CHEMICAL INDUCTION OF FETAL HEMOGLOBIN PRODUCTION : POTENTIAL TREATMENT FOR β-THALASSEMIA

PREEYACHAN LOURTHAI

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Thesis
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…….
Miss Preeyachan Lourthai
Candidate

…….
Prof. M.R. Jisnuson Svasti, Ph.D.
Major-Advisor

…….
Prof. Suthat Fucharoen, M.D.
Co-Advisor

…….
Prof. Prapon Wilairat, Ph.D.
Co-Advisor

…….
Assoc. Prof. Supan Fucharoen, Ph.D.
Co-Advisor

…….
Prof. Banchong Mahisavariya, M.D.
Dean
Faculty of Graduate Studies
Mahidol University

…….
Assoc. Prof. Tuangporn Suthiphongchai, Ph.D.
Chair
Doctor of Philosophy Programme in Biochemistry
Faculty of Science
Mahidol University

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March 25, 2009

…….
Miss Preeyachan Lourthai
Candidate

……	…….
Prof. M.R. Jisnuson Svasti, Ph.D. Member	Assoc. Prof. Tuangporn Suthiphongchai, Ph.D. Chair
……	…….
Prof. Prapon Wilairat, Ph.D. Member	Prof. Suthat Fucharoen, M.D. Member
……	……
Assoc. Prof. Supan Fucharoen, Ph.D. Member	Assoc. Prof. Issarang Nuchprayoon, M.D., Ph.D. Member
……	……
Prof. Banchong Mahisavariya, M.D. Dean Faculty of Graduate Studies Mahidol University	Prof. Skorn Mongkolsuk, Ph.D. Dean Faculty of Science Mahidol University

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Preeyachan Lourthai

CHEMICAL INDUCTION OF FETAL HEMOGLOBIN PRODUCTION : POTENTIAL TREATMENT FOR β-THALASSEMIA

PREEYACHAN LOURTHAI 4637298 SCBC/D

Ph.D. (BIOCHEMISTRY)

THESIS ADVISORY COMMITTEE: M.R. JISNUSON SVASTI, Ph.D., SUTHAT FUCHAROEN, M.D., PRAPON WILAIRAT, Ph.D., SUPAN FUCHAROEN, Ph.D.

ABSTRACT

Augmentation of fetal hemoglobin (HbF) synthesis can reduce the severity of β-thalassemia and β-globin chain hemoglobinopathies by improving the imbalance between α- and non-α-globin chains. This is supported by observations that β-thalassemia patients with Hereditary Persistence of Fetal Hemoglobin (HPFH) are transfusion independent. A number of pharmacological agents have also been found to induce HbF through various mechanisms. However, all known HbF inducers have a low efficacy and specificity and may result in long-term toxicity.

Reporter assays in human erythroleukemia K562 cell line and transgenic mice based on a bacterial artificial chromosome (EBAC) containing the human β-globin locus with an enhanced green fluorescence protein (EGFP) reporter cassette in-frame replacement at the $^{G}\gamma$- to $^{A}\gamma$- globin genes (pEBAC/148β:: $\Delta^{G}\gamma^{A}\gamma$ EGFP) were developed. An episomal reporter assay for the screening of agents that specifically mimic the effect of the HPFH mutation on HbF expression was created by the introduction of the -175(T→C) HPFH mutation into the construct. This assay was also used to compare the level of EGFP expression from this construct with the parent construct. HPFH mutation gave approximately a 2-fold increase of EGFP expression compared to that of the wild type.

K562 cell lines carrying stably integrated γ-globin reporter assay construct was used in high throughput screening of a 2132 chemical library. Corresponding pharmacological increases in γ-globin mRNA expression and HbF were confirmed in reporter assay, native K562 cell line and human primary erythroid progenitor cells. Fourteen out of 29 compounds were validated using both cell lines. Two nucleoside analogs showed increased ratios of HbF by 20% in primary erythroid culture derived from β-thalassemia/HbE patients.

In transgenic mice carrying the γ-globin reporter assay transgene, EGFP expression decreased during mouse γ- to β- globin switching, but persisted throughout the adult stage. Murine erythroid culture derived from fetal livers showed a response to induction by HbF-inducing compounds.

The cellular and transgenic reporter assays will greatly facilitate the identification and evaluation of HbF-inducing agents. This screening has successfully identified active compounds for further mechanistic and preclinical evaluation as potential therapeutic agents for β-thalassemia treatment.

KEY WORDS: β-THALASSEMIA / HEMOGLOBIN F / HPFH / DRUG DISCOVERY / TRANSGENIC MICE

230 pages

การกระตุ้นการสร้างฮีโมโกลบินเอฟด้วยสารเคมีเพื่อการรักษาเบต้าธาลัสซีเมีย
(CHEMICAL INDUCTION OF FETAL HEMOGLOBIN PRODUCTION : POTENTIAL TREATMENT FOR β-THALASSEMIA)

ปรียจรรย์ เหล่าไทย 4637298 SCBC/D

ปร.ด. (ชีวเคมี)

คณะกรรมการที่ปรึกษาวิทยานิพนธ์: ม.ร.ว. ชิษณุสรร สวัสดิวัตน์, Ph.D., สุทัศน์ ฟู่เจริญ, พ.บ., ประพนธ์ วิไลรัตน์, Ph.D., สุพรรณ ฟู่เจริญ, Ph.D.

บทคัดย่อ

โรคเบต้า-ธาลัสซีเมียเป็นโรคที่เกิดจากการสร้างเบต้าโกลบินที่น้อยลงหรือสร้างไม่ได้เลย ซึ่งทำลายความสมดุลระหว่างสายแอลฟ่า สายเบต้าโกลบิน การลดภาวะไม่สมดุลโดยการกระตุ้นการแสดง ออกของยีนแกมม่าโกลบินทำให้มีการสร้างฮีโมโกลบินเอฟเพิ่มขึ้นจะสามารถบรรเทาอาการของโรคได้ ในธรรมชาติผู้ป่วยซึ่งมียีนเบต้า-ธาลัสซีเมียร่วมกับเอ๊ชพีเอฟเอ๊ชซึ่งเป็นสภาวะทางพันธุกรรมที่พบปริมาณฮีโมโกลบินเอฟในเลือดสูงในผู้ใหญ่นั้นจะมีระดับความรุนแรงของโรคน้อยกว่าผู้ป่วยโฮโมซัยกัสเบต้า-ธาลัสซีเมียที่มียีนเบต้า-ธาลัสซีเมียอย่างเดียว สารกระตุ้นฮีโมโกลบินเอฟที่พบจากการศึกษาที่ผ่านมาส่วนใหญ่มีประสิทธิ ภาพ ความจำเพาะต่ำ และยังมีความเป็นพิษสูง จึงไม่สามารถนำมาใช้ในการรักษาได้

งานวิจัยนี้ได้ทำการสร้างแบบจำลองเพื่อวิเคราะห์การกระตุ้นการสร้างแกมม่าโกลบินโดยใช้ชิ้นส่วนของกลุ่มยีนเบต้าโกลบินของมนุษย์ทั้งชนิดปกติและที่มีการกลายพันธุ์ตำแหน่ง -175 ของแกมม่ายีนเป็นเอ๊ชพีเอฟเอ๊ช มีการแทนที่เนื้อยีนแกมม่าโกลบินด้วยยีนสร้างโปรตีนเรืองแสง (EGFP) ทั้งในเซลล์ K562 และหนูทรานส์จีนิค พบว่าเซลล์ที่มียีนเอ๊ชพีเอฟเอ๊ชเรืองแสงมากกว่าเซลล์ปกติ 2 เท่า และเซลล์แบบจำลองดังกล่าวถูกนำมาใช้เพื่อคัดกรองสารกระตุ้นฮีโมโกลบินเอฟจากสารทั้งหมด 2,132 ตัวอย่าง ซึ่งประกอบด้วยยาและสารสกัดจากธรรมชาติ สาร 14 ตัวอย่างจาก 29 ตัวอย่างที่ได้ผ่านการคัดกรองขั้นต้นถูกนำมาทดสอบการกระตุ้นการสร้างเมสเซนเจอร์อาร์เอ็นเอ (mRNA) ของแกมม่าโกลบินในเซลล์ K562 และการสร้างฮีโมโกลบินเอฟในเซลล์เม็ดเลือดแดงตัวอ่อนจากผู้ป่วยเบต้าธาลัสซีเมีย/ฮีโมโกลบินอี พบว่าสารต้านไวรัส 2 ชนิดในกลุ่ม nucleoside analog สามารถเพิ่มการแสดงออกของฮีโมโกลบินเอฟได้ถึงประมาณร้อยละ 20

สำหรับหนูทรานสจีนิกที่เกิดจากการสอดใส่ชิ้นยีนกลุ่มเบต้าโกลบินที่มี EGFP ได้มีการสร้างโปรตีนเรืองแสงในเม็ดเลือดแดงแตกต่างกันในระยะต่างๆ ของการเจริญเติบโต และพบว่าเซลล์เม็ดเลือดแดงตัวอ่อนของหนูมีการตอบสนองต่อการกระตุ้นด้วยสารกระตุ้นฮีโมโกลบินเอฟจากดังกล่าวด้วย

การศึกษานี้ได้พัฒนาแบบจำลองในเซลล์ และหนูทรานส์จีนิคสำหรับการตรวจหาสารกระตุ้นฮีโมโกลบินเอฟ ซึ่งนำไปสู่การค้นพบสาร 2 ชนิดที่มีแนวโน้มในการใช้เพื่อการรักษาโรคเบต้า-ธาลัสซีเมียในอนาคต

230 หน้า

CONTENTS

CONTENTS (cont.)

CONTENTS (cont.)

CONTENTS (cont.)

CONTENTS (cont.)

CONTENTS (cont.)

CONTENTS (cont.)

CONTENTS (cont.)

CONTENTS (cont.)

LIST OF TABLES

LIST OF FIGURES

LIST OF FIGURES (cont.)

LIST OF FIGURES (cont.)

LIST OF FIGURES (cont.)

LIST OF ABBREVIATIONS

°C	Degree Celsius
%EGFP +ve	Percentage of EGFP positive cells
%inc	Percentage increase
5-Aza	5-Azacytidine
A	Adenine
A-cell	Hemoglobin A containing cell
Amp	Ampicillin
AmpR	Ampicillin resistance/resistant
ARMS-PCR	Allele specific polymerase chain reaction
ASHP	a-hemoglobin stabilizing protein
BAC	Bacterial artificial chromosome
BFU-e	Erythroid burst-forming units
bp	Base pair
BSA	Bovine serum albumin
C	Cytosine
cAMP	Cyclic adenosine triphosphate
cDNA	Complementary deoxyribonucleic acid
cfu	Colony forming unit
CFU-e	Erythroid colony-forming units
cGMP	Cyclic guanosine triphosphate
cAMP	Cyclic adenosine triphosphate
Cm	Chloramphenicol
CmR	Chloramphenicol resistance/resistant
CMV	Cytomegalovirus
CsCl	Cesium Chloride
ddH_20	Distilled deionized water

LIST OF ABBREVIATIONS (cont.)

dH_20	Distilled water
DMEM	Dulbecco modified Eagle medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide 5' triphosphate
EBNA1	Epstein-Barr nuclear antigen-1
EBV	Epstein-Barr virus
EDTA	Ethylene diamine-tetra-acetic-acid
EGFP	Enhanced green fluorescent protein
EKLF	Erythroid Krüppel-like factor
E_{max}	Maximal induction efficiency
EPO	Erythropoietin
ET	recE, and recT
F(E)	EGFP fluorescence value
F(V)	Viability assay fluorescence value
F-cell	Hemoglobin F containing cell
FCS	Fetal Calf Serum
FDA	Food and Drug Administration
FISH	Fluorescent in situ hybridization
fl	Femtoliter
FL1	Fluorescence channel 1
FSC	Forward scatter
G	Guanine
g	Gram
g	Gravity
g/kg	Gram per kilogram
g/ml	Gram per mililitre

LIST OF ABBREVIATIONS (cont.)

GG	$\Delta^G\gamma^A\gamma$-globin EGFP transgenic mice
Glu	Glutamic acid
GM-CSF	Granulocyte-macrophage colony-stimulating factor
GTP	Guanosine triphosphate
Hb	Hemoglobin
HbA	Hemoglobin A
HbA_2	Hemoglobin A two
HbE	Hemoglobin E
HbF	Hemoglobin F
HbH	Hemoglobin H
HbS	Sickle hemoglobin
HCT	Hematocrit
HDAC	Histone deacetylase
HGB	Hemoglobin
HPFH	Hereditary persistence of fetal hemoglobin
HPLC	High performance liquid chromatography
hr	Hour
HS	Hypersensitive site
HSA	Human serum albumin
HU	Hydroxyurea
Hyg	Hygromycin
Hyg^R	Hygromycin resistant
IC_{50}	Half maximal inhibitory concentration
IgG	Immunoglobulin G
IL-3	Interleukin-3
IMDM	Iscove Modfied Eagle Media
IMP	Inosine monophosphate

LIST OF ABBREVIATIONS (cont.)

IV	Intravenous
IVS	Intervening sequence
K	Constant
Kan	Kanamycin
Kan^R	Kanamycin resistant
kb	Kilobase pair
kDa	Kilodalton
KEB	K562 cell line stably express EBNA1 protein
kV	Kilovolt
l	Liter
LB	Luria-Bertani medium
LCR	Locus control region
Lys	Lysine
M	Molar
mA	Milliampere
MAPK	Mitogen activated protein kinase
MCH	Mean corpuscular hemoglobin concentration
MCHC	Mean corpuscular hemoglobin concentration
MCV	Mean corpuscular volume
μF	Microfarad
μg	Microgram
mg	Miligram
mg/ml	Miligram per mililiter
μg/ml	Microgram per mililiter
min	Minute (s)
ml	Mililiter
μl	Microliter

LIST OF ABBREVIATIONS (cont.)

mM	Milimolar
μM	Micromolar
MPF	Median peak fluorescence
mRNA	Messenger ribonucleic acid
n	Sample size
Neo	Neomycin
Neo^R	Neomycin resistant
ng	Nanogram per microliter
ng/μl	Nanogram per microliter
nm	Nanometer
nM	Nanomolar
NO	Nitric oxide
nt	Nucleotide
OD	Optical density
O_2	Oxygen
ORG	Olfactory receptor genes
PAC	P1-derived artificial chromosome
PAGE	Pulse field gel electrophoresis
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PI	Propidium Iodide
pmol	Picomole
qRT-PCR	Quantitative reverse transcriptase polymerase chain reaction
RBC	Red blood cell
RDW	Red cell distribution width

LIST OF ABBREVIATIONS (cont.)

RETIC	Reticulocyte
rhEPO	Recombinant human erythropoietin
rhIL-3	Recombinant human interleukin-3
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rpm	Revolutions per minute
RT-PCR	Reverse transcriptase polymerase chain reaction
SCD	Sickle cell disease
SCF	Stem cell factor
SCFAD	Short chain fatty acid derivative
SD	Standard deviation
SDS	Sodium dodecyl suphate
sec	Seconds
sGC	Soluble guanylyl cyclase
siRNA	Small-interfering RNA
SNP	Single nucleotide polymorphism
SSC	Side scatter
T	Thymine
TEMED	Tetramethylethylenediamine
Tet	Tetracyclin
Tet^R	Tetracyclin resistant
TGF-b	Transforming growth factor beta
tk	Thymidine kinase
TSS	Transformation and storage solution
U	Unit
U/ml	Unit per mililiter
UDP	Uridine diphosphate

LIST OF ABBREVIATIONS (cont.)

UTR	Untranslated region
UV	Ultraviolet light
V	Volt
W	Ohm
w/v	Weight per volume
w/w	Weight per weight
WT	Wild type
YAC	Yeast artificial chromosome

PREFACE

The work described in this study was mainly carried out in the laboratory of the Cell and Gene Therapy (CAGT) group, Murdoch Childrens Research Institute (MCR), Royal Children's Hospital, Parkville, Australia and the laboratory of the Thalassemia Research Center, Institute of Science and Technology for Research and Development, Mahidol University, Thailand. The high throughput screening was carried out at the WEHI High Throughput Chemical Screening Facility, Bundoora, Australia. I certify that this work has not been submitted to any other university.

Some sections of the work have been presented at national and international conferences as give in the list below. Manuscripts are in preparation.

Oral Presentations:

1. Lourthai P, Vadolas J, Wardan H, Winichagoon P, Svasti J, Fucharoen S, Ioannou P. (2004). Development of an HPFH-specific assay for HbF inducers. Abstract in the 5th HUGO Pacific Meeting & 6th Asia Pacific Conference on Human Genetics, Biopolis, Singapore, November 17-20, 2004. Student Award
2. Lourthai P, Vadolas J, Wardan H, Winichagoon P, Svasti J, Fucharoen S, Ioannou P. (2004). Development of an HPFH-specific assay for HbF inducers. Abstract in the 17th FAOBMB Symposium/ 2nd IUBMB Special Meeting/ 7th A-IMBN Conference, Bangkok, Thailand, November 22-26, 2004.
3. Lourthai P, Wardan H, Fucharoen S, Parisot J, Street I, Vadolas J. (2006). Development of Fluorescence-Based Genomic Reporter Assay for High-Throughput Screening of Potential Fetal Hemoglobin Inducers. Abstract in the 6th Institute of Science of Science and Technology for Research and Development Conference: Molecular Medicine 2006" November 27-30, 2006. Institute of Science and Technology for Research and Development, Mahidol University, Nakornpathom, Thailand. Oral Presentation Award.

Poster Presentations:

1. Lourthai P, Vadolas J, Wardan H, Winichagoon P, Svasti J, Fucharoen S, Ioannou P. (2004). Development of an HPFH-specific assay for HbF inducers. Abstract in the 5th HUGO Pacific Meeting & 6th Asia Pacific Conference on Human Genetics, Biopolis, Singapore, November 17-20, 2004.
2. Lourthai P, Vadolas J, Wardan H, Winichagoon P, Svasti J, Fucharoen S, Ioannou P. (2005). Development of an HPFH-specific assay for HbF inducers. Abstract in the 4th Australasian Gene Therapy Society Meeting, Melbourne, Australia, April 27-29, 2005.
3. Wardan H, Vadolas J, Lourthai P, Orford M, Williamson R, Ioannou PA. (2005). Development of Fluorescence-Based Genomic Reporter Assay for Evaluation of Potential Fetal Hemoglobin Inducers. Abstract in the 4th Australasian Gene Therapy Society Meeting, Melbourne, Australia, April 27-29, 2005.
4. Lourthai P, Wardan H, Fucharoen S, Parisot J, Street I, Vadolas J. (2006). Development of Fluorescence-Based Genomic Reporter Assay for High-Throughput Screening of Potential Fetal Hemoglobin Inducers. Abstract in the 10th International Conference on Thalassaemia & Haemoglobinopathies & 12th International TIF Conference for Thalassaemia Patients and Parents, 2006.
5. Lourthai P, Wardan H, Fucharoen S, Parisot J, Street I, Vadolas J. (2006). Development of Fluorescence-Based Genomic Reporter Assay for High-Throughput Screening of Potential Fetal Hemoglobin Inducers. Abstract in the 11th International Congress of Human Genetic, Brisbane, Australia, August 6-10, 2006

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CHAPTER I
INTRODUCTION

Thalassemia is an inherited disorder occurring from the reduced or absent globin chain synthesis. It is the most common genetic blood disease in Thailand and many other countries in the world. β-thalassemia occurs when there is a quantitative reduction of β-globin chains, the component of adult hemoglobin (HbA, $\alpha_2\beta_2$). Pharmacological stimulation of fetal hemoglobin (HbF, $\alpha_2\gamma_2$) production is a potential approach to therapy for β-thalassemia as well as sickle cell anemia. The rationale for this approach is based on observation that HbF hemoglobin compensates for the unbalanced α/β globin chain ratio in β-thalassemia. This is confirmed by the evidence that β-thalassemia patients with hereditary persistence of fetal hemoglobin (HPFH) have milder thalassemia symptoms. Despite a number of clinical trials investigating potential HbF inducers, currently available inducers such as 5-azacytidine, hydroxyurea, or butyrate either have low efficacy or high toxicity. Therefore, the identification of new pharmacological agents, including Thai natural products, that can induce HbF with greater efficacy and less toxicity is urgently needed.

In order to accomplish this goal, a major limitation is the lack of screening assays that can conveniently detect novel HbF inducers with high efficiency, and high specificity, therefore the improved screening assay is needed to be developed first. In this study, the fluorescence-based (EGFP) genomic reporter on K562 erythroleukemia cell line is used in both high-throughput and low-throughput screening. In addition, the fluorescence-based genomic reporter for β-globin, and γ-globin chain on transgenic mice were generated and characterized for the later use in studying in HbF induction and regulation studies.

Then, screening and validation of the HbF inducers was performed at the mRNA, and protein levels in both K562 cellular model and transgenic mouse model. Last, the

upregulation of HbF was studied in erythroid progenitor culture (CD 34^{+}) from β-thalassemia/HbE patients. From starting of over 2132 compounds, through the whole screening process, at the end two novel promising HbF inducer compounds were identified. However, the mechanism which leads to upregulation of γ-globin gene of the identified compounds still needs to be further studied.

Overall, from this study, the system for screening of HbF inducers has been developed and used. The validation of hits compounds from the screening verified the fidelity of this system. This provides an ideal system for additional identification of potential novel inducers of fetal hemoglobin for the treatment of β-hemoglobinopathies if required. In addition, the transgenic mice and cellular assay also provided excellent tools for γ-globin regulation study.

CHAPTER II
OBJECTIVES

The aims of this thesis are:

1. To develop and characterize an HbF inducer screening system in both a cellular model and an animal model.
2. To generate and characterize transgenic mice created using a 185 kb human genomic DNA fragment with an EGFP reporter replacing γ-globin gene in the context of the entire, intact human β-globin locus.
3. To screen a 2000 bioactive compound library and 132 Thai natural products and their derivatives using a high-throughput system.
4. To validate the compounds as potential novel γ-globin inducers. Initial assessments will be based on expression of EGFP fluorescent reporter gene. Levels of γ-globin mRNA and protein will then be analyzed in the K562 cellular model. Final confirmation will be obtained by studying effects in primary erythroid cells from the transgenic mice and cultured primary erythroid cells obtained from β-thalassemia/HbE patients.

CHAPTER III
LITERATURE REVIEWS

3.1 Human Hemoglobin

Hemoglobin is the protein of the red blood cells that transports oxygen from lungs to the tissues, and carbon dioxide from the tissues back to the lungs. The normal hemoglobin molecule is composed of tetramers of a pair of α-like globin chains, and another pair of β-like globin chains non-covalently bound (Figure 1). Each globin chain binds a prosthetic group, heme, which consists of an iron atom located at the centre of a porphyrin ring.

3.1.1 Types of hemoglobin

The component of human hemoglobin changes during development. The initial embryonic hemoglobins, Hbs Gower1 and 2 and Hb Portland (1-4) is confined to the yolk-sac stage of development. This is then replaced by fetal hemoglobin (HbF) until shortly before term. After birth, HbF is replaced by adult hemoglobin (HbA) which is a major hemoglobin comprising about 97 % of the total, and a minor component, HbA_2 which accounts for 2-3 % (5). However, in normal adult, amounts of HbF, consisting ~1% of the total hemoglobin continue to be produced (6). The production of these different hemoglobins is a reflection of physiological adaptations to different oxygen requirement at each stage of development.

Adult and fetal hemoglobins have α chains that are combined with β-globin chains (HbA, $\alpha_2\beta_2$), δ-globin chains (HbA_2, $\alpha_2\delta_2$) or γ-globin chains (HbF, $\alpha_2\gamma_2$), whereas in the embryo α-like globin chains called ζ-globin chains combine with γ (Hb Portland, $\zeta_2\gamma_2$) or ε-globin chains (Hb Gower1, $\zeta_2\varepsilon_2$), and α-globin chains combine with ε chain to form Hb Gower 2 ($\alpha_2\varepsilon_2$) (Figure 2).

Synthesis of individual globin chains of hemoglobin is a tightly regulated process. In order to generate adequate levels of hemoglobin to facilitate survival and

growth, the α- and β-globin chains must be very highly expressed during erythrocyte development. However, it is essential that the two globin chains are produced at the balanced levels since any disruptions resulting in an excess of either chain has significant damaging effects on red cell survival, a situation which is clearly illustrated by the thalassemia.

3.1.2 Chromosome organization of globin gene clusters

The globin genes of humans and most other species are organized into two clusters.

3.1.2.1 α-globin locus

The 80 kb human α-like globin locus locates on the short arm of the chromosome 16 in band p13.3. It includes an embryonic gene (ζ2), two fetal/adult genes (α2 and α1), three pseudo genes (ψζ1, ψα2, ψα1) and a gene (θ) of unidentified function. Each haploid genome in human contains the two α1 and α2 globin genes (7) (Figure 3).

3.1.2.2 β-globin locus

The 70 kb human β-globin locus locates on the short arm chromosome 11 in band p15.5. It includes an embryonic gene (ε), two fetal genes ($^{G}\gamma$, and $^{A}\gamma$), two adult genes (δ and β) and a pseudo gene (ψβ) (Figure 3).

The order of genes from 5' to 3' along each locus reflects the developmental regulation of globin genes. The α- and β-globin loci have upstream regulatory regions. In the β-globin locus, it is called the locus control region (LCR), which is crucial for high level expression of the globin genes. Five DNaseI hypersensitive sites (HS) have been identified upstream of the β-globin locus (8). Most of the activity of the LCR resides in the core elements of the DNase hypersensitive sites, which contain binding motifs for erythroid-specific and ubiquitous transcription factors (9). In the α-globin, the upstream regulatory region is called HS-40 region (Figure 3). There is another HS approximately 20 kb 3' to the β-globin gene. The two extreme HS sites flanking the β-globin complex have been suggested to mark the boundaries of the β-globin gene domain. The β-globin complex is embedded in a cluster of olfactory

receptor genes (ORG), part of the family of approximately 1000 genes that are widely distributed throughout the genome.

3.1.3 Hemoglobin Switching

The developmental regulation of the globin genes reflects their sequential activation in a 5'-3' direction (Figure 3). In humans, yolk sac erythropoiesis happens between week 3 and 8 of gestation. During this period the embryonic globins ($\varepsilon_2\zeta_2$) are the predominant species but all the globin genes are expressed at low (γ-) or very low (β-) levels (10). By the 10th week of gestation this hematopoiesis is replaced by erythropoiesis in the fetal liver: the ε- and ζ-globin genes are progressively silenced and the α- and γ-genes become predominant, which give fetal hemoglobin (HbF) ($\alpha_2\gamma_2$). This HbF is about 95% of the total hemoglobin in fetal blood. The β-gene is slightly expressed, giving HbA (α2β2) as a minor component.

The second globin switch occurs around birth. The amount of β-globin chains, which had been increasing slowly throughout fetal development, sharply increase as erythropoiesis switches to the bone marrow. The production of HbA persists throughout adult life under normal conditions. During the switch, there is disproportionate change in the expression of two γ-globin genes. Expression of the $^{G}\gamma$-globin gene reduces faster than that of the $^{A}\gamma$ globin gene. Consequently, the ratio of Gγ- to Aγ-globin chains falls from 3:1 at the time of birth to 2:3 by approximately 5 months of age. A low level of HbF also continues to be produced, consisting of about 1% of the total hemoglobin (11). This expression of γ-globin genes is restricted to a sub-population of red cells termed F cells. Altogether, α-like globin genes switch only once at the time of the ζ → α switch, whereas the β-like globin genes switch twice: ε → γ → β.

Hemoglobin production has been extensively studied as a model of developmental gene regulation. However, the ways in which these developmental switches are controlled during globin gene expression are still not fully understood. It is believed that globin switching is not under hormonal control. Metabolic influence

was reported, for example, α-amino-n-butyric acid, a metabolite found in the plasma of hyperinsulinemic infants of diabetic mothers, can inhibit the γ to β switch. On a molecular level, globin gene switching is accompanied by changes in chromatin structure, DNA methylation, and transcription factor binding profile.

3.2 Regulation of globin gene expression

The human α- and β-globin gene clusters are subjected to several levels of regulation. The expression of the genes is regulated by complex interactions between *cis*-acting sequences within and surrounding the globin genes and *trans*-acting factors. The *cis*-acting elements located either in proximity or at remote regions are referred to as HS-40 in the α-globin gene cluster, and the β-, whereas *trans*-acting factors include both transcription factors and chromatin remodeling activities (12). The developmental expression is believed to rely on gene silencing and gene competition, which are mediated by the different transcription factors in embryonic, fetal and adult hematopoietic cells. The LCR plays a critical role in β-like globin gene expression by maintaining an open chromatin state and acting as an enhancer of globin gene transcription. The tissue- and developmental-specific expression of the individual globin gene is controlled by the direct physical interaction between each globin gene promoter and the LCR. Despite crucial role of LCR for globin gene activation and hemoglobin switching, a recent study of LCR function in transgenic mice has shown that the LCR region is not required for chromatin opening or developmental regulation of the locus (13, 14). This finding opened the possibility that the larger regions upstream of the globin gene cluster may be required for switching (15).

The promoters of globin genes are relatively alike but each one also contains unique sequences that may be responsible for convey the developmental stage specificity. Previous work has demonstrated that lineage-specific transcription factors play essential roles in erythroid development (16), including function in transcriptional activation, mediation of the interaction between the LCR and promoters and alteration of chromatin structure (17). These transcription factors includes the erythroid specific zinc finger transcription factor named GATA-1 and

NF-E2 (nuclear factor (erythroid-derived 2)) (18). Erythroid Krüppel-like factor (EKLF) is essential for activation of the adult β-globin gene and also has significant interactions with the β-LCR (19, 20). While GATA-1 and NF-E2 are not differently active in fetal and adult cells, EKLF is active predominantly in adult-stage cells, and specifically enhances β-globin and not γ-globin gene expression. Therefore, EKLF is critical for the switch in expression from fetal to adult β-globin during erythropoiesis. Normal β- to γ-globin switching probably requires developmental stage-specific changes in transcription factors and/or chromatin remodeling activities, in addition to the roles of GATA-1, NF-E2, and EKLF. Many of the actions of these DNA-binding proteins are achieved through the involvement of protein-protein interaction with other factors for binding to targeted DNA sequences (21). For instance, GATA-1 interacts with multiple proteins including FOG-1, EKLF , SP1,CBP/p300 and PU.1 (21). The mechanisms by which these interactions influence GATA-1 function remain incompletely understood.

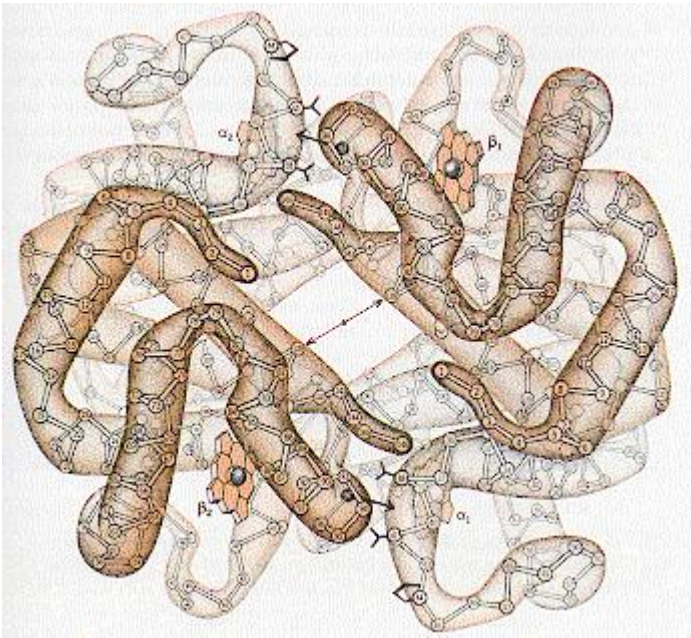


Figure 1: Human hemoglobin heterotetramer

The two α-like globin chains and two β-like globin chains are held together by non-covalent ionic bonds.

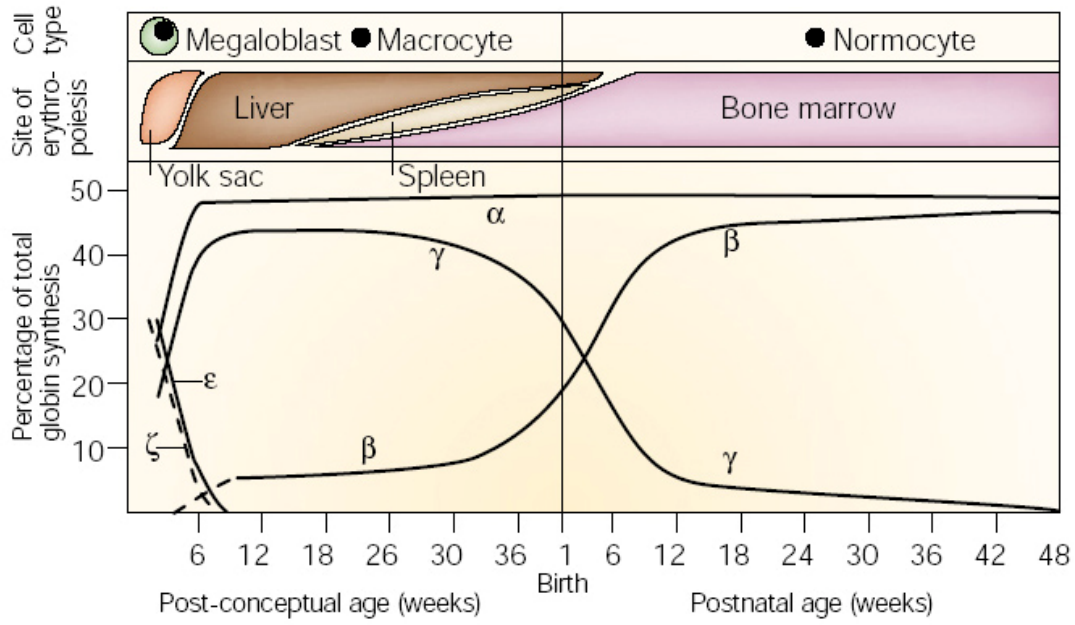


Figure 2: Predominant components of hemoglobin throughout developmental stages (22)

The lines represent the sequential activation of the embryonic, fetal and adult globin genes in α- and β-globin loci. Both the α-like and β-like chains of hemoglobin undergo switching during development. The α-like chains switch from ζ-globin in early embryonic stage to α-globin which is then maintained into adulthood. The β-like chains undergo two switches from ε-globin in early embryonic stage to γ-globin in the fetus and finally to adult β-globin a few weeks after birth.

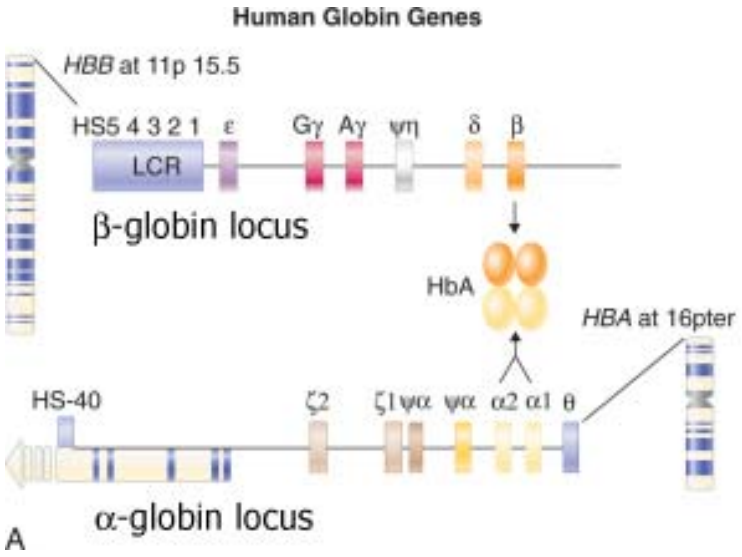


Figure 3: Structure of the human α- and β-globin loci on chromosome 16, and 11 respectively

The globin genes and locus control region (LCR) of β-globin locus, and HS-40 of α-globin locus were depicted (23).

3.3 Thalassemia

Thalassemia is the most common hereditary disease found in many Mediterranean countries, the Middle East, Africa and Southeast Asia. Thalassemia is caused by reduced or absent globin chain synthesis. There are various types of thalassemia, according to the type of globin chain that is affected. Two major types of thalassemia that have clinical significance are α-and β-thalassemia.

3.3.1 The α-thalassemias

The α-thalassemias are characterized by a reduced rate of α-globin synthesis resulting in the presence of excess β-like chains that associate to form the abnormal tetramers, Hb Bart's (γ_4) and Hb H (β_4). The majority of α-thalassemias appear to be due to gene deletions but some non-deletion types have also been found. Since each α-globin locus encodes two functional copies of α-globin with a total of four copies in a diploid genome, the classification of α-thalassemia is based on the number of gene deleted. The deletions of one or two α-globin genes are referred to as a silent carrier stage and α-thalassemia trait, respectively, and neither result in a clinically significant anemia. Inheritance of three defective α-globin genes results in hemoglobin H disease, which is less severe than β-thalassemia major (24). The excess β-globin chains in α-thalassemia form characteristic β-globin tetramers (Hb H). Deletion of all four α-globin genes (Hb Bart's hydrops fetalis) results in death either *in utero* or at the time of delivery.

An α-thalassemia phenotype also results from the unstable α-globin mRNA, e.g. Hb Constant Spring which occurs from a mutation at the terminator codon. The mutated mRNA allows the entry of the ribosome into the 3' untranslated region where the formation of an α-globin mRNA-specific stability determinant may be interfered.

3.3.2 The β-thalassemias

In contrast to the α-globin locus where deletions are common, the majority of molecular defects associated with β-thalassemia syndromes are due to point mutations

within the β-globin gene or its immediately flanking regions. Only a minority of β-thalassemias are caused by extensive gene deletion. Over 200 mutations which affect the β-globin locus and result in β-thalassemia have been described (25).The majority of these mutations located either in β-globin gene or the immediate flanking regions on human chromosome 16 (26).Mutations affecting the upstream promoter elements result in decrease transcriptional output while mutations in the coding region, splice sites or termination codons affect RNA translation. All result in result in reduced production of β-globin at a variable degree. In β^{+}-thalassemia (e.g. IVSI-110 splicing mutation), decreased amounts of β-globin are present, whereas in β^{0}-thalassemia (e.g. 4bp deletion –TTCC at codons 41-42) there is no β-globin chain production (27).

The imbalance in α versus β-globin chain synthesis is responsible for thalassemia phenotype. This difference between α- and β-thalassemia is partially attributed to the different characteristics of the α-globin and β-globin chains. The excess β-globin chains in α-thalassemia form characteristic β-globin tetramers (Hb H) which are non-functional but soluble. In contrast, the excess α-globin chains in β-thalassemia are highly unstable and precipitate in red cells and their precursors. The thalassemia phenotype includes the severe anemia, which is a composite of marked ineffective erythropoiesis, as well as hemolysis of peripheral red blood cells. Previous studies in β-thalassemia have described the postulated mechanisms of hemolysis, including precipitation of the excessive unmatched α-globin chains and unbound iron. These result in the increased susceptibility of β-thalassemic red blood cells (RBC) to oxidative stress, red cell damage, hemolysis, ineffective erythropoiesis, removal via the reticuloendothelial system, and anemia (Figure 4).

3.4 Hemoglobin E

Hemoglobin E is the Glu → Lys mutation at codon 26 (GAG → AAG) of the β-globin gene. The abnormal gene partially activates an adjacent cryptic splice site towards the 3' end of exon 1, resulting in a proportion of abnormally spliced mRNA and β^{E}-globin. The homozygous HbE usually result in a less severe phenotype (28).

3.5 Current therapies for β-thalassemia

3.5.1 Blood transfusion

The symptomatic treatments for thalassemias are primarily focused on the correction of anemia and the suppression of intense erythropoiesis. Regular blood transfusions to keep the hemoglobin level above 10 g/dl at all times, together with regular iron chelation, are required for the patients with high severity of anemia to facilitate growth and improve the quality of life. However, chronic transfusion is complicated by high rates of allo-immunization, blood borne infectious diseases and iron overload.

3.5.2 Iron chelation

As the accumulation of iron in vital organs results in tissue damage and accounted for the major cause of death in thalassemia major. The purpose of iron chelation is to decrease the iron overload in thalassemia that results from either regular blood transfusions or from excessive gastrointestinal iron absorption. The most effective iron chelating agent available is desferioxamine.

3.5.3 Bone marrow transplantation

Bone marrow transplantation is curative and has been carried out in over 800 patients. The procedure is associated with an overall morality of around 20 percent, which depends on the clinical well being of the patient prior to transplantation (29). However, the proportion of matched sibling donors and its high cost are the limiting factors that make bone marrow transplantation non-practical, especially in third world and developing countries. The bone marrow transplantation complications include chronic graft-versus-host disease.

3.6 Potential therapies for β-thalassemia

The understanding of the pathophysiology of β-thalassemia has led to the development of new strategies in attempts to cure or ameliorate the disease. The potential targets for therapy can be divided into three categories; which are 1)

correction of β-expression level, 2) reducing α:β globin imbalance, and 3) promoting erythroid survival, decreasing iron overload, and antioxidants (Figure 5) (30).

Correction of β-globin expression includes hematopoietic stem cell transplantation, and lentiviral vector based gene therapy (31). There were also reports of using the antisense against the aberrant mRNA products for β-thalassemia caused by aberrant splicing, (32) and small interfering mRNA (siRNA) to the negative transcription regulator (33).

Reducing α:β-globin imbalance can be accomplished mainly be reactivation of γ-globin that binds to excess α-globin chain and produce functional HbF. This strategy was approached by lentiviral vector-based gene therapy(34), manipulation of the transcription factor (35, 36), or using pharmacological agents such as hydroxyurea(37), butyrate (38) or 5-azacytidine(39). Another aspect of reducing g α:β-globin is by suppression of α-globin level, potentially through inhibition of gene expression by siRNA(40-42) or promotion of α-globin chain degradation by manipulation of α-hemoglobin stabilizing protein level (ASHP) (43) or ubiquitination(44).

The third level of targets for β-thalassemia therapies includes correction of the downstream pathophysiology of β-thalassemia such as promotion of erythroid survival by erythropoietin supplementation, decreasing iron overload by hepcidin supplementation (45), and reduction of oxidative stress by using vitamin C , E (46) or curcumin derivatives(47).

These potential treatment options provide more understanding in the molecular mechanisms underlying the disease. Three treatment options are far more progress than the others: pharmacological induction of HbF, gene therapy using lentiviral vectors and nonmyeloablative bone marrow transplant. The lentiviral-mediated gene therapy for the β-hemoglobinopathies has reached first phase I/II clinical trial for SCA and β-thalassemia major in 2006. Non-myeloablative transplants offer great potential for high-risk patients but a major problem remains – titrating immunosuppression to obtain an optimal balance of stable mixed chimerism. However these two therapies are likely to be extremely expensive.

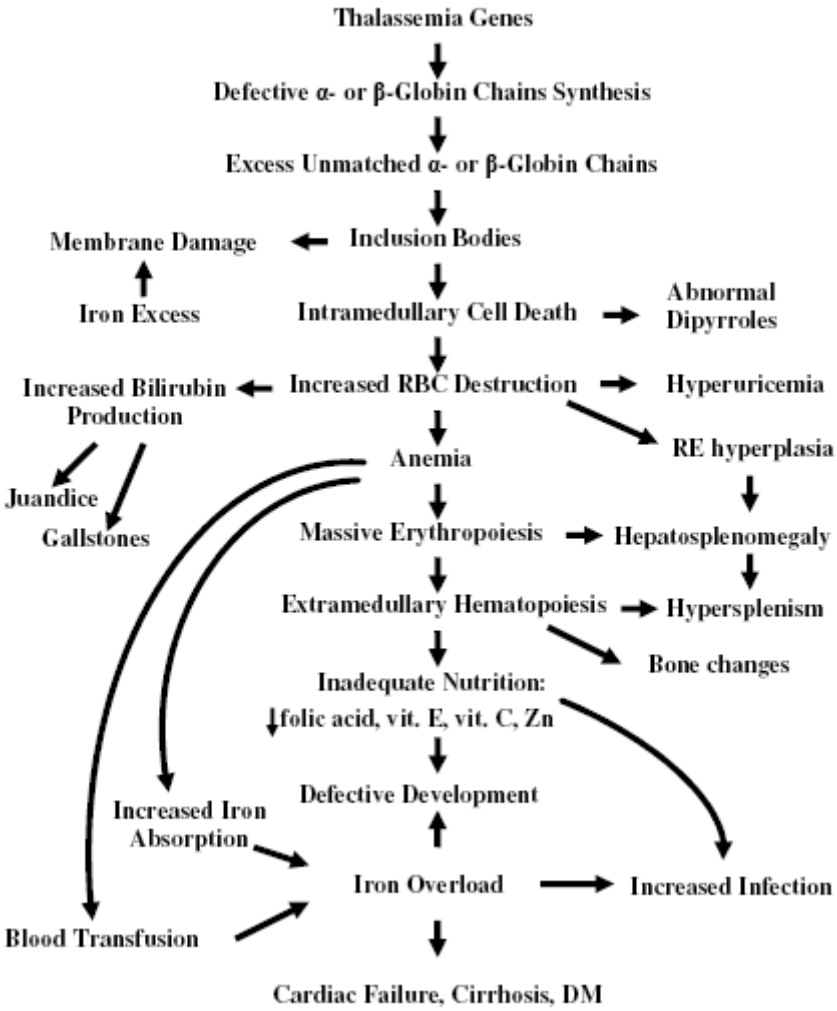


Figure 4: Diagram showing pathophysiology of thalassemia (25)

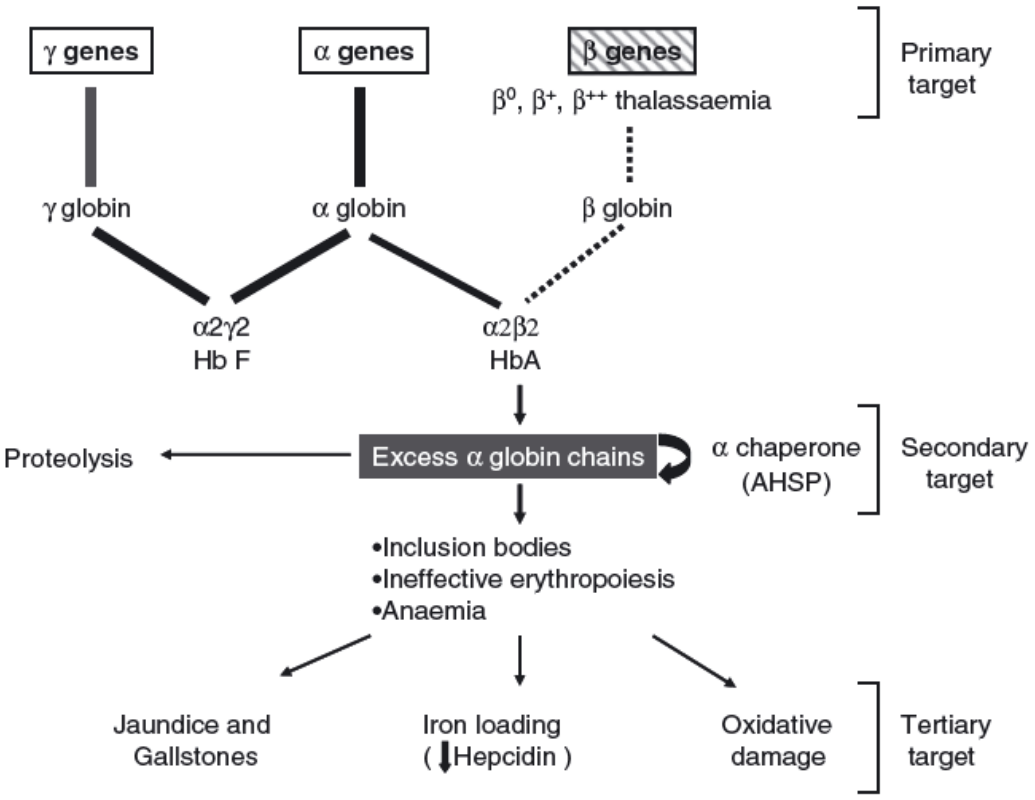


Figure 5: Levels of potential molecular therapy for β-thalassemia (30)

3.7 Fetal hemoglobin production

3.7.1 Fetal hemoglobin (HbF)

HbF ($\alpha_2\gamma_2$), the predominant form of hemoglobin expressed in the developing fetus, appears a few weeks post-conception and persists for a few months post-birth. HbF exhibits a higher average oxygen affinity than HbA. This is beneficial for transportation of oxygen from the maternal circulation to the fetal circulation since the high oxygen affinity ensures that oxygen will ultimately flow in the direction of the fetal circulation. However, an adult with homozygous for the different forms of hereditary persistence of fetal hemoglobin (HPFH) with HbF levels of almost 100%function normally as adults.

In normal individuals, Hb F levels are typically very low (1-2% of total hemoglobin) but it appears that there is an increase in γ-globin production in cases of β-thalassemia. The increased HbF helps ameliorate the phenotype for homozygous β-thalassemia patients who are not able to produce any HbA. The mild phenotype of the disease is due to an ability of γ-globin to bind excess α-globin to generate functional hemoglobin.

Evidence for the potential therapeutic benefit of HbF was recognized from the report that infants with sickle cell anemia had the milder symptoms because of the presence of a high HbF level in infant blood (48). β^0-Thalassemia who inherits the ability to synthesize large amounts of HbF also have a milder symptom than what was expected (49).

3.7.2 Regulation of γ-globin gene expression

The γ-globin gene expression primarily depends on its correspond promoter and gene-proximal and distal regulatory regions with transcription factor binding at those regions. This is shown by the point mutations occurring in transcription factor binding motifs, that produce the hereditary persistence of fetal hemoglobin (HPFH) phenotype. At the region, common sequence motifs found in many other genes, such as TATA, CCAAT and CACCC motifs are also present. TATA and CCAAT motifs, which are conserved in species that express the γ-globin gene in the fetal stage, are the

binding site of the stage selector protein complex (SSP) providing a competitive advantage for γ-globin gene over β-globin gene. SSP is composed of ubiquitously expressed factor CP2 and NF-E4, which is erythroid specific and activates γ-globin gene expression (50, 51). CCAAT motif alone is the binding site for several proteins; CP1, a ubiquitous protein that acts as a transcription activator, CAAT displacement protein (CDP), a transcription repressor which is a competitive binding of CP1(52), and the lineage-specific zinc-finger protein GATA-1, which is inconclusive as gene suppressor(53).

The CACCC box plays a crucial role in gene expression at the fetal stage of definitive hematopoeisis when the major synthesis of fetal hemoglobin takes place in humans. It contains the binding site of Krüppel-like zinc finger protein family, which is essential in erythroid lineage. This zinc finger protein family includes the ubiquitous proteins Sp1, YY1, EKLF, and BKLF/FEF-2 (54).

Other important sites in the upstream promoter have also been revealed by HPFH mutants. The -175HPFH mutation alters the interaction with GATA-1 and octamer-1(55, 56), but the relevance of these in vitro effects to the HPFH phenotype remains unclear. Several HPFH mutations have been found in the -200 region, which was speculated to be the binding site for a repressor complex (57). It has been shown that GATA-1 has protein-protein interaction with EKLF *in vitro* and *in vivo,* and synergistically activates expression of the linked gene. The potential binding site for GATA-1 and YY1 can be found throughout the β-globin locus. It is possible that GATA-1 and YY1 proteins binding may mediate the formation of the higher order protein DNA complexes.

The potential binding sites located upstream of the promoter (-382 to -730), which believed to localize a silencer (58) and a butyrate response element (59) are also reported. Moreover, an element located downstream of $^{A}\gamma$ gene has been shown to be sensitive to nuclease digestion in erythroid cells. It contains binding sites for GATA-1, and believed to stabilize the interaction between the LCR and the γ-globin gene. However, the actual function is not fully clear.

3.7.3 Erythropoiesis and HbF production

The erythropoiesis process started with early erythroid progenitors (burst forming units-erythroid, BFU-e), then to late erythroid progenitors (colony forming units-erythroid, CFU-e). Erythroid progenitors are characterized by their ability to give rise to colonies of erythroblasts in semisolid culture media. The CFU-e was differentiated into proerythroblast. The transitions from BFU-e to CFU-e and from CFU-e into proerythroblast do not appear to be separated by obvious discrete differentiation events. The maturative-proliferative proerythroblasts was matured into polychromatophilic erythroblasts, followed by the basophilic stage. The differentiation continued to maturative nonproliferative polychromatophilic erythroblasts which mature through the reticulocyte stage into the final erythrocytes (Figure 6).

Erythropoiesis is under the control of series of hematopoietic growth factors that interact to determine the rate of erythrocyte production. The *kit* ligand, interleukin-3 and granulocyte-macrophage colony-stimulating factor (GM-CSF) are active predominantly on early erythroid progenitors to stimulate proliferation of primitive progenitors The erythroid-specific factor erythropoietin activity is exerted during the later stage of progenitor differentiation mainly at the level of CFU-e.

Even though the developmental switch from fetal to adult globin synthesis is effectively completed at 12 weeks post gestation, with HbA comprising 97% of the circulating hemoglobin, HbF is still detectable during the maturation process of individual adult erythroid cells. A 2.7% of normal adult human erythrocytes contain HbF (F-cells) of approximately 20% of total hemoglobin per cell (60), thus comprises less than 1% of total hemoglobin. The number of F cells and the amount of HbF in individual cells appears to be under genetic control. Regulation of F-cell formation in adults appears to be mediated during early erythropoiesis. The molecular mechanisms are still not fully understood.

Correlations between HbF and erythropoiesis have been reported in studies involving erythroid-directed cytokines (61). The *in vitro* erythropoietin induced erythroid differentiation studies showed that transition through BFU-e, CFU-e and proerythroblast phases was accompanied by a switch from γ- to β- globin expression in individual cells (62). In these cultures, γ-globin synthesis occurs mainly in

proerythroblast and basophilic normoblasts at day 7, whereas β-globin synthesis occurs later, maximum at day 14. This pattern of globin expression mimics the developmental switching in a shorter timeframe.

Besides erythropoietin, stem cell factor (SCF) has also been shown to regulate erythroid growth and HbF production *in vivo* and in primary cell cultures (63, 64). The HbF response to SCF at the highest magnitude in the late progenitor stage as correlated with surface CD117 expression (65). The SCF and transforming growth factor beta (TGF-β) had synergistic effects on HbF production by pancellular reactivation of γ-globin gene of hemoglobinized erythroblasts from human adults (66). It was speculated that the SCF may regulate The transcriptional regulation study of these cells showed an increase in GATA-1, RBTN2 and EKLF expression simultaneously with γ- to β-globin switching (67). This is accompanied by a reciprocal decrease in GATA-2 levels. This GATA expression profiles are correlated with those in mice and chickens erythroid differentiations (21). It is possible that the factors which regulate the developmental switch may also affect the pattern of globin gene expression in adult erythropoiesis.

Despites the previous studies, the globin expression profiles are greatly depending on the conditions within the culture medium, which are responsible for different results obtained by various laboratories.

3.7.4 HbF containing cell (F-cells)

Rapid regeneration or expansion of the erythroid marrow induces F-cell production (68). These observations suggested that the kinetics of erythroid regeneration determine whether a cell will become an F-cell or express only HbA. Previous reports, which showed that F-cells and non F-cells could be derived from a single progenitor, support the hypothesis that early progenitors encode a program allowing expression of fetal globin genes. The programs are changed to adult globin expression during the downstream differentiation and maturation of erythroid progenitor cells, possibly through alteration of *trans*-acting factors profiles (69, 70). In acute erythropoietic stress as seen in acute anemia and chemotherapeutic marrow ablation, accelerated erythropoiesis increases the chance of premature commitment resulting in enhanced production of F-cells (71) (Figure 7). Cytotoxic agents such as

hydroxyurea also kills late erythroid progenitor cells triggering rapid erythroid regeneration and inducing F-cell formation (72).

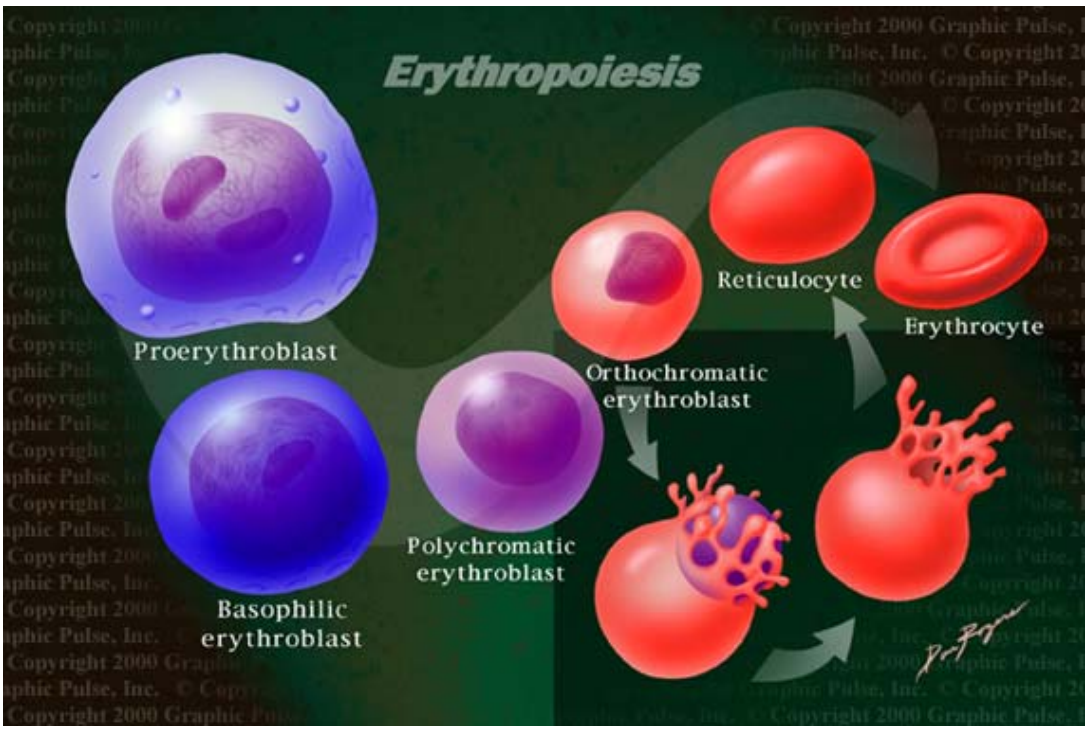


Figure 6: Maturation of red blood cells

The production of red blood cells follows the sequential formation, from erythroid progenitors to proerythroblasts, basophilic, polychromatic and orthochromatic erythroblast. Then, orthochromatic erythroblast undergoes terminal maturation leading to enucleation and the cells become reticulocytes and mature erythrocyte, respectively.

(Image from Israels, L. and Israels, E. Erythropoiesis. In: Mechanisms in Hematology. Manitoba: The University of Manitoba; 1997)

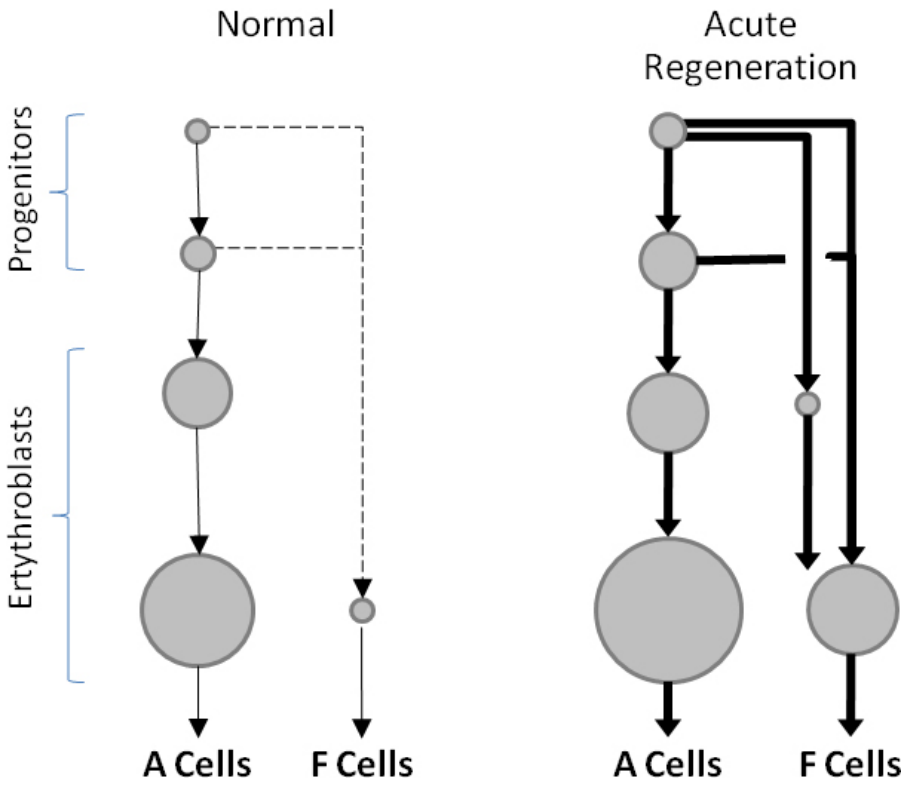


Figure 7: Model of formation of F cells in the normal adult and in acute erythroid regeneration

In normal adult erythropoiesis, minimal amount of F cells were produced, in contrast, in acute regeneration, the cells were forced to commit prematurely to replenish the peripheral demand, resulting in increased F cell population in blood stream.

3.8 Genetic factors affecting HbF production

Production of γ-globin can vary widely and more than 90% of this variation in Hb F levels is responsible by genetic factors. They can be classified by the factors linked to β-globin locus, and unlinked to β-globin locus.

3.8.1 Hereditary Persistence of Fetal Hemoglobin (HPFH)

Hereditary persistence of fetal hemoglobin (HPFH) is a group of disorders characterized by the presence of a substantial elevation of HbF in RBCs (5-30% of total hemoglobin) with no associated phenotype of hypochromia and microcytosis in heterozygotes. The increased HbF in heterozygous HPFH is distributed in a relatively uniform (pancellular) fashion among all of the red cells rather than the heterocellular fashion in the F cells. HPFH is caused by deletions in the β-globin locus or point mutations in γ-globin gene promoters.

3.8.1.1 Deletional type of HPFH

The HPFH deletion can be relatively short from ~3 kb 5' to the δ gene to 0.7 kb 3' to β gene, ~50 kb, or even up to ~100 kb deletion. There are three molecular mechanisms proposed for the elevated level of γ-globin gene expression in deletional HPFH: a) loss of γ-globin silencer sequences; b) translocation to closer proximity to the γ-globin genes of distal enhancer elements; c) increasing the ability of the γ-gene to compete more effectively with adult globin genes (δ-, and β-) for binding to the LCR.

3.8.1.2 Non-deletional type of HPFH

Non-deletional HPFH mutations are generally found in either the Gγ or the Aγ-globin promoters. The mutations are clustered in three regions of the promoters of the γ-globin genes (Figure 8, Table 1). These mutations are thought to affect the expression of the γ-globin gene by alter the binding of a number of *trans*-acting factors to the γ-globin gene promoters. The alteration could be either prevention the binding of negative regulatory factors or enhancement the binding of positive regulatory factors and thereby cause the up-regulation of γ-globin gene expression.

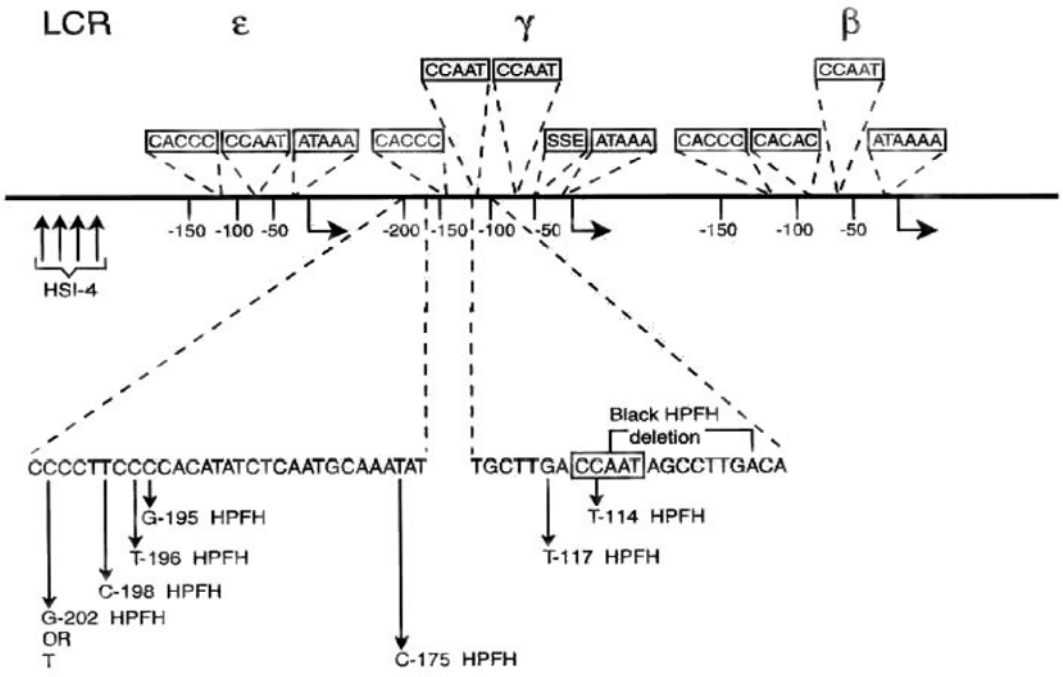
First region is around 200 bp from the transcription initiation site; -202 (C→T), -198 (T→C), -196 (C→T), -195 (C→G). It was found that there is a binding site for Sp1 at –200 position. The -202 (C→T) mutation creates a binding site for the stage selector protein (SSP), a protein complex that is thought to be an important regulator of γ-globin gene expression. Second region is located at position –175. This region contains a sequence that is the binding site of ubiquitous trans-acting factor, OCT-1, and erythroid specific factor, GATA-1. Third region is in the area of CCAAT box, which is the well known regulatory element of globin and other genes. The CCAAT box region is known to be the binding site of a number of trans-acting factors, including the ubiquitous factors CCAAT binding protein (CP1) and CCAAT displacement factor (CDP) as well as the erythroid-specific factor NF-E3. These mutation are -114(C→T), -117(G→T).

3.8.2 γ-globin genes rearrangement

The presence of multiple copies of γ-globin genes from 3-5 genes showed the level of HbF from 0.7-3.8% in adults (73).

3.8.3 *Xmn*I polymorphism

Approximately 30% with increased γ-globin can be linked to the presence of a common *Xmn*1-$^{G}\gamma$ polymorphism (74). It is called polymorphism because it was found more than 1% of normal population which was not always related with high HbF. This polymorphism involves a C → T substitution at position -158 in the $^{G}\gamma$ gene and is usually silent in normal individuals (75). However, under conditions of hematopoietic stress such as β-thalassemia, the presence of the *Xmn*1-$^{G}\gamma$ polymorphism leads to a moderate increase in γ-globin (76). The mechanism of binding of a regulatory protein at the mutation location related to increasing activity of $^{G}\gamma$-globin gene is not clearly.



Type and Racial Group	Mutation in Globin Gene	Percentage of Hb F in Heterozygotes
Pancellular $^{G}\gamma$ HPFH		
Black	$^{G}\gamma$: -202 (C→G)	15–25
Black	$^{G}\gamma$-: 175 (T→C)	20–30
Sardinian	$^{G}\gamma$: -175 (T→C)	17–21
Japanese	$^{G}\gamma$: -114 (C→T)	11–14
Pancellular $^{A}\gamma$ HPFH		
Southern Italian	$^{A}\gamma$: -196 (C→T)	12–16
Chinese	$^{A}\gamma$: -196 (C→T)	14–21
Black	$^{A}\gamma$: -175 (T→C)	36–41[a]
Greek	$^{A}\gamma$: -117 (G→A)	10–20
Sardinian	$^{A}\gamma$: -117 (G→A)	12–16
Black	$^{A}\gamma$: -117 (G→A)	11–16
Black	$^{A}\gamma$: -114 to -102 deleted	30–32
Pancellular $^{G}\gamma^{A}\gamma$ HPFH		
Chinese	Unknown	20–25
Heterocellular $^{G}\gamma$ HPFH		
Black (Atlanta)	$^{G}\gamma^{G}\gamma$: -158 (C→T)	2.3–3.8
Japanese	$^{G}\gamma$: -114 (C→T)	11–14
Heterocellular $^{A}\gamma$ HPFH		
Black	$^{A}\gamma$: -202 (C→T)	1.6–3.9
British	$^{A}\gamma$: -198 (T→C)	3.5–10
Brazilian	$^{A}\gamma$: -195 (C→G)	4.5–7
Black (Georgia)	$^{A}\gamma$: -114 (C→T)	2.6–6
Heterocellular $^{G}\gamma^{A}\gamma$ HPFH		
Swiss	Unknown	1–4
Black (Seattle)	Normal γ-gene promoters	3–8

Figure 8, Table 1: Position and phenotype of non-deletional HPFH (77)

Association of high HbF production in adult unlinked to the β-globin gene has been investigated because the high HbF level in some cases could not be explained by mutation or deletion on the β-globin locus. Previous genome-wide association studies conducted in independent populations have identified few SNPs in different chromosomal loci that are associated with varying expression level of HbF. Numbers of genes unlinked to the β-globin locus which result increased γ-globin production, however, approximately half of the genes remain undefined (78). These elusive genes appear to affect factors which act in *trans*.

3.8.4 Polymorphism at the HBS1Lloci

The study on the heterocellular HPFH, sickle cell disease, and β-thalassemia (79) showed the role of genetic determinant located at chromosome 6q (80, 81), which encompassed several genes, including HBS1L. HBS1L is a member of the GTP-binding protein family that is expressed in erythroid progenitor cells. Its expression correlates with high F cell alleles which supports a role for the HBS1L-related genetic variants in HbF (82, 83).

3.8.5 Polymorphism at the BCL11A loci

Genetic association studies have identified sequence variants that influence HbF levels in the intron of gene BCL11A in chromosome 2, which encodes a protein that represses transcription in the B lymphoid lineage. It is hypothesized that BCL11A might repress γ-globin gene expression. The short isoform of the BCL11A can be found in cells that express high amounts of HbF, including human embryonic erythroleukemia cell and in primary human fetal liver cells. However, the molecular mechanism of γ-globin gene repression by BCL11A is still unknown (82, 84).

3.8.6 Polymorphism located on chromosome Xp 22.2

The numbers of F-cell and levels of HbF was affected by gender, in normal females had slightly higher F-cell than those in males. This elucidation leads to linkage analysis using polymorphic restriction sites on the X chromosome, mainly in

Jamaican families. The study has localized the F-cell production locus on chromosome Xp22 (85).

3.8.7 Polymorphism located on chromosome 8q

The genome-wide linkage studies showed a genetic interaction between the *Xmn*I-$^{G}\gamma$ site and a locus on chromosome 8q which influence level of adult F-cell (86, 87).

3.9 Non-genetic factors affecting HbF production

3.9.1 Pregnancy

A modest increase in HbF can be found during second trimester of pregnancy, and 6% increase in hydatidiform moles. It is believed that hormones secretion during pregnancy might play a role in these HbF increase, but the mechanism is still unclear (88).

3.9.2 Diabetic mellitus

The delayed γ- to β- globin switching was reported in infants of diabetic mothers (89). This resulted from elevated γ-aminobutyric acid level in blood of diabetic mothers. This finding leads to discovery of short chain fatty acids as the HbF inducers.

3.9.3 Other medical conditions

Numbers of other medical conditions besides hemoglobinopathies have been reported to associate with high HbF. Down syndrome and other chromosomal translocations were reported to result in accelerated γ- to β- globin switching (90). Many studies showed relationship of elevated HbF level to sudden infant death syndrome (SIDS) (91). Increased HbF was found in acute anemia, as mentioned previously. Juvenile chronic myeloid leukemia and Fanconi anemia were found to be accompanied by increase HbF level (92). Correlation to HbF was also reported in the case with solid tumors, hematologic malignancies and paroxysmal nocturnal hemoglobinuria.(93, 94)

3.10 Pharmacological induction of HbF

The characteristic of β-thalassemia is imbalanced α- to non-α-globin chain synthesis. Increasing the ability to synthesize more γ-globin chains and HbF reduces this imbalance and decreases the accumulation of free α- globin chains that cause most of the pathophysiology of β-thalassemia, coexistence of HPFH with homozygous β-thalassemia. There has been increasing interest in the possibility of either reactivating or further stimulating HbF production in these conditions as a potential therapeutic approach for hematologic diseases, including β-Thalassemia and sickle cell anemia (95-97).

The first reported HbF inducer is 5-azacytidine, which acts through the hypomethylation of critical regulatory elements of the γ-globin genes (98). Hydroxyurea, a cytotoxic drug, induces Hb F by altering the kinetics of erythropoiesis, and the nitric oxide-dependent activation of soluble guanylyl cyclase (99, 100). Another group of drugs is the histone acetylase inhibitors (101, 102), including short chain fatty acids (103), such as butyrate and its derivatives Hydroxyurea (104, 105) and butyrate (106) have been in clinical trials, and found to reduce the need for blood transfusions. Combination drugs were found to have synergistic effects (107). In spite of a number of HbF inducing agents discovered, the responses to the drugs are not consistent, and vary in different patients. Moreover, concerning of the mode of actions, hydroxyurea and butyrate seem to globally affect the transcriptional regulation of many other genes, The long term side effects of these drugs have been an issue of continuous concern (108, 109). Beside these drugs, various agents capable of augmenting HbF had also been identified, including hormones such as erythropoietin, which acts by inducing rapid erythroid regeneration, hematopoietic cytokines, and DNA binding drugs such as cisplatin (110), mithramycin (111) and tallimustine.

The reported potential HbF inducers can be categorized in several classes according to the mechanism of action such as histone deacetylase (HDAC) inhibitor, cytotoxic agents, and DNA-binding drugs (Figure 9). The potential HbF inducers reported up to date were listed in Table 2.

Table 2: Reported γ-globin inducing agents

Agent	Studying model	Reference
DNA methyltransferase inhibitors (n = 4)		
5-Azacytidine	c, m, p, hc, hv	(112, 113)
Decitabine	c, m, p, hc, hv	(114)
5,6 dihydro-5-Azacytidine	hv	(115)
S110	p, hc	(116)
Cytotoxic agents (n = 23)		
DNA alkylators		
Busulfan	p, hv	(117)
Cisplatin	c	(110)
Streptozotosin	c	(118)
Nucleoside analogue		
Cytosine arabinoside	c, p, hc, hv	(119)
Inosine monophosphate dehydrogenase inhibitors		
Ribavirin	c	(120)
Mycophenolic acid	c	(120)
Tiazofulin	c	(120)
Ribonucleotide reductase inhibitors		
Didox	c, m	(118, 121)
Hydroxyurea	c, m, p, hc, hv	(37, 122, 123)
Resveratrol	hc	(124)
Trimidox	c	(118)
DNA intercalating agents		
Aclarubicin	c	(125)
Chromomycin	c	(126)
Distamycin	c	(127)
Doxorubicin	c	(125)
Mithramycin	c, hv	(126)
Tallimustin	c	(127)

Table 2: Reported γ-globin inducing agents (cont.)

Agent	Studying model	Reference
Psoralens ± UVA irradiation		
Angelicin	c, hc	(128)
5-Methoxypsoralen	c, hc	(129)
Trimethyl angelicin	c, hc	(128)
Dihydrofolate reductase inhibitor		
Methotrexate	p	(130)
Microtubule inhibitor		
Vinblastine	p	(131, 132)
Protein synthesis inhibitor		
Anisomycin	c	(133)
Short chain fatty acids and derivatives (n = 25)		
Butyrate	c, m, p, hc, hv	(38, 134)
Phenyl butyrate	c, hc, hv	(105)
α-aminobutyric acid	c, p, hc	(135)
di-methylbutyric acid	c, m	(103)
Tributyrin	c	(136)
Acetate	c, m, p, hc	(137)
Phenylacetate	c, hv	(138)
Phenoxyacetic acid	c, m	(139)
Butyryl-hydroxamate	c, m, hc	(140)
α-Methylhydrocinnamic acid	c, m	(139)
Caproate	c	(141)
Heptanoic acid	hc	(142)
Hexanoic acid	hc	(142)
Isobutyramide	m, p, hc, hv	(143)
Nonanoic acic	hc	(142)
Octanoic acid	hc	(142)
Pentanoic acid	p, hc	(142)

Table 2: Reported γ-globin inducing agents (cont.)

Agent	Studying model	Reference
Propionic acid	p, hc	(142)
Dimethoxyphenyl propionic acid	c	(103)
Propional hydroxamate	c, m, hc	(144)
RB7	c, hc	(145)
RB4, RB9, RB29	c	(146)
Valproic acid and derivatives	c, hc, hv	(147, 148)
Histone deacetylase inhibitors (n = 11)		
Apicidin	c, hc	(101, 149)
Compounds "24" and "29"	c, hc	(150)
FK228	c, hc	(151)
Helminthsporium toxin	c, hc	(102)
MS-275	c, hc	(151)
SAHA (Vorinostat)	c, hc	(101, 144)
SBHA	c, hc	(144)
Scriptaid	c, hc	(152, 153)
Trapoxin	c, hc	(102, 152)
Trichostatin A	c, hc	(102, 154)
Imunomodulatory drugs (n = 3)		
Thalidomide	hc	(155)
Revlimid	hc	(156)
Pomalidomide	hc	(156)
Hormonal agents (n = 2)		
Nomegestrol	hv	(157)
Progesterone	hv	(158)
Cytokines (n = 3)		
Erythropoietin	c, m, p, hc, hv	(159)
Stem cell factor	p, hc	(65, 160, 161)
TGF-β	hc	(162)

Table 2: Reported γ-globin inducing agents (cont.)

Agent	Studying model	Reference
mTOR inhibitors (n = 2)		
Rapamycin	c, hc	(163, 164)
Everolimus	c, hc	(165)
Oligonucleotides (n = 2)		
Triple-helix oligonucleotide	c	(35)
Peptide nucleic acids (PNA)	c	(166)
Miscellaneous (n = 4)		
Zileuton (5-lipoxygenase inhibitor)	c	(167)
Vanadate (phophatase inhibitor)	hc	(168)
FG-2216 (HIF-prolyl hydroxylase inhib)	p, hc	(169)
CysNO (nitric oxide donor)	c, hc	(100)
Wheat grass	hv	(170)
Sesquiterpene Aminoquinones (from a marine sponge)	c	(171)
Chebulinic acid (ellagitannin in plant)	c	(172)
Nicotinic acid	c	(173)

Table modified from (174)

c : immortalized erythroid cell line
hc : human primary cell culture
hv : human *in vivo*
m : murine model
mTOR : mammalian target of rapamycin
p : nonhuman primate *in vivo*
TGF-β : tumor promoting graft factor-beta
UVA : ultraviolet A

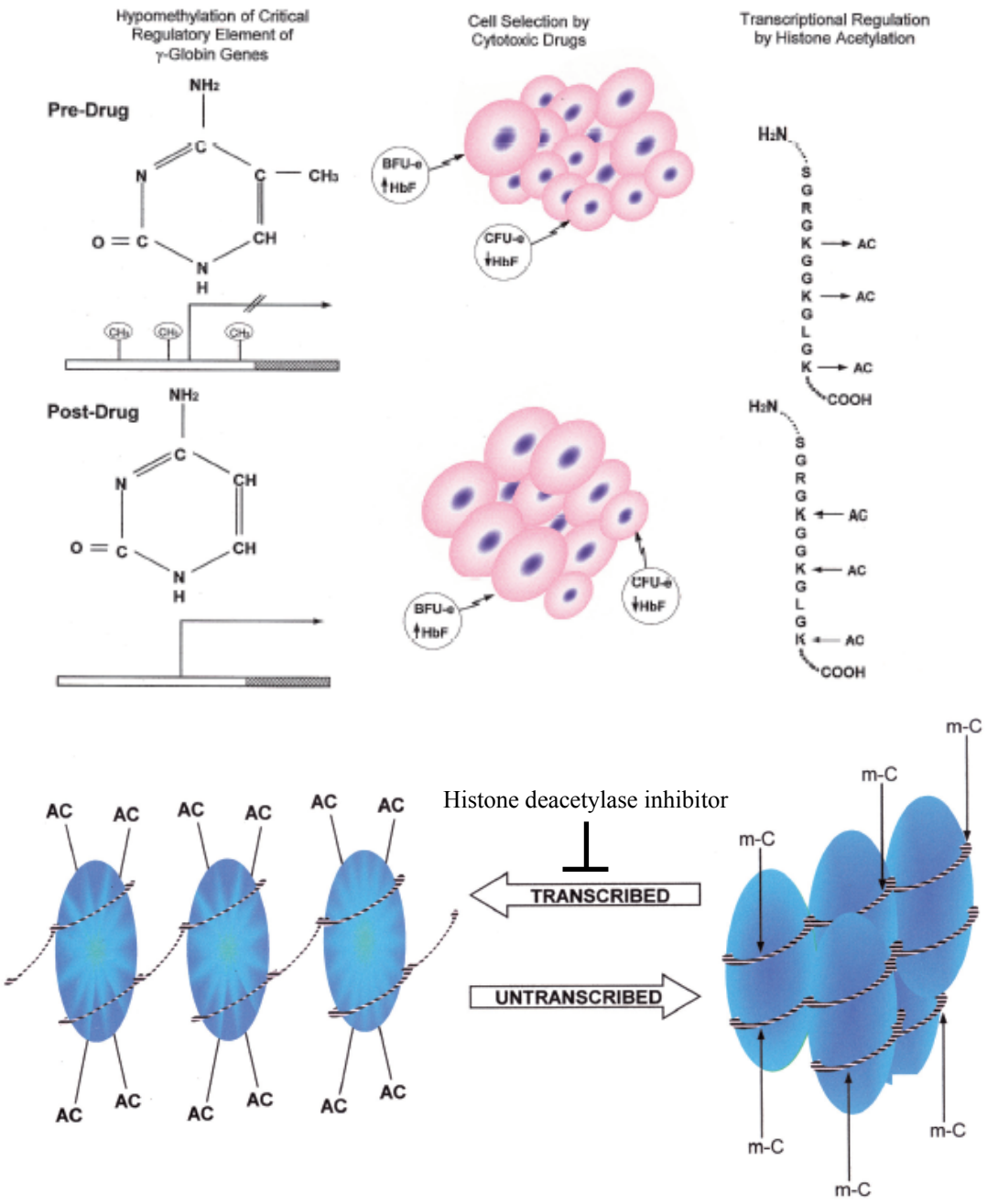


Figure 9: Major mechanisms of HbF induction

DNA methyltransferase inhibition, F-cell production during stress erythropoiesis, and histone deacetylase inhibition are proposed mechanisms of HbF induction by 5-azacytidine, hydroxyurea, and butyrate, respectively.

3.10.1 DNA methyltransferase inhibitors

Cytosine demethylation at CpG dinucleotides may increase transcription. Hypomethylation can be induced by cytidine analogs like 5-azacytidine, which has a nitrogen in the 5-position of the pyridine ring. Incorporation of 5-azacytidine into DNA inhibits DNA methyltransferase and prevents methylation, allowing transcription to occur.

5-azacytidine (5-Aza), the first HbF inducing agent studied, was first done in anemic juvenile baboons (112). The study was extended to a patient with severe homozygous β-thalassemia, who was found to have 7-fold increase in γ globin mRNA synthesis and elevated hemoglobin concentration after receiving 5-azacytidine treatment for 7 days (113, 175). Induction of fetal hemoglobin was subsequently demonstrated in patients with homozygous Hb S, or S/β-thalassemia. Despite of the solid clinical evidence to support their capability as HbF inducing compound, concerns about risks of carcinogenicity restricted this compound to only experimental use. Later, introduction of decitabine as a non-carcinogenic derivative of 5-aza (176), together with approval of 5-aza and decitabine for the treatment of patients with myelodysplatic syndromes, leaded the renewed interest into these agents as HbF inducers.

The mechanism of DNA demethylation at the 50 bp upstream of the transcriptional start site of γ-globin gene by 5-azacytidine has been shown to result in facilitating the binding of an activating protein complex (175, 177, 178) However, the actual mechanism of action of 5-aza in HbF induction has been debated for decades. Globin gene expression correlates with DNA methylation (179), but DNA methylation does not appear to be a primary mechanism controlling globin gene switching (180). Therefore, the alternative hypothesis based on the cytotoxic properties that destroy most actively cycling erythroid cells has been proposed (181, 182). The resulting decrease in late erythroid progenitor cells could trigger rapid erythroid regeneration and induce F-cell formation (Figure 7). This hypothesis was supported by experiments showing that other cytotoxic agents, which were not DNA methyltransferase inhibitors including hydroxyurea, also increased HbF production (119). Nevertheless, recent study brought another skeptic into both models, based on findings that HbF induction in primary human erythroid differentiation could still be

attained at dose of 5-aza that did not alter erythroid kinetics, cell cycle distribution or global DNA methylation levels. Besides the hypomethylation of the γ-globin promoter was inadequate to induce HbF in primary human erythroblasts (183). It is likely that the combined effect on erythropoiesis and DNA methylation is the cause for the superior *in vivo* induction of Hb F by 5-aza.

The DNA methyltransferase inhibitors have shown exceptional clinical evidence of HbF induction. Some challenges such as side effects, including myelosuppression and carcinogenicity, are still to be overcome. Moreover, the issue about their nonspecific alteration of the global DNA methylation patterns is still concerned.

3.10.2 Cytotoxic agents

Hydroxyurea (HU), S-phase specific cytotoxic ribonucleotide reductase inhibitor, has effects on DNA by blocking the conversion of ribonucleotides into deoxyribonucleotides. HU has long been used to treat myeloproliferative diseases.

The discovery of Hb F induction by HU was a consequence of the debate on the mechanism of action of 5-aza. Stress erythropoiesis induced by altering the kinetic of erythroid differentiation, leads to F-cell production (182). In situations of acute bone marrow stress such as recovery from the cytotoxic effects of HU, the early erythroid progenitors are selectively recruited and proliferate maximally, resulting in an enhanced proportion of Hb F in the new red cell population. However, similarly to 5-azacytidine, the detailed mechanism of action of HU remains uncertain. Recently it has been shown that HU can be oxidized by heme groups to reduce the free-radical gas molecule NO. Thus, it has been proposed that HU induces fetal hemoglobin by the nitric oxide-dependent activation of soluble guanylyl cyclise (100, 184).

Hydroxyurea is found to augment HbF production and inhibits *in vitro* growth of erythroid colonies from the blood of anemic nonhuman primates and patients with sickle-cell anemia. HU can raise γ-globin levels on both early progenitors and late erythroid precursors. It increases total intracellular hemoglobin in the human K562 erythroleukemia cell line and preferentially increases γ-globin mRNA levels in these cells. In two-phase liquid culture of peripheral-blood mononuclear and erythroid

progenitor cells from normal donors, HU significantly increases both γ-globin mRNA levels and HbF.

Even though FDA-approval for SCD, the efficacy of HU in thalassemia is controversial, few reports of clinical efficacy in the most severe, transfusion-dependent types of β-thalassemia suggested that HU was not a generally useful treatment for these disorders (37). In some types of β-thalassemia intermedia there may be a moderate effect on total hemoglobin concentration and HbF (106, 185, 186). Hemolysis may be reduced and extramedullary hematopoiesis may regress, but drug response is greatly varied among each individual. Altogether, the future for HU as a sole treatment of severe β-thalassemia major does not look promising.

3.10.3 Butyrate and Short chain fatty acid derivatives (SCFADs)

Several observations have indicated that a number of SCFADs and butyrate are able to influence the developmental program of globin synthesis. It has been shown that infants of diabetic mothers have a markedly delayed γ to β globin switch as a consequence of elevated level of α amino-n-butyric acid in these infants (89). This evidence led to discovery of butyrate as HbF inducer.

Butyrate and short-chain fatty acid derivatives (SCFADs) have been found to possess inhibitory activity on histone deacetylase, resulting in global hyperacetylation of ε-amino groups of lysine residues in histones. This leads to a decreased association of basic core histone proteins with the DNA, rendering certain genes more accessible to the transcriptional machinery. However, recent study showed that while butyrate resulted in increased acetylation of the γ-globin promoter histones, it also decreased β-globin promoter histone acetylation. The study in transgenic mice showed butyrate responsive element located between position -382 and -730 region of the Aγ gene promoter (187). This raised the possibility that there are more specific mechanisms other than global histone hyperacetylation mediate SCFAD induction of HbF (188). Additional experiments reported the effect of butyrate on translational level by increasing the association of γ-globin mRNA with ribosomes (189), and on activation of p38 mitogen activated protein kinase (MAPK) (133) and cyclic nucleotide signaling pathways(190).

Butyrate is also known to have several effects in culture and in vivo, such as cellular growth arrest in the G1 phase through induction of p21, induction of protein synthesis, changes in cell morphology and cytoskeleton, induction of differentiation of erythroleukemia cell lines. Altogether, the global histone hyperacetylation inhibition may not be the primary mechanism underlying SCFAD stimulation of HbF.

Butyrate and other short-chain fatty acids have been shown to stimulate fetal and embryonic globin gene expression in experimental models. These models include preferential γ-globin expression from the β-globin locus transfected into Xenopus oocytes (191). In fetal sheep the infusion of butyric acid in utero during the time of the normal gene switch stimulates the γ globin gene (192). Butyrate was showed to enhance fetal globin gene expression in erythroid cells cultured from normal fetuses (193), patients with β-thalassemia or SCD (138, 194), baboons (107, 134) and transgenic mice. Reporter gene assays have demonstrated that several fatty acids and an amide derivative, isobutyramide, stimulate γ-globin gene expression through a proximal region of the γ-globin gene promoter.

In clinical studies, patients showed increased fetal globin expression at the mRNA, protein or cellular levels in patients treated with intermittent doses of arginine butyrate and urea cycle disorder drug, phenylbutyrate (38, 195). Limitations of the available SCFADs as therapeutics, however, include their short *in vivo* half-life, thus requiring continuous administration by the intravenous route or with such large oral doses that compliance is limited in many patients. An orally bioavailable SCFAD that induces γ globin at lower doses than butyrate or phenylbutyrate and has longer biologic activity would be of value as a Hb F-inducing therapeutic agent(196, 197). The small human trials of isobutyramide and valproic acid were also performed (147, 198). However, signs of rapid drug tolerance and erythropoiesis suppression side effect were observed. Besides the half-life problem, requirement of large amounts of the drug to be administered; offensive odors of many derivatives make these compounds even less suitable for a long-term patient compliance.

3.10.4 Other histone deacetylase inhibitors

Besides butyrate and short chain fatty acid derivative, a number of histone deacetylase inhibitors have been studied as a consequence of the finding that HbF

induction mechanism of butyrate involved in histone acetylation-deacetylation. In the beginning, three HDAC inhibitors, including trichostatin A, trapoxin and HA toxin, were found to induce γ-globin gene expression in erythroid cell culture (102). Later, many additional HDAC inhibitors have been shown to induce HbF in variety of experimental systems (Table 2). Due to these HDAC inhibitors studies, evidences which supported more specific mechanisms, other than global hyperacetylation, were considered to be accounted for HbF induction. Cao et al showed that the degree of γ-globin gene induction was not dependent of the potency of HDAC inhibition(199). In addition, numbers of reports have shown the importance of activation of p38 MAPK to HbF induction by HDAC inhibitors.

Several HDAC inhibitors were shown to induce HbF in human primary erythroid system, but none have reached the *in vivo* study in human.

3.10.5 DNA-binding drugs

Many DNA binding drugs have been used for therapy. The interaction of DNA-binding drugs with the minor groove of DNA might perturb DNA structure and inhibit the interactions between transcription factors and target DNA elements. Study on the effects of DNA-binding drugs on gene expression have been reported by several groups, demonstrating that DNA-binding drugs exhibit important biological activities in many cellular model systems.

The GC-rich binders, chromomycin and mithramycin, are used in anticancer therapy and in hypercalcemia, while the AT-rich binder, tallimustine and its analogues, are anticancer and antiviral agents. Angelicin are used in psoalens plus ultra violet A (PUVE) light therapy of psoriasis. Cisplatin, which is the intrastrand cross-link of the *cis*-Pt(NH3)2 unit to cellular DNA at two neighboring guanine bases, is used in anticancer therapy.

In HbF induction aspect, it has been demonstrated that tallimustine and some cisplatin analogues, as well as chromomycin and mithramycin, can induce erythroid differentiation in the human leukemic K562 cell line and augment the expression of γ-globin mRNA. In normal or β-thalassemia human erythroid progenitor cells,

mithramycin, angelicin and tallimustine were also proved to elevate γ-globin gene expression.

However, despite having a large clinical use, some of the DNA-binding drugs, such as cisplatin, exhibit severe side-effects, the most significance of which is acute and chronic nephrotoxicity as well as optic neuropathy and ototoxicity.

3.10.6 Erythropoietin (EPO) and some other cytokines

This group of compounds is mostly used as the adjuvant treatment of cytotoxic compounds. EPO was used to stimulate F cell production according to the hypothesis that this compound induces rapid erythroid regeneration accompanied by an increase in HbF production. In baboons, high doses of EPO were used as to produce acute regeneration kinetics, and caused sharp elevations of F reticulocytes. In contrast, EPO produced insignificant increase of F cells in patients with SCD. Other hematopoietin cytokines that can expand the early cell pools progenitor on which EPO acts have also been studied. However, GM-CSF, IL-3 or SCF showed uncertain results. The possible use of erythropoietin in combination with true HbF inducers is expected to cause both increase of erythropoiesis and preferential induction of HbF.(200)

3.10.7 Other inducing agents

Two reports have shown that progestins, nomegestrol and progesterone could induce HbF in female sickle cell disease, and in primary erythroid culture. The FDA-approved immunomodulatory drugs, such as thalidomide and lenalidomide, were used for treatment of myelodysplastic syndrome and multiple myeloma. The anticancer mechanism is still unknown. Three of immunomodulatory drugs have shown to induce HbF in K562 and primary erythroid culture. It was proposed that the mechanism for HbF induction might be through histone acetylation (156), ROS production, and p38 MPK activation (155). Additional agents are listed in Table 2.

3.10.8 Problems with currently available HbF inducers

Few HbF inducers have reached clinical trial, but none of these clinically active agents exhibit efficacy, specificity, safety, and ease of use to make them valid to the patients worldwide, especially the developing countries. The potent DNA

methyltransferase inhibitors which proved to be beneficial to both β-thalassemia and SCD patients, must be administered by injection or infusion over several days each month and cause hematopoietic suppression. Butyrate derivatives have obstacle of inconvenience of administration due to daily IV infusions or many pills requirement. Erythropoiesis suppression is also found in patients received butyrate. The inexpensive orally administered hydroxyurea showed response in only half of SCD patients, and even less in β-thalassemia patients (201-203). Its side effects includes low blood counts due to its cytotoxicity, risk of malignancy (204).Concerns about non-specific mechanisms of the HbF inducers, which can chemically modify DNA and cause global histone acetylation, thus change the epigenetic structure of cycling cells, raised the fear for cancer risk in long-term treatment.

3.11. Experimental models for studying of HbF induction

A diverse of experimental systems has been used to study HbF induction, and to identify new inducing agents. These included normal and genetically modified immortalized cell line, mice, primary cell cultures, and *in* vivo non-human primate and human studies. The ideal models, which are best representative of HbF induction in human and convenience for studying, are sought after, but none of the model can obtain both. The appropriate model must be chosen for the best outcome. The flow-chart portraying the necessary steps for the characterization of a potential useful clinical HbF inducers was shown in Figure 10 (205).

3.11.1 Native human erythroleukemia cell line (K562)

Human and mouse erythroid-like cell lines, such as K562 (206), HEL (207) and MEL cell lines have been used for study of globin gene regulation. The K562 cell line is the most widely used among these. The K562 cell line which is a cell line derived from a patient who had chronic myeloid leukemia in terminal blast cell crisis. These cells have been shown to have embryo-fetal erythroid characteristics (208). Because of their leukemic origin and long history in culture, they do not recapitulate all aspects of erythropoiesis, such as non-responsiveness to EPO and the physiological erythroid hormone. The most important is that these cells do not produce adult hemoglobin;

hence this model does not represent the pathology of β-thalassemia cells such as excess of α-globin chains. The human erythroleukemia K562 cell line can be induced to undergo erythroid differentiation after treatment with a variety of chemical compounds. Differentiation was found to be associated with an increase in the synthesis of γ-globin mRNA, suggesting that these compounds could be considered as potential inducers of γ-globin gene expression. However, it is often not clear whether the observed increases in γ-globin mRNA or HbF are the result of erythroid differentiation with the activation of many erythroid genes or are equivalent to the induction of γ-globin genes seen in differentiating human erythroblasts, which already express a wide range of erythroid genes. Despite of only about 20% of HbF inducing agents were tested *in* vivo in human experiments, each of these agents is also active in immortalized human cell line. Altogether, due to its ease of use, K562 cell line is valuable for drug screening, evaluation and preliminary mechanistic experiments.

3.11.2 Other genetic modified human erythroleukemia cell line (K562)

Many studies described the use of reporter genes under the transcriptional control of the ^{G}γ-globin gene promoter to simplify the γ-globin expression measurement for HbF induction study. A number of published studies have focused on the screening for HbF inducers using various reporters in small plasmid constructs, in combination with some of the regulatory elements from the β-globin locus (109). In a study, the reporter DNA construct composed of the coding sequences of two different luciferase reporter genes, firefly and renilla, and substituted for those of human ^{G}γ-globin and β-globin genes, respectively (Figure 11). The activity of these genes can be measured by enzymatic assay in cell lysates. Specific γ-globin gene inducers are recognized by their ability to mainly increase γ-firefly luciferase gene activity.

While some of these assays gave interesting results, it is questionable whether the use of globin regulatory elements out of their natural context can recapitulate the requirements for therapeutic HbF reactivation in the erythropoietic compartment. To overcome these limitations some studies introduce the testing of drugs in the context of the human β-globin locus in YAC transgenic mice (121, 209, 210). However,

YACs are difficult to manipulate and do not facilitate the screening of the HbF inducers in convenient cellular assays.

3.11.3 K562:: $\Delta^{G}\gamma^{A}\gamma$EGFP in β-globin locus context

Most of the genetic modified K562 cell lines were in episomal format, which are not convenient for high throughput screening due to (1) the continuous requirement for antibiotic selection, (2) the variation in the proportion of expressing cells, (3) the heterogeneity in the cell population with respect to episome copy number, and (4) the likelihood of random integration of the transgene after long periods of selection. The random integration of the transgene subject to regulatory constraints imposed by flanking genomic sequences (211, 212). The intact β-globin locus has been shown to be isolated from such position effects and to produce regulated levels of globin gene expression in transgenic animal models (213) In this study, the cellular reporter assay developed by introducing enhanced green fluorescence protein (EGFP) genes under the Gγ-globin promoter within an intact β-globin gene locus : pEBAC/148β::ΔGγAγEGFP, into K562 cell line (Figure 11) (214). This stably transfected K562:: $\Delta^{G}\gamma^{A}\gamma$EGFP cell line demonstrated a maintenance of uniform basal level of EGFP expression over long periods of continuous culture, and that induction of GFP expression parallels the induction of the endogenous γ-globin genes.

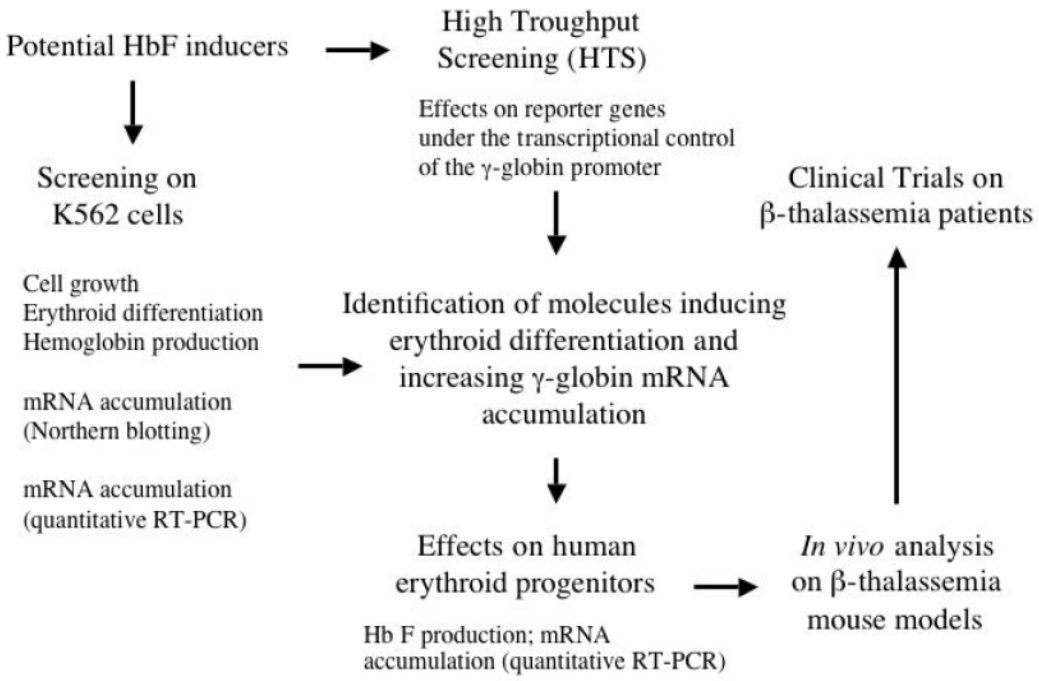


Figure 10: Scheme depicting the progress through the different strategies for the development of HbF inducers of possible therapeutic interest in β-thalassemia

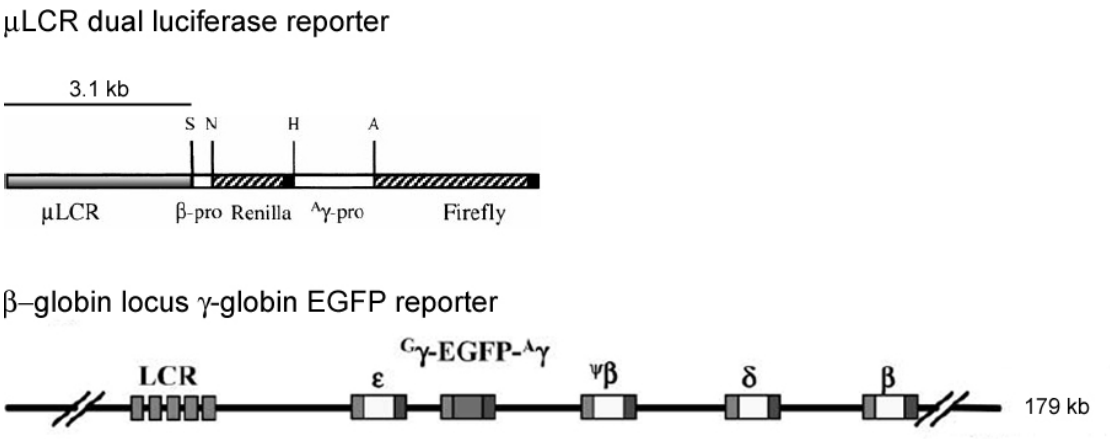


Figure 11: Example of γ-globin reporter constructs used for HbF induction studies.

The micro LCR, γ-globin promoter linked to the luciferase reporter gene was shown on the top panel. The reporter construct used in this study contained the complete β-globin LCR with the enhanced green fluorescence protein (EGFP) replaced at the γ-globin genes.

3.11.4 Primary erythroid culture

The primary erythroid culture obtained from isolation of erythroid progenitors (CD34+) from peripheral blood provided a good *in vivo* model for HbF induction study. The system recapitulates many aspects of *in* vivo erythropoiesis including globin RNA metabolism, cell cycle kinetics, expression of cell surface antigens, iron and ferritin metabolism and transcription factors (138). Numbers of culturing condition, both semi-solid and liquid, were described (215, 216). Different culture conditions may affect the cell characteristic, including HbF expression profile. This is due to the cytokines (such as erythropoietin and stem cell factor) supplemented in the culture medium.

In order to study HbF induction by chemical agents, the compounds can be added on different days when the culture consists of cells at specific stages of maturation. Peripheral blood cells are used in this method because of the availability of blood from the subjects, and the homogeneity of the erythroid progenitors (BFU-e) in peripheral blood, in contrast to the various stage progenitors found in the bone marrow.

The Hb profile of the erythroid cells can be measured by many techniques, such as alkaline denaturation, benzidine staining, immunofluorescent staining detected by flow cytometer and HPLC. Due to the more troublesome method for detecting γ-globin expression and HbF in the primary erythroid culture, this system is normally used in the later steps of HbF inducer identification studies.

3.12 Mouse models for human diseases

Production of the genetically engineered animals can facilitate understanding of the consequences of increased or decreased gene expression and pathogenic mechanisms in human. Furthermore, these models can be used to test new genetic and drug therapies for the disorders.

3.12.1 Transgenic mice

Transgenic mice are produced by stable integration of foreign DNA into the mice genome, either by pronuclear microinjection or embryonic stem cell method. The most direct way to generate transgenic mice is by microinjection of a DNA solution into the pronucleus of fertilized oocytes (Figure 12). The DNA fragments were later integrated into the mouse genome and replicate with the endogenous chromosome, hence becoming part of the newly forming embryo. Normally, the integration occurs at only one chromosome, resulting in the hemizygous transgene in the founder mouse. There is a 10-30% chance that the integration of the transgene occurs during the multiple cell stage, resulting in the mosaic for the transgene of the founder (217). The actual mechanism of integration is remained unclear. It is believed that it involves double strand breaks of the genomic DNA during the microinjection procedure (218).

3.12.2 Pronuclear microinjection method

The DNA fragment containing the desirable gene was introduced into the pronucleus of fertilized mouse oocytes by microinjection. The superovulated female, which was injected with hormones to increase the number of available fertilized eggs, are mated to intact males. The pre-implanted fertilized eggs, containing pronucleus of female and male separately, are flushed from oviducts and collected. The transgene is microinjected into the male pronucleus. After the pronuclei fuse to form the diploid zygote nucleus, the zygote will double by mitosis. Approximately 10-40 zygotes are transplanted into the oviduct of a pseudopregnant foster mother mouse, induced by mating with the vasectomized male at the night before. The foster mother will deliver a litter of pups in 3 weeks. Approximately 20-25% of the pups are expected to be transgenic, screened by using small piece of tissue acquired from the pups for the presence of the transgene.

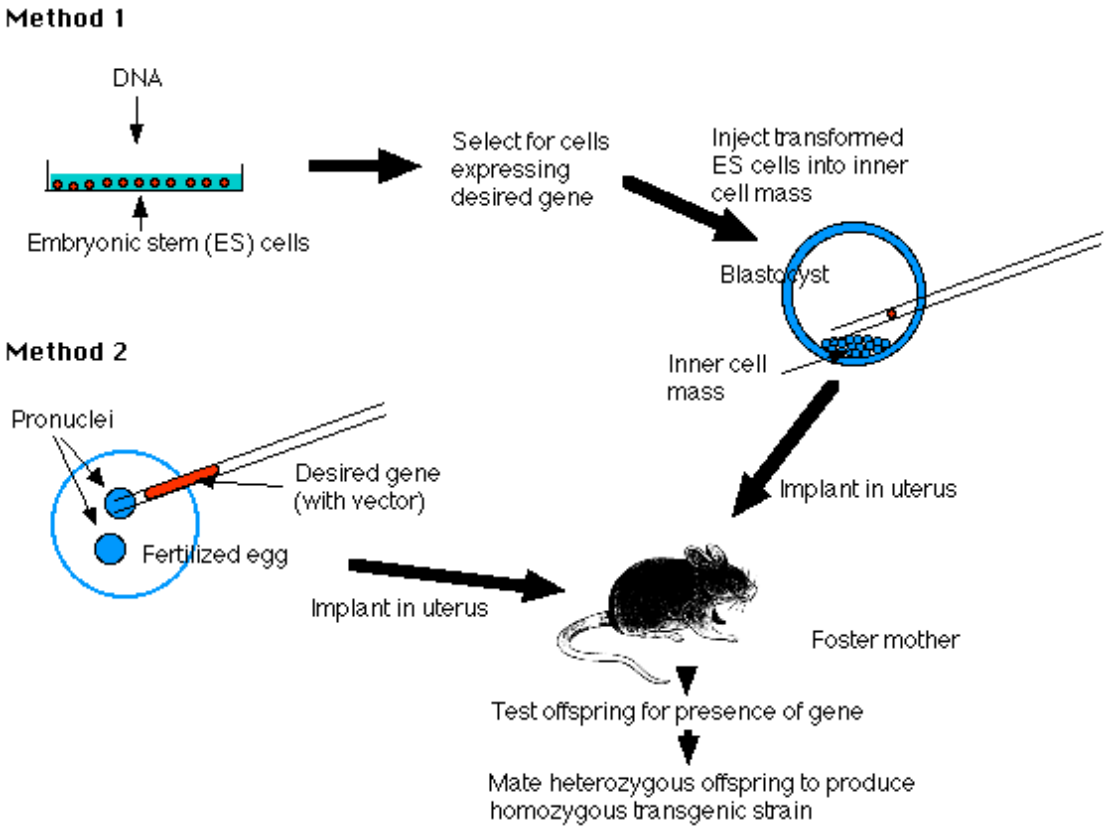


Figure 12: Creation of transgenic mice by ES cells method and microinjection method

ES cells method shown in method 1 is performed by introduction of a transgene fragment into the mouse ES cells, while microinjection method shown in method 2 is performed by injection of the DNA fragment into the pronucleus of a fertilized mouse egg.

3.12.3 Factors influencing the efficiency of transgenesis

Properties of DNA are the major influence of the efficiency of transgenesis. DNA quality is one of the most crucial factors for success transgenic mice generation, since the oocytes are highly sensitive to the toxic substances, such as phenol, EDTA and bacterial endotoxin. Contamination of dust particles also causes the blockage of the microinjection needle. Therefore, special care should be considered on preparation of DNA solutions for microinjections.

Structure of the microinjected DNA also affects the success; linear DNA gives better efficiency than circular (supercoiled) DNA (219). The DNA concentrations are commonly used at 0.01 – 50 ng/μl range with the optimum at 2 ng/μl. The DNA concentration can affect the efficiency since too high amounts of DNA could be toxic to oocytes, thus decrease the number of surviving oocytes.

3.12.4 Choice of mouse strain

The mouse strain is another factor to be taken into consideration. The mouse strain with characterized genetic background is preferred over the outbred mouse strain for generating transgenic mice, since the unexpected genetic background could disguise the actual phenotype causing by the transgene (220). The mouse strains that are suitable for the generation of transgenic mice include C57BL/6J (219) and FVB/N (221)

3.12.5 Factors affecting transgene expression

Besides DNA construct design, other incontrollable factors can still affect the expression of the transgene. These factors include the position effect, which is the most influential factor caused by genomic sequences flanking the site of integration and can have several effects on the transgene (222).This effect may reduce or entirely abolish transgene expression either by specific silencing elements or simply because the transgene is inserted into a transcriptionally inactive region of the genome. On the other hand, the transgene can also be upregulated by enhancer of the neighboring genes (223)

It is also possible that the transgene random integration occurs within a transcriptional region of endogenous genes, resulting in an insertional mutation and

disruption of the locus (224). The phenotype of the disrupted genomic locus normally presents in a proportion of the homozygote transgenic line, not the heterozygote.

In addition to the different genetic factors, environmental and dietary inconsistency may contribute to ambiguous results.

3.12.6 Globin gene expression in mouse

The mouse β-globin gene cluster on chromosome 7 has four functional β-globin genes: βh1, an early embryonic globin gene; ε^{y}, a late embryonic globin gene; and two adult globin genes, b1 (β^{major}) and b2 (β^{minor}) (225, 226). Hemoglobin switching in mice is as followed; mouse embryonic hemoglobins are first expressed at 9.5 days of gestation in the yolk sac and later in fetal liver. The switch from predominantly embryonic to predominantly adult hemoglobins is completed between days 14 and 15 of gestation in the normal fetus (227). The *b1* and *b2* genes are then expressed in fetal liver and spleen and, finally, in bone marrow during adult life. The *b1* gene is reported to be responsible for ~80 % and *b2* for ~20 % of adult β-globin production (Figure.13)

3.12.7 Transgenic mice carry human β-globin locus

The use of BAC transgenic mice was developed as in vivo model systems as a consequence of the availability of human genome on BAC clone. Large genomic inserts are more likely to contain the whole set of regulatory elements necessary for tissue-specific expression and position-independent expression. A number of transgenic mice carrying the human β-globin locus have been generated using cosmids (228), YAC (213, 229, 230). The disadvantages of these systems are limitation of size at 40 kb of cosmids, and tendency to rearrange upon integration of large YAC constructs.

The BAC DNA vector system which can handle inserts of up to 700 kb while BACs was also used for generating transgenic mice. (231)

The transgenic mice carrying the human β-globin locus have been reported (232, 233). The previous work by Vadolas, et al. has shown the creation of transgenic mice carrying 179 kb of transgene with the normal human β-globin locus. The study

demonstrated that the hemizygosity for the β-human transgene on a heterozygous β-mouse knockout background ameliorates hematological abnormalities associated with the heterozygous knockout mutation, and confirmed the function of the human transgene on the mouse background.

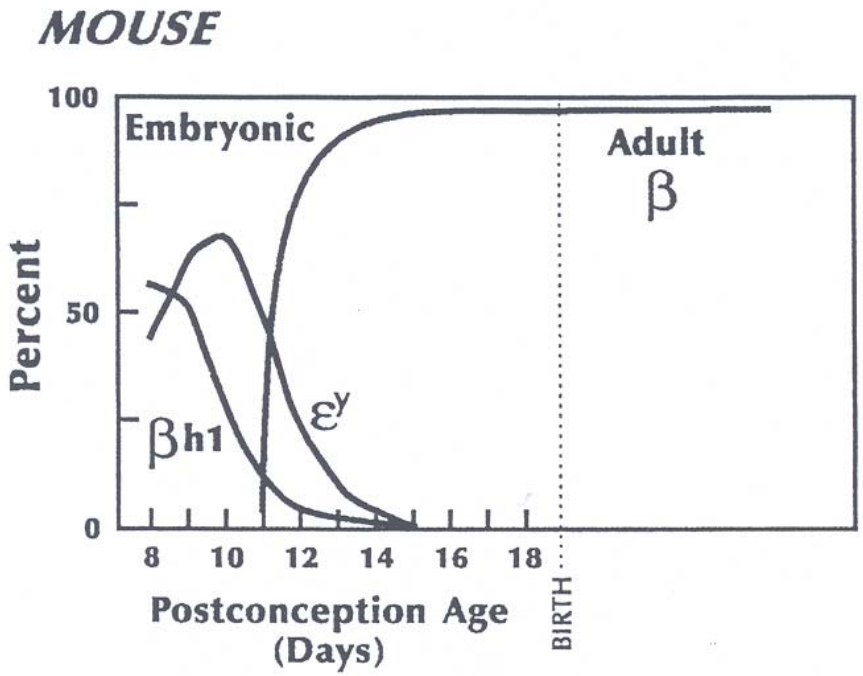


Figure 13 Hemoglobin switching in mice (234)

The murine ε^y gene is homologous to human ε. The βh1 gene is homologous to γ-globin gene while the murine β minor and β major are homologous to human β-globin gene.

3.13 Bacterial artificial chromosome (BAC)

Since the completion of the sequencing of the human and many other genomes, it is becoming clear that the complexity in higher organisms is achieved primarily not by an increase in the number of genes, but by the development of complex regulatory networks. Most studies on gene regulation over the last 20 years have usually relied on the cloning of short DNA fragments with an appropriate reporter gene in small plasmid vectors, followed by transient transfection studies in eukaryotic cells. These approaches have been helpful in identifying promoters and other regulatory elements, but difficult to clarify the regulation of gene expression *in vivo* under a variety of physiologically relevant conditions.

The development of the bacterial artificial chromosome (BAC) cloning systems (235) overcame many of the limitations of other large plasmid, such as YAC.. The BAC vectors are circular plasmid DNA molecules that are derived from the F factor from *Escherichia coli* and normally existed as single copy plasmids in bacterial cells. The very low levels of rearrangement and the high stability of BAC clones in recombination deficient strains of *E.coli*, together with the ease of isolation of large quantities of DNA from bacterial cultures, made BAC clones the systems of choice for gene mapping, characterization and sequencing. The large insert size of BAC clones (up to 300 kb) enabled to contain most human genes together with all their flanking regulatory sequences in single DNA fragments.

3.13.1 Modification of BAC clones

Unfortunately, the large size of BAC vectors makes the conventional cloning methods that rely on the occurrence of unique restriction sites unsuitable for the BAC modification. The developed modification methods are depended mainly on the use of homologous recombination systems derived from bacteria or λ bacteriophage which are normally absent in host cells. This requires either the transfer of the target BAC to a suitable bacterial strain containing the specific recombination machinery. The BAC engineering technique involves the *Red* bacteriophage genes *exo (or RedA), bet (or Redβ),* and *gam* function. The *exo* gene product has 5'-4' exonuclease activity and *bet* gene product is a single strand DNA binding protein that promotes annealing, and

gam gene product inhibits the *recBCD* nuclease from degrading linear DNA fragments (Figure 14) (236). *Exo* and *bet* are equivalent to RecE and RecT in the original ET recombination prophage, respectively. An *E.coli* strain, DY380, harboring a defective prophage carrying these genes under the control of a temperature sensitive repressor, was integrated into the genome of an *E. Coli* strain (237, 238). Following the transfer of BAC DNA into this strain, recombination machinery can be transiently supplied by shifting the cultures to 42°C for a short period of time. The recombination occurs through homology regions between the two molecules that recombine.The length of homology required for efficient recombination is 30-50 bases. The homology arms can be easily added to the DNA fragment by PCR.

3.13.2 Epstein-Barr virus and EBV-based vectors

The Epstein-Barr virus (EBV) is a γ-herpesvirus that was first described in 1964 following its discovery in cells cultured from a B-cell-derived tumour, termed Burkitt's lymphoma. Its genome exists episomally in latently infected cells, where expression of only one viral protein, the Epstein-Barr Nuclear Antigen-1 (EBNA1), is required for its persistence.EBV-based vectors are plasmids that have been engineered to carry the EBNA1 and *oriP* sequences derived from EBV. EBNA1 and *oriP* act together to facilitate both the replication and episomal persistence of either the EBV genome or EBV-based plasmid vector in the nucleus of target cells (239, 240).EBNA1 contains a DNA (*oriP*)-binding which enables the protein to bind to binding site within the *oriP* sequence. The binding facilitates the episomal maintenance and segregation of EBV-based plasmid vectors in dividing cells, by binding to cellular chromosomes and enabling the tethering of *oriP*-bearing plasmids to sister chromatids (241).

EBV-based vectors carrying only EBNA1 and *oriP* sequences have not been shown to produce infectious particles.

3.13.3 K562-EBNA1 cells (KEB cells)

EBV-plasmids can be shuttled between bacterial and human cells. A number of previous studies have reported significant increases in the transfection efficiency of EBV-based plasmids following delivery into human cell lines that constitutively

express EBNA1 (242).Therefore, in order to improve the transfection of the BACs in human cells, the K562 erythroleukemia cell line constitutively expresses the EBNA1 protein was developed. Subsequently, transfection efficiency was shown to be improved by up to 10-fold following the transfection of the BAC construct into KEB cells, comparing with the native K562 cells (243). However, the mechanism by which EBNA1 facilitates the transfection of EBV-based vectors is remained to be investigated.

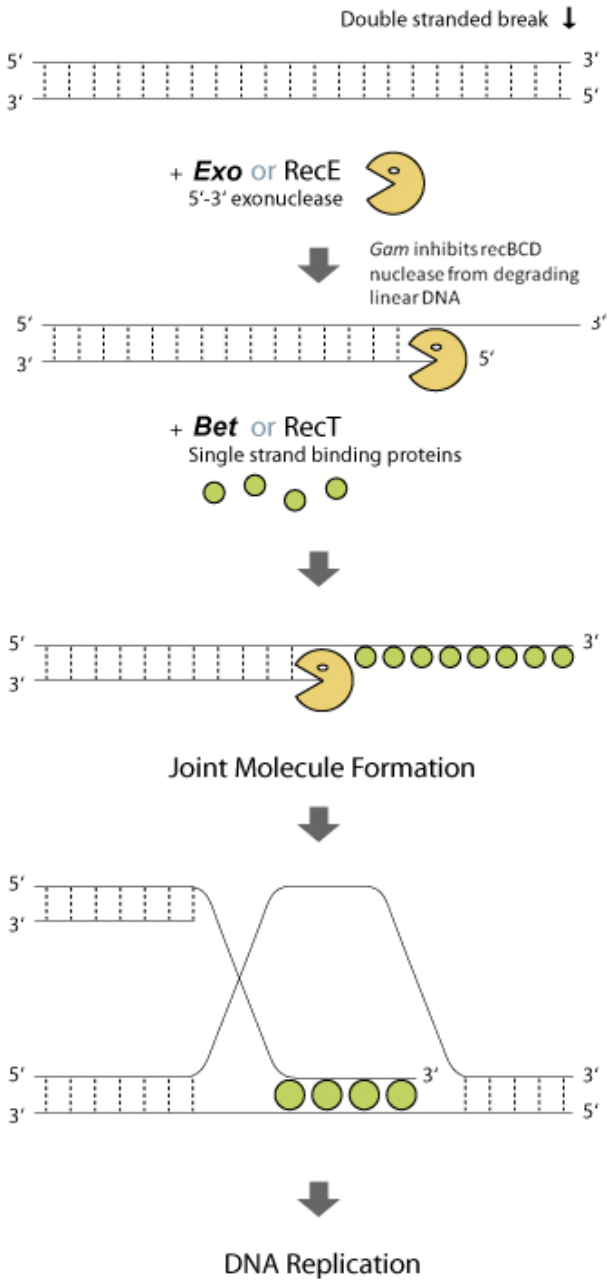


Figure 14: Schematic representation of the bacteriophage λ recombination system (Image modified from Gene Bridges GmbH and (244))

Exo has 5’ to 3’ exonuclease activity resulting in 3’ overhangs. *Bet can* bind single-stranded DNA and promote annealing while the *gam* gene product inhibits recBCD nuclease from degrading linear DNA fragment. These machineries facilitate recombination of the linear DNA fragment into the target.

CHAPTER IV
MATERIALS

4.1 Bacterial Culture

4.1.1 Bacterial strain

4.1.1.1 *E. coli* strain DH10B

The BACs and small plasmids were routinely maintained and propagated in the *E. coli* strain DH10B (F^- *mrc*A Δ*(mrr-hsd*RMS-*mcr*BC)Φ80d*lacZ* ΔM15 Δ*lac*X74 *end*A1 *rec*A1 *deoR* Δ*(ara, leu)*7697 *araD139 galU galK nupG rpsL* λ^-*)* (Invitrogen). DH10B cultures were normally grown overnight at 37°C in an orbital shaker at 220 rpm in Luria-Bertani (LB) liquid culture or on LB agar (1.5%) plates supplemented with antibiotics as required (ampicillin (100 μ g/ml), kanamycin (25 μ g/ml) or chloramphenicol (12.5 μ g/ml) (Table 4).

4.1.1.2 E.coli strain DY380

Homologous recombination studies were performed in the *E. coli* strain DY380. This modified DH10B strain harbours a defective λ-prophage carrying the *red* genes under the tight control of the temperature-sensitive λ-cI857 repressor (237). Incubation of DY380 cells at 42°C results in the inactivation of the temperature-sensitive λ repressor, and the production of the exo (α), bet (β) and gam (γ) proteins enabling recombination. DY380 cultures were grown overnight at 30°C in LB liquid culture supplemented with Tetracyclin antibiotic at 100 μg/ml.

4.1.2 Bacterial growth media

LB liquid culture and LB agar plates used to grow *E. Coli* DH10B and DY380 cells carrying BACs and small plasmids were prepared as shown in Table 3. SOC medium was used to incubate bacterial cells after electroporation or TSS transformation.

4.1.3 Antibiotics

Antibiotics were supplemented into the bacterial growth media at the following concentrations shown in Table 4.

Table 3: Bacterial growth media

Media	**Components**
LB medium	1% (w/v) bacto-tryptone, 0.5% bacto-yeast extract, 1% (w/v) NaCl
LB agar	1.5% (w/v) of bacto-agar in LB medium
SOB medium	2% (w/v) bacto-tryptone, 0.5% bacto-yeast extract, 0.05% (w/v) NaCl, 10mM $MgCl_2$
SOC medium	20mM glucose in SOB medium
Freezing medium	50% LB broth, 50% glycerol

Table 4: Antibiotics used in the homologous recombination experiments

Antibiotics	Stock concentration	Solvent	Final concentration
Chloramphenicol (Cm)	12.5 mg/ml	dH_20	12.5μg/ml
Ampicillin (Amp)	100 mg/ml	100% ethanol	100 μg/ml
Kanamycin (Kan)	25 μg/ml	dH_20	25 μg/ml
Tetracyclin (Tet)	100 mg/ml	dH_20	100 μg/ml

4.2 Plasmids

4.2.1 pEGFP-N22

The pEGFP-N22 plasmid was derived from pEGFP-N2 vector (Clontech, Palo Alto, CA) by removing the multicloning site located at the 5'end of the EGFP gene using *Bgl*II and *Bam*HI double digest, and by blunting the *Not*I site at downstream of the EGFP gene. This 4693bp construct was used as a control plasmid in mammalian cell transfection. (Figure 15)

4.2.2 pEBAC160G

The pEBAC160G plasmid was generated from pEBAC140 (Figure 3) by the insertion of a modified pUC19 sequence into the multicloning site, and by the blunt end cloning of an AflIII-AflII fragment from pEGFP-N22 into the Bst1107I site (243). (Figure 16)

4.2.3 pEBAC/148β

The pEBAC/148β is the second generation of the BAC clone containing the entire β-globin locus (about 73 kb), as well as additional sequences at both the 5' and3' ends (245). This plasmid was generated from PAC/148β, a PAC clone isolated from the RPCI 2 human total genomic PAC library (246). The 185 kb PAC/148β genomic insert was isolated as a single *Not*I fragment and cloned into the *Not*I site of pEBAC140, to generate pEBAC/148β. The hygromycin (Hyg^R) and thymidine kinase (*tk*) genes enable selection in eukaryotic cells, while the *oriP* and *EBNA-1* genes from Epstein-Barr virus facilitate episomal maintenance (Figure 17)

4.2.4 pEBAC/148βG

The pEBAC148βG vector was generated by retrofitting the 185-kb globin genomic fragment into the pEBAC 160G vector. The expression of EGFP, driven by the CMV promoter, from the pEBAC 160G backbone made this 205 kb vector ideal for monitor transfection efficiency of large BACs following transfection of mammalian cells (Figure 16).

4.2.5 pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP

The pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP is an EGFP-modified pEBAC/148β construct (243, 247). The 2.7 kb EGFP-Neo/Kan cassette was amplified from pEGFP-N22 by PCR. The EGFP-Neo/Kan PCR product, designed to be flanked by 50 nt of pEBAC/148β homology, was introduced into the pEBAC/148β via homologous recombination. The 5' end of the EGFP-Neo/Kan expression cassette was placed in frame at the start codon of the $^{G}\gamma$-globin genes, while the 3' end of the EGFP-Neo/Kan expression cassette was inserted at the termination codon of the $^{A}\gamma$-globin gene, resulting in the replacement of the sequence from the $^{G}\gamma$- to $^{A}\gamma$- globin genes by the EGFP-Neo/Kan cassette.

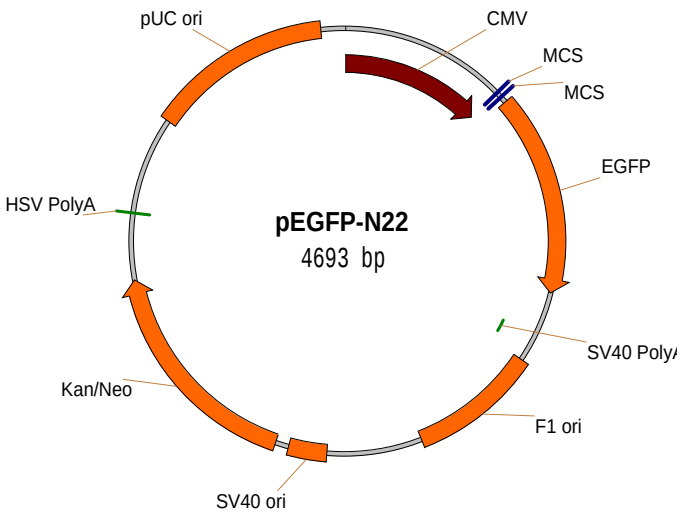


Figure 15: Map showing the key features of pEGFP-N22

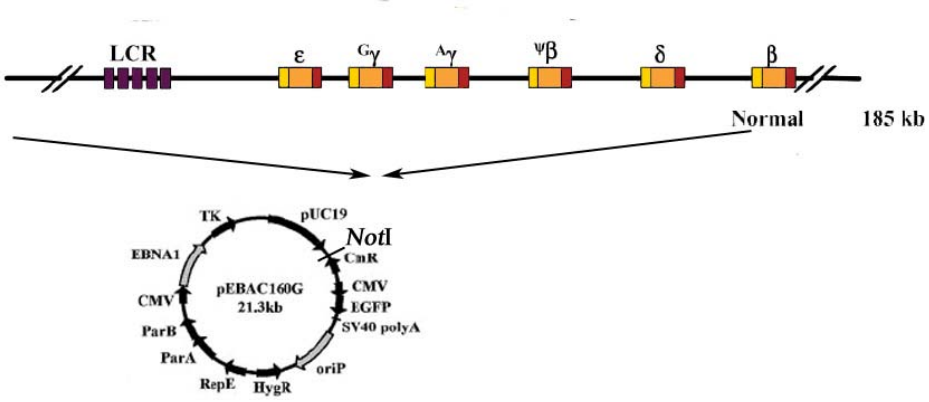


Figure 16: Map showing the key features of the pEBAC160G and pEBAC148βG

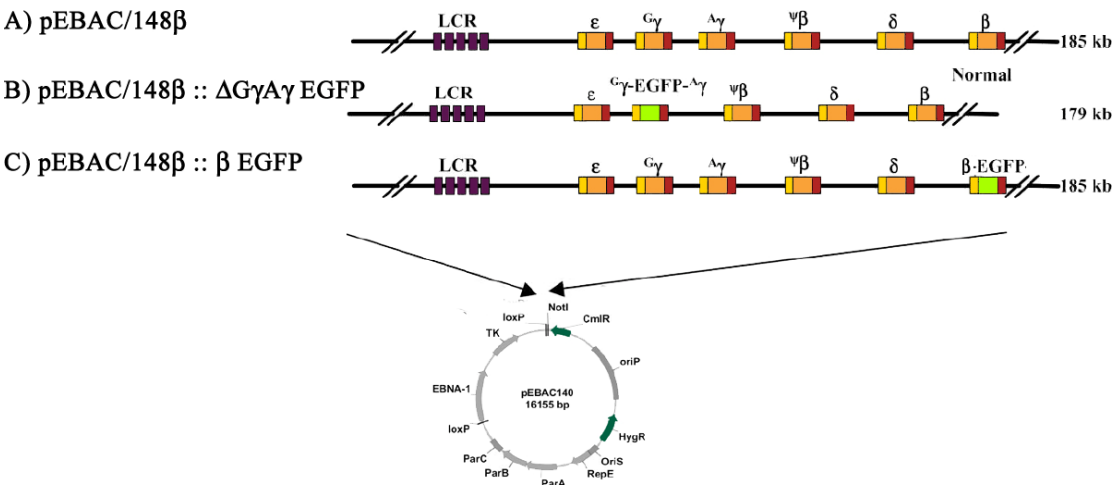


Figure 17: Map showing the key features of the pEBAC/148β and derivatives

Each fragment represents the β-globin locus genomic insertion into the pEBAC160

A) Normal β-globin locus genomic insertion of pEBAC/148β

B) EGFP replaced GγAγ-globin gene genomic insertion of pEBAC/148β :: ΔGγAγEGFP

C) EGFP replace β-globin gene genomic insertion of pEBAC/148β :: βEGFP

4.3 PCR Primers

PCR primers were purchased from Invitrogen (Melbourne, Australia). The primers sequences used in this study are shown in Table 5.

Table 5: Primer sequences

Primers for HPFH recombination

Primer	**Sequence (5'→3') / description**
s175WF	CCCTTCCCCACACTATCTCAATGCACAT
	ARMS-PCR forward primer for wild type -175HPFH (underlined) at human γ–globin 5' promoter with additional mismatch at 3rd base from 3' end.
s175MF	CCCTTCCCCACACTATCTCAATGCACAC
	ARMS-PCR forward primer for (T→C) -175HPFH (underlined) at human γ–globin 5' promoter with additional mismatch at 3rd base from 3' end.
GProR	GGAACTGCTGAAGGGTGCTTCCTTTTATTC
	ARMS-PCR Rev primer for -175 HPFH screening, bind at human γ–globin 5' promoter
175mutF	AAAATTAAGCAGCAGTATCCTCTTGGGGGCCCCTTCCCCAC ACTATCTCAATGCAAACATCTGTCTGAAACGGTC
	Forward primer with -175HPFH point mutation at human γ–globin 5' promoter for amplification of EGFP-Kan recombination cassette
Givs1R	TTGATAACCTCAGACGTTCCAGAAGCGAGT
	Rev primer located at 5'UTR of human γ-globin
G-EGFP-R	TCTCCTACACCATTTACTCCCACTTGCAGA
	Rev primer at human ^{A}γ–globin 3'promoter for amplification of EGFP-Kan recombination cassette
sEGFP-F	CTATATCATGGCCGACAAGCAGAAG
	Forward primer for screening recombination at 3'junction of EGFP-Kan cassette. Binds in the EGFP coding region

Primer	**Sequence (5'→3') / description**
sEGFP-R	TGATCTCACAGTGCTGGTCTGTTTC
	Rev primer for screening recombination at 3'junction of EGFP-Kan cassette, bind at the human $^{A}\gamma$–globin 3' promoter globin
GProSEQ2	ACTGGAGCTACAGACAAGAAGGTGAAA
	Forward primer for amplifying $^{G}\gamma$ 3' promoter region sequencing fragment, binds at the human $^{G}\gamma$–globin promoter
GPspecF	AAAGGCTATAAAAAAAATTAAGCA
	Forward primer binds specifically at-241 of the human $^{G}\gamma$-globin promoter (last 4 bases are $^{G}\gamma$-globin specific)

Primers for genotyping of transgenic mice

Primer	**Sequence (5'→3') / description**
EGFP-Probe_F	CCCTTCCCCACACTATCTCAATGCACAT
	Forward primer for EGFP genotyping, bind at EGFP coding region.
EGFP-Probe_R	CCCTTCCCCACACTATCTCAATGCACAC
	Reverse primer for EGFP genotyping, bind at EGFP coding region.
APRT5'	ATGGTGAGCAAGGGCGAGGAGCTGTT
	Forward primer for mouse internal control in multiplex PCR with EGFP-probe primers, binds at mouse adenine phosphoribosyltransferase gene.
APRT3'	ACAGGCCGCAAACATGGCTC
	Reverse primer for mouse internal control in multiplex PCR with EGFP-probe primers, bind at mouse adenine phosphoribosyltransferase gene.
LUG Fwd	GTCTGTTTCCCATTCTAAACTGTA
	Forward primer for human β-globin genotyping, binds at human β-globin coding region.

Primer	**Sequence (5'→3') / description**
LUG Rev	ACAAGACAGGTTTAAGGAGACCA
	Reverse primer for human β-globin genotyping, binds at human β-globin coding region.
mouseβ-Fw	TGAGAAGGCTGCTGTCTCTTG
	Forward primer for mouse internal control in multiplex PCR with LUG primers, binds at mouse β-globin gene.
mouseβ-Rev	CAGAGGATAGGTCTCCAAAGCTA
	Reverse primer for mouse internal control in multiplex PCR with LUG primers, binds at mouse β-globin gene.
human-γ- FW	GACCGTTTTGGCAATCCATTTC
	Forward primer binding at human γ-globin gene
human-γ-Rev	GTATTGCTTGCAGAATAAAGCC
	Reverse primer binding at human γ-globin gene

Primers for real-time RT-PCR

Primer	Sequence (5'→3') / description
hu_cDNA_BetaGlob2_F	TGGATCCTGAGAACTTCAGGCT
	Forward primer for human β-globin cDNA real-time PCR
hu_cDNA_BetaGlob2_R	GCACCACTTTCTGATAGGCA
	Reverse primer for human β-globin cDNA real-time PCR
h_gammaG_cDNA_F	AAGGCTCCTGGTGTCTACCCA
	Forward primer for human ^{G}γ-globin cDNA real-time PCR
h_gammaG_cDNA_R	TCAGCACCTTCTTGCCATGTG
	Reverse primer for human ^{G}γ-globin cDNA real-time PCR

Primer	Sequence (5'→3') / description
hu_cDNA_betaACTIN_F	CCAGCTTAACGGTATTTGGAGG
	Forward primer for human β-actin cDNA real-time PCR,as internal control
hu_cDNA_betaACTIN_R	TCGCCCACATAGGAATCCTT
	Reverse primer for human β-actin cDNA real-time PCR as internal control

4.4 Mammalian Cell Culture

4.4.1 Cell lines

4.4.1.1 Human erythroleukemia K562 cell line

K562 cell line (209) was provided by CAGT group, MCRI, Australia. The K562 cells are originally derived from a patient with a chronic myelogenous leukemia. These cells display an early embryonic erythroid phenotype with the embryonic ε β-like chains and ζ α-like chains being the predominant globin chains expressed with relatively smaller amounts of fetal hemoglobin. These characteristics as well as the ability to indefinitely grow in culture have made the K562 cell line one of the best characterized and utilized human erythroid cell lines.

4.4.1.2 KEB Cells

The KEB cells are K562 cells constitutively expressing the Epstein-Barr Nuclear Antigen-1 (EBNA1) protein. The KEB cell line was generated by transfection of an EBNA1/Neo expression cassette in the K562 cells (248). KEB cells were provided by CAGT group, MCRI, Australia

4.4.1.3 K562 :: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP

This cell line was generated by the stable transfection of is KEB cells with linearized pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP construct. This cell line will be referred to as the fluorescence based cellular genomic reporter assay (GRA), with the green

fluorescence protein gene placed under the control of the γ-globin promoter in the intact human β-globin locus. This cell line has been proved to respond to known HbF inducers as measured by an increase EGFP expression using flow cytometry analysis (214). The cell line was provided by CAGT group, MCRI, Australia

4.4.2 Culture media

Table 6 and 7 summarize the various reagents and additives used in the routine maintenance and culture of the mammalian cell lines used in this study..

Table 6: Cell culture reagents

Reagents/ Buffer	**Components/ Source**
PBS	26.67 mM KCl, 14.71 mM KH_2PO_4, 1.38 M NaCl and 80.60 mM Na_2HPO_4 (GIBCO-Invitrogen, Grand Island, NY, USA)
RPMI-1640 containing L-glutamine	GIBCO-Invitrogen (Grand Island, NY, USA)
Dulbecco modified Eagle's Media (DMEM)	GIBCO-Invitrogen (Grand Island, NY, USA)
Heat-inactivated fetal calf serum (FCS)	GIBCO-Invitrogen (Grand Island, NY, USA)
10,000 U/ml of penicillin streptomycin solution	GIBCO-Invitrogen (Grand Island, NY, USA)
L-glutamine	GIBCO-Invitrogen (Grand Island, NY, USA)
Trypan blue solution	0.4% trypan blue, 0.8% NaCl and 0.06% KPO_4
Bovine Serum Albumin (BSA)	Stem Cell Technologies Inc (Vancouver, BC, Canada)
Hygromycin B solution	GIBCO-Invitrogen (Grand Island, NY, USA)
G-418,Geneticin solution	GIBCO-Invitrogen (Grand Island, NY, USA)

Table 7: Mammalian cell culture media

Cell type	Media Components
K562 cells	DMEM or RPMI-1640 media containing 10% FCS, 100 U/ml penicillin, and 100 mg/ml streptomycin
KEB cells	DMEM or RPMI-1640 media containing 10% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin and 400 μg/ml G-418
KEB :: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP cells	DMEM or RPMI-1640 media containing 20% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin and supplemented with an antioxidant mix (1mM sodium pyruvate, 50 mM athioglycerol and 20 nM bathocuprionedisulfonate)
Transfection media	Opti-MEM containing hygromycin as indicated in the protocol
KEB cells transfected with pEBAC	DMEM containing 20% FCS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 200 μg/ml of hygromycin selection
Mouse tissue fibroblast	DMEM or RPMI-1640 media supplemented with 10% FCS

4.5 Human Erythroid Progenitor Culture

4.5.1 Peripheral Blood

β-thalassemia/HbE patients recruited in this study exhibited both mild and severe clinical symptoms. Informed consent forms were completed by all participants. Peripheral blood (20 ml or 50 ml) were collected from β-thalassemia/HbE patients or normal volunteers respectively. Sodium heparin was used as an anticoagulant. Since the diagnosis of β-thalassemia/HbE is based on clinical manifestation, red cell indices and Hb typing, complete blood counts and red blood cell indices were obtained by an automated blood cell counter (ADVIA120, Bayer Diagnostic, Tarrytown, NY, USA.).

Hemoglobin analysis was performed and quantified by an automated HPLC (VARIANT ^{TM}; Bio-Rad Laboratories, Hercules, CA, USA). Multiplex Gap-PCR and allele specific PCR were used to screen and exclude participants with α-thalassaemia traits (249, 250).

4.5.2 CD34+ cell selection and erythroid cell culture

CD34+ Human erythroid progenitor cells were isolated from participants' blood. Table 8 summarizes the reagents, instruments, and culture media and buffers utilized for the isolation and culturing of these cells.

Table 8: Reagents and instruments for CD34+ cell selection and cell culture

Reagents/ Buffer	Components/ Source
PBS	26.67 mM KCl, 14.71 mM KH_2PO_4, 1.38 M NaCl and 80.60 mM Na_2HPO_4 (GIBCO-Invitrogen, Grand Island, NY, USA)
20% Human serum albumin (HSA)	(Kedrion, Gallicano, Italy)
CD 34+Selection Buffer	PBS containing 10% Human serum albumin (HSA), and 2mM EDTA
Lymphoprep	density 1.077±0.001 g/mL (Axis-Shield Poc AS, Norway)
Red Cell Lysis Buffer	0.16 M NH_3Cl, 10 mM $KHCO_3$, 5 mM EDTA
Anti-CD34 Immunomagnetic bead	(Miltenyi Biotech, Auburn, CA, USA)
LS separation column	(Miltenyi Biotech, Auburn, CA, USA)
Multi stand	(Miltenyi Biotech, Auburn, CA, USA)
Iscove's modified Dulbecco's medium (IMDM)	(GIBCO-Invitrogen, Grand Island, NY, USA)
rhEPO	(EPREX, Brussel, Belgium)
rhIL-3	(Kirin-Brewery, Tokyo, Japan)
GM-CSF	(Promokine, Heidelberg, Germany)
CD34+ day0 culture media	IMDM supplemented with 15% FCS, 15% human AB serum, 2 U/ml erythropoietin (EPO), 20 ng/ml stem cell factor (SCF), 10 ng/ml interleukin(IL)-3, 100 U/ml penicillin, and 100 mg/ml streptomycin
CD34+ day≥3 culture media	IMDM supplemented with 15% FCS, 15% human AB serum, 2 U/ml erythropoietin (EPO), 20 ng/ml stem cell factor (SCF), 100 U/ml penicillin, and 100 mg/ml streptomycin

4.6 Compounds Tested for HbF Induction

A number of compounds were assayed for their potential to induce γ-globin gene expression in the GRA. The list of compounds as well as the diluents used to prepare the solutions are listed in Table 9. Following their preparation all stock solutions were kept in the dark at 4 °C.

Table 9: Compound for HbF induction

Agents	**Source**	**Solvent for stock solution**	**Stock Concentration**
Butyrate	Sigma (St. Louis, MO)	ddH_2O	
Cisplatin	Sigma (St. Louis, MO)	ddH_2O	3.3mM
Hemin	Sigma (St. Louis, MO)	NaOH/Tris base/BSA	5mM
Hydroxyurea (HU)	Sigma (St. Louis, MO)	ddH_2O	50 mM

4.6.1 Hemin

Hemin (Sigma, St Louis, MO) 5 mM stock solution was prepared by dissolving 32.5mg bovine hemin in 0.5ml 1M NaOH for 30 min and mixed with 0.5 ml of 0.5M Tris base, followed by 10 ml of 10% BSA. The mixture was neutralized by addition of 0.5 ml of 1 M HCl. The pH was verified and then filtered through a 0.45 μm filter. The solution was stored at 4°C for no more than 1 week prior to use (251).

4.6.2 Compound library

The GRA was utilized for a high throughput screen of a MicroSource Discovery Systems, Inc. (Gaylordsville, CT) chemical compound library. This 2000 compounds library from their Spectrum collection includes compounds of a wide range of biological activities and structural diversity. Approximately 50% of the library consists of drugs that have been introduced into the United States, Europe and Japan. 30% of the library is natural products with unknown biological properties. The remaining 20% of the compounds are other bioactive representatives such as non-drug

enzyme inhibitors, receptor blockers, membrane active compounds, and cellular toxins that have not reached development or are toxic.

4.6.3 DNA binding drugs

Tallimustine, G2, G3 and G4 DNA binding drugs was kindly provided by Prof. Roberto Gambari, Department of Biochemistry and Molecular Biology, Via Luigi Borsari, Italy. Compounds were prepared as previously reported(127).

4.6.4 Wheat grass extract

Wheat grass extract, and fractions were provided by Dr. R.K. Marwaha. The extract was prepared according to the previous report(170).

4.6.5 Thai Natural compound

Thai natural compounds and their derivatives were kindly provided by Prof. Apichart Suksamran, Department of Chemistry, Faculty of Science, Ramkhamhaeng University, Bangkok, 10240, Thailand. Compounds were dissolved in 100% DMSO into final concentration of 2 mg/ml.

4.7 Mouse Strain

4.7.1 C57BL/6 mice

The murine C57BL/6J strain is the most widely used inbred strain. They are used in a wide variety of research areas, and commonly in the production of transgenic mice. This is because C57BL/6J mice breed well, are long-lived, and have a low susceptibility to tumors. These mice were maintained by the Disease Model Unit at the MCRI, Melbourne, Australia. Fertilized oocytes of the C57BL/6J mouse strain were also used for microinjection to generate transgenic mice maintained on a C57BL/6J background.

CHAPTER V
METHODS

5.1 Construction of -175 HPFH pEBAC::ΔGγAγ EGFP by Homologous Recombination System

All plasmids were maintained and propagated in the *E.coli* strain DH10B. Bacterial strains were cultured in LB broth or on LB agar and grown in a 30°C or 37°C for DY380 strain or DH10B strain respectively for 12–16 hr. The medium was supplemented with antibiotic as previously described. All strains were stored at –70°C in sterile 1:1 LB/glycerol solution.

5.1.1 Small scale alkaline lysis BAC DNA extraction

Bacteria carrying the BAC of interest were cultured in 5ml of LB in the presence of appropriate antibiotic selection overnight in a 30°C or 37°C orbital shaking incubator. The culture was aliquoted into eppendorf tubes and cells harvested by centrifugation at 13,000 rpm for 30 sec. The cells were resuspended in Buffer P1 (50 mM Tris (pH 8.0), 10 mM EDTA, 100 μg/ml DNase-free RNase A) and were then lysed by adding 300μl of Buffer P2 (200 mM NaOH, 1% SDS) followed by gentle inversion. Following a 5 min incubation at room temperature, 300 μl of Buffer P3 (3 M potassium acetate, pH 5.5) were added and mixed by gentle inversion then incubated on ice for 20 min. The lysate was then centrifuged at 13,000 rpm at 4°C for 10 min and the supernatant collected. Isopropanol (0.6 volume) was added to the supernatant, mixed by inversion and incubated on ice for 5 min. The precipitated DNA was collected by centrifugation at 13,000 rpm for 10 min at 4°C. The DNA pellet was with 70% ethanol by centrifugation at 13,000 rpm for 5 min at 4°C. The washed pellet was air dried then resuspended in 20-30 μl of 0.5X TE buffer and stored at 4°C or -20°C until use.

5.1.2 Large scale BAC DNA extraction

BAC DNA extraction was also performed using the alkaline lysis method with minor alteration from the small-scale BAC DNA extraction protocol to improve the yield and purity of DNA. *E. coli* DH10B or DY380 cells carrying the required BAC were grown in 1 liter of LB medium (4 x 250 ml in sterile 2-liter flasks) containing appropriate antibiotic selections and incubated at 37°C or 30°C respectively for 12-14 hrs. The cells were harvested by centrifugation at 4000 rpm at 4°C for 10 min. After discarding the culture medium, the cells were resuspended in 30 ml of Buffer P1 (50mM Tris (pH 8.0), 10mM EDTA, 100 μg/ml DNase-free RNase A) per 250 ml of culture. Cells lysis was performed by adding 30 ml of Buffer P2 (200 mM NaOH, 1% SDS) and gently mixed by inversion and incubated at room temperature for 30 min. The lysis solution was precipitated by addition of 30 ml of Buffer P3 (3 M potassium acetate, pH 5.5), gently mixed by inversion and incubated on ice for 30 min. The precipitated solution was centrifuged for 30 min at 13000 rpm at 4°C and the supernatant collected. Isopropanol (0.6 volume) was added and gently mixed by inversion and incubated on ice for 30 min. The BAC DNA pellet was recovered by centrifugation at 13,000 rpm for 30 min at 4°C. The DNA pellet was washed twice with 10 ml of 70% ethanol by centrifugation at 13,000 rpm for 15 min at 4°C. The pellet was air dried for 5 min at room temperature then resuspended in 2 ml of 1x TE buffer. In order to obtain high purity BAC DNA for microinjection, BAC DNA was then purified by cesium chloride (CsCl) ultracentrifugation method.

5.1.3 Purification of BAC DNA by CsCl ultracentrifugation

This method is suitable for large scale purification of plasmids >10 kb. BAC DNA extracted from 1 liter of *E.coli* culture was resuspended in 2ml of 1x TE buffer. CsCl was added to the DNA solution at a ratio of 1.1 g CsCl: 1ml DNA solution. Ethidium bromide was then added to the DNA/CsCl mixture at a ratio of 100 μl of 10 mg/ml ethidium bromide per 2.6 ml DNA solution. The solution was transferred to a 5 ml ultracentrifugation tube and topped up with CsCl/TE solution (1.1 g CsCl / ml) and heat sealed. The samples were centrifuged at 80,000 rpm for 16 hr at 14°C in a

Beckman Coulter ultracentrifuge. The resulting plasmid band (lower band) was extracted using a 20-gauge needle and 1 ml syringe and washed 5 times in saturated NaCl/TE/isopropanol to remove the ethidium bromide. The BAC DNA was then dialyzed overnight using Slide-A-Lyzer 7K MWCO Dialysis Cassettes (Thermo Fisher Scientific, Rockford, USA) in 3 L of TE solution at 4°C to remove CsCl. BAC DNA was quantitated using eppendorf Bio Photometer 6131. The quality of BAC DNA is evaluated by digesting a 10µl aliquot of DNA solution with *Not*I and *Xho*I and run on EFGE together with undigested BAC DNA and a Low Range PFG Marker (New England Biolabs, MA, USA). The purified BAC DNA was stored at 4°C until use.

Notes: a) Avoid disturbing or shaking tube while taking out from the centrifuge as these can disturb the chromosomal and BAC DNA bands; b) Avoid freeze-thawing of the BAC DNA solution, since it can lead to the low quality DNA resulting from DNA shearing.

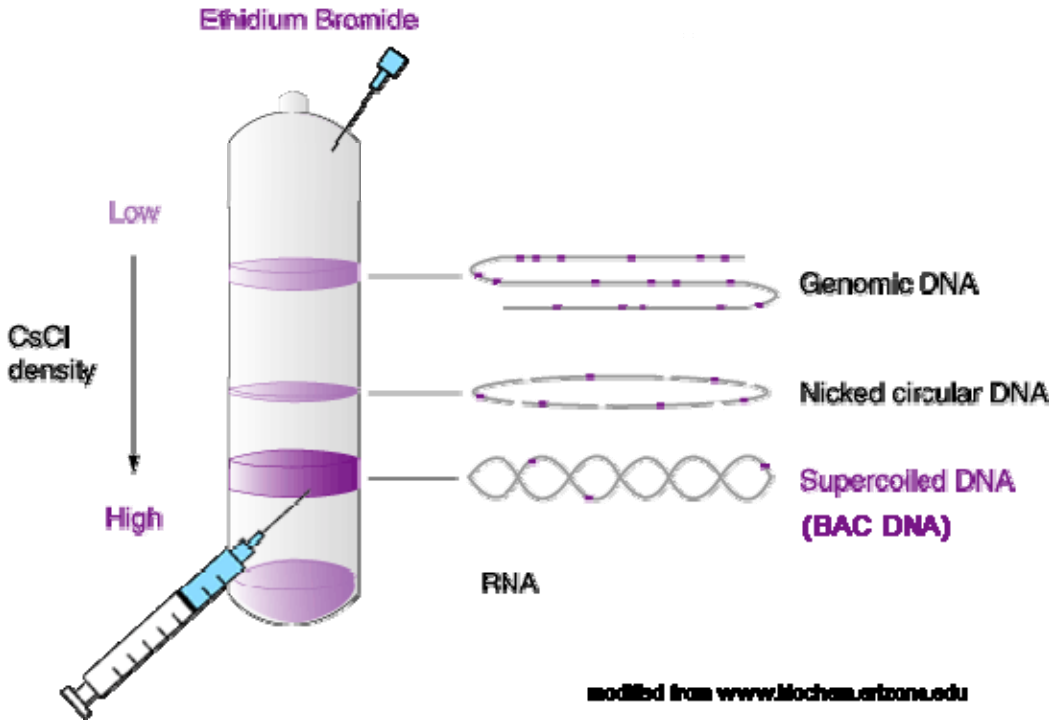


Figure 18: Collecting BAC DNA from a CsCl gradient

The upper band is composed of bacterial chromosomal DNA and the lower band is composed of the supercoiled BAC plasmid. (Image modified from www.biochem.arizona.edu)

5.1.4 TSS method for transformation of E.*coli*

The TSS (transformation and storage) method was used for the introduction of large plasmids into the DY380 strain of E.coli. A single colony of *DY380* cells was cultured in 5 ml LB medium for 16 hrs at 30°C in an orbital shaking incubator at 200 rpm. 10 ml of LB were inoculated with 0.2 ml culture and incubated at 30°C until optical density at 600 nm is between 0.5 and 0.6. Cells were harvested by centrifugation at 5000 g at 4°C for 10 min. The pellet was resuspended in 1 ml TSS solution (1X LB medium, 10 %(w/v) PEG3350, 5% (v/v) DMSO, 20mM $MgCl_2$, 20mM $MgSO_4$, pH 6.5). After mixing, cells were incubated on ice for 5 min before the addition of 100 µl sterile 100 mM KCl, 30 mM $CaCl_2$, 50 mM $MgCl_2$ solution containing 1 µg of CsCl purified BAC DNA. The bacterial suspension was then mixed thoroughly and placed on ice for 20 min followed by 20 min incubation at room temperature. The mixture was then immediately added to 0.8 ml of LB medium and placed in a 30°C orbital shaker for 1 h. The cell suspension was then centrifuged at 13,000 rpm for 30 sec, resuspended in 1 ml of LB and spread on agar plates containing the appropriate antibiotic**.**

5.1.5 Single step non-counterselection homologous recombination of -175 HPFH $^{G}\gamma$ denatured PCR fragment on the pEBAC 148b:: $\Delta^{G}\gamma^{A}\gamma$EGFP

5.1.5.1 PCR reaction

Sense and antisense 75-mer oligonucleotides were designed to target the modified -175 HPFH on the $^{G}\gamma$ promoter of the pEBAC 148β:: $\Delta\,^{G}\gamma^{A}\gamma$ EGFP (T→C at the base -175 of $^{G}\gamma$ gene). There is no addition of selection gene into the construct. The 265 bp of this -175HPFH $^{G}\gamma$ promoter was amplified using sense oligo: 175mutF (5'- AAA ATT AAG CAG CAG TAT CCT CTT GGG GGC CCC TTC CCC ACA CTA TCT CAA TGC AAA CAT CTG TCT GAA ACG GTC-3'); antisense oligo: Givs1R (5'- TTG ATA ACC TCA GAC GTT CCA GAA GCG AGT-3'). The underlined base corresponds to the -175HPFH point mutation. The PCRs were performed in a PTC-200 thermal cycler (MJ research). The Expand High-Fidelity PCR system (Roche), which incorporates a proofreading DNA polymerase, was used for the amplification of DNA fragments that were required for homologous

recombination, and also sequencing reactions. The 50ul PCR reaction comprised of 1X PCR buffer without $MgCl_2$, 2mM $MgCl_2$, dNTPs (200 μM of dATP, dTTP, dCTP and dGTP), 0.1μM of the oligonucleotides, 100 ng of template DNA pEBAC/148β:: $\Delta^G\gamma^A\gamma$ EGFP and 0.75 μl Expand High Fidelity enzyme mix (containing Taq polymerase). Reactions containing no template DNA were also included as a negative control. PCR reactions were performed in 50ul for 30 cycles (94°C 30 sec; 69°C 30 sec; 72°C 30 sec). The PCR products were examined by agarose gel electrophoresis for product size and PCR specificity.

5.1.5.2 Electrophoresis

The routine separation and analysis of DNA was performed followed electrophoresis on 0.8–2% (weight/volume) agarose gels. Agarose was dissolved in 0.5 x TBE buffer (1 x TBE: 45 mM Tris, 45 mM borate, 1 mM EDTA). DNA samples were mixed with 1/5th volume of loading dye (0.025% w/v bromophenol blue, 0.025% xylene cyanol, 30% w/v glycerol, 0.1 M EDTA) before loading into wells. Electrophoresis was performed at 80–110 V for 1–2h. DNA bands were visualized by staining gels in an ethidium bromide bath (0.5–1 μg/ml). Stained gels were imaged by UV transillumination using the Gene Genius Bioimaging System (Syngene). The DNA Molecular Weight Marker X (Roche Applied Science) was generally used to verify DNA size, and λ *Hind*III was used to quantitate the amount of PCR products.

5.1.5.3 Gel purification of PCR product

PCR products were purified for *E.*coli electroporations and also for sequencing reactions. DNA fragments were separated and excised following gel electrophoresis in 1% Seakem GTG agarose (Rockland, ME, USA). DNA was visualized using an UV transilluminator and recovered using a QIAquick Gel Extraction Kit according to manufacturer's specifications (Qiagen). Briefly, the DNA fragment was excised using a sterile scalpel, trimmed of excess gel and placed into a 1.5 ml Eppendorf tube. Three volumes of buffer QG was added to one volume of gel (100 mg ~ 100 μl) prior to incubation at 50°C for 10 min. The sample was applied to a QIAquick column and centrifuged for 5 min at 16,000 g. Flow through was discarded and 0.5 ml buffer QG was added to column prior to centrifugation for 2 min to remove all traces of agarose. After the addition of 0.75 ml buffer PE (wash buffer)

the column was allowed to stand for 1 min before centrifugation for a further 2 min. Flow through was discarded and column was centrifuged for an additional 1 min before placing into a clean 1.5 ml microfuge tube. The DNA was eluted by adding 30–100 μl 0.5X TE buffer to centre of column followed by 15 min incubation at room temperature and centrifugation at 16,000 g for 5 min. The DNA was quantitated and stored at -20°C until use.

5.1.5.4 Preparation of electrocompetent cells

Electrocompetent cells were prepared by inoculating 2ml of DY380 culture into 100 ml LB medium with Cm at 30°C, with shaking at 200rpm until an OD_{600}, of 0.5- 0.6 was reached. Cells were harvested in 50ml Falcon tubes and pelleted by centrifugation at 5,000 rpm for 10 min at 4°C in a Beckman Coulter JLA-10.500 rotor. Expression of *exo*, *bet* and *gam* genes was then induced by incubation for 20 min at 42°C, and then a further 20 min on ice. Cells were harvested in 50ml tubes and pelleted by centrifugation at 5,000 rpm for 10 min at 4°C, and subsequently washed four times with 50ml ice-cold 10% glycerol. The final pellet was resuspended in 480 μl of ice-cold 10% glycerol. Aliquots (30 μl) were used for electroporation immediately.

5.1.5.5 Electroporation of bacterial cells

To minimize the effects of any secondary structure, the recombination cassette was pre-heated at 95°C for 2min and cooled rapidly on ice just before use. The electrocompetent DY380 cells harboring pEBAC 148β:: ΔGγAγ EGFP were mixed with 10-100ng purified PCR product on ice. The PCR product was pre-denatured at 95°C for 2 min, and snap-frozen in dry ice/Ethanol. Electroporation was performed in a pre-chilled 0.1cm cuvette (BioRad) with a Bio-Rad Gene Pulser (BioRad Labs, Hercules, CA, USA) set at 1.8 kV, 25 μF, with a pulse controller of 200Ω. Cells were immediately diluted with 1ml SOC medium, and incubated in a shaker at 30°C for 1hr, and later spread onto LB agar plate with appropriate selective antibiotic.

5.1.5.6 Electroporation efficiency

An aliquot of the electrocompetent DY380 cells was mixed and electroporated with a standard know amount of a commercially available plasmid, e.g.

10 pg of pUC19. Cells were than diluted in 1 ml SOC media and incubated at 30°C for 1 hr before plating serial dilution of the cells onto LB agar plates containing appropriate antibiotics (Amp for pUC19). Following overnight incubation at 30°C, the number of colonies was counted. The electroporation efficiency was estimated as the number of colony forming units per 1 μg DNA electroporated (cfu/μg DNA)

5.1.5.7 Screening of recombinant clones using allele specific PCR

Screening to identify recombinant clones was performed with allele specific PCR (ARMS-PCR) on pooled bacterial colonies. Mutation specific primers were designed to include mutated base(s) at the 3' end. Screening of colonies for the -175HPFH point mutation was performed with the primers: GProR: 5'- GGA ACT GCT GAA GGG TGC TTC CTT TTA TTC-3' and s175MF (mutant): 5'- CCC TTC CCC ACA CTA TCT CAA TGC ACA C-3' (the mutated base expected in the recombinant clones is underlined) or s175WF (wild type): 5'- CCC TTC CCC ACA CTA TCT CAA TGC ACA T-3' (wild type base is underlined). PCR was performed in 50μl reactions for 30 cycles (94°C 30 sec; 70°C 30 sec; 72°C 30 sec). The PCR reaction comprised of 5 μl 10X PCR buffer without $MgCl_2$ (Roche), 2 mM $MgCl_2$, dNTPs (200 μM of dATP, dTTP, dCTP and dGTP), 0.2 μM oligonucleotides, 0.2 μl AmpliTaq DNA polymerase (Roche), 1μl of heat inactivated of overnight culture of pooled bacterial colonies. The size of expected PCR product is 200 bp.

Note: The overnight pooled bacterial cultures were heat inactivated (95°C for 5 min) prior to adding to the PCR mix. The heat denaturation improved the PCR reaction supposedly by degradation of inhibition enzymes or nucleases.

5.1.6 Counter selection homologous recombination of -175 HPFH $\Delta^G\gamma^A\gamma$ EGFP-Neo/Kan cassette on pEBAC148β

The 3.1 kb of -175 HPFH $\Delta^G\gamma^A\gamma$ EGFP cassette was amplified from the pEBAC 148β:: $\Delta^G\gamma^A\gamma$ EGFP using primers: 175mutF (5'- AAA ATT AAG CAG CAG TAT CCT CTT GGG GGC CCC TTC CCC ACA CTA TCT CAA TGC AAA CAT CTG TCT GAA ACG GTC-3') and G-EGFP-R: (5'- TCT CCT ACA CCA TTT ACT CCC ACT TGC AGA -3'). The underlined base corresponds to the -175HPFH point

mutation. The 50ul PCR reaction comprised of 1X PCR buffer without $MgCl_2$, 2mM $MgCl_2$, dNTPs (200 μM of dATP, dTTP, dCTP and dGTP), 0.1μM oligonucleotides, 100 ng template DNA 148β:: $\Delta^G\gamma^A\gamma$ EGFP and 0.75 μl Expand High Fidelity enzyme mix. PCR reactions were performed in 50ul mixture for 30 cycles (94°C 30 sec; 69°C 30 sec; 72°C 30 sec). The PCR products were examined by agarose gel electrophoresis, purified, and quantitated the same way as in the preparation of -175 HPFH $^G\gamma$ promoter oligonucleotide for single step non-counterselection recombination system**.**

5.1.6.1 Electroporation of bacterial cells

Electrocompetent DY380 cells harboring pEBAC 148β were mixed with 100 purified PCR products on ice. 10pg pUC19 was used as a electroporation efficiency control. The electroporation was performed as previously described.

5.1.6.2 Screening of recombinant clones

The colonies, which showed kanamycin resistance resulting from insertion of kanamycin gene on the -175 HPFH $\Delta^G\gamma^A\gamma$ EGFP-Neo/Kan cassettes, were picked and streak-purified on LB agar supplemented with kanamycin. The colonies from streak-purified overnight culture were then subcultured on LB broth for maintaining and small-scale BAC DNA extraction. The DNA from small scale alkaline lysis BAC DNA extraction was used for the recombinant BAC validation.

5.1.6.2.1 PCR reactions

The PCR reactions were performed across 3' junction of the homologous recombination fragment. Screening was performed with the primers: sEGFP-F: 5'- CTA TAT CAT GGC CGA CAA GCA GAA G -3' and sEGFP-R: 5'- TGA TCT CAC AGT GCT GGT CTG TTT C -3'. PCR was performed in 50μl reactions for 30 cycles (94°C 30 sec; 64°C 30 sec; 72°C 75 sec). The PCR reaction comprised 5 μl 10X PCR buffer without $MgCl_2$ (Roche), 2.5 mM $MgCl_2$, dNTPs (200 μM of dATP, dTTP, dCTP and dGTP), 0.15μM oligonucleotides, 0.2 μl AmpliTaq DNA polymerase (Roche).

5.1.6.2.2 Restriction endonuclease digestions

Restriction endonucleases were obtained from New England Biolabs or Roche. The enzymes were used according to manufacturers' recommendations.

The reaction of restriction endonuclease digestion was composed of DNA, 1x restriction endonuclease buffer, restriction endonuclease and sterile distilled water. The optimum condition for digestion was varied according to each restriction endonuclease's manufacturer.

5.1.6.2.3 Pulsed field gel electrophoresis

Pulsed field gel electrophoresis (PFGE) was used for the analysis of large DNA fragments, such as BACs and genomic inserts. Restriction analyses of BACs were carried out by a pulsed field gel electrophoresis (PFGE) (CHEF-DRII, BIO-RAD) according to the manufacturer's recommendations. BAC DNA was mixed with loading dye and loaded on 0.8% PFG agarose gels (Bio-RAD). The Low Range PFG Marker (New England Biolabs) was used to verify DNA size. Gels were run at 180 V, (6 V/cm, angle 120°, linear ramping 1–20 sec) in 0.5x TBE buffer at 14°C for 14-16 hrs. The gels were then stained in an ethidium bromide bath (0.5x TBE containing 0.5 μg/μl ethidium bromide) for 30 min. De-staining was performed by gently shaking the gel in distilled water for an additional 30 min to remove unbound ethidium bromide. The gel was then visualized and photographed by UV transillumination using the Gene Genius Bioimaging System (Syngene).

5.1.6.2.4 DNA sequencing reactions

The DNA was amplified by PCR at the sequence area of interest. The PCR products were gel purified according to the protocol mentioned above. The purified products were used in the DNA sequencing reaction, which was performed using ABI Prism Big Dye Terminator cycle sequencing. Sequencing reactions contained 100–500 ng DNA, 8 μl Big Dye sequencing mix (Amersham) and 3.2 pmol of an appropriate oligonucleotide. The total reaction volume was adjusted to 20 μl by adding distilled water. Sequencing reactions were placed into a thermocycler and subjected to 25 cycles of 10 sec at 95°C, 15 sec at 50°C and 4 min at 60°C. Reaction products were ethanol precipitated and the DNA was pelleted by centrifugation. The pellet was then washed with 70% ethanol, air dried and submitted to the Applied Genetic Diagnostics division of the Department of Pathology at the University of Melbourne. DNA sequence data was analyzed using Chromas Version 2.22 software (Technelysium).

5.1.6.3 E.coli glycerol stock

Single colony of E.*coli* strain carrying the BAC of interest was cultured overnight at an appropriate temperature in LB broth containing the required antibiotics. The overnight culture was centrifuged at 4000 rpm for 5 min. The cell pellet was resuspended in 1ml of 50% LB Broth/ 50% Glycerol. The stocks were kept at -70°C.

5.1.7 Mammalian cell culture, transfection and analysis

In order to perform functional analysis of the recombined EBAC, the constructs were transfected and analyzed in the human erythroleukemia cell line K562 and derivative KEB.

5.1.7.1 Maintenance of human erythroleukemia cell lines

K562 and KEB cells were maintained in continuous culture in the proper cell culture medium (Table 7). Cells were incubated at 37°C and subcultured every 3–4 days by adding the confluent culture to the growth medium in a ratio of 1:10 at the minimum total volume of 10 ml. The antibiotic selection was withdrawn 2 days prior to transfection, and cells were seeded at 1 x 10^5 cells/ml 12 hours prior to transfection. The cells were counted and cell viability was determined by Trypan Blue dye exclusion assay.

5.1.7.2 Human erythroleukemia cell lines lipofection

K562 and KEB cells were transfected with DMRIE-C reagent (Invitrogen) according to the manufacturer's instructions. Briefly, in a 6-well-plate, CsCl purified DNA (1–10 µg) was diluted in OptiMEM reduced serum medium (Invitrogen) to a final volume of 500 µl. 2–10 µl DMRIE-C was also diluted in OptiMEM to a final volume of 500 µl. The two solutions were mixed by swirling the plate and then incubated at room temperature for 30 min to allow formation of lipid-DNA complexes. During this period cells were washed twice with OptiMEM and resuspended to a final volume of 10^7 cells/ml. 2 ml of the cell suspension was added to each well of a 6-well plate containing the lipid-DNA complexes solution and gently mixed. Plates were then placed in a humidified CO_2 incubator at 37°C for 4 hrs. Subsequently, 2 ml of the culture media (Table 7) were added to the cells in each well after 4 hrs and 24 hrs of incubation. For the establishment of episomal maintenance

cultures, 48 hrs following lipofection 400 μg/ml of hygromycin selection or 50 μg/ml of G-418 were added to transfected cells according to the transfected construct. The selection was maintained throughout the growth of the cultures.

Note: The lipid-to-DNA ratio for each construct was optimized in order to achieve highest transfection efficiency. Generally, the ratio of 2:1 was used for large BAC such as pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP. The ratio of 4:1 was used for constructs <25 kb insize such as pEGFP-N22 which was used as a positive control.

5.1.7.3 Flow cytometry analysis

Following transfection, cells were analyzed by using a LSR II flow cytometer (Becton Dickson, Franklin Lakes, CA, USA). Briefly, cells (0.1–1 x 10^6) were washed once in PBS supplemented with 1% FBS and resuspended in a final volume of 0.5 ml. Viability assay was performed by adding propidium iodide at a final concentration of 0.25 μg/ml directly to the cell suspension prior to analysis. For the detection of EGFP reporter gene expression, analysis was performed only on viable cells. The percentage of EGFP expressing cells and the median peak fluorescence (MPF) of EGFP were measured. Data acquisition and analysis were performed using BD FACsDiva software (Becton Dickson). Rainbow Fluorescent Particles, 6.5-8.0 μm (Spherotech Inc., IL, USA) were used for flow cytometer calibration. The transfected cells were assayed two days following transfection and grown for up to fifty days in media containing 200 μg/ml hygromycin.

5.1.7.4 Fluorescent activated cell sorting (FACS)

Cells were cultured under selection until there was sufficient numbers for cell sorting were reached. The cell were harvested and suspended at 5 x 10^6 - 5 x 10^7 cell in 1 ml ice-cold PBS supplemented with 1-2% FBS. Subsequently, cell suspensions were filtered through a 100 μm nylon mesh (Becton Dickinson). Cells were sorted for 4 hours by fluorescence-activated cell sorting (MoFlow, Cytomation), and EGFP positive cells were collected. Cells transfected with pEBAC/148β without

EGFP were used as negative control. The collected EGFP positive cells were maintained in culture with selection as described earlier.

5.2 High Throughput Screening of HbF Inducing Compounds

High throughput screening was optimized and carried out at WEHI High Throughput Chemical Screening Facility, WEHI Biotechnology Centre, Bundoora, Australia.

5.2.1 Cultures condition

K562 :: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP, K562, and KEB cells were maintained as previously described in the proper medium for each cell type (Table 7). Briefly, cells were incubated at 37°C and subcultured every 3–4 days by adding the confluent culture to the growth medium in a ratio of 1:10. One day prior to treatment, cells were seeded at 5 x 10^5 cells/ml without antibiotic selection in the culture flask. Cell viability was determined by Trypan Blue dye exclusion assay.

5.2.2 Compound library

The compound library used in this screening is the 2000 compounds library from the Spectrum collection of MicroSource Discovery Systems, Inc. (Gaylordsville, CT). This library is made up of known drugs, bioactives and natural products as mentioned earlier. The libraries are stored in sealed, 384-well microtiter plates at 10°C. The compounds are stored in 100%DMSO at a concentration of 1 mg/mL.

5.2.3 Compounds treatment preparation

On the day of the treatment, the log-phase K562 :: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP and KEB cells were washed by centrifugation at 1300 rpm for 5 min at room temperature. The cells were resuspended at a concentration of 1.5 X 10^5 cells/ml in the appropriate media without selection. Then 100µl of cell suspension was dispensed into each well of a 384-well-plate (white, clear-flat-bottom plate, Greiner) (Figure 19) by the automated Multidrop 384 (Thermo Fisher Scientific) (Figure 20). Barcodes were applied to the

cell suspension plates. The 0.1μl of compounds to be tested were added into each well of the cell plate by automated 384-pin tool dispenser (CaliperLS SciClone ALH 3000 pipetting workstation, A V&P Scientific 384 Pin Tool) integrated with Zymark Twister II robotic arm plate handler with stackers and barcode reader (Caliper Life Sciences) (Figure 21 and 22). This gave the final concentration of the compounds at 10 μM, and 0.1% DMSO. The cell-compound plates were transferred to a 37°C / 5% CO_2 incubator. In the incubator, the plates were placed in another layer of humidified boxes in order to minimize evaporation and uneven O_2 distribution. 10μM Cisplatin was used as a positive control. KEB cells were also treated to eliminate the auto-fluorescence effect from the compounds themselves. The cells were incubated in the presence of the compounds for 5 days.

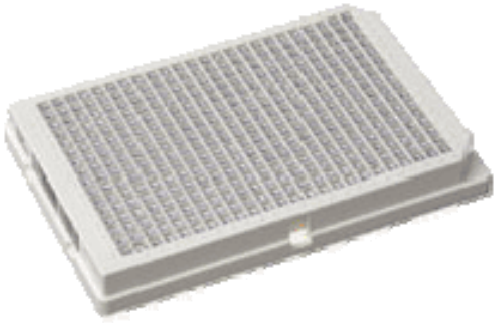


Figure 19: 384-well plate used in high throughput screening

The white-384-well plate clear flat bottom (Greiner) was used for thigh throughput screening of HbF induction compounds.

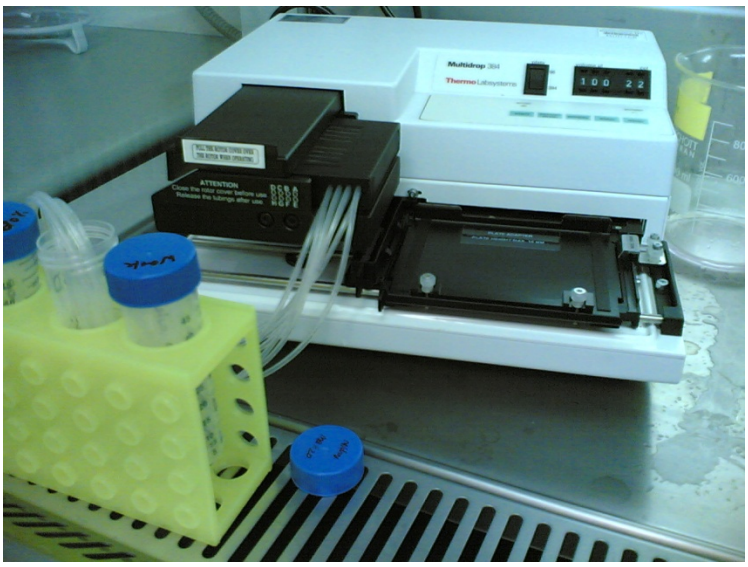


Figure 20: Multidrop 384 dispenser

Figure 21: Zymark Twister II robotic arm with stackers

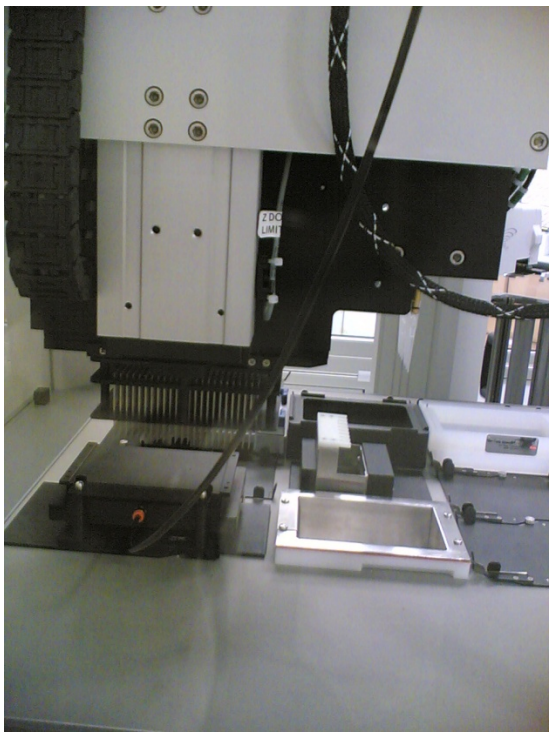


Figure 22: Automated 384-pin tool dispenser at CaliperLS SciClone ALH 3000 pipetting workstation

Figure 23: Envision Fluorescence plate reader

5.2.4 EGFP analysis

On day 5, the plates were taken for EGFP measurement. The 6 standard titrations of cell number ranging from 0-160000 cells/well were also included in each plate on the read out day. Each titration has been performed in quadruplicate. The plates were shaken prior to measurement. EGFP was measured by Packard FUSION^{TM} Universal Microplate Analyzer (Packard BioScience).

5.2.5 Viability assay

Following EGFP measurements, the viability assay was performed to normalize the EGFP value to the cell amount in each well. 5 μl of 37°C CellTiter-Blue^{TM} cell viability assay (Promega) was dispensed to each well by Multidrop 384 (Thermo Fisher Scientific) (Figure 20) onto the screening plates. The assay was incubated at 37°C for 2 hours. The plates were shaken for 10 sec, and cell-titer blue viability assay signal was recorded at fluorescence 560/590nm by top-reader Envision fluorescence microplate reader (Perkin-Elmer) with plate lid off. Data was also analysed by Fusion v3.5 software (Figure 22).

5.3 Small Scale HbF Induction

5.3.1 Cultures condition

K562 :: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP, K562 and KEB cells were maintained as previously described in high throughput section.

5.3.2 HbF inducers treatment

The cells were resuspended at 3 x 10^5 cells/ml and aliquoted in a ml aliquots per well of a 24-well plate. The compounds to be tested were prepared at 2x the final concentration, then 1 ml was added to the appropriate wells of the 24-well plate, resulting in a final volume of 2 ml/well. The plates were incubated at 37°C in a humidified 5% CO_2 incubator for 3 or 5 days. The culture medium was not changed during the induction period.

5.3.3 Flow cytometry analysis

After 3 or 5 days incubation, treated cells were harvested and analysed by flow cytometry as described earlier.The percentage of EGFP expressing cells and the median peak fluorescence (MPF) of EGFP were measured. Data acquisition and analysis were performed using BD FACsDiva software (Becton Dickson).

5.3.4 Reverse transcriptase-polymerase chain reaction (RT-PCR) and real-time quantitative RT-PCR

5.3.4.1 RNA extraction

Following 3 or 5 days of induction, the cells were harvested and RNA extracted using Tri Reagent® (Molecular Research Center) according to the manufacturer's specifications. Briefly, 5-10 x 10^6 cells were harvested without washing. Cells were homogenised in 1 ml of Tri reagent by repeatedly pipetting and incubated at room temperature for 5 min. 0.2 ml of chloroform were added and shook vigorously for 15 sec. The mixture was incubated at room temperature for 2-15 min and centrifuged at 12,000 g for 15 min at 4°C. The upper colorless aqueous phase was transferred to a fresh eppendorf tube with 0.5 ml of isopropanol, and incubated at room temperature for 5-10 min. The solution was centrifuged at 12,000 g for 8 min at 4-25 °C. The pellet was washed with 1 ml of 75% ethanol by vortexing and subsequent centrifugation at 7,500 g for 5 min at 4-25 °C. The RNA pellet was air dried for 3-5 min and dissolved in 15µl RNase-free water (Ambion). RNA concentration and purity were checked using Nanodrop1000 (Thermo Fisher Scientific). 2 µl of RNA was quantified by measuring the absorbance at 260 nm. Purity was analyzed. A_{260}/A_{280} indicated protein contaminantion if a value < 2, while A_{230}/A_{280} indicated contamination of polysaccharide or guanidine thiocyanate if a value < 2. The RNAs were confirmed by gel electrophoresis and the RNA solutions were stored at -70°C.

5.3.4.2 Reverse transcriptase-polymerase chain reaction (RT-PCR)

First-strand cDNA was systhesised by RT-PCR using SuperscriptII First-strand cDNA Synthesis system for RT-PCR (Invitrogen) according to the manufacturer's instruction. Briefly, the mixtures of RNA/primer were prepared in tubes as follows : 5 μg of total RNA, 1μl of 10 mM dNTP mix, 1 μl of $Oligo(dT)_{12-18}$ (0.5 μg/μl), and RNase-free water to total volume of 10 μl. The mixture was incubated at 65°C for 5 min, and then placed on ice for at least 1 min. Meanwhile, the reaction mixture was prepared as follows: 2 μl 10x RT buffer, 4 μl 25 mM $MgCl_2$, 2 μl 0.1 M DTT, and 1 μl RNaseOUT™ recombinant Rnase inhibitor. The 9 μl reaction mixture was added to RNA/primer mixture. The mixture was mixed gently, collected by brief centrifugation, and incubated at 42°C for 2 min. 1 μl (50 units) of SuperScript™ II RT were added, mixed and incubated at 42°C for 50 min. The reactions were terminated at 70°C for 15 min, and chilled on ice. The reaction was collected by brief centrifugation. 1μl of RNase H was added to each tube to remove RNA and incubated at 37°C for 20 min. The cDNAs were stored at -20°C.

5.3.4.3 Real-time quantitative RT-PCR

All samples were analyzed for α-globin, β-globin, γ-globin and β-actin expression with primers designed using Primer Express software (Applied Biosystems, Foster City, CA, USA). The primer sequences are shown in Table 3. Reactions were performed in 25 μl using SYBR® Green PCR Master Mix (Applied Biosystems) on a 7300 Real-Time PCR System (Applied Biosystems). The 25 μl reaction mixture composed of 25 ng cDNA, 12.5 μl 2X SYBR® Green PCR Master Mix, 1 μl 2 μM forward and reverse primers each, H_2O to total volume of 25 μl. Each sample reaction was performed in triplicate. The formula $2^{-\Delta Ct}$ was applied to give fold-difference between each globin mRNA relative to the reference gene. Relative gene expression between induced and uninduced samples were determined by dividing fold-differences in treated cells against fold-differences in uninduced cells.

5.4 Primary Erythroid Progenitor Cell Culture

5.4.1 CD34+ cell selection and erythroid cell culture

CD34+ cells were enriched by positive immunomagnetic selection method using CD34 MicroBead kit (Miltenyi Biotech, CA, USA) (Figure 24). The separation was performed according to manufacturer's instructions. 50 ml of normal donor peripheral blood or 20 ml of β–Thalassemia/HbE donor peripheral blood were collected in heparin tubes. Whole blood was centrifuged at 600 g for 7 min at 22°C. The upper phase was removed, the rest were diluted 2-4 times with ice-cold CD34+ selection buffer (PBS containing 10% Human serum albumin (HSA), and 2mM EDTA). The diluted blood was layered on top of Lymphoprep (Axis-Shield Poc AS, Norway, density 1.077±0.001 g/mL) with ratio of Lymphoprep: diluted blood at 1:2, and centrifuged at 400g for 30 min at 22°C. The cloudy interphase was extracted into a fresh tube and washed once with 10 ml ice-cold CD34+ selection buffer by centrifugation at 400g for 30 min at 22°C. The cell pellet from first wash was resuspended with 2 ml of red cell lysis buffer (0.16 M ammonium chloride, 10 mM potassium bicarbonate, 5 mM EDTA) and incubated at 37 °C for 5 min. The mononuclear cells were washed twice with ice-cold CD34+ selection buffer. After the last wash, the cell pellet was resuspended in 300 µl of CD34+ selection buffer. 100 µl of FcR Blocking Reagent was added followed with 100 µl CD34 MicroBeads and mixed. The cell-antibody solution was incubated on ice for 30 min in the dark. The cell mixture was added to a LS separation column, attached to a magnetic stand (Miltenyi Biotech, CA, USA), and previously equilibrated with 3ml CD34+ selection buffer. The unbound / unlabeled cells were washed with 3 consecutives washes using 3 ml of ice-cold CD34+ selection buffer. Following the washes the LS column was detached from the magnetic stand and the cells the bound CD34+ cells eluted with 5 ml of ice-cold CD34+ selection buffer. The eluted cells were counted and centrifuged at 400 g for 7 min at 4°C. The cells were resuspended at 1 x 10^5 cells/ml with culture media supplemented with cytokines (IMDM supplemented with 15% FCS, 15% human AB serum, 2 U/ml erythropoietin (EPO), 20 ng/ml stem cell factor (SCF), 10 ng/ml interleukin(IL)-3, 100 U/ml penicillin, and 100 mg/ml streptomycin) (Table 6)

and aliquoted into a well of a 12-well-plate. The culture was maintained at 37°C in a humidified 5% CO_2 incubator. On day 3 and 7 of culture the cells were subcultured with culture media without IL-3 and maintained at 1 x 10^5 cells/ml. The cultured cells were collected on day 12 for HbF induction assays.

5.4.2 HbF inducers treatment for primary erythroid culture

On day 7 of culture, cells were harvested, and 2ml of 2 x 10^5 cells/ml in culture media without IL-3 (Table 8) were seeded into each well of a 12-well-plate. The cultures were supplemented with compounds to be tested for their HbF inducing potential at desired concentrations and incubated at 37°C in a humidified 5% CO_2 incubator for 5 days. The medium was not changed during the induction period.

5.4.3 HbF quantification by HPLC

After 5 days of induction, the cells were harvested. Hemoglobin was analyzed and quantified by an automated high-performance liquid chromatography (HPLC) (VARIANT ^{TM}; Bio-Rad Laboratories, Hercules, CA, USA) according to manufacturer's instruction.

5.4.4 HbF Immunofluorescent staining for flow cytometry analysis

The cultured cells were harvested, and washed with PBS by centrifugation. 3 x 10^5 cells were fixed with 4% paraformaldehyde at room temperature for 10 min, and then centrifuged at 300xg for 3 min. Supernatant was removed, and cells were permeabilized using 1ml of methanol: acetone, 1:4 v/v. Cells were washed twice with PBS, followed by staining with FITC-conjugated monoclonal mouse anti-human HbF antibody (BD Bioscience, San Jose, CA, USA) or Isotype Mouse IgG1, κ as a control (BD Biosciences, San Jose, CA, USA) in 0.5 ml of 1% BSA in PBS at 4°C for 45 min. Cells were harvested and resuspended in 0.5 μl PBS for further flow cytometry analysis on FACSCalibur (BD Biosciences, San Jose, CA, USA).

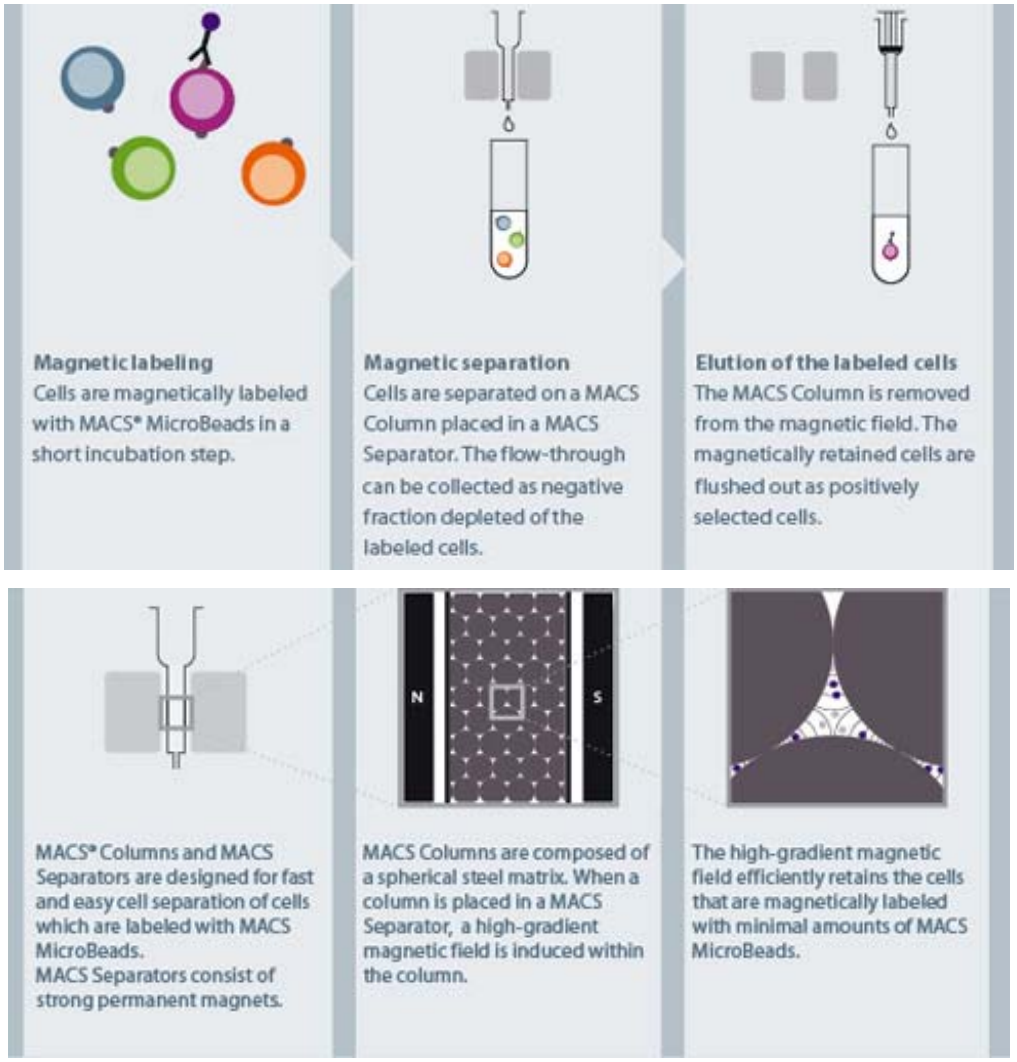


Figure 24: Principle schematic of CD34+ positive immonomagnetic selection

(Image from Miltehnyibiotec)

5.5 Creation of Globin-EGFP Transgenic Mice

5.5.1 Preparation of BAC DNA for microinjection

Large volume and high purity BAC DNA was prepared by large-scale BAC DNA extraction followed by Cesium Chloride gradient ultracentrifugation as described earlier. For microinjection purposes, DNA sample should be freshly prepared and used without prolonged storage.

5.5.2 BAC DNA digestion and purification

The 185 kb genomic insert containing the EGFP modified human β-globin locus was released from the vector backbone of pEBAC 148β:: $\Delta\,{}^{G}\gamma^{A}\gamma$ EGFP by *Not*I digestion of 50 μg purified BAC DNA. Digested DNA was separated by pulsed field gel electrophoresis (PFGE). The DNA-gel mixture was loaded into the gel outside electrophoresis chamber before submerged into the 0.5x TE buffer. Loading dye was not included. The gel was run at 180 V with switching time of 20 sec for 16 hrs at 14°C as described earlier. The 185 kb linearized fragment was localized and excised from the gel without exposure of the DNA to ethidium bromide or UV light. An aliquot of the sample DNA was also loaded and run in the same gel. The aliquot lane was excised from the main gel for ethidium bromide staining. The position of the band was marked onto the gel, and used as a reference for excision of the sample DNA. The left over gel pieces were also checked with ethidium bromide staining under UV light.

5.5.3 Electroelution of the DNA fragment in pulsed field gel

The DNA in the excised gel fragment was extracted by electroelution (Figure 25). The gel fragment was transferred into dialysis tubing, sealed with clips ensuring no air bubbles are trapped inside the tube. The dialysis tube with gel strip was submerged in sterile 0.5 x TBE buffer in a sterile electrophoresis tank. The gel strip was placed horizontally against the electric current. Current was applied at 70V for 4.5 hrs, and under reversed current direction for additional 10 min to remove DNA attached to the dialysis tube. The sealed dialysis tubing was then dialysed in 3 L of

sterile 1x TE Buffer for 1 hr at 4°C, then transferred to a fresh dialysis tank with sterile 3 L of TE buffer and dialysed overnight at 4°C.

5.5.4 Microdialysis

Following the overnight dialysis, the DNA solution was extracted from the dialysis tubing and concentrated by microdialysis(Figure 26) in microinjection buffer (10 mM Tris-HCl (pH 7.4), 0.2 mM EDTA, 100 mM NaCl, made up with water for embryo transfer (Sigma)) on a microdialysis filter (#VMW P02500, 0.05 μM, white VMWP 25 mm Millipore Corporation, Massachusetts) overnight at 4°C. The microdialysis filters were equilibrated by floating on 15 ml of microinjection buffer for 1 hr in sterile petri dish prior to loading 200 μl of the extracted DNA for microdialysis. No buffer was allowed on the upper side of the filter. Subsequently, the microdilaysed DNA solution was carefully aspirated, noting that the final volume would have decreased by 50% from the original volume loaded.

5.5.5 Evaluation of microinjection DNA

The microdialysed DNA was quantitated by spectrophotometry followed by pulsed field gel elctrophoresis alongside λ *Hin*dIII as a standard. The final concentration of the microinjection DNA was adjusted to 0.4 ng/μl and microinjected into the fertilized mouse oocytes (C57BL/6J) at the Walter and Eliza Hall Institute of Medical Research Microinjection Facility. (Parkville, VIC, Australia).

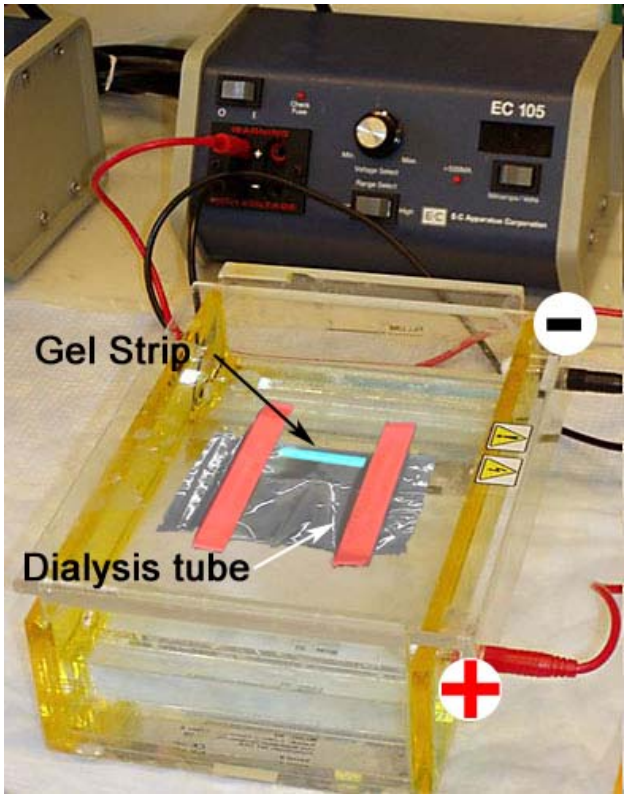


Figure 25: Electroelution of DNA-Gel Strip

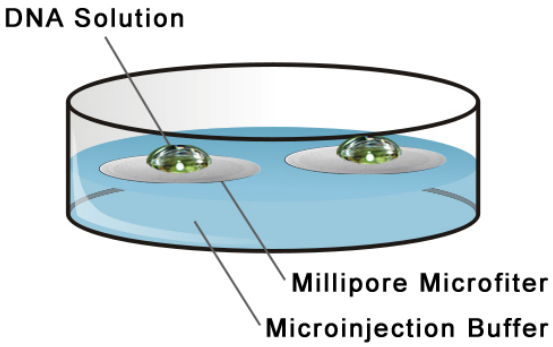


Figure 26: Schematic showing microdialysis

5.6 Characterization of Globin-EGFP Transgenic Mice

5.6.1 Mouse tail genomic DNA extraction

Genomic DNA was isolated from a tail biopsy. The tails were digested at 55 °C in 500 µl tail lysis buffer (50 mM Tris-HCl pH 8.0, 100 mM EDTA, 0.5% SDS) and 5 µl 20 mg/ml Proteinase K (Roche) overnight. After digestion, approximately 200 mg of silicone grease (Beckman) and 300 µl phenol/chloroform solution (Invitrogen) were added to the tail solution and thoroughly mixed by inversion. After centrifuge at 13,000 rpm for 5 min at room temperature, the top phase was decanted into an eppendorf tube containing 0.8 ml of 100% ethanol and 8 µl 5 M NaCl in order to precipitate the genomic DNA. The lower organic phase was still trapped beneath the silicone grease. The tube was gently mixed by inversion and spin at 8000 rpm for 5 min to recover the DNA pellet. The supernatant was gently discarded by decanting. The DNA pellet was washed with 1ml of 70% Ethanol by centrifugation at 8000 rpm for 5 min. The DNA pellet was air dried for 5 min then dissolved in 300 µl ddH_2O. The samples were then stored at -20°C.

5.6.2 Screening and breeding of transgenic mice

Transgenic mice were housed at the Melbourne University Animal Facility. Founder mice identified by PCR were bred with wild type C57BL/6J females or males to generate F1 progeny with germ-line transgene integration. Transgenic mice were maintained as hemizygous colonies by breeding F1 progeny with C57/BL/6J mice. Mice were genotyped by PCR analysis using genomic DNA. Multiplex PCR with EGFP-ProbeF/EGFP-ProbeR primers (615bp product) was used to detect EGFP gene, and APRT5'/APRT3' primers was used for internal control (252). Primers sequences were listed on Table 3, and PCR condition was shown in Table 10. 10 µl aliquots of PCR products were electrophoresed on a 1% Agarose MP gel in 0.5 x TBE buffer at 90V for 1 hr alongside MarkerX (Roche) for size estimation. PCR products were stained with ethidium bromide and visualized using UV fluorescence (Figure 27).

Table 10: EGFP-ProbeF/EGFP-ProbeR multiplex PCR condition

Component	**Volume**	**Final concentration**
PCR master mix	10.0 μl	1x PCR buffer, 2.0 mM Mg_2Cl, 200 μM of each dNTP
EGFP-ProbeF/EGFP-ProbeR (20μM of each primer)	1.0 μl	0.4 μM of each primer
APRT5'/APRT3' (10μM of each primer)	1.0 μl	0.2μM of each primer
Taq DNA polymerase (Roche) (5 units/μl)	0.2 μl	1 unit
Genomic DNA template (~100 ng/μl)	1.0 μl	100 ng
ddH_2O	to total volume of 50 μl	
PCR cycle: 30 cycles (94°C 30 sec; 61°C 30 sec; 72°C 30 sec)		

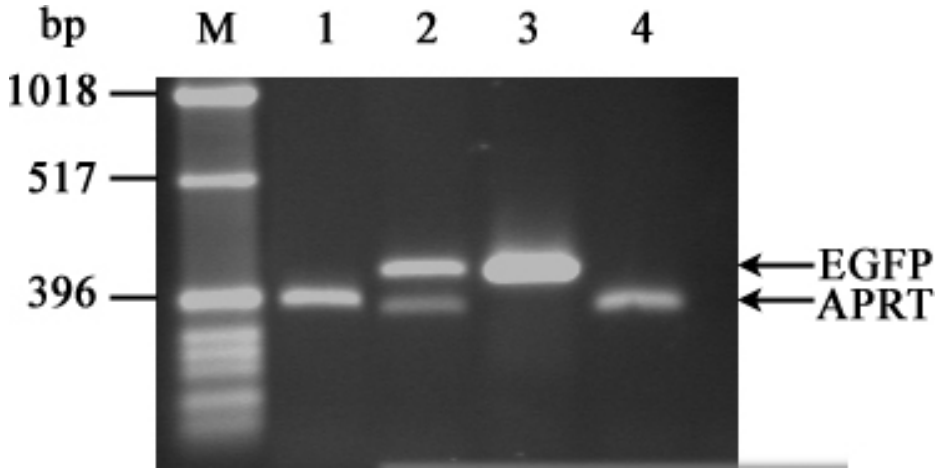


Figure 27: Genotyping of transgenic mice by EGFP multiplex PCR

Multiplex PCR using two primer pairs specific to the EGFP transgene, and the mouse APRT gene were used to genotype mice.

Lane M: Molecular weight marker X (Roche)

Lane 1: Wild type mouse

Lane 2: $\Delta^{G}\gamma^{A}\gamma$-EGFP transgenic mouse

Lane 3: Positive control for EGFP: pEGFP-N22

Lane 4: Positive control for APRT: genotyped mouse genomic DNA

5.6.3 Overall integrity of transgene fragment

The integrity of the transgene was determined by multiplex PCR using LUGFwd/LUGRev primers (447 bp product), human-γ- FW/human-γ- Rev primers (165 bp product) designed to detect the human β-globin, γ-globin respectively, while the mouseβ-Fwd/mouseβ-Rev primers (260 bp product) were included to detect the endogenouse murine β-globin as an endogenous control (253). Primers sequences were listed on Table 5 and the PCR conditions for the various multiplex PCRs performed are listed in Tables 11 and 12 below. 10 μl aliquots of PCR products were electrophoresed on a 1% Agarose MP gel in 0.5 x TBE buffer at 90V for 1 hr alongside MarkerX (Roche) for size estimation. PCR products were stained with ethidium bromide and visualized using UV fluorescence.

Once founder were identified and were found to be transmitting the transgene to their F1 progeny, only multiplex PCRs with EGFP-Probe primers were performed for genotyping purposes coupled with EGFP detection by flow cytometry in blood collected from the tail biopsies.

5.6.4 Flow cytometer analysis of EGFP expression of blood cells

In order to monitor the EGFP expression pattern in the transgenic mice whole blood, bone marrow cells and fetal liver cells (obtained from livers of pups obtained from timed matings) were harvested and analysed by flow cytometry.Whole blood from tail biopsies was collected into EDTA tube, then 20 μl of the blood samples were diluted into 500μl of PBS supplemented with 1% FBS then analysed by flow cytometry as described earlier. Cells extracted from bone marrow, and fetal livers were washed with PBS then resuspened in 500 μl PBS supplemented with 1 % FBS. The cells were then analysed by flow cytometry as described earlier.

Table 11: LUGFwd/LUGRev multiplex PCR condition

Component	Volume	Final concentration
PCR master mix	6.0 µl	1x PCR buffer, 2.5 mM Mg_2Cl, 200 µM of each dNTP
LUG Fwd/LUG Rev (20 µM of each primer)	0.5 µl	0.4 µM of each primer
Mouseβ-Fw/Mouseβ-Rev (10 µM of each primer)	0.5 µl	0.2 µM of each primer
Taq DNA polymerase (Roche) (5 units/µl)	0.1 µl	0.5 units
Genomic DNA template (~100 ng/µl)	1.0 µl	100 ng
ddH_2O	to total volume of 25 µl	
PCR cycle: 30 cycles (94°C 30 sec; 55°C 30 sec; 72°C 30 sec)		

Table 12: human-γ- FW/human-γ- Rev PCR condition

Component	Volume	Final concentration
PCR master mix	11.0 µl	1x PCR buffer, 2.5 mM Mg_2Cl, 200 µM of each dNTP
human-γ- FW/human-γ- Rev (10µM of each primer)	1.0 µl	0.2 µM of each primer
Taq DNA polymerase (Roche) (5 units/µl)	0.2 µl	1 units
Genomic DNA template (~100 ng/µl)	1.0 µl	100 ng
ddH_2O	to total volume of 50 µl	
PCR cycle: 30 cycles (94°C 30 sec; 55°C 30 sec; 72°C 30 sec)		

5.6.5 Fluorescent *in situ* hybridization (FISH)

The site of integration of the human transgene in the murine genome was determined by Fluorescent I*n Situ* Hybridization (FISH). A tail biopsy (1cm) or skin biopsy was cut into small pieces and incubated at 37°C in a humidified 5% CO_2 5% O_2 incubator in 3 ml of DMEM culture medium containing 20% FCS and penicillin/streptomycin with high HCO_3. The shredded pieces of the tissue were left to attach to the culture flask surface undisturbed. Within a couple of days fibroblasts can be seen emanating from the attached biopsy fragments. Culture media was refreshed as needed. Once cells reach 50 – 70% confluency, the cells are rinsed with PBS and detached by incubation with pre-warmed trypsin/EDTA (0.025%) at 37°C for 5 min. the cells were resuspeded into a single cell suspension by repetitive pipetting then fresh media was added and cells split into multiple flasks as needed. For FISH analysis of metaphase spreads, the cells were harvested during the exponential growth phase. Prior to harvest the cells were treated with 0.1 ml of 10μg/ml colcemid (Invitrogen) at 37°C for 3 hrs. The cells were then scraped from the surface of the flask and collected by centrifugation at 1,300 rpm for 10 min. The supernatant was removed retaining ~0.5 ml in the tube, then 6 ml of 37°C 0.56% KCl were added and incubated at 37°C for 10 min. The cells were collected by centrifugation at 1,300 rpm for 10 min. The supernatant was removed retaining ~0.5 ml in the tube. The cells were then resuspended in 6 ml of fixative solution (methanol: acetic acid, 3:1) which was added dropwise as the tube was being vortexed. The cells were then collected by centrifugation an the fixation protocol repeated three times. The cells were retained in 0.5 ml of fixative. Approximately 10 μl of the fixed cells' suspension was dropped onto a slide, air-dried and checked for cell concentration byphase contrast microscopy. The excess fixative solution was removed from the spread cells by passing dried slides through 70%-, 85%-, 95%-ethanol for 1 min at each soncentration. The slides were drained and air-dried.

The metaphase chromosomes were denatured by immersion in 70% formamide in 2x SSC at 73°C for 2 min, then the reaction was stopped by placing in 70% ethanol ad -20°C. The probe was prepared by labeling purified pEBAC/148β DNA with digoxigenin using nick translation according to the manufacturer's instructions

(Roche). The labeled DNA was ethanol precipitated together with 30 μg CoT-1 DNA and resuspended in 50% formamide, 10% dextran sulphate and 2x SSC to a concentration of 40 ng/μl. The probe was denatured by heating at 70°C for 8 min, followed by incubation on ice. Hybridization was performed at 37°C for 16 hrs followed by washing in 2x SSC at 70 °C for 10 min. The digoxigenin labeled pEBAC/148β probe was detected with anti-digoxigenin (Roche) followed by fluorescein conjugated anti-mouse antibody. The slides were mounted in Vectashield (Vector Laboratories) containing DAPI counterstain. The cells were analyzed by Lucille Voullaire (CAGT Group, MCRI, Australia), using Zeiss epifluorescence microscope with appropriate filters and captured by Cytovision imaging equipment and software (Applied Imaging Corp., CA). Ten metaphases were analysed for individual signal.

5.6.6 Hematological analysis

Blood samples were collected from the retro-orbital sinus of the transgenic mice into EDTA tubes. Full blood examination was performed using an automated Roche Cobas Helios hematological analyzer at the Walter and Eliza Hall Institute, Melbourne.

5.6.7 Murine primary erythroid culture system

Cells extracted from the fetal livers (day 13.5 of gestation) of transgenic mice werecultured according to previously described method with minor alterations (254). Briefly, primary erythroid cells were resuspended in 1 ml serum-free stem cell expansion medium (StemPro-34TM; Life Technolo-gies Gibco BRL). Cells were passed through a 70 mm Nylon cell strainer (Falcon #2350, Becton Dickinson), washed and seeded at 1 x 10^6 cells/ml into Stem-Pro-34TM medium supplemented with 1 μM dexamethasone (Sigma), 20 ng/ml insulin like growth factor 1 (Promega, Madison, WI), 2 U/ml Aranesp (Amgen, Thousand Oaks, CA) and murine recombinant stem cell factor (180 ng/ml). Partial changes of medium supplemented with fresh factors were done daily. Cell density was maintained at 1×10^6 cells/ml by daily media changes. In order to drive differentiation of the cells, differentiation

media ,StemPro-34 medium (Invitrogen) containing 200 ng/ml biotin (Sigma), 8.3 ng/ml hypoxanthine (Sigma), 10 U/ml Aranesp (Amgen), 4 x 10^{-4} IU/ml insulin (Actrapid HM, Novo Nordisk, Bagsvaerd, Denmark), 1 mg/ml iron-saturated human transferrin (Sigma), 1 μM mifepristone (Invitrogen) and 100 μl/ml foetal calf serum (Thermo Electron, Melbourne, Australia), was used.

5.6.8 Fluorescence Microscopy

Cultured murine primary erythroid cells were examined using phase contrast and fluorescence microscopy. Microscopy was performed on an Olympus IX70 Fluorescence Microscope (Olympus) using SPOT Advanced Image Capture software (SciTech) for data acquisition. EGFP was visualized by illumination using a 490 nm excitation laser.

5.6.9 HbF inducer treatment of murine primary erythroid culture

For induction studies, cultured murine primary erythroid cells were harvested and washed once with PBS, then resuspended at concentration of 1 x 10^6 cell/ml in culture media. 200 μl of cell suspension with inducing compound at desired concentration were dispensed into each well of a 96-well plate. The culture was incubated at 37°C humidified 5% CO_2 incubator and cells were assayed on day 2 or 3. The plates were centrifuged at 1,200 rpm for 5 min. The supernatant was aspirated out. Then the cells were resuspended in 300 μl PBS supplemented with 1% FBS and assayed by flow cytometry as described earlier.

CHAPTER VI
RESULTS AND DISCUSSION
SCREENING OF FETAL HEMOGLOBIN INDUCERS

In this chapter, the results of the optimization of the high throughput system, and screening of the ~2100 compounds using the optimized throughput system were described. Validation of the hit compounds from high throughput screening was carried out by small scale induction using both the cellular EGFP reporter models. The functional studies were performed in original K562 cell line and primary erythroid culture.

6.1 Cellular genomic reporter screening of HbF inducers

6.1.1 Optimization of High throughput screening system for screening of HbF inducers

The cellular EGFP reporter model has been shown to respond to both pharmacological induction (214, 243) and genetic modification obtained by the introduction of -175HPFH point mutation in the transiently transfected cell described in the next chapter. The stable cellular model (K562 :: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP) has so far been utilised in a small-scale screening of a limited number of compounds. A number of factors need to be optimized prior to the use in high throughput screening of HbF inducing agents, such as type of the plate reader, microtiter plate specification and, most important, method for cell viability assay.

In the small-scale induction studies the EGFP expression was monitored by flow cytometry, allowing EGFP analysis per cell. The high throughput assay utilizing the microtiter plate reader provides EGFP data per well. A consistent and

reproducible viability assay was needed to allow the standardization of the high throughput system which is very crucial for the fidelity of the assay.

In this high throughput system, the screening was performed in 384-well-plate format to facilitate mass screening. The cell viability assay was performed by using Cell-Titer Blue (Promega) that is based on the ability of living cells to convert a redox dye (resazurin) into a fluorescent end product (resorufin) ($579_{Ex}/584_{Em}$). Nonviable cells rapidly lose metabolic capacity and thus do not generate a fluorescent signal. The resorufin fluorescent product is in the pink color range, minimizing the interference by EGFP fluorescence (Figure 28).

In order to identify the range of cell numbers per well to be working with, the simulated induction of the K562 :: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP cells was prepared in the 384-well plate format. A 100µl volume of $3x10^5$ cell/ml culture was seeded in addition with cisplatin (0-15 µM) for 5 days. Cell numbers were evaluated on day 3 and day 5 using a hemocytometer, and cell viability was determined by trypan blue dye exclusion assay. The results showed a minimum cell density at 2.95 x 10^5 cells/ ml on day 5 of 15µM cisplatin treatment, and a maximum cell density at 2.94 x 10^6 cells/ml on day 5 of untreated cells (Figure 29). These results indicate that cell growth under the restricted area of a 384-well plate resemblesthat of normal culturing conditions in the culture flasks. This also provided the range of cell number for optimization of the viability assay.

The obtained range of cell density was then used for viability assay optimization. In this experiment, total volume of the culture (50, 100µl), volume of CellTiter Blue (5-20µl), Cell Titer Blue incubation time (1-4 hrs) were optimized in order to provide the linear relationship between the cell number and viability assay fluorescence (F(V)) in the range of working cell number.

The total EGFP fluorescence (F(E)) standard curve was plotted in relationship to the cell number in each well. The standard curves derived from the top reader and the bottom reader was compared. Despite the higher read-out F(E) value from the top reader, the result showed that the bottom reader presented a more linear trend (Figure 30). Therefore, the bottom reader was chosen for EGFP measurement.

None of the viability assay standard curves acquired from 50µl of cell culture showed linear relationship between cell number and F(V) (Figure 31). However, the more convex curve was obtained with the longer incubation. This is probably because the conversion of the resasurin into resorufin had reached saturation. In contrast, the plots showed linear relationship in many conditions with 100µl of cell culture (Figure 32). All of 5µl Cell Titer Blue conditions (1, 2, 3, 4 hrs incubation time) and 10µl Cell Titer Blue at 3 and 4 hrs incubation time provided linear relationship. To select the best condition among the 6 linear-relationship conditions, the constant value (K) was determined. K value derived by dividing EGFP fluorescence value (F(E)) by viability assay fluorescence value (F(V));

$$K = F(E)/F(V)$$

The K value is the representative value of EGFP expression per a cell. In this experiment, the cells came from the same source, without treatment by HbF inducing compounds. Therefore, the K value should be identical in every well. Using this criterion, the procedure using 5µl Cell Titer Blue incubated for 2 hours was selected as it would present the least fluctuation value and least inclination of trend line (Figure33).

The ability of the system to detect induction of EGFP expression was confirmed by the 5-day treatment of the K562 γ-globin EGFP reporter cell using cisplatin (0-20µM) (Figure 34). The system was able to pick up the induction at the sensitivity of 154.34% EGFP increase (10µM), 183.27% EGFP increase (15µM) and 112.79% EGFP increase (20µM). Subsequently, type of 384-well plate was studied, and the black clear-bottom 384 well plate (Greiner) was chosen instead of the white one because it gave a higher sensitivity for EGFP upregulation (data not shown). However, one difficulty of the system is the inconsistency of the read-out around the area at the edge of the plate, mostly at the first and last row/column. This edge effect is due to an uneven evaporation rate and uneven distribution of O_2 during the cell culture incubation period, which is 5 days. The result showed that viability assay was greatly affected by this edge effect (Figure 35) which the EGFP expression assay was

not affected (Figure 36). To minimize the problem, cultures were performed in another layer of humidified box, placing inside the incubator, to eliminate uneven evaporation and O_2 distribution. Also, the outermost wells of the plate were omitted from the assay.

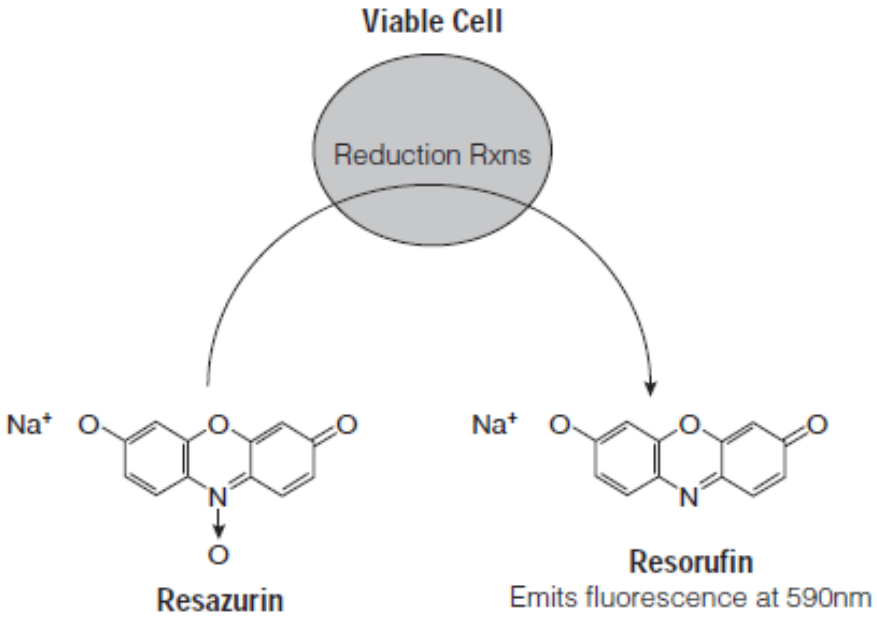


Figure 28: Cell Titer-Blue viability assay based on conversion of resazurin to resorufin by metabolically active cells

(Image from product leaflet, Promega**)**

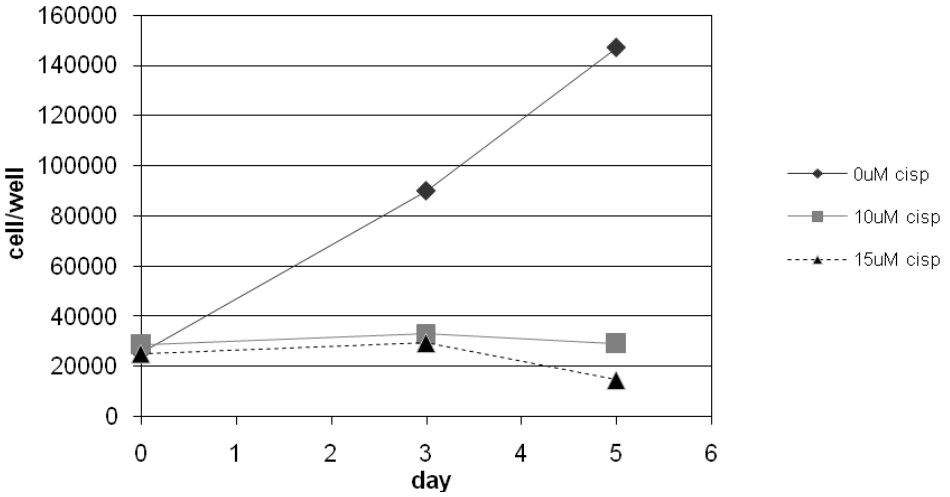


Figure 29: K562 :: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP cell growth under cisplatin treatment in 384-well plate

The graph represents cell number in each well of 384-well plate under 0 μM cisplatin (solid black line), 10 μM cisplatin (solid grey line), and 15 μM cisplatin (dash black line) over 5 days period. The cell number in 100μl culture of each well was converted to cell density per 1 ml culture.

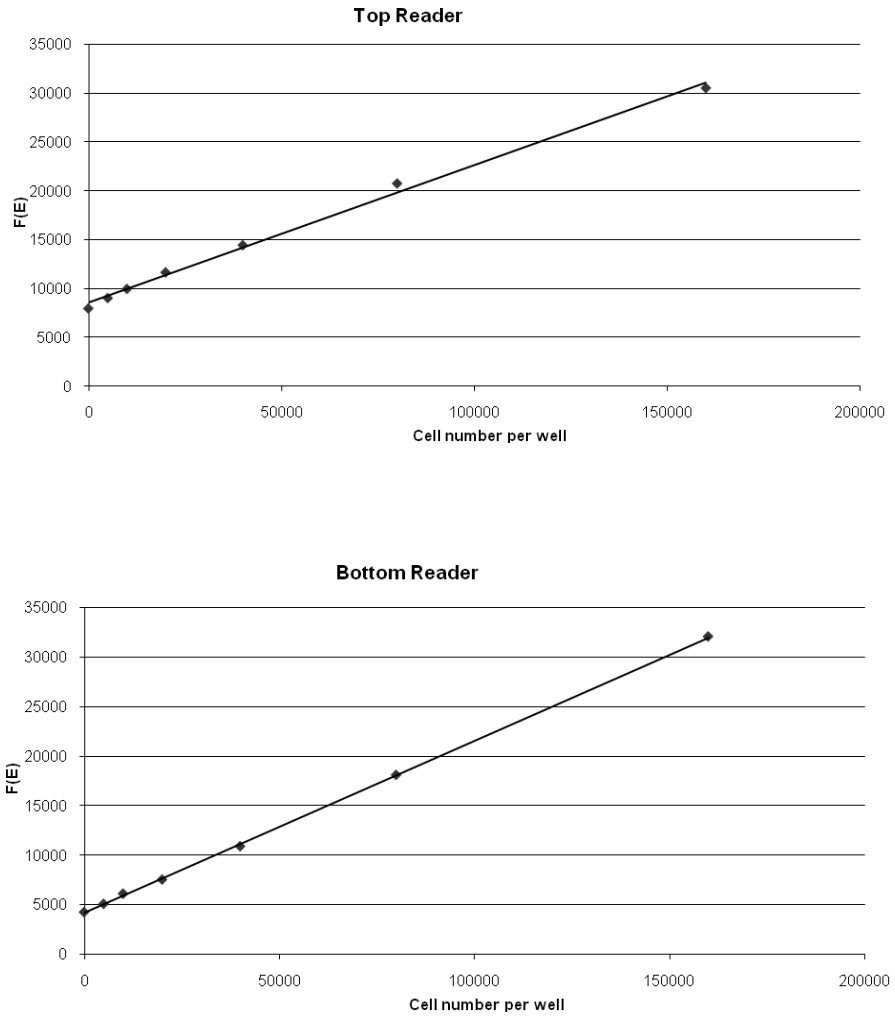


Figure 30: EGFP fluorescence standard curve comparison between top and bottom plate reader

The standard curves of EGFP fluorescence (F(E)) were compared between top (upper panel) and bottom (lower panel) plate readers. The x-axis represents F(E) value, while y-axis represents cell number per well.

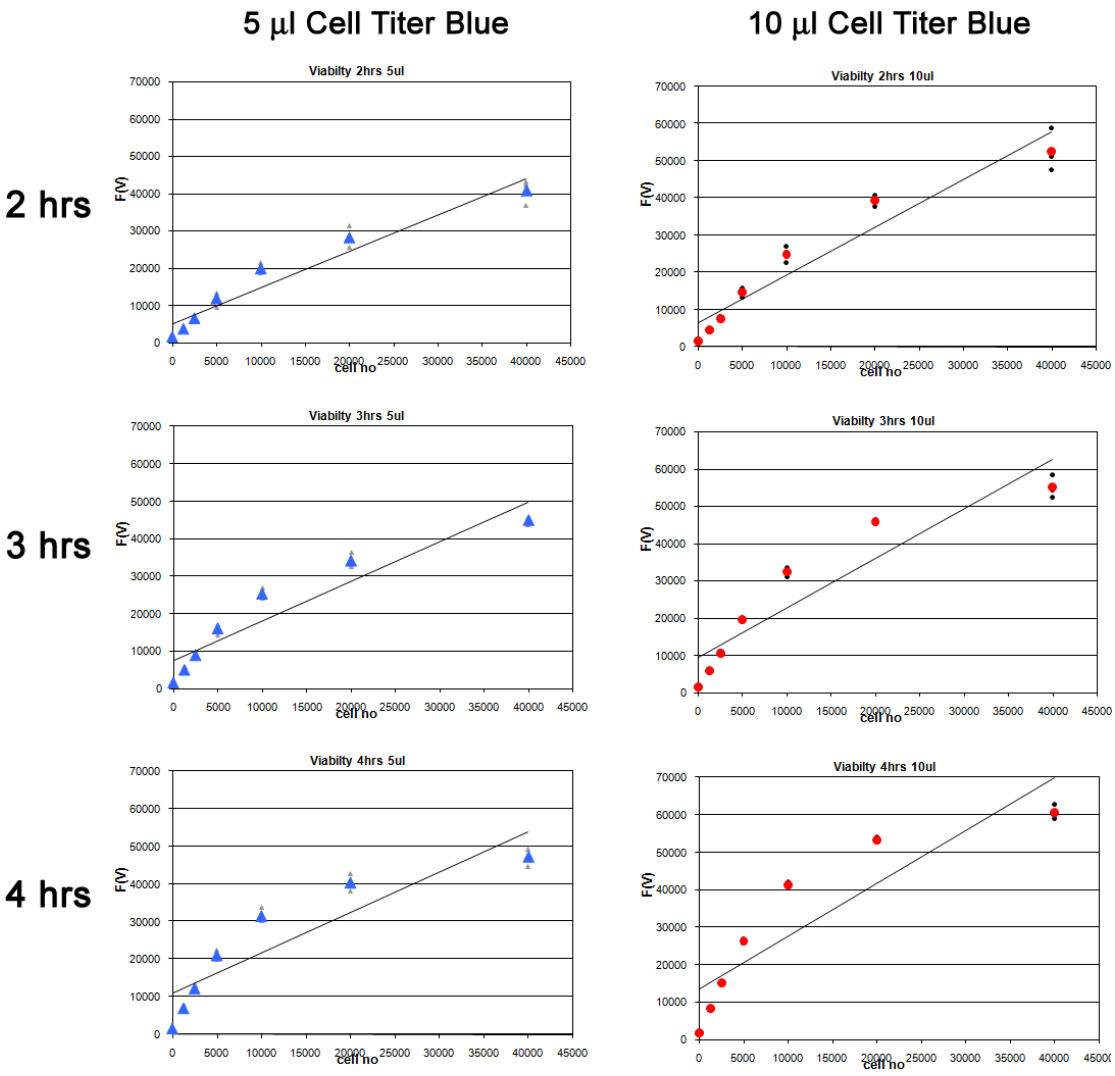


Figure 31: Viability Assay optimization for 50µl culture in 384-well plate

Figure 49 shows scattergram representing relationship of cell number/well (X-axis) to mean viability assay fluorescence; F(V) (Y-axis). Cells were assayed with 5µl (left panel), and 10µl (right panel) Cell Titer Blue for 2 hrs (upper panel), 3 hrs (middle panel), and 4 hrs (lower panel). Experiment was performed in triplicate and the convex curve relationship between the cell number/well and mean viability was observed.

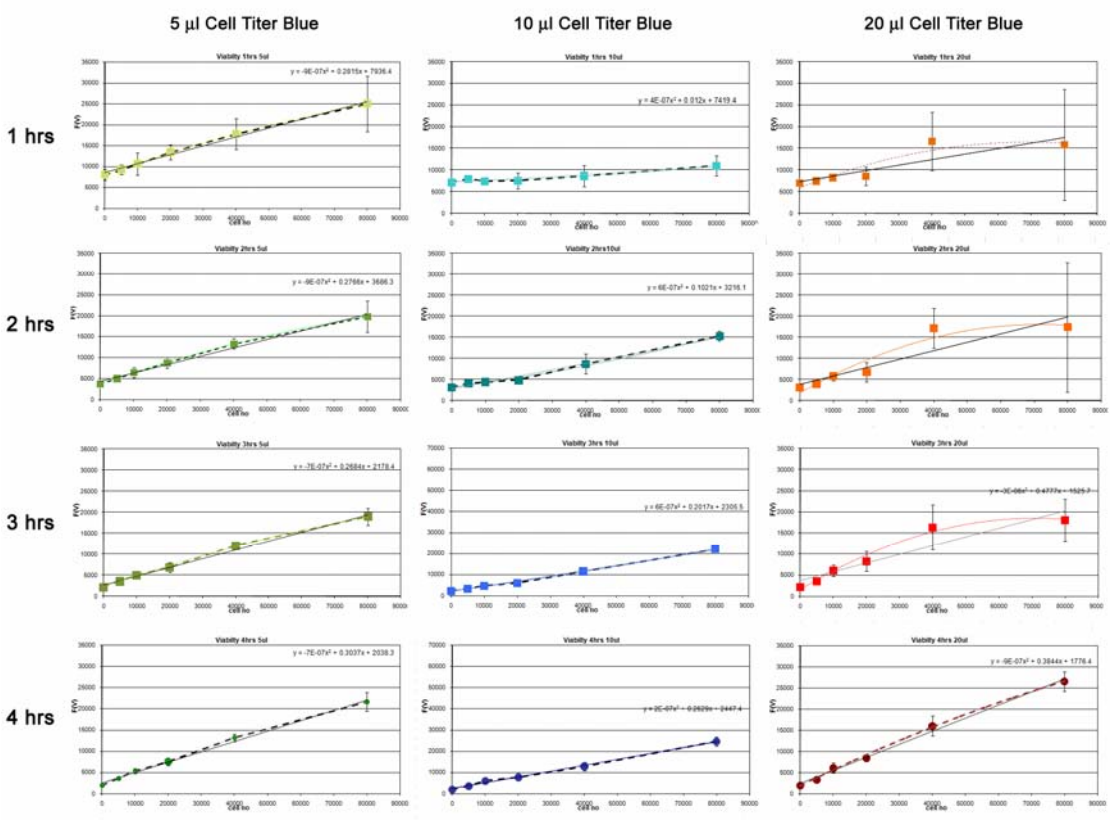


Figure 32: Viability Assay optimization for 100µl culture in 384-well plate

Figure 50 shows scattergram representing relationship of cell number/well (X-axis) to mean viability assay fluorescence; F(V) (Y-axis). Cells were assayed with 5µl (left panel), 10µl (middle panel), and 20µl (right panel) Cell Titer Blue for 1 hrs (1st row), 2 hrs (2nd row),3 hrs (3rd row) and 4hrs (4th row). Experiment was performed in triplicate and trend of linear relationship was observed.

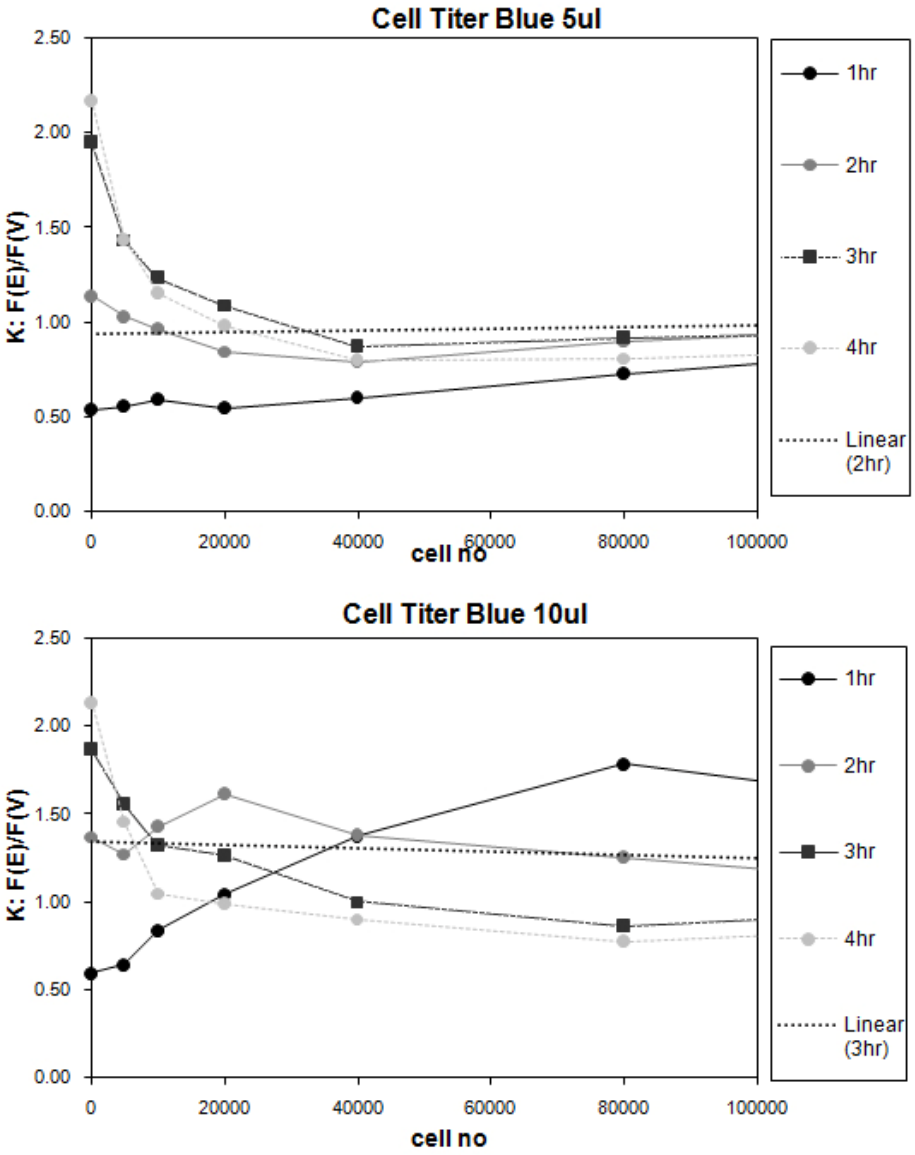


Figure 33: EGFP expression per cell in viability assay optimization

The EGFP expression per cell (K Value) was calculated by dividing EGFP fluorescence value by viability assay fluorescence value. The K value (Y-axis) was plotted against cell number per well (X-axis). 5µl (upper panel) and 10 µl (lower panel) of Cell Titer Blue were incubated for 1, 2, 3, and 4 hours (shown in legends box).

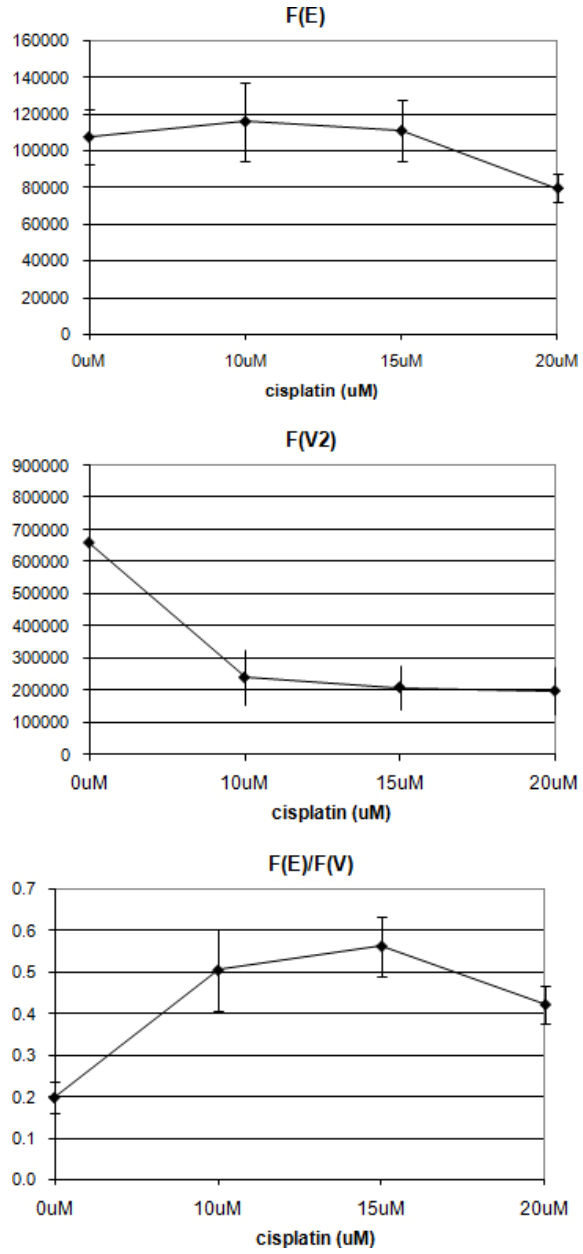


Figure 34: Validation of the high throughput screening system using cisplatin-induced K562 :: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP cell line

The K562 :: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP cell line was treated for 5 days with cisplatin (0-20μM) as indicated in the figure. The EGFP fluorescence profile (F(E)) was shown on upper panel while the viability assay fluorescence profile (F(V)) was shown in the middle panel. The lower panel showed the constant value (F(E)/F(V)) representing EGFP expression/cell.

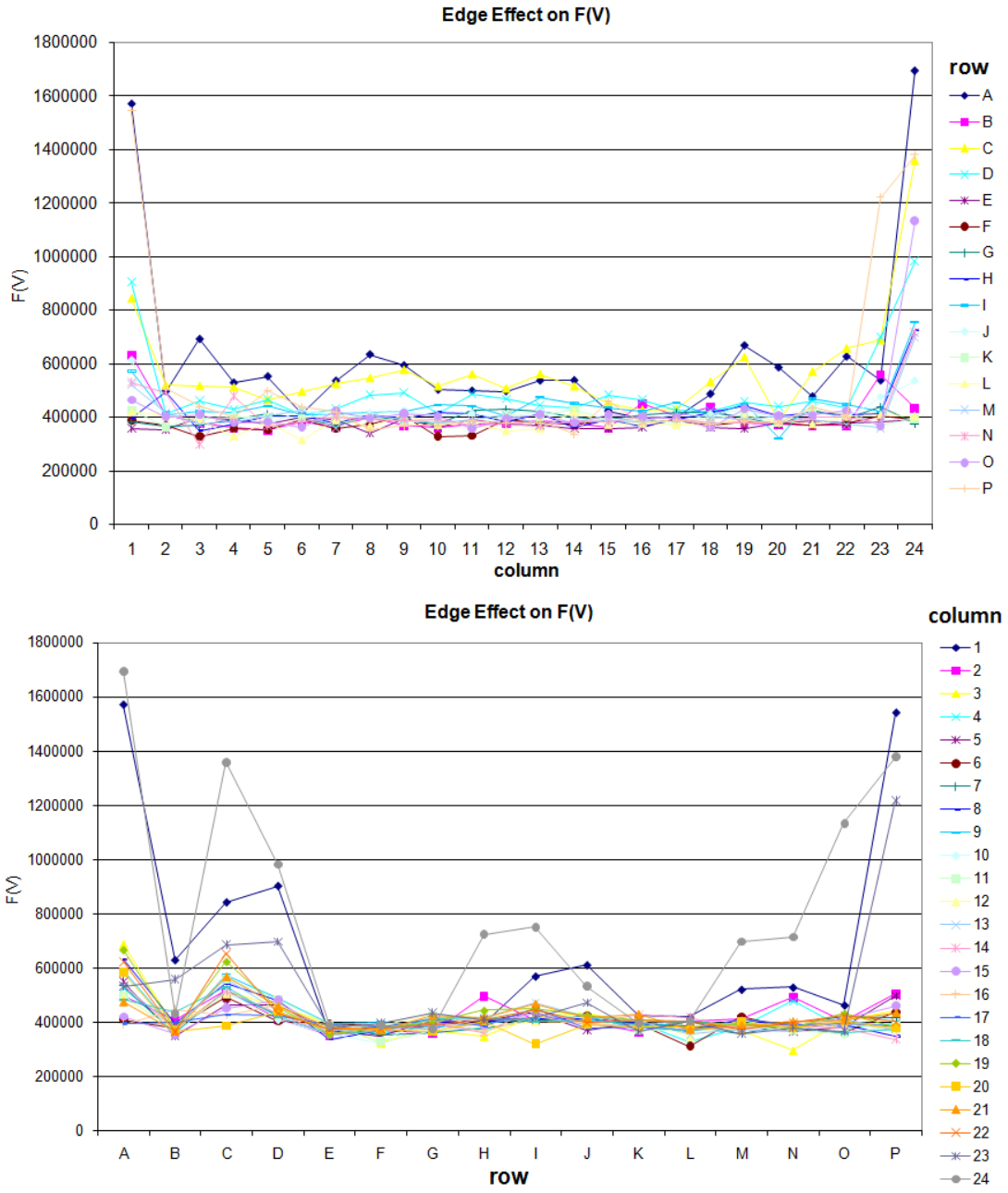


Figure 35: Edge effect on viability assay read-out across 384-well plate

The fluorescence of viability assay (F(V)) was plotted against the cell culture in each well cross the 384-well plate. The cell plate was incubated at 37°C for 5 days. Upper panel shows the edge effect across each column of the wells, each line represents each row of wells, and vice versa in lower panel. F(V) was apparently increase in the well on the edge of the plate.

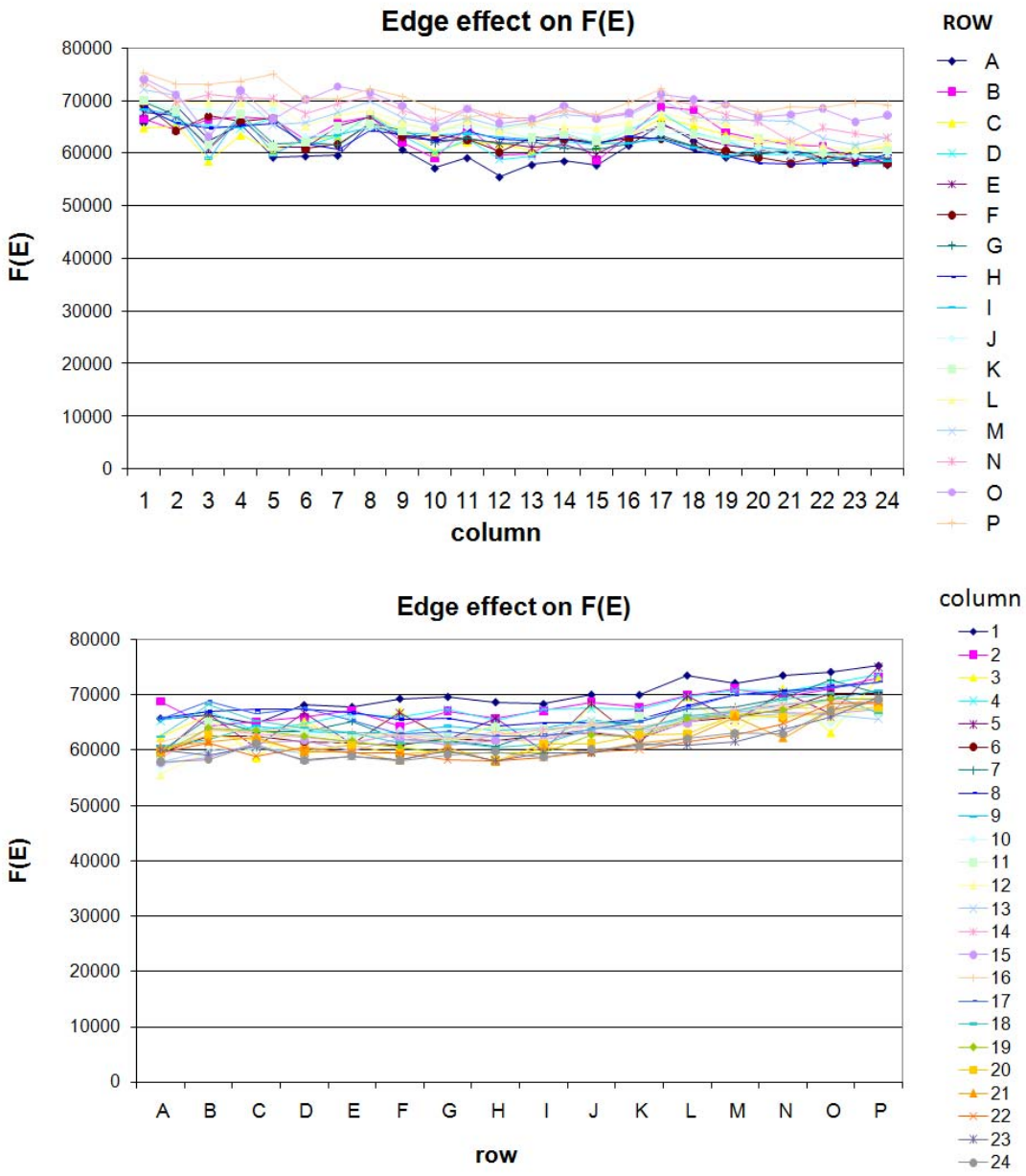


Figure 36: Edge effect on EGFP assay read-out across 384-well plate

The fluorescence of EGFP (F(E)) was plotted against the cell culture in each well cross the 384-well plate. The cell plate was incubated at 37°C for 5 days. Upper panel shows the edge effect across each column of the wells, each line represents each row of wells, and vice versa in lower panel. No edge effect was detected in the F(E) assay.

6.1.2 High throughput screening system for screening of HbF inducers

Once the protocol for the high throughput screening was set, the screening of 2000-compound library, plus extra 130 natural products was performed according to the optimized protocol. Briefly, the K562:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP cells were seeded at 3 x 10^5 cells/ml in 100μl volume with addition of 0.1μl of the compound solution, which gave the final concentration of the compound at 10 μM. The cell seeding concentration was maintained similarly to previous small-scale induction protocol (214). This gave the final concentration of DMSO at 0.1%, which is an acceptable concentration (below 1%) without cell interference to the cell-based assay. Moreover, the concentration of 10μM is generally the standard concentration to start screening because its relevance to the drug concentration in the physiological system. The detailed protocol was provided in chapter V. Fourteen wells of cisplatin (10μM) induction and untreated cells were included as controls in each plate. The compound treatment on non-fluorescent KEB cell was also performed in parallel as a control. This is to eliminate the auto-fluorescence background of the compounds. The compounds, that gave the higher EGFP expression than that of cisplatin induction subtracted by its 1 standard deviation (mean -1 SD), were selected as hit compounds. The scattergram from one of the experiments was shown in Figure 37.

Twenty nine hits from all triplicate screening were identified, accounting for ~10% of the total screened compounds. Three of which were auto-fluorescent. These compounds fall into diverse range of biological activities and structures.

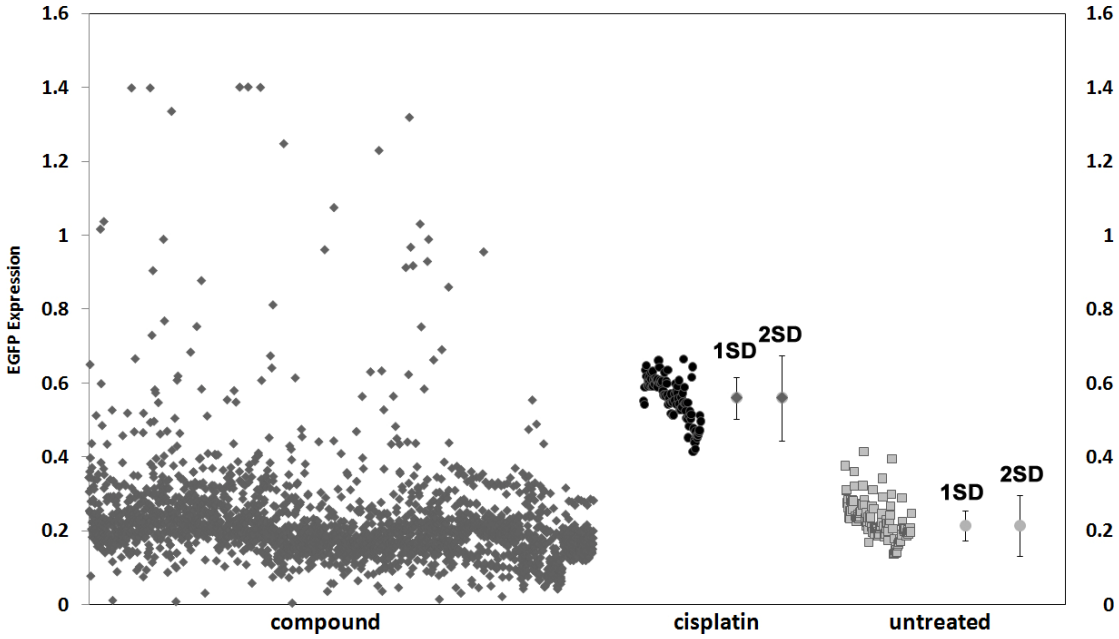


Figure 37: Result of high throughput screening of 2130 compounds

The result from one of the high throughput screening experiments was shown. The scatter gram represents EGFP expression upregulation (F(E)/F(V); Y-axis) of the K562 :: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP cell line treated with compounds for 5 day. Each dot represents each well that was treated with 10µM compound (diamond dot). The 10µM cisplatin induced (round dot) and untreated (square dot) cell were included as the controls. The standard deviation (SD) error bars were included as a reference for hit compound selection.

6.2 Validation of the hit compounds from high throughput screening by small-scale induction

Fourteen high throughput hits compounds were further assessed by the small-scale validation. Since three of the hit compounds from high throughput screening were found to be the nucleoside analog antiviral drugs. Consequently, range of 34 reported nucleotide analog antiviral drugs were investigated. In addition, five commercially available antiviral drugs were incorporated into the list of the compounds for small-scale validation, bypassing the high throughput screening step.

Due to commercial availability limitation, fourteen high throughput hits and five additional antiviral drugs were validated on the small-scale induction. This The test is a 24-well plate format based on flow cytometry analysis of the K562 :: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP cell line, and mRNA quantitative analysis of original K562 cell line. The dose-dependent response and cytotoxicity of each compound were evaluated after 5 days of treatment. Four of high throughput hits and two of the additional antiviral drugs gave no induction property, hence they were dropped out. The list of validated compounds was shown in Table 14. The efficacy ($E_{max)}$, and cytotoxicity ($IC_{50)}$ of each hit compounds were also shown. All of the validated compounds had dose-dependent response. The inducing efficacy is varied throughout every group of compounds.

Nucleoside analogs are the majority of the validated compounds. These four compound structures are generally identical, except for some of the side chain or functional groups (Figure 38). However, the applications of the compounds are on various types of virus. The overall anti-viral mechanism of these nucleoside analogs is that; the antiviral agent is converted to an active form by the virus itself, and the virus then uses the active form of the antiviral agent, rather than normally use nucleoside, for DNA synthesis during viral replication. Incorporation of active antiviral agent into new viral DNA inhibits the further production of the DNA by viral DNA polymerase. A report has shown the anti-sickling property of the two antiviral agents (255). However, none of the mechanism of the antiviral agents for gene regulation was reported. It has been shown that nucleoside analog C is a potent inhibitor of IMP dehydrogenase (256), which is involved in the de novo synthesis of GMP, and

significantly decrease intracellular GTP in K562 cell (120). Previous reports also showed the role of soluble guanylate cyclase (sGC) and cGMP-dependent protein kinase (PKG) pathway in upregulation of γ-globin gene by hydroxyurea, hemin, and butyrate *in vivo,* and *in vitro* in both β-thalassemia, and sickle cell anemia(99, 100, 190, 257, 258).

Considering the structure of the validated antiviral agents in this study, they are all guanine nucleoside analogs, which correlates with the previous evidences. Therefore, it is hypothesized that the nucleoside analog antiviral agents might involve in GTP pool or cGMP pathway to alter γ-globin gene expression.

The second majority of the validated compounds and some of the high throughput hits without validation were the flavonoids. Flavonoid, an organic compound, is a member of a class of biological pigments that are found in many plants. The structure backbone of flavonoid was shown in Figure 39. Because of the compounds pigmentation properties, it was questionable whether the upregulation of EGFP expression would be seen on the high throughput, and small-scale cellular reporter assay was actually an interference of the EGFP signal by the compounds. However, the result of the control treatment using EGFP negative KEB cell, showed no increase in EGFP fluorescence value, both by fluorescence plate reader and flow cytometer. This ensures the authenticity of the EGFP upregulation of the cellular assay.

Besides their vivid color, the abilities of flavinoid to modify the organism's reaction to allergens, viruses, and carcinogens were reported. They also show anti-inflammatory(259), anti-microbial and anti-cancer(259) activity. However, flavonoids are most commonly known for their antioxidant activity.

There were reports showing that oxidative stress was accounted for hydroxyurea-induced (260, 261), and butyrate-induced(262) erythroid differentiation. Furthermore, the Nrf2 (NF-E2-related factor 2) transcription factor, which was originally identified as a binding protein of locus control region of β-globin gene, binds to the antioxidant responsive element (ARE) sites leading to up-regulation of downstream genes of antioxidant proteins, such as glutathione *S*-transferases(263). This information may serves as another link between globin regulation and oxidative

stress. At this stage, the antioxidant mechanism may be the best speculate for γ-globin upregulation of the flavonoid, and possibly the aromatic cyclic compound as well.

The hypothesized mechanism of the remaining of the compounds could be in the wide range. The mechanism of organophosphorus alkylating antineoplastic could be through the phosphorylation/dephosphorylation of the signal transduction mediator, or transcription factor, based on the phosphorus characteristic, which is quite similar to phosphate group. Due to the alkylating property which causes DNA breakage or double strand cross-link, nitrogen mustard alkylating antineoplastic A might bind to the DNA and alter transcription profile. Another possible mechanism for these two antineoplastic compounds is by their myelosuppression property, such as hydroxyurea, which causes hematopoietic stress and drives the F-cell population into the circulation. The estrogen derivative compound, due to its hormonal characteristic, might somehow affect a certain pathway that, by chance, cross-talk with pathway involving γ-globin gene expression. Last, the chloroacetanilide herbicide A was believed that herbicide mode of action is an effect on protein biosynthesis. Anyhow, concerning its high toxicity, the application of this compound for upregulation of γ-globin gene for β-thalassemia patient is out of consideration.

Altogether, despite all these speculation of γ-globin gene upregulation mechanism by the validated compounds, further investigation is needed to conclude the real mechanism of these compounds.

Table 13: List of validated compound by small-scale induction

Compound	Molecular mass	IC50 (uM)	Emax(concentration)
Nucleoside analog A	225.20	>2200	1022.8 (2200uM)
Nucleoside analog B	255.23	1000	829.8 (2uM)
Nucleoside analog C	244.20	180	594.2 (108uM)
Nucleoside analog D	324.34	1250	998.9 (2uM)
Nucleoside analog E	390.82	>1250	551.2 (1250uM)
Flavonoid A	302.24	49	479.2 (36uM)
Flavonoid B	286.24	27	383.4 (30uM)
Flavonoid C	256.25	60	1031.6 (54uM)
Aromatic organic A	110.11	189	870 (81uM)
Organophosphorus alkylating antineoplastic A	189.22	60	657.4 (72uM)
Nitrogen mustard alkylating antineoplastic A	305.20	30	710.7 (30uM)
Estrogen derivative A	296.40	15	229 (10uM)
Chloroacetanilide herbicide A	211.69	20	754.5 (10uM)

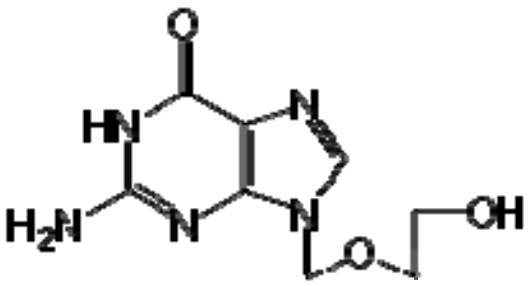


Figure 38: Structure of nucleoside analog backbone

This figure depicts the acyclic guanine nucleoside analog

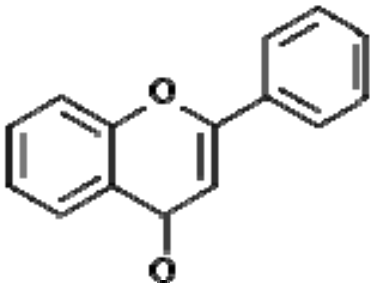


Figure 39: Structure of flavonoid backbone

6.3 Functional study of the candidate HbF inducing compounds

6.3.1 Relative expression of the globin genes mRNA in K562 cell induced by candidate HbF inducers

Even though, the upregulation against the K562 :: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP cell line was promising, the EGFP expression under the γ-globin gene of the BAC construct was not entirely representative of endogenous γ-globin of the original K562 cell line. Site of integration of the linear genomic BAC DNA as well as the stress caused by the foreign EGFP protein, might probably affect the outcome. In order to further investigate the genuine effect of the validated compounds on γ-globin induction, the globin mRNA analysis was performed on small-scale induced K562 cell line. An optimum concentration of each compound was tested.

Quantitative RT-PCR using primers specific for β-globin, γ-globin, and β-actin genes were used to determine the relative expression of the globin genes. The 112 bp, 119 bp, and 53 bp products were produced from β-globin, γ-globin, and β-actin genes, respectively. The $2^{-\Delta\Delta Ct}$ formula was used for calculating fold induction of indicated globin mRNA from treated cells compared to untreated cells. Most of the compounds showed upregulation of both β-globin and γ-globin expression, with higher degree on day 5 of induction (Figure 40). The level of EGFP expression in the cellular assay treated with the validated compounds in the previous experiment did not reflect the level of γ-globin mRNA in this experiment. This may be true since the mRNA level does not totally reflect the protein level. The translational regulation and protein stability may have a role on this. The result also showed non-specificity toward γ-globin gene. This is not surprising since majority of the HbF inducing compounds, such as hemin and butyrate, induce differentiation of K562 cell line, hence increase overall hemoglobin production (264, 265). However, the upregulation magnitudes in this study seem to be higher in γ-globin mRNA, except for nucleotide analog C, D, and flavonoid A. In this study, it showed that the DMSO used as the compound solvent, had no effect on γ-, and β-globin mRNA expression. The compounds, which showed drastic increase in γ-globin expression compared to untreated ($2^{-\Delta\Delta Ct} = 0.47$),

include the positive control cisplatin (14.66), nucleoside analog A (9.78), nucleoside analog C (9.65), nucleoside analog D (5.19), organophosphorus antineoplastic A (19.09), and nitrogen mustard antineoplastic A (19.43). Therefore the nucleoside analog group was chosen for further investigation in primary cell culture. As for antineoplastic compounds, the high toxicity of the compounds hinders the clinical application for the treatment of β-thalassemia; consequently the compounds were dropped out.

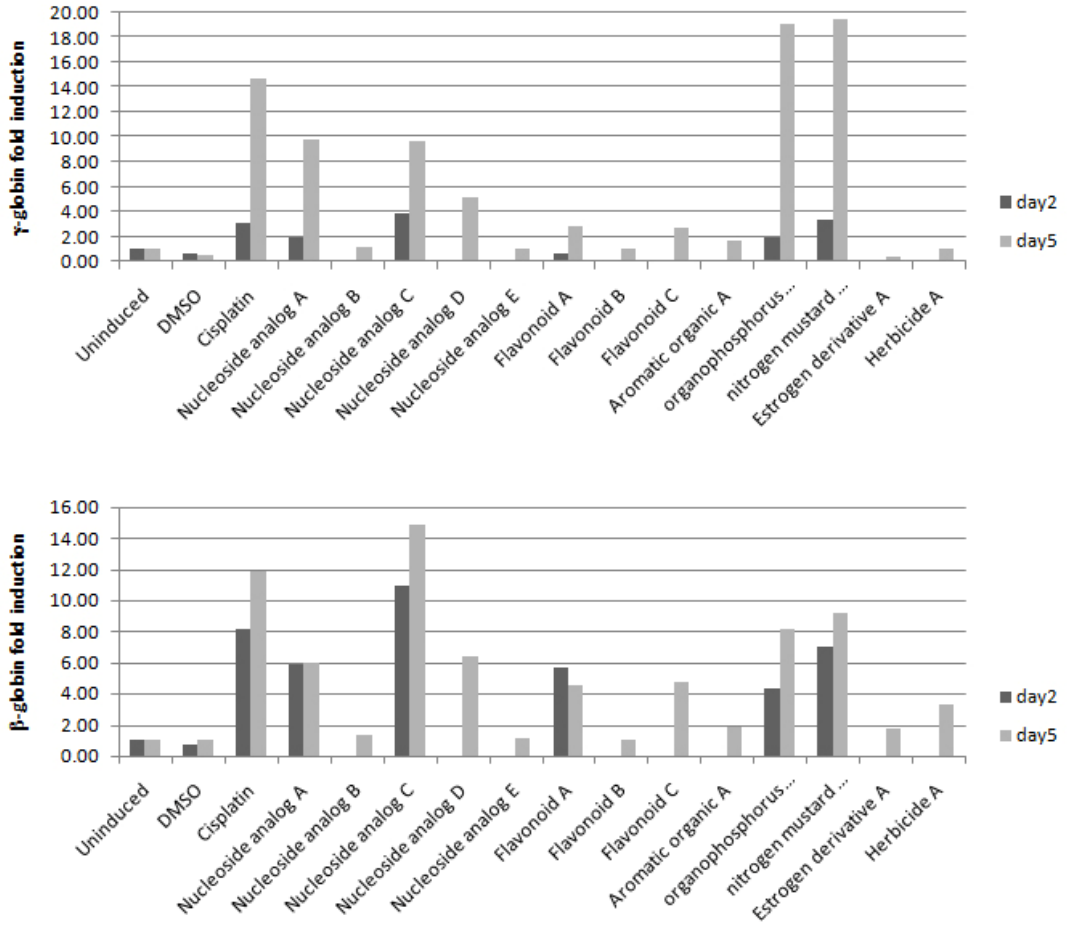


Figure 40: Relative globin mRNAs expression profile of K562 cell line treated with the candidate compounds.

The relative γ-and β-globin mRNA normalized by using to β-actin mRNA as the internal control, after 2-day (black bar) and 5-day (grey bar) treatment with the validated compounds, was analyzed by real-time RT PCR. The uninduced and DMSO were included as negative control, and vessel control. Each compound was used at the optimum concentration shown in Table 14.

6.3.2 HbF production in human primary erythroid culture

In order to more closely model physiological conditions, it is necessary to shift from cell lines to primary cell models which more closely correspond to erythroid progenitors *in vivo.* Four validated compounds were selected for further study in this system, because of limitation in the blood collection volume and, hence, amount of cell obtained. The nucleoside analog compounds were chosen based on (1) their HbF induction potency shown in previous experiments, (2) their well-documented properties that facilitate the mechanism study and applications in the future, and (3) their low cell toxicity.

Two normal and 6 β-thalassemia/HbE subjects were recruited for the study, which composed of 2 mild, and 4 severe clinical phenotypes. The $CD34^+$ cells were isolated from peripheral blood by immunomagnetic positive cell selection using anti-CD34 immunomagnetic bead. The erythroid progenitor culture was maintained in the media supplemented with human AB serum and stem cell factor (SCF) according to the method previously described (266). Both normal and β-thalassemia/HbE erythroid progenitor cells on day 7, consisted predominantly of proerythroblasts on day 7, were treated with the compounds for 5 days. On day 12, the erythroid cells, which were differentiated into polychromatophilic erythroblasts, and partially orthochromatophilic erythroblasts, were analyzed for the ratio of HbF to total Hb by HPLC.

The basal %HbF in erythroid culture on day 12 was at 22.7 ± 5.73% for normal and 31.9 ± 7.8% for β-thalassemia/HbE. There was no significant difference in basal %HbF between mild (31.7 ± 4.7%) and severe (31.9±9.8%) phenotypes. However, the overall basal %HbF levels in β-thalassemia/HbE subject were increased, which is correlated with the fact that HbF level in peripheral blood from β-thalassemia patients are elevated. The standard deviations of the basal %HbF are broad, while they are normal in primary erythroid culture derived from different individuals. It is apparent that the basal %HbF in this primary erythroid progenitor culture may be higher than what was detected in peripheral blood (~1% in normal subject). It has been reported that stem cell factor (SCF), in the presence of erythropoietin, can increase both %HbF from approximately 1% to 20%, and cell proliferation much like that occurred during

stressed erythropoiesis (65, 66, 267). This suggested that circulating progenitor cells in adult humans are capable of producing more than 20 percent HbF under the appropriate cytokine conditions. However, this condition created *in vitro* is not physiologically practical since the cytokines have effects in multiple tissues and could harm the host. In terms of this study, it was uncertain whether this effect would interfere with the study of HbF reactivation or not. However, the study of hydroxyurea using this erythroid culture system has already been reported(123).

For compound treatment, the human primary erythroid cells were more sensitive to the drug toxicity than the K562 cell line, Thus the compound concentrations previously used in K562 cell line were re-optimized and used. They were cisplatin 0-2.5μM, nucleoside analog A at 0-1100μM, nucleoside analog B at 0-2μM, nucleoside analog C 0-100μM, and nucleoside analog D 0-1250μM. The hemoglobin lysates extracted from the cultures demonstrated that the erythroid cultures from both normal and β-thalassemia/HbE subjects exposed to the candidate compounds for 5 days, 2 out of 4 nucleoside analogs resulted in the increase of ratio of HbF to total HbF (%HbF) in 2 out of 4 nucleoside analogs.

Nucleoside analog A showed dose-dependent response and gave the highest increase at 1100μM concentration; with 63.80 % increase of %HbF in normal subject, and 18.29 ± 5.48% increase in β-thalassemia/HbE subjects (Figure 41). Nucleoside analog D also showed dose-dependent response and gave the highest increase at 1250μM concentration; with 26.22 % increase of %HbF in normal subject , and 21.17 ± 2.92% increase in β-thalassemia/HbE subjects (Figure 42). The broad % increase of % HbF is partly responsible by wide deviation of the basal % HbF of the erythroid cultured obtained from each individual. There was significant difference of response to the inducing compounds between erythroid culture from severe phenotype, and mild phenotype oϕ β-thalassemia/HbE.

The efficacy compared between two identified nucleoside analogs was shown in Figure 43. The %HbF profile using erythroid culture from the same β-thalassemia/HbE subjects was increased in both nucleoside analog A and D, with slightly higher degree in nucleoside analog D (Figure 43). However, the average %increase in %HbF from three subjects is not different (Figure 43B). The difference

in degree of %HbF increase between single experiment and average from multiple experiments was due to diversity in basal %HbF and response to the compounds treatment from one individual to another, which was previously reported in hydroxyurea treatment for β-thalassemia (268).

In vitro study of sickling inhibition of the sickle cell anemia erythrocyte by nucleoside analogs, such as acyclovir, ganciclovir showed that, despite of structure similarity, penciclovir could not exert the inhibition. It was believed that the absence of an oxygen alpha to the N9 of the guanine moiety in the inactive penciclovir might be responsible for the inactivity of the compound (255). This result is somewhat resembling with ours. Although the mechanism for the anti-sickling of antiviral compound is still unknown, regarding to our work, it is possible that they work through up-regulation of HbF. Numbers of studies showed that upregulation of HbF can ameliorate symptom of sickle cell anemia by reduction of sickling hemoglobin precipitation. Hydroxyurea is now an approved drug for sickle cell anemia treatment.

The effective windows of concentration of these nucleoside analog compounds were broad. Even through the highest upregulation of HbF was observed at 1250 μM, the upregulation could still be detected in concentration as low as 10 μM range. Low toxicity properties of these compounds are greatly beneficial for potential treatment. Well-documented properties of the compounds would assist further investigation of the compounds both in mechanism of action and to clinical approach. The structural difference in active and inactive nucleoside analog may be used as a guide to search for other, possibly more effective HbF inducers.

It is not surprising that not all of the validated compounds could increase %HbF in primary erythroid culture. Even though K562 cell line is a good model for preliminary study, it may not serve as the best representative of human erythroid cell because of its neoplastic property and chromosomal abnormality (chromosome11 trisomy and chromosome 16 tetrasomy) (269). Besides, the K562 cell line also has an embryonic expression program and therefore expresses mostly ε-globin and ζ-globin. α-globin expression is detectable but very low and is not truly representative of normal expression (209).

Only part of the validated compounds was studied in the primary erythroid culture due to the cell limitation. However, considering the obtained positive result, it is very encouraging for further study of the rest of the validated compounds in this primary culture system.

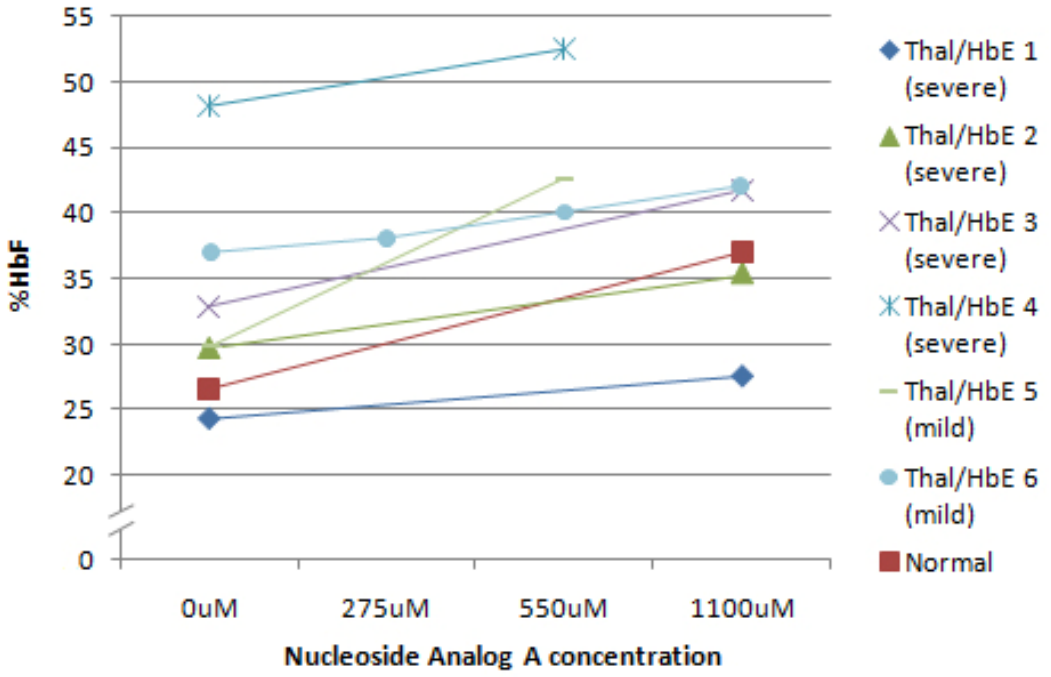


Figure 41: Nucleoside analog A effect on primaryerythroid culture

The graph showed HbF ratio to total Hb (%HbF) at indicated concentration of nucleoside analog A. Each line represents each subject.

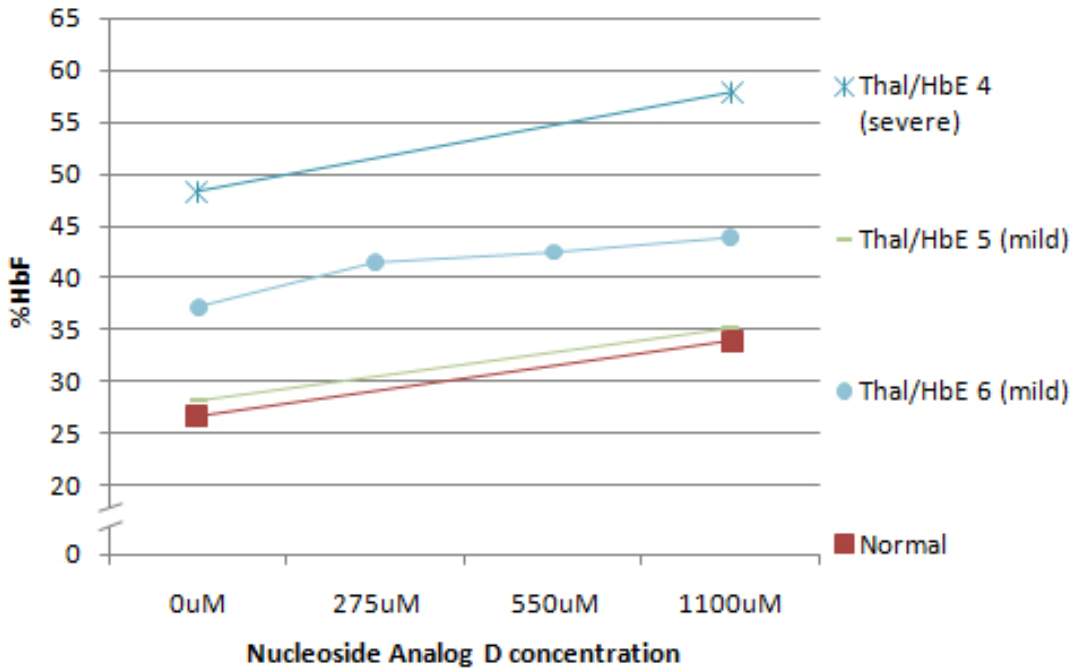
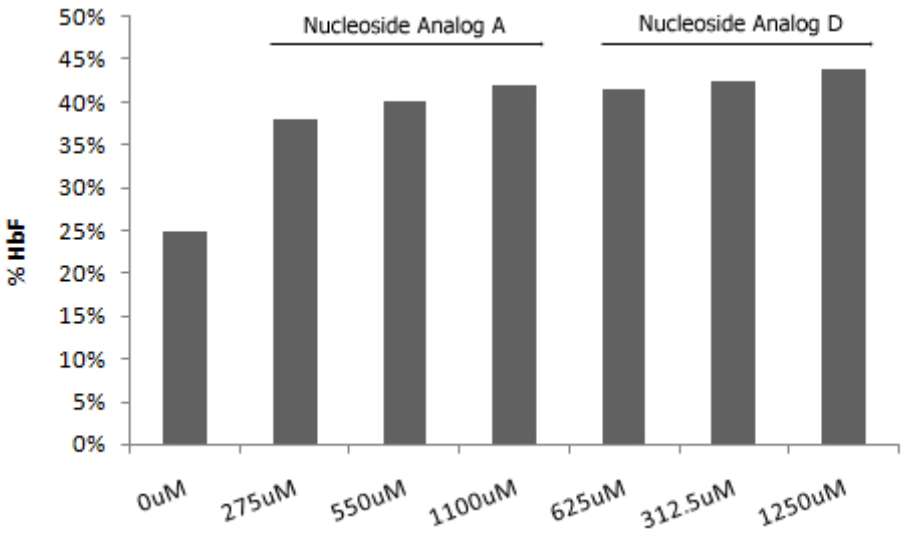


Figure 42: Nucleoside analog effect on primaryerythroid culture

The graph showed HbF ratio to total Hb (%HbF) at indicated concentration of nucleoside analog A. Each line represents each subject.

A)



B)

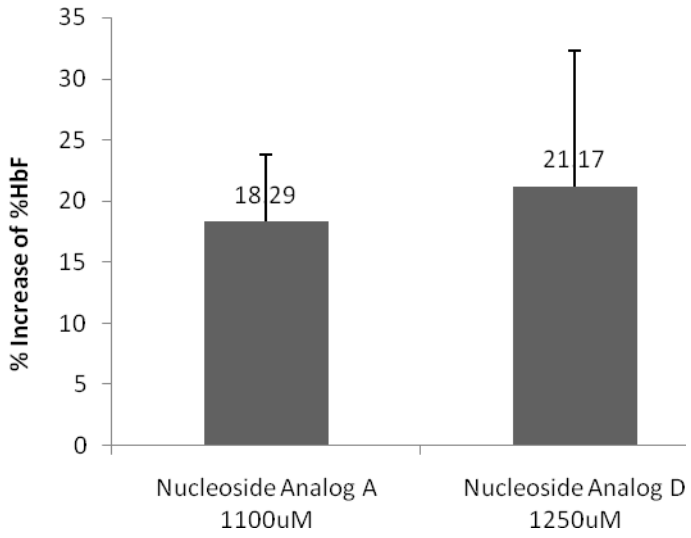


Figure 43: Comparative profile of HbF% induced by two nucleoside analogs

A) The % HbF profile of one of the primary erythroid culture derived from β-thalassemia/HbE subjects treated with indicated concentration of nucleoside analogs for 5 days.

B) The average % increase of %HbF in the β-thalassemia/HbE erythroid culture treated with the nucleoside analogs at the indicated concentration. The experiment was performed in triplication.

CHAPTER VI
RESULTS AND DISCUSSION
CONSTRUCTION OF -175HPFH EGFP BAC AND FUNCTIONAL STUDY

The K562 :: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP cellular genomic reporter has been previously shown to respond to pharmacological induction of γ-globin (214, 243). However, γ-globin expression can also be upregulated by other factors aside from pharmacological induction such as genetic polymorphisms which result in HPFH. It was therefore of interest to determine if this system would also respond to genetic modifications in addition to pharmacological inductions. To test this hypothesis and further validate this system, an HPFH mutation was introduced into K562 cells.

Although deletional HPFH is more common in Thai populations, a non-deletional HPFH polymorphism was selected for use in this study for the following reasons:

1) It has been reported that globin genes in β-globin locus function in an integrated manner(6). Non-deletional HPFH mutations require less drastic modifications at the gene locus compared to deletional forms. Minimising disruptions to surrounding genes also minimises potential interference with gene interactions and allows a more focused analysis of γ-globin expression.

2) Synthesis of oligonucleotides containing point mutations is less technically challenging and easier to achieve using commercially available oligonucleotide synthesis strategies.

Out of ten possible non-deletional HPFH mutations, a -175 (T → C) HPFH polumorphism was selected because this mutation results in the highest % HbF in heterozygous individuals compared to other forms of non-deletional HPFH (Table 1).

It is anticipated that the relatively higher expression levels will maximise ease of detection and any potential γ-globin upregulations. This could give a clear γ-globin upregulation outcome in the EGFP reporter assay. Furthermore, this HPFH mutation is active in both the $^{G}\gamma$, and $^{A}\gamma$ promoters which offers greater flexibility in experimental design.

7.1 Single step non-counterselection homologous recombination of -175 HPFH $^{G}\gamma$ denatured PCR fragment with pEBAC 148β::$\Delta^{G}\gamma^{A}\gamma$EGFP

The selective modification of bacterial artificial chromosomes (BACs) has been previously achieved using the beta protein of bacteriophage λ to catalyse homologous recombination with an exogenous complementary oligonucleotide sequence containing the desired alterations (270, 271). The *bet* protein is thought to bind the exogenous oligonucleotides and facilitate the annealing of exogenous nucleotide sequences with complementary regions of the BAC near the replication fork, leading to the formation of recombinants. A major advantage of this strategy over more standard plasmid modification tenchniques is that no operational sequences from the recombination event are left behind, resulting in modified nucleotide sequences which are completely identical to those seen in human patients. Furthermore, this recombination system has a relatively high degree of efficeincy with up to 6% success rate in treated cells. Therefore, a single step recombination using denatured PCR fragments was used to introduce the -175 (T → C) HPFH mutation into pEBAC 148β::$\Delta^{G}\gamma^{A}\gamma$EGFP.

The strategy used for recombination has been illustrated in Figure 44. In brief, a 75 nucleotide primer containing the -175 (T→C) mutation was commercially synthesised and used to PCR amplify pEBAC 148β::$\Delta^{G}\gamma^{A}\gamma$EGFP. The resulting 265 bp PCR product included the $^{G}\gamma$ 5'promoter region modified with the HPFH mutation. This PCR product was then denatured into ssDNA before electroporation into a DY380, modified strain of E. coli, which harboured the target BAC vector (wild type

pEBAC 148β::$\Delta^{G}\gamma^{A}\gamma$EGFP). Recombination was activated by inducing bacteriophage λ recombination machinery integrated within DY380 cells. As the recombined BAC only differed from the WT unmodified BAC by one base pair, it was not possible to screen for successful recombinants by antibiotic selection. Instead, an ARMS-PCR specific for -175 (T→C) $^{G}\gamma$ HPFH was developed and used to screen pools of clones.

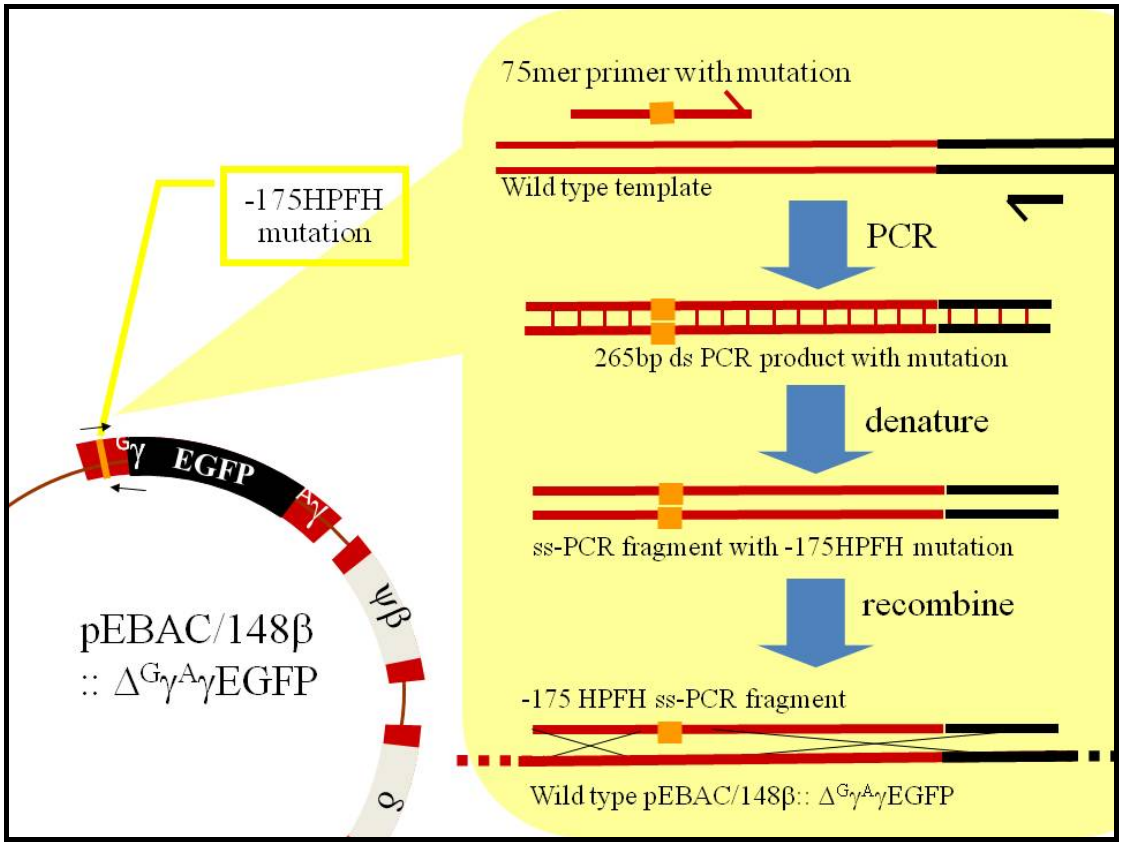


Figure 44: Strategy schematic of single step non-counterselection homologous recombination of -175 HPFH $^{G}\gamma$ oligonucleotide on the pEBAC 148β::ΔGγAγEGFP

7.1.1 Optimization of ARMS-PCR for detection of -175 (T→C) HPFH point mutation

Forward primers were designed which contained either wild type or mutated HPFH sequences in the last position at the 3' end in addition to a mismatch at the 3rd base upstream from the 3' end (Table 5). PCR conditions for screening wild type and HPFH point mutation were optimized by adjusting factors such as $MgCl_2$ concentrations, primer concentrations, annealing temperatures and clone conditions (Figures 45 and 46).

Specificity of the mutant primer was tested on heat inactivated DY380 harboring wild type pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP in the presence (+) or absence (-) of 10 ng of -175 HPFH mutant PCR fragment (Figure 45). WT DY380 was included to simulate expected PCR conditions of the screening assays which are likely to include a mix of both WT and modified vectors. Optimized PCR reactions are expected to result in specific PCR products only when the mutant template is present.

In general, allele-specific PCR requires higher annealing temperatures compared to standard PCR so two different temperatures, 65°C and 70°C, were evaluated for ability to yield a HPFH mutant-specific product. At annealing temperatures (T_A) of 65°C, the mutant primer set was unable to discriminate between WT (-) or HPFH mutant (+) templates and WT DNA was amplified with equal efficiency as HPFH modified DNA templates. However, a T_A of 70°C resulted in a significantly higher degree of specificity with amplification occurring only in the presence of HPFH template DNA. Therefore, a T_A of 70°C was utilised in this ARMS-PCR protocol

PCRs performed using 1 µl of DY380 overnight culture at dilutions of 1:0, 1:10 or 1:100 did not have any detectable effects on the specificity or efficiency of PCR reactions. Therefore, undiluted (1:0) overnight cultures were utilized as the template for ARMS-PCR.

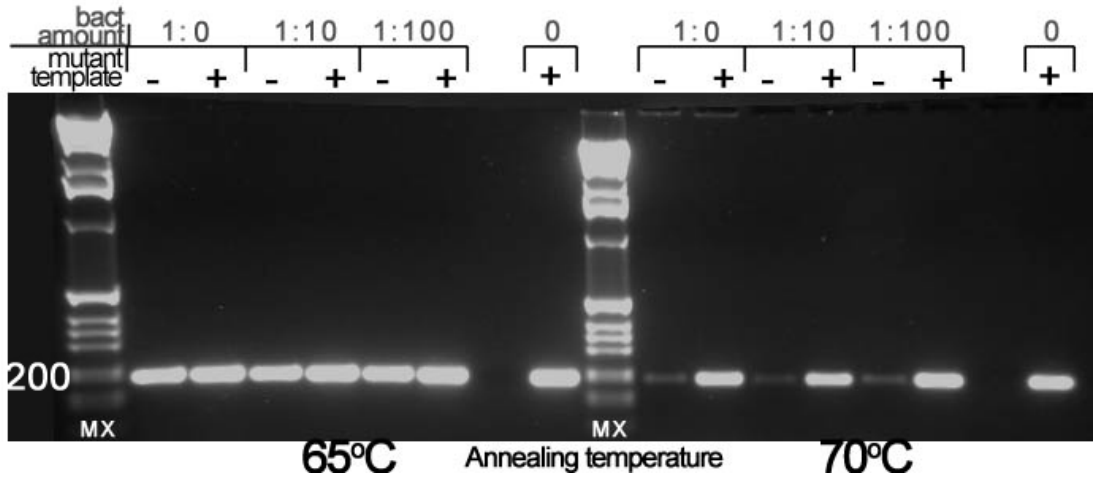


Figure 45: Effect of annealing temperature, and amount of bacteria overnight culture to ARMS-PCR specificity.

The PCR products (200bp) were amplified reaction using mutant primer sets with (+) and without (-) mutant template on the wild type DY380 background (1:0, 1:10, 1:100 dilutions). Lane MX represents molecular weight marker X (Roche). Lane 0 represents positive control, using only mutant PCR fragment.

Optimizations of $MgCl_2$ and primer concentrations in PCR reactions were performed by using both wild type primers (W) and mutant primers (M) to amplify DY380 harboring wild type pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP at annealing temperatures of 70°C (Figure 46). A 200 bp PCR product was expected from reactions with wild type primers and no product was expected from the mutant primers.

The results showed that PCR efficiency decreased with decreasing $MgCl_2$ concentrations (2.5 mM, 2.0 mM, and 1.5 mM). No PCR products were detected with concentrations of 1.5 mM. Ultimately, 2.0 mM was selected as the optimum $MgCl_2$ concentration for ARMS-PCR as it resulted in the clearest discriminations between wild type and mutant primers. Primer concentrations of 0.1 μM and 0.2 μM were also tested to determine optimal concentrations. As primer concentrations of 1 μM resulted in poor amplification efficiency in both $MgCl_2$ concentrations, 0.2 μM of primer was selected for further experiments.

To further maximize amplification efficiencies, three different template purities were assessed including 1) purified DNA (Wild type pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP (WT) and -175 HPFH mutant recombination PCR fragment (-175)), (2) unmodified overnight bacterial culture and (3) overnight cultures heat inactivated at 95°C for 5 minutes. Overnight cultures were once again assessed at three different dilutions (1:0, 1:1, 1:100). Both wild type primers (W) and mutant primers (M) were used to amplify DY380 harboring wild type pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP at annealing temperature of 70°C (Figure 47). A 200 bp PCR product was expected from the reaction by wild type primers and no product was expected from the mutant primers.

Of these three different template purities, the heat inactivated overnight cultures resulted in greatest specificity and highest yields. While PCR specificity did not differ significantly between heat inactivated and fresh cultures, PCR yields appeared higher with the heat inactivated template. The higher yields from heat inactivated cultures are thought to be due to degradation of inhibitory enzymes and nucleases which are released from bacteria cultures, resulting in higher PCR efficiency. As a result, the heat inactivated PCR templates were still able to amplify with high efficiency even with low amounts of template (1:100 dilution).

In conclusion, the optimized PCR conditions identified in this study were as follows; 5 µl of 10 x PCR buffer without $MgCl_2$ (Roche) combined with 2 mM $MgCl_2$, 200 µM dNTPs (200 µM each dATP, dTTP, dCTP and dGTP), 0.2 µM primers, 0.2 µl AmpliTaq DNA polymerase (Roche) and 1 µl heat inactivated overnight cultures of pooled bacterial colonies to a final volume of 50 µl. Amplification was performed in 30 cycles with the following conditions : 94°C 30 sec; 70°C 30 sec; 72°C 30 sec.

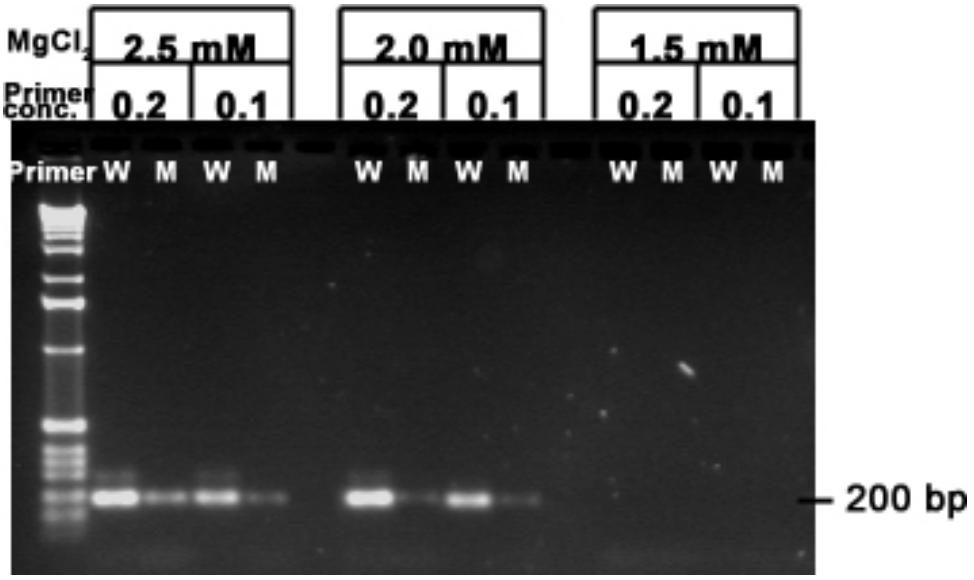


Figure 46: Effect of $MgCl_2$ concentration, and primer concentration to ARMS-PCR specificity.

The PCR amplification of the wild type pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP template using wild type (W), and mutant (M) primers. The $MgCl_2$ concentration, and primer concentration were used as indicated. MX represents molecular weight marker X (Roche) was included.

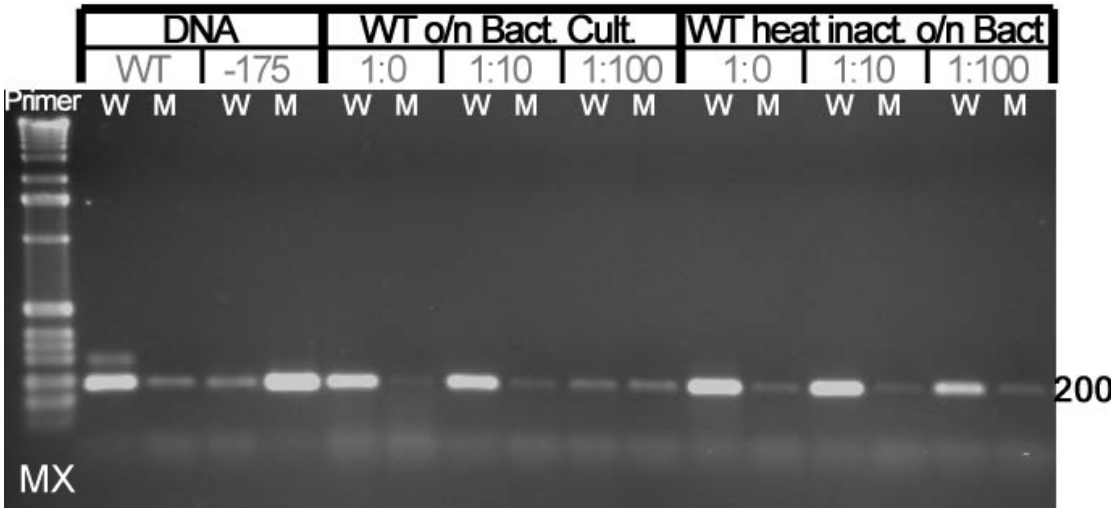


Figure 47: Effect of PCR templates to ARMS-PCR

The 200 bp PCR product were compared between the three form of templates as indicated (DNA, WT o/n Bact Cult., WT heat inact. o/n Bact.) using wild type (W), and mutant (M) primers. The template concentrations were used as indicated (1:0, 1:10, 1:100). MX represents molecular weight marker X (Roche) was shown on the first lane.

7.1.2 TSS transformation of pEBAC148 β::ΔGγAγEGFP in DY380

To enable recombination, the target BAC DNA vector (pEBAC148 β::ΔGγAγEGFP) had to first be transferred from DH10B stock E. coli vectors into the modified DY380 strain which contained integrated inducible recombination machinery. pEBAC148 β::ΔGγAγEGFP was extracted from DH10B cells by large scale alkaline lysis and purified using a cesium chloride gradient. A TSS transformation method was then used to transform between 300-1500 ng of pEBAC148 β::ΔGγAγEGFP into DY380 cells. Transformants were selected by plating onto LB agar plates containing chloramphenicol antibiotic. As a positive control, 10 pg of pUC19 small plasmid (2686 bp, Fermentas) was also transformed into DY380 and plated onto ampicillin containing selective media. DY380 transformed with no DNA and plated chloramphenicol was used as a negative control.

Following 24 hours of incubation at 30°C, DY380 transformed with 300 ng of BAC DNA gave rise to 29 chloramphenicol resistant colonies. DY380 transformed with 900 ng and 1500 ng of BAC DNA resulted in 51 and 89 resistant colonies, respectively. DY380 mock transformed with no DNA did not give rise to any colonies. The transformation efficiency for each of these concentrations was then estimated by calculating the number of colony forming units per 1 µg of DNA (cfu/µg DNA) (Figure 48). From this experiment, it appears that increasing DNA concentrations resulted in lower transformation efficiency as calculated by colonies per µg of DNA. This apparently contradictory result may have arisen either due to saturation of DNA uptake by bacterial cells or toxicity due to high concentrations of DNA.

Eight of these resistant colonies were selected and propagated and DNA was extracted by alkaline lysis for further analysis. The DNA extracted from each clone was digested with both *Not* I and *Xho* I restriction enzymes and samples were separated overnight on a pulsed-field gel (Figure 49). Restriction profiles of DNA extracted from newly transformed colonies were higher similar to that of previously identified pEBAC148β::ΔGγAγ EGFP, confirming the presence of the plasmid in all eight clones.

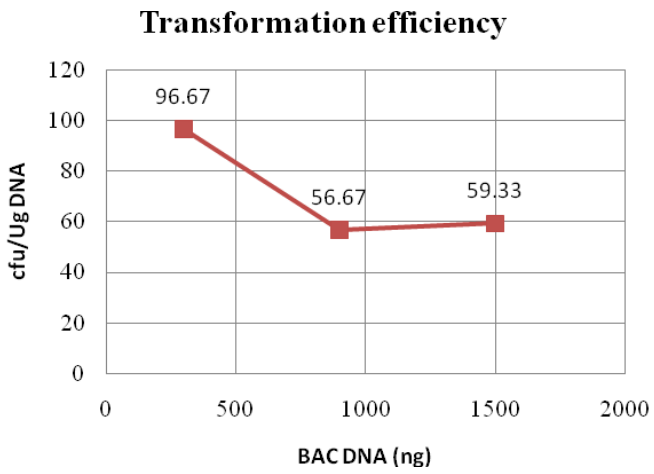


Figure 48: Graph showing relationship between transformation efficiency and amount of BAC DNA

The transformation efficiency (cfu/1 μg DNA) value is represented in Y-axis, while the amount of BAC DNA (ng) was represented in X-axis.

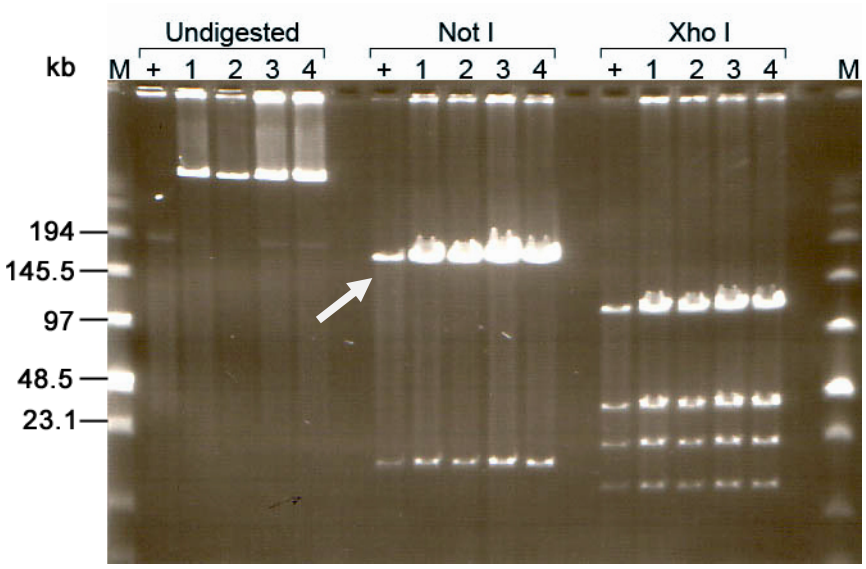


Figure 49: Pulsed Field Gel Electrophoresis of restriction analysis of DNA from TSS transformed DY380 chloramphenicol resistant clones.

The electrophoresis gel shows four out of eight selected resistant clones digested by *Not*I and *Xho*I restriction enzymes. *Not*I digestion released 185 kb genomic fragment (arrow) from the 15 kb vector backbone. Undigested DNA was also included to analyze integrity of the whole BAC.

Lane	M	:	Low molecular weight PFGE marker (Biolabs)
Lane	+	:	Previously identified β::ΔGγAγEGFP Undigested panel:
Lane	1-4	:	Chloramphenicol resistant clone 1-4

7.1.3 Homologous recombination using denatured PCR product

A 265 bp PCR fragment containing the -175 (T→C) HPFH mutation was PCR amplified using a 75 bp oligonucleotide sequence as previously described. The PCR fragment was then gel purified, denatured, and electroporated into electrocompetent DY380 harboring pEBAC148β::ΔGγAγEGFP. As there were no counterselection markers for selection of recombinant clones, electroporated DY380 cells were plated in serial, 20-fold, dilutions across six LB-agar plates (up to 1:160000 dilution factor). Following overnight incubation, single colonies were isolated and picked into individual wells of 96-well plates containing 50 µl LB broth and chloramphenicol. Pools of clones were created by combining each row and each column of the 96-well plate into single cultures of LB broth containing chloramphenicol then cultured overnight at 30 °C.

The estimated efficiency of successful recombination events without the use of counter selection markers is approximately 1%. Therefore, identification of successful recombinants which occur with such relatively low efficiency may prove labor intensive if colonies are screened individually. To overcome this, mutation-specific PCR screenings were performed on pools of clones obtained by combining each column or each row of the 96-clone plates. Overnight cultures representing each column and each row were then screened for the presence of the HPFH mutation using optimized ARMS-PCR conditions. In this mass screening approach, illustrated in (Figure 50), the presence of positive clones in a pooled row and a pooled column should give rise to intersecting co-ordinates which identify individual positive colonies.

This recombination strategy was repeated three times and up to one thousand clones were screened but no mutant colonies were identified. There is some evidence which indicates that recombination efficiency can diverge up to 40-fold depending on the mutation introduced (271).and factors which are known to influence recombination rates include the length of homologous regions as well as the type of mispairing which is formed (270). In this case, the PCR fragments and target BAC DNA only differed by a single base pair and shared almost 100% homology so recombination efficiency was expected to be high. However, the high homology between the recombination cassette and the target DNA may also have resulted in

recombination of partial PCR cassettes which omitted the HPFH point mutation. Ultimately, in order to increase the chances of isolating modified colonies, a counterselection approach appeared preferable over this attempted single strand oligonucleotide strategy.

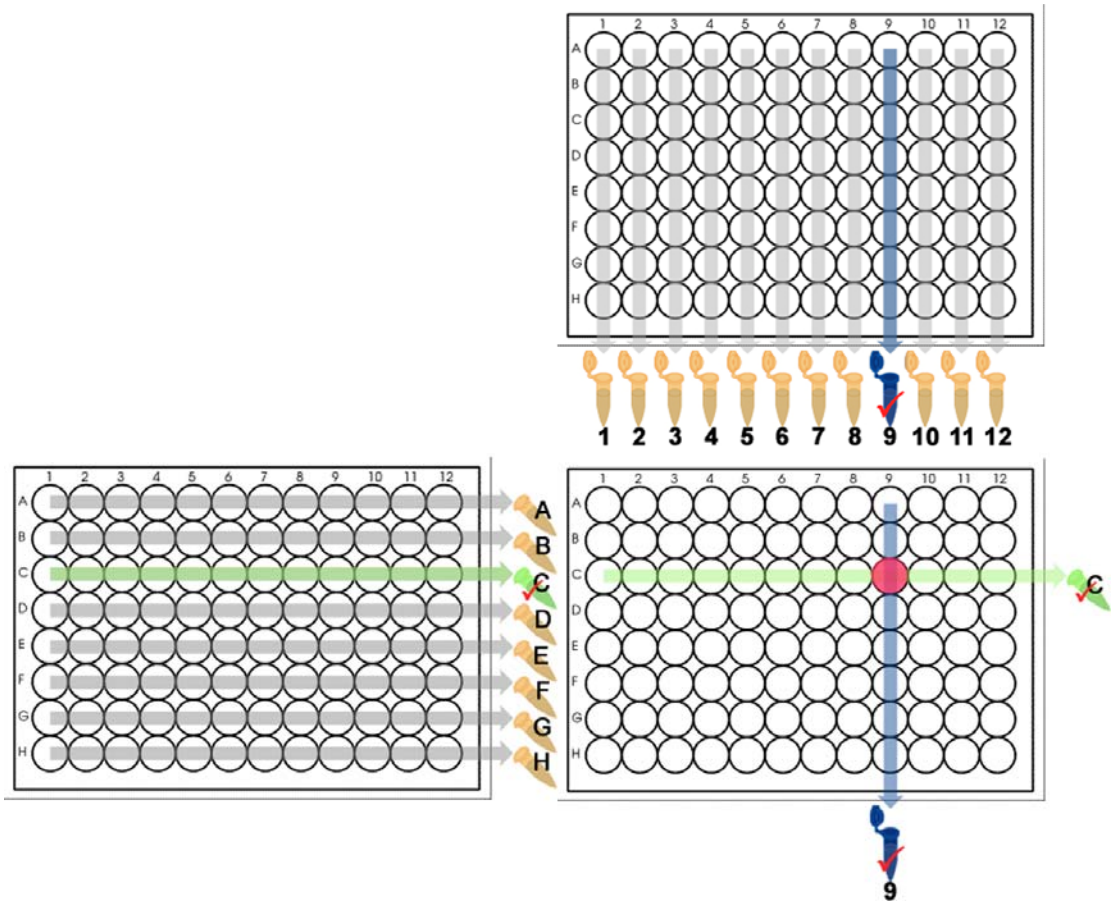


Figure 50: Diagram showing allele-specific PCR pool of clones screening strategy

According to Figure 50, Pools of clones from each row, and each column were ARMS-PCR screened. The coordination of the recombinant clone is at the intersection point of positive row, and positive column. By this strategy, it helps enormously decrease screening workload and cost.

7.2 Counter selection homologous recombination of -175 HPFH $\Delta^{G}\gamma^{A}\gamma$ EGFP-Neo/Kan cassette on pEBAC148β

In order to introduce -175 (T→C) HPFH point mutation into pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP, a simpler counter selection homologous recombination strategy was designed (Figure 51). The -175 HPFH point mutation was incorporated into the EGFP/Kan^{R} cassette using the same 75 bp HPFH mutation-containing primer from the previous experiment to amplify pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP template DNA. This double-stranded EGFP/Kan^{R} cassette containing the HPFH mutation was then recombined with pEBAC/148β target DNA. This resulted ultimately in a modified construct with an HPFH containing EGFP/Kan^{R} cassette forming an in-frame replacement of the $^{G}\gamma$- and $^{A}\gamma$-globin genes. This construct would be identical to the originally envisaged HPFH -175 (C→T) containing pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP but this method of recombination allowed counter selection by kanamycin.

The target BAC (pEBAC/148β) was again transferred from DH10B stock into DY380 by TSS transformation as previously described. Confirmation of the pEBAC/148β construct in transformed cells was again verified by restriction digest and pulsed field gel electrophoresis (data not shown). Homologous recombination was then performed by electroporating 430 ng of the -175 HPFH EGFP/Kan^{R} PCR cassette into DY380 harboring pEBAC/148β. Fifty kanamycin resistant colonies were detected, giving an estimated recombination efficiency of 8.33 x 10^{3}cfu/ 1 μg DNA.

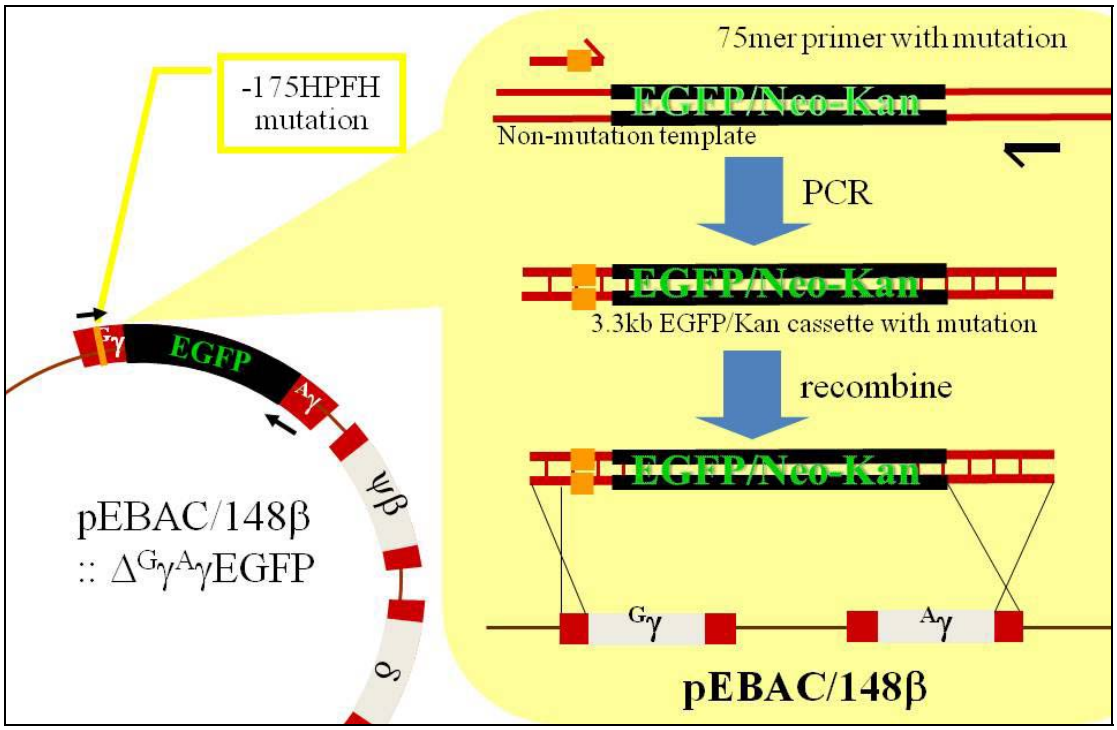


Figure 51: Strategy schematic of counter selection homologous recombination of -175 HPFH $\Delta^{G}\gamma^{A}\gamma$ EGFP-Neo/Kan cassette on pEBAC148β

7.2.1 High resolution restriction analysis

Fourteen resistant colonies were randomly selected and streak purified overnight. BAC DNA was then extracted by alkaline lysis and characterized by *Eco*RI restriction digest. An *Eco*RI digest of the unmodified pEBAC/148β construct is expected to yield 3 extra fragments (2642 bp, 1581bp, 555bp) compared to the successfully recombined -175HPFH $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP BAC (Figure 52 and arrows in Figure 53). Of the 14 resistant colonies selected for analysis, 11 gave a digestion pattern consistent with those expected from successful recombination and creation of -175HPFH $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP (lane E+, Figure 53).

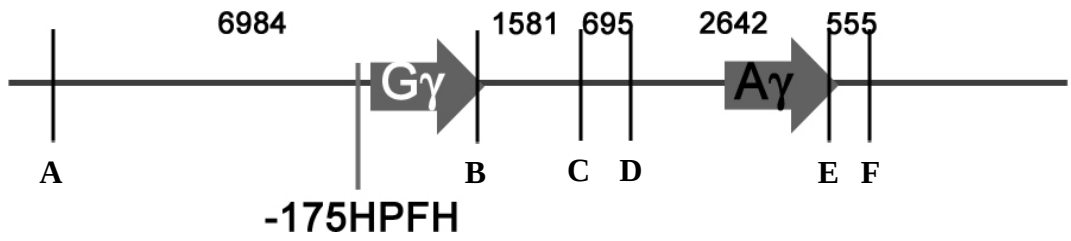


Figure 52: *EcoR*I digestion mapping at γ-globin genes of pEBAC/148β

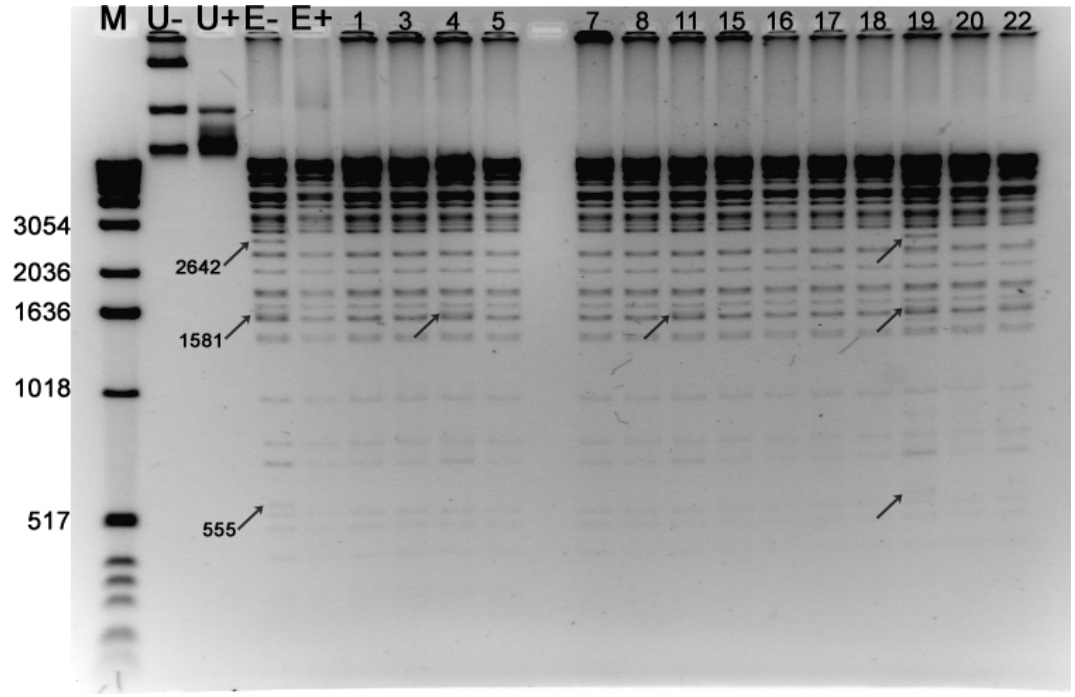


Figure 53: High resolution restriction analysis of the recombinant BAC clones after the introduction of the -175 HFPH $\Delta^{G}\gamma^{A}\gamma$ EGFP fragment.

The -175HPFH recombinant BAC gave the same *Eco*RI digestion pattern as pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP . *Eco*RI digested 14 kanamycin resistant BAC clones showed that eleven clones showed to have pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP restriction pattern in comparison to pEBAC/148β, and pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP DNA control. Lane 19 showed pEBAC/148β pattern by having additional 2642 bp, 1581 bp, 55bp bands (arrows). Lane 4, 11 showed additional 1581bp bands (arrows) comparing to pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP.

Lane	M	:	Molecular weight marker X (Roche)
Lane	U-	:	Undigested pEBAC/148β
Lane	U+	:	Undigested pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP
Lane	E-	:	*Eco*RI digested pEBAC/148β
Lane	E+	:	*Eco*RI digested pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP
Lane	1-22	:	Individual recombinant BAC clones

7.2.2 Low and medium resolution restriction analysis

The overall integrity of the modified BAC construct was further confirmed by medium and low resolution restriction digestion. Modified BAC DNA was isolated and subjected to digestion by *Not* I and *Xho* I restriction enzymes followed by pulsed-field gel electrophoresis (PFGE) separation. Although the fragment released by *Not* I digestion of the -175 HPFH recombined construct is expected to be 3609 bp smaller than *Not* I digestion of the parent (pEBAC/148β) construct, this relatively small size difference was not easily observable by PFGE. However, of the 11 recombinant clones subjected to further analysis, 9 yielded a *Xho* I digestion pattern corresponding to pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP control DNA. This indicates that a successful recombination event had occurred and the -175 HPFH mutation had likely been introduced (Figure 54).

7.2.3 PCR analysis of 3' junction

As there is significant sequence homology between the $^{G}\gamma$-globin and $^{A}\gamma$-globin genes, it was possible that one of four recombination events had occurred (see Figure 55). Therefore, it was necessary to determine the exact site of integration of the -175 HPFH EGFP cassette. In order to achieve this, PCR primers used to amplify the 3' junction of the recombination site. Forward primers (sEGFP-F) were designed to bind the EGFP gene within the HPFH cassette while reverse primers (sEGFP-R) were designed to bind specifically at the 3' promoter of the $^{A}\gamma$-globin gene but not the $^{G}\gamma$-globin gene. Expected PCR products for each recombination event has been illustrated in Figure 55. PCR analyses of the six recombinant clones previously identified by *Eco* RI, *Not* I and *Xho* I restriction digestion were all positive for the desired PCR product (Figure 56), meaning these six colonies very likely contained the desired modification.

7.2.4 Allele-specific PCR screening

As the successful recombinant is anticipated to differ from pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP by only a single (T → C) base, correct restriction digestion patterns and correct 3' junction integration did not necessarily guarantee the presence of the -175 HPFH (T → C) point mutation. Therefore, the ARMS-PCR previously developed for the detection of -175 HPFH was used to screen the previously verified clones (Figure

57). The results from this sensitive PCR detection method revealed that only four out of the six clones were positive for the -175 HPFH (T → C) point mutation. It is possible that this anomaly arose during the homologous recombination process. The recombination event may have occurred somewhere between the -175 HPFH mutation and the EGFP start codon, resulting in the omission of the sequence containing the -175 HPFH sequence.

7.2.5 Sequencing reaction

Two of these positive recombinant clones were then selected and sequenced to confirm the presence of the HPFH -175 mutation. Sequencing results showed both clones contained the -175 (C → T) HPFH point mutation in the expected position at the $^{G}\gamma$-globin 5' promoter. No other sequence alterations were found in the region of recombination (Figure 58).

7.2.6 Analysis of false positive clones

Three kanamycin resistant colonies which gave unusual digestion patterns when digested with *Eco* RI (Figure 53) were subjected to further analysis in order to identify potential problems which might occur with this recombination method. *Eco*RI digestion of clones 4 and 11 resulted in an extra 1518 bp digestion product compared to expected patterns for pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP, while digestion of clone 19 gave rise to a pattern very similar to those expected from unmodified pEBAC/148β. Sequence analysis of the Gγ and Aγ genes, the intergenic sequences and the immediate flanking sequences revealed the presence of six *Eco* RI recognition sites (Figure 52), while the EGFP/Kan^{R} replacement cassette contains no *Eco*RI binding sites. Therefore, the position of recombination will determine the number and pattern of *Eco*RI sites which are removed.

For example, the desired recombination event would result in the removal of five of these *Eco* RI sites (B-F) and absence of the 1581, 695, 2642 and 555 bp fragments. Recombination occurring only at the $^{G}\gamma$-globin gene would result in removal of *Eco* RI recognition site (B) and therefore no 6984 bp or 1581 bp fragments. And finally, recombination of the EGFP/Kan^{R} genes with the $^{A}\gamma$–globin gene would result in the removal of *Eco* RI sites (E) and (F) and subsequent absence of the 2642 bp and 555 bp fragments while the 1581 fragment remains intact. This latter pattern of digestion is consistent with the patterns observed for clones 4 and 11

and indicates that in these two clones, the -175 HPFH EGFP cassette had replaced only Aγ-globin gene.

Subsequent *Xho* I and *Not* I digestion of clones 4 and 11 resulted in the expected digestion patterns, highly similar to pEBAC/148β, as the replacement of ^{A}γ-globin by EGFP would only result in a relatively minor size alteration (Figure 54). In addition, the results from a 3' junction PCR further supported the theory that these recombination events had occurred at the ^{A}γ-globin gene (data not shown). ARMS-PCR also indicated that both clones did not contain the -175 HPFH mutation. The *Xho*I digest of clone 19 was different from anticipated patterns (Figure 54) and excludes the possibility that kanamycin resistance in this colony arose from a contaminating carry-over of the BAC (pEBAC/148β:: Δ^{G}γ-^{A}γ EGFP) template DNA used to amplify the -175 HPFH cassette. In addition, the 3' junction PCR also gave a negative result (data not shown), suggesting that recombination had occurred elsewhere in the β-globin locus. Homologous recombination between two γ-globin genes has been previously reported (247) and the high homology between the globin genes in the β-globin locus could theoretically have resulted in recombination of the -175 HPFH cassette at any of the β-like globin genes.

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7.3 EGFP expression in K562-EBNA1 (KEB) cell line.

The -175 HPFH recombinant clone 3 was cesium purified and BAC DNA integrity was reconfirmed prior to transfection in mammalian cells. As these extremely large BAC DNA constructs are highly susceptible to shearing and degradation, post-purification validation of vector integrity is particularly important. EGFP expression of both the -175 HPFH BAC and the pEBAC/148β:: Δ^{G}γ-^{A}γ EGFP with wild-type γ-globin promoter were assessed by transient transfections into a modified human erythroleukaemic K562 cell line which stably expresses EBNA-1 (KEB cells). The KEB cell line was utilised as it has been reported to support extrachromosomal replication and episomal maintenance of oriP containing plasmids.

In addition, the stable expression of EBNA-1 has also been demonstrated to improve transfection efficiencies of BAC vectors ranging from 20-200 kb in size (272).

A lipofection strategy was utilised to transfect BAC DNA into KEB cells. The uptake of a 4.7 kb pEGFP-N22 small plasmid was monitored to determine transfection efficiencies. In addition, the 200 kb pEBAC/148βG BAC was also included as a large BAC transfection control, since the CMV promoter driven EGFP on the backbone is expressed in all cell types. Unfortunately, the lipofection strategy was not particularly efficient and transfection levels of BAC DNA remained very low even after optimization of lipofection conditions. After 17 independent experiments, transfection efficiency of BAC DNA remained at 0.1% as detected by flow cytometry 48 hours post-transfection. The control pEGFP-N22 small plasmid displayed an average transfection efficiency of 10.81 ± 2.67% in KEB cells and 2.55 ± 0.78% in K562 cells.

Due to the low transfection efficiency of KEB cells with BAC DNA, it was necessary to isolate and selectively propagate the small proportion of transfected cells for further analyses. In order to achieve this, cells were lipofected with wild type or -175 HPFH BAC DNA and successfully transfected cells were selected by culturing in the presence of hygromycin for 21 days. Once suitable cell numbers were available, the cells transfected with BAC DNA were analyzed by flow cytometry for EGFP expression. The results indicated that the presence of the -175 HPFH point mutation resulted in approximately two-fold increase in EGFP expression as compared to a wild-type promoter (Figure 59 and Figure 60) in the pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP construct. This correlated well with previous reports in the K562 cell lines (273) and demonstrated that this EGFP reporter system responds to both pharmacological and HPFH upregulation of γ-globin gene expression.

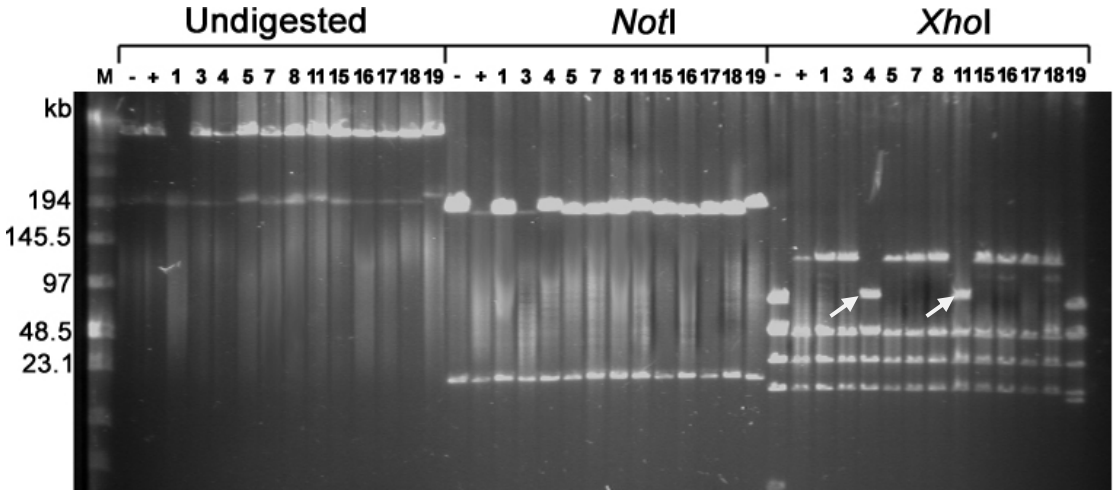


Figure 54: Low and medium resolution restriction analysis of the -175HPFH recombinant BAC clones

The recombinant clones were digested with *Not*I and *Xho*I restriction digestion. The restriction patterns were compared with pEBAC/148β, and pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP DNA control. Undigested BAC DNA was also included. In *Not*I digestion, the entire 185 genomic insert was released but the 3609 bp difference could barely be detected. *Xho*I pattern of lane 4 and 11 was similar to that of pEBAC/148β. *Xho*I digested recombinant clone in the lane 19 did not match any pattern of the two controls.

Lane M	:	Low Range Molecular weight Marker (Biolab)
Lane -	:	pEBAC/148β DNA as negative control
Lane +	:	pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP DNA as positive control
Lane 1-19	:	Individual recombinant BAC clones

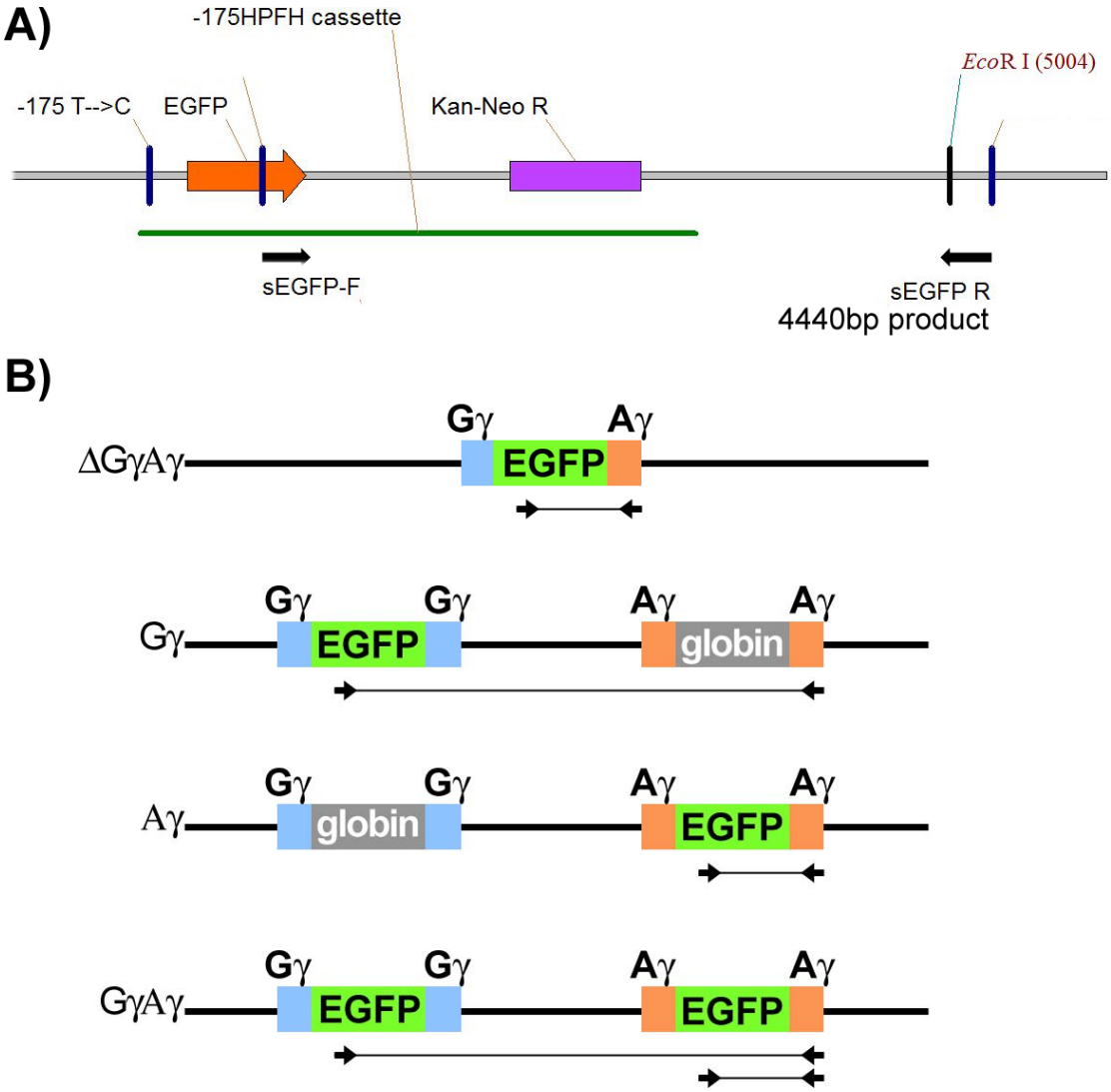


Figure 55: Schematic of 3'junction PCR

A) Mapping of -175 HPFH cassette insertion area of pEBAC/148β :: Δ ^{G}γ-^{A}γ EGFP . -175 HPFH cassette was shown in thin green line. The PCR forward and reverse primers were represented as black arrows.

B) Illustration showing possible -175HPFH EGFP cassette site of integrations at γ-globin genes promoter. 3'junction PCR primers were shown as black arrows.

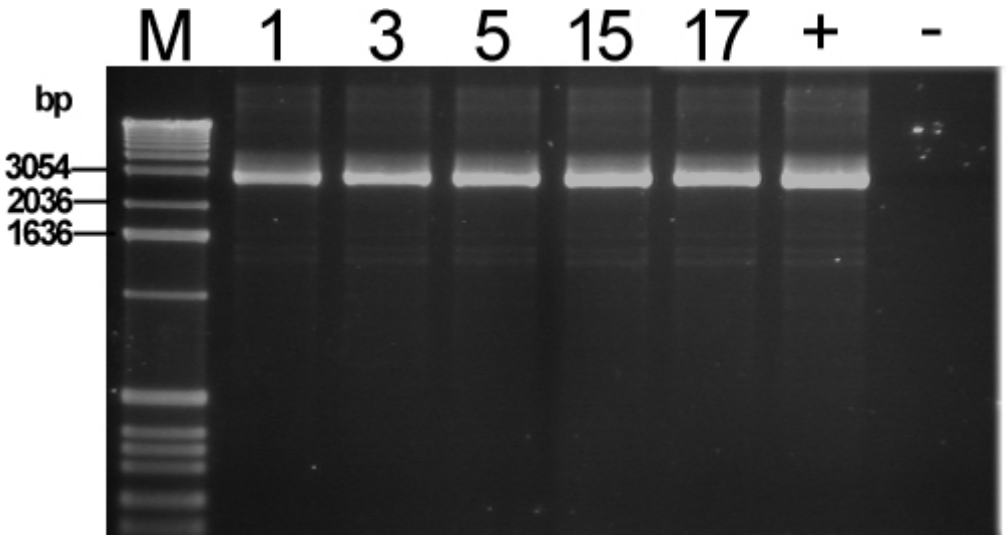


Figure 56: PCR analysis of 3' junction of recombination cassette integration site

The recombinant clones were amplified across 3'junction of -175HPFH recombination cassette. Forward primer binds at EGFP gene inside the cassette, while reverse primer binds at 3'promotor of $^{A}\gamma$-globin gene, outside the cassette.

Lane	M	:	Molecular weight Marker X (Roche)
Lane	+	:	pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP DNA
Lane	-	:	pEBAC/148b DNA
Lane	1-17	:	Individual recombinant BAC clones

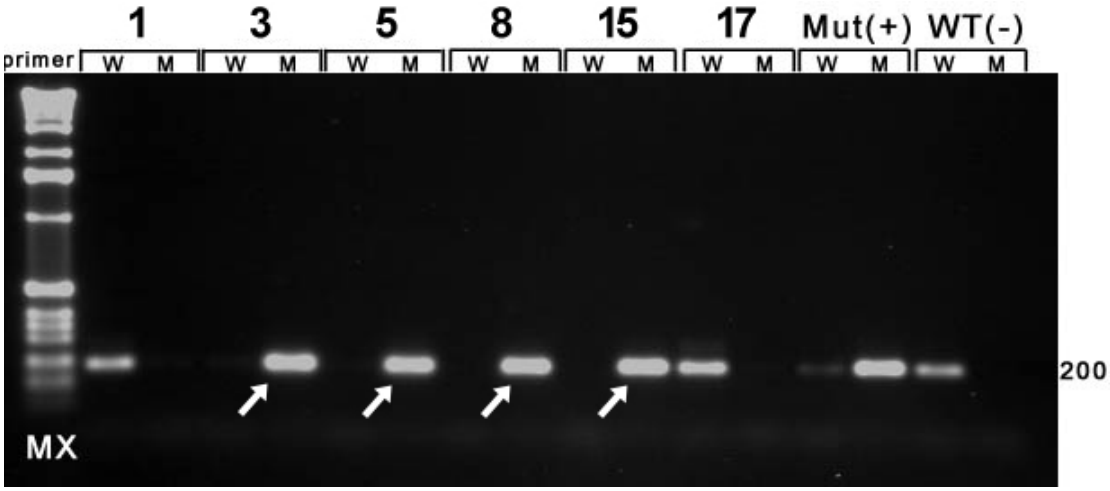


Figure 57: Allele specific PCR (ARMS-PCR) screening of the recombinant clones for -175 (T→C) HPFH point mutation

Allele specific PCR using both wild type (W), and -175HPFH mutant (M) primers for recombinant BAC clone screening was shown. Six recombinant clones (1, 3, 5, 8 15, 17) were screened. -175HPFH PCR fragment was used as a mutant control (Mut (+)), and pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP was used as a wild type control (WT (-)). The 200bp PCR product of clone 3, 5, 8, 15 which contained -175HPFH (T→C) point mutation was marked (arrow).

Lane	MX	:	Molecular Weight Marker X (Roche)
Lane	Mut(+)	:	-175HPFH recombination cassette as template
Lane	WT (-)	:	pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP as template
Lane	1-17	:	Individual recombinant BAC clones

Primer W	:	Reaction using primer specific for wild type
Primer M	:	Reaction using primer specific for -175HPFH mutation

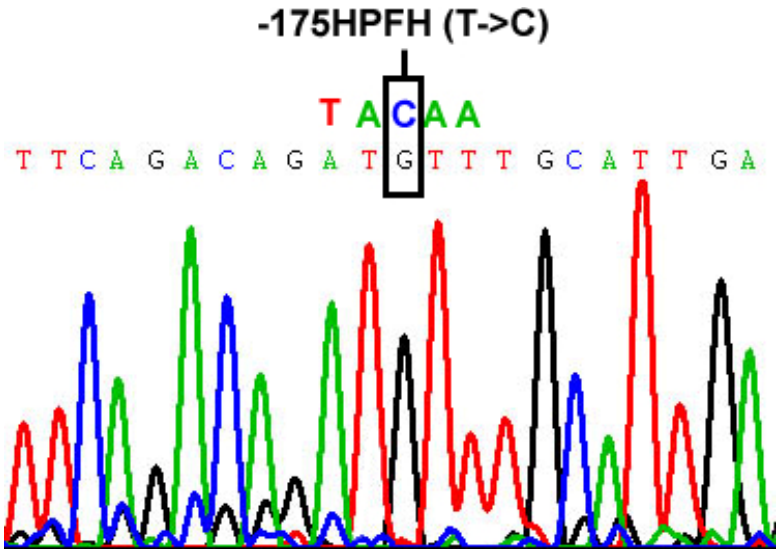


Figure 58: Sequence analysis of a recombinant BAC clone with -175 HPFH point mutation

Sequencing chromatogram demonstrates the -175HPFH (T→C) point mutation at position -175 upstream of $^{G}\gamma$ promoter.

Note: Reverse primer was used for sequencing reaction; hence the sequence acquired was the complementary strand sequence.

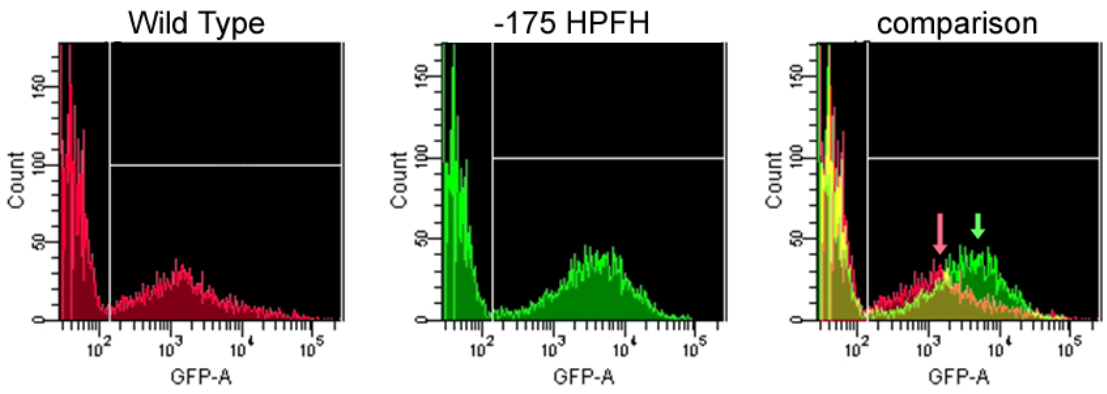


Figure 59: Histogram comparing EGFP expression profile between wild type, and -175 HPFH pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP transient transfected cells.

Wild type (red), and -175 HPFH (green) pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP transient transfected KEB cell were analyzed by flow cytometer. Median peak fluorescence (MPF) values were obtained from viable EGFP expressing cell population. Comparison panel shows overlay of wild type and -175 HPFH histograms. The positions of the peaks were marked by arrows coloring accordingly.

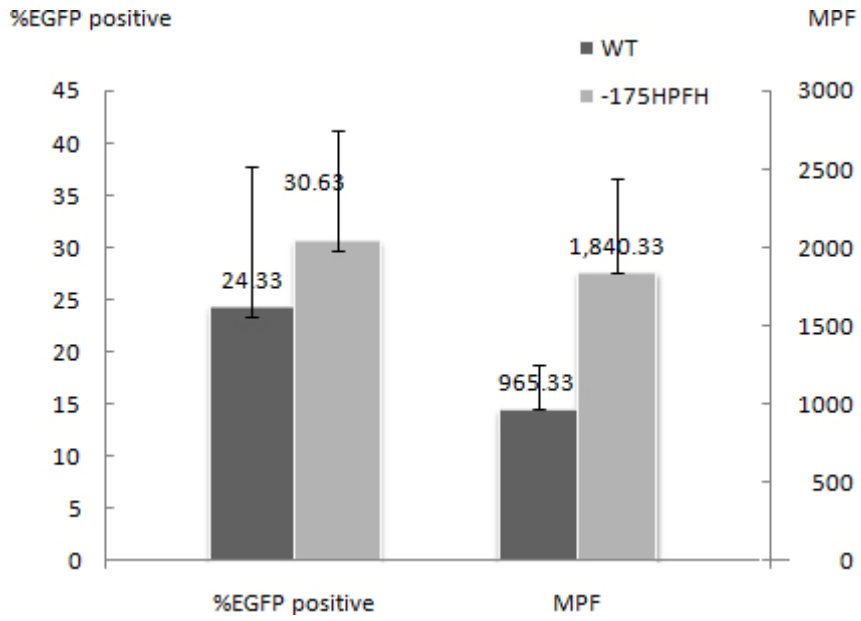


Figure 60: EGFP expression in KEB transient transfected with wild type and -175HPFH pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP

Percentage of EGFP expressing cell and median peak fluorescence (MPF) comparing between KEB cell transient transfected by wild type pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP(WT); were shown in dark grey column, and -175 HPFH pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP were shown in light grey column. Data values were shown on top of each column.

7.4 Pharmacological induction of -175 HPFH cell

The -175 HPFH mutation which results in upregulation of γ-globin is believed to act by modifying interactions between the DNA promoter and associated transcription factors. For example, a number of studies have demonstrated that the presence of the -175 HPFH sequence results in altered affinity of the promoter for erythroid-specific transcription factors Oct-1 and GATA-1 (273-275). Therefore, the inclusion of the -175 HPFH point mutation on a γ-globin screening BAC construct could potentially help elucidate the mechanisms of action of some γ-globin inducers. Hypothetically, a γ-globin inducing compound which is unable to generate any further increases in EGFP expression from the -175 HPFH BAC, is likely to be acting through pathways which are related to the -175 HPFH mechanisms (i.e. exerting effects by disrupting transcription factor/DNA interactions). Alternatively, if inducing compounds remain effective and are capable of further upregulating EGFP expression in the -175 HPFH BAC, this would suggest the inducers act through a different pathway to increase γ-globin expression.

In order to test the validity of this theory, a number of compounds previously shown to induce γ-globin expression were evaluated using this novel EGFP-based genomic reporter assay. K562-EBNA1 (KEB) cells were transiently transfected with either a wild type or an HPFH -175 modified pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP construct. Transfected cells were then flow sorted to obtain a 100% EGFP positive population and inductions were performed as previously described for normal K562 small scale inductions. In brief, experiments were performed in duplicates and transfected cells were incubated with previously identified compounds for five days. Four different concentrations of each compound was also tested in order to identify optimal concentrations (Appendix 1). EGFP expression levels were determined by flow cytometry analysis following induction (Table 14 and Figure 61).

The results of these assays indicated that the γ-globin inducers can be roughly separated into five major categories based on induction capabilities. First, hemin (0-100 μM) is able to induce extreme levels of EGFP expression in both wild type and HPFH clones, justifying the inclusion of this compound as a positive control. The second class are strong inducers of γ-globin promoters (100-200% increase) and

include the DNA binding drugs cisplatin (20 μM) and G4 (10 μM). These compounds were also more active in the wild type clone compared to the HPFH clones. Third, a group of moderate inducers (47-74% EGFP increases in wild type and 15-25% increases in HPFH clones) also included DNA binding drugs such as Tallimustine, G2 and G3. Again, these compounds had a more substantial inducing effect on wild type clones compared to HPFH clones. Fourth, hydroxyurea (100 μM) demonstrated moderate inducing capabilities but did not discriminate between WT and HPFH clones, inducing both promoters by approximately 28%. The final two compounds, butyrate and wheat grass extract both had minimal or no γ-globin inducing properties.

Our original hypothesis was that agents which act through the same mechanisms as the -175 HPFH mutation should demonstrate induction capabilities against the WT γ-globin promoter but should have no effects on the -175 HPFH γ-globin promoter. Unfortunately, initial results from this screen indicate that none of the tested compounds fulfill these criteria exactly. However, a number of compounds which were active against the -175 HPFH promoter nevertheless demonstrated substantially improved induction capabilities when tested against the wild-type promoter. In addition, many of these compounds (cisplatin, tallimustine, G2, G3 and G4) are DNA binding drugs, suggesting that their mechanism of action at least partially overlaps with the -175 HPFH pathways. Alternatively, it is possible that the -175 HPFH mutation is insufficient to upregulate γ-globin expression to its full extent, therefore, the addition of inducing compounds could stimulate further upregulation of γ-globin expression.

Previous studies have reported that some DNA-binding drugs are capable of interfering with transcription factor binding in a sequence-dependent manner (110, 276, 277). Furthermore, certain DNA-binding drugs, such as mithramycin and chromomycin, have been shown to induce γ-globin expression by interacting specifically with the γ-globin promoter in the same regions bound by transcription factors, which are also the regions found mutated in non-deletional HPFH (126). It is also possible that cisplatin, tallimustin, G2, G3 and G4 could act by altering protein binding affinities in or around the -175 HPFH mutation. This may result in increased affinity for positive regulatory factors, decreased affinity for inhibitory factors or a combination of both. More specifically, the -175 position of the γ-globin promoter

contains the binding site for GATA-1 transcription factor as well as the octamer proteins (Oct1) and the presence of the -175 HPFH mutation in this position has been demonstrated to result in altered affinities for these two factors (273-275). Therefore, it is tempting to speculate that these DNA binding drugs in our assay also acts through a similar pathway, interfering with GATA-1 and/or Oct1 negative regulation and thereby upregulating γ-globin gene expression.

Hemin-induced upregulation of γ-globin expression has been linked to a number of different pathways including repression of special AT-rich binding protein 1 (SATB1) nuclear protein (278), activation of cGMP-dependent and cAMP-dependent pathways (279), and stimulation of glutathione S-transferase P1-1 (GSTP1-1) expression. In addition, hemin induction of γ-globin has also been associated with a drastic decrease in GATA-1 mRNA expression as well as reduced promoter binding activity of the GATA-1 protein (280). In this study, hemin was found to act on both the WT and -175 HPFH γ-globin promoters but generated lower relative induction levels when the HPFH mutation was present. As the -175 mutation is believed to result in decreased GATA-1 binding, it is possible that the addition of hemin was not able to generate greater increases in γ-globin expression by further reducing GATA-1 expression and binding, resulting in lower relative induction. However, as hemin is able to induce γ-globin expression through other pathways, expression of EGFP was nonetheless increased in cells with the -175 HPFH construct.

Hydroxyurea showed similar degree of EGFP increase between wild type, and HPFH cells. The mechanism responsible for HbF induction is believed to be mainly by its cytotoxicity, triggering rapid erythroid regeneration and leads to selection of HbF-producing erythroid progenitors(48). Recently, numbers of mechanism involved in hydroxyurea induced HbF stimulation have been reported, such as activation of soluble guanylate cyclase (sGC)(257), and GATA-1, GATA-2(281, 282). Since GATA-1 transcription factor and bind to the -175 area of the γ-gobin promoter, it could be speculated that that action through -175 HPFH is one of many mechanisms of hydroxyurea-induced γ-globin induction. The similar degree of EGFP increase between the wild type and HPFH cells does not give the clear cut of the -175HPFH specificity of the compound. This is because, despite the similar % EGFP increase

parameter between the wild type, and the -175 HPFH assay, the median peak fluorescence (MPF) was still higher in the -175 HPFH one.

Both wheat grass and butyrate have been previously reported to stimulate γ-globin expression so the results obtained were slightly unexpected. It is possible that the mix of components utilized in the wheat grass extract might interfere with the inducing properties of the wheat grass itself. However, since many of the components in the extract as well as information regarding their properties are unknown, it is not possible to interpret this data. Similarly, due to inadequate data about butyrate induction in wild type cells, we are unable to draw any conclusions at this point .

In conclusion, the -175 HPFH mutation was found to increase EGFP expression of pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP by ~2 fold in KEB cells compared to the wild type promoter. Therefore, the -175 HPFH reporter assays used in combination with the wild type assay provide a good model for screening Hb F inducers which act specifically through the HPFH mechanism. However, the unstable nature of these episomal reporter assays would not be convenient for high-throughput screening of potential inducing compounds. Future developments and improvements of this assay should involve integration of these constructs to form stable reporter systems. Nonetheless, this system could still be beneficial for studying mechanisms of γ-globin upregulation of newly identified compounds in future investigations.

Table 14: Induction of -175 HPFH transient transfected cell with HbF inducers

EGFP expression and percentage of cell viability of K562-EBNA1 (KEB) cell transient transfected by wild type and -175 HPFH EGFP BACs induced by indicated compounds for 5 days were shown. Experiments were repeated twice.

WT = Wild type pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP

HPFH = -175 HPFH pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP

MPF = Median peak fluorescence

% Inc = Percentage of EGFP expression increase

$((MPF_{ind}-MPF_{unind})/MPF\ un_{ind}) \times 100$

%Viab = Percentage of viability cell detected by PI staining

		WT			**HPFH**		
compound	**Concent-ration**	**MPF**	**% Inc**	**%viab**	**MPF**	**% Inc**	**%viab**
Hemin	75uM	9965	754.95	29.0	17322	549.49	46.3
Hydroxyurea	100uM	1490	27.84	76.9	3416	28.08	72.9
Butyrate	500uM	1330	14.07	83.9	2873	7.72	80.9
Cisplatin	20uM	3251	178.89	23.0	5533	107.44	28.5
Tallimustine	100nM	1778	47.510	47.9	3077	16.07	45.3
Wheat Grass	4%	1455	24.839	62.6	2172	-18.56	52.5
G2	100nM	2039	74.946	58.2	3191	19.648	56.4
G3	7.5uM	1836	57.529	62.7	3357	25.87	54.5
G4	10uM	2876	146.761	49.6	3445	29.17	46.6

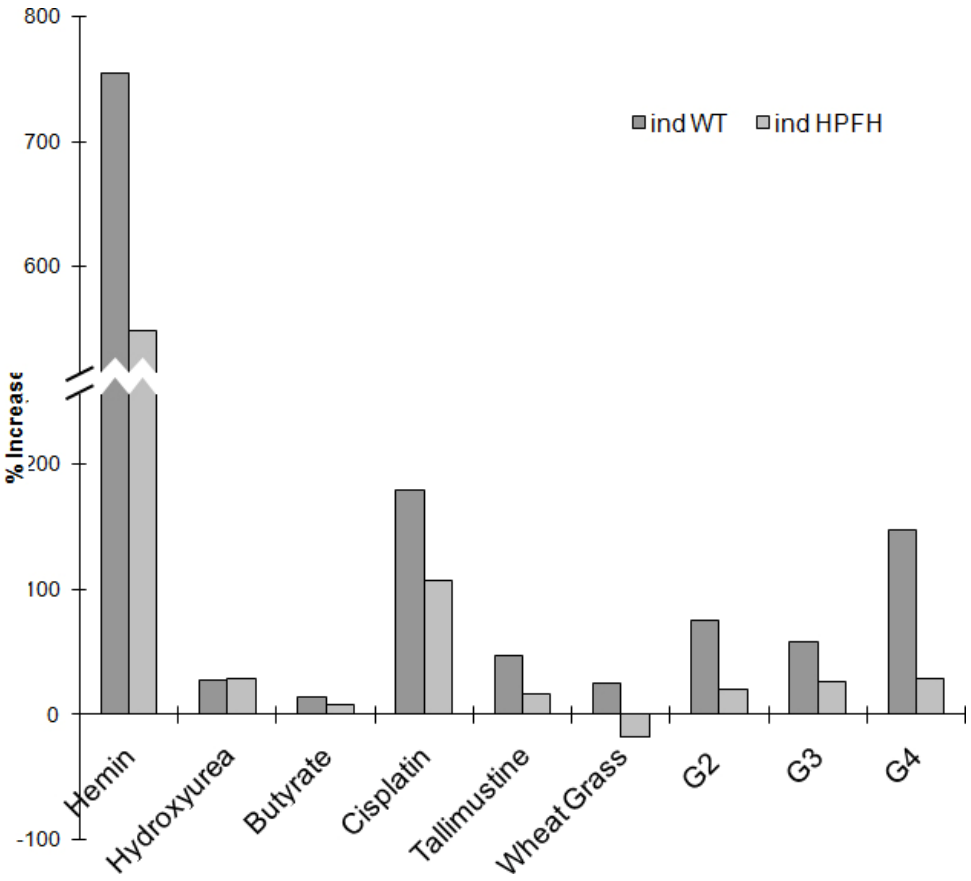


Figure 61: The comparative EGFP expression of the wild type and -175 HPFH BAC transfected cells induced by several HbF inducers previously reported (data were derived from Table 13).

Bars represents increased percentage of EGFP comparing induced and uninduced wild type (dark grey) or HPFH (light grey) cells.

WT = Wild type pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP

HPFH = -175 HPFH pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP

Ind = Percentage of EGFP expression increase

$((MPF_{ind}-MPF_{unind})/MPF\ un_{ind}) \times 100$

CHAPTER VIII
RESULTS AND DISCUSSION
PRODUCTION OF TRANSGENIC MICE

Transgenic mice provide a good *in vivo* system for studying of gene function or, in this case, upregulation of fetal hemoglobin. Because the K562 :: $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP cellular genomic reporter proved to be a useful model for screening of the HbF inducing compounds, the same genomic fragment of the pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ was introduced into the fertilized oocytes of the C57BL/6 mouse to create the transgenic mice with EGFP reporter for γ-globin expression. In this chapter, the results showed the production, characterization and *in vitro* induction of the erythroid culture derived from the transgenic mice by HbF inducing agents.

8.1 Production of transgenic mice

The pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ clone was used to generate transgenic mice. The linear 185 kb β-globin genomic fragment was separated from the 17 kb vector backbone by digestion with *Not*I and PFGE. The fragment was then purified and concentrated by micro-dialysis before microinjection into C57BL/6 fertilized oocytes. Eight oocytes were transferred, yielding 45 pups, of which 4 (6.67%) were transgenic. Founder mice were identified by PCR screening of tail DNA specific for EGFP gene (Figure 62A) and human β-globin gene (Figure 62B) to ensure the transgene integrity in both ends. The result showed that 3 of transgenic lines were positive for both genes, while one of which was positive for EGFP gene only. The PCR screening was reconfirmed using skin biopsy. This was assumed that there was a partial transfer with the 5' end missing in the genomic fragment. Subsequently, the partial transferred mice were dead because of the complication from skin biopsy. Afterward, another founder died due to difficulty giving birth, genotyping of the dead pups showed the presence

of the transgene, indicating transgene transmission. The two remained identified founder mice were mated with C57BL/6 females or males to generate F1 progeny with germ-line transgene transmission. One of the remained founder lines failed to transmit the transgene to the F1 generation. These animals were assumed to be germ-line mosaics. Only one transmitting founder line was bred with C57BL/6 mice to establish independent colony. The line is named GG.

8.2 Characterization of transgene mice

8.2.1 Transgene integration site

Fluorescent *in situ* hybridization (FISH) analysis performed from fibroblasts using the pEBAC/148β probe showed the single integration site of the human β-globin locus with $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP transgene in the mouse chromosome 17 in the transmitting founder line (Figure 63).

8.2.2 Hematological studies

The hematologic indices of transgenic and control animals from transmitting founder line at 6-8 weeks of age were determined on samples extracted by retro-orbital eye bleeds (Table 15, Figure 64). Raw data was shown in Appendix B. Heterozygous γ-globin EGFP mice showed insignificantly more anemic than the wild type, as is reflected with slight decrease in hemoglobin concentration (HGB), hematocrit (HCT), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and red blood cell count. HCT, which is determined by the size and numbers of red blood cells, was decreased by 3.77% in heterozygous $\Delta^{G}\gamma^{A}\gamma$-globin EGFP compared to WT mice. MCH, the average concentration of hemoglobin in each cell, was reduced by 11.82% in transgenic mice to WT littermates. Red blood cell (RBC) counts were about the same in both transgenic and wild type mice. However, there were markedly increase in red cell distribution width (RDW) ($p = 0.00188$), and reticulocyte count (%RETIC) ($p = 0.00037$). The red cell distributions widths (RDWs) represent the regularity of cell shape and volume and can be an indirect measurement of membrane damage. In WT mice, red cells are a consistent size and

volume which is represented by a low RDW value. In heterozygous $\Delta^{G}\gamma^{A}\gamma$-globin EGFP mice, membrane damage and cellular disruptions result in significantly increased RDWs of 1.7-fold. The % circulating reticulocytes is an indirect indicator of enhanced erythropoiesis which can be stimulated by chronic anemia. In slight anemic $\Delta^{G}\gamma^{A}\gamma$-globin EGFP mice, % reticulocytes increased by 2.09-fold as compared to WT mice. Altogether, the abnormalities of the hematological indices could be caused by (1) EGFP expression, or (2) presence of human β-globin gene on the mouse globin background. Expression of human β-globin in the transgene has been reported to be accounted for 10% of total β-globin production in the transgenic mice carrying the normal human β-globin locus (from pEBAC/148β) (231). The studies of hybrid molecule of human β-and mouse α-globin in the transgenic mice showed higher oxygen affinity than the mouse native hemoglobin molecule (Kongsaeree, P. *et al.*, manuscript in preparation). If the hypothesis was correct, the hematological parameter should be somewhat the same, unfortunately it was not. Besides, the reported cases of the high oxygen affinity usually accompanied by the elevated hemoglobin concentration (283), which is also opposite of what was found in the transgenic mice. However, it was shown that the high oxygen affinity hemoglobin induced erythropoietin production, which leads to increase erythropoiesis. This may explain the increased RDW and %RETIC in the $\Delta^{G}\gamma^{A}\gamma$-globin EGFP mice.

8.2.3 EGFP expression in adult peripheral blood

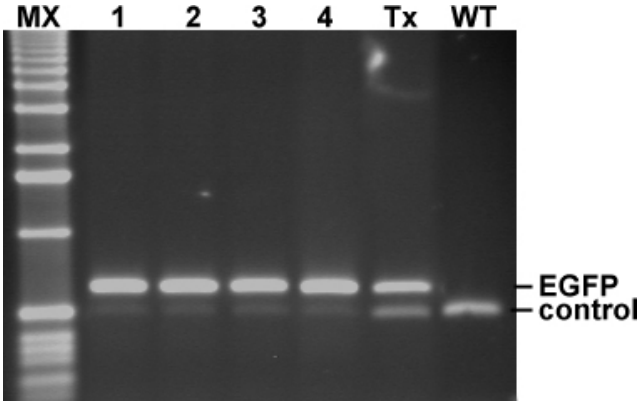
Transgenic mice carrying the normal human β-globin locus (from pEBAC/148β) (231) showed normal ε- to γ globin switching on day 10.5, and γ- to β-globin switching on day 13.5. Hence, the EGFP expression under the regulation of γ-globin promoter in the context of β-globin locus should express in the same manner. However, the EGFP was still detected in adult peripheral blood. It was presumed that site of integration and absence of intergenic region between $^{G}\gamma$- and $^{A}\gamma$-globin of the genomic fragment from pEBAC/148β:: $\Delta^{G}\gamma$-$^{A}\gamma$ might have a role on this. Under the fluorescence microscope, the EGFP seemed to be expressed specifically in erythroid cell. The EGFP expression of the peripheral blood cells of the heterozygous $\Delta^{G}\gamma^{A}\gamma$-globin EGFP mice was investigated using flow cytometry analysis (Table 16). The

EGFP index was calculated by percentage of EGFP positive cells multiply by median peak fluorescence (%EGFP x MPF). The percentage of EGFP positive cell and median peak fluorescence vary among each mouse, even among the littermates. The mice with high percentage of EGFP positive cell have a tendency for high median peak fluorescence.

The correlation between hematological indices and EGFP index of the heterozygous $\Delta^{G}\gamma^{A}\gamma$-globin EGFP mice was investigated, and no significant change in hematological indices as the EGFP index increased, except for mean corpuscular volume (MCV). The MCV increased as EGFP index rose, which probably was a result from the higher protein content in the blood cell. Since elevated red cell distribution width (RDW) and percentage of reticulocyte were not proportionate to the EGFP expression in the red blood cell, it is believed that the degree of EGFP expression is not responsible for the abnormalities in RDW and %reticulocyte as speculated earlier. This is supported by the study showing that EGFP expression has no side effect on the organ tissue (284).

The EGFP expression in the peripheral blood obtained during tail biopsy remained at the same range as the mice were continuous bred into the next generation (data not shown).

A)



B)

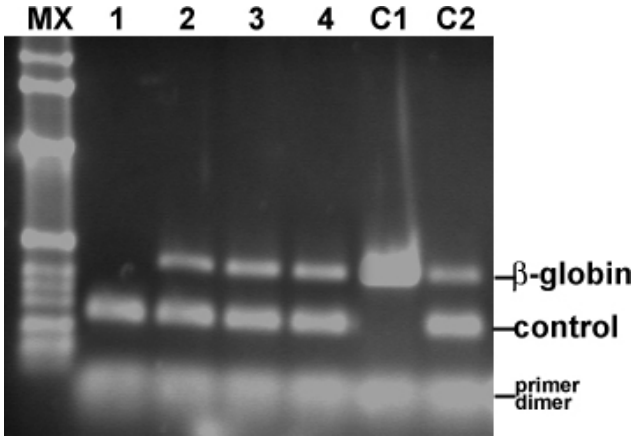


Figure 62: Genotyping and determination of transgene integrity

PCR using primer pairs specific to EGFP, and human β-globin sequence of the transgene fragment wa used for genotyping and determination of transgene integrity. A) EGFP-specific multiplex PCR, B) β-globin-specific multiplex PCR

Lane MX : Molecular weight marker X (Roche)

Lane 1-4 : Founder transgenic mice line 1-4

Lane Tx : Positive control for EGFP using genomic DNA from transgenic mouse containing EGFP gene

Lane WT : Negative control for EGFP using genomic DNA from wild type C57BL/6 mouse

Lane C1 : Positive control for human β-globin using pEBAC/148β

Lane C2 : Positive control for human β-globin using genomic DNA from transgenic mouse containing human β-globin gene

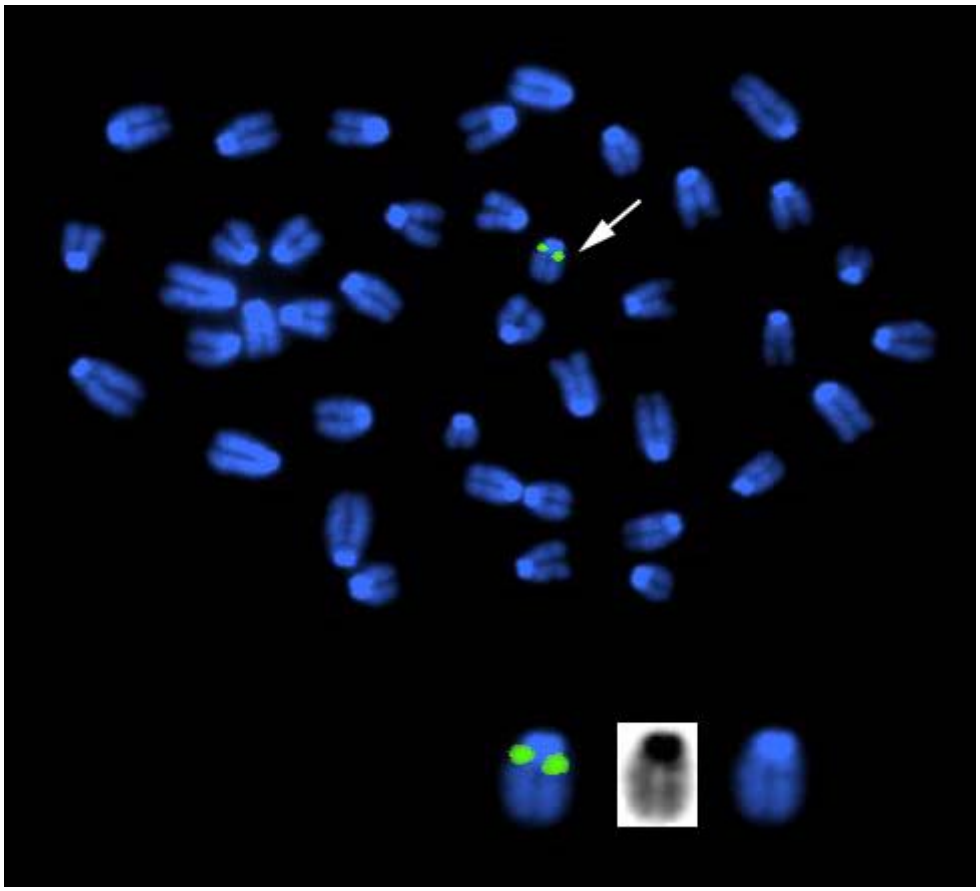


Figure 63: FISH analysis of ΔGγAγ EGFP transgenic mouse

Transgene integration site in transmitting transgenic line was determined by FISH using pEBAC/148β as a probe. The arrow indicates the single integration site of the human β-globin locus containing ΔGγAγEGFP modification transgene in mouse chromosomes. The enlarged chromosomes depicted chromosome 17 with homozygous transgene.

Table 15: Hematological indices of $\Delta^G\gamma^A\gamma$-globin EGFP transgenic mice.

Genotype	n	RBC (10^6/μl)	HGB (g/dl)	HCT (%)	MCV (fl)	MCH (pg)	RDW (%)	RETIC (%)
GG	11	10.84 ± 0.64	13.97 ± 0.60	48.21 ± 1.75	44.59 ± 2.47	12.93 ± 0.87	23.66 ± 2.72	6.53 ± 1.06
WT	5	10.34 ± 0.34	15.16 ± 0.51	50.10 ± 1.72	48.44 ± 0.24	14.66 ± 0.05	13.84 ± 0.99	3.13 ± 0.94

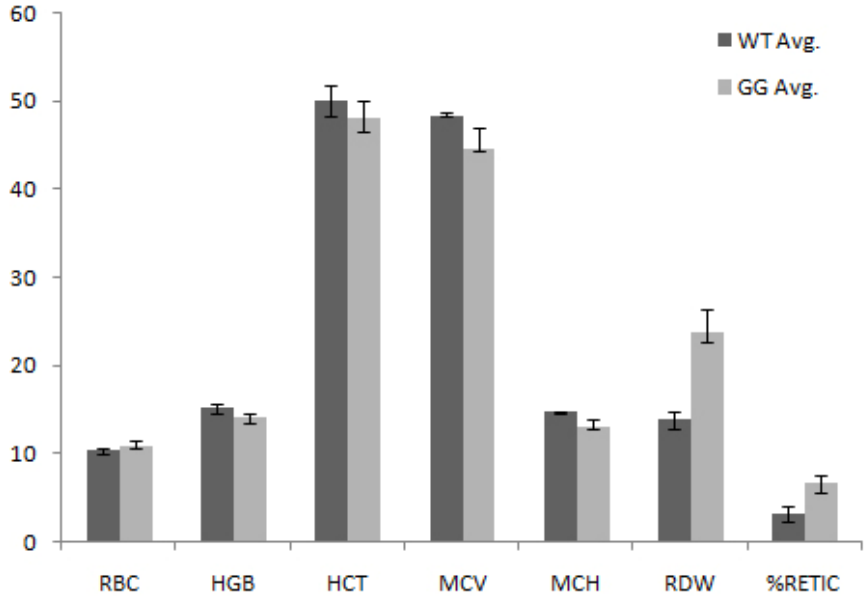
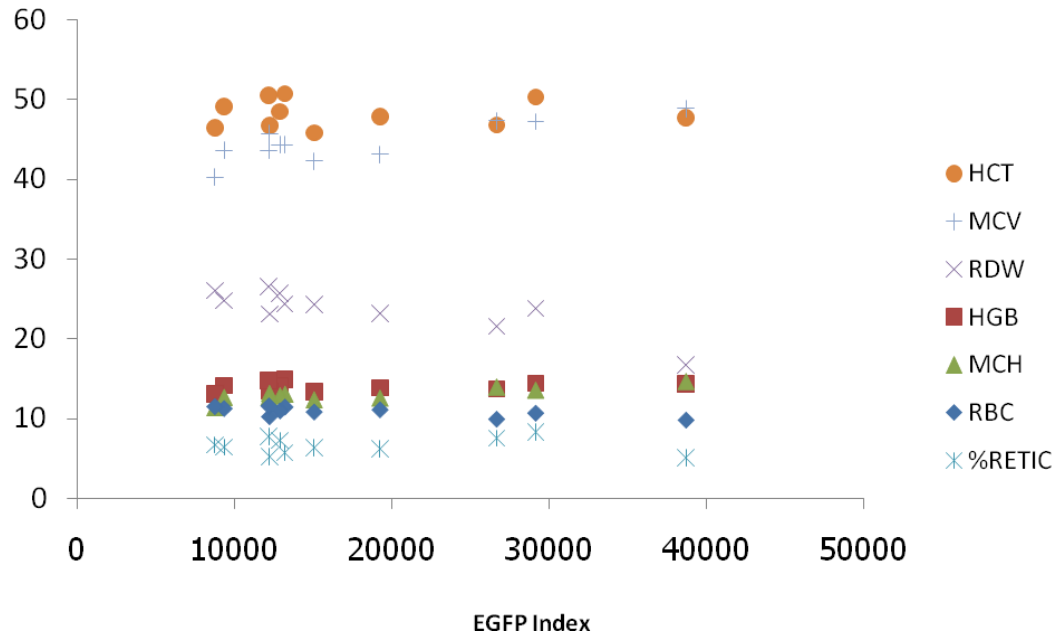


Figure 64: Hematological indices of heterozygous $\Delta^G\gamma^A\gamma$-globin EGFP transgenic mice compared to wild type mice.

Average value of hematological parameters of 11 heterozygous γ-globin EGFP mice (grey bar), and 5 wild type C57BL/6 mice (black bar) were shown.

RBC	:	Red blood cell
HGB	:	Hemoglobin concentration
HCT	:	Hematocrit
MCV	:	Mean corpuscular volume
MCH	:	Mean corpuscular hemoglobin
RDW	:	Red cell distribution width
%RETIC	:	Percentage of reticulocyte

Table 16: EGFP expression and hematological indices of the $\Delta^{G}\gamma^{A}\gamma$-globin EGFP transgenic mice



The retro-orbital eye bleeds were analyzed for EGFP expression by flow cytometer and hematological indices by full blood examination. All the samples were simultaneously analyzed. The EGFP index was calculated by percentage of EGFP positive cell (%EGFP +ve) multiplied by median peak fluorescence (MPF). The hematological parameter of each individual were shown in relative to the EGFP index.

8.3 EGFP expression in murine primary erythroid culture

In order to closely investigate the EGFP expression during erythroid differentiation, the primary culture from each time point of mice development were acquired and followed throughout the culture time. Later on, the ability of the cell culture to respond to chemical induction of HbF was investigated. Dolznig *et al.* (285) have previously reported an *in vitro* murine culture system, which is capable of recapitulating many of the normal cellular processes of erythroid precursor proliferation and maturation. In this culture system, the most immature erythroid precursor cell, the burst-forming unit for erythropoiesis (BFU-e), can be isolated from murine fetal liver or adult bone marrow and selectively proliferated in serum-free media containing optimized concentrations of stem cell factor (SCF),erythropoietin (EPO) and dexamethasone, a glucocorticoid which suppresses differentiation (286). Following *in vitro* expansion, BFU-Es can be induced to differentiate along the erythroid lineage by culturing with high concentrations of Epo and insulin (287) resulting in four to five rapid CFU-Es cell divisions and accumulation of hemoglobin (288). In general, this culture system is able to recapitulate *in vivo* erythropoiesis to a reasonable extent.

According to previous report of transgenic mice carrying the normal human β-globin locus (from pEBAC/148β) (231) showed γ- to β- globin switching on day 13.5. Therefore erythroid cell cultures isolated from fetal liver of day 13.5 transgenic mice were investigated, and the exponential pattern of the cumulative erythroid cell growth over 8 days observed (Figure 65). The EGFP expression drastically decreased over time, which was reflex by the EGFP index (%EGFP positive cell x median peak fluorescence) (Figure 66B) accounted by MPF value. The EGFP positive population was also slightly decreased (Figure 66A). The decline EGFP index was unexpected since hemoglobinization should occur as cell differentiates. Another possibility is that the expressed γ-globin gene gradually switched off as cell matures. The increase %EGFP positive population on the first day could be explained by contamination of the hepatocytes during erythroid isolation process. The fluorescence microscopic

morphology of murine primary erythroid cells were shown in Figures 67 and 68. The erythroid progenitor from liver and peripheral blood at day 17.5, which is the half-way of γ- to β-globin switching, was investigated. The MPF of cells from liver showed the lower MPF compared to that of day13.5 by 5-8 folds (Figure 69, 70). This corresponded with the silencing of γ-globin gene expression during the switching. There were no significant difference in MPF between liver cells and peripheral blood, which is not correlated with *in vitro* culture differentiation result. The *in vivo* similarity of MPF between two sites might infer that the γ-globin expression in erythroid progenitor cells and mature cells were somehow balanced. The %EGFP positive cells between two sources of cells were unable to be compared, due to possible contamination of the hepatic cells. The adult bone marrow cells at day 21.5 transgenic mice were also acquired, however, the amount of cell obtained was too low and no culture was performed.

It was perplexing that the EGFP expression in each stage of development fluctuated enormously among the transgenic mouse line, or even among the littermates. In addition, the persistence of EGFP expression in adult peripheral blood was still questionable. Unfortunately, since only one founder was obtained from microinjection, the site of integration effect could not be investigated.

8.4 Chemical induction of murine primary erythroid culture

Some of known HbF inducers and identified compound from high-throughput screening were tested on the primary erythroid culture from transgenic mice. The cells cultured for 3 days were treated for another 3 days with the varied concentration of compounds. This murine erythroid culture exerted dose-dependent response. The %increase in MPF and %increase in EGFP positive cell population of each compound at the concentration which gave highest induction was shown in Figure 71. The EGFP induction effect of chemical treatment exerted mostly on MPF value and slightly on %EGFP expressing cells. This indicated that the compounds were likely to further upregulate the cell with γ-globin gene expression, rather than to turn on the gene in the silence cells. It is worth mentioning that the EGFP induction efficacy pattern of

the compounds in this murine primary erythroid culture is quite similar to that of the γ-globin mRNA in K562 hugely different degress of induction from one model to another. This emphasizes the influence of the model to the accuracy for the drug screening. However, further investigation of this $\Delta^{G}\gamma$-$^{A}\gamma$ transgenic mouse model was needed; this includes transgene copy number determination, transgene globin mRNA expression study, and also *in vivo* treatment of HbF inducing compounds.

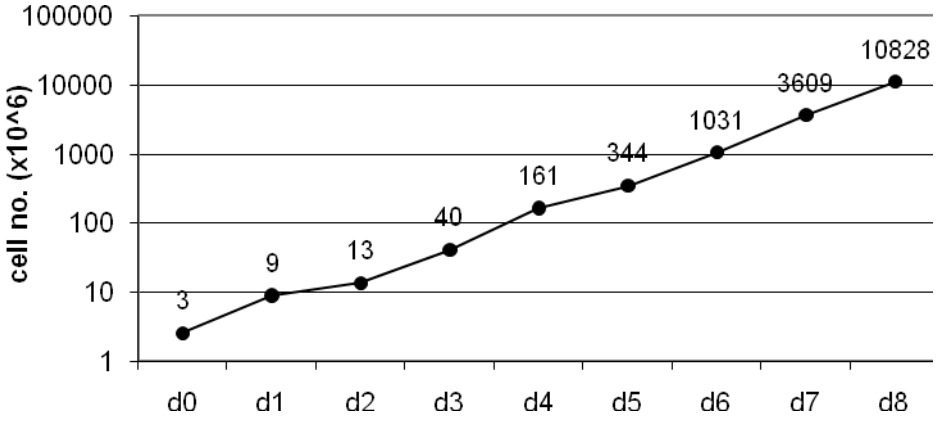
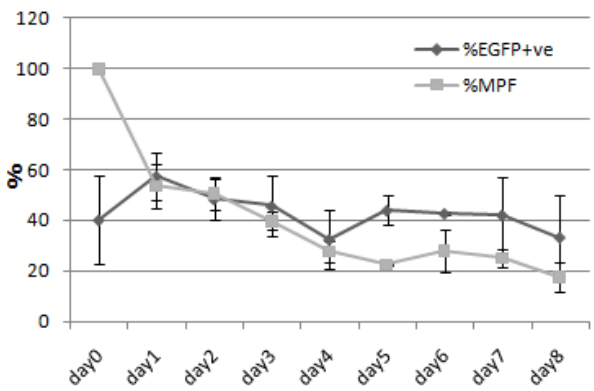


Figure 65: Murine erythroid culture cumulative cell growth

The cumulative cell number (Y-axis) over 8-day culture (X-axis) was in exponential trend.

A)



B)

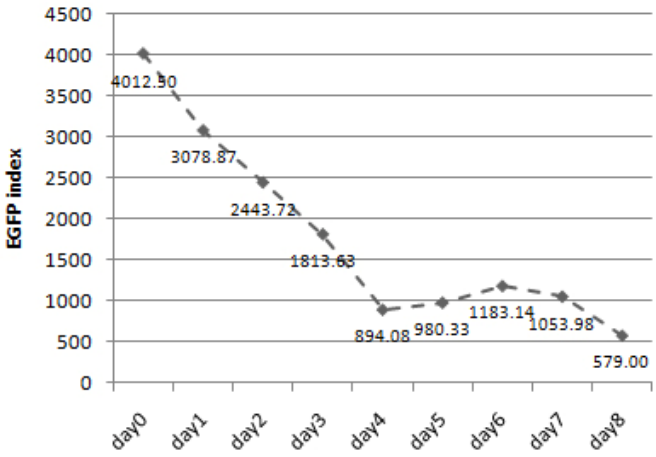


Figure 66: EGFP expression profile of murine primary erythroid culture over 8 days

A) %EGFP positive cell (black line) was slightly decreased. The % median peak fluorescence (%MPF) (grey line), which was calculated by dividing MPF at the indicated day by that of day 0, was clearly decreased.

B) EGFP index, calculated by multiplying %EGFP positive cells with MPF, showed significant decline on first 4 days of culture.

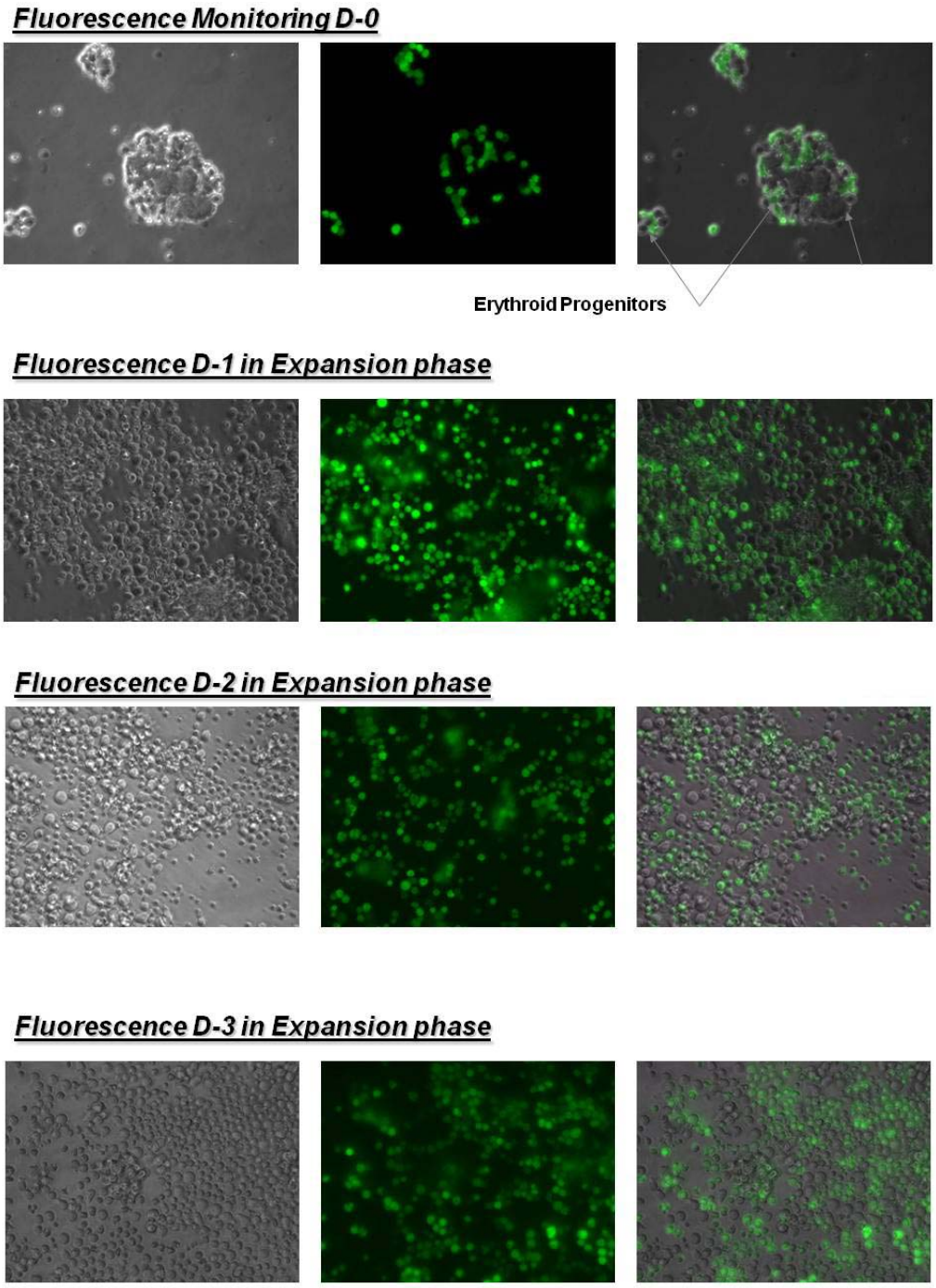


Figure 67: Murine erythroid culture under fluorescence microscope.

The transgenic mice primary erythroid cultures of the indicated day were shown. The magnification power is X40. EGFP was visualized (middle panel) and merged with the normal light (right panel) showing EGFP positive cells in total cell population.

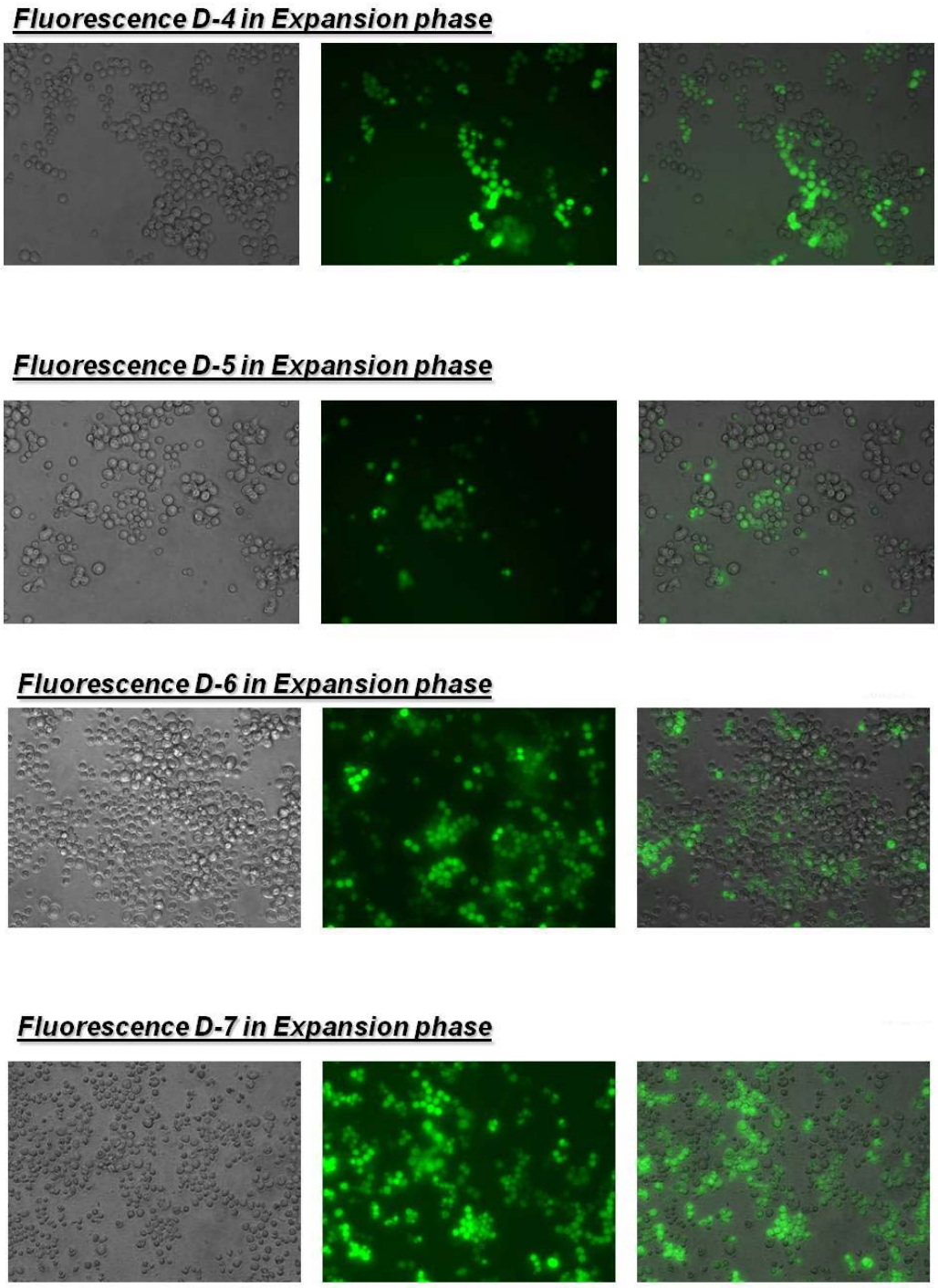


Figure 68: Murine erythroid culture under fluorescence microscope (continue)

The transgenic mice primary erythroid cultures of the indicated day were shown. The magnification power is X40. EGFP was visualized (middle panel) and merged with the normal light (right panel) showing EGFP positive cells in total cell population.

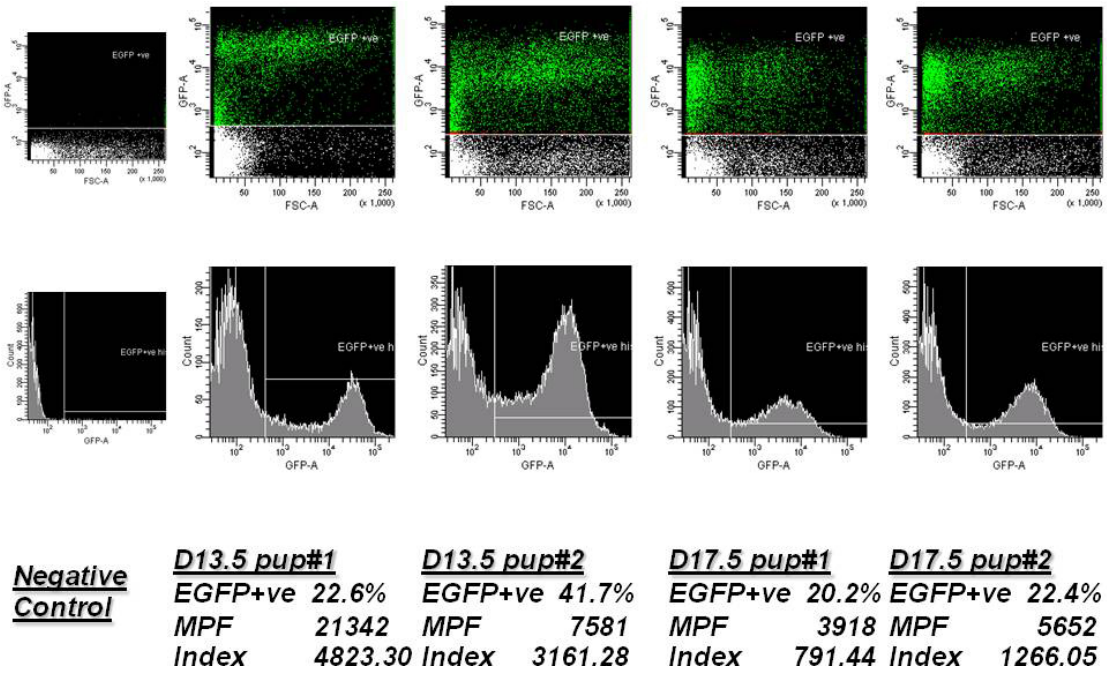


Figure 69: Flow cytometry analysis of fetal liver cell from day 13.5 and 17.5 transgenic pups

Top panel shows flow cytometry analysis scattergram representing EGFP expression (Y-axis) and size of the cell (FSC-A; X-axis). Bottom panel shows histograms of EGFP expression. The data was obtained from 4 littermates, comparing to the wild type control littermate. Index values represent EGFP+ve x MPF

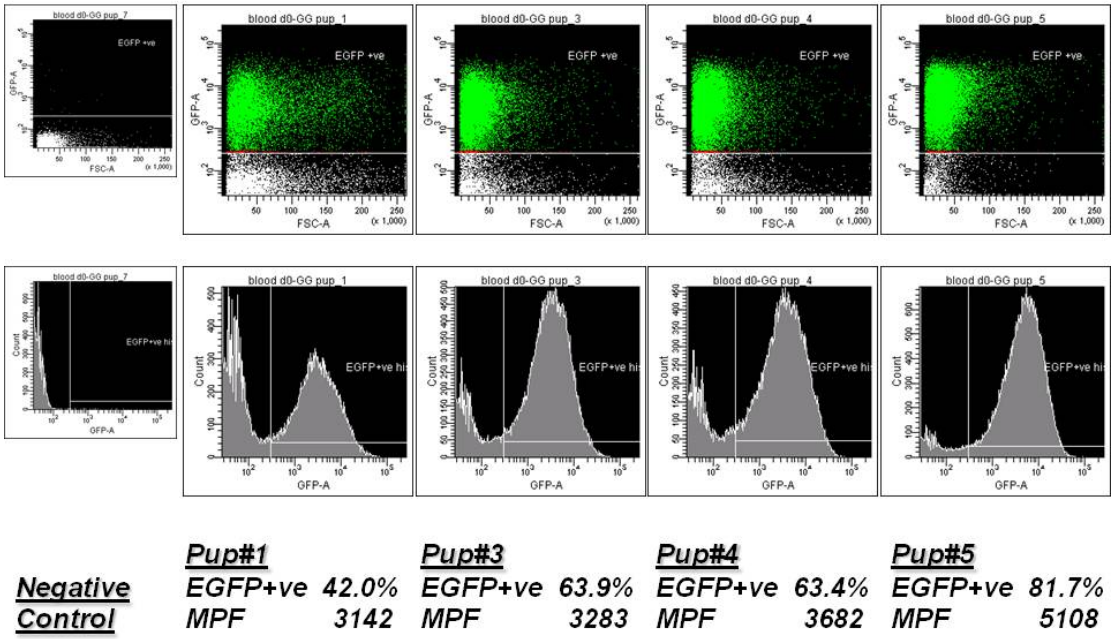


Figure 70: Flow cytometry analysis of peripheral blood from day 17.5 transgenic pups

Top panel shows flow cytometry analysis scattergram representing EGFP expression (Y-axis) and size of the cell (FSC-A; X-axis). Bottom panel shows histrogram of EGFP expression. The data was obtained from 4 littermates, comparing to the wild type control littermate.

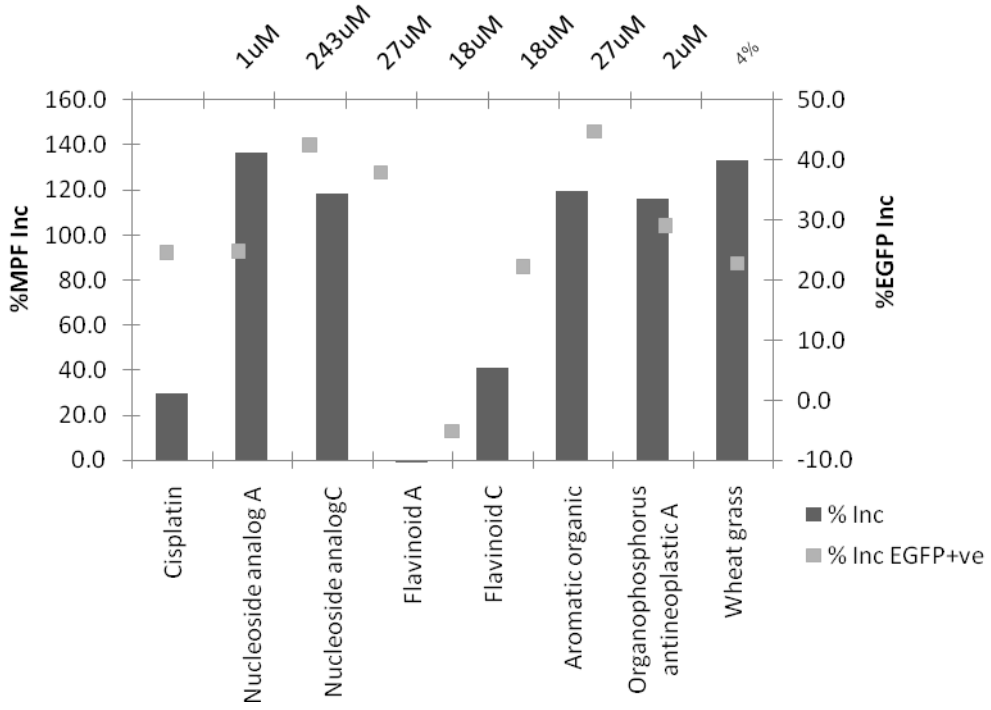


Figure 71: Chemical induction of transgenic mice erythroid culture.

Primary erythroid cultures from $\Delta^{G}\gamma$-$^{A}\gamma$ EGFP transgenic mice cultured for 3 days were treated with known inducers and high throughput validated compounds at indicated concentration. The %MPF increase (bar; left Y-axis), and % EGFP positive cell increase (square dot; right Y-axis) compared to uninduced cells were shown.

CHAPTER IV
CONCLUSION

β-Thalassemia is a major health problem in Thailand and many other countries. Chemical induction of HbF has been pointed out as a very promising therapy for β-thalassemia patients, especially in developing countries where the high-cost clinical management seems unapproachable.

Here, three fluorescence-based experimental models for studying and screening of HbF inducer were developed including the episomal -175 HPFH specific γ-globin reporter cellular model, stable γ-globin reporter cell-based high-throughput screening system, and the γ-globin reporter transgenic mice for *in vivo* study.

All models are based on the use of the large BAC carrying 175 kb genomic insertion with whole β-globin locus and the neighbouring olfactory receptor genes, and the ET recombination was employed for the BACs modifications. The -175 HPFH point mutation was successfully introduced by using counter selected homologous recombination. Two-fold increase in EGFP expression was obtained with HPFH construct in K562 cells, comparing to that of the wild type. The responses to each group of known HbF inducers by the HPFH and WT construct were distinct. The DNA-binding drugs exerted higher EGFP increase in the WT construct than the HPFH, indicating tendency for specificity toward -175 HPFH mechanism of HbF upregulation. The non-specific compounds such as hemin and hydroxyurea showed the opposite outcome. This system, once further developed in to the stable cell format, would greatly benefit screening of specific HbF inducers and investigation of the HPFH mechanism of γ-globin upregulation.

In order to identify novel HbF inducing agents, the high throughput screening based on the stable γ-globin reporter cells were optimized and performed. 2132 compounds, composed of FDA-approved drugs, natural products, bioactive compounds, and cellular toxins, were screened and 29 hit compounds were acquired.

Thirteen out of selected 19 compounds were verified by small-scale validation using the cellular reporter model. These validated compounds composed mainly of antiviral nucleoside analogs, and flavonoids. These two groups were outstanding in two different aspects; the antiviral nucleoside analogs represent the approved drugs with well studied background, while the flavonoids represent the natural-derived compounds well-known in the alternative medicine. Besides these compounds, the anticancer agents, hormone derivative, and even herbicides were identified.

Unfortunately, only a few validated compounds were specific solely for γ-globin mRNA upregulation in K562 cell line, while most of the compounds increase both γ- and β-globin mRNA. Coincidently, two specific compounds are also the DNA-binding anticancer drugs, which correlated with the results obtained from the -175 HPFH screening system. Only nucleoside analogs were progressed to the study in primary erythroid culture acquired from β-thalassemia/HbE patients, and two compounds were able to increase ratio of HbF by approximately 20%. An understanding of the mechanism of action would greatly facilitate the development of the compounds for clinical use.

Besides the cellular model, the 175 kb genomic fragment of the constructs carrying the γ-globin EGFP reporter gene was used to generate transgenic mice. One transmitting transgenic line was obtained and analyzed. EGFP expression could be detected in fetal liver, adult bone marrow and peripheral blood. EGFP level dropped during γ- to β-globin switching, but unexpectedly EGFP continued to be expressed throughout adult stage. Further study to investigate the site of integration and/or absence of intergenic sequence between $^{G}\gamma$- and $^{A}\gamma$-globin genes might contribute to this persistence EGFP expression. The transgene resulted in insignificant anemia, and strikingly induced anisocytosis and increased reticulocytes in the blood. The primary erythroid culture of transgenic mice fetal liver cells showed gradual decline of EGFP expression as the cells differentiated, supporting the model of HbF expression during erythropoiesis. The murine primary erythroid culture also showed response to the γ-globin induction of the newly identified compounds. The response of the transgenic mice erythroid culture seems to reflect the γ-globin mRNA level in the K562 model.

The developed screening systems proved to be useful in identifying compounds which stimulate γ-globin transcription and promote HbF production. The cellular models are invaluable models for screening and studying of the HbF induction, while the mouse models will be useful for *in vivo* and pathophysiological studies of HbF induction. The novel HbF inducer agents identified here can serve as another aspect of development of new HbF elevating compounds. Moreover, they further provide tools by which the multiple mechanisms of pharmacological up-regulation of γ-globin transcription may be investigated.

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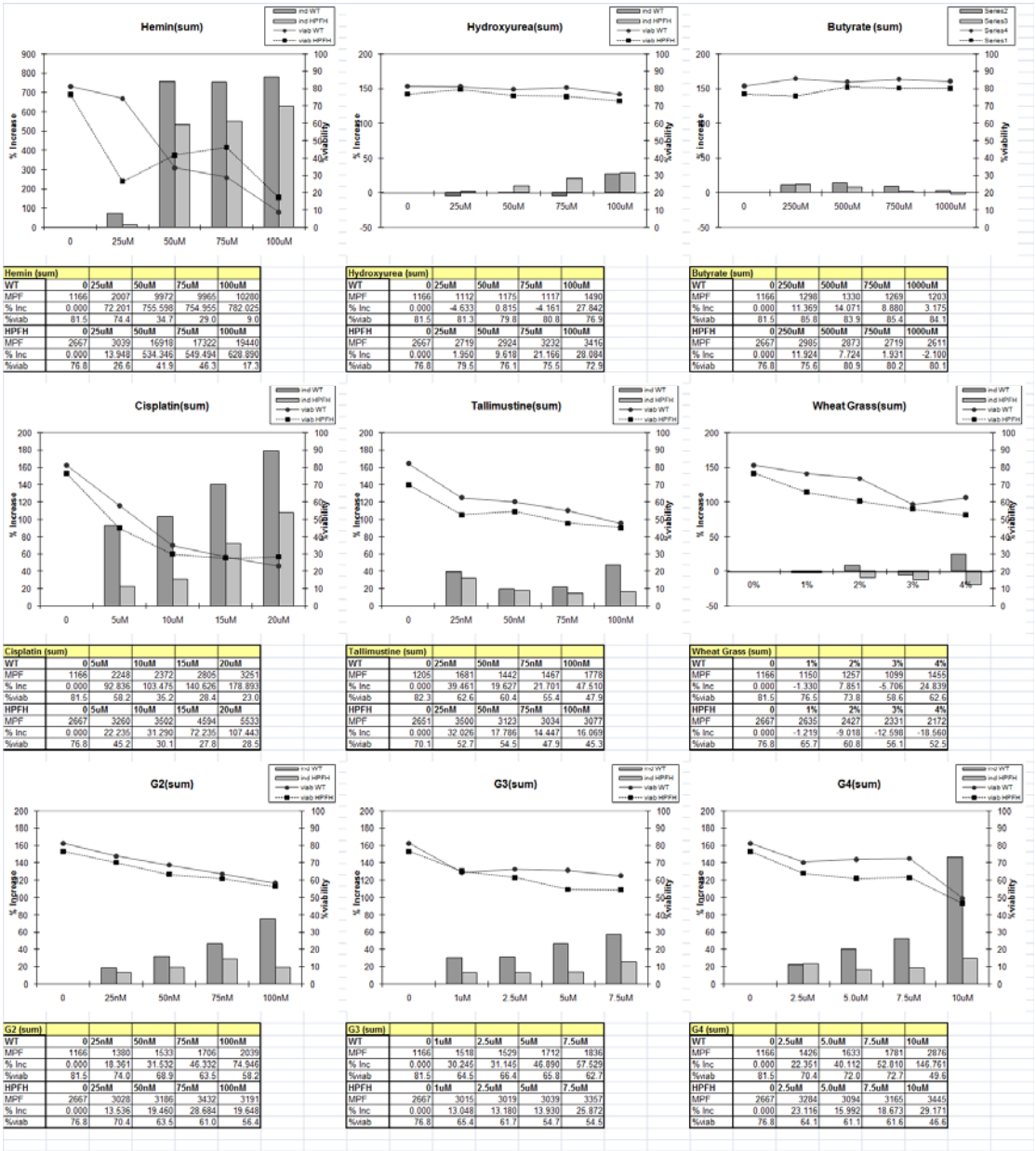
APPENDIX

APPENDIX A

Raw data of -175 HPFH BACs transfected cell EGFP expression induced by HbF inducers



Hemin (sum)					
WT	0	25uM	50uM	75uM	100uM
MPF	1166	2007	9972	9965	10280
% Inc	0.000	72.201	755.598	754.955	782.025
%viab	81.5	74.4	34.7	29.0	9.0
HPFH	0	25uM	50uM	75uM	100uM
MPF	2667	3039	16918	17322	19440
% Inc	0.000	13.948	534.346	549.494	628.890
%viab	76.8	26.6	41.9	46.3	17.3

Hydroxyurea (sum)					
WT	0	25uM	50uM	75uM	100uM
MPF	1166	1112	1175	1117	1490
% Inc	0.000	-4.633	0.815	-4.161	27.842
%viab	81.5	81.3	79.8	80.8	76.9
HPFH	0	25uM	50uM	75uM	100uM
MPF	2667	2719	2924	3232	3416
% Inc	0.000	1.950	9.618	21.166	28.084
%viab	76.8	79.5	76.1	75.5	72.9

Butyrate (sum)					
WT	0	250uM	500uM	750uM	1000uM
MPF	1166	1298	1330	1269	1203
% Inc	0.000	11.359	14.071	8.880	3.175
%viab	81.5	85.8	83.9	85.4	84.1
HPFH	0	250uM	500uM	750uM	1000uM
MPF	2667	2985	2873	2719	2611
% Inc	0.000	11.924	7.724	1.931	-2.100
%viab	76.8	75.6	80.9	80.2	80.1

Cisplatin (sum)					
WT	0	5uM	10uM	15uM	20uM
MPF	1166	2248	2372	2805	3251
% Inc	0.000	92.836	103.475	140.626	178.893
%viab	81.5	58.2	35.2	28.4	23.0
HPFH	0	5uM	10uM	15uM	20uM
MPF	2667	3260	3502	4594	5533
% Inc	0.000	22.235	31.290	72.235	107.443
%viab	76.8	45.2	30.1	27.8	28.5

Tallimustine (sum)					
WT	0	25nM	50nM	75nM	100nM
MPF	1205	1681	1442	1467	1778
% Inc	0.000	39.461	19.627	21.701	47.510
%viab	82.3	62.6	60.4	55.4	47.9
HPFH	0	25nM	50nM	75nM	100nM
MPF	2651	3500	3123	3034	3077
% Inc	0.000	32.026	17.786	14.447	16.069
%viab	70.1	52.7	54.5	47.9	45.3

Wheat Grass (sum)					
WT	0	1%	2%	3%	4%
MPF	1166	1150	1257	1099	1455
% Inc	0.000	-1.330	7.851	-5.706	24.829
%viab	81.5	76.5	73.8	58.6	62.6
HPFH	0	1%	2%	3%	4%
MPF	2667	2635	2427	2331	2172
% Inc	0.000	-1.219	-9.018	-12.598	-18.560
%viab	76.8	65.7	60.8	56.1	52.5

G2 (sum)					
WT	0	25nM	50nM	75nM	100nM
MPF	1166	1380	1533	1706	2039
% Inc	0.000	18.361	31.532	46.332	74.946
%viab	81.5	74.0	68.9	63.5	58.2
HPFH	0	25nM	50nM	75nM	100nM
MPF	2667	3028	3186	3432	3191
% Inc	0.000	13.536	19.460	28.684	19.648
%viab	76.8	70.4	63.5	61.0	56.4

G3 (sum)					
WT	0	1uM	2.5uM	5uM	7.5uM
MPF	1166	1518	1529	1712	1836
% Inc	0.000	30.245	31.145	46.890	57.529
%viab	81.5	64.5	66.4	65.8	62.7
HPFH	0	1uM	2.5uM	5uM	7.5uM
MPF	2667	3015	3019	3039	3357
% Inc	0.000	13.048	13.180	13.930	25.872
%viab	76.8	65.4	61.7	54.7	54.5

G4 (sum)					
WT	0	2.5uM	5.0uM	7.5uM	10uM
MPF	1166	1426	1633	1781	2876
% Inc	0.000	22.351	40.112	52.810	146.761
%viab	81.5	70.4	72.0	72.7	49.6
HPFH	0	2.5uM	5.0uM	7.5uM	10uM
MPF	2667	3284	3094	3165	3445
% Inc	0.000	23.116	15.992	18.673	29.171
%viab	76.8	64.1	61.1	61.6	46.6

APPENDIX B

Raw data of full blood examination of the γ-globin EGFP transgenic mice and wild type mice

Sample	RBC	HGB	HCT	MCV	MCH	RDW	%RETIC	%EGFP +ve	MPF	index
Transgenic										
GG1.20	11.10	13.8	47.8	43.1	12.5	23.2	6.1	82.2%	23402	19236.44
GG1.21	11.51	13.0	46.4	40.3	11.3	26.1	6.7	68.3%	12756	8712.35
GG1.25	10.22	13.4	46.7	45.7	13.1	23.1	5.1	68.5%	17802	12194.37
GG2.3	10.82	13.3	45.8	42.3	12.3	24.3	6.3	73.8%	20380	15040.44
GG2.7	9.78	14.3	47.7	48.8	14.6	16.7	5.0	90.0%	43037	38733.30
GG2.8	9.90	13.7	46.8	47.3	13.9	21.6	7.5	81.0%	32923	26667.63
GG2.11	11.45	14.9	50.7	44.3	13.0	24.4	5.6	77.2%	17075	13181.90
GG2.13	11.59	14.7	50.5	43.6	12.6	26.6	7.7	60.6%	20063	12158.18
GG2.14	10.95	14.1	48.5	44.3	12.8	25.7	7.2	59.9%	21476	12864.12
GG2.18	11.26	14.1	49.1	43.6	12.6	24.8	6.4	66.7%	13949	9303.98
GG2.28	10.67	14.4	50.3	47.2	13.5	23.8	8.3	80.0%	36443	29154.40
GG Avg.	**10.84**	**13.97**	**48.21**	**44.59**	**12.93**	**23.66**	**6.53**	**73.5%**	**23573.27**	**17931.56**
GG SD	**0.64**	**0.60**	**1.75**	**2.47**	**0.87**	**2.72**	**1.06**	**9.5%**	**9716.00**	**9586.24**
Wild type										
BL6.1	10.86	16.0	52.9	48.7	14.7	13.4	4.6			
BL6.2	9.94	14.6	48.2	48.5	14.7	15.6	2.0			
BL6.3	10.33	15.1	49.9	48.3	14.6	13.5	3.2			
BL6.4	10.18	15.0	49.5	48.6	14.7	13.5	2.9			
BL6.5	10.39	15.1	50.0	48.1	14.6	13.2	3.0			
WT Avg.	**10.34**	**15.16**	**50.10**	**48.44**	**14.66**	**13.84**	**3.13**			
WT SD	**0.34**	**0.51**	**1.72**	**0.24**	**0.05**	**0.99**	**0.94**			

BIOGRAPHY

NAME	Miss Preeyachan Lourthai
DATE OF BIRTH	26 January 1982
PLACE OF BIRTH	Bangkok, Thailand
INSTITUTIONS ATTENDED	Mahidol University, 1999-2002 Bachelor of Science (Medical Science) Mahidol University, 2003-2008 Ph. D. (Biochemistry)
POSITION&OFFICE	Thalassemia Research Center Institute of Science and Technology Mahidol University, Salaya Campus Nakornpathom, Thailand Tel: 02-8892557-8 E-mail: Preeyachan@yahoo.com
HOME ADDRESS	172 soi Sungthong10 Phaholyothin 35 road Ladyao Sub-district Jatujak District Bangkok, Thailand Tel: (02) 9397441