การศึกษาวิธีการกระตุ้นเซลล์เม็ดเลือดแดงผู้ใหญ่ให้มีการแสดงออกของลักษณะเซลล์เม็ดเลือดแดงตัวอ่อนด้วยยีนบำบัด

นางสาวเนตรชนก ลีลาอดิศร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาวิทยาศาสตรมหาบัณฑิต
สาขาวิชาวิทยาศาสตร์การแพทย์
คณะแพทยศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย
ปีการศึกษา 2559

REACTIVATE EXPRESSION OF FETAL HEMOGLOBIN IN CD34+ PRIMARY ADULT ERYTHROID CELLS BY GENE THERAPY

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A Thesis Submitted in Partial Fulfillment of the Requirements
for the Degree of Master of Science Program in Medical Science
Faculty of Medicine
Chulalongkorn University
Academic Year 2016

Thesis Title	REACTIVATE EXPRESSION OF FETAL HEMOGLOBIN IN CD34+ PRIMARY ADULT ERYTHROID CELLS BY GENE THERAPY
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Field of Study	Medical Science
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เนตรชนก ลีลาอดิศร : การศึกษาวิธีการกระตุ้นเซลล์เม็ดเลือดแดงผู้ใหญ่ให้มีการแสดงออกของลักษณะเซลล์เม็ดเลือดแดงตัวอ่อนด้วยยีนบำบัด (REACTIVATE EXPRESSION OF FETAL HEMOGLOBIN IN CD34+ PRIMARY ADULT ERYTHROID CELLS BY GENE THERAPY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร. นพ.นิพัญจน์ อิศรเสนา ณ อยุธยา, 37 หน้า.

ในกลุ่มของผู้ป่วยเบต้าธาลัสซีเมียที่มีอาการร่วมกับการเกิด Hereditary persistence of fetal hemoglobin (HPFH) ผู้ป่วยยังคงมีการแสดงออกของเซลล์เม็ดเลือดแดงตัวอ่อน (Hb F) อยู่ ซึ่งช่วยบรรเทาความรุนแรงของโรคได้ ในงานวิจัยนี้ ผู้วิจัยได้ใช้วิธีการตัดลำดับเบสด้วยเทคนิค CRISPR/Cas9 ใน erythroleukemia cell line (K562) ที่จะทำให้เกิด DNA deletion ขนาด 3.5 kb ณ ตำแหน่ง γ-δ *globin* gene ซึ่งเป็นการเลียนแบบการกลายพันธุ์ทางธรรมชาติลักษณะหนึ่ง จากการ designed sgRNA ทำงานร่วมกับ Cas9 สามารถทำให้เกิด DNA double strand break (DSB) และตัดลำดับเบสขนาด 3.5 kb ในตำแหน่งที่ต้องการได้อย่างมีประสิทธิภาพและผู้วิจัยยังประสบความสำเร็จในการตัดลำดับเบสขนาดเล็ก 69 bp ซึ่งคลอบคลุมตำแหน่งการเกาะของ *BCL11A* ซึ่งเป็น transcription factor ที่สำคัญในการควบคุมการแสดงออกของ Hb F นอกจากนี้ ผู้วิจัยยังได้ใช้เทคนิค CRISPR ที่มีการต่อกับ Transcriptional activator เป็นอีกวิธีหนึ่งที่สามารถใช้ในการกระตุ้นการแสดงของ Hb F ได้ ทั้งนี้ genetic engineered K562 และ designed CRISPR sgRNA ที่สร้างได้จะเป็นประโยชน์ในการนำมาใช้สำหรับศึกษากลไกการควบคุมการแสดงออกของ Hb F และพัฒนาวิธีการกระตุ้น Hb F สำหรับไปใช้ในการรักษาผู้ป่วยต่อไป

สาขาวิชา วิทยาศาสตร์การแพทย์ ลายมือชื่อนิสิต .....

ปีการศึกษา 2559 ลายมือชื่อ อ.ที่ปรึกษาหลัก .....

5674040230 : MAJOR MEDICAL SCIENCE
KEYWORDS: FETAL HEMOGLOBIN / BCL11A / CRISPR/CAS9

NETCHANOK LEELA- ADISORN: REACTIVATE EXPRESSION OF FETAL HEMOGLOBIN IN CD34+ PRIMARY ADULT ERYTHROID CELLS BY GENE THERAPY. ADVISOR: ASST. PROF. NIPAN ISRASENA, M.D.Ph.D, 37 pp.

Beta thalassemia patients with the co-inheritance of hereditary persistence of fetal hemoglobin (HPFH), the patients' red blood cells still express fetal hemoglobin (Hb F) which has been found to alleviate the severity of the symptoms. In our study, CRISPR/Cas9 technique was used to modify the DNA, which mimicked the natural deletion in HPFH patients, in erythroleukemia cell line (K562). Designed CRISPR sgRNA with Cas9, used for cutting specific DNA sequences, could induce DNA double strand break (DSB) and site-specific deletion of 3.5 kb in γ-δ *globin* gene. Additionally, designed CRISPR sgRNA, which could cut a small region of DNA sequences (69 bp) including binding site of *BCL11A*, was successfully developed. This binding site is an important transcription factor, which involves in a silencer of fetal hemoglobin. Moreover, we have achieved in using CRISPR-tagged transcriptional activator to impulse an expression of Hb F. These genetically-engineered K562 and designed CRISPR sgRNA would allow the further studies on the controlling mechanism of fetal hemoglobin expression and reactivation, which might eventually become a therapeutic option in will be available for further studies of control mechanism of Beta-thalassemia patients.

Field of Study: Medical Science Student's Signature .....

Academic Year: 2016 Advisor's Signature .....

ACKNOWLEDGEMENTS

First at all, I would like to express my gratitude to my advisor Asst. Prof. Nipan Israsena who gave me the golden opportunity to do this valuable project in my master's degree study. I am appreciated for his patience and suggestion. I would also like to thank my thesis committee Prof. Apiwat Mutirangura, Asst. Prof. Chalisa louicharoen Cheepsunthorn and Dr. Piya Rujkijyanont for scarifying their time, insightful comments and suggestion.

I would also like to acknowledge Prof. Ponlapat Rojnuckarin of the Division of hematology, King Chulalorgkorn Memorial Hospital. For his support on hemoglobin typing.

I would like to thanks our members in Stem cells and cell therapy research unit for their helps and supports, especially to Dr. Praewphan Ingrungruanglert who always suggest and guide me in the right direction.

Finally, I must express my very profound gratitude to my parents and to my brilliant sister for providing me with unfailing support and continuous encouragement throughout my years of study and through the process of researching and writing this thesis. This accomplishment would not have been possible without them. Thank you.

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CHAPTER I

INTRODUCTION

Beta-Thalassemia is an inherited blood disorder caused by either *beta globin* gene mutation or deletion, which affects the adult hemoglobin. The patients who have thalassemia major suffer from severe anemia, chronic hemolysis, and needed incessant blood transfusions. Chronic blood transfusions can lead to subsequent problems. (1) The effective cure is a bone marrow transplantation from a matched family or unrelated donor or cord blood transplantation from a related donor. Nevertheless, only few patients can undergo this procedure, because in the absence of HLA compatible, the vast patients lack of well-match with unrelated donor. (2) The previous study reported that the increasing level of fetal hemoglobin expressed in Beta-Thalassemia patients with Hereditary Persistence of Fetal Hemoglobin (HPFH) could ameliorate the clinical severity. (3-5) These data suggested the important role of fetal hemoglobin in Beta-Thalassemia, leading to an attempt to reactivate fetal hemoglobin Beta-Thalassemia in order to treat the symptoms.

Hereditary persistence of fetal hemoglobin (HPFH) is caused by point mutation in ***γ*** *globin* gene or region deletion in ***δ-β*** *globin* gene. The fetal hemoglobin level is depending on patterns of mutation.(6) and level of fetal hemoglobin is differ from Beta-Thalassemia. The published data was showed that HPFH with regional deletion expressed high level of fetal hemoglobin.(6) There are many types of deletion, for example, patients with homozygous 7.2 kb deletion of intergenic ***δ-β*** *globin* gene (Corfu deletion) which fetal hemoglobin level is around 80% with only mild anemia (7, 8) and patients with the deletion of 101 kb at ***γ-β*** *globin* gene which the level of fetal hemoglobin is 20%.(9) Nowadays, it is still unclear that why the deletion in those regions has affected to fetal hemoglobin expression. The previous studies shown that there is a single nucleotide polymorphism at *BCL11A* gene in non-anemic HPFH patient. (5, 10, 11) *BCL11A* is a transcription factor which involves in silencing fetal hemoglobin. It has been reported that *BCL11A* has many binding sites on ***β***-globin locus, (12-15), however it is still unable to identify the specific site to suppress fetal hemoglobin. In

2011, Sankaran et al reported a case of a Sri Lanka HPFH patient who has deletion of 3.5 kb intergenic ***γ-δ*** *globin* gene, which the patient had level of fetal hemoglobin of more than 40% with no anemia. Interestingly, there is also a *BCL11A* binding site within that 3.5 kb intergenic ***γ-δ*** *globin* gene. (13) However, there has not been a report regarding the role of 3.5 kb and *BCL11A* that bind in this region.

In this study, the genome editing method, using CRISPR/Cas9, to cut a large region of DNA sequences in erythroleukemia cell line (K562), has been developed to mimic the natural deletion in HPFH patients. The designed CRISPR sgRNA with Cas9 could induce DNA double strand break (DSB) and site-specific deletion of 3.5 kb in ***γ-δ*** *globin* gene. Moreover, the CRISPR sgRNA designated to cut a small region of DNA sequences (69 bp around *BCL11A* binding site) has been created. Interestingly, the deletion of 69 bp resulted in increasing level of fetal hemoglobin as well as the 3.5 kb deletion. In addition, the level of fetal hemoglobin was successfully enhanced by using CRISPR with transcriptional activator (CRISPR-On). Taken together, genetically engineered K562 and designed CRISPR sgRNA that we established might be the foundation for the study of 'Expression Control Mechanism in Fetal Hemoglobin' and 'Fetal Hemoglobin Reactivation' could be useful for the discovery of any possible therapeutics for the Beta-thalassemia patient.

CHAPTER II

BACKGROUND AND LITERATURE REVIEW

Beta-thalassemia and hereditary persistence of fetal hemoglobin

Beta-thalassemia is an inherited blood disorder caused by more than 200 different *β-globin* gene mutations which resulted in reduced or abolished *β-globin* chains. Beta-zero (β^0) thalassemia is referred to mutations in the *β-globin* gene that prohibit the production of any *β-globin* gene. Other mutations allow some *β-globin* gene to be produced but in a decreased level, is called Beta-plus (β^+) thalassemia. Classifying by the symptoms, Beta-thalassemia is divided into two subtypes, thalassemia major and thalassemia intermedia. The patients who have thalassemia intermedia or minor, presented milder anemia that rarely requires blood transfusion. within contrast, patients with thalassemia major suffer from severe anemia, chronic hemolysis, and needed incessant blood transfusions to restore their red blood cell supply. Chronic blood transfusions can lead to iron overload that affects the liver, heart, and hormone, subsequently causing problems.(1) The effective cure is a bone marrow transplantation from a matched family or unrelated donor or cord blood transplantation from a related donor. However, it has been reported that Beta-thalassemia patients who have co-inheritance with hereditary persistence of fetal hemoglobin (HPFH) can show reduced severity of the symptoms.(3-5)

Hereditary Persistence of Fetal Hemoglobin (HPFH) is an inherited blood disorder where patients' red blood cells express fetal hemoglobin in adulthood without harmful effects. In 1955, Edington and Lehmann are the first groups who found HPFH patients in West African. HPFH could be found worldwide. Although, genotypes of HPFH patients contain mutations similar to δβ thalassemia, HPFH patients only have mild hypochromia and microcytosis or no symptom. Previous studies classified mutation of HPFH into 2 groups, non-deletion and deletion groups. Patterns of deletion form in HPFH might be different for each patient. Most of the time, we found that deletion occurred in *δ* and *β globin* gene with difference sizes. Moreover, there are

crossing over deletion in Hb Lepore and Hb Kenya.(16) The cause of Non-deletion form in HPFH is a single point mutation in ***γ-globin*** gene. We found that point mutation occurred around promoter of *G**γ*** and *A**γ***. (6, 13, 17, 18)

Previous studied showed that each type of mutation led to different expression level of fetal hemoglobin. Studies about the patterns of deletion helped establish a data base to compared level of fetal hemoglobin.(19)

Hemoglobin and hemoglobin switching

Hemoglobin is a group of protein molecules in red blood cells that involve in transporting O_2 to every part of the body. Hemoglobin is composed of 2 polypeptide chains, alpha globin chain and beta globin chain, and heme group which has porphirin and Fe (Figure1). Genes that control synthesis of beta globin chain are on chromosome 11. There are group of beta globin gene cluster, consisting of ***β-globin*** locus control region (***β***-LCR), which plays roles in chromatin opening to regulate the expression of globin genes by direct bind at the promotes, the epsilon globin gene; ***ε***, gamma globin gene; G***γ***, A***γ***, delta globin gene; ***δ***, and beta globin gene; ***β***. Alpha globin chain is on chromosome 16 consist of alpha globin gene cluster; zeta globin gene; ***δ***, and alpha globin gene; ***α***1 and ***α***2 (Figure2). (20) When both of the globin chains combine together, different types of hemoglobin will be generated (Table1).

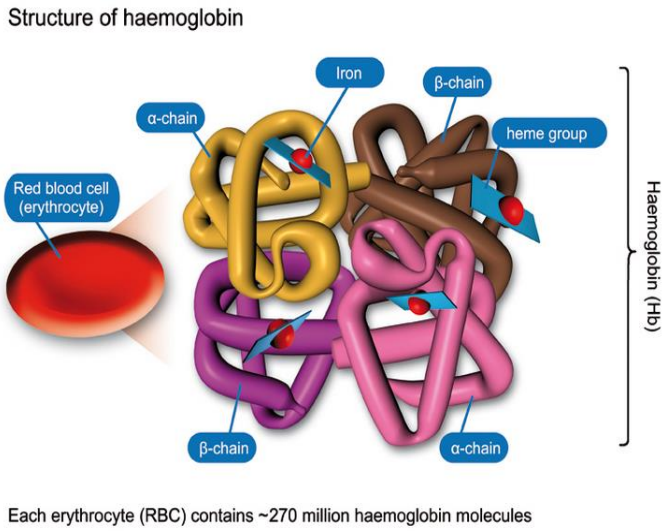


Figure 1 Structure of hemoglobin (http://www.fmcenalpharma.com/anaemia.htm)

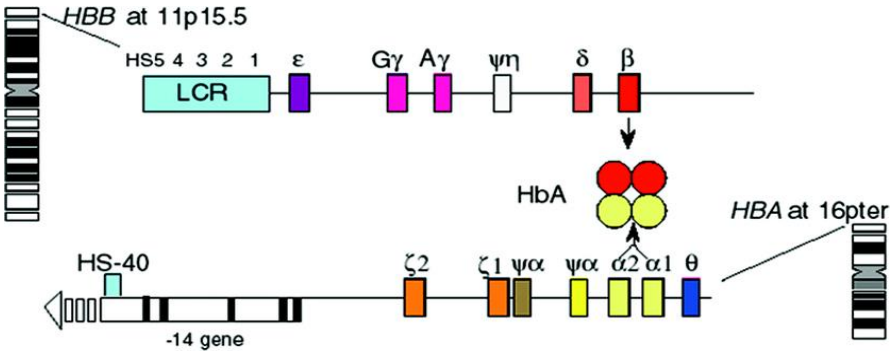


Figure 2 The gene order of *β-globin* gene on chromosome 11 and *α-globin* gene on chromosome 16. (21)

Table 1 Each type of hemoglobin during human development

	Embryo	Fetus-Newborn	Infant-Adult
Hb Gower I (ζ2ε2)	Present	0	0
Hb Gower II (α2ε2)	Present	0	0
Hb Portland (ζ2γ2)	Few	Few	-
Hb F (α2γ2)	Few	60-80%	1%
Hb A (α2β2)	-	40-20%	96-98%
Hb A2 (α2δ2)	-	2%	1-3%

In human, hemoglobin synthesis occurs after fertilization of the embryo. In this period, Embryo hemoglobin (Hb Gower) is the first type of hemoglobin that is synthesized in the yolk sac. After 7-10 weeks of pregnancy, stem or progenitor cells of blood cells begin to synthesize Fetal hemoglobin (Hb F), to replace Hb Gower in fetal liver. After birth, newborn will have Hb F around 50-95% and hemoglobin synthesis will switch to a synthesis in bone marrow to generate adult hemoglobin (Hb A). This process is referred to as γ-to-β switching which is regulated by LCR form loops to interaction with β- *globin* promoter. Transcription factors that are involved in mechanism of switching include GATA1, TAL1, E2A, LMO2 and LDB1. The level of Hb F will be decreased, whereas level of Hb A will be increased. After 1-2 years, the level of Hb A in the baby will become similar to the adult. (Figure3) (2)

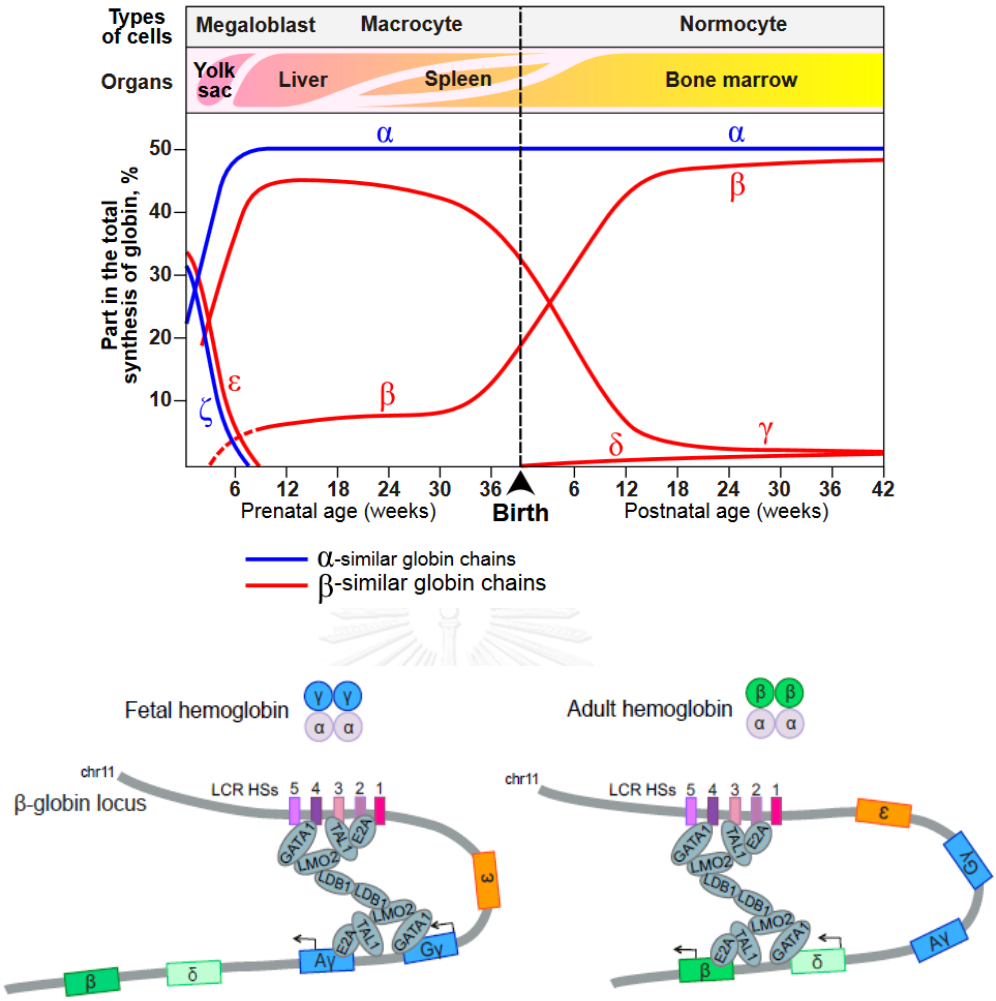


Figure 3 Developmental stage of hemoglobin switching (upper). Schematic showed process of γ-to-β switching by LCR formed chromosomal looping interaction with transcription factor that involved in mechanism of switching. (lower) (2)

Reversing of hemoglobin switching

Previous data showed that in Beta-thalassemia patients who had expressed fetal hemoglobin, the severity of the disease was ameliorated. The researchers suspected that the expression of fetal hemoglobin led to such an observation. Epigenetic marks, such as DNA methylation, were identified as an inducer using 5-azacytidine.(22-24) However, it was still unclear through which mechanisms the drugs worked. To induce more fetal hemoglobin, molecular regulators were studied by cloning the globin genes and studying transcription factors such as GATA1, KLF1, and SCL/TAL1.(25) However, the results suggested that those transcription factors were

related to normal erythropoiesis and globin gene expression more than specific to production of fetal hemoglobin. Therefore, they collected blood samples to examine natural human variation traits such as non-anemic HPFH patients who have express high level of fetal hemoglobin. A genome-wide association study (GWAS) was performed to study pattern of polymorphism. The results showed a single nucleotide polymorphism (SNPs) occurred in the sequence of the *BCL11A* gene which associated with an increase of fetal hemoglobin.(5) Thus, researchers focus more intensively in studying the role of *BCL11A*. Researchers found the relationships between expression of *BCL11A* proteins and developmental stage which is in primitive stage and fetal hemoglobin in fetus liver are highly expressed **γ**-*globin* gene in contrast of *BCL11A* expression.(12) Thus, they hypothesized that *BCL11A* played a role as a repressor of **γ**-*globin* gene. In order to test this hypothesis, *BCL11A* gene was knocked down by shRNA and showed that the reduction of *BCL11A* could reactivate fetal hemoglobin expression in adult red blood cells without destroying or disrupting the functions of red blood cells.(12, 14, 26)

Transgenic mouse model was used to determine how *BCL11A* was involved in hemoglobin switching. By breeding mice with human ***β*** *globin* locus and mice with *Bcl11a* gene knock out. Researchers were able to show that normal erythropoiesis could occur in mice without expression of *Bcl11a* but ***γ*** *globin* gene could not be silenced. (27) From this research, the importance of the *BCL11A* was confirmed in hemoglobin switching. Subsequently, immunoprecipitation was performed to search *BCL11A* binding site in the ***β***-*globin* gene cluster. They found that *BCL11A* could bind to multiple sites on ***β***-*globin* gene cluster. (12-14) However, it is uncertain which site is critical to suppress fetal hemoglobin expression.

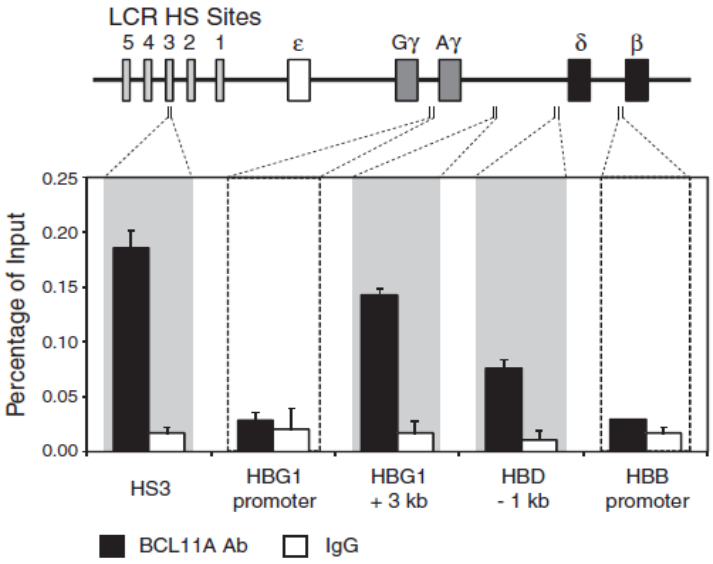


Figure 4 Schematic showed multiple regions of *BCL11A* binding site on *β-globin* locus.

An analysis of Beta-thalassemia and HPFH patient were performed by the mutational mapping. The authors divided deletion types into 3 groups which are Sri Lankan HPFH, Sri Lankan $(\delta\beta)^0$ thalassemia and Kurdish β^0– thalassemia. When they compared pattern of deletion and level of fetal hemoglobin expression, the region 3.5 kb intergenic *γ-δ globin* gene is probably involved in stimulation of *γ-globin* gene. In Sri Lankan $(\delta\beta)^0$ thalassemia and Kurdish β^0– thalassemia, which did not have the deletion at 3.5 kb intergenic *γ-δ globin* gene, showed level expression of fetal hemoglobin at 10% and 2%, respectively. On the other hand, Sri Lankan HPFH with the deletion at 3.5 kb showed level of fetal hemoglobin at 40% (13)

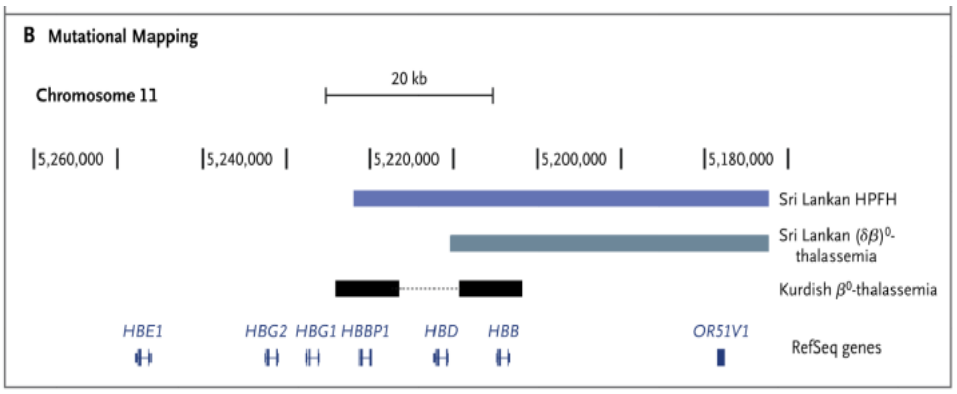


Figure 5 Schematic showed mutational mapping in Sri Lankan HPFH, Sri Lankan $(\delta\beta)^0$ thalassemia and Kurdish β^0– thalassemia. (13)

Researchers hypothesized that 3.5 kb intergenic *γ-δ globin* gene region might have transcription factors that are involved in suppression of fetal hemoglobin. To test hypothesis, immunoprecipitation was performed and they found that this region had expression of *BCL11A* which is consistent with previous research that *BCL11A* acts as a repressor of fetal hemoglobin.

CHAPTER III
MATERIALS AND METHODS

Cell line

This research used human erythroleukemia K562 cells. K562 cells were also cultured in RPMI-1640, which supplemented with 10% Fetal Bovine Serum (FBS), 1% Antibiotic-Antimycotic, and 1% Glutamine. Cells were maintained in an incubator with 5% CO_2 at 37 ℃.

Generation of CRISPR/Cas9 and CRISPR ON

According to the research of Sankaran et al in 2011, an interesting region might be involved in silencing fetal hemoglobin gene. Based on the particular research, we used bioinformatics tools to find the sequences and generating CRISPR specifically for 3 regions; 3.5 kb intergenic ***γ-δ globin*** gene, dissect 3.5 kb into 3 parts, 69 bp around *BCL11A* binding site. We designed sgRNA by 20bp upstream a NGG PAM sequence. Then, sgRNA guides that have a high scored of non-off target site were chosen. After that, we have cloned sgRNA guides into plasmid pSpCas9(BB)-2A-GFP (PX458) was a gift from Feng Zhang (Addgene plasmid # 48138) which contained Cas9 and GFP site. In part of CRISPR ON, we generated sgRNA guides that bind to upstream of promoter site. We designed sgRNA that specific for gamma hemoglobin (*HBG*), beta hemoglobin (*HBB*) and *BCL11A* promoter. We have cloned sgRNA guides into plasmid pAC154-dual-dCas9VP160-sgExpression which was a gift from Rudolf Jaenisch (Addgene plasmid # 48240). Both CRISPR/Cas9 and CRISPR On, cloning technique was followed Zhang's protocol. Briefly, the specific site of *Bbs*I in px458 and pAC154 were digested by restriction enzyme and sgRNA was cloned into the cut site. Then, 3 sub clones were selected from each sgRNA for plasmids extraction. Before DNA sequencing, restriction enzyme *Bbs*I was used for digesting and confirming whether the selected clones were the corrected ones. The corrected clones were ones that are not digested in the process of restriction enzyme. To help identify the corrected clones, agarose gel

electrophoresis was preceded for band of plasmid. Through DNA sequencing, each plasmid, which showed single band, confirmed as a plasmid with insert sgRNA.

Transfection of CRISPR/Cas9 and CRISPR On in K562

To test efficiency of CRISPR/Cas9 and CRISPR On, we tested in K562 because K562 is cell line in type of erythroleukemia. It can express hemoglobin and have potential to differentiate to early-stage of erythrocyte. We transfected CRISPR/Cas9 plasmid into K562 by using SF Cell Line 4D-Nucleofector® X Kit L. A total of 1x106 K562 cells were transfected with 2 μg of plasmid containing each sgRNA. CRISPR/Cas9 plasmid were combined together.

Table 2 showed combined gRNA that transfected into K562

	CRISPR sgRNA 1	CRISPR sgRNA 2
3.5 kb intergenic ***γ-δ*** *globin* gene	3.5 A Begin	3.5 A End
Dissect 3.5 kb into 3 parts	3.5 A Begin	3.5 Mid 1A
	3.5 Mid 1A	3.5 Mid 2 A
	3.5 Mid 2A	3.5 A End
69 bp around *BCL11A* binding site	HBG 20 #1	HBG 20 #3

Flow Cytometry

After transfection for 48 hours, K562 cells containing of CRISPR were sorted by flow cytometry using BD FACSAria II (Becton Dickinson, Franklin Lakes, NJ, USA). All of K562 cells which expressed GFP were collected as mix population. After that, we cultured each clone of K562 cells and extracted genomic DNA to observe pattern of DNA sequence.

Serial dilution to select single cell

After we collected total GFP positive cells as mix population. We would like to observe pattern of DNA after editing with CRISPR/Cas9 from each cell. Serial dilution

was performed to select single cell. We used 96-well plate, the procedure followed figure6.

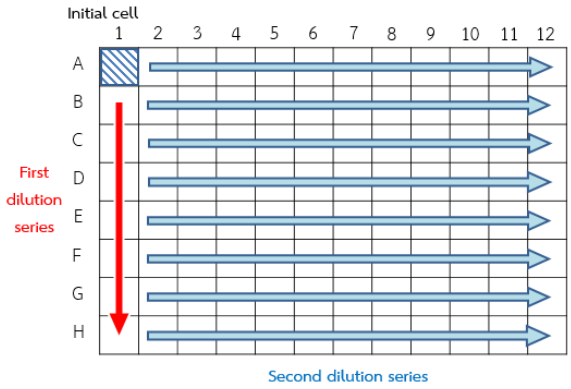


Figure 6 Schematic showed the procedure of serial dilution.

Reverse transcription-polymerase chain reaction (RT-PCR) and real-time PCR

TRI reagent (Molecular Research Center, Cincinnati, OH, USA) was used for RNA extraction. In the next process, the isolated RNA was reverse transcribed with RevertAid™ H Minus M-MuLV (Fermentas, Glen Burnie, MD, USA). Then, using cDNA, real-time PCR assay was performed on ABI 7500 Fast Real-time PCR System using Maxima SYBR Green/ROX qPCR Master Mix (2X) (Fermentas). Also, GAPDH was used as an internal control.

Red blood cell differentiation

K562 has potentiated to differentiate to red blood cells and normally expressed *HBG*. K562 cells were treated in 30uM hemin for 3 days. After that cells were collected and performed hemoglobin typing.

Hemoglobin typing

After cell pellet were collected, we used VARIANT II Hemoglobin testing system to analysis type of hemoglobin. Cell pellets were resuspended with dilution buffer and then analyzed which was assistance by Division of hematology, King Chulalorgkorn Memorial Hospital.

CHAPTER IV
RESULTS

Using CRISPR/Cas9 to establish the natural deletion at 3.5 kb intergenic *γ-δ globin* gene

Mutation analysis of *β- globin* locus deletions found in HPFH and δβ-Thalassemia has identified a 3.5 kb intergenic *γ-δ globin* gene representing a potential fetal hemoglobin silencer. Without this 3.5 kb deletion, in Kurdish *β*0-Thalassemia, fetal hemoglobin expression level is lower (4%) compared to other mutation types (Figure7). Based on these analyses, we hypothesized that deletion of 3.5 kb intergenic *γ-δ globin* gene might be sufficient to activate fetal hemoglobin expression. To test this hypothesis, we made 2 sets of sgRNAs targeted 5' and 3' of 3.5 kb intergenic *γ-δ globin* gene region. 3.5 A begin, end and 3.5 B begin, end were designed (Figure8 A, B). Nucleofection of gRNA and Cas9- GFP into K562 erythroblastic cell line demonstrated transfection efficiency of 37.2% based upon GFP expression (Figure9 A-C).

Deletion of 3.5 kb intergenic *γ-δ globin* gene region was evaluated within transfected GFP positive cells by amplifying the genomic DNA at 3.5 kb region using primer outside gRNA targets. 612 bp PCR product instead of 4010 bp was observed in GFP positive cells transfected with 3.5 A begin and 3.5 A end but not in 3.5 B set and control with deletion efficiency 68.85%. DNA sequencing of 612 bp PCR product confirmed the absence of 3.5 kb intergenic *γ-δ globin* gene region (Figure10 A, B).

These results indicated that, using CRISPR/Cas9 system, we could generate erythroblast model with 3.5 kb intergenic *γ-δ globin* gene deletion which referred as K562 $^{-3.5\ kb}$

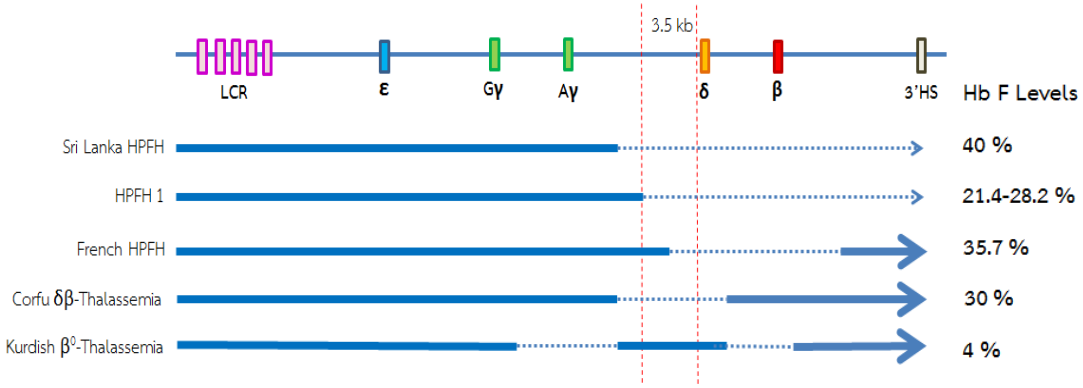


Figure 7 Schematic described genomic deletion in common HPFH and their expression of fetal hemoglobin (Hb F) level referred to published data (19). Region with blue dash line presented deletion region. Interestingly, HPFH with deletion of 3.5 kb (region between red dash line) expressed high level of fetal hemoglobin.

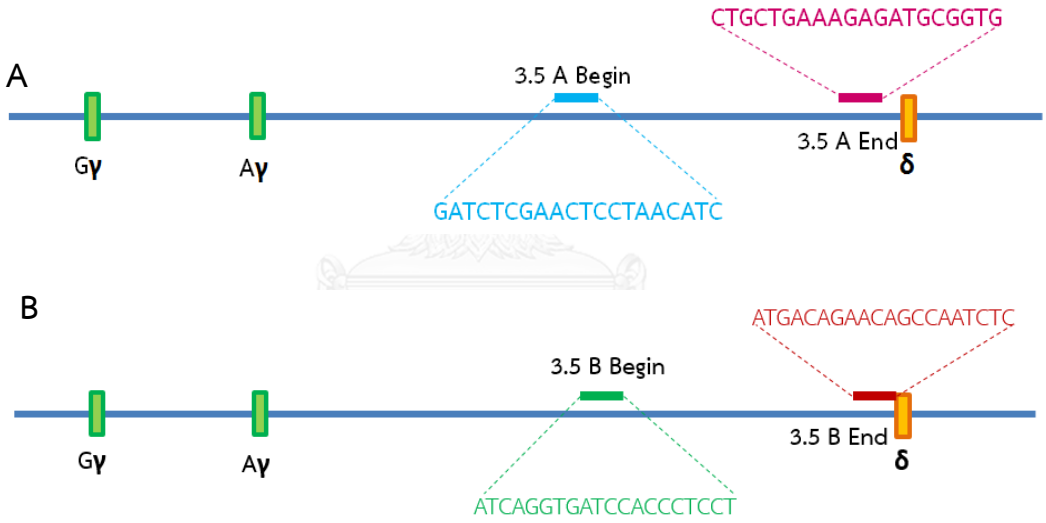


Figure 8 Schematic representation to 2 sets of sgRNA that specific in 3.5 kb intergenic γ-δ globin gene. (A) sgRNA 3.5 A Begin was specific to 5' of 3.5 kb and sgRNA 3.5 A End was specific to sequence upstream δ globin gene. (B) Also in sgRNA 3.5 B Begin + End.

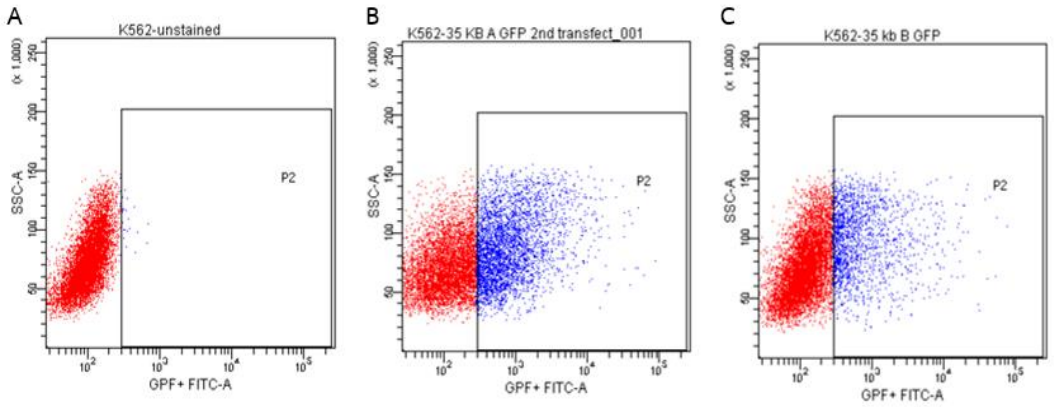
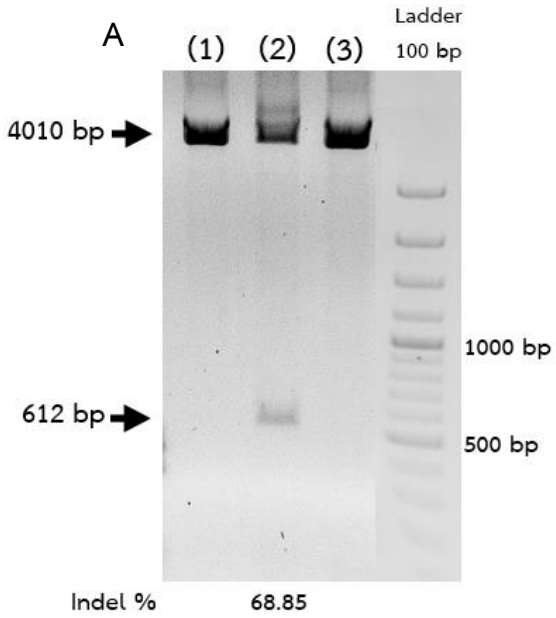


Figure 9 K562 transfected with sgRNAs (3.5 A Begin + 3.5 A End) and Cas9-GFP. GFP positive cells (P2) were sorted, 48 hours after transfection, by flow cytometry. (A) Populations of K562 without transfection (Control), (B) K562 with 3.5 A Begin + 3.5 A End. Population P2 were collected. Efficiency of transfection showed 37.2%. (C) K562 with 3.5 B Begin + 3.5 B End. Efficiency of transfection showed 20% and population P2 were collected.



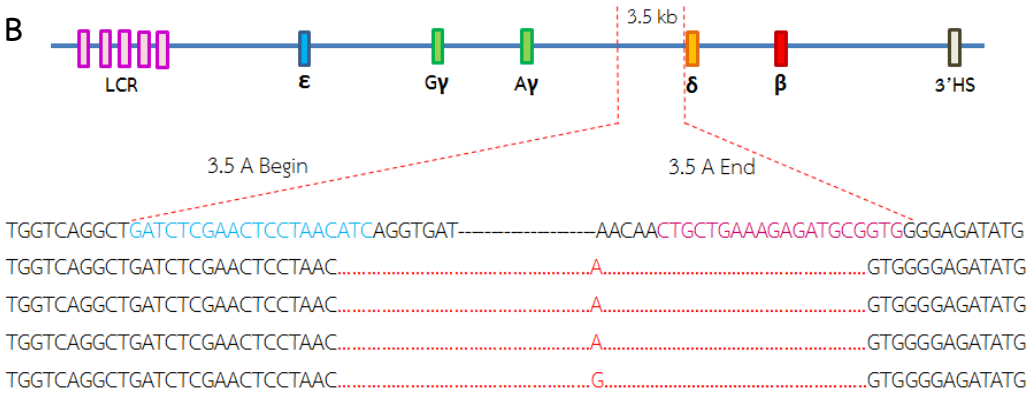


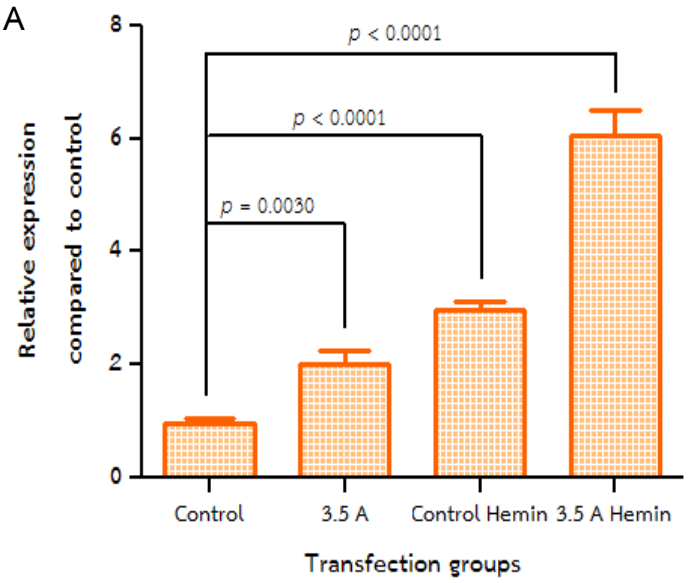
Figure 10 Detection of genomic DNA after transfected with sgRNA. (A) Genomic DNA was amplified by using primer cover region of 3.5 kb. Lane (1) control amplified band 4010 bp, (2) K562 with sgRNA 3.5 A induced DNA double strand break could amplify band 612 bp, (3) K562 with sgRNA 3.5 B could not induce DNA double strand break, amplify band 4010 bp. (B) Sequence alignment of 612 bp after non-homologous end joining process by DNA sequencing. In between of two sgRNA, there is a region ~3.5 kb that was deleted in four sub clones with insert A and G for 3 and 1 sub clones, respectively.

Next, we test whether deletion of 3.5 kb intergenic ***γ-δ globin*** gene region could activate fetal hemoglobin expression. First, K562 and K562 $^{-3.5\ kb}$ were differentiated to erythrocyte by treated with hemin. mRNA expression level of fetal hemoglobin was determined using qRT-PCR. Interestingly, the expression level of fetal hemoglobin in K562 $^{-3.5\ kb}$ and K562 $^{-3.5\ kb}$ treated with hemin (K562 $^{-3.5\ kb}$ hemin) were increased to 2-fold and 6-fold, respectively, compared to K562. Hemoglobin typing analysis using HPLC also showed an increase of fetal hemoglobin level from 35.2% in K562 hemin to 50.7% in K562 $^{-3.5\ kb}$ hemin. These data demonstrated that deletion of 3.5 kb intergenic ***γ-δ globin*** gene region could activate fetal hemoglobin expression in K562 cell line, implying the important role of this 3.5 kb region in controlling fetal hemoglobin expression (Figure11 A-D).

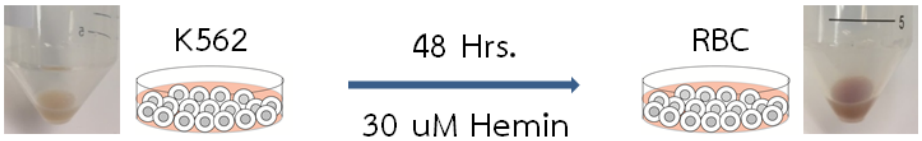
Moreover, we further performed sequential deletion to identify a minimum region that essential for ***γ globin*** repression. 3.5 kb region was divided into three parts, 1118 bp, 1104 bp and 1236 bp (Figure12). sgRNA targeted each dissected region were successfully designed and proved efficient. However, deletion of targeted sequences

observed in GFP positive enriched population is lower than 50% (expected DNA size 2912, 2926 and 2794 bp) (Figure13). So, we are currently in the process of establishing clonal K562 cell line with target sequence deletion for further fetal hemoglobin expression analysis.

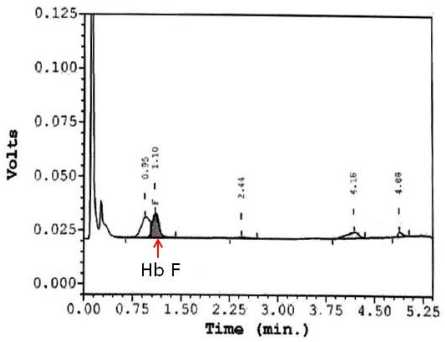
A



B

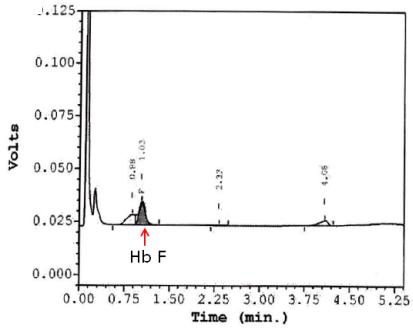


C



Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	41.2	0.95	90495
F	35.2	---	1.10	76804
Ao	---	2.9	2.44	6435
Unknown	---	16.4	4.18	35987
Unknown	---	4.6	4.88	10131

D



Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
P1	---	32.3	0.88	48947
F	50.7	---	1.03	76172
Ao	---	0.9	2.33	1437
D-window	---	16.4	4.08	24770

Figure 11 Evaluated level of fetal hemoglobin expression in K562 control, K562 Hemin, K562 $^{-3.5\ kb}$ and K562 $^{-3.5\ kb}$ hemin. (A) Level of HBG expression was measured by qRT-PCR. Result are mean±SD. Significance of difference between each transfection was calculated using t-test, n=3. (B) Schematic showed K562 treated with hemin to differentiate into red blood cells. Hemoglobin typing was performed to evaluate level of fetal hemoglobin in both treated with hemin K562 (C) and K562 $^{-3.5\ kb}$ (D) by HPLC.

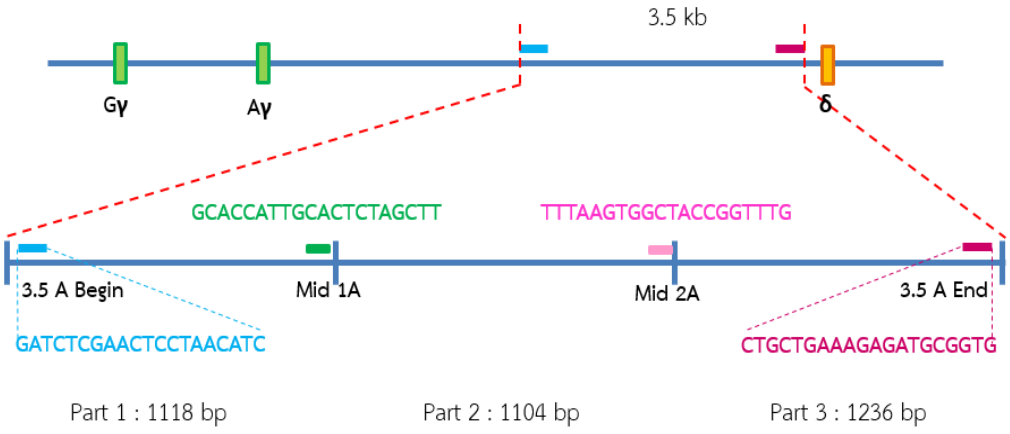


Figure 12 Schematic representation of designed sgRNAs to sequential deletion 3.5 kb for identified the minimum region that critical for **γ** globin repression. 3.5 kb was dissected into 3 parts; 1) 3.5 A Begin–Mid 1A, 2) Mid 1A-Mid 2A and 3) Mid 2A-3.5 A End, size of each part, 1118 bp, 1104 bp and 1236 bp, respectively.

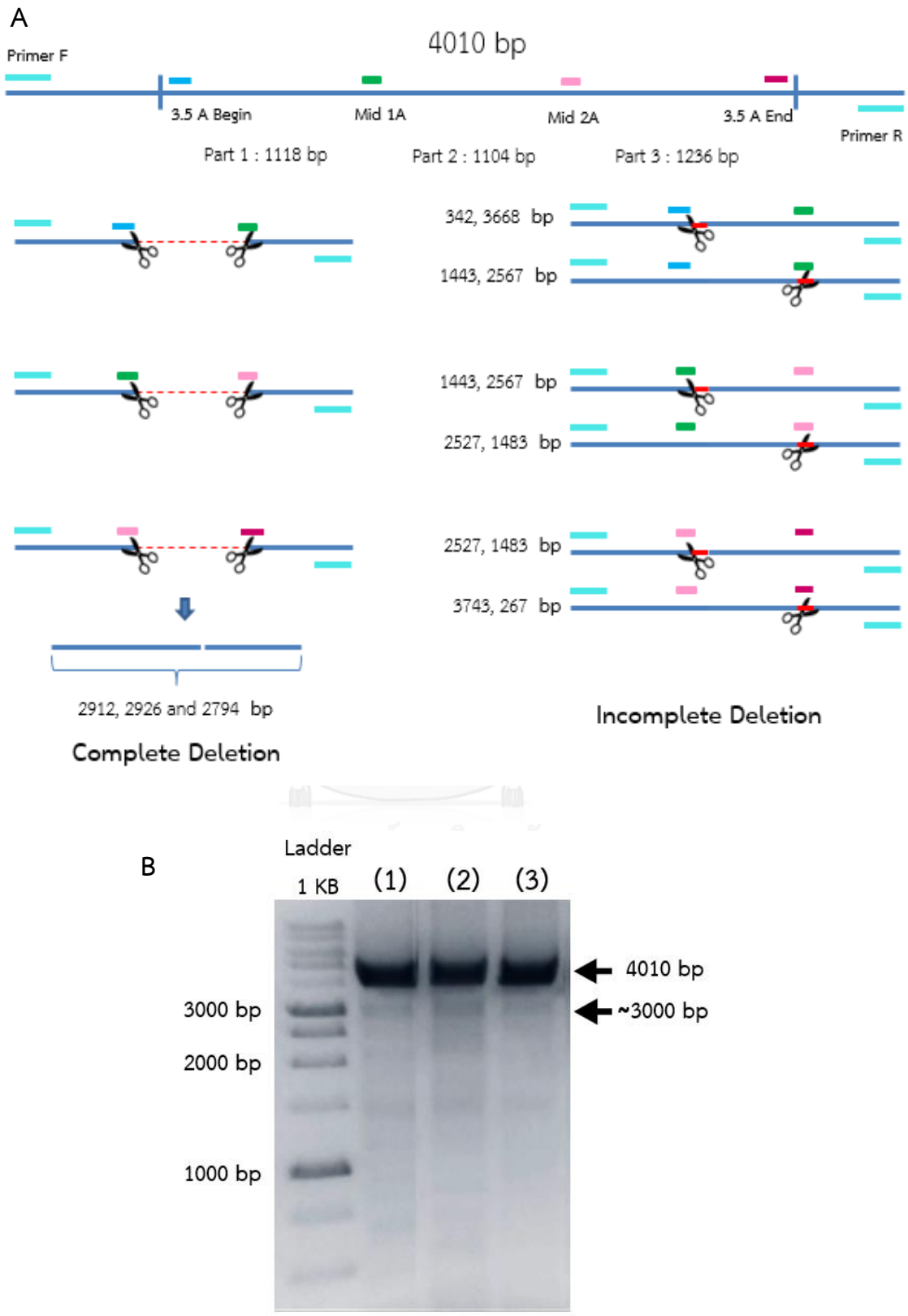


Figure 13 Schematic showed specific primer for amplifying DNA size 4010 bp. PCR was performed to confirm DNA double strand break (DSB). In case of complete deletion, DNA size 2912, 2926 and

2794 were amplified. But if CRISPR/Cas9 could work only one side, DNA size in group of incomplete deletions were amplified instead (A). Agarose gel electrophoresis was performed to separate each band of DNA. The band of DNA ~ 3000 bp were showed but with low efficiency. Specific primer amplified DNA size 4010 bp in case of sgRNA could not induce DSB. Lane (1) Deletion of the first part of 3.5 kb. (2) Delete sequence in the middle. (3) Delete sequence at end of 3.5 kb (B).

Eliminating of *BCL11A* binding site could lead to the fetal hemoglobin expression

Persistence of Hb F in HPFH was associated with the mutations in regions of ***β-globin*** locus that regulate transcription of Hb F. In order to find critical region that once mutated could trigger ***γ globin*** gene expression, we focused on the binding site of major transcription repressor *BCL11A*, which plays a vital role in Hb F silencing. Study by Xu et al, in 2013, demonstrated multiple binding of *BCL11A* and its corepressor on ***β-globin*** locus using ChIP-PCR technique. (Figure14 A, B) Highest occupancy level of *BCL11A* protein was observed in ***γ-δ*** intergenic region (position D). We hypothesized that deletion of 69 bp ***γ-δ*** intergenic region (position D) which contains *BCL11A* binding site could enhance ***γ globin*** gene expression in K562 cell line. To test this hypothesis, sgRNAs targeted 69 bp around position D were co-transfected with Cas9-GFP into K562 cell line. PCR amplified 500 bp region around surrounding the predicted cut sites followed by T7 endonuclease analysis was used to analyze genomic mutation introduced by designed sgRNA and Cas9. Two types of mutations were observed in GFP+ transfected K562. PCR analysis showed higher incidence of indel mutation (338 bp and 93 bp) than 69 bp deletion (431 bp). 431 bp PCR band was rarely observed in mix population. Subsequently, K562 clones were derived from sorted GFP+ cells. PCR analysis of 7 K562 $^{-69\ bp}$ clones obtained from single cell culture showed unique pattern of mutation in each clone except clone 1 (Table3). 431 bp PCR product which indicates 69 bp deletion was detected only in clone 2 and clone 5 (Figure15 A-D). The presence of 500 bp band in these 2 clones indicated heterozygous deletion in ***β-globin*** allele. Based on intensity of 338 bp band, clone 2 was selected for further analysis. After erythrocyte differentiation, interestingly, expression level of Hb F in K562 $^{-69\ bp}$ clone 2 was 5-fold higher than K562 control which comparable to K562 $^{-3.5}$ kb hemin.

Correlated with mRNA results, percentage of fetal hemoglobin also increased to 50.1% in K562 with 69 bp deletion (Figure16 A-C). These results demonstrated that small deletion of 69 bp ***γ-δ*** intergenic region which contain *BCL11A* binding site can elevated ***γ*** *globin* gene expression level, resembling that observed in 3.5 kb intergenic region deletion. These data also imply the role of *BCL11A*.

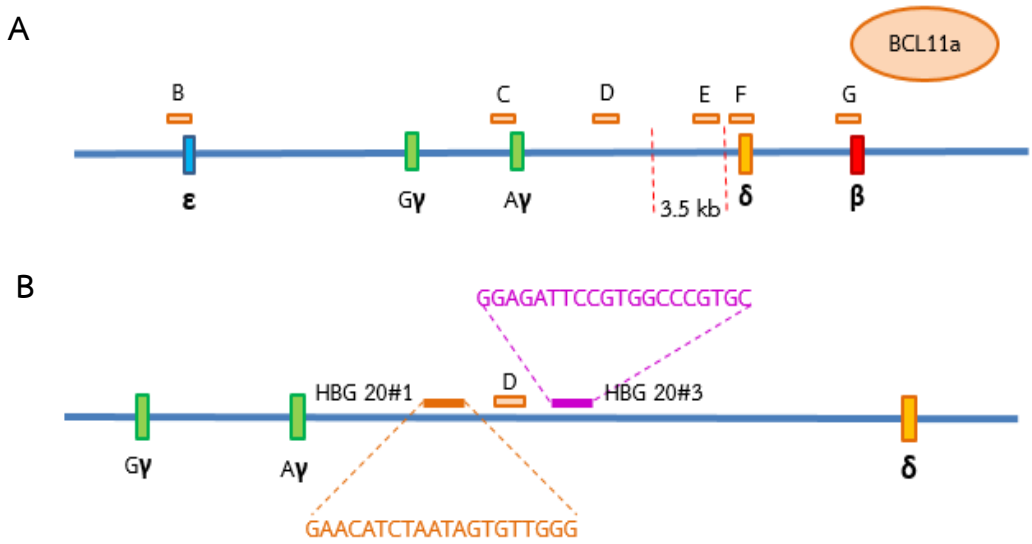
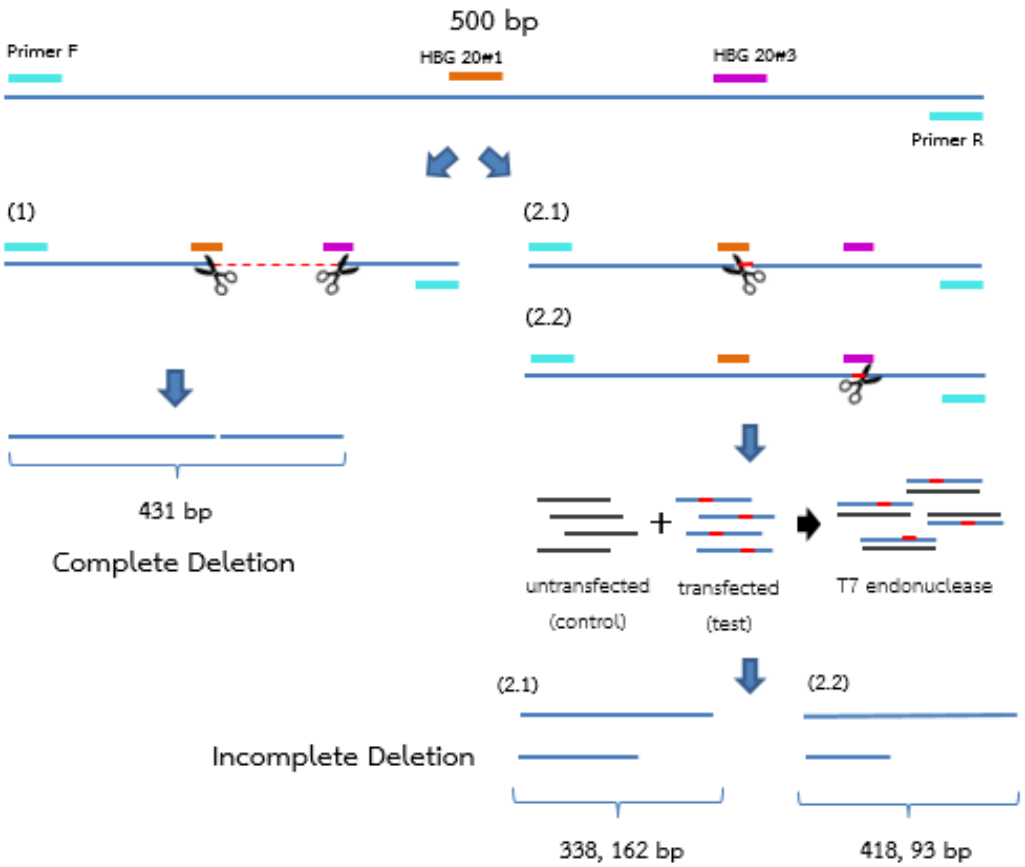
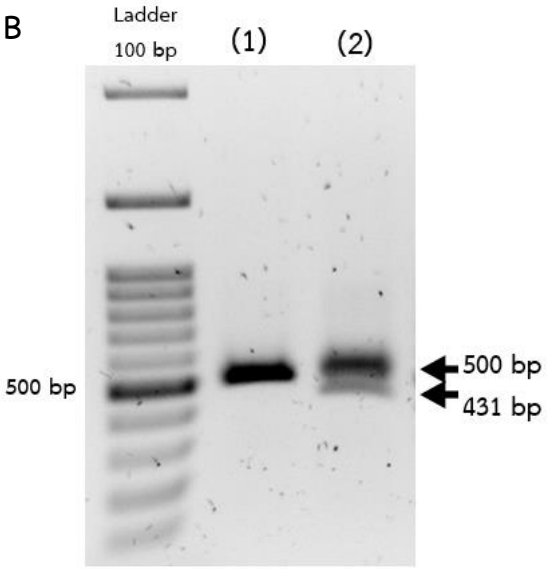


Figure 14 (A) Schematic representation of multiple binding of BCL11A in β-globin locus, regarding Jain Xu et al, in 2013. (B) At site D, had high level of BCL11A binding, was chosen to be designed in between of sgRNA guides, HBG 20#1 and HBG 20#3.

A


500 bp
Primer F
HBG 20#1
HBG 20#3
Primer R
(1)
(2.1)
(2.2)
431 bp
Complete Deletion
untransfected
(control)
transfected
(test)
T7 endonuclease
(2.1)
(2.2)
Incomplete Deletion
338, 162 bp
418, 93 bp

B


Ladder
100 bp
(1)
(2)
500 bp
500 bp
431 bp

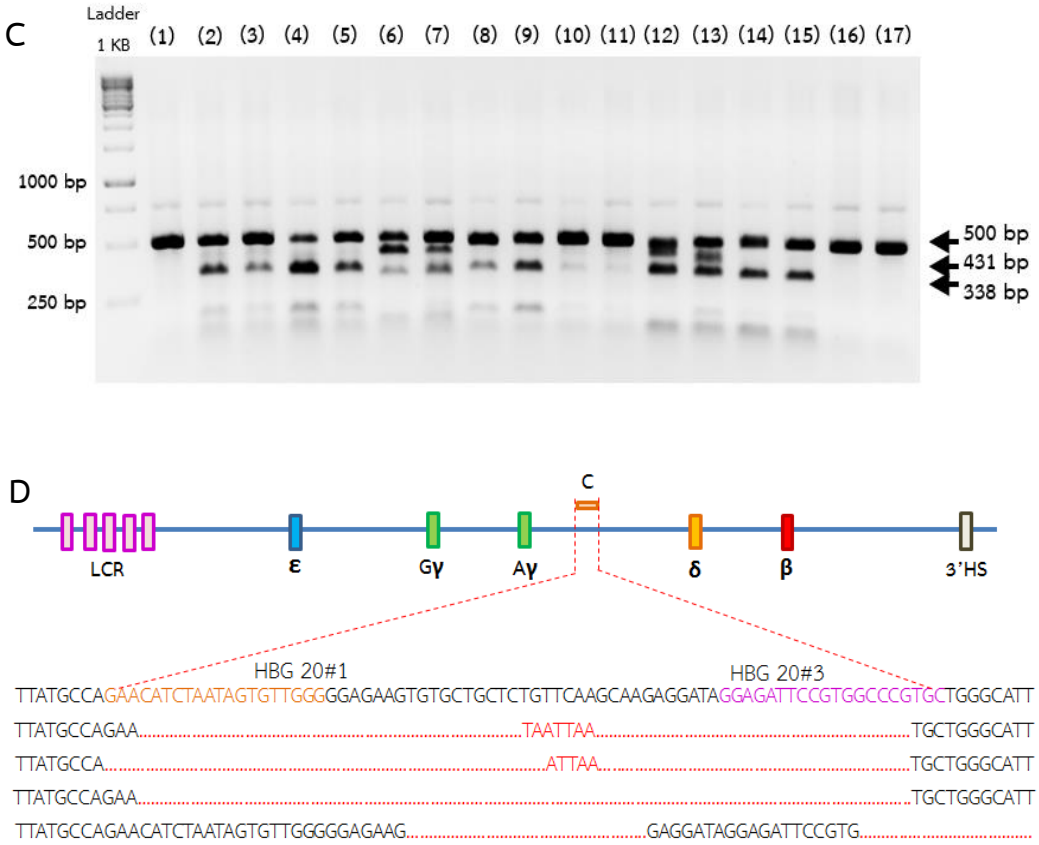
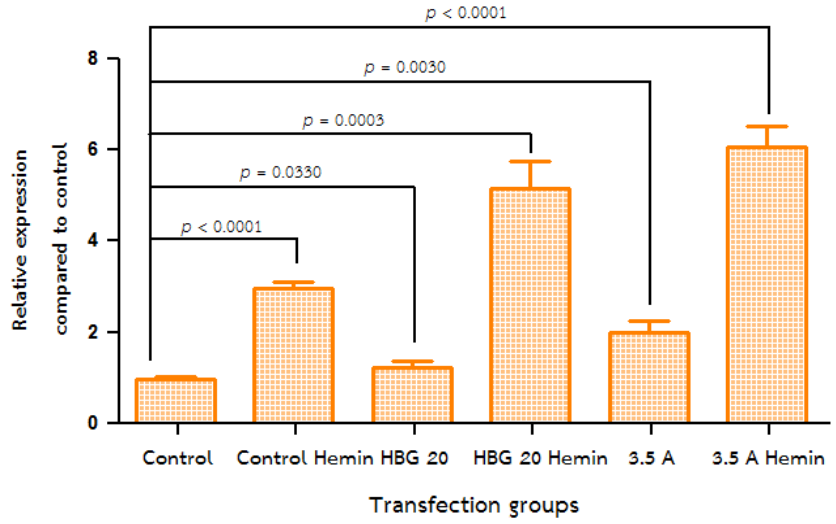


Figure 15 sgRNA HBG 20#1 + #3 were transfected into K562. (A) Schematic described after 69 bp deletion. In case that, both of CRISPR could work, DNA in red dash line have been remove and after NHEJ could amplify DNA size 431 bp (A1). If CRISPR only work only one side, T7 endonuclease could detect mismatch after annealing between control and K562 with 69 bp deletion. DNA size (338, 162) and (418, 93) bp were amplified (A2). (B) Agarose gel electrophoresis showed band DNA size 431 bp after complete deletion by CRISPR/Cas9. (C) Then, serial dilution was performed to select single cell. We could select 7 clones. Genomic DNA with 69 bp deletion from each clone was amplified using specific primer and detected double strand break by T7 endonuclease. Product size from each clone showed in Table3. (D) Sequence alignment of DNA band 431 bp after non-homologous end joining processes. In between of two sgRNA, region 69 bp around BCL11A binding site was removed (red dash line). There are nine sub clones in total. We separated to 4 groups. 1) with insertion 7 bp, 2) with insertion 5 bp, 3) without any indel and 4) CRISPR/Cas9 incompletely deletion.

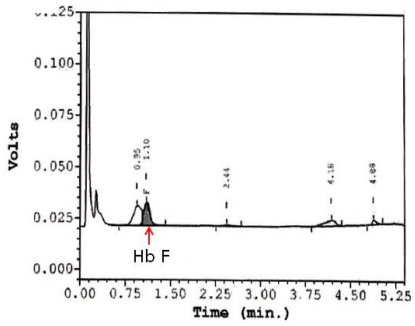
Table 3 showed size of PCR product after detecting mismatch by T7 endonuclease.

Genomic DNA	Product Size	Genomic DNA	Product Size
(1) Control + T7	500 bp	(10) Clone 4 + T7	500 bp
(2) Mix pop + T7	500, 338 bp	(11) Clone 4 + control +T7	500 bp
(3) Mix pop + control + T7	500, 338 bp	(12) Clone 5 + T7	500, 431, 338 bp
(4) Clone 1 + T7	500, 338 bp	(13) Clone 5 + control +T7	500, 431, 338 bp
(5) Clone 1 + control +T7	500, 338 bp	(14) Clone 6 + T7	500, 338 bp
(6) Clone 2 + T7	500, 431, 338 bp	(15) Clone 6 + control +T7	500, 338 bp
(7) Clone 2 + control +T7	500, 431, 338 bp	(16) Clone 7 + T7	500 bp
(8) Clone 3 + T7	500, 338 bp	(17) Clone 7 + control +T7	500 bp
(9) Clone 3 + control +T7	500, 338 bp		

A

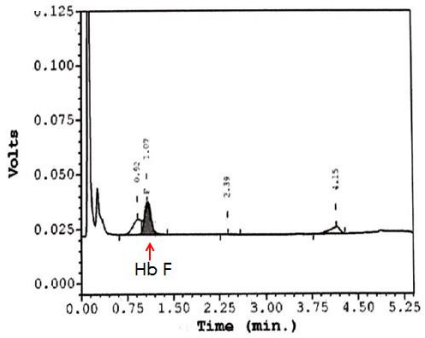


B



Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	41.2	0.95	90495
F	35.2	---	1.10	76804
Ao	---	2.9	2.44	6435
Unknown	---	16.4	4.18	35987
Unknown	---	4.6	4.88	10131

C



Peak Name	Calibrated Area %	Area %	Retention Time (min)	Peak Area
Unknown	---	32.3	0.92	66770
F	50.1%	---	1.07	102892
Ao	---	0.6	2.39	1260
D-window	---	17.4	4.15	35909

Figure 16 Measurement of HBG expression in K562 control, K562 Hemin, K562 $^{-3.5\ kb}$, K562 $^{-3.5\ kb}$ hemin, K562 $^{-69\ bp}$, K562 $^{-69\ bp}$ hemin. (A) qRT-PCR was performed to compare level of HBG expression between 3.5 kb and 69 bp deletion. Data presented in mean±SD. Statistically significant differences are indicated as p = 0.0330, p = 0.0030, p = 0.0003 and p < 0.0001 as determined by using t-test, n=3. To evaluate level of fetal hemoglobin expression by using hemoglobin typing in K562 Hemin (B) and K562 $^{-69\ bp}$ hemin (C).

CRISPR-On with transcriptional activator promoted expression of fetal hemoglobin.

CRISPR/Cas9 system has been commonly known as an efficient tool for genome editing. In addition to this aspect, generation of catalytically inactive Cas9 (dCas9) fusing with transcription activators such as VP160 (dCas9-VP160) has been successfully used to activate endogenous gene expression. dCas9-VP160 binds to regulatory region of interested gene via sgRNAs and then trigger mRNA expression. The advantage of using CRISPR-On is that this technique does not required modification of genomic DNA. For this reason, we first tested whether CRISPR-On can enhance *HBG* expression in K562. 3 *HBG* promoter specified sgRNA were designed and cloned into pAC154-dual-dCas9-VP160-SgExpression vector (Figure17). Transfection of single sgRNA-dCas9-VP160 (#1, #2, #3) slightly upregulated *HBG* mRNA expression levels. Interestingly, when transfected with triple sgRNA vectors, *HBG* mRNA expression was significantly increased by approximately 20-fold (Figure18). We next asked whether this upregulation was specific to *HBG*. Expression level of *HBB* which located in same genomic locus was evaluated. We found that while high expression level of *HBG* was detected, *HBB* mRNA

levels was slightly changed (Figure18). These data demonstrated that using sgRNA targeted proximal region of *HBG* promoter with dCas9- VP160 can efficiently upregulated *HBG* mRNA expression level in K562.

As *HBG* is normally expressed in K562 which implied the active form of its promoter, we, next, tested whether CRISPR-On can activated the expression of gene that did not express in K562 cell line. *BCL11A*, a potent *HBG* silencer, and *HBB* were inactive in K562 which were chosen. 3 sgRNAs targeted proximal promoter region of each gene were designed. Similar to *HBG*, transfection of triple sgRNA-dCas9-VP160 specifically activated *BCL11A* and *HBB* by approximately 3000- fold and 6- fold, respectively (Figure19A, B and Figure20A, B). These results demonstrated the potential of CRISPR-On in activating transcription of both active and inactive gene. The difference in activation level might be the reflection of gene silencing mechanism. Together with the result, CRISPR- On activation system represents as an alternative tool for *HBG* activation.

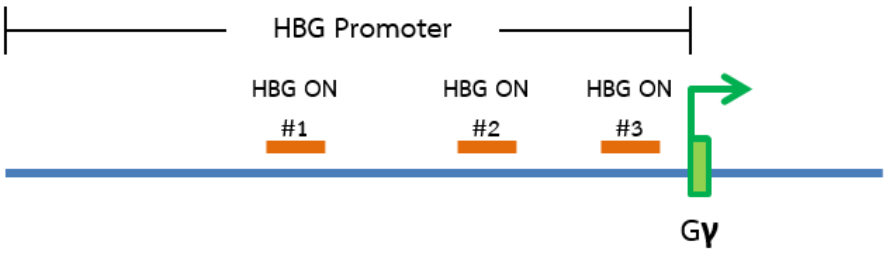


Figure 17 Schematic showed designed sgRNA that specific to HBG promoter. Guide HBG ON#1, 2 and 3 are on reverse strand.

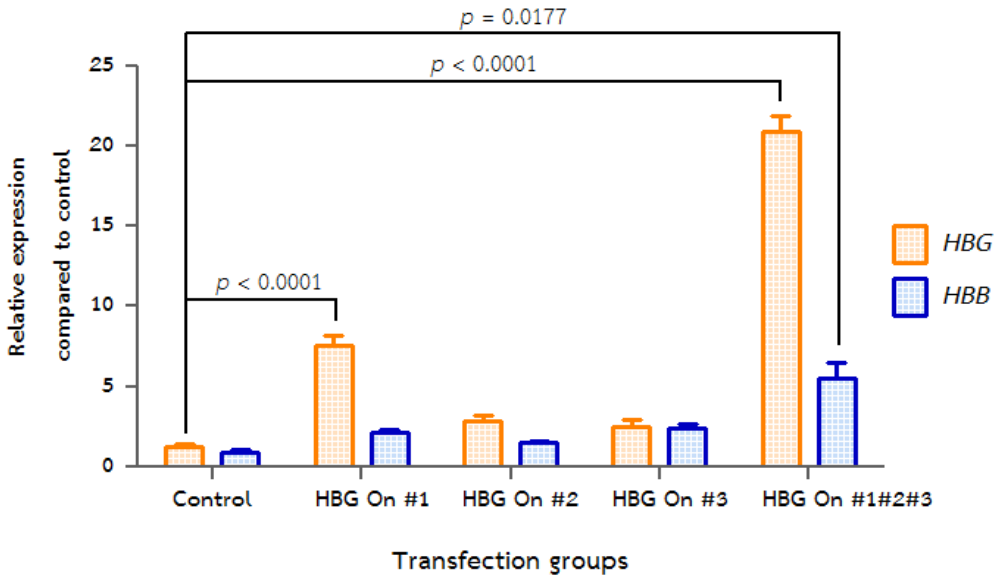
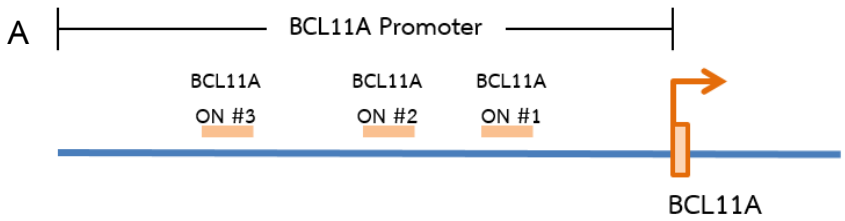


Figure 18 CRISPR-On at HBG promoter were transfected in K562. To compare level of HBG expression between transfected with single sgRNA (HBG On#1, HBG On#2, and HBG On#3) and combined sgRNA (HBG On #1, #2, and #3) by qRT-PCR relative expression compared to control and also measured level of HBB to confirm that have slightly affect to HBB expression. Data presented in mean±SD. Statistically significant differences are determined by using t-test, n=3.



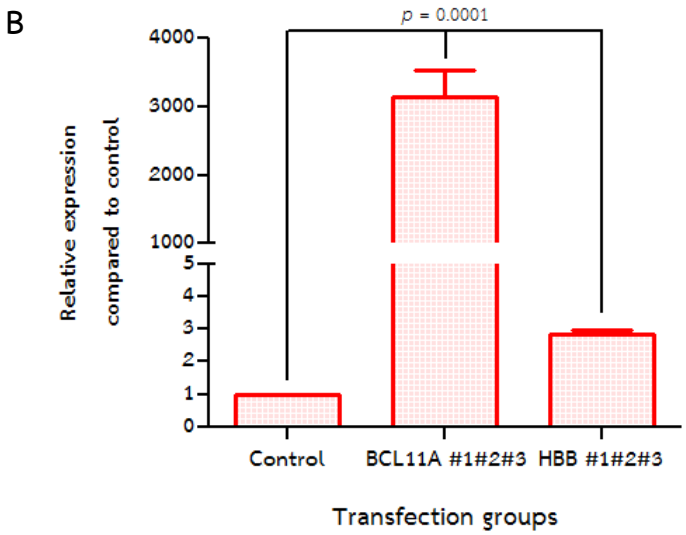


Figure 19 K562 transfected with combined CRISPR-On to activate expression of BCL11A. (A) Schematic showed each site of sgRNA that specific to BCL11A promoter. Guide BCL11A ON#1, 2, 3 are on reverse strand. (B) After transfected with combined sgRNA (BCL11A #1, #2, and #3), qRT-PCR was performed to measure level of BCL11A, compared between K562 control, combined sgRNA BCL11A. Data presented in mean±SD. Statistically significant differences are determined by using t-test, n=3.

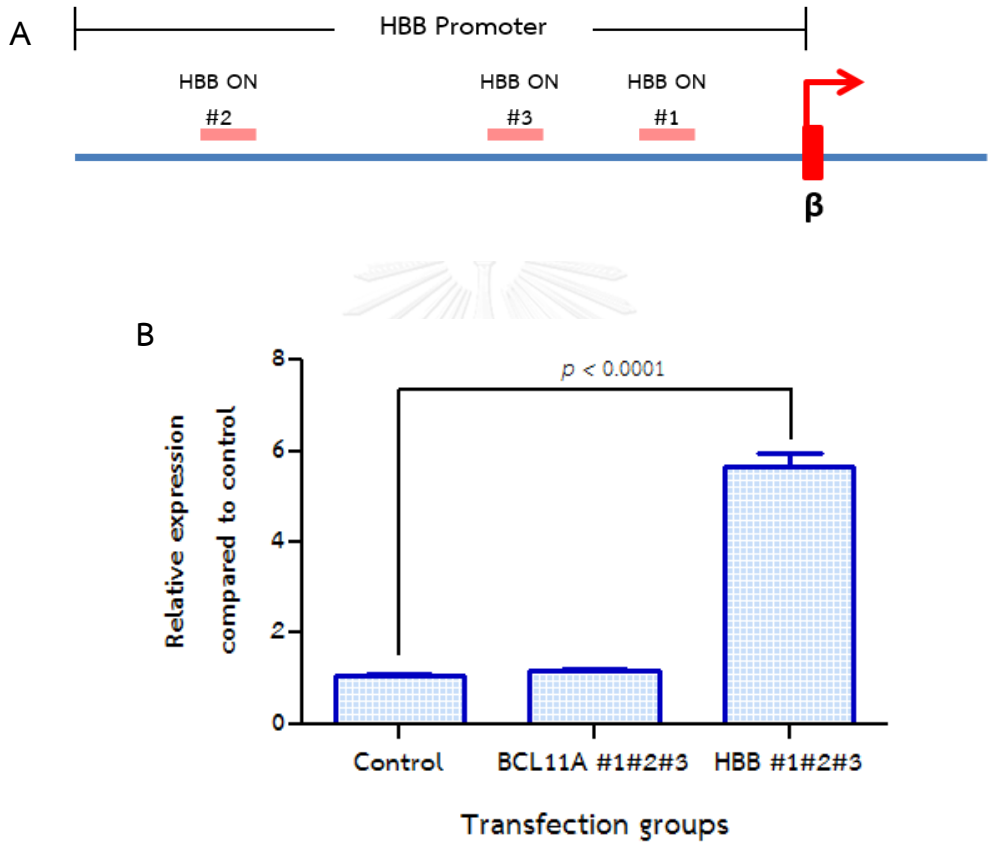


Figure 20 (A) Guide HBB ON#1, 2, 3 were designed specifically to HBB promoter. (B) mRNA of K562 with HBB #1#2#3 was analyzed by qRT-PCR. Result provided significantly different in statistic by using t-test, n=3.

CHAPTER IV
DISCUSSION

Beta- Thalassemia is one of the health problem issue in the developing countries. The effective cure is allogenic transplantation of hematopoietic stem cells (HSCs). However, the limitation of this procedure was the absence of a well-matched HLA between patients and donors. (2) The observation in Beta-Thalassemia patients who have a mutation of hereditary persistence of fetal hemoglobin (HPFH), can be ameliorated the severity of beta- thalassemia disease through increased fetal hemoglobin expression.(3-5) The researchers attempted to mimic the natural deletion. Recently, CRISPR/Cas9 has been used for genome editing tool to delete 13 kb on ***β-globin*** locus which occurring in Sicilian HPFH mutation.(28) The imitating of naturally deletion can reactivate fetal hemoglobin. However, deletion of a large region might not appropriate for clinical treatment. (29) It could delete sequences that effect to other functions in erythropoiesis or globin genes. From the published data has been report that a deletion in individual HPFH patient has increased level of fetal hemoglobin which including region of 3.5 kb intergenic ***γ-δ globin*** gene. (13) The role of 3.5 kb intergenic ***γ-δ globin*** gene has not been reported. Thus, we chose this region and designed sgRNA. Our study was successful to use CRISPR/Cas9 deleted a region of 3.5 kb intergenic ***γ-δ globin*** gene to generate genotype mimicking the natural deletion and elevated level of *HBG* mRNA and fetal hemoglobin expression in K562. There are some researches which support a potential of 3.5 kb intergenic ***γ-δ globin*** gene that might involve in expression of fetal hemoglobin. (5, 8, 13, 14) Even deletion of 3.5 kb intergenic ***γ-δ globin*** gene could increase level of fetal hemoglobin, but the exact mechanism in 3.5 kb is remain unclear. To study more about the role of 3.5 kb intergenic ***γ-δ globin*** gene, the region was dissected into 3 parts. CRISPR/Cas9 was used to delete each part of 3.5 kb. However, the efficiency of transfection was extremely low. T7 endonuclease could detect few DNA double strand break. Recently, CRISPR/Cas9 had an improvement for increasing transfection rate. To transfect Cas9 protein with in vitro transcript gRNA called ribonucleoprotein complex could elevate

transfection rate in HSCs to 75%. (30) This method might increase efficiency of transfection in our study.

The mechanism of hemoglobin switching is still unclear. Many researches have been study about fetal hemoglobin silencing. (20, 25, 31-36) There are some transcription factors that involved in suppression of fetal hemoglobin. *BCL11A* had played a major role as a repressor of fetal hemoglobin. (27, 32, 35, 37) Previous studies provided that knock down or knock out *BCL11A*, had resulted in increasing level of ***γ*** *globin* gene expression. (12, 13, 15, 26, 27) However, *BCL11A* also involved in lymphoid development (38, 39) and adult neural homeostasis.(40) Thus, directly deletion of *BCL11A* would be suboptimal for clinical translation. (29) According to Jain Xu et al, in 2013, showed that *BCL11A* has many binding sites on ***β****-globin* locus (14), and it is still unknown which site is the major role to suppress fetal hemoglobin. Therefore, we interested in disrupted function of *BCL11A* through its binding site on ***β****-globin* locus. Specific sgRNA were designed to target at 69 bp around *BCL11A* binding site. We were also successful using CRISPR/Cas9 to delete a region of *BCL11A* binding site and our result showed that level of *HBG* mRNA and protein in K562 with 69 bp *BCL11A* binding site deletion was increased as well as K562 with 3.5 kb deletion. These results provided an interesting data. It supported that the region 69 bp around *BCL11A* binding site has strong potential to suppress fetal hemoglobin. Furthermore, CRISPR/Cas9 were used to disrupt a minimal region at erythroid specific enhancer in *BCL11A* (41) and to delete 13 bp upstream *HBG* promoter (42), also increased level of fetal hemoglobin. Next, we would like to search for a critical base which we mutate one base and can promote level of fetal hemoglobin.

After, we confirmed that K562 with CRIPR/Cas9 deletion could promote fetal hemoglobin expression in K562. As developmental of adult CD34+ bone marrow cells already have completed hemoglobin switching, genome editing might not enough to reactivate fetal hemoglobin. Recently, there were developing CRISPR that tagged with transcriptional activator (CRISPR-On) (43). It used to impulse expression of endogenous genes. We achieved to promote level of *HBG* in K562 compare to control. Due to *HBG* was a normally expressed in K562, but *HBB* and *BCL11A* were scarcely expressed as an inactive gene. We confirmed a potential of CRISPR-On by impulsion of *HBB* and *BCL11A*

gene. Our result has confirmed the ability of CRISPR-On, we had increasing level of *BCL11A* and *HBB* when compared to control. Our result provided an effective method using CRISPR-On to promote *HBB* and *BCL11A*. It might be useful when we have planned to activate adult hemoglobin in red blood cell differentiated from human induced pluripotent stem cells (hIPSCs). Afterward, we desire using CRISPR/Cas9 and CRISPR-On to drive fetal hemoglobin expression in adult CD34+ bone marrow cells and adult hemoglobin expression in hIPSCs.

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APPENDIX

VITA

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