CHAPTER V

DISCUSSION

DSCR1 spans nearly 45 kb and contains 7 exons, 4 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 partial trisomy 21 indicating genes associated with the phenotypic features of Down syndrome to a segment of chromosome 21, the Down syndrome critical region (DSCR). Among the genes located in Down syndrome critical region, *DSCR1* is overexpressed in the brain of Down syndrome fetuses, and interacts 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 indicated that the overexpression of *DSCR1* is associated with mental retardation and memories in Down syndrome human. It's possible that normalizing *DSCR1* expression may restore normal brain function in Down syndrome individual.

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. In this experiment, siRNA was designed to specifically and perfectly match of *DSCR1*. The hypothesis of this study, *DSCR1* is decreased by siRNA. As well as, siRNA was designed to avoid the repetitive sequence of genomic mRNA by blast to NCBI database. Furthermore, siRNA was designed to complementary with all of three isoforms of *DSCR1* mRNA. In this study, siRNA was cloned into a plasmid vector and expressed under U6 promoter, RNA polymerase III recognition sequence. In intracellular, siRNAs were transcribed as fold-back steam-loop structure called shRNA. The loop was cleaved by DICER, endogenous RNAse III nuclease, to form duplex

siRNA. siRNA is unwided and used guide strand to form a complex called RISC (RNA Induced Silencing Complex). This guide strand is complementarities with mRNA of *DSCR1* gene and activated compartment of RISC to cleave the mRNA target. A comparison result of *DSCR1* mRNA and protein quantification indicated the study of *DSCR1* gene silenced by siRNA in fibroblast cells.

The trasfection efficiency is assured by cloning of RFP, red fluorescence protein, into siRNA plasmid vector. The result of RFP signal could be refer to the function of siRNA, and indicating that siRNA was transfected into fibroblast cell lines. The signal was detected within 5 days of post-transfection due to the cytosolic division of cell lines could be excluded siRNA in the new divided cells. With the numbers of cell division, siRNA could be faded away from cell lines. Furthermore, the siRNA protocol from Promega indicated that siRNA could be effective when cell lines should be collected within 96 hours after transfection. While, the limitation of cell growth, cell lines could not be measure mRNA and protein within 5 days after transfection. Due to the lipofectamine 2000 was known to be toxic to many cells and causing slowly grown of cell lines. In addition, fibroblast cell is mortal cell line, with various subculture and age of cell line, can generate the cell division period to longer than the primary passage of cell line. By these reasons, the long period of post-transfection, siRNA could not be effective to knockdown gene expression. In this experiment, total RNAs were extracted in the 14^{th} day, and proteins were extracted in the 28^{th} day of post-transfection because the limitation of cell growth.

A result of relative quantitative PCR, from Table 20, SD value of 10 controls sample (SD±0.4178) is less than 2, indicate that the *DSCR1* gene expression level are no significant differences among 10 control samples. Conversely, from Pr value in table 23 (t test, Pr=0.0107) is less than 0.05, indicate that *DSCR1* gene expression level in fibroblast cell in control samples are higher than case samples even though the copy number of *DSCR1* gene are less. 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. Since, *DSCR1* overexpression in brain is a brain specific isoform derivative from alternative splice site

in four exons. It is possible that the highly expression of *DSCR1* is only from the brain specific isoform, the leading cause of mental retardation. However, *DSCR1* expression is regulated by Ca^{2+} / Calmodulin/ Calcineurin dependent pathway. *DSCR1* is a negative feed back loop controlled by dephosphrylation of transcription factor. The amount of *DSCR1* and calcineurin are balanced in normal human. In fibroblast cells from patients with Down syndrome, it is possible that *DSCR1* might be suppressed at low level in every 3 copies resulting lower expression than normal human, and might be due to reduction of transcription factor to transcribe *DSCR1* gene. Based on this evidence, this is a new finding that is no report about *DSCR1* gene expression in fibroblast cells from patients with Down syndrome.

As expected, The *DSCR1* gene expression level in control samples were not changed by the effect of siRNA (Table 24, 25 and 26, t test, Pr=0.61, 0.5626 and 0.3947, respectively >0.05) due to the long period of post-transfection. However, the RFP signal was confirmed that siRNA were transfected into cells. It is possible that neither siRNA could knock down *DSCR1* on the earlier date of transfection and cell turned to be as same as untreated cells with siRNA nor siRNA did not affect on control samples. Because of the limitation on cell line growth, this experiment could not measure mRNA in the earlier period. This hypothesis could be finding out by increasing the batch of transfection, collection cell lines and measurement the mRNA within 5 days of post-transfection.

Interestingly, siRNA affected on case samples at the concentration of siRNA 0.5 and 1 fold of concentration that recommended from manufactory. At 0.5x and 1x concentration of siRNA, *DSCR1* expression levels were significantly difference from untreated cells (from Pr values in table 27 and 28 t test, Pr=0.0203 and 0.0054, respectively are less than 0.05), indicating that *DSCR1* gene expression level in treated cells at siRNA concentration of 0.5 and 1 fold were higher than untreated cells at 95% confidence. It is possibility caused by 3 hypotheses that, firstly siRNAs decrease *DSCR1* expression level on early date (result from RFP signal) and interfere the balance of *DSCR1* product and calcineurin. Therefore, cells attempt to keep this balance and restore the level of *DSCR1* by producing extra *DSCR1*. However, western blot analysis

indicate that after shooting of *DSCR1* level in the 14th day, *DSCR1* protein are decreased to the level of untreated cells in the 28th day after transfection. With the information from Gennady Elmark (94) indicate that amyloid beta (*APP* gene, 21q21.3) stimulates *DSCR1* activity, leading to paired helical filaments formation, and linking the formation both neuritic plaques and neurofibrillary tangles, leading cause of Alzheimers disease. The secondary hypothesis, *DSCR1* level is decreased by siRNA, then *APP* stimulates *DSCR1* activity and resulting the over shooting of *DSCR1* gene by Amyloid ß protein activation. As this hypothesis, could be discovered by study the expression of *APP* gene. Finally, the off-target effect of siRNA should not be omitted even though the 23-nt siRNA is designed to perfectly match with mRNA of *DSCR1*, as only a stretch of 7 nucleotides of sequence complementarity between the guide strand of siRNA and its target could be enough to get RISC activation, it may be impossible to design sequences with no off-target potential. This phenomenal can give rise the off-target effect of any gene to induce the overexpression of *DSCR1*. The siRNA effect of nearby and other genes in fibroblast cells could be finding out by microarray to study the gene expression.

Considering with treated cell at siRNA concentration of 0.5 and 1 fold, the Pr value (t test, Pr =0.0522) were less than 0.06, indicated that *DSCR1* gene expression level of treated cell with siRNA 0.5 fold were higher than *DSCR1* gene expression level of treated cell with siRNA 1 fold at 94% confidence. As this result can be indicated that siRNA at concentration 0.5 fold was most affected to *DSCR1* expression in fibroblast cells.

On the other hand, the comparison of treated Down syndrome cells with 1.5 folds of siRNA and untreated Down syndrome cells do not significantly differene between both groups (Table 29) indicate that siRNA does not affect on cases sample at the 1.5 folds concentration of siRNA. Since, cell lines at this concentration grow slower than the other; it is possible that more concentration of lipofectamine 2000 is toxicity with transfected cell lines. As a result of lipofectamine 2000 effect, the survival cells must be untransfected cells resulting to similar gene expression of treated and untreated cell line. However, the high concentration of siRNA might induce the other dephosphorylation pathway of transcription factors to regulate *DSCR1* gene expression.

Conversely, the high-level expression of shRNA can lead to competition for and possibly saturation of endogenous cellular components involved in RNAi. This could lead to non effective of siRNA at 1.5 fold concentration.

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. With 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 groups.

Nevertheless, siRNA could not decrease the *DSCR1* expression in fibroblast cell but interestingly, the new finding of DSCR1 gene expression in fibroblast cells from Down syndrome patients is lower than normal human, and siRNA effect on Down syndrome cell line might lead to understand siRNA actions on fibroblast cell 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 improvement of this experiment is collection of cell line after transfection within 5 days by increasing the batch of transfection for sufficient mRNA and protein measurement to ensure that siRNA can decrease gene expression level on target gene. Moreover, cell lines should be collected in serial date after transfection to certify the overshooting effect of siRNA.

Importantly, this experiment can give raises 2 new finding that *DSCR1* gene expression in fibroblast cells from patient with Down syndrome is lower express than normal human even though the copy numbers are more. And, the construction of siRNA with tagged by RFP is valuable for further study of siRNA effect.

The future study of siRNA in short term effect, effect of over shooting of siRNA in other cell types, and microarray of the other activate transcription factor pathway are necessary. However, this finding raises these questions;

1. Are there any changes in gene expression of nearby genes such as *APP*?
2. Does siRNA affect on Down syndrome only?
3. What the associated gene is the off-target effect of siRNA?