing Extension	 94°C/ 30 sec 65°C / 60 sec 72°C/ 45 sec
3. Final extension	72°C/ 10 min

3. The amplification product is analyzed on a 1% agarose gel.

B. Ligation of amplified siRNA into psiLentGene vector

1. PCR product is cleaned up by DNA purification Kit (Qiagen) and ligated into psiLentGene vector (Promega).
2. Set up ligation reactions as described below.

Table 4 ; Component for ligation

No.	Ligation component	Standard reaction	Negative control (minus insert)
1	Nuclease-Free Water	variable	variable
2	2 X Rapid Ligation Buffer (Promega)	5 µl	5 µl
3	PCR product ~15ng	variable	-
4	3 units/ µl of T4 DNA Ligase (Proligo)	2 µl	2 µl
5	psiLentGene™ Vector (50ng)	1 µl	1 µl

3. Mix the reactions by pipetting and incubate the reactions overnight at 4°C.

E. Transformation of *E.coli*

E.coli strain JM109 competent cells are used for transformation of ligated reactions to produce a number of siRNA constructions.

Procedure

1. Prepare 2 LB/ampicillin/IPTG/X-Gal plates for each ligation reaction and control transformation. Equilibrate the plates to room temperature before plating.
2. Remove the frozen high-efficiency competent cells from –70°C storage and place them in an ice bath until just thawed (about 5 minutes). Mix the cells by gently flicking the tube.
3. For each ligation reaction and transformation control, carefully transfer 50 µl of the cells to a sterile 15 ml tube.
4. Briefly centrifuge the tubes containing the ligation reactions. Add 10 µl of each

ligation reaction to the tube prepared in Step 3. To perform a transformation control, add 0.1ng of supercoiled plasmid DNA (Promega) to 100 µl freshly thawed competent cells in a sterile 15ml tube.

5. Gently flick the tubes to mix and place them on ice for 20 minutes.
6. Heat-shock the cells for 45–50 seconds in a water bath at exactly 42°C. Do Not Shake.
7. Immediately return the tubes to ice for 2 minutes.
8. To the tubes containing cells transformed with the experimental ligations, add 950 µl of room temperature SOC medium. For the transformation controls, add 900 µl of room temperature SOC medium.
9. Incubate for at 37°C with shaking (approximately150rpm) for 1.5 hours.
10. Centrifuge cells at 1,000 × g for 10 minutes and resuspend in 200 µl of SOC medium.
11. Plate 100 µl of each transformation onto duplicate LB/ampicillin/ IPTG/X-Gal plates.
12. Incubate the plates overnight (16–24 hours) at 37°C.

F. Screening Transformants for Inserts

Successful cloning of an insert into the psiLentGen Vectors interrupts the coding sequence of ß-galactosidase; recombinant clones can usually be identified by color screening on indicator plates. The siLentGen U6 Cloning Upstream Primer includes stop codons in all possible reading frames to facilitate producing white colonies from inserts.

G. Purifying recombinant plasmid DNA

Standard miniprep procedure is used for isolating plasmid DNA.

Procedure

1. Pick up a single white colony and streak onto LB/ampicillin plate as a primary plate.
2. Incubate the plate overnignt (16-24 hours) at 37°C.
3. Pick up a single colony and streak onto LB/ampicillin plate as a secondary plate.
4. Incubate the plate overnignt (16-24 hours) at 37°C.
5. Pick up a single colony in 5 ml of LB/ampicillin broth and incubate on shaker with maximum speed at 37°C overnight (16-24 hours).
6. Pellet 1-1.5 ml aliquots of late log or stationary phase *E. coli* cultures for 30 minute in a microcentrifuge at 4°C maximum speed.
7. Remove the supernatant with a pipet tip.
8. Thoroughly resuspend the bacterial pellet in 100 µl of P1 buffer by pipetting the mixture up and down.
9. Add 200 µl of P2 buffer. Mix the contents by inverting the tube 4-6 times until the cell suspension “clears”.
10. Add 150 µl of P3 buffer. Mix the contents by inverting the tube 4-6 times. A white precipitate consisting of cellular debris and chromosomal DNA will appear.
11. Incubate mixture on ice for 5 minutes.
12. Pellet cellular debris by spinning in a microcentrifuge at maximum speed for 10 minutes at 4°C.
13. Transfer the supernatant solution to a clean tube. Be careful not to take any white precipitate to the next tube.
14. Add 900 µl of 100% Ethanol for precipitation of plasmid DNA. Mix the contents by inverting the tube and incubate at room temperature for 2 minutes.
15. Spin the tube in a microcentrifuge at maximum speed for 10 minutes at 4°C.
16. Carefully remove the ethanol with a pipet tip.

17. Wash pellet twice with 70% Ethanol being careful not to lose the plasmid DNA and centrifuge at maximum speed for 2 minutes at 4°C.
18. Dry the pellet at room temperature and resuspend with 50 µl of sterile water.
19. Measure the DNA concentration by absorbance at 260 nm.
20. Aliquots purified recombinant plasmid DNA for siRNA sequencing.

3. siRNA transfection into lymphoblast cell lines

Lipofectamine2000 reagent (Invitrogen) is used to transfect recombinant plasmid DNAs into lympholast cells with cationic lipid of lipofectamine2000 condenses DNA to compact structure within DNA-lipid complexes. These positively charged complexes are binding to and are entering the cell by endocytosis. The DNA-lipid-complexes acts as a proton-sponge that buffers the endosomal pH, since the hydrophilic unit contains several sites which can be protonated. Continuous proton influx induces osmotic swelling and rupture. Nucleic acids are released simultaneously from the DNA-lipid complex by progressive proton-assisted lipid layer disintegration.

Procedure

1. Prepare 0.5-1 x 10^6 cells in 500 µl of RPMI supplemented with 10% (v/v) FBS into 24-well plate.
2. Dilute recombinant plasmid DNA in 50 µl of RPMI without serum using concentration as follows:

Table 5 : Plasmid DNA and lipofectamine2000 concentration

DNA Concentration	DNA (µg) in media 50 µl	Lipofectamine2000 (µl) in media 50 µl
0.5-folds of DNA is recommend by manufactory	0.4	1
1-folds of DNA is recommend by manufactory	0.8	2
1.5-folds of DNA is recommend by manufactory	1.2	3

3. Mix Lipofectamine2000 gently before use, then dilute the appropriated amount in 50 µl of DMEM without serum.
4. Incubate for 5 minutes at room temperature.
5. After 5 minutes incubation, combine the diluted DNA with diluted Lipofectamine2000 (total volume 100 µl). Mix gently and incubate for 20 minutes at room temperature.
6. Add the 100 µl of complexes to each well containing cells and medium.Mix gently by rocking the plate back and forth.
7. Incubate cells at 37°C in a CO_2 incubator and subculture into new 25 cm^3 cultured flask.

4. siRNA transfection into fibroblast cell lines

Lipofectamine2000 reagent (Invitrogen) is used to transfect recombinant plasmid DNAs into fibroblast cells with cationic lipid of lipofectamine2000 .

Procedure

1. One day before transfection, plate 0.5-2 x 10^5 cells in 500 µl of DMEM supplemented with 10% (v/v) FBS into 24-well plate so that cells will be 90-95% confluent at the time of transfection.
2. Dilute recombinant plasmid DNA in 50 µl of DMEM without serum using concentration as follows:
3. Mix Lipofectamine2000 gently before use, then dilute the appropriated amount in 50 µl of DMEM without serum.

4. Incubate for 5 minutes at room temperature.

5. After 5 minutes incubation, combine the diluted DNA with diluted Lipofectamine2000 (total volume 100 µl). Mix gently and incubate for 20 minutes at room temperature.

6. Add the 100 µl of complexes to each well containing cells and medium. Mix gently by rocking the plate back and forth.

7. Incubate cells at 37°C in a CO_2 incubator and subculture into new 25 cm^3 cultured flask.

5. Selection of transfected cell lines

The psiLentGene-2 U6 hairpin cloning system-Neomycin (Human) kit (Promega) is consisted of neomycin antibiotic selection marker. Cell lines vary in the level of resistance to antibiotics, so the level of resistance of a particular cell line must be tested before attempting stable selection of the cells. A kill curve will determine the minimum concentration of the antibiotic needed to kill the untransfected cells. The antibiotic concentration for selection will vary depending on the cell type and growth rate. When cells are highly confluent, they are more resistant to antibiotic selection, so it is important to maintain the cells at a subconfluent level. The typical effective ranges and lengths of time needed for selection are given in Table 6.

Table 6 : Conditions for selection of stable transfectants.

Vector	Antibiotic	Effective antibiotic concentration	Time needed for selection
psiLentGene™-Neomycin	G-418	100 - 1,000 µg/ml	4 - 14 days

Procedure

1. Vary G-418 at concentrations of 0, 100, 200, 400, 600, 800 and 1,000 µg/ml in DMEM+ 10% (v/v) FBS.
2. Subculture nontransfected fibroblast cells at low concentration (30-50% Confluent) in 500 µl of DMEM with antibiotics from step 1 into 24-well plate.
3. Change the medium every 2-3 days until all nontransfected cells are dead.
4. Use the appropriate drug concentration and time for selection of transfected cell lines.

6. Uptake rate of siRNA into cell lines

Coding region of red florescence protein (RFP), 700 based pairs, are cloned into recombinant plasmid DNA and expressed under SV40 promoter by restriction site of enzyme NheI (Biolabs) and XhoI (Biolabs). RFP is amplified using DsRed2 plasmid (Takara Bio) as a template with recognition sequence of enzyme NheI (Biolabs) and XhoI (Biolabs) for the restriction endonuclease included at the 5' end of the primer. The primer sets, PCR components and PCR condition are shown as follow;

Table 7 : Primer sets for RFP cloning

Name	Restrinction enzyme	recognition site	Sequence (5' - 3')
RFP-NheI-F	NheI	5′...C▼TCGAG...3′ 3′...GAGCT▲C...5′	ATCGGCTAGCCGC CACCATGGCCTCCT
RFP-XhoI-R	XhoI	5′...G▼CTAGC...3′ 3′...CGATC▲G...5′	ATCGCTCGAGGTT CCTGTAGCGGCCGC

Table 8 : Reaction components for PCR/ 20 µl final volume

No.	Components	Stock concentration	Final concentration
1	Sterile dd H_2O	variable	-
2	Buffer(QIAGEN)	10 X	1 X
3	MgCl2(Qiagen)	-	-
4	dNTP(Biolabs)	25 mM	0.2 mM
5	Forward primer (Proligo)	10 µM	0.5 µM
6	Reverse primer(Proligo)	10 µM	0.5 µM
7	HotstarTaq(Qiagen)	5 unit/µl	0.625 unit
8	pDsRed 2 (Takara Bio)	100 pg-100 ng	

Recombinant plasmid vector and RFP PCR product are cleaved with enzyme NheI (Biolabs) and XhoI (Biolabs). After digestion, recombinant plasmid and PCR product are detected by 1% agarose gel electrophoresis and purified by Qiaquick® gel extraction kit (Qiagen).

Table 9 : Reaction component for digestion/ 50 µl final volume.

Digestion component	Stock concentration	Final concentration
NEBuffer 2 (Biolabs)	10X	1X
Bovine Serum Albumin-BSA (Biolabs)	100X	1X
XhoI (Biolabs)	20,000 U/ml	1 U/DNA 1 µg
NheI (Biolabs)	10,000 U/ml	1 U/DNA 1 µg
DNA	variable	
ddH2O	variable	

The digested PCR product and recombinant plasmid are ligated with enzyme T4 ligase. Ligated condition and component are shown as follow;

Table 10 : Reaction components for ligation/ 20 µl final volume

No.	Ligation component	Standard reaction	Negative control (minus insert)
1	Nuclease-Free Water	variable	variable
2	10 X Rapid Ligation Buffer (Promega)	2 µl	2 µl
3	PCR product ~15ng	variable	-
4	3 units/ µl of T4 DNA Ligase (Proligo)	1 µl	1 µl
5	psiLentGene™ Vector (50ng)	variable	variable

Recombinant RFP plasmids are selected by PCR amplification of RFP product using RFP-NheI forward primer and RFP-XhoI reverse primer and confirmed by sequencing.

7. RNA extraction

RNA extractions were performed using QIAamp RNA miniprotocol for isolation of total RNA from cultured cells QiAamp® RNA blood mini Kit.

Procedure

1. Centrifuge 10-ml cultured cells at 300 g for 5 minutes, remove supernatant.
2. Wash pellets two times with 1X PBS by pipette up and down, centrifuge at 300 x g for 10 minutes.
3. In final wash, rest 1 ml PBS for pellet resuspension.

4. Transfer suspension into new sterile 1.5-ml centrifuge tube.
5. Disrupt cells by add 600 µl of buffer RLT+ β mercapto-ethanol, mix by pipet
6. Pipet lysate into QIAshredder spin column sitting in a 2-ml collection tube, centrifuge at 13,000 rpm. Discard QIAshredder spin column and save homogenized lysate.
7. Add 1 volume of 70% ethanol to the homogenized lysate and mix well by pipetting.
8. Pipet sample in to a new spin column sitting in a 2-ml collection tube. Centrifuge for 15 seconds at 13,000 rpm.
9. Transfer the QIAamp spin column into a new 2-ml collection tube. Add 700 µl Buffer RW1 into spin column, centrifuge for 15 second at 13,000 rpm.
10. Transfer the spin column into a new collection tube. Pipet 500 µl buffer RPE in to the spin column and centrifuge for 15 seconds at 13,000 rpm.
11. Add 500 µl Buffer RPE, centrifuge at 13,000 rpm for 3 minutes.
12. Transfer the spin column into a new 1.5-ml collection tube and pipet 50 µl of RNase-free water directly on to the membrane, centrifuge for 1 minute at 13,000 rpm for elution.
13. Use immediately or store at -80°C until use.

8. DNase-I treatment

The mRNA samples are treated with DNase-I to eliminate the residue genomic DNA which might interfere with subsequent processes.

9. Reverse transcription

The DNase-I treated mRNA samples are transcribed to cDNA by reverse transcriptase using oligo dT primer. To prevent RNase contamination, DEPC-treated water and RNase inhibitor are applied.

Procedure

A. Target RNA and primer combination and denaturation

1. Place sterile thin-walled dilution tubes and reaction tubes on ice.
2. Prepare the reaction as follow:

Table 11 : Components for RNA-Primer combination and denaturation

No.	Component	Volume
1	RNA template	Up to 1 Ug
2	Oligo-dT Primer	20pmol or 0.5 Ug
3	Nuclease free water	Adjust to 5 Ul

3. Incubate at 70ºC for 5 minutes.
4. Chill on ice immediately at 4ºC for 5 minutes.

B. Reverse transcription

1. Prepare reaction mix (table 12)

Table 12 : Components for reverse transcription/ 15 µl total volume

No.	Component	Stock concentration	Final concentration
1	Nuclease-free water	variable	-
2	ImProm-II™ reaction buffer	5X	1X
3	$MgCl_2$	25mM	3mM(1.5-8)
4	dNTP mix	25mM	0.5mM
5	Recombinant RNasin®Ribonuclease inhibitor		20 unit
6	Vortex the mixture, add Improm-II™Reverse Transcriptase		

2. Aliquot reaction mix to each reaction tube on ice. Add 5 µl of RNA and primer mix to each reaction.
3. Anneal by incubate at 25°C for 5 minutes.
4. Extend by incubate at 42°C for one hour.
5. Inactivate enzyme by incubate at 70°C for 15 minutes
6. Proceed next step or store at -20°C until use.

10. RT-PCR

The primer sets were design to cover *DSCR1* mRNA exon 3 and 4.

Table 13 : Components for RT-PCR

No.	Components	Stock concentration	Final concentration
1	Sterile ddH2O	variable	-
2	Buffer(QIAGEN)+15 mM MgCl2	10 X	1 X
3	MgCl2 (Qiagen)	25mM	0.5 mM
4	dNTP(Biolabs)	25 mM	0.2 mM
5	Forward primer (Proligo)	10 µM	1 µM
6	Reverse primer(Proligo)	10 µM	1 µM
7	HotstarTaq(Qiagen)	5 unit/ µl	2.5-5 unit
8	cDNA	variable	< 500 ng/ reaction

Table 14 : RT-PCR condition

Step	Temperature and incubation time
1. Initial denaturation	95°C/ 15 min
2. 35 cycles of	
Denaturation	94°C/ 1 min
Annealing	62°C / 1 min
Extension	72°C/ 1 min
3. Final extension	72°C/ 10 min

11. Quantitative real-time PCR

Since relative quantification is used for determine the expression level of interested gene compare with the house-keeping gene, the PCR efficiency must be controlled. Before proceed to a relative quantification, the validation of multiplexed PCR must be completed.

Table 15 : Probe-primer mixture preparation

No.	Components	Stock concentration	Final concentration
1	RNase-free water	-	-
2	Forward primer	100 µM	8 µM
3	Reverse primer	100 µM	8 µM
4	Probe	100 µM	4 µM

Table 16 Genes, primer sets and probes for quantitative real-time PCR

Gene	Region	Selected position	Name	Sequence	Annealing Temperature (ºC)	Size (bp)
DSCR1	CDS	6, 7	DSCR1F	GCCAAATCCAGACAAGCAGTTT	56	149
			DSCR1R	CGTGCAATTCATACTTTTCCCCT	56	
			DSCR1P	CCCTCCCGCCTCTCCGCCAGT	69	
GAPDH		5'UTR-exon3	GAPDHF	CGACAGTCAGCCGCATCTTC	56	104
			GAPDHR	CGCCCAATACGACCAAATCCG	56	
			GAPDHP	CGTCGCCAGCCGAGCCACATCG	72	

Note: The 5' terminal of *DSCR1* probe was labeled with FAM, while 3' was labeled with TAMRA.

The 5' terminal of GAPDH probe was labeled with Cy5, while 3' was labeled with BHQ (Black hole quencher).

Table 17 : Multiplexed-PCR components/ 20 µl total volume

No.	Components	Stock concentration	Final concentration
1	RNase-free water	-	-
2	Quantitect PCR master mix	2X	1X
3	Probe-primer mix (*GAPDH*)	8 µM	0.4 µM
4	Probe-primer mix (*DSCR1*)	8 µM	0.4 µM
5	cDNA	variable	< 500 ng/ reaction

Table 18 : Multiplexed PCR condition

Step	Temperature and incubation time
1. Initial denaturation	95°C/ 15 min
2. 40 cycles of	
Denaturation	94°C/ 15 sec
Annealing/extension	60°C / 30 sec
Read plate	

11. Agarose gel electrophoresis

1% agarose gel is used for observe the PCR products from RT-PCR and multiplexed PCR.

12. Protein Extraction

Protein extractions were performed the using miniprotocol for Isolation of cytoplasmic and nuclear protein from cultured cells.

Procedure

1. Centrifuge 10-ml cultured cells at 300 x g for 5 minutes, remove supernatant.
2. Wash pellets two times with ice cold 1X PBS by pipette up and down, centrifuge at 300 x g for 10 minutes.
3. The final washing, rest 1 ml PBS for pellet resuspension.
4. Transfer suspension into new sterile 1.5-ml centrifuge tube.
5. Disrupt cells by add 200 µl of freshly prepared lysed buffer.
6. Briefly mix and incubate on ice for 20 minutes.
7. Centrifuge at 5000 rpm at 4^{o}C for 5 minutes.
8. Save supernatant for cytoplasmic compartment on ice.
9. Wash nuclear cell pellet twice with 400-600 µl of ice cold 1X PBS and centrifuge at 5000 rpm at 4^{o}C for 5 minutes.
10. Resuspend cell pellet in 200 µl of freshly prepared lysis buffer.
11. Mix by vortex and incubate on ice for 10 minutes.

12. Save product for nuclear compartment on ice.

13. Incubate product from step 8 and 12 for 10 seconds with 1 not sonicator 3 Times and chill on ice immediately.

13. Protein assay

Bradford method is used to measure protein by BIORAD kit.

Procedure

1. Prepare standard protein solution with BSA at concentration 1.5mg/ml, 1.2 mg/ml, 1 mg/ml, 0.8 mg/ml, 0.4 mg/ml, and 0.2 mg/ml.
2. Prepare reagent A' = reagent A 1ml + reagent S 20 μl
3. Prepare solution with reagent B 400 μl + reagent A' 50 μl + sample 10 μl
4. Measure standard and sample protein at O.D. 750nm

14. Calcineurin activity assay

The calcineurin activity assay is a kit that used for measurement of calcineurin activity by incubation with RII phophopeptide, a known substrate for calcineurin, and the free phosphate released is detected based on the malachite green assay. In cellular extracts RII phosphopeptide is also cleaved by other phosphatases; i.e. PP1, PP2A and PP2C. Using calcineurin activity assay may not a good choice for the measurement of calcipressin quantification.

15. Western blot

A western blot (alternately, immunoblot) is a method in molecular biology/biochemistry/immunogenetics to detect protein in a given sample of tissue homogenate or extract. It uses gel electrophoresis to separate native proteins by shape and size. The proteins are then transferred out of the gel and onto a membrane (typically

nitrocellulose), where they are "probed" using antibodies specific to the protein. As a result, researchers can examine the size, processing, or amount of protein in a given sample and compare several groups.

A. Polyacrylamide gel preparation

Procedure

1. Prepare 6% and 18 % polyacrylmide gel and pour into gel gradient former chamber A and B.
2. Set glass plate cassette and open valve to pour gel into the cassette.
3. 100 µg of protein samples and 1X loading dye solution are loaded into each well.
4. Proteins are separated by size at 200V for 50 minutes.

B. Transfer and blocking

Proteins are transferred from gel to nitrocellulose membrane by semi-dry blotter. The other space of nitrocellulose membrane is filled with protein in blocking solution.

Procedure

1. Gel is washed with transfer buffer on shaker for 5 minutes and placed on Saturated membrane and filter paper. Roll out all air bubbles.
2. Transfer protein from gel to membrane at 250 mA for 30 minutes.
3. Membrane is washed with 1XTBST for 5 minutes two times.
4. Incubate membrane with blocking solution for 1 hour at room temperature.

C. Antibody blotting

DSCR1 protein is attached with *DSCR1* antibody as primary antibody and then bound to Donkey anti-goat antibody as secondary antibody.

Procedure

1. Goat *DSCR1* antibody is used as primary antibody and diluted with blocking solution at 1:200.
2. Donkey anti-goat antibody is used as secondary antibody and diluted with blocking solution at 1:2000.
3. Wash membrane with 1XTBST for 5 minutes 3 times on shaker.
4. Seal membrane in plastic bag and fill with 5ml-diluted goat *DSCR1* antibody.
5. Incubate over night at 4°C on shaker.
6. After incubation, wash membrane with 1XTBST for 5 minutes 3 times.
7. Seal membrane in plastic bag, fill with 3 ml-diluted Donkey anti-goat antibody and incubate at room temperature for 1 hour.

D. Detection

The secondary antibody is conjugated with horse radish peroxidase (HRP) enzyme and detected by western blotting luminol reagent (SantaCruz), a non-radioactive, light-emitting system for detection proteins on a membrane. HRP mediates oxidation of luminol in the presence of hydrogen peroxide and this reaction produces an iridescent light, is detected by X-ray film.

Procedure

1. Wash membrane with 1XTBST for 5 minutes 3 times.
2. Mix 4ml per each of western blotting luminol reagent A and reagent B (Santa cruz).

3. Incubate membrane with homogeneous solution for 5 minutes at room temperature.
4. Remove the exceed reagent and enfold membrane with plastic wrap.
5. Medical X-ray film is placed on the membrane in dark room and contained by film cassette for 1 Hour.
6. Film is washed with developer solution for 10 second, washed with water 2 times and fixed by fixer solution for 10 minutes.

16. Coomasie blue gel staining

Coomassie (also known as Brilliant Blue, Brilliant Blue G, Acid Blue 90, C.I. 42655, or Brilliant Blue G 250) is a blue dye commonly used in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). The gel is soaked in dye and then destained. This treatment allows the visualization of bands indicating the protein content of the gel. The visualization on the gel usually contains a set of molecular weight marker so that protein molecular weight can be determined in an unknown solution.

Procedure

1. Fix gel with fixing solution for 30 minutes.
2. Gel is stained by coomasie working solution for 25 minutes.
3. Destain gel with destaining solution on shaker until background is cleared.

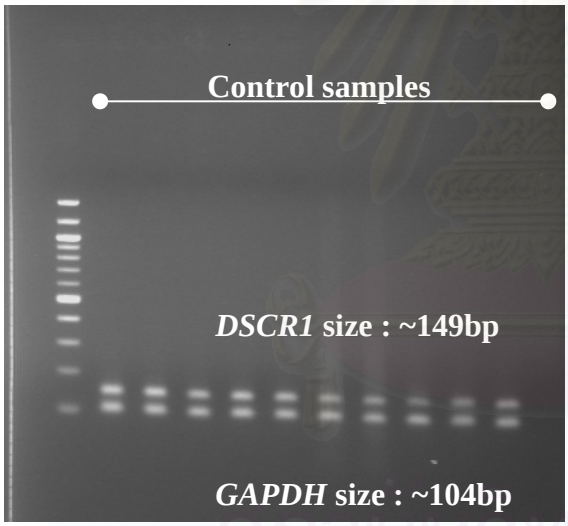
CHAPTER IV

RESULTS

1. RT-PCR

The total RNAs were extracted from treated cell line at 14 days of post-transfection with siRNA. The mRNA samples were successfully transcribed without any contamination of residue genomic DNA by application of DNase-I treatment and contained with *DSCR1* gene.

A house keeping gene, *GAPDH*, was also expressed in both case and control samples as expected



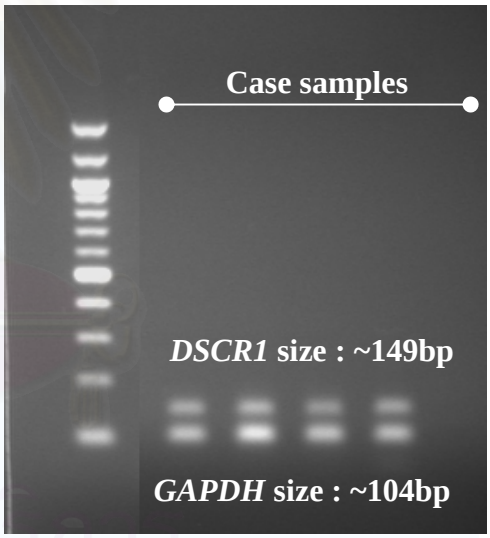


Figure 8 RT-PCR from control cDNA samples. **Figure 9** RT-PCR from case cDNA samples.

2. Real-time PCR

Duplex RT-PCR were successfully done in all cDNA samples before proceed to real-time PCR. The result from real-time PCR in controls showed no signs of probes degradation or genomic DNA contamination. The 10 normal control samples average Ct of *GAPDH* and *DSCR1* were shown in Table 19.

2.1 No significant differences among 10 normal control samples

Relative quantification of 10 control samples showed no significant differences among ten normal control samples (Table 19 and Fig 11).

Table 19 : The average C_t of *GAPDH* and *DSCR1*.

Sample	Note	FAM (c_t)	CY5 (c_t)	Δc_t	Gene expression	Cal1	Cal2	Cal3	Cal4	Cal5	cal6	cal7	cal8	cal9	cal10
550	Control	23.39	19.45	3.94	0.869197	1	1.3566043	0.5946036	0.7022224	0.6925547	1.5800826	0.4796321	1.0069556	1.6021398	0.9201877
554	Control	24.78	20.4	4.38	0.6407152	0.7371346	1	0.4383029	0.5176325	0.5105061	1.1647336	0.3535534	0.7422618	1.1809927	0.6783022
618	Control	22.63	19.44	3.19	1.4618093	1.6817928	2.2815274	1	1.1809927	1.1647336	2.6573716	0.8066418	1.6934906	2.6944672	1.547565
619	Control	24.22	20.79	3.43	1.2377802	1.4240502	1.9318727	0.8467453	1	0.9862327	2.250117	0.6830201	1.4339552	2.2815274	1.3103934
622	Control	25.3	21.89	3.41	1.2550589	1.4439292	1.9588406	0.8585654	1.0139595	1	2.2815274	0.6925547	1.4539725	2.3133764	1.3286858
640	Control	23.91	19.31	4.6	0.5500959	0.6328783	0.8585654	0.3763117	0.4444213	0.4383029	1	0.3035487	0.6372803	1.0139595	0.5823668
644	Control	23.83	20.95	2.88	1.8122162	2.0849315	2.8284271	1.2397077	1.4640857	1.4439292	3.2943641	1	2.0994334	3.3403517	1.9185282
648	Control	23.62	19.67	3.95	0.863193	0.9930925	1.3472336	0.5904963	0.6973718	0.6877709	1.5691682	0.476319	1	1.591073	0.9138315
649	Control	22.24	17.62	4.62	0.5425226	0.6241653	0.8467453	0.3711309	0.4383029	0.4322686	0.9862327	0.2993697	0.6285067	1	0.5743492
654	Control	23.34	19.52	3.82	0.9445867	1.0867349	1.4742692	0.6461764	0.7631296	0.7526234	1.7171309	0.5212329	1.0942937	1.7411011	1

Note: The gene expression is the average value of normalized target gene expression level in sample using all 10 control samples as calibrators.

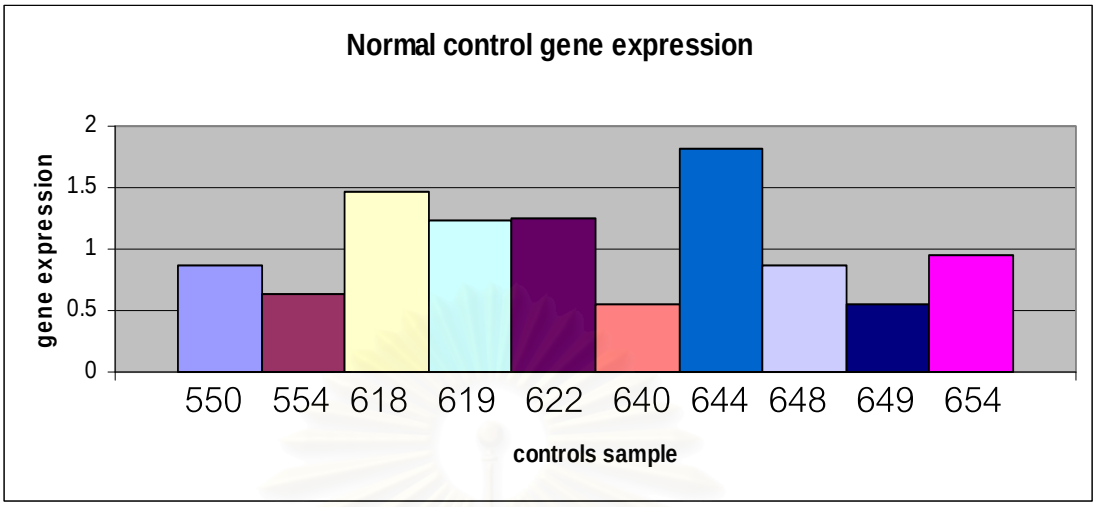


Figure 10 Normal controls *DSCR1* gene expression

Table 20 : Statistic calculation of standard derivative of 10 control samples.

Variable	N	Mean	Std Dev
Gene expression	10	1.0177	0.4178

The standard deviation was less than 2 indicate that there were no significant differences among 10 normal control samples at 95% confidence (Table 20).

2.2 Gene expression using five control samples as calibrators

In experiment, 5 control samples were selected from 10 control samples to compare with treated siRNA sample and harvested at 14^{th} day. The average C_t of *GAPDH* and *DSCR1* were shown as follow;

Table 21 : The average c_t of *GAPDH* and *DSCR1*

Sample	Note	FAM(Ct)	CY5(Ct)	dCt	OVERALL MEAN	Cal1	Cal2	Cal3	Cal4	Cal5
648	Normal	21.55	14.4	7.15	3.942509249	1	10.05611	4.055838	3.031433	1.569168
644	Normal	32.28	21.8	10.48	0.392051243	0.099442	1	0.403321	0.301452	0.156041
622	Normal	21.86	12.69	9.17	0.97205789	0.246558	2.479415	1	0.747425	0.386891
618	Normal	30.54	21.79	8.75	1.300543036	0.329877	3.317278	1.337928	1	0.517632
550	Normal	21.26	13.46	7.8	2.512483531	0.63728	6.408559	2.584706	1.931873	1
954	Down	29.05	21.82	7.23	3.729841022	0.946058	9.513657	3.837056	2.86791	1.484524
953	Down	31.85	24.61	7.24	3.704077128	0.939523	9.447941	3.810552	2.8481	1.474269
1502	Down	28.30	20.33	7.97	2.233202598	0.566442	5.696201	2.297397	1.717131	0.888843
1500	Down	27.01	20.42	6.59	5.812320024	1.474269	14.82541	5.979397	4.469149	2.313376
1318	Down	32.45	24.21	8.24	1.852038564	0.469761	4.723971	1.905276	1.42405	0.737135
1266	Down	29.88	22.71	7.17	3.888231559	0.986233	9.917662	4	2.989698	1.547565
1211	Down	26.18	16.44	9.74	0.654794514	0.166086	1.670176	0.673617	0.503478	0.260616
1126	Down	36.31	27.17	9.14	0.992482892	0.251739	2.531513	1.021012	0.76313	0.395021
1122	Down	26.73	19.20	7.53	3.029572308	0.768438	7.727491	3.116658	2.329467	1.205808
1040	Down	30.01	24.22	5.79	10.11983694	2.566852	25.81254	10.41073	7.78124	4.027822

Table 21 : The average c_t of *GAPDH* and *DSCR1* (continue)

Sample	Note	FAM(Ct)	CY5(Ct)	dCt	OVERALL MEAN	Cal1	Cal2	Cal3	Cal4	Cal5
550 0.5	Normal 0.5	20.98	13.15	7.83	2.460777367	0.624165	6.276673	2.531513	1.892115	0.97942
618 0.5	Normal 0.5	27.96	21.71	6.25	7.356982397	1.866066	18.76536	7.568461	5.656854	2.928171
644 0.5	Normal 0.5	24.13	13.57	10.56	0.370903076	0.094078	0.946058	0.381565	0.285191	0.147624
648 0.5	Normal 0.5	23.18	13.22	9.96	0.562183938	0.142595	1.433955	0.578344	0.432269	0.223756
662 0.5	Normal 0.5	23.42	13.83	9.59	0.726540003	0.184284	1.853176	0.747425	0.558644	0.289172
550 1	Normal 1	24.5	18.3	6.2	7.616425821	1.931873	19.42712	7.835362	5.856343	3.031433
618 1	Normal 1	23.4	14.71	8.69	1.355771585	0.343885	3.458149	1.394744	1.042466	0.539614
662 1	Normal 1	30.41	19.83	10.58	0.365796744	0.092783	0.933033	0.376312	0.281265	0.145592
644 1	Normal 1	24.29	15.4	8.89	1.180267717	0.29937	3.010493	1.214195	0.907519	0.469761
648 1	Normal 1	24.15	14.03	10.12	0.503168719	0.127627	1.283426	0.517632	0.386891	0.200267
550 1.5	Normal 1.5	25.94	22.6	3.34	55.29641862	14.02569	141.0439	56.88593	42.51795	22.00867
618 1.5	Normal 1.5	28.33	17.43	10.9	0.293028753	0.074325	0.747425	0.301452	0.225313	0.116629
662 1.5	Normal 1.5	23.13	12.7	10.43	0.4058769	0.102949	1.035265	0.417544	0.312083	0.161544
644 1.5	Normal 1.5	23.46	13.74	9.72	0.663935105	0.168404	1.693491	0.68302	0.510506	0.264255
648 1.5	Normal 1.5	23.32	12.99	10.33	0.435008091	0.110338	1.109569	0.447513	0.334482	0.173139

Table 21 : The average c_t of *GAPDH* and *DSCR1* (continue)

Sample	Note	FAM(Ct)	CY5(Ct)	dCt	OVERALL MEAN	Cal1	Cal2	Cal3	Cal4	Cal5
954 0.5	Down 0.5	21.95	14.69	7.26	3.653082004	0.926588	9.317869	3.758091	2.80889	1.453973
953 0.5	Down 0.5	23.01	17.47	5.54	12.03458209	3.052518	30.69645	12.38052	9.253505	4.789915
1502 0.5	Down 0.5	22.59	14.48	8.11	2.026674135	0.514057	5.169411	2.084932	1.558329	0.806642
1122 0.5	Down 0.5	24.21	18.34	5.87	9.573949123	2.42839	24.42015	9.849155	7.361501	3.810552
1040 0.5	Down 0.5	22.57	15.99	6.58	5.852747907	1.484524	14.92853	6.020987	4.500234	2.329467
1126 0.5	Down 0.5	24.35	15.59	8.76	1.291559529	0.327598	3.294364	1.328686	0.993092	0.514057
1211 0.5	Down 0.5	22.76	15.94	6.82	4.955786855	1.257013	12.64066	5.098243	3.810552	1.972465
1266 0.5	Down 0.5	23.37	18.83	4.54	24.06916419	6.105037	61.3929	24.76104	18.50701	9.57983
1318 0.5	Down 0.5	23.2	18.09	5.11	16.21339308	4.112455	41.35529	16.67945	12.46663	6.453134
1500 0.5	Down 0.5	21.37	14.48	6.89	4.721070868	1.197479	12.04197	4.85678	3.630077	1.879045
953 1	Down 1	30.9	24.69	6.21	7.563815325	1.918528	19.29293	7.78124	5.81589	3.010493
954 1	Down 1	23.33	16.07	7.26	3.653082004	0.926588	9.317869	3.758091	2.80889	1.453973
1040 1	Down 1	22.52	14.61	7.91	2.328037245	0.590496	5.938094	2.394957	1.79005	0.926588
1502 1	Down 1	24.98	21.37	3.61	45.85840078	11.63178	116.9704	47.17661	35.26096	18.25222
1122 1	Down 1	35.23	28.14	7.09	4.109930904	1.042466	10.48315	4.228072	3.160165	1.635804

Table 21 : The average c_t of *GAPDH* and *DSCR1* (continue)

Sample	Note	FAM(Ct)	CY5(Ct)	dCt	OVERALL MEAN	Cal1	Cal2	Cal3	Cal4	Cal5
1318 1	Down 1	31.57	25.05	6.52	6.1012893	1.547565	15.56248	6.276673	4.69134	2.42839
1500 1	Down 1	29.89	25.11	4.78	20.38045195	5.169411	51.98415	20.96629	15.67072	8.111676
1211 1	Down 1	23.54	19.9	3.64	44.91464854	11.3924	114.5632	46.20573	34.5353	17.87659
1266 1	Down 1	24.33	16.77	7.56	2.967224612	0.752623	7.568461	3.052518	2.281527	1.180993
1126 1	Down 1	22.48	15.03	7.45	3.202312585	0.812252	8.168097	3.294364	2.462289	1.274561
953 1.5	Down 1.5	21.3	14.28	7.02	4.314263038	1.094294	11.00433	4.438278	3.317278	1.717131
1040 1.5	Down 1.5	22.56	15.31	7.25	3.678491199	0.933033	9.38268	3.784231	2.828427	1.464086
954 1.5	Down 1.5	22.57	16.37	6.2	7.616425821	1.931873	19.42712	7.835362	5.856343	3.031433
1502 1.5	Down 1.5	24.27	17.63	6.64	5.614331067	1.42405	14.3204	5.775717	4.316913	2.234574
1122 1.5	Down 1.5	25.21	16.35	8.86	1.205067651	0.30566	3.07375	1.239708	0.926588	0.479632
1318 1.5	Down 1.5	23.48	19.86	3.62	45.54163366	11.55143	116.1625	46.85074	35.0174	18.12614
1500 1.5	Down 1.5	23	20.55	2.45	102.4740027	25.99208	261.3791	105.4197	78.79324	40.78594
1211 1.5	Down 1.5	20.46	13.92	6.54	6.017291047	1.526259	15.34823	6.19026	4.626753	2.394957
1266 1.5	Down 1.5	24.43	17.21	7.22	3.755784118	0.952638	9.57983	3.863745	2.887858	1.494849
1126 1.5	Down 1.5	26.07	21.48	4.59	23.2492801	5.897077	59.30164	23.91759	17.87659	9.253505

2.3 Gene expression using five control samples as calibrators

In experiment, 5 control samples were selected from 10 control samples to compare with treated siRNA sample and harvested at 5th day. The average $\mathbf{C_t}$ of *GAPDH* and *DSCR1* were shown as follow;

Table 22 : The average c_t of *GAPDH* and *DSCR1*

Sample	Note	FAM(Ct)	CY5(Ct)	dCt	OVERALL MEAN	Cal1	Cal2	Cal3	Cal4	Cal5
648	Normal	21.55	14.4	7.15	3.942509249	1	10.05611	4.055838	3.031433	1.569168
644	Normal	32.28	21.8	10.48	0.392051243	0.099442	1	0.403321	0.301452	0.156041
622	Normal	21.86	12.69	9.17	0.97205789	0.246558	2.479415	1	0.747425	0.386891
618	Normal	30.54	21.79	8.75	1.300543036	0.329877	3.317278	1.337928	1	0.517632
550	Normal	21.26	13.46	7.8	2.512483531	0.63728	6.408559	2.584706	1.931873	1
550-1	Si 1x	21.89	13.76	8.13	1.998772314	0.50698	5.098243	2.056228	1.536875	0.795536
550-2	Si 1x	22.43	14.37	8.06	2.098144644	0.532185	5.35171	2.158456	1.613284	0.835088

Table 22 : The average c_t of *GAPDH* and *DSCR1* (Continue)

Sample	Note	FAM(Ct)	CY5(Ct)	dCt	OVERALL MEAN	Cal1	Cal2	Cal3	Cal4	Cal5
550-3	Si 1x	22.15	13.49	8.66	1.384259228	0.351111	3.530812	1.42405	1.06437	0.550953
614-1	Si 1x	22.37	13.11	9.26	0.913270501	0.231647	2.329467	0.939523	0.702222	0.363493
614-2	Si 1x	22.42	12.94	9.48	0.784102486	0.198884	2	0.806642	0.602904	0.312083
614-3	Si 1x	22.22	13.21	9.01	1.086067778	0.275476	2.770219	1.117287	0.835088	0.432269
617-1	Si 1x	25.34	15.85	9.49	0.778686295	0.19751	1.986185	0.80107	0.598739	0.309927
617-2	Si 1x	22.2	13.03	9.17	0.97205789	0.246558	2.479415	1	0.747425	0.386891
617-3	Si 1x	23.12	13.47	9.65	0.696943756	0.176777	1.777685	0.716978	0.535887	0.277392
640-1	Si 1x	22.35	13.43	8.92	1.155978159	0.293209	2.948538	1.189207	0.888843	0.460094
640-2	Si 1x	22.59	13.93	8.66	1.384259228	0.351111	3.530812	1.42405	1.06437	0.550953
640-3	Si 1x	23.52	14.5	9.02	1.07856576	0.273573	2.751084	1.109569	0.82932	0.429283
648-1	Si 1x	22.26	13.43	8.83	1.230388684	0.312083	3.138336	1.265757	0.946058	0.48971
648-2	Si 1x	22.89	14.11	8.78	1.273778247	0.323088	3.24901	1.310393	0.97942	0.50698
648-3	Si 1x	22.36	13.4	8.96	1.124367875	0.285191	2.86791	1.156688	0.864537	0.447513

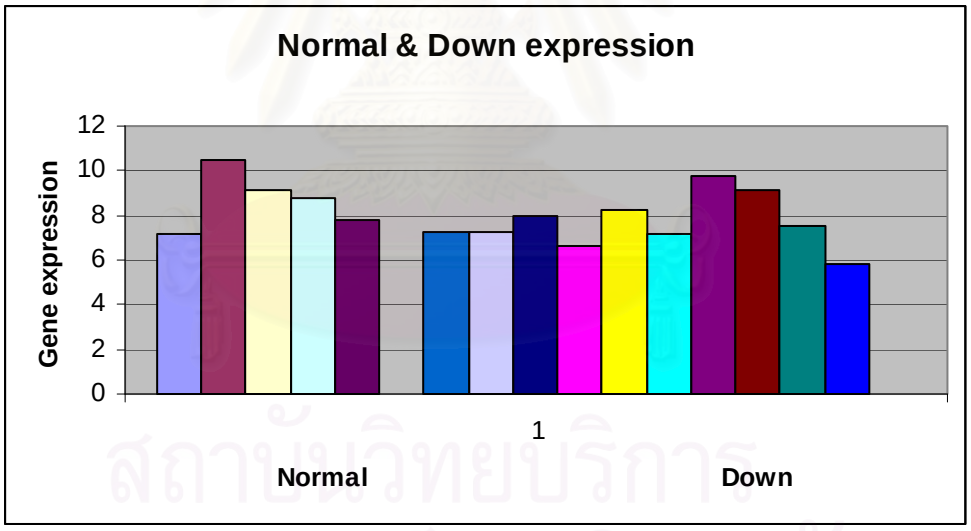
2.4 DSCR1 gene expression same as case samples and control samples

In untreated condition, *DSCR1* gene expression of case samples are not different control samples at 95% confidence (Table 22) even though the copy number of *DSCR1* in case samples are higher.

Table 23 : F test DSCR1 gene expression of case samples

Source	DF	Anova SS	Mean Square	F Value	Pr > F
treatment	1	10.53418522	10.53418522	1.80	0.2031

Figure 11 Normal controls gene expression compare with cases gene expression



2.4 siRNAs did not affect on control samples

In treated and untreated control groups, there were no significant differences between both groups (Table 24-26).

Table 24 : T-tests between untreated control samples group and treated with siRNA 0.5 fold in control samples group.

T-Tests			
Difference	**DF**	**t Value**	**Pr > |t|**
Control 0.5X-Control	4	0.29	0.7878

Table 25 : T-tests between untreated controls samples group and treated with siRNA 1 fold in control samples group.

T-Tests			
Difference	**DF**	**t Value**	**Pr > |t|**
Control 1X - Control	4	0.40	0.7099

Table 26 : T-tests between untreated control samples group and treated with siRNA 1.5 fold in control samples group.

T-Tests			
Difference	**DF**	**t Value**	**Pr > |t|**
Control 1.5X - Control	4	0.92	0.4103

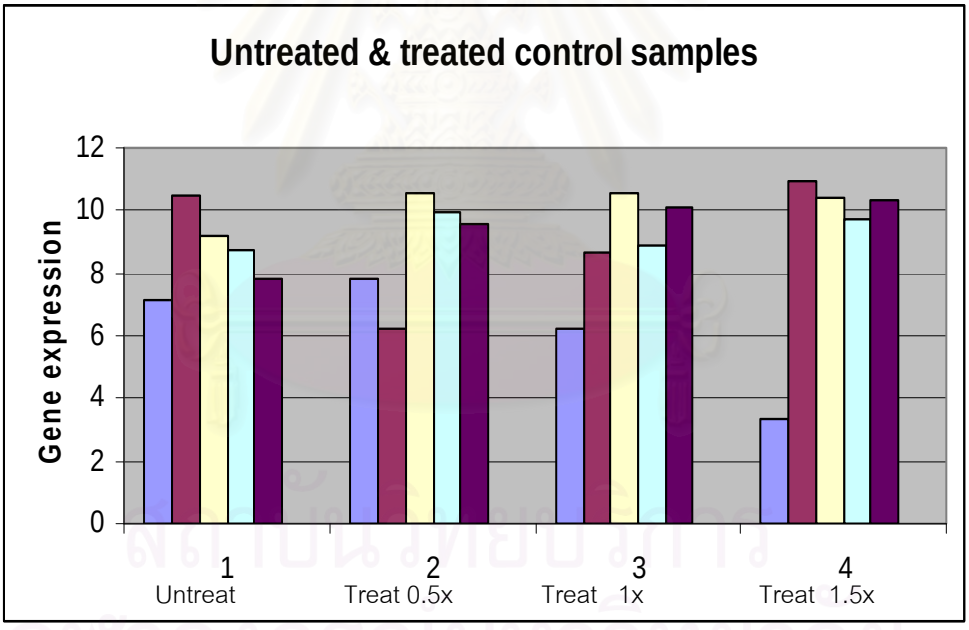


Figure 12 Untreated controls gene expression compare with treated controls gene expression.

2.5 siRNAs did not affected on case samples at all concentrations

In treated and untreated control groups, there were no significant differences between both groups (Table 27,28) .

Table 27 : T-tests between untreated case samples group and treated with siRNA 0.5 fold in case samples group.

T-Tests			
Difference	**DF**	**t Value**	**Pr > |t|**
Case 0.5X - Case	9	0.29	0.7878

Table 28 : T-tests between untreated case samples group and treated with siRNA 1 fold in case samples group.

T-Tests			
Difference	**DF**	**t Value**	**Pr > |t|**
Case 1X - Case	9	0.40	0.7099

Table 29 : T-tests between untreated case samples group and treated with siRNA 1.5fold in case samples group.

T-Tests			
Difference	**DF**	**t Value**	**Pr > |t|**
Case 1.5X - Case	9	0.92	0.4103

Figure 13 Untreated cases gene expression compare with treated cases gene expression

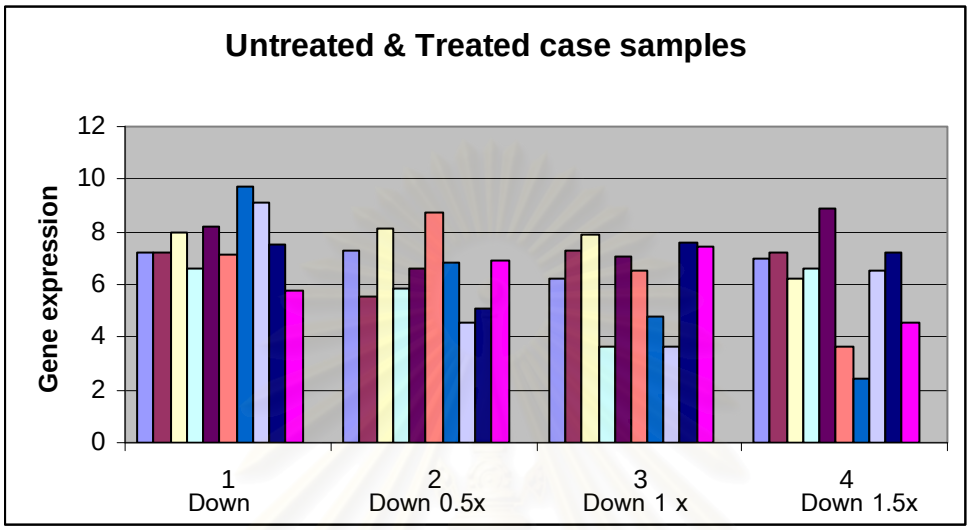


Figure 14 Untreated control samples gene expression are harvested at 96 hours compare with treated control samples gene expression

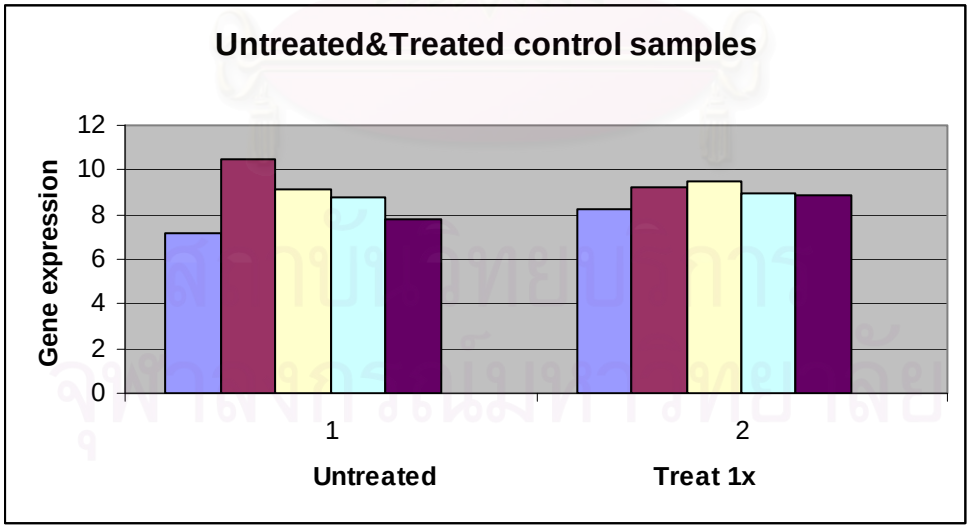


Table 30 : T-tests between untreated control samples group and treated with siRNA 1 fold in control samples group at 5 th day.

T-Tests			
Difference	**DF**	**t Value**	**Pr > |t|**
Control 1X - Control	4	0.63	0.5626

3. siRNA designing and cloning

The down stream primer of siRNA was designed by Promega software. The 300 bp of siRNA and U6 promoter was amplified, and successfully cloned into plasmid vector with confirmed by sequencing (Fig 14). siRNA sequence was blasted by NCBI database with no sequence matching to unwanted target. siRNA concentration was varied from 0.5, 1, 1.5 folds of concentration that recommended from manufactory and transfected into controls and cases fibroblast cells for determining the appropriate concentration of si-RNA to silence *DSCR1* gene in fibroblast cells. The size of inserted RFP siRNA plasmid is as alike as siRNA plasmid alone due to cutting of the ~900bp of neomycin resistant gene and inserting the~700bp of RFP gene. Consequently, RFP is transcribed by SV40 promoter. The closely size of two plasmids can refer that uptake rate of two plasmids are the same. However, in experiment of siRNA transfection, RFP was excluded from plasmid to ensure that the changing of *DSCR1* expression was caused by siRNA only.

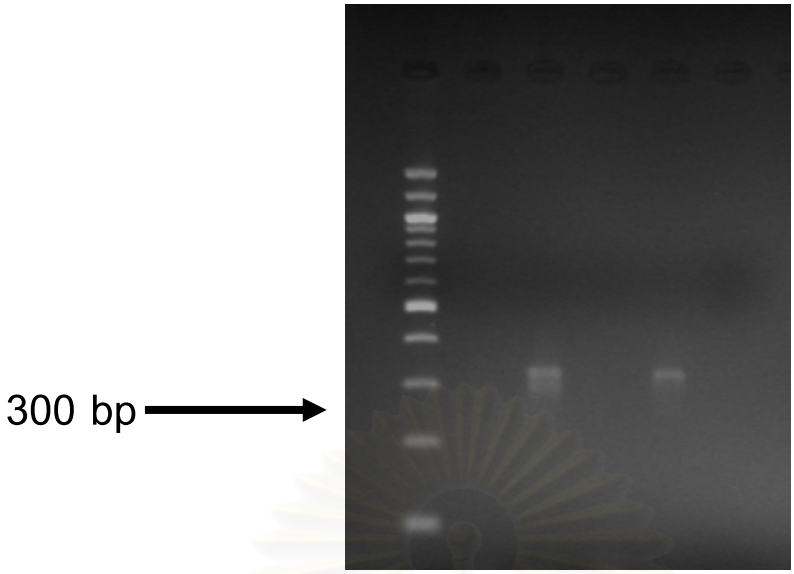


Figure 15 The 300 bp PCR product of siRNA and U6 promoter

4. Selection of transfected cell lines

G-418 at concentrations of 100, 200, 400, 600, 800 and 1,000 µg/ml in RPMI+ 10% (v/v) FBS were used for selection of transfected cell lines within 14 days but there were no existent cells.

5. Uptake rate of siRNA into cell lines

The 700 bp of RFP gene was successfully amplified, cloned into siRNA plasmid, and confirmed by sequencing (Fig 15).

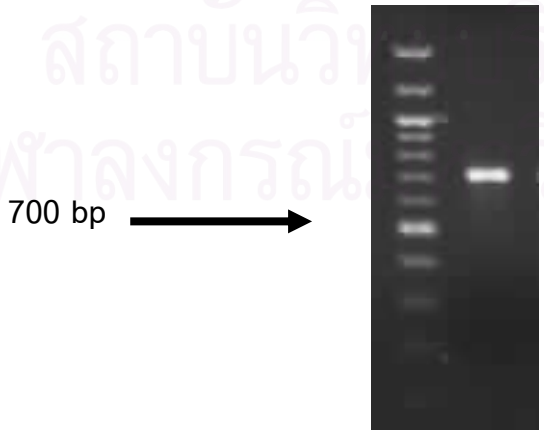


Figure 16 The 700 bp PCR product of RFP gene

RFP protein was detected using florescent microscopy. Fibroblast cell lines were effectively obtain plasmid vector about 60% of treated cells with siRNA 0.5x fold (Figure 16), and about 80% of treated cells with siRNA 1x and 1.5x fold (Figure 17,18). The signal of RFP was detected within 5 days of post-transfection.

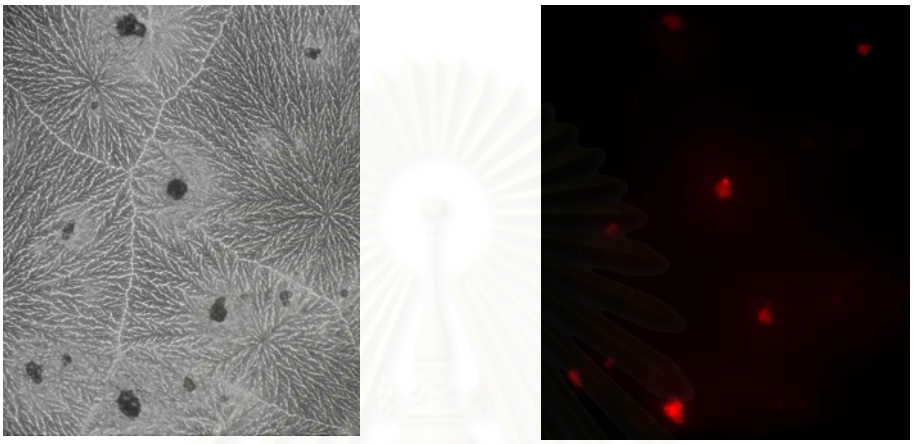


Figure 17: Uptake rate of treated lymphoblast cells with siRNA 0.5x fold. A: Bright field B: Spectrum red

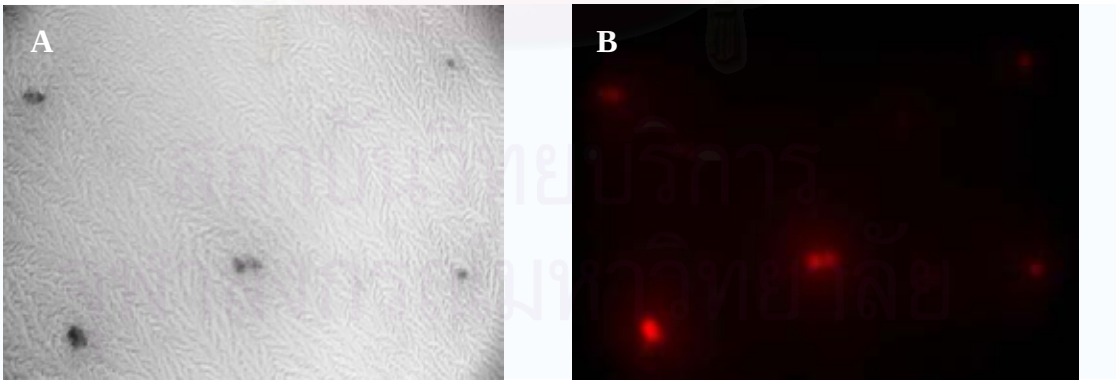


Figure 18: Uptake rate of treated lymphoblast cells with siRNA 1x fold. A : Bright field B : Spectrum red

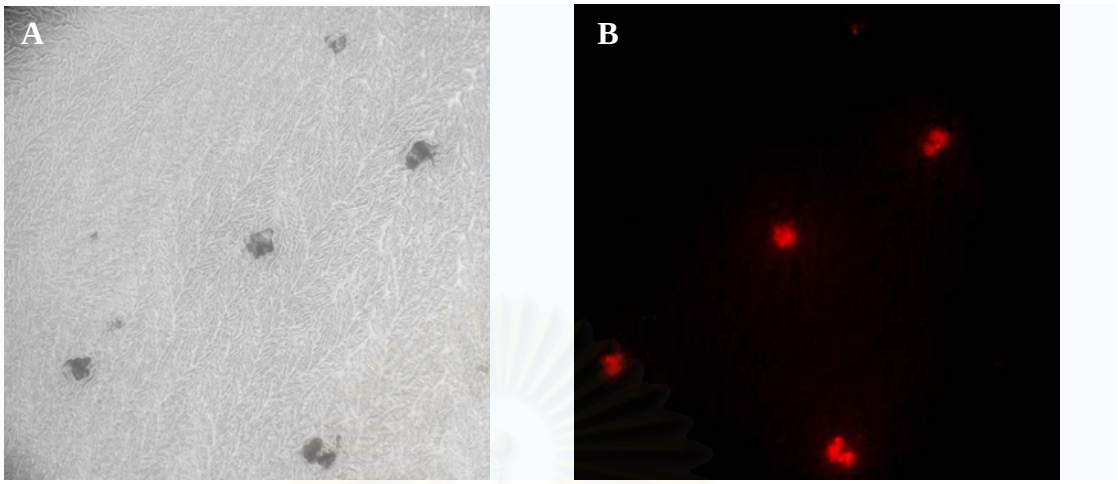


Figure 19: Uptake rate of treated lymphoblast cells with siRNA 1.5x fold. A : Bright field B: Spectrum red.

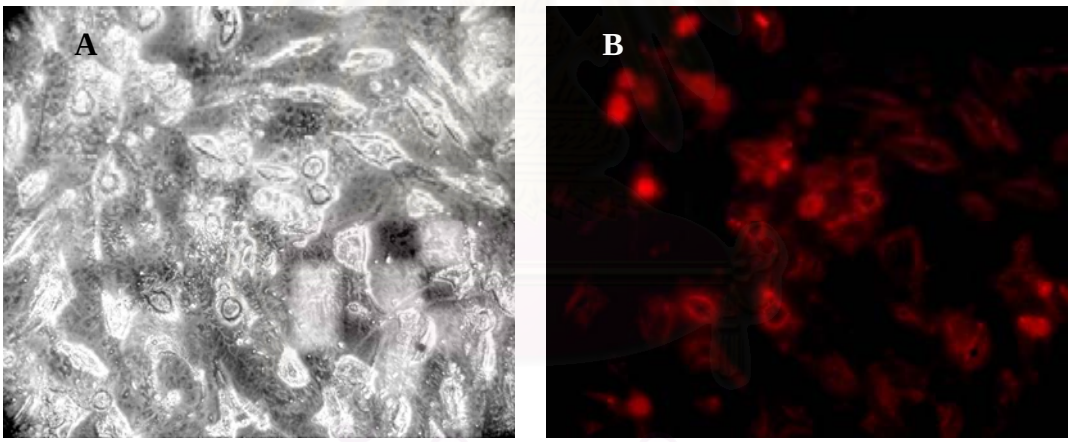


Figure 20 Uptake rate of treated fibroblast cells with siRNA 1x fold. A: Bright field B: Specrum red.

6. Coomasie blue gel staining

Nine protein samples were selected from control and case samples, and 5 µg of proteins were loaded and stained with coomasie blue. The coomasie gel staining was shown that all the samples were similarly quantity loaded (Fig 19)

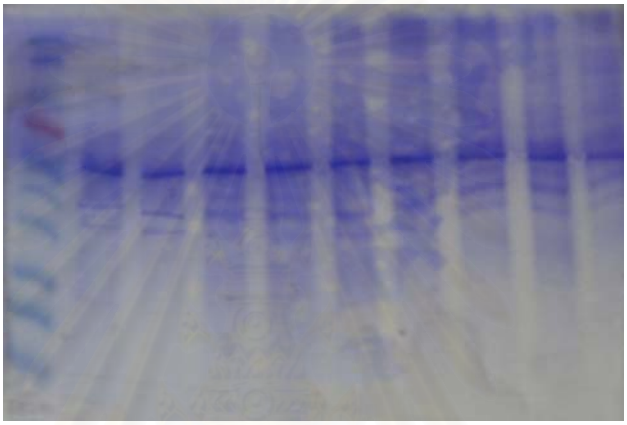


Figure 21 The coomasie gel staining

7. Western Blot

All treated case samples of No. 1431 and 1439 were not able to detect with western blot assay because cell lines slowly grown. All treated and untreated of 5 controls and 8 cases cell line were extracted protein at 28 days of post-transfection with siRNA and successfully done in western blot analysis. The ~22 KDa protein size of *DSCR1* product was detected by *DSCR1* antibody and shown in Figure 18-21. By semi-quantitative analysis, all treated proteins are seemed to be as same as untreated protein.

Figure 22 The ~22 KDa of *DSCR1* protein was detected in untreated 5 control samples.

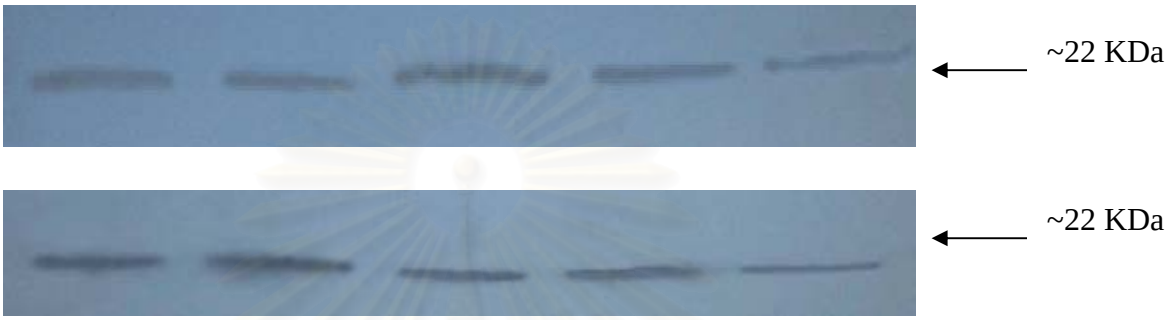


Figure 23 The ~22 KDa of *DSCR1* protein was detected in untreated 10 case samples.

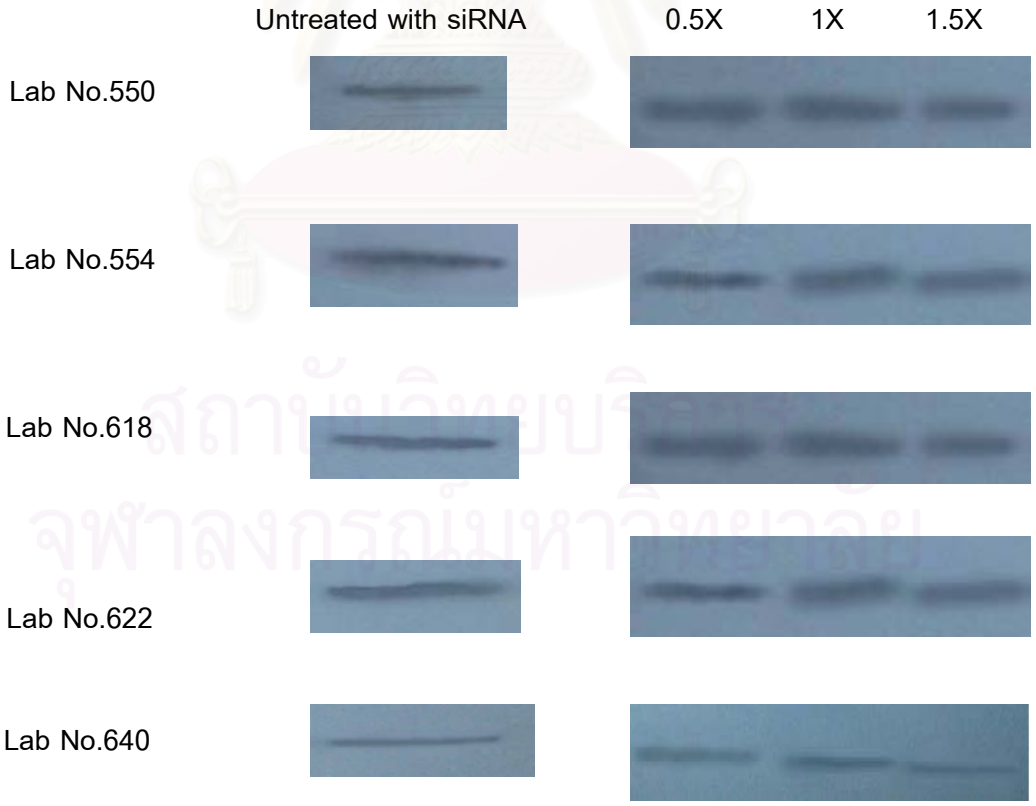


Figure 24 The ~22 KDa of *DSCR1* protein was detected in treated 5 control samples

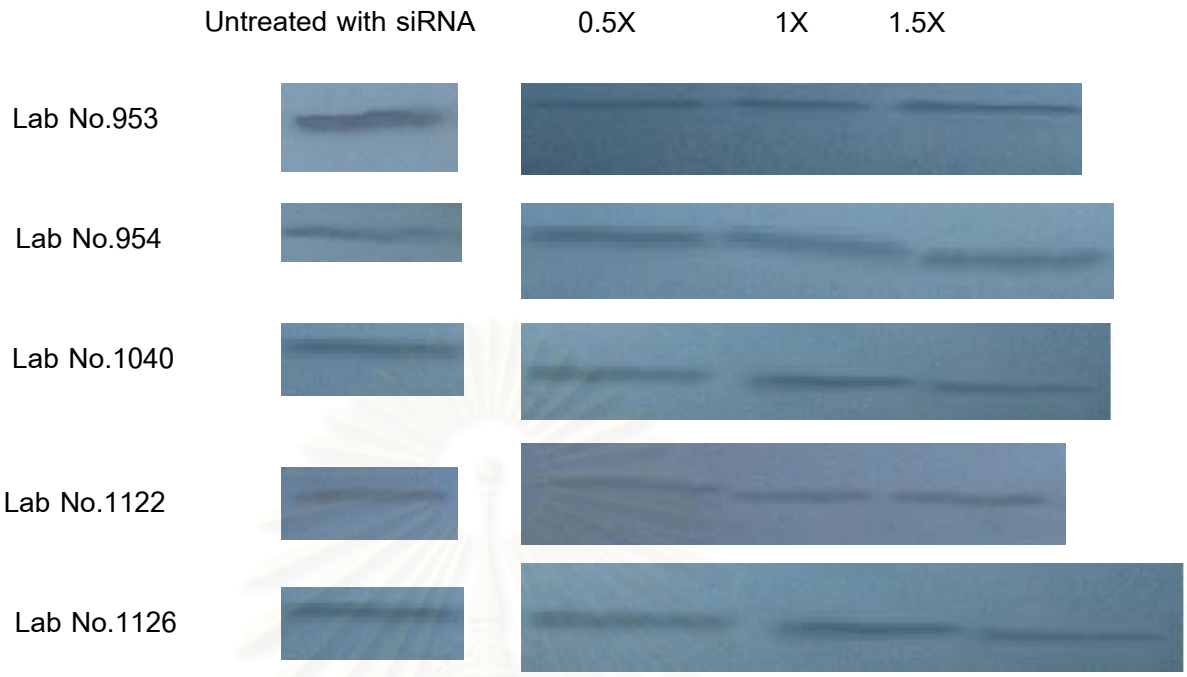


Figure 25 The ~22 KDa of *DSCR1* protein was detected in treated 10 case samples.

CHAPTER V

DISCUSSION

DSCR1 gene spans nearly 45 kb and contains 7 exons. Four of which are alternative first exons, giving 3 isoforms of tissue-specific expression patterns for the alternative transcripts. The studies of Down syndrome patients with trisomy 21 indicating genes associated with the phenotypic features of Down syndrome to a extra segment of chromosome 21. The exsentive studies on DS patients with partial trisomy21 have allowed researcher to narrow the search of genes associated with the phenotypic features of DS to a segment of chromosome 21 , the Down syndrome critical region (*DSCR*).The interested gene located in Down syndrome critical region is the *DSCR1* gene, *DSCR1* is normally expressed in the central nervous system and the heart, but overexpressed in the brain of Down syndrome fetuses, *DSCR1* belongs to a highly conserved calcineurin inhibitor family called calcipressin. Biochemical studies have shown that calcipressin can interact physically and functionally with calcineruin A , a serine/threonine phosphatase responsible for transition from short- to long-term memory, and *DSCR1* is associated with mental retardation in *Drosophila*, it can be indicated that the overexpression of *DSCR1* is associated with mental retardation and memories in Down syndrome human. It is possible that decreased *DSCR1* gene expression by using appropiate siRNA suitably may restore normal brain function in Down syndrome individual.

Gene silencing using siRNAs is a novel and promising technology for therapeutic applications of various decrease that involves upregulation of several genes.Bases on efficiency of RNAi/siRNA technology, can be applied for post-transcriptional gene silencing of any over-expressive gene. *DSCR1* is an interesting gene that associated with mental retardation, learning and memory in human especially in Down syndrome's patient, who has over-expression of *DSCR1* gene. RNAi/siRNA technology has been adapted for silencing this abnormal gene and studied the effect of siRNA on *DSCR1* gene. Using this technology, we generated shRNA which has advantage that each can replicate themselves and driven by individual pol III promoters,

to develop siRNA form a 20-25 nucleotide dsRNA .which is further processed by dicer enzyme ,The siRNAs assemble into endonuclease containing complexes known as RNA-induced silencing complexes (RISCs) that unwind the duplex siRNA. The antisense strand subsequently guides the RISCs to complementary RNA molecules that initiate ATP-independent endunuclease activity resulting in cleavage and destruction of the cognate mRNA that effected the level of *DSCR1* mRNA and calcipressin protein.

Because *DSCR1* gene is very important to human's memory and learning process, researcher must design the shRNA which specifically and perfectly match to *DSCR1* gene in order to study knock-down of *DSCR1* gene, by observing the decreasing level of mRNA and protein which canl be measured by real time PCR and Western Blotting respectively. The hypotheses of this study are that the *DSCR1* gene expression is decreased by siRNA, and we can find out the appropriate concentration of siRNA for suppression *DSCR1* gene. From theory, siRNA is designed to avoid the repetitive sequence of genomic mRNA by blasting with NCBI database. Furthermore, siRNA was designed to complementary with all of three isoforms of *DSCR1* mRNA for suppress all isoforms. In this experiment, shRNA was cloned into a plasmid vector contain the Amp^r gene, which confers resistance to amplicillin and allows selection in E.coli are expressed under human U6 promoter, RNA polymerase III recognition sequence, Additionally the vectors allow blue/white screening of transformants in E.coli.

In previous findings that *DSCR1* gene is highly expressed in different areas of the adult human brain and *DSCR1* gene function has been associated with calcineurin inhibition. It is also interesting that the highest level of calcineurin expression in mammalian tissues is found in the brain. Our new detailed studies demonstrate that *DSCR1* gene is normally expressed in lymphoblast cells for both control and case samples by analyzing the level of *DSCR1* mRNA at the cellular level and no association in the age or sex.

Quantitative real time PCR analyses revealed expression of *DSCR1* gene, from Table 20, SD value of 10 control samples (SD±0.4178) is less than 2 It can be indicate that the *DSCR1* gene expression level are no significant differences among 10 control samples. Moreover, it can be said, from Pr value in table 23 (t test, Pr=0.2031 is more than 0.05, that *DSCR1* gene expression level in lymphoblast cell in control samples are not different from case samples even though the copy number in case samples of *DSCR1* gene is more than control samples 1 copy. It is correlated with a study of *DSCR1* gene expression in trisomy mouse model; the expression of *dscr1* in trisomy mouse was not express at 1.5 fold of diploid normal mouse in other tissues except brain tissue.

From results, We demonstrate that the level of *DSCR1* mRNA in both control samples and case samples are not significantly changed after transfection with siRNA , In control samples (Table 24, 25 and 26, t test, Pr=0.7878, 0.7099 and 0.4103, respectively >0.05) and in case samples (from Pr values in table 27,28 and 29 t test, Pr=0.0999, 0.0968 and 0.1381 respectively>0.05) .

Apart from mRNA measurement, protein was detected by western blotting to confirm the function of *DSCR1*. Western blot is a very semi-quantification using shade of banding of interesting protein. The shade depends on quantity of protein. Protein was similarly load with the protein assay for measurement the total protein quantity. As similar loading of protein, the shade of band depends on gene expression and compares between treated and untreated protein. The protein loading was confirmed by coomasie gel staining. The result of western blotting supports Real time PCR result, but anyway the difference date of harvest cell lines to extract RNA and protein, could not compare the result from mRNA and protein quantity. Since, the banding of protein by western blot was very semi-quantification and non sensitivity method, the protein quantity in untreated and treated with siRNAs samples are seem to be similarity in both sample groups.

The target time point and transfect conditions can predict the variation in suppression efficiency, The transfection efficiency is considered as *DsRed2* protein, a varient of original red fluorescence protein,modified through six point mutations.These mutations improve the solubility of *DsRed2* by reducing its tendency to form aggregates, and decrease the time from transfection to detection. A marker for measure percentage of shRNA transfected cells by ligate RFP gene into siRNA plasmid vector. The result of RFP signal could be indicated to transfection percentage of shRNA into lymphoblast cell lines. The signal was detected within 8 days of post-transfection. Furthermore, the siRNA protocol from Promega indicated that the maximum suppression time can vary from 48-96 hours. It means that shRNA could be effective within 96 hours after transfection, but signal of RFP can detected until 8 days. It shows half life of RFP take longer than siRNA and RFP gene. Anyway, we proved recombinant plasmid can transfected to lymphoblast cells optimizely.

The problem of this research is that we used the long period of post-transfection, total RNAs were extracted in the 14^{th} day, and proteins were extracted in the 21^{th} day of post-transfection because the limitation of cell growth . From handbook siRNA can not effect to knockdown gene expression after 96 hours.

Here the RFP signal was confirmed that siRNA were transfected into cells and period of signal less than the day of harvest cells, It is possibility caused by 3 hypotheses that, firstly siRNAs can suppress *DSCR1* gene but decreasing happened on early date (result from RFP signal) after interfering the balance of *DSCR1*,Cells attempt to keep this balance and restore the level of *DSCR1*. Result of western blot analysis will be support this hypothesis because calcipressin level is not difference between treated and untreated cells, Secondly, this target sequence of siRNA cannot induce mRNA degradation and finally all components involved RNAi pathway ex. Dicer , exportin 5 , RISC are not enough to process effectively RNAi pathway from more shRNAs.

We research continuously for solving time point problem by decrease time period. We collect cell lines 5 samples from normal and measure mRNA at 96 hours of post-transfection for test *DSCR1* suppression. After adjust the time to 96 hours, we

found that siRNA can not knockdown *DSCR1* gene eventhough it is treated by appropriate siRNA concentration (1 fold). It can be summarized that time point is not involved knockdown efficiency in this experiment, It means, We can release the first hypothesis which is explained that the time point is not optimal lead to non suppression.

Moreover we tested transfection efficiency in both of fibroblast and lymphoblast cells from same cases. The result can demonstrate that the percentage of transfection efficiency and period of signal in both cell types are not significantly difference.

Even though, siRNA can not decrease the *DSCR1* expression in lymphoblast cells but interestingly, there is the new finding. The DSCR1 gene expression in lymphoblast cells from Down syndrome patients are not overexpress, even though the number of copy is more than normal 1 copy. And siRNA which effect on Down syndrome cell line might lead to understand siRNA actions on lymphoblast cells in long term effect. Moreover, the construction of siRNA plus RFP plasmid could be valuable for following up the function of siRNA effect in many further studies. In future studies, we suggest to increasing the siRNA target sequence at least 3-6 target sequences for finding the highest suppression to ensure that which siRNA can decrease gene expression level on target gene efficiently and add important components to RNAi pathway for process effectively.

We have observed that the target,time point and transfection conditions can result in variation in suppression efficiency, The transfection efficiency is considered as DsRed2 protein, a varient of original red fluorescence protein, modified through six point mutations. These mutations improve the solubility of *DsRed2* by reducing its tendency to form aggregates,and decrease the time from transfection to detection, A marker for measure percentage of shRNA transfected cells by ligate RFP gene into siRNA plasmid vector. The result of RFP signal could be indicated to transfection percentage of shRNA into lymphoblast cell lines. The signal was detected within 8 days of post-transfection. Furthermore, the siRNA protocol from Promega indicated that the maximum suppression time can vary from 48-96 hours, It means shRNA could be effective within 96 hours after transfection but signal of RFP can detected until 8 days. It shows half life of RFP take

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We research continue for solve time point problem by decrease time period, collect cell lines 5 samples from normal and measure mRNA at 96 hours of post-transfection for test DSCR1 suppression . After adjust the time to 96 hours, We found siRNA cann't knockdown DSCR1 gene are treated by appropriate siRNA concentration (1 fold). We revealed time point is not involved knockdown efficiency in this experiment, It means , We can release the first hypothesis which is explained the time point is not optimal lead to non suppression.

Moreover we tested transfection efficiency in both of fibroblast and lymphoblast cells from same cases. We demonstrate that the percentage of transfection efficiency and period of signal in both cell types are not significantly different .

Nevertheless, siRNA could not decrease the *DSCR1* expression in lymphoblast cells but interestingly, the new finding of DSCR1 gene expression in lymphoblast cells from Down syndrome patients are not overexpression eventhough the number of copy is

more than normal 1 copy , and siRNA effect on Down syndrome cell line might lead to understand siRNA actions on lymphoblast cells in long term effect. Moreover, the construction of siRNA plus RFP plasmid could be valuable for following up the function of siRNA effect in many further studies. The future studies, We suggest that increasing siRNA target sequence at least 3-6 target sequences for finding the highest suppression to ensure that which siRNA can decrease gene expression level on target gene efficiently and add important components to RNAi pathway for process effectively.

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APPENDICES

APPENDIX A

BUFFERS AND REAGENT

1. Lysis Buffer I

Sucrose	109.54	g
1.0 M Tris-HCl	10	ml
1.0 M $MgCl_2$	5	ml
Triton X-100	10	ml
Distilled water to	1,000	ml

Sterilize the solution by autoclaving and store at 4°C

2. Lysis Buffer II

5.0 M NaCl	10	ml
0.5 M EDTA (pH 8.0)	48	ml
Distilled water to	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

3. 10% SDS solution

Sodium dodecyl sulfate	10	g

Distilled water to	100	ml

Mix the solution and store at room temperature.

4. 20 mg/ml Proteinase K

Proteinase K	2	mg
Distilled water	1	ml

Mix the solution and store at -20°C

5. 1.0 M Tris-HCl

Tris base	12.11	g

Dissolve in distilled water and adjusted pH to 7.5 with HCl

Distilled water to	100	ml

6. 0.5 M EDTA (pH 8.0)

Disodium ethylenediamine tetraacetate.$2H_2O$	186.6	g

Dissolve in distilled water and adjusted pH to 8.0 with NaOH

Distilled water to	1,000	ml

Sterilize the solution by autoclaving and store at room temperature.

7. 1.0 M $MgCl_2$

Magnesium chloride.6 H_2O	20.33	g
Distilled water to	100	ml

Dispense the solution into aliquots and sterilize by autoclaving.

8. 5 M NaCl

Sodium chloride	29.25	g
Distilled water to	100	ml

Dispense the solution into aliquots and sterilize by autoclaving.

9. 10X Tris borate buffer (10X TBE)

Tris base	100	g
Boric acid	55	g
0.5 M EDTA (pH 8.0)	40	ml

Adjust volume to 1,000 ml with distilled water. The solution was mixed and stored at room temperature.

10. 7.5 Ammonium acetate

Ammonium acetate	57.81	g

Distilled water	80	ml

Adjust volume to 100 ml with distilled water and sterilize by autoclaving.

11. 6X loading dye

Bromphenol blue	0.25	g
Xylene Cyanol	0.25	g
Glycerol	50	ml
1M Tris (pH 8.0)	40	ml
Distilled water to	100	ml

Mix and stored at 4°C

11. 1% agarose gel (w/v)

Agarose	1.0	g
1x TBE	100	ml

Dissolve by heating and occasional mix until no granules of agarose gel are visible.

12. 12% polyacrylamide gel (w/v)

1.5M Tris-HCl pH 8.8	1	ml
10% SDS	100	µl

40% Acrylamide/Bis	3	ml
10% APS	100	µl
TEMED	50	µl
Distilled water	3.7	ml

12. 6% polyacrylamide gel (w/v)

0.5M Tris-HCl pH 6.8	500	µl
10% SDS	100	µl
40% Acrylamide/Bis	750	µl
10% APS	50	µl
TEMED	25	µl
Distilled water	5.9	ml

13. Lysed buffer (for cytoplasm lysis)

5% Tween20	10	ml
50mM PIPES pH8.0	10	ml
1M KCl	8.5	ml

Distilled water	71.5	ml

Mix and store at room temperature.

When using, add 100X Proteinase inhibitor to final concentration at 1X and keep on ice.

14. Lysis buffer (for nuclear lysis)

10% SDS	10	ml
1M Tris-HCl pH8.1	5	ml
100mM EDTA	10	ml
Distilled water	75	ml

Mix and store at room temperature.

When using, add 100X Proteinase inhibitor to final concentration at 1 and keep on ice.

15. Ethidium Bromide

Ethidium Bromide	10	mg
Distilled water	1	ml

Mix the solution and store at 4°C

BIOGRAPHY

Miss Pantipa Trichantong was born in Chantaburi province on the 2nd of July 1981. She graduated her bachelor degree from Thammasat University for Faculty of Science and Technology; major biotechnology in 2003. Consequently, she started to studying her master degree in the Faculty of Science; major Genetic. In 2007, Finally she received her master degree in Genetics from Faculty of Science, Chulalongkorn University.

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