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THESIS

RED CELL SURVIVAL AND HEMATOLOGIC STUDY IN MOUSE MODEL FOR β-THALASSEMIA

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The major cause of anemia in β-thalassemia, secondary to imbalanced globin chain synthesis, is its deleterious effect of the excess α–globin chain on erythroid maturation, RBC membrane is rigid leading to instability and short red cell survival in patients. A mice model mimics β^0-thalassemia/ HbE disease in human have been developed from the heterozygous βIVSII-654 knockin mouse, being generated by replacement of 21 kb regions encompassing of the mouse β^{major} and β^{minor} with 5.7 kb DNA fragment of human β-globin gene contained IVSII-654 splicing mutation. The second β^0-thalassemic model was β-knockout mice which carried a 20 kb deletion encompassing the entire mouse β^{major} and β^{minor}. The objective of creating HbE transgenic mice has been made up for mouse model of homozygous β^0-thalassemia / HbE or rescued mice were generated by breeding the β^0-thalassemic models with HbE transgenic, these transgenic mice were used to study red cell survival by determining the number of biotin labeled red cells. The red cell mean half-life survival in wild type mice and the transgenic mice that carried the human β^E-globin locus transgene was 25 days. Red cell life span in the mice model for heterozygous β^0-thalassemia, the βIVSII-654 knockin mice was 15 days. Double heterozygous mice which carried 4 copies of the human β^E-globin transgene on heterozygous βIVSII-654 mutation background had 20 days of red cell life span. The rescued mice of both genotype (homozygous βIVSII-654 knockin / HbE mice and hemizygous βIVSII-654 knockin / β^{mouse} –knockout / HbE mice), had red cell survival at 14 days of red cell life span. Although a red cell life span of rescue mice was similar to heterozygous β^0-thalassemic mice, the hematological indices and pathophysiologic study had indicated that rescue mice had an active erythropoiesis which higher RBC turnover rate. Therefore rescued mice may be used as a model of β^0-thalassemia/ HbE to understand the mechanism of pathophysiology, such as ineffective erythropoiesis, and to develop the novel treatment for thalassemia patients.

Student's signature

Thesis Advisor's signature 1/04/2006

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TABLE OF CONTENTS

Page

TABLE OF CONTENTS (Con' d)

Page

TABALE OF CONTENTS (Con' d)

Page

TABALE OF CONTENTS (Con' d)

Page

LIST OF TABLES

LIST OF FIGURES

Figure **Page**

LIST OF FIGURES (Con' d)

Figure **Page**

LIST OF FIGURES (Con' d)

Figure **Page**

Appendix Figure

LIST OF ABBREVIATIONS

ATP	=	Adenosine triphosphate
bp	=	Base pair
C	=	Degree Celsius
Ca	=	Calcium
cm	=	Centimeter
DH	=	Double heterozygous
DNA	=	Deoxyribonucleic acid
dNTP	=	Deoxynucleotide 5' triphosphate
EDTA	=	Ethylene diamine-tetra-acetic-acid
FSC	=	Forward scatter
g	=	Gram
g	=	Gravity
g/dl	=	Gram per decilitre
g/kg	=	Gram per kilogram
GET	=	Gam, recE, and recT
Hb	=	Hemoglobin
HbA	=	Hemoglobin A
HbCS	=	Hemoglobin Constant Spring
HbE	=	Hemoglobin E
$h\beta^E$	=	Human β^E globin
HbF	=	Hemoglobin F
HbH	=	Hemoglobin H
HBSM	=	HEPES buffered saline for mouse
HCl	=	Hydrochloric acid
Hct	=	Hematocrit
HPLC	=	High performance liquid chromatography
i.g.	=	Intragastric
i.p.	=	Intraperitoneal

LIST OF ABBREVIATIONS (Con' d)

kb	=	Kilobase pair
kDa	=	Kilodalton
LCR	=	Locus control region
Lys	=	Lysine
mA	=	Milliampere
mα	=	Mouse α globin
mβ	=	Mouse β globin
MCH	=	Mean corpuscular hemoglobin
MCHC	=	Mean corpuscular hemoglobin concentration
MCV	=	Mean corpuscular volume
mα	=	Mouse α globin
mg	=	Milligram
mRNA	=	Messenger ribonucleic acid
n	=	Number
PAGE	=	Polyacrylamide gel electrophoresis
PCR	=	Polymerase chain reaction
PE	=	Phycoerythrin
pH	=	Negative logarithm of the effective hydrogen-ion concentration
PAGE	=	Polyacrylamide gel electrophoresis
R	=	Rescued mice
RBC	=	Red blood cell
RBCs	=	Red blood cells
RDW	=	Red blood cell distribution width
RET	=	Reticulocyte count
RNA	=	Ribonucleic acid
ROS	=	Reactive oxygen species

LIST OF ABBREVIATIONS (Con' d)

RT-PCR	=	Reverese-transcriptase polymerase chain reaction
S.D.	=	Standard deviation
SDS	=	Sodium dodecyl sulphate
SE	=	Southeast
sec	=	Second
$T_{1/2}$	=	Half-time
UTR	=	Untranslated region
V	=	Volt
w/v	=	Weight by volume
w/w	=	Weight by weight
WT	=	Wild-type
μg	=	Microgram
μg/ml	=	Microgram per millimetre
μl	=	Microliter
μm	=	Micrometer
μM	=	Micromolar

RED CELL SURVIVAL AND HEMATOLOGIC STUDY IN MOUSE MODEL FOR β-THALASSEMIA

INTRODUCTION

Thalassemia, a diverse group of genetic disorders of hemoglobin synthesis, is characterized by the abnormal globin gene expression resulting in total absence or quantitative reduction of α- or β-globin chain synthesis in human erythroid cells. The imbalance of globin chain production leading to an overall deficit of hemoglobin accumulation in red cells and cause hypochromic, microcytic anemia with a low mean corpuscular hemoglobin concentration. The β-thalassemia is characterized by reduction (β^{+}) or absence (β^{0}) in the synthesis of β-globin chain. The continued synthesis in normal amount of an unaffected α-globin chain result in the accumulation, within the erythroid cells, of excessive amount of these chains. Not finding complementary globin chains to bind, these excess α-globin chains form insoluble aggregates and precipitate within the cell causing membrane damage and premature destruction of the red cells.

A mouse model mimics the β-thalassemia/HbE disease in human have recently been developed. This is modified from the plasmid construct carrying the ~ 148 kb of whole β-globin gene cluster, which was first developed by Narayanan (Narayanan *et al*., 1999). Using the recombination technique the β^{A}-globin gene of the construct was replaced by β^{E}-globin gene. This newly construct plasmid was successfully transfected into the fertilized ovum that was later integrated into mouse chromosome (Jamsai *et al*., 2004). β^{E}-Globin gene expression was noted in the double heterozygote and rescued mice after cross breeding between hemizygous HbE transgenic mice and heterozygous βIVSII-654 knockin mice (β-thalassemic mice) (Lewis *et al*., 1998). Hematologic data

and red cell survival study will be carried out in double heterozygous βIVSII-654 knockin with HbE transgene and the rescued mice (HbE transgene on homozygous βIVSII-654 knockin background) in comparison with the wild-type mouse. This information will be used as the background for future intervention study such as antioxidant, antisense therapy.

OBJECTIVES

1) To create the rescued mice, by breeding the heterozygous β^0-thalassemic mice, either the knockin βIVSII-654 or the knockout mice with HbE transgenic mice through breeding scheme that generated the feature of β^0-thalassemia/ HbE in patients.

2) To compare phenotypes of HbE transgenic mice, double heterozygotes and rescued mice by hematological data.

3) To study the red cell survival in each mouse genotypes in order to relate HbE transgene expression and the age of red blood cells.

LIERATURE REVIEWS

The genetic disorders of hemoglobin can be broadly classified into three groups: (i) the thalassemias, in which there is impaired synthesis of normal globins; (ii) hemoglobin variants, in which there is a structural alteration in one of the globin chains; and (iii) hereditary persistence of fetal hemoglobin (HPFH), in which there is a defect in the developmental progression from fetal to adult hemoglobin production.

1. Thalassemia

Thalassemia is a diverse group of genetic disorders of hemoglobin synthesis characterized by an imbalance in globin chain production (Weatherall and Clegg, 1981). These defects range from decreased synthesis to the absence of the affected globin chains leading to the accumulation of the excess unaffected globin chains which aggregate and precipitate within the red blood cell precursors and their progeny. This leads to intramedullary destruction, ineffective erythropoiesis, and hemolytic anemia, which are characteristic of all the thalassemias (Weatherall and Clegg, 1996) (Fig.1). Thalassemias can be divided into two major groups, the β-thalassemias, in which β-globin chain synthesis is impaired and the α-thalassemias, in which α- globin chain synthesis is reduced or absent

1.1 β-thalassemias

β-thalassemias is characterized by reduction or absence in the synthesis of β-globin chain. The basic pathophysiology of premature hemolysis of red blood cells in the peripheral circulation and more particularly of extensive destruction of erythroid precursors in the bone marrow and

extramedullary sites (ineffective erythropoiesis) results from the excess of α-globin chains due to the basic of β-globin gene genetic defect. The imbalanced α-/non α-globin synthesis is the major factor in determining the severity of the disease (Weatherall, 2001). The severe ineffective erythropoiesis results in erythroid marrow expansion leading to the characteristic deformities of the skull and face, as well as osteopenia and focal defects in bone mineralization. Marrow hyperplasia leads ultimately to increased iron absorption and progressive deposition of iron in the tissues (Weatherall *et al*., 1981) (Fig.1).

Most β-thalassemias syndromes are caused by mutations affecting gene regulation or expression rather than gene deletion (Hardison *et al*., 2002). Despite having a relatively limited range of clinical, hematological and biochemical phenotypes, β-thalassemias are due to a surprisingly large number of different genetic defects (Hardison *et al*., 2002). The present distribution of β-thalassemias spans all continents but is particularly common in Mediterranean, Middle Eastern, Indian sub-continent and Southeast Asian regions (Weatherall, 2001).

The β-thalassemias are caused by more than 200 mutations in the functionally important regions of the β-globin gene which result in a deficit of β-globin chain production (Hardison *et al*., 2002). The defect ranges from minimal (mild β^{+}-thalassemias alleles) to a complete absence (β^{0}-thalassemias alleles). With rare exceptions heterozygotes for β-thalassemias, including β^{0}-thalassemias, are clinically asymptomatic with minor hematological abnormalities, whereas homozygotes or compound heterozygotes for β^{0}-thalassemias have severe disease and are transfusion dependent (Weatherall and Clegg, 1981). The mutations can be classified according to the type or category of defect in gene expression.

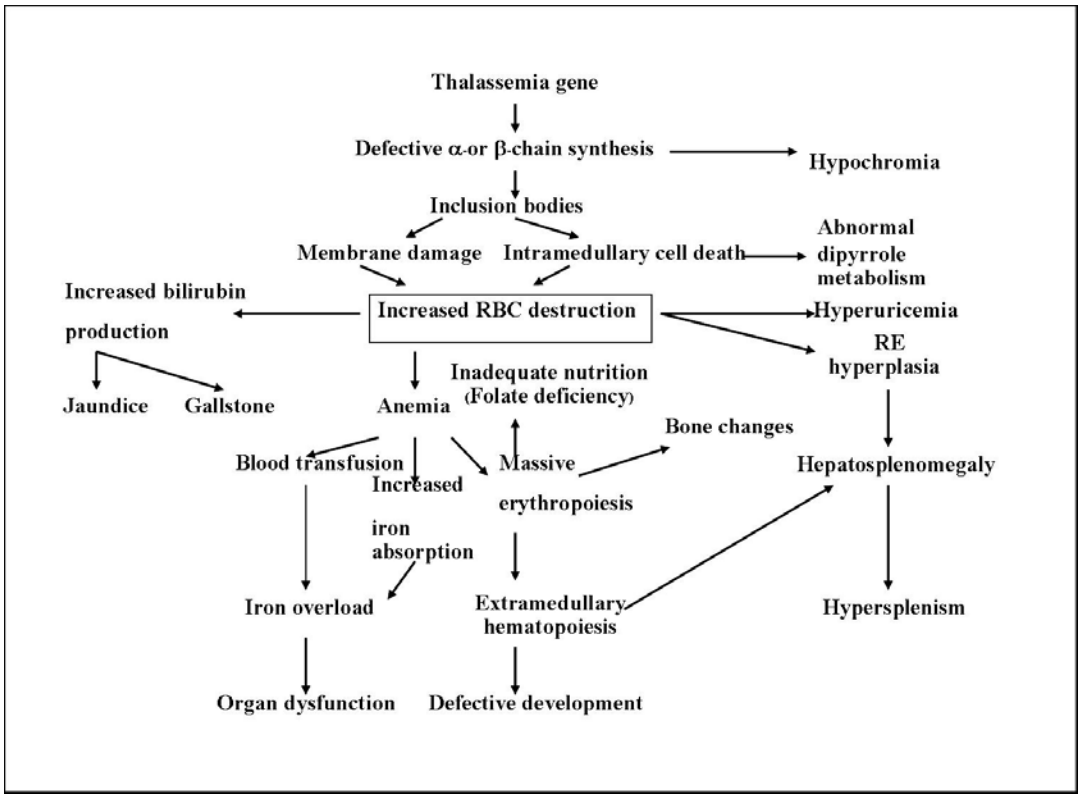


Figure 1 Pathophysiology of β-thalassemia. The precipitation of excess unbound α-globin gene leads to a number of consequential pathophysiological defected in β-thalassemia patients.

Source: Olivieri (1999)

1.1.1 Frameshift mutations

Frameshift mutations result from the deletion or insertion of one or more nucleotides (other than multiples of three) in the protein-coding region of the gene. This causes a change in the normal reading frame of the encoded mRNA at the site of the mutation. Such mutations usually allow the continued translation of mRNA for some distance, yielding a novel abnormal amino acid sequence, until a chain termination codon is encountered in the new reading frame. Chain termination usually occurs at a position before the normal termination codon and results in the synthesis of a truncated, unstable mutant globin chain that is presumably rapidly degraded the 4 bp deletion (-TTCT) at codon positions 41 and 42 is a particularly common mutation among patients in Southern China and Thailand, accounting for 40% of β-thalassemias in some regions (Chan *et al*., 1987). The deletion causes a frameshift and creates a stop codon at the new codon 59 position, thus resulting in β^0-thalassemia (Kimura *al*., 1983; Kazazian *et al*., 1984). Although most frameshift mutations are associated with typical β-thalassemias, those that occur relatively far into the coding sequence in exon 3 are associated with the phenotype of dominant β^0-thalassemias, including moderately severe hemolytic anemia, splenomegaly and inclusion body formation. This abnormality occurs presumably because of the absence of nonsense-mediated mRNA decay and the mutant β-globin chains synthesized can bind heme and produce aggregates that are relatively resistant to proteolytic degradation (Thein *et al*., 1990; 1992).

1.1.2 Nonsense mutations

Nonsense mutations result from single base substitutions that change a codon for a given amino acid to a chain termination codon and thus cause premature cessation of mRNA translation. Truncated globin chains

should be produced as a result of such mutations, but they are not usually detected, presumably because they are very unstable and rapidly degraded. The phenotype is that of β^0-thalassemias with total absence of normal β-globin chain synthesis from the affected gene. A number of nonsense mutations have been reported to cause β-thalassemias worldwide (Hardison *et al*., 2002). The most common nonsense mutations are codon 17 AAG→TAG (Chang and Kan, 1976) which is common in the Southeast Asian region and codon 39 CAG→TAG (Trecartin *et al*., 1981), which is common in patients of Mediterranean ancestry.

1.1.3 Splicing mutations

Mutations within splice junctions at the consensus sequences reduce the efficiency of normal splicing to varying degrees and produce a β-thalassemia phenotype that ranges from mild to severe anemia. The invariant dinucleotides GT and AG present at the 5' and 3' spice junction, respectively, are necessary but not sufficient to ensure efficient and accurate splicing. They are normally part of large consensus sequence that contains a number of other conserved sequence features. Mutations of certain residues in this consensus sequence result in a partial block of normal mRNA splicing, the severity of which varies with the site of the mutation within the consensus sequence. As a group, these mutations are generally associated with a phenotype of β^+-thalassemias. The G→C and G→T mutations at position 5 of The first intervening sequence (IVS I) reduce splicing at the mutated donor site. They appear to activate cryptic donor sites, two in exon 1 and one in IVSI, which are utilized preferentially to the normal donor site leading to the severe β^+-thalassemia phenotype (Treisman *et al*., 1983).

Mutations within the second intron have also been identified (Treisman *et al*., 1983; Docbkin *et al*., 1983). An interesting but as yet unexplained feature of these mutations is the activation of alternative splicing by the C→T mutation at position 654. All mRNA is processed by splicing from the normal intron 2 donor to the activated cryptic receptor and from the mutated new donor to the normal intron 2 acceptor (Cheng *et al*., 1984). This transcript retains 73 nucleotides from the second intron, rendering its functionless with phenotype of severe β^+-thalassemia phenotype. In the mutations involving position 705 and 745, some normal RNA is made in addition to the abnormally spliced products resulting in a mild phenotype of β^+-thalassemia (Treisman *et al*., 1983; Docbkin *et al*., 1983). Accordingly, the mutation at the 654 position is characterized as β^0-thalassemias. However, how these mutations activate a cryptic acceptor site upstream from the mutation is unclear, as is why a cryptic acceptor is used in preference to an unaltered acceptor splice site farther downstream. The IVS II-654 mutation is frequent among patients in China and Thailand, accounting for 20% of β-thalassemias in some regions (Cheng *et al*., 1984; Takihara *et al*., 1984).

1.2 α-Thalassemia

In contrast to the β-thalassemia most α-thalassemias are caused by the deletion of one or both the α-globin genes in the α-globin locus (Higgs *et al.,* 1989). α-Thalassemias can be divided into two forms: the severe form, α-thalassemias 1, and a mild form, α-thalassemias 2. α-Thalassemias 1, which resulted from the loss of both linked α-globin genes leads to no α-globin chain synthesis. α-Thalassemias 2 is caused by the loss of only one α-globin gene and results in reduced synthesis of α-globin chains (Weatherall *et al*., 1970; Todd *et al*., 1970).

2. Hemoglobin Variants

Structure alterations of hemoglobin include single and double amino acid substitutions, amino acid deletions, insertions and additions, and fusions formed of two polypeptide chains. About 90% of hemoglobin variants are single amino acid substitutions in the α-, β-, γ- or δ-globin chains; no embryonic variants are known. The most common hemoglobin variants are Hbs E, C and S (Eastman *et al*., 1999). The carrier (heterozygous) states for all three are, for the most part, symptomless. However, there are a few structural hemoglobin variants that are synthesized ineffectively and so have a thalassemia phenotype (Huehns and Bellingham, 1969).

3. Hemoglobin E

Hemoglobin E (β26 Glu→Lys) is one of the most common hemoglobin variants worldwide. It is most prevalent in the rapidly expanding population of Southeast Asia, and it is also encountered with increasing frequency in the immigrant populations of Europe, North America, and Australia (Kazazian *et al*., 1984). Although HbE was first identified in 1954 (Frischer and Bowman, 1975) there are still uncertainties about many aspects of its pathophysiology. The heterozygous state, although clinically silent, is associated with slight microcytosis and target erythrocytes. No anemia or reticulocytosis is noted. Hemoglobin electrophoresis reveals 20 to 35% HbE. Homozygosity of HbE is characterized by prominent microcytosis and significant morphologic alterations but little or no anemia. No physical abnormalities are observed. HbE accounts for 92 to 98% of total hemoglobin, HbF is normal or only slightly increased. Red cell survival is slightly reduced, and osmotic fragility is decreased. Shortened cell survival may be resulted, in part, from the instability of HbE, a property attributed to the tendency for β^{E}-dimers to dissociate into

monomers, thereby exposing reactive SH groups (Frischer and Bowman, 1975).

The thalassemia phenotype of the β^E gene is a result of the activation of a cryptic donor splice site by the codon 26 G→A mutation (Orkin, *et al*., 1982). The new cryptic splice site at codon 25 competes with the normal donor splice site at the beginning of the first intron, thereby reducing β^E mRNA generation (Traeger *et al*., 1980; Benz *et al*., 1981). The abnormally spliced mRNA is non-functional since part of exon 1 is missing and a new stop codon is generated. Thus, the β^E-globin is produced in reduced amounts and results in a mild β^+ thalassemia phenotype. Decreased synthesis of β^E-globin may be compounded by β^E mRNA instability. In addition, *in vitro* experiments have shown that HbE is mildly unstable and may be susceptible to oxidant damage (Traeger *al*., 1982).

4. β-Thalassemia/HbE Diseases

Thalassemias and structural hemoglobin variants occur together at a high frequency in many populations (Weatherall and Clegg, 1996). It is not uncommon for an individual to inherit genes for both types of condition. The compound heterozygous state of β-thalassemias/HbE disease leads to the phenotype ranging from mild anemia to the most severe form of β-thalassemia major (Benz *et al*., 1981; Fucharoen *et al*., 1987; Yang *et al*., 1989; Thein *et al*., 1990). β-Thalassemia /HbE patients have hemoglobin levels varying from 3 to 11 g/dl (Fucharoen *et al*., 1987; Winichagoon *et al*., 1993). The presence of β^+-thalassemia, α-thalassemia and homozygosity for the *XmnI* cleavage site at position -158 $^G\gamma$-globin gene are disease severity modifying factors. However, the extreme variation remains unexplained (Winichagoon *et al*., 1993).

5. Chromosome Organization of Globin Gene Clusters

The globin genes of humans and most other species are organized into two clusters. In humans the β-globin cluster spans 70 kb on the short arm of chromosome 11 in band p15.5, which includes an embryonic gene (ε), two fetal genes ($^{G}\gamma$, and $^{A}\gamma$), two adult genes (δ and β) and a pseudogene (ψβ) (Collins and Weissman, 1984) (Fig. 2). The human α-globin cluster spans about 80 kb on the short arm of chromosome 16 in band p13.3 (Turcinov *et al*., 2000). It includes an embryonic gene (ζ2), two fetal/adult genes (α2 and α1), three pseudogenes (ψζ1, ψα2, ψα1) and a gene (θ) of unidentified function (Higgs *et al*., 2000) (Fig.2). Both α- and β-globin loci have important upstream regulatory regions. In the β-globin locus, this is called the locus control region (LCR) (Grosveld *et al*.(1987a, 1987b); Tuan *et al*., 1985; Forester *et al*., 1987) whereas HS-40, located 40 kb upstream of ζ-globin gene, is the major control element of α-globin gene family (Vyas *et al*., 1992). Five DNase hypersensitive sites have been identified upstream of the β-globin locus. The most 5' site (HS5) does not show tissue specificity, while HSs1-4, which together form the LCR, are largely erythroid-specific and establish a transcriptionally active domain that spans the entire β-globin gene cluster. In the α-globin HS-40 region, there are various erythroid-specific and ubiquitous DNA-binding protein binding sites with the 300 bp core region (Vyas *et al*., 1992), similar to the locus control region of the human β-globin gene cluster. Their functions, however are somewhat different from each other because HS-40 is designated as a positive control element of the human α-globin gene cluster (Craddock *et al*., 1995; Liebhaber and Russell, 1998).

The developmental regulation of the globin genes reflects their sequential activation in a 5'-3' direction. Furthermore, there is an important

difference between the α-like globin genes, which undergo a single developmental switch (embryonic→fetal/adult) and the β-like globin genes, which undergo an additional switch around the time of birth (embryonic→fetal→adult) (Orkin, 1983). The way in which this development switch is controlled in globin gene expression is still not fully understood (Grosveld *et al*., 1987).

6. Globin Gene Structure

The coding region of the globin genes in humans and other animal is interrupted at two positions by considerable stretches of noncoding DNA called intervening sequences (IVS) or introns. In the β-like globin genes, the intervening sequences interrupt the sequence between codons 30 and 31 and between codons 104 and 105; in the α-like globin gene family, the intervening sequences interrupt the coding sequence between codons 31 and 32 and between codons 99 and 100 (Collins *et al*., 1984) (Fig.3). Although the precise codon position numbers at which the interruption occurs differ between the α- and β-like globin genes, the introns occur at precisely the same position relative to the regions of the primary structure of the α- and β-globin chains that are homologous. The first intervening sequence (IVS I) is shorter than the second intervening sequence (IVS II) in both α- and β-globin genes, but the IVS II of the human β-globin gene is much larger than that of the α- globin gene (Collins and Weissman, 1984) (Fig.3).

7 Human Hemoglobin

The structure of human hemoglobin changes during development (Weatherall, 2001). All the normal hemoglobins are tetramers of two pairs of unlike globin chains. Adult (HbA) and fetal (HbF) hemoglobins have α-globin chains that are combined with β-globin chains (HbA, α_2 and β_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-chains combine with γ-globin chains (Hb Portland, $\zeta_2\gamma_2$) or ε-globin chains (Hb Gower 1, $\zeta_2\varepsilon_2$), and α-globin chains and ε-globin chains form Hb Gower 2 ($\alpha_2\varepsilon_2$). Embryonic hemoglobin is confined to the yolk-sac stage of development and thereafter is replaced by HbF until shortly before term. After birth, HbF is replaced by HbA and HbA_2, over first year of life.

8 Clinical Classification of Thalassemia

Based on clinical manifestations, the thalassemia can be divided into three groups.

8.1 Thalassemia minor

Thalassemia minor is an asymptomatic illness with mild or no anemia and usually represent the carrier states or traits.

8.2 Thalassemia intermedia

Signs and symptoms of thalassemia intermedia are comparable to those of thalassemia major but of a lesser magnitude. Although chronically anemic, individuals with thalassemia intermedia do not require transfusion, except in association with intercurrent illness. Growth and development during childhood is relatively uncompromised, pubescence takes place normally and fertility is preserved (Aksoy,1970).

8.3 Thalassemia major

Thalassemia major is the most severe form of thalassemia disease which life can only be sustained by regular blood transfusion. Patients develop all signs and symptoms associated with severe anemia, growth retardation, hepatosplenomegaly and thalassemia faces. β-Thalassemia major usually results either from the compound heterozygous state for two different β-globin mutations or homozygous stage of the mutation (Weatherall and Clegg, 1981; Anand *et al*., 1988; Raiola *et al*., 2003).

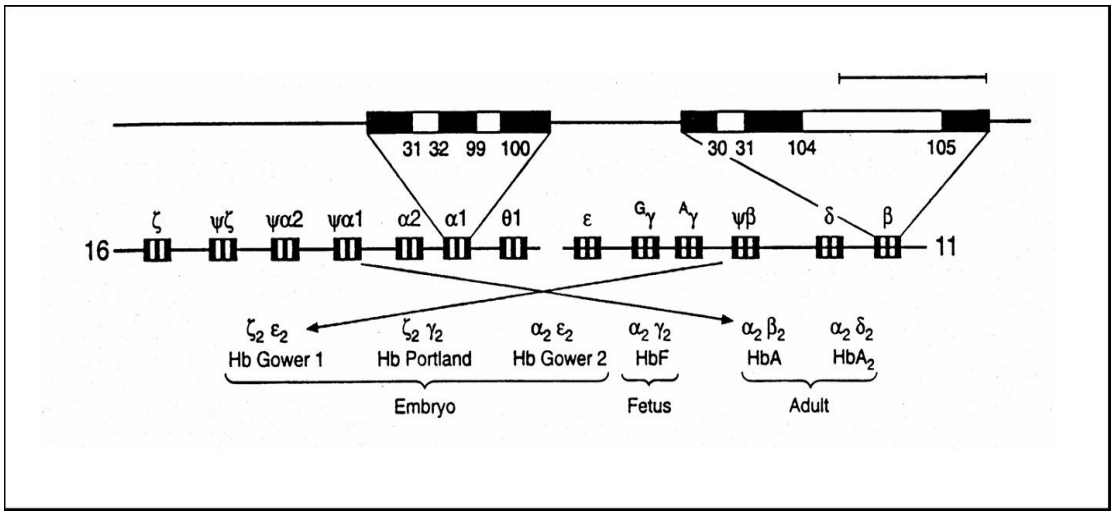


Figure 2 The α and β –globin gene clusters. The genes that regulate the synthesis and structures of different globins are organized in two separate clusters. The α-like globin genes, which are encode on chromosome 16, are found in the order 5'-ζ-ψζ-ψ α2-ψ α1- α2- α1-θ-3'. The β-like globin genes, on chromosome 11, occur in the order 5'-ε-^{G}γ-^{A}γ- ψβ-δ-β-3'. In the figure, exons are shaded dark and introns are unshaded.

Source: Weatherall (2001)

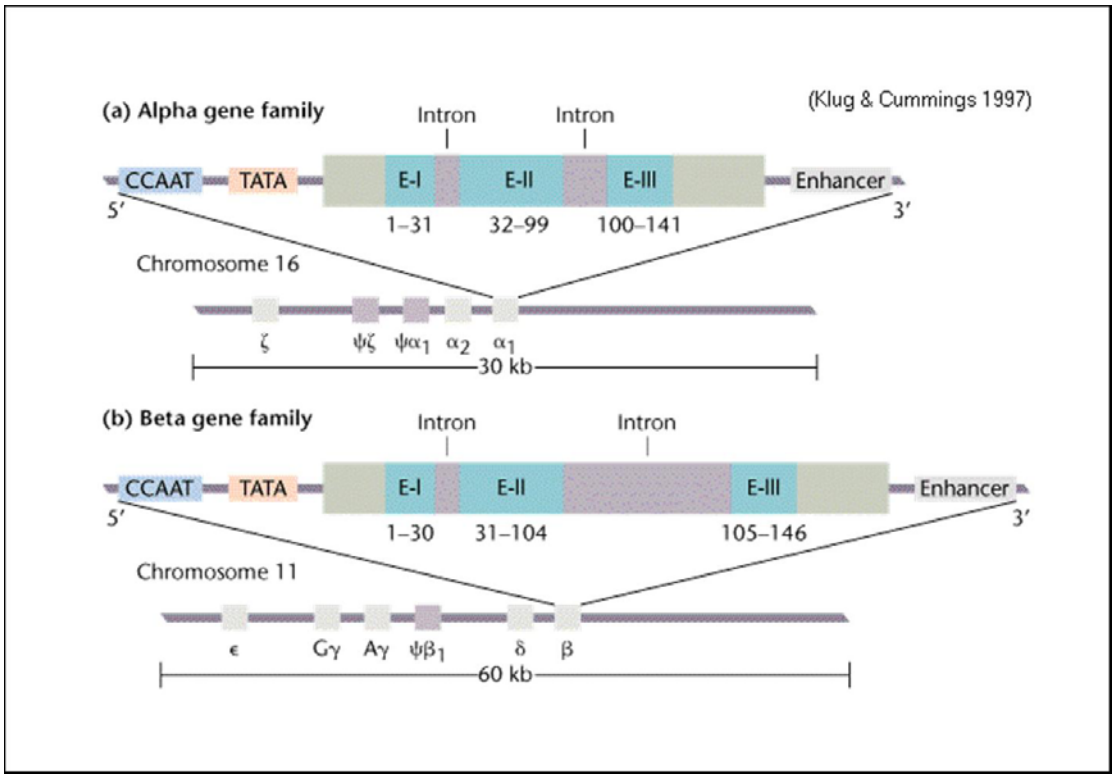


Figure 3 Globin gene structure. The blue blocks represent the coding regions of the gene. The gray blocks at either end present the 5' and 3' untranslated sequence, the purple blocks represent the IVSI and IVSII, which interrupt the coding sequences of the gene. The numbers below the diagram indicated that the amino acid codon positions of the coding sequence.

Source: Bunn and Forget (1986)

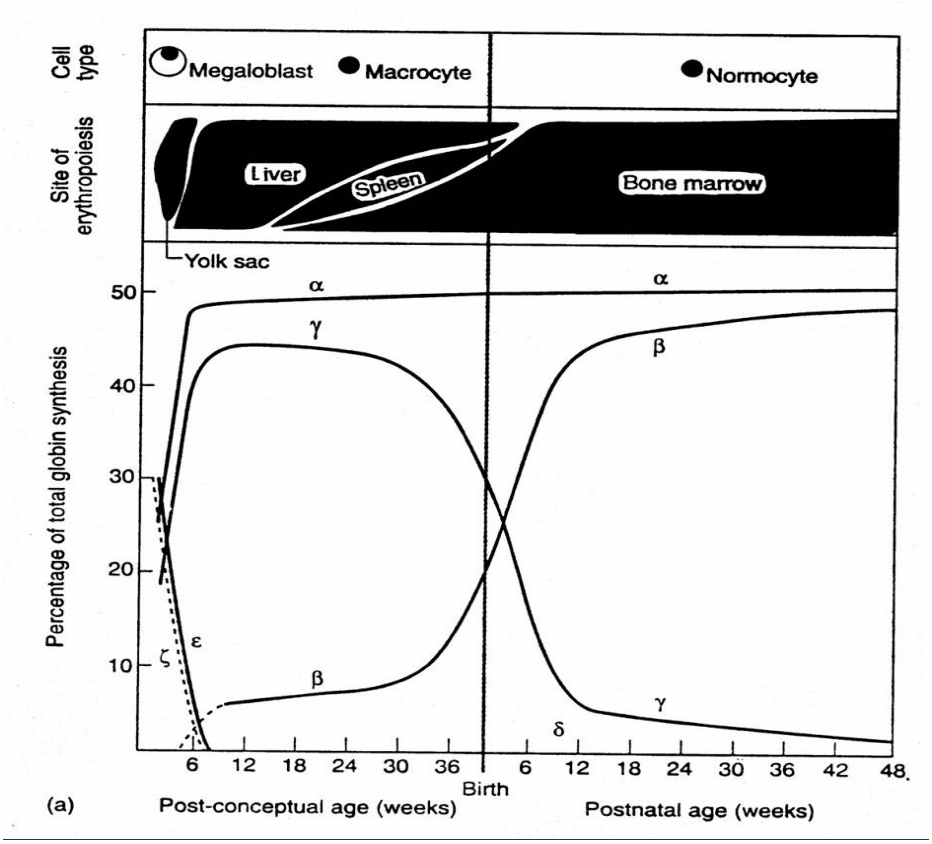


Figure 4 Developmental changes in hemoglobin production. The sites of erythropoiesis during development and different globins chains produced at each stage are different.

Source: Weatherall (2001)

9. Mouse β-Globin Gene Cluster

The mouse β-globin gene cluster located 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}) (Whitelaw *et al*., 1990; Jahn *et al*., 1980). Organization of the mouse β-globin locus is illustrated in Figure 5. Mouse embryonic hemoglobins are first expressed at 9.5 days of gestation in the yolk sac and later in the fetal liver. The switch from predominantly embryonic to predominantly adult hemoglobins is completed between days 14 and 15 of gestation in the normal fetus (Craig and Russell, 1964; Barker, 1968). The *b1* and *b2* genes are then expressed in the 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 (Russell, 1979).

10. Mouse Models for Human Disease

Three types of mouse models that have been used extensively for various studies, (1) knock out models that the endogenous genes of interest are deleted by homologous recombination, (2) knock in models, in which the specific mutation is introduced into the mouse gene, and (3) transgenic models, in which the gene(s) of interest are introduced into the mouse genome.

Production of these genetically engineered animals can facilitate more complete understanding of the consequences of increased or decreased gene expression in human. Information from these studies can lead to a better understanding of the pathogenic mechanisms of human genetic and infectious

disease as well as provide animal models that can be used to test new genetic and drug therapies for these disorders.

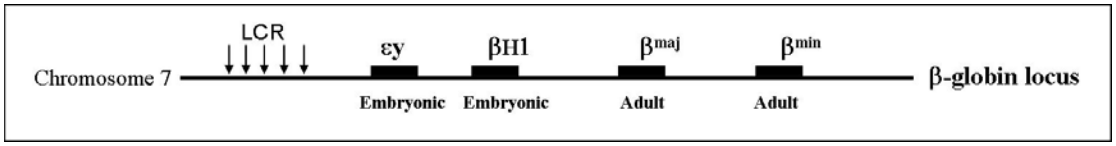


Figure 5 The mouse β-globin locus. The mouse β-globin gene cluster on chromosome 7 has four functional β-globin genes: βH1, an early embryonic globin gene; ε^{y}, a late embryonic globin gene; and two adult globin genes, β major and β minor. The LCR is marked by four arrows indicating DNase I hypersensitive site.

Source: Whitelaw (1990)

10.1 Mouse models for β^+-thalassemia

Two mouse models of β^+-thalassemia have previously been reported (Skow *et al*., 1983; Shehee *et al*., 1993). First was the mouse with a spontaneous DNA deletion of ~3.7 kb in the β-globin locus including the entire *b1* (β major) gene and its adjacent upstream sequences (Hbb^{th-1}). Mice homozygous for the deletion (Hbb^{th-1}/Hbb^{th-1}) survived to adulthood at ~60 % of the numbers of the wild type mice. The heterozygous mice showed a mild phenotype comparable to that of thalassemia trait in humans (Skow *et al*., 1983). The second model for β^+-thalassemia generated by targeted gene disruption of the mouse *b1* gene (Hbb^{th-2}/Hbb^{th-2}) were severely anemic and did not survive more than a few hours after birth. A possible explanation for the difference between the deletion and the disruption mouse models of thalassemia is that non-globin producing promoters in the disrupted β-globin locus can compete for access to the locus control region needed for transcription, thereby reducing transcription from the normal *b2* gene. In the deletion animals, *b2* (β minor) gene has no competitors. Similar differences in transcription have been observed in human thalassemic patients with deletions or mutations in the promoter region of adult β-globin gene compared to patients with deletions or mutations that do not affect the promoter region (Shehee *et al*., 1993).

11. Deletion and Replacement of the Mouse Adult β-Globin Genes by a Plug and Socket Method to Generate Mouse Models for β^0-Thalassemia

A two-step targeting strategy, called the plug and socket, have been used to introduce the human β-globin gene in to the mouse genome. The first step in the "plug and socket" strategy used conventional targeting to introduce a nonfunctional portion of the selectable marker (the "socket") close to the

chosen locus. The second step modified the locus by homologous recombination with a targeting construct (the "plug") designed to have two functions. First, the plug modified the target gene in the desired manner. Second, the plug recombined with the nonfunctional marker and converted it into a functional form. The plug and the socket are designed to contain different non-reversible mutations (deletions) in the marker gene. Consequently restoration of function is absolutely dependent upon homologous recombination.

The general scheme for the plug and socket strategy is illustrated in Fig. 6. In the first step of this two-steps procedure, conventional gene targeting is used to insert a construct that we call a socket near the gene to be altered. A socket consists of a functional positively selectable marker (Ml) and a nonfunctional portion of a different positively selectable marker (ΔM2). Southern analysis or PCR to identify clones containing the correctly targeted socket. In the second step of the procedure, a vector that we call a plug alters the locus containing the socket and supplies the missing portion of the positively selectable marker M2. To accomplish this, the plug contains mouse DNA sequences from the gene to be altered and a nonfunctional portion of the second marker (M2Δ) which overlaps ΔM2. Recombination between ΔM2 and M2Δ, mediated by their shared homology, reconstructs a functional M2. The two positively selectable markers, 1 and 2, must be different (for example, *hyg* and *neo* or *neo* and *HPRT*). The two nonfunctional versions of M2, M2Δ and ΔM2, should have non-reversible but complementary mutations, preferably deletions. Since the plug and socket steps are independent events, different plugs can be used to make different modifications starting with the same cell line which contains an appropriately targeted socket.

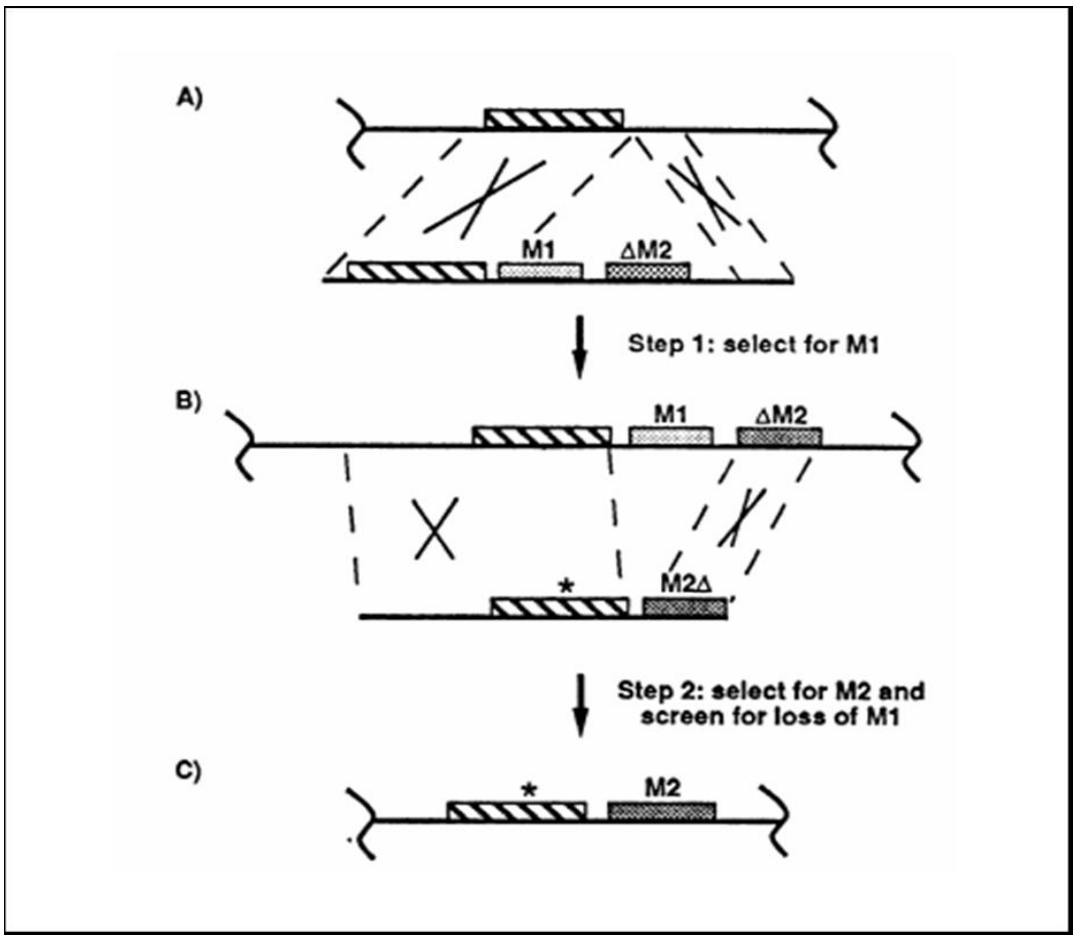


Figure 6 Schematic diagram illustrating the plug and socket strategy. The gene to be altered is shown as a cross-hatched box, and the two selectable marker genes are shown as stippled boxes. The first step involves insertion of the socket targeting construct containing a functional marker gene (MI) and a different nonfunctional marker gene (ΔM2) flanked by homology to the target area. Homologies are indicated by connecting dashed lines with each crossover shown as an X. Insertion of the socket in the first step requires selection for M1 and screening for and cloning of an ES cell line containing the socket at the target locus. The second step involves the completion of the nonfunctional M2 gene by recombination between the socket in the ES cell genome and the plug targeting construct. The plug contains homology to the target locus and a different nonfunctional portion of M2 (M2Δ) which overlaps ΔM2. The desired change is represented by an asterisk. The second step requires selection for a functional M2 and screening for the loss of MI. (A) Recombination event to insert the socket. The upper line represents the gene to be modified in the chromosome, and the lower line shows the socket targeting construct. (B) Recombination event to alter the gene and complete M2. The upper line represents the socket in the mouse chromosome, and the lower line represents the plug targeting construct. (C) The desired change in the chromosome of the ES cell.

Source: Detloff (1994)

11.1 Specific socket insertion 3' of the mouse β-globin locus.

In 1994, Detloff *et al*., have used the mouse adult β-globin locus to test the plug socket strategy and to produce valuable embryonic stem (ES) cell mutants. The socket-containing construct, β-socket (Fig. 7), was electroporated into the BK4 subclone of E14TG2a cells which lack HPRT function due to a deletion of part of the endogenous *Hprt* gene (Hooper *et al*., 1987). M1 in this construct is the *neo* gene; ΔM2 is Δ*HPRT,* part of an *HPRT* minigene. Furthermore, since the plug and socket steps are independent, one ES cell line with an appropriate socket (β-socket) can be transformed repeatedly with a variety of plugs using direct positive selection.

11.2 Deletion of the adult β-globin genes to generate β^{mouse}-knockout mice.

The presence of mouse β -globin chains affects the phenotypes of transgenic mice expressing mutant forms of human hemoglobin (Ryan *et al*., 1990). Although there are some mutations that lower the level of adult hemoglobin in mice (Shehee *et al*., 1993; Skow *et al*., 1983), no null mutation exists for both the adult β-globin genes, β^{maj} and β^{min}. So, In 1995 *Yang, B.* was generated a mouse heterozygous for such a double null mutation for both the adult β-globin gene, β^{maj} and β^{min} , would consequently be valuable for breeding to mice harboring human β-globin transgenes with the aim of subsequently obtaining offspring that depend solely on the transgene for their adult β-globin. A plug that deleted both the β^{maj} and β^{min} genes, βΔplug1.5, contains 4kb of homology upstream of β^{maj} and 1.5 kb of homology to the socket sequences located downstream of the locus. The desired Ω-type (replacement-type) recombination event shown in Figure 8, deletes 20 kb of the ES cell genome (containing the β^{maj}, β^{min} genes and *neo* genes) while

completing the *HPRT* minigene. Three independently targeted ES cells were injected into host B6 blastocysts. Chimeric mice generated from two of them transmitted the targeted gene to their offspring. Mice homozygous for this deletion die perinatally similar to the most severe form of Cooley anemia in humans. Mice heterozygous for the deletion appear normal, but their hematologic indices show characteristics of severe thalassemia.

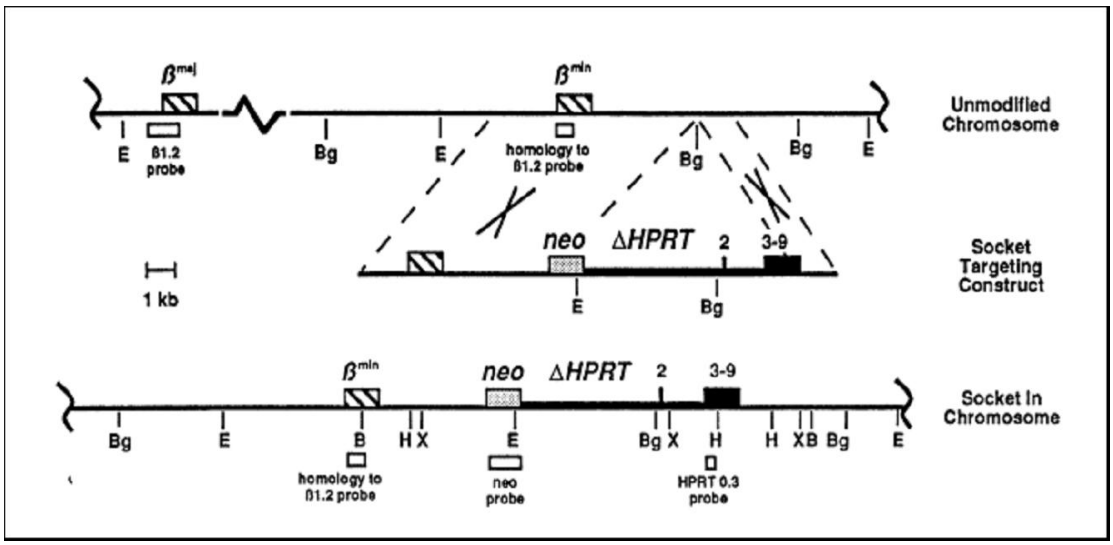


Figure 7 Target locus, socket-containing targeting construct (βsocket), and the chromosome resulting from insertion of the socket downstream of the β-globin locus. Representations are as in Fig. 6, with the following additions. The stippled box represents the neo gene, thick lines indicate HPRT introns, and black boxes represent HPRT exons. Relevant restriction sites for *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hind*III (H), and *Xba*I (X) are shown. Various probes are shown as open boxes.

Source: Detloff (1994)

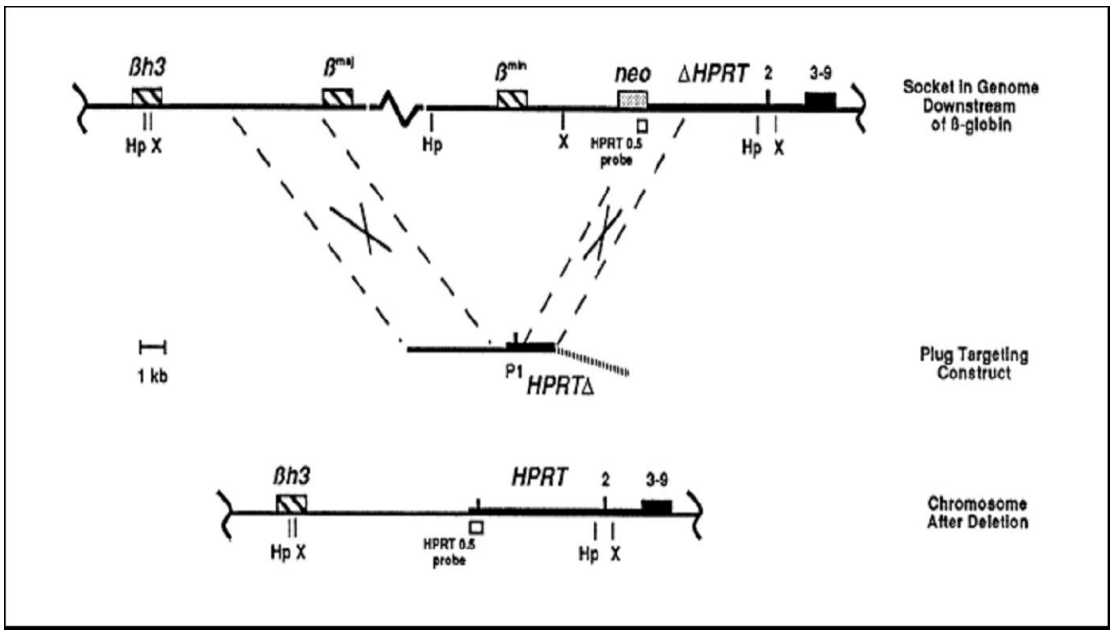


Figure 8 The socket-containing chromosome, the linearized plug targeting construct for deletion, and the chromosome obtained after deletion. Representations are as in Fig. 7, with the following additions: (i) the thick broken line of the targeting construct represents plasmid sequences, and (ii) Hp and X represent HpaI and XbaI restriction sites, respectively.

Source: Yang (1995)

11.3 Replacement of the adult β-globin genes with human βIVSII-654 splicing mutation gene to generate βIVSII-654 knockin mice.

The first step was applied to the murine β-globin locus to produce a generally useful "socket"-containing ES cell line, B20. The B20 cell line, derived from a HPRT-deficient ES cell line, contains a neomycin resistance (*neo*) gene and a partial *HPRT* (ΔHPRT) downstream of the murine β^{maj} and β^{min} genes (Fig.9A). For the second step, a "plug" targeting construct (Fig. 9B) was used to replacement the 21-kb of mouse genomic DNA containing the murine β^{maj} and β^{min} genes with a 5.7 kb DNA fragment containing the human βIVSII-654 gene. The desired mutation (Fig.9C) was designated $Hbb^{th\text{-}4}$ (abbreviated to th-4) (Lewis *et al*., 1998).

The construct, th-4 plug, includes a 5.7-kb genomic *Hind*III-*Xba*I fragment of human β-globin gene, in which the human βIVSII-654 mutation was introduced by site-directed mutagenesis (Kim and Smithies, 1988). The th-4 targeting construct also contains a 3.9-kb *Bam*HI-*Hind*III fragment of BALB/c mouse DNA inserted 5' to the murine adult β-globin genes and a 1.9-kb *Cla*I-*Xho*I fragment, which contains the promoter, and exon 1 of *HPRT* minigene inserted 3' to the mouse genes. The β-globin and *HPRT* gene are in the same transcriptional orientation. Th-4 targeting DNA was introduced into B20 cells by electroporation and selection by hypoxanthine-aminopterin-thymidine (HAT)-resistant. Three germline transmitting chimeras were generated from one of three targeted ES cell lines that were isolated. Chimeras were bred to C57BL/6J (B6) mice to produce F1 offspring. No homozygous mice ($Hbb^{th\text{-}4}$/ $Hbb^{th\text{-}4}$) survive postnatally. Heterozygous mice ($Hbb^{th\text{-}4}$/ Hbb^{+}) mice carrying this mutant gene produce reduce amounts of the mouse β globin chains and no human β globin, and have a moderate form of β thalassemia.

11.4 Introduction of transgene fragment into mouse ES cells

Embryonic stem cells are derived from early mouse embryos and have the capacity to contribute complete development of animal. The fertilized eggs from a pregnant mouse were obtained and cultured for 3 days before harvesting the inner cell mass (ICM) from blastocysts. Culturing of the ICM on feeder cells was performed to develop to ES cell lines (Dinkel *et al*., 1999). Desired DNA is constructed and introduced into ES cells. Several methods can be used to introduce DNAs into ES cells such as transfection and electroporation (Kaufman *et al*., 1999). An antibiotic selectable marker was usually introduced in the transgene fragment for the selection of ES cells containing the transgene. If there is no selectable marker, molecular methods such as PCR and Southern blot analysis can be used for the identification of transgene ES cell lines. The transgenic ES cells were injected into the blastocoel of a new 3-day old host blastocysts and embryo were implanted in the uterus of the surrogate mother. The injected ES cells combined with the host ICM and contributed to the developing embryo. The first generation offspring are chimeras somatic and germ cells composed of both transgenic ES cells and host cells. The transgene in germ cell linage can be transmitted to offspring (Fig. 10) (Gersenstein *et al*., 2002).

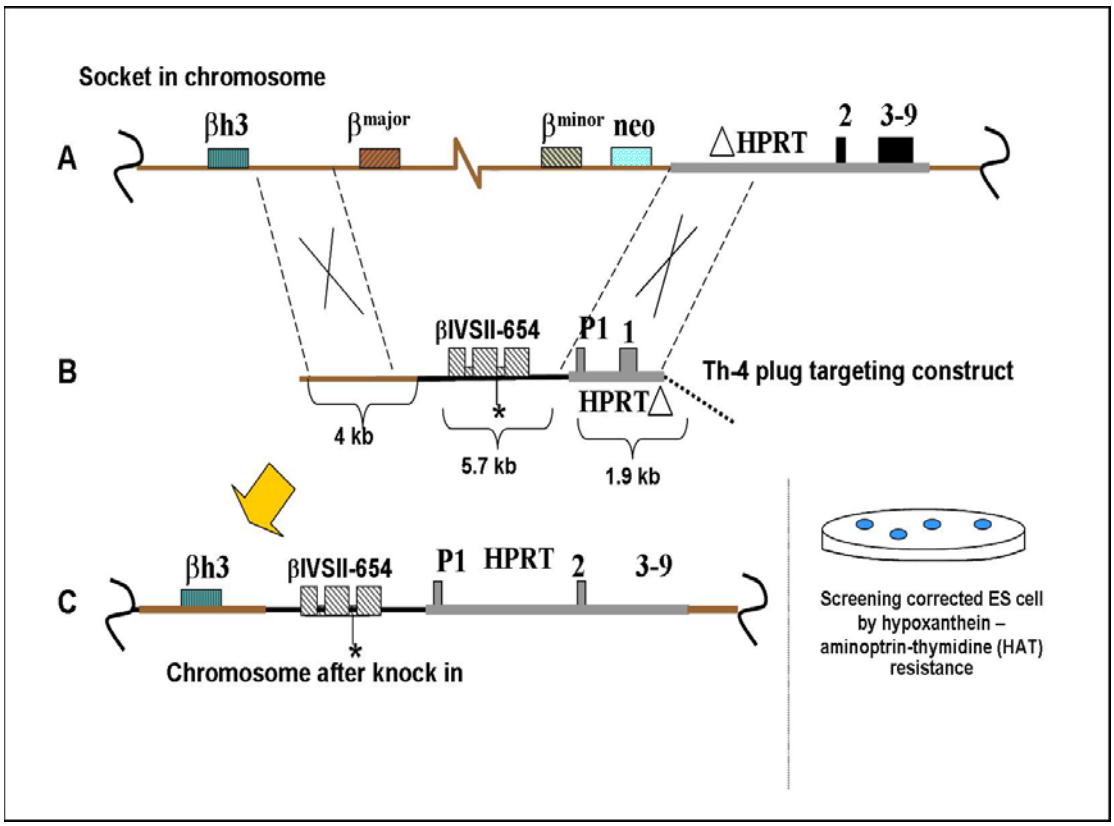


Figure 9 Replacement of the murine adult β-globin genes by the human βIVSII-654 gene. The socket-containing chromosome (A), the th-4 plug targeting construct (B), and correctly targeted chromosome (C) are shown. The exons and introns of genes are represented as boxes and thick line, respectively. The human β-globin gene is cross-hatched with the position of the IVSII-645 mutation shown an asterisk. Promoter (p) and exons 1-9 of HPRT are marked. Upstream and downstream sequences that are identical or homologous in the targeting construct and the target chromosome are demarcated by dashed lines. βh3 is a b-globin pseudogene. Recombination (indicated by Xs) occur between the target genes, β major and β minor. Additionally, the *neo* gene is removed and the function of *HPRT* gene is created by the correct targeting. The HPRT gene and globin genes are transcribed from left to right in figure, the *neo* gene is transcribed from right to left.

Source: Lewis (1998)

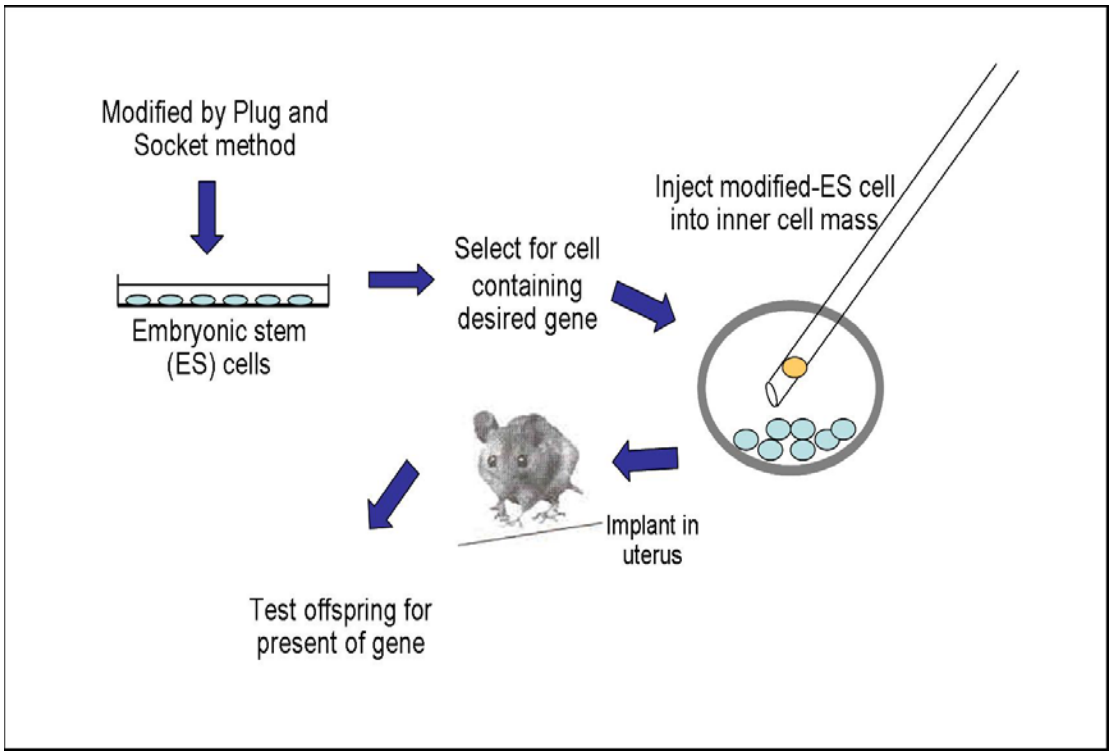


Figure 10. The modified β-socket embryonic stem (ES) cell, by plug and socket method, was injected into the blastocoel of a new 3-day old host blastocysts and embryo are implanted in the uterus of surrogate mother.

Source: Houddbine (2003)

12. Insertion of HbE Mutation into the Human β-Globin Locus BAC clone using GET Recombination System to Generated the HbE Transgenic Mice

The large size of genomic inserts in PAC/BAC clones makes them ideal for the physical mapping of large chromosomal regions, the isolation of complete genes including distal regulatory elements, and as intermediates in the sequence of entire genome (Shizuya *et al*., 1992, Ioannou *et al*., 1994). pEBAC/148β which is a second generation BAC clone contains the entire β-globin locus (about 73 kb) as well as additional sequences at both the 5' and 3' end (Narayanan *et al*., 1999). This plasmid was generated from PAC/148β, a PAC clone isolated from the RPCI 1 human total genomic PAC library (PA Ioannou and PJ Dejong, Roswell Park Institute, Buffalo, NY, USA). 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 generated pEBAC/148β. The hygromycin (Hyg^R) and thymidine kinase (*tk*) genes enable selection in eukaryotic cells. (Fig. 11)

In 2003, Jamsai *et al*. had generated the cell lines having the insertion of HbE mutations in pEBAC/148β clones,. The *GET* recombination system in combination with *Eco*RI endonuclease gene as a counterselection marker was used to introduce HbE mutation (codon 26, GAG→AAG) which found in high frequency in South-East Asia (Fig. 12, 13). In the first stage of GET recombination, a single copy of the *Eco*RI/kan^R cassette was introduced into the β-globin gene of pEBAC/148β. The *Eco*RI/Kan^R cassette was amplified and targeted into IVSI of the β-globin gene, with the deletion of 9 bp in between the homology regions (Fig.14)

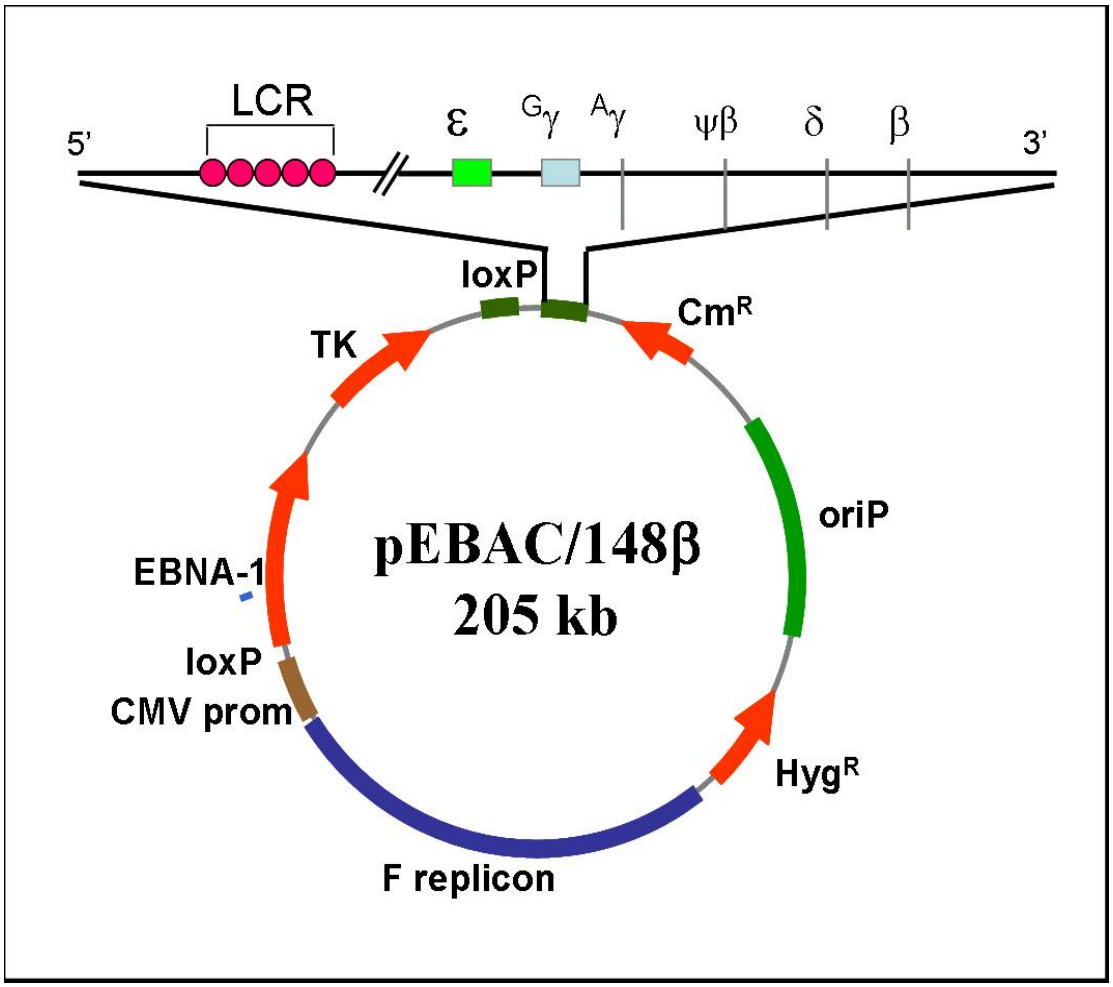


Figure 11 The pEBAC/148β is based on the backbone of the F plasmid pBeloBAC11. The hygromycin (hyg^R) and thymidine kinase (tk) genes enable for selection in eukaryotic cells, while the *ori*P and *EBNA*-1 genes from Epstein-Barr virus facilitate episomal maintenance. The chloramphenicol resistant gene (Cm^R) gene for selection in bacterial cells.

Source: Narayanan (1999)

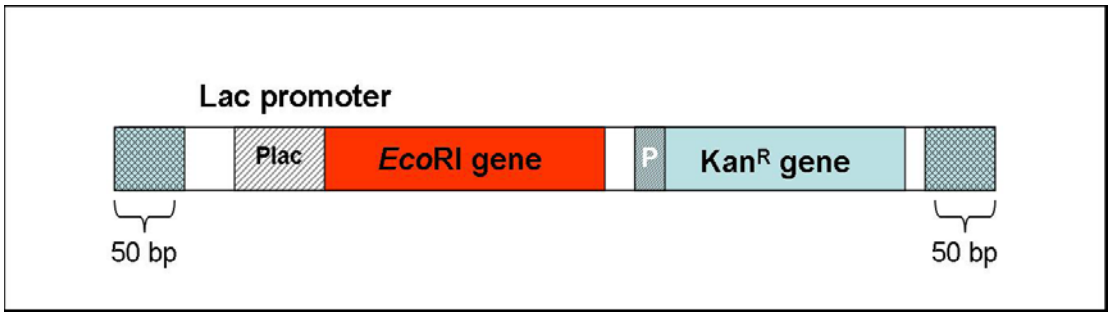


Figure 12. *Eco*RI endonuclease counterselection cassette was used in *GET* recombination system. 50 bp sequence flanked at 5'and 3' end was homologous into IVS I of β-globin gene. Efficient counterselection depended upon the tight regulation of the highly toxic *Eco*RI endonuclease gene by expression of *lacI*.

Source: Jamsai (2003)

The deletion of the counterselection cassette in the second round of *GET* recombination used to introduce DNA fragment containing HbE mutation, 732 bp amplified product from genomic DNA of homozygous HbE (codon 26, GAG→AAG), into pEBAC/148β:: *Eco*RI/Kan^R and pGETrec2 (Fig15). The *Eco*RI/Kan^R cassette was knocked out and HbE mutation DNA fragment was integrated into human β-locus BAC clone.

BAC DNA extraction was also performed using the alkaline lysis method. The 185 kb genomic insert containing the human β^E-globin locus was released from vector backbone by digestion with *Not*I endonuclease and microinjection into C57BL/6 fertilized oocytes (Posfai *et al*., 1999). Three transgenic lines were obtained and confirmed by fluorescence *in situ* hybridization (FISH) analysis, which showed the integration of human β-globin transgene in only one site of mouse chromosome. Transgene copy number determined by Southern blot hybridization with the human β-globin probe showed that transgene copy number was four for line 1, and two for lines 2 and 3. The intact β-globin locus was demonstrated in all transgenic lines by seven PCR primers covering the T7 and SP6 ends of the β-globin genomic fragment, although HbE transgenic line 2 appeared to have a deletion close to the T7 end. However, the possibility of some internal rearrangement or deletions in one or more of the transgene copies could not be excluded (Jamsai, 2004).

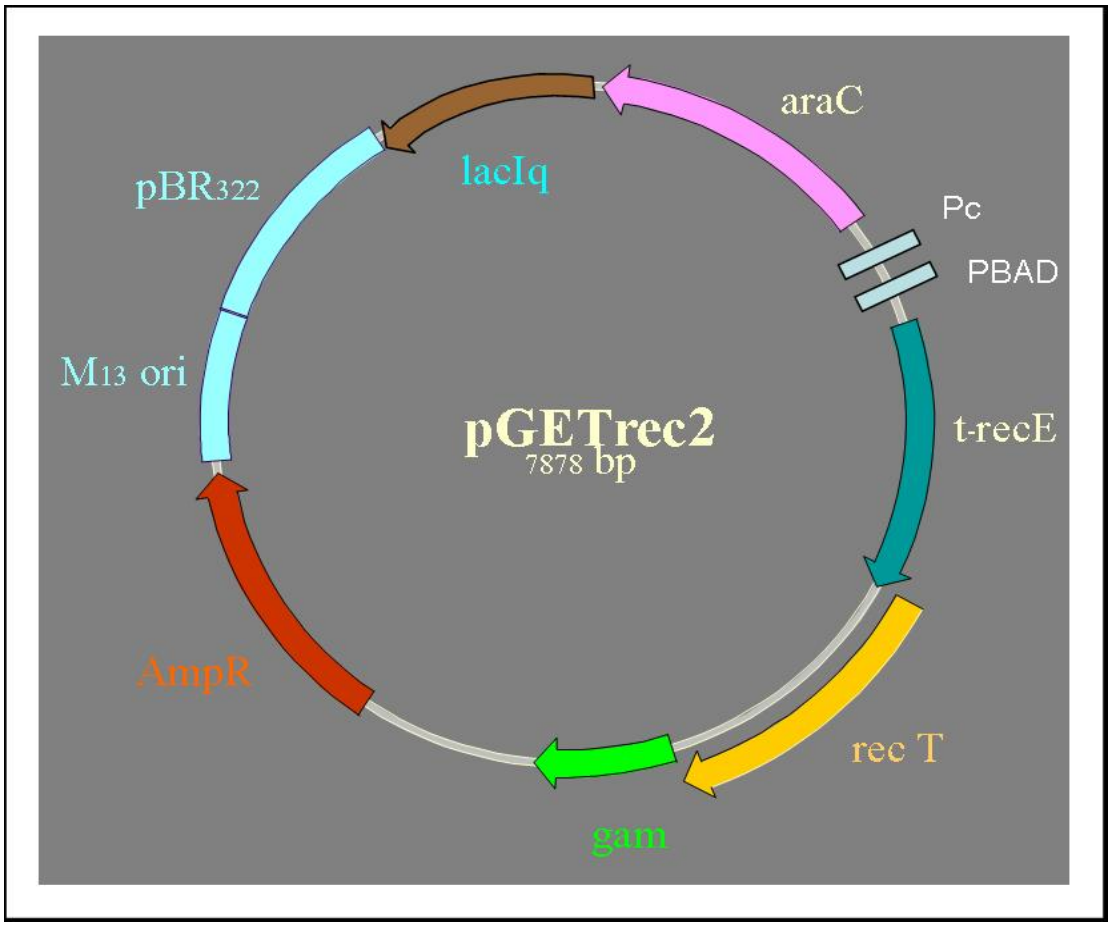


Figure 13 Map showing the main features of pGETrec2 plasmid. The *lacI*^{q} gene with own constitutive promoter. Bacteriophage λ gam gene was inhibit nuclease in *E.coli*. *RecE* and *recT* genes, facilitates homologous recombination between DNA fragments and BAC clone.

Source: Jamsai (2003)

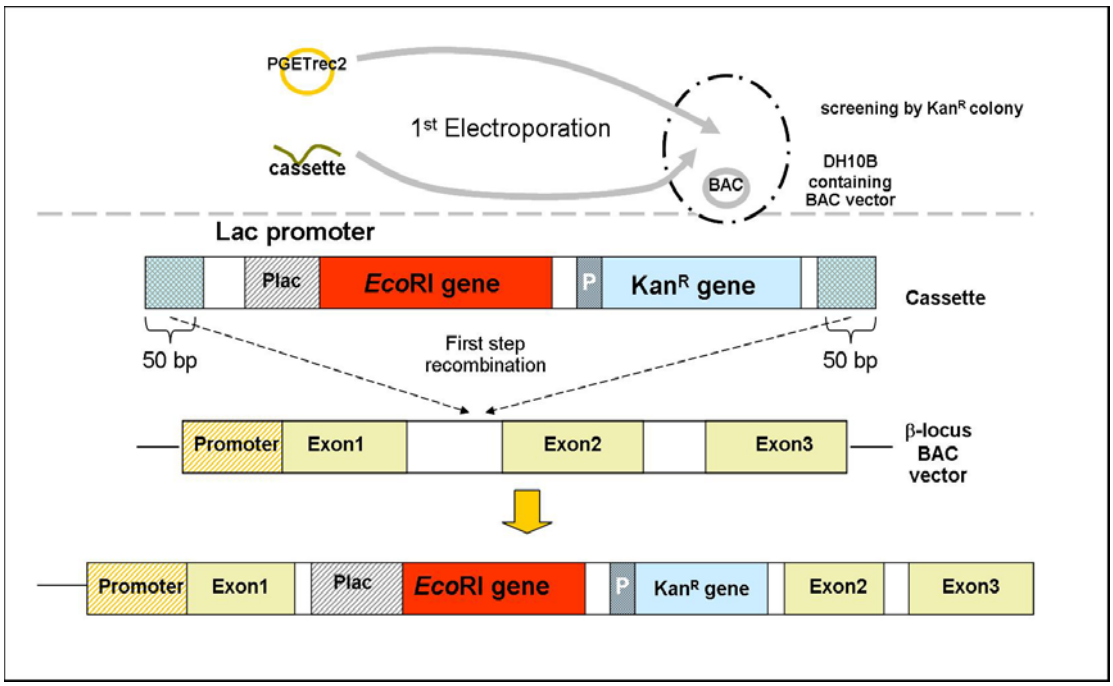


Figure 14 Diagram illustrating the insert of disease-causing mutation into a 205 kb globin BAC clone using the *Eco*RI/Kan^R cassette in two-stage *GET* recombination. In the first stage, *Eco*RI/Kan^R cassette was inserted into intron1 of the β-globin gene using homology arms corresponding to the targeted region of pEBAC/148β. Recombinant clones are identified on Kan plates, while expression of *Eco*RI is replaced by constitutive expression from the pGETrec2 vector.

Source: Jamsai (2003)

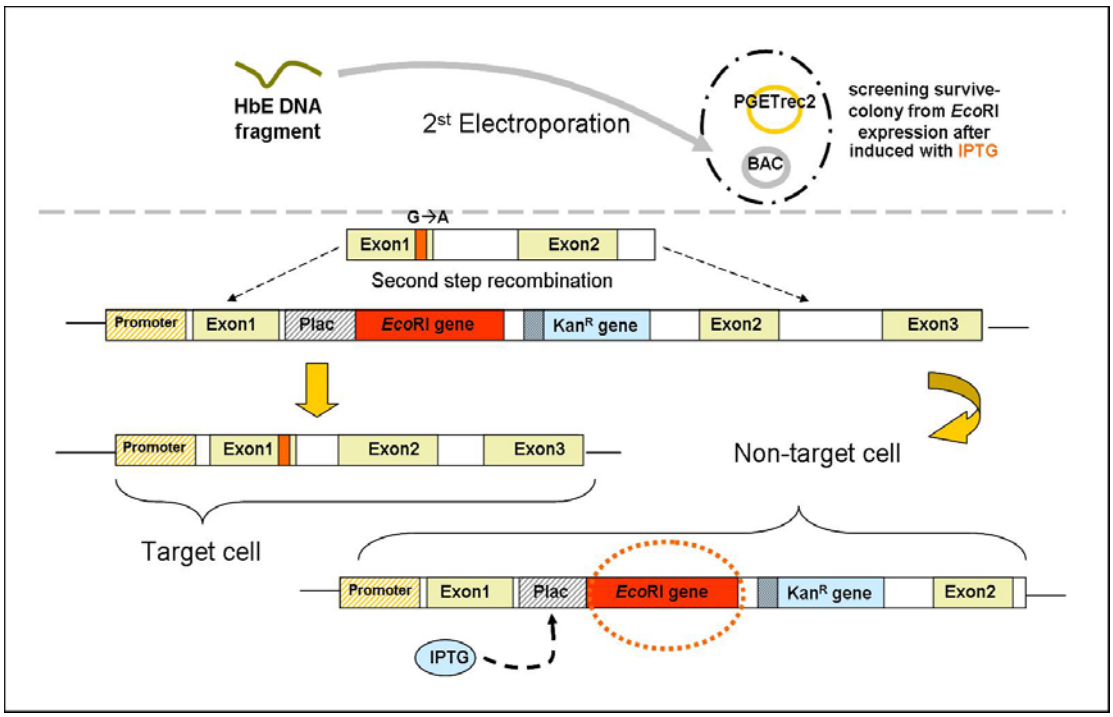


Figure 15 In the second stage of recombination, *Eco*RI/Kan^R cassette was knocked out by PCR fragments carrying the HbE mutation. Non recombinant clones were eliminated by induction of the *Eco*RI gene by plating the cells on IPTG.

Source: Jamsai (2003)

METERIALS AND METHODS

Materials

1. Mice

The founder β^E-transgenic (TG) mice [Tg (LCR$\varepsilon^G\gamma^A\gamma\delta\beta^E$) *Hbb*$^+$*/Hbb*$^+$] carrying the entire human β-globin locus with codon 26 Glu → Lys mutation were generated on a C57BL/6J background by using GET recombination system at Murdoch Childrens Research Institute (MCRI) **(**Jamsai, 2004).

The heterozygous βIVSII-654 mice, principle of β^0-thalassemic mice model, was generated by "Plug and Socket" method, in which replacement of 21 kb region encompassing of the mouse β^{major} and β^{minor} with 5.7 kb DNA fragment of human β-globin gene contains IVSII-654 splicing mutation (Lewis *et al*., 1998). Spliced βIVSII-654 mRNA retained nucleotides 580-652 of second intron and as a result did not encode a functional β-globin polypeptide.

The β-thalassemic Hbb^{th-3}/Hbb$^+$ or β-knockout mice, kindly provided by MCRI, Australia, have a heterozygous deletion of adult murine β-globin gene (β^{major} and β^{minor})

The mother lines were maintained in the animal facility of National Laboratory Animal Centre (NLAC), Mahidol University. Colonies were bred at Thalassemia Research Center (TRC) animal facility.

2. Chemicals

2.1 Chemicals for polyacrylamide gel electrophoresis for globin chain s separation

- Acrylamide (acrylic acid amide) (C_3H_5NO), electrophoresis grade, M.W. 71.08, Sigma Chemical Company, U.S.A.
- Ammonium persulphate ($H_8N_2O_4S$), electrophoresis grade, M.W. Sigma Chemical Company, U.S.A.
- Mercaptoethanol (C_2H_6OS), reagent grade, M.W. 78.13, Sigma Chemical Company, U.S.A.
- Coomassie Brilliant Blue, Sigma Chemical Company, U.S.A.
- Glacial acetic acid (CH_3COOH), electrophoresis grade, MW. 60.05, Sigma Chemical Company, U.S.A.
- N,N'-Methylene bis-acrylamide ($C_8H_{12}N_2O_4$), electrophoresis grade, MW. 200.19, Sigma Chemical Company, U.S.A.
- N,N,N',N'-Tetramethylethylenediamine (TEMED), electrophoresis grade, MW. 200.19, Sigma Chemical Company, U.S.A.
- Urea crystal (NH_2CONH_2), extra pure, M.W. 60.60, Sigma Chemical Company, U.S.A.

2.2 Chemicals for cellulose acetate electrophoresis

- Trizma base, Tris (Hydroxymethyl) Aminoethane, reagent grade, M.W. 121.10, Sigma Chemical Company, U.S.A.
- Ethylenediaminetetraacetic acid (EDTA) ($C_{10}H_{14}N_20_8Na_2.H_20$), M.W. 372.24, BDH Laboratory Supplies, England.
- Boric acid (H_3BO_3), M.W. 61.83, BDH Laboratory Supplies, England.

2.3 Chemical for red cell survival study

- Biotin 3-sulfo-N-hydroxy-succinimide ester sodium salt ($C_{14}H1_8N_3NaO_8S_2$), M.W. 443.4, Sigma Chemical Company, U.S.A.

- Streptavidin-phycoerythrin (PE) conjugated (SAV-PE) (0.5 mg/ml) is a second step reagent for the indirect immunofluorescent staining of cells in combination with biotinylated primary antibodies for flow cytometric analysis. Excitation at 488 nm leads to a fluorescence emission maximum of ~575 nm, BDapplication, U.S.A.

3. Instruments

- Automated blood cell analyzer Advia 120, Bayer, U.S.A.
- Densitometer GS710, Biorad, U.S.A
- FACSort flow cytometer, Becton Dickinson, U.S.A.
- Super Z-12 Applicator Kit, Helena laboratories, U.S.A.
- Zip Zone camber, Helena laboratories, U.S.A.
- Titan III-H cellulose acetate samples, Helena laboratories, U.S.A.

Methods

1. Breeding Scheme to Generate the Mouse Model for β^0-Thalassemia/HbE Disease

Mouse model for β^0-thalassemia/HbE disease or rescued mice (HbE transgene on homozygous β^0-thalassemia background) were generated through breeding scheme by using the mouse model for both β^0-thalassemia and HbE transgenes. Different genotypes of rescued mice were produced by two types of breeding scheme (Fig. 16-19). In the first scheme, mouse carrying HbE transgene is in the homozygous βIVSII-654 knockin type. While in the second scheme the mouse is compound heterozygous βIVSII-654 knockin /β^{mouse} knockout with HbE transgene.

1.1 Breeding scheme to generate the rescued mice for homozygous βIVSII-654 knockin with HbE transgene

1.1.1 The first breeding was to produce mice carrying HbE transgene on the heterozygous βIVSII-654 mutation background (double heterozygous βIVSII-654 mice). This was performed by breeding hemizygous transgenic mice line 1 which carries 4 copies of HbE transgene (Jamsai *et al*., 2004) with heterozygous βIVSII-654 knockin mice (Lewis *et al*., 1998) (Fig. 16). Mouse tail DNA and whole blood from the tip of tails from all pups were examined for double heterozygous βIVSII-654 phenotype by multiplex PCR and cellulose acetate electrophoresis. The double heterozygous βIVSII-654 mice that have been checked were used as parents for next breeding.

1.1.2 The second breeding were to produce the mice carrying HbE transgene on homozygous βIVSII-654 knockin mutation background (rescued mice). This was performed by breeding together the two double heterozygous βIVSII-654 knockin / HbE mice (Fig. 17). Mouse tail DNA from all pups born were examined by multiplex PCR to search for the genotype of the rescued mice.

1.2 Breeding scheme to generate the rescued mice for compound heterozygous βIVSII-654 knockin /β ^{mouse} knockout with HbE transgene

1.2.1 The first step of mating was to produce double heterozygous βIVSII-654 knockin mice by mating heterozygous βIVSII-654 knockin mice with hemizygous transgenic mice line 1 which carry 4 copies of HbE transgene (Fig. 18). The next step, the heterozygous β ^{mouse} knockout mice (Yang *et al.*, 1995) mated with HbE transgenic to generate double heterozygous β ^{mouse} knockout mice. Mouse tail DNA and whole blood of all pups were used for searching both genotypes of double heterozygous mice by multiplex PCR and cellulose acetate electrophoresis.

1.2.2 Mice carrying HbE transgene on compound heterozygous βIVSII-654 knockin /β ^{mouse} knockout background (rescued mice) were produced by breeding the double heterozygous βIVSII-654 with double heterozygous β ^{mouse} knock out mice (Fig. 19). Mouse tail DNA of all pups born were checked by multiplex PCR to search for the rescued mice.

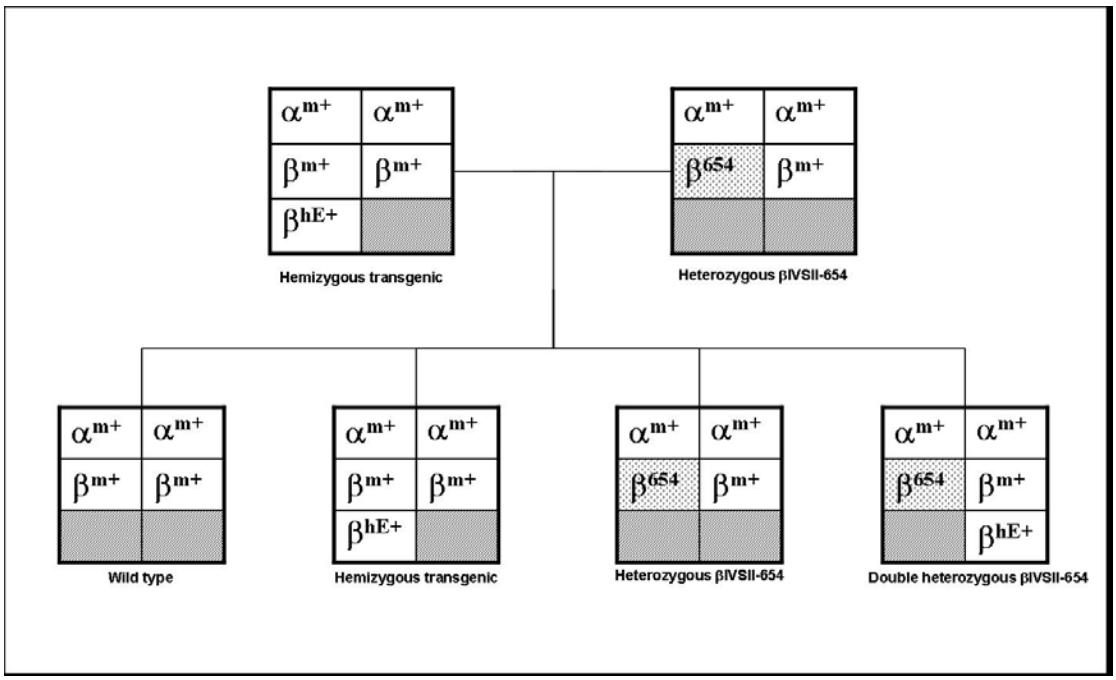


Figure 16 Breeding scheme to generate the double heterozygous IVSII-654. Each mouse genotype is represented by a large square box containing 6 small boxes. Upper panels (right and left) are expressed as a pair of homologous mouse α-globin alleles on the chromosome 11. Middle panels are the homologous alleles of mouse β-globin gene on the chromosome 7, which is the site for knockin by human βIVSII-654. Lower panels represent the integrated whole construct of human β^{E}-globin locus, previously found on the chromosome 2 by *in situ* hybridization.

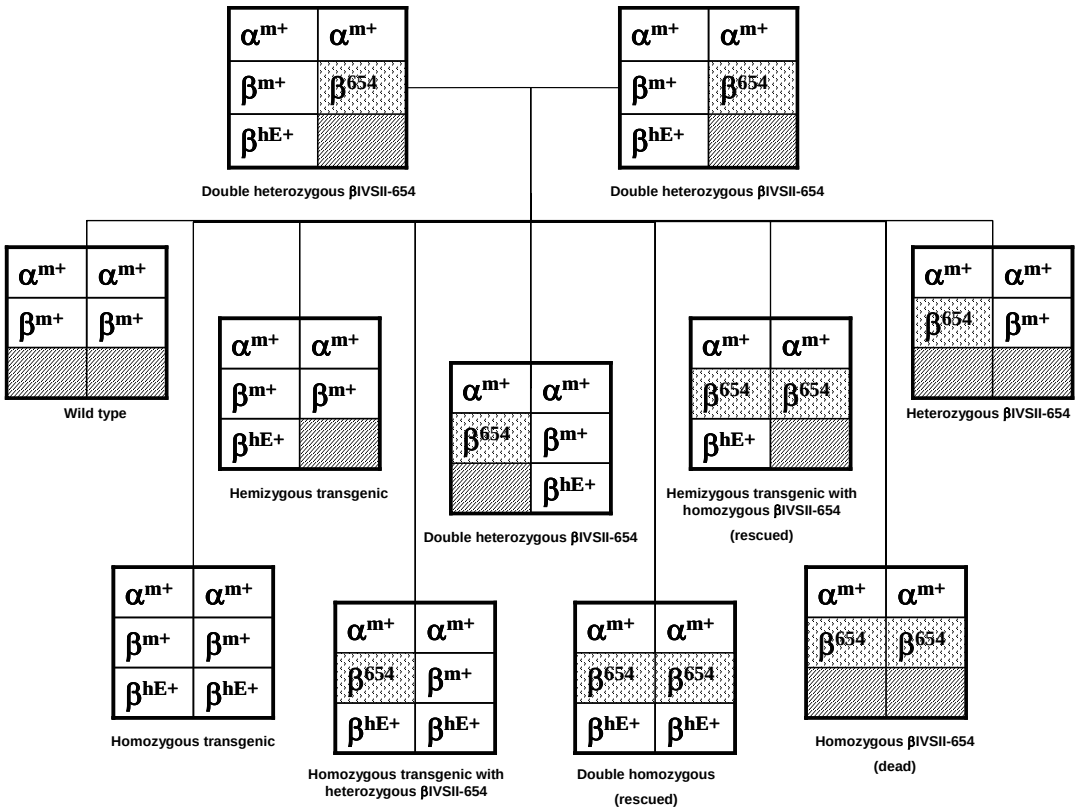


Figure 17 Breeding scheme to generate the rescued mice using double heterozygous βIVSII-654 mice as parents. Only one-site integration of human β^{E} transgene or β^{hE+} (and one dark-filled square) means 'hemizygous' condition, in addition, double-integratation haplotype defines as 'homozygous' condition.

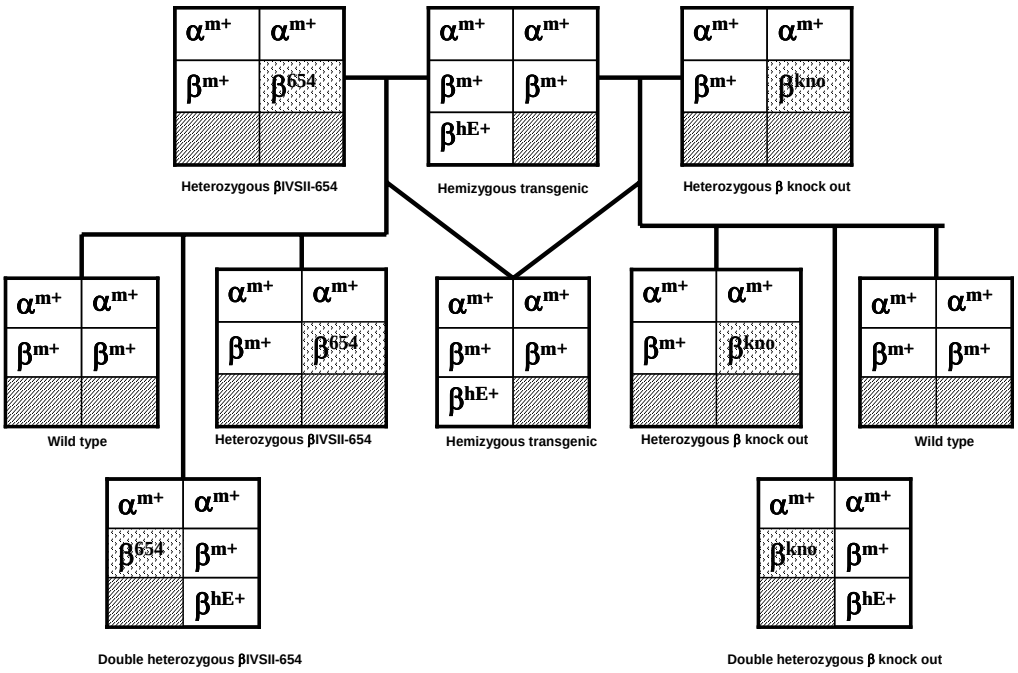


Figure 18 Breeding scheme to generate the double heterozygous βIVSII-654 and double heterozygous β-knockout mice. Each mouse genotype is represented by a large square box containing 6 small boxes. Upper panels (right and left) are expressed as a pair of homologous mouse α-globin alleles on the chromosome 11. Middle panels are the homologous alleles of mouse β-globin gene on the chromosome 7 which the site for knock in by human βIVSII-654 and β-knock out gene. Lower panels represent the integrated whole construct of human β^{E}-globin locus.

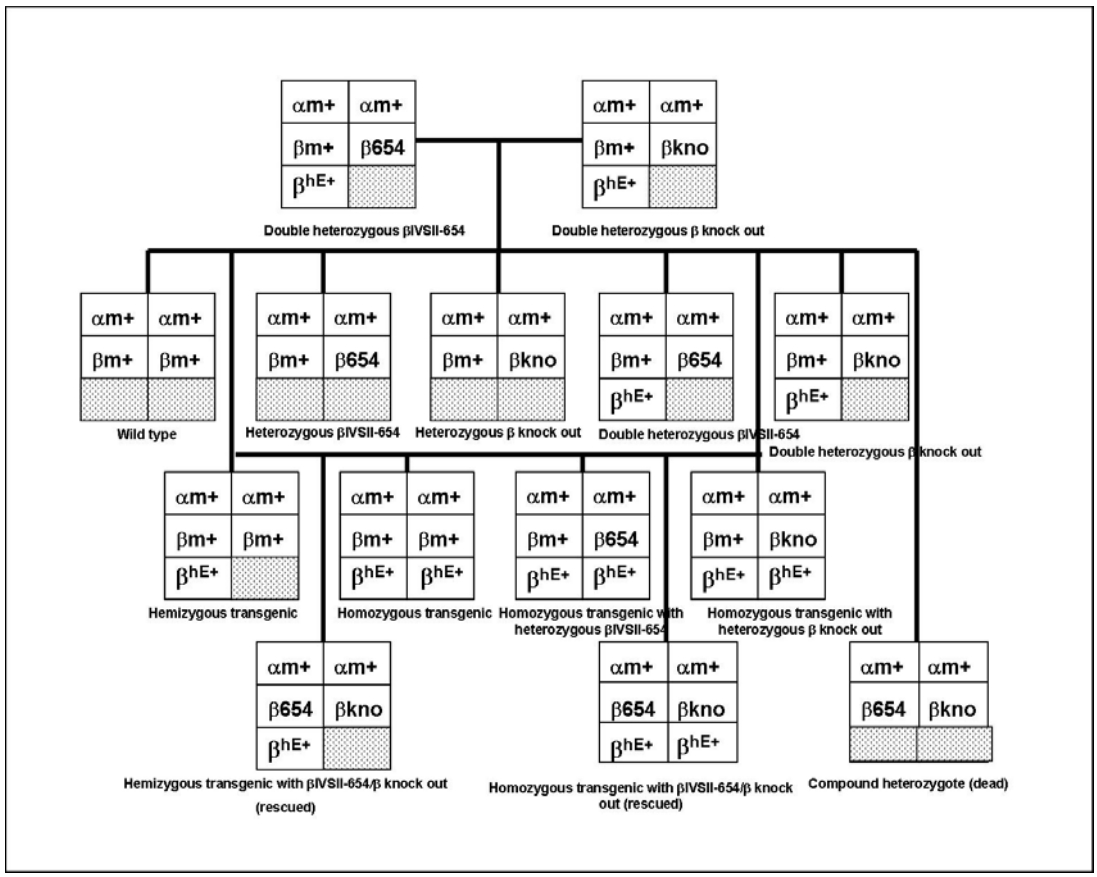


Figure 19 Breeding scheme to generate the rescued mice using double heterozygous βIVSII-654 and double heterozygous β knock out mice as parents. Only one-site integration of human β^{E} transgene or β^{hE+} (and one dark-filled square) means 'hemizygous' condition, in addition, double-integratation haplotype defines as 'homozygous' condition.

2. Characterization of Thalassemic Mice

2.1 Mouse tail genomic DNA extraction

This is a rapid phenol-chloroform based extraction method optimized for preparation of good quality genomic DNA from multiple samples in a relatively short time. Genomic DNA was isolated from a tail biopsy (~0.5-1.0 cm). The tails were digested at 55 °C in 500 µl of tail lysis buffer (50 mM Tris-HCl, pH 8.0, 100 mM EDTA, 0.5 % SDS) and 5 µl of 20 mg/ml Proteinase K for 5 h or overnight. After digestion, approximately 200 mg of vacuum grease and 300 µl of phenol/chloroform (1:1 v/v) solution were added to the tail solution and mixed inversely. After centrifuging at 11,700 ×g for 5 min, the top phase was removed to a new tube and 300 µl of chloroform was subsequently added (this step can be performed twice if the quality of DNA is not satisfactory with one treatment). The sample was then centrifuged again at 11,700 ×g for 5 min. The supernatant was again collected to a new tube and then 40 µl of 5 M NaCl and 1 ml of absolute ethanol were added to precipitate the genomic DNA. DNA pellet was obtained by centrifuged at 11,700 ×g for 5 min and cleaned once with 70 % ethanol solution and then air-dried for 3 h. Finally, DNA was dissolved in 200 µl of DNA hydration solution (PUREGENE®) and store samples at -20 ^{0}C.

2.2 Identification of mice genotypes by multiplex PCR

Genotyping of mice in breeding schemes was performed by multiplex PCR using three primer pairs (Table 1); LUG1A/LUG2A (447-bp product) for detecting human β-globin gene, HPRT-FW/HPRT-RW (315-bp product) representing HPRT minigene in β-knockout and β-humanIVSII-654 knockin construct and Mouseβ-FW/Mouseβ-RW (260-bp product) for mouse

β-globin gene. Multiplex PCR reaction (Table 2) was carried out in 25 μl reactions by starting the first cycle at 94 °C for 5 min, followed by 30 cycles (94 °C 30 sec; 55 °C 30 sec; 72 °C 30 sec) and finished with the last cycle at 72 °C 10 min. Aliquots 10 μl of each reaction product were run on a 2 % agarose gel. In the case of wild-type mice, only one PCR product was produced from Mouseβ primers. Two PCR products were amplified from Mouseβ and HPRT primers as represented heterozygous β-knockout mice. Hemizygous transgenic mice on the normal mouse background produced two PCR products with Mouseβ and LUG primers. Three PCR products, amplified from heterozygous βIVSII-654 mice, had the same result as HbE double heterozygous βIVSII-654 and double heterozygous knockout mice, which showed three PCR products amplified by Mouseβ, HPRT and LUG primers. Rescued mice produced two PCR products with HPRT and LUG primers because of a lack of mouse β-globin gene (Jamsai, 2004).

2.3 Identification of heterozygous βIVSII-654 mice and double heterozygous βIVSII-654 mice by cellulose acetate electrophoresis.

Heterozygous βIVSII-654 and double heterozygous βIVSII-654 mice were unable to identify by multiplex PCR, since both genotype had the same PCR products from Mouse β, HPRT and LUG primer. But mouse hemoglobin and chimeric hemoglobin expressed from the two genotypes were different, and their hemoglobin could be distinguished by cellulose acetate electrophoresis.

20 μl of whole blood from tail vain was centrifuge at 3,000 rpm for 10 min, and plasma was aspirated. The packed erythrocyte were washed twice with 0.9% NaCl, and 5 μl of the sample was lysed in 20 μl distillated water with vortex. Hemolysate (the destruction of RBCs resulting in the release of

hemoglobin protein from the cells) were run on cellulose acetate membranes (Helena Laboratories) at 300V for 20 min, stained with Ponceau S (Sigma) in 5% trichloroacetic acid for 10 min, and de-stained twice in 7% acetic acid for 10 min. The gels were fixed twice in methanol for 5 min, and placed in clear solution (15 ml of glacial acetic acid, 35 ml of methanol) for 7.5 min, and dried under a microhood for 10 min.

2.4 Characterization of double heterozygous β-knockout and double heterozygous βIVSII-654 mice.

2.4.1 Allele specific PCR

Allele specific PCR (Old *et al.,* 2001) was applied to identify genotype of double heterozygous β-knockout and double heterozygous βIVSII-654 mice by detecting βIVSII-654 mutation. Two primers specified βIVSII-654 mutation, βIVSII-654 normal and βIVSII-654 mutant primer, reacted with common primer D by separating the PCR reaction into two tubes containing normal primer + primer D and mutant primer + primer D (Table 3). Genomic DNA was isolated from a tail biopsy, mouse's DNA of each sample was diluted to 44 ng/ul (approximated by OD_{260}). Allele specific PCR reaction (Table 4) was carried out in 25 μl reactions by starting the first cycle at 95 °C for 15 min, followed by 30 cycles (94 °C 30 sec; 55 °C 55 sec; 72 °C 45 sec) and finished with the last cycle at 72 °C 10 min. 10 μl of each reaction product was run on a 2 % agarose gel. In case of double heterozygous knock out mice, only one PCR product was produced from IVSII-654 normal primers. The 829 bp PCR products were amplified from IVSII-654 normal and mutant primers as represented double heterozygous IVSII-654.

Table 1 Primer sequences from multiplex PCR used for detect mice genotypes

PCR primer	Sequence (5′→3′)	PCR product
LUG1A	ACAAGACAGGTTTAAGGAGACCA	Humanβ -globin gene
LUG2A	GTCTGTTTCCCATTCTAAACTGTA	
HPRT-FW	GATGGGAGGCCATCACATTGTAG	HPRT marker gene
HPRT-RW	GCGACCTTGACCATCTTTGGATTA	
Mouse β-FW	TGAGAAGGCTGCTGTCTCTTG	Mouse β-globin gene
Mouse β-RW	CAGAGGATAGGTCTCCAAAGCTA	

Source: Jamsai (2004)

Table 2 Multiplex PCR component

Reagent	concentration	
	stock	working
PCR buffer	10 ×	1 ×
Magnesium chloride	25 mM	2.5 mM
dNTP	2 mM	200 μM each
LUG1A/LUG2A	20 μM	0.4 μM
HPRT-Fw/HPRT-Rev	15 μM	0.3 μM
MouseBeta-Fw/MouseBeta-Rev	10 μM	0.2 μM
Taq DNA polymerase	5 units/μl	0.5 units
Genomic DNA template	100 ng/μl	100 ng

Table3 Primer sequences used for the detection of the βIVSII-654 mutation by the allele-specific priming technique

PCR primer	Sequence (5′→3′)	product size (bp)
IVSII-654 (normal sequence)	GAATAACAGTGATAATTTCTGGGTTAACGC	829
IVSII-654 C→T (mutant sequence)	GAATAACAGTGATAATTTCTGGGTTAACGT	829

The above primers are coupled as indicated with primer D:
5'-GAGTCAAGGCTGAGAGATGCAGGA-3'.
Source: Old (2001)

Table 4 Allele specific PCR component

Component	Volume	Final concentration
10 x PCR buffer	2.5 µl	1x
2 mM dNTP	2.5 µl	0.2 mM
50 mM $MgCl_2$	3.0 µl	6 mM
Primer D (4 pmol/µl)	1.0 µl	160 mM
IVSII-654 normal (4 pmol/µl)	1.0 µl	160 mM
IVSII-654 normal (4 pmol/µl)	1.0 µl	160 mM
Taq DNA polymerase (Hot start)	0.15 µl	200-500 ng
Genomic DNA template (approximately 100 ng/ul)	1.0 µl	100 ng
Distilled water	13.85 µl	
Total	25.00 µl	

3. Polyacrylamide Urea Gel Electrophoresis

Hemolysate samples were used for studying globin chain expression. Approximately 31.25 g/ml of hemolysate protein (determine by Drabkin method, see appendix) was run onto two-phase polyacrylamide gel containing a separating gel (12 % polyacrylamide gel containing 6 M urea and 2 % Triton-X 100, in 5 % glacial acetic acid) and a stacking gel (2 % polyacrylamide gel). The gel was pre-electrophoresed in 5 % glacial acetic acid for 2 h at 250 V (constant voltage). After pre-electrophoresis, the acid solution was removed and fresh 5 % acetic acid solution was added again. The samples containing equal amounts of protein were loaded and the electrophoresis was performed for 17 h at constant current of 7 mA. The gel was stained in the staining solution with 0.06 % Coomasie Blue for 1 hour and destained.

3.1 Gel mixture prepare

Solution B	16 ml
Solution A	4 ml
TEMED	200 μl
15% ammonium persulphate	200 μl

Put the mix in to the glass, insert the comb, and then overlay with the water. Gelling occurred within 30 min but left the gel for 2 hours prior to use.

3.2 Preparation of hemolysate

Mouse tail blood was collected by heparinized capillary into a new Eppendorf tube and washed with 3-5× volumes of deionized HBSM. After 11,700 ×g centrifugation for 12-15 sec, supernatant was discarded and replaced with 1× volume sterile water. The blood sample was then mixed well with vortex and spinned down again at 11,700 ×g for 5 min. The clear supernatant was collected in a new Eppendorf tube. Mix 5 min at room temperature of 90 μl sample buffer with 10 μl Hemolysate

3.3 Staining and destaining

After electrophoresis, the gel was stained with staining solution for 1 h and then destained with destaining solution for 1 h on a shaker. The destaining solution was changed twice during destaining process. The gel was analyzed on GS710 densitometer before it was air-dried on cellophane membrane.

4. Hematological Analysis

4.1 Blood collection method

4.1.1 Retro-orbital sinus puncture

This technique was used for the collection of blood samples of alive rescued mice during hematologic study. The mouse was manually restrained, and heparinized capillary tube was inserted at the medial or lateral canthus of the eyes. The tube was gently rotated and directed caudally at 30^0 angle. As the sinus was ruptured, blood (~300 μl) will flow back into and through the tube (Figure 20a). After the tube was withdrawn, mild pressure should be applied with cotton or a gauze sponge to stop bleeding. If personals were properly trained and skilled in this technique, it could be accomplished with little trauma and would followed by rapid healing. If there were any question about the training and skilled of the person performing the procedure, the mouse should be anesthetized. Retro-orbital blood sampling could be performed more than once at the same site, although the risk of permanent damage increasing each time the procedure was performed. It was preferable to allow at least two weeks between sampling from the same sinus.

4.1.2 Tail laceration

This technique was used in studying red cell survival by using a little amount of blood sample. The mouse was restrained on a flat surface or placed in a restrain device with the tail protruding. A cut may be made on the ventral surface of the tail to lacerate the artery, or the tip of the tail might be amputated. Warming the animal or its tail beforehand would increase the flow blood, which would generally not exceed a few drops.

4.1.3 Cardiac puncture

This should be done only as a terminal procedure in an anesthetized mouse. The animal was placed on its back on a flat surface (Figure 20b). A 24-gauge needle was either (1) inserted through the diaphragm lateral to the xiphoid cartilage and directed forward and medially toward the heart; or (2) inserted between the fifth and six ribs on the left side and directed forward toward the heart. This method requires training and skill, but could be used to obtain comparatively large volumes of blood to study hematologic.

4.2 RBC morphology analysis

4.2.1 Cleaning the slide

Slides used for blood smears must be free of grease and dust. Slides may be purchased precleaned or may be washed with soap and water, rinsed thoroughly in hot water and then distilled water, dipped in 95% ethanol, and polished with a clean, lint-free cloth. Clean slides may be stored in 95% ethanol and should be handled by the edge only. Slides with frosted ends are preferred because they could easy to label.

4.2.2 Collecting the blood specimen

A satisfactory smear might also be made from cardiac puncture of anesthetized mice which had the anticoagulation EDTA added to it. Tubes of aniticoagulated blood must be mixed for at least two minutes by mechanical mixer or inverted gently sixty times by hand before the sample was taken. Smear was made within two hours of collection.

4.2.3 Making the smear

The blood smear could be prepared by placing a small drop of well-mixed blood about one-half to three quarters of an inch from the right end of precleaned slide placed on the flat surface. The end of a second "spreader" slide was brought to rest at 30-35^0 angles in front of the drop of blood. The spreader was then brought back into the drop of blood until the drop spread along three-quarters of the edge of the spreader slide. This should be performed in a smooth, quick sliding motion. As soon as the blood spreads along the edge of the spreader, the spreader is pushed to the left with a quick, steady motion to spread a blood into the thin film. The smear is placed in a slide-drying rack and allowed to air-dry as quickly as possible. It is then ready for straining or preserving.

4.2.4 Preserving and staining the smear

Dried smears should be stained immediately. If this was not possible, the dried smear may be immersed in methanol for 30-60 seconds and then allowed to air dry. The methanol was a fixative that prevents changes or deterioration of the cellular component. A smear is placed on staining rack and flooded with Wright stain. Fixation occurs in this step because of the methyl alcohol in the stain. After approximately one to three minutes, an equal volume of phosphate buffer, pH 6.8-7.0, is added drop wise to the stain, mixing the stain and buffer together. A green metallic shield appears when solution was mixed, usually within two to four minutes. Times may vary according to stain and buffer used. The slide was rinsed gently, allowed to air-dry, and can then be examined using microscope.

4.3 Complete blood count

Murine EDTA blood was drawn by cardiac puncture of anesthetized mice. Red blood cell indices and reticulocyte count were measured using the automated hematological analyzer Advia[120]. The two independent Student *t*-test was used for calculating statistic significant of hematologic values of HbE transgenic, IVSII-654 heterozygous mice, double heterozygous mice, rescued mice in comparison with those of wild-type mice.

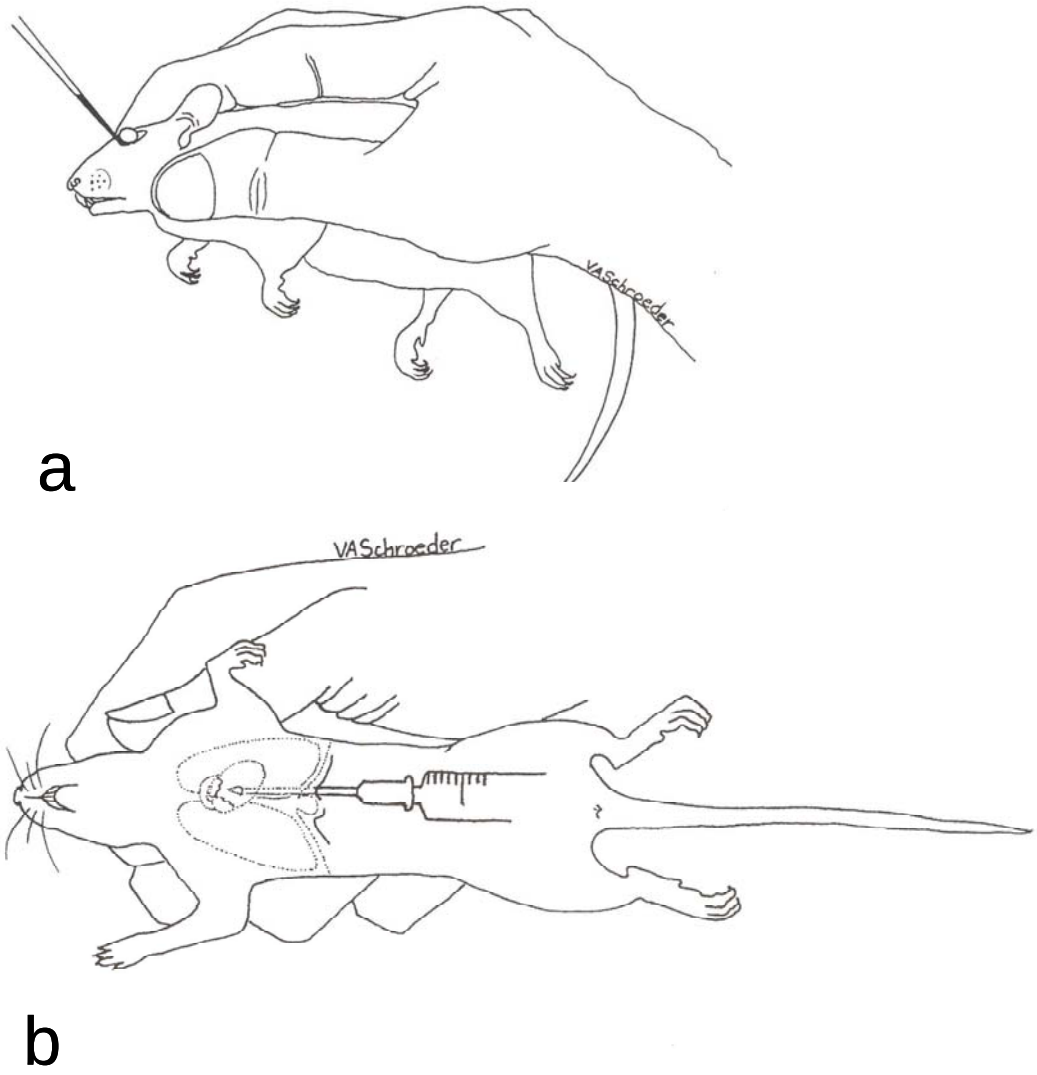


Figure 20 a) Blood collection from the retro-orbital sinus. A glass capillary tube is inserted at the medial canthus and gently rotated until the sinus is penetrated and blood flows into the tube.

b) Intracardiac blood collection. The needle is inserted just lateral to the manubruim and directed through the diaphragm and into the cardiac ventricle. Diaphragm, lungs and heart are all represent by dot lines.

Source: Suckow (2001)

5 RBC Survival Study

Survival study was performed using *in vivo* biotinylation of the entire RBCs and determination of the number of biotinylated RBCs was performed by quantitative flow cytometry (Jong *et al*., 2001). Briefly, the mice were injected via tail vein with 200 μl of 15 mg/ml sulfo-*N*-hydroxysuccinimide biotin.

Flow cytometry was a tool which could rapidly measure complicated physical and biological properties of large numbers of individual cells in a very short time. It was realized quickly that the most efficient way to achieve that was to prepare cells in a suspension and to slowly introduce this suspension into a fast flowing stream of fluid which would then surround the cells like a "sheath" and "center" the cells in the middle of the flowing sheath. This principle of "sheath-flow" is what is widely used in flow cytometry to precisely align cells in front of a beam of laser light. This approach allows hundreds or even thousands of cells per second to pass through the point of intersection between the sheath and laser light.

An important requirement for flow cytometry is the need to specifically label cell constituents with fluorescent molecules, which are then used to identify cells carrying this "label". Cell constituents can be any of a number of cellular components including DNA, which can be labeled by different dyes. Unique "markers" or proteins (Antigens) on the cell surface can be labeled with monoclonal antibodies (mAb) conjugated with one of many fluorescent dyes (fluorochromes) such as fluorescein iso-thiocyanate (FITC) or phycoerythrin (PE). Using these labels, it then becomes possible to identify "positive" and "negative" cells.

For flow cytometric analysis, 2-3 μl tail blood was weekly obtained by tail vein puncture with heparinized capillary tube. The sample of 5×10^6 cells were labeled with 5 μg/ml PE-conjugated streptavidin in 0.1 ml HBSM and determined by FACSort flow cytometer (BD application). Twenty-thousand events were acquired and data were analyzed using CellQuest software. The RBC survival curve was then created using SPSS software. The survival half-time ($T_{1/2}$) means how many days are taken to have 50 % biotinylated RBC reduction.

5.1 Preparation of biotin solution

The purified biotin of approximately 3 mg was weighed in a new Eppendorf tube and dissolved with HBSM in a volume of ~200 μl. The biotin solution was freshly prepared for each experiment.

5.2 Preparation of mice before biotin injection

In order to label the murine RBCs, the biotin solution has to be introduced via mouse tail vein, which is quite small and tough to insert the needle into the vessel. Mice were warmed under light for 3-5 min to enlarge the tail vessel before injection (Fig.21A).

5.3 Biotin injection method

When the tail vein was fully dilated, the prepared mouse which was ready for biotin injection, was immediately moved into the plastic holder 'restrainer' in order to limit its movement (Fig.21B). Only the mouse tail stuck out of the restrainer and the tail vein was then identified. The site of injection should be around 1-2 cm from tip of tail (Fig.21C).

5.4 Detection of biotinylated red blood cells

After biotin was introduced into the mouse circulation, the injected mouse was left for at least 4 hours to have a complete conjugation of biotin to surface components of the RBC outer membrane of. The first blood collection using a heparinized capillary tube was performed afterward as day 0. Other blood sampling was weekly carried out until the percentage of biotinylated RBCs declined to zero.

a) Phycoerythrin-conjugated streptavidin labeling

As mentioned earlier, the whole blood sample from mouse tail was collected by tail vein puncture using heparinized capillary tube. Only 2-3 μl whole blood was required for labeling assay. The biotinylated RBCs in whole blood sample were detected by labeling with phycoerythrin (PE) conjugated streptavidin which is specific bind to biotin probed on the RBC surface.

In a total volume of 100 μl, 1 μl of whole blood and 1 μl of PE-streptavidin were added and mixed gently. The blood solution was incubated for 1 h at room temperature then washed with 1ml HBSM and spinned down at 11,700 ×g for ~10-15 sec. The pellet was resuspended with 500 μl HBSM and transferred into a 5-ml polystyrene tube for flow cytometric analysis.

b) Analysis of biotinylated RBCs using flow cytometer

Twenty thousand intact cells were taken by flow cytometer to calculate the cellular size and their granularity as expressed on FSC-SSC window. Only intact red blood cells were subsequently selected to interpret the

fluorescence intensity on FL1 histogram (Figure 22). The first marker (M1) was created to identify the positive cells as referred to biotinylated RBCs. The result was expressed as the percent of positive RBCs.

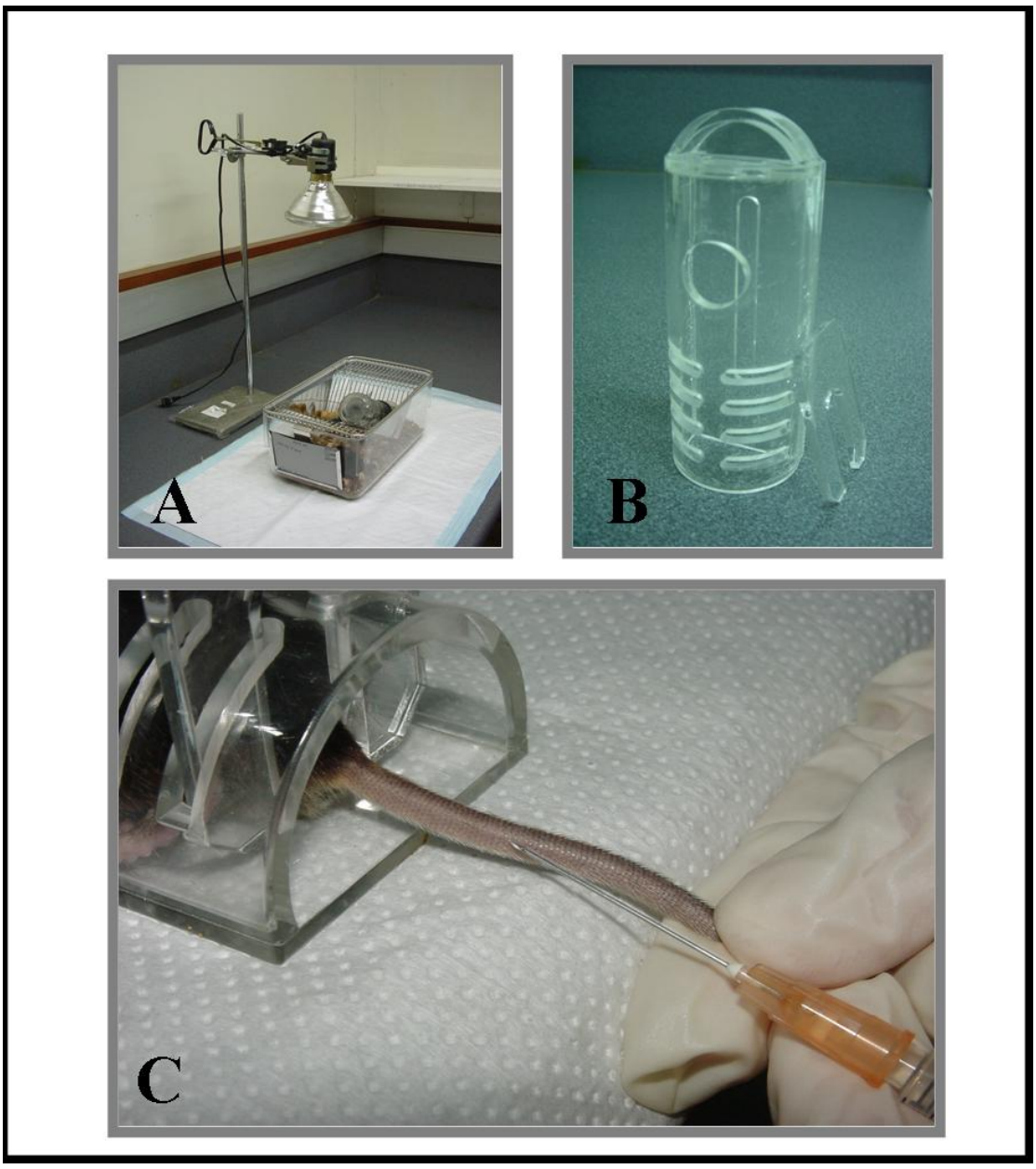


Figure 21 Biotin injection method for determined a red cell survival. Preparation of mice before biotin injection by warmed under light for 3-5 min to enlarge the tail vessel (A). Handling a mouse in to the plastic holder 'restrainer' in order to limit its movement (B). When the tail vein was identified, the site of injection should be around 1-2 cm from tip of tail (C).

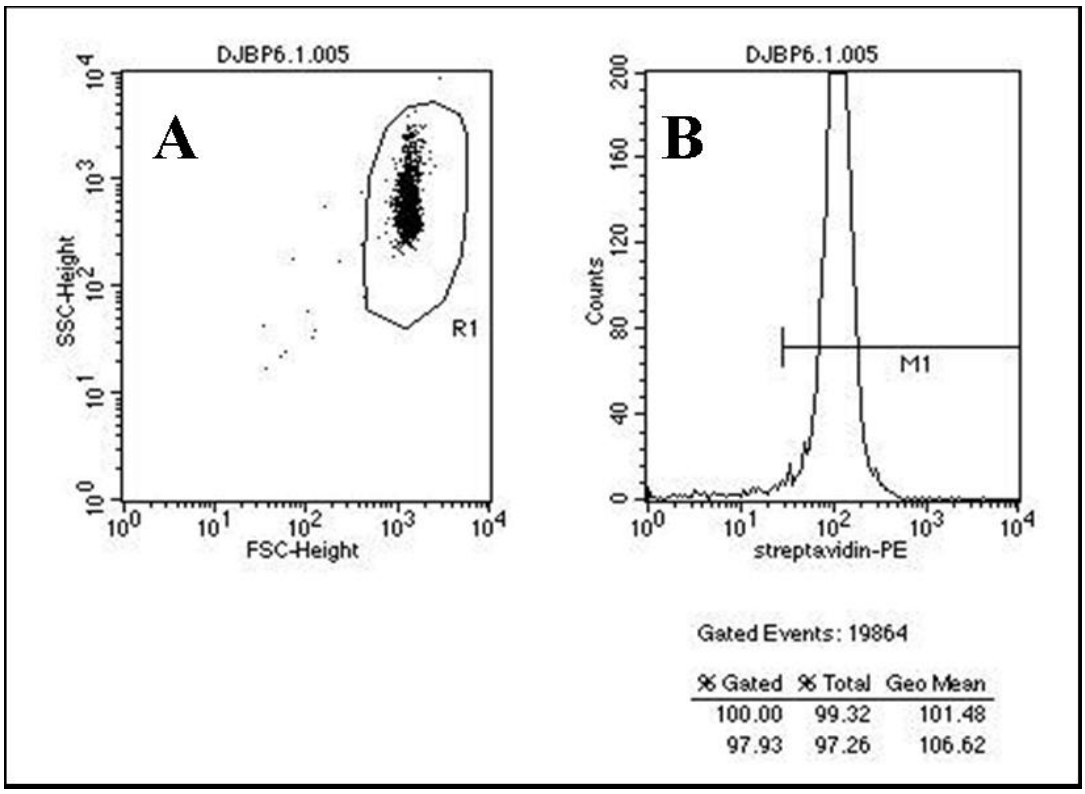


Figure 22 Analysis of biotinylated RBCs using flow cytometer. Identified red blood cell population, R1, by size and their granularity as expressed on FSC-SSC window (A). Selected only RBC population and interpret the fluorescence intensity from a bound phycoerythrin-conjugated streptavidin (B). M1 represented the positive cells as referred to biotinylated RBCs.

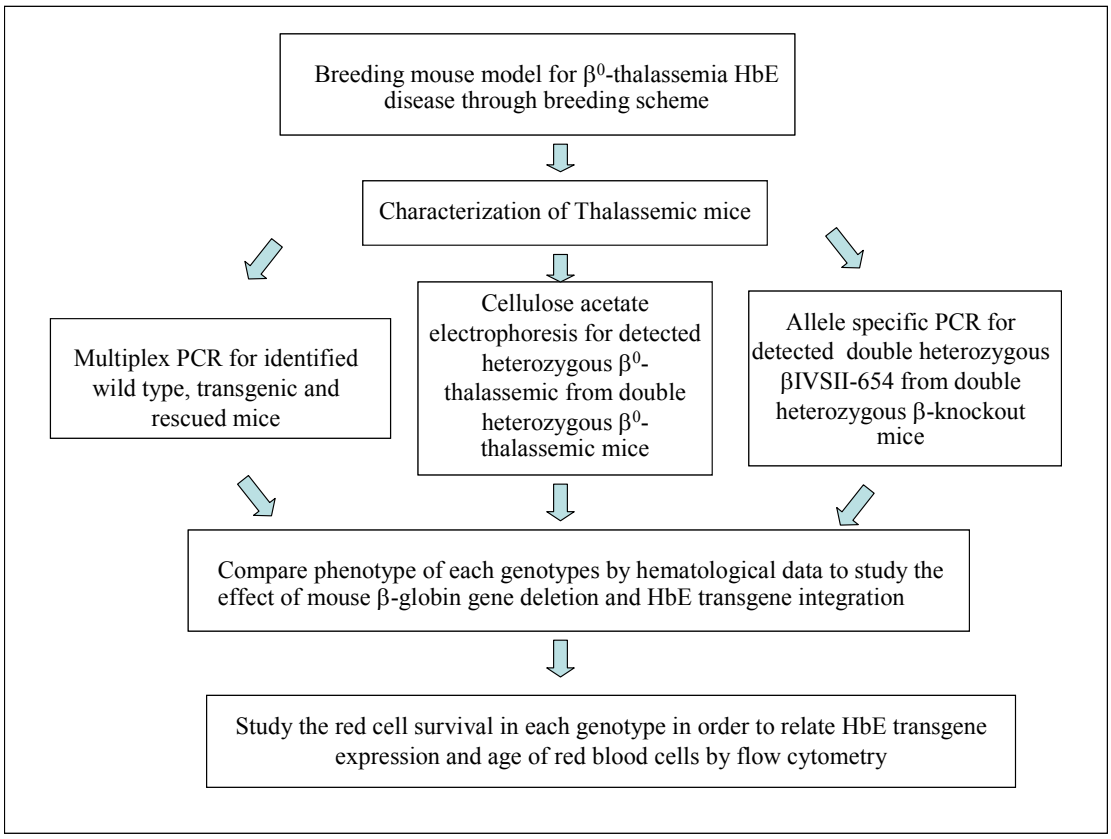


Figure 23 Flow chart representation of all methods in the context of a studying mouse model for β^0-thalassemis/ HbE disease.

RESULTS

1. Breeding Scheme to Generate the Rescue Mice

The objective of creating HbE transgenic mice is to generate a mouse model of homozygous β^0-thalassemia/HbE or rescued mice by breeding the β^0-thalassemic models with HbE transgenic mice (Fig. 16-19). The first breeding scheme (Fig.16) resulted in a mouse model carrying 4 copies of HbE transgene on heterozygous βIVSII-654 knockin background (double heterozygous βIVSII-654 knockin mice). The breeding scheme to generated double heterozygous βIVSII-654 knockin mice resulted in the genotype frequency offspring for wild type, transgenic, heterozygous βIVSII-654 and double heterozygous βIVSII-654 mice was 0.37, 0.26, 0.08 and 0.29 respectively (Fig.23). This was not follow the law of Segregation and Independent Assortment

Cross-breeding of these double heterozygous βIVSII-654 knockin mice (4 copies of HbE transgene) generated mice with HbE transgene on homozygous βIVSII-654 background (rescued mice) (Fig.17). As shown in Figure 24, the genotype frequency of offspring was not follow the law of Segregation and Independent Assortment, in which frequency of double heterozygote > transgenic > heterozygote > wild type. In this experiment the genotype frequency of double heterozygous βIVSII-654, transgenic, heterozygous βIVSII-654 and wild type mice was 0.6, 0.29, 0.07 and 0.03 except the rescued mice that acquired only 0.01 of genotype frequency.

By corporation with *Suwanmanee,* 2002. The second breeding scheme (Fig.18-19) generated the mice carrying HbE transgene on compound

heterozygous βIVSII-654 /β ^{mouse} knock out background (rescued mice) by breeding the double heterozygous βIVSII-654 (4 copies of HbE transgene) with double heterozygous β ^{mouse} knock out mice(4 copies of HbE transgene) . The genotype frequency of these rescued mice was 0.02. The rescued mice from both breeding schemes were able to survive after birth because the expression of HbE locus transgene compensated the defected mouse β-globin genes (Fig.30 lane 3).

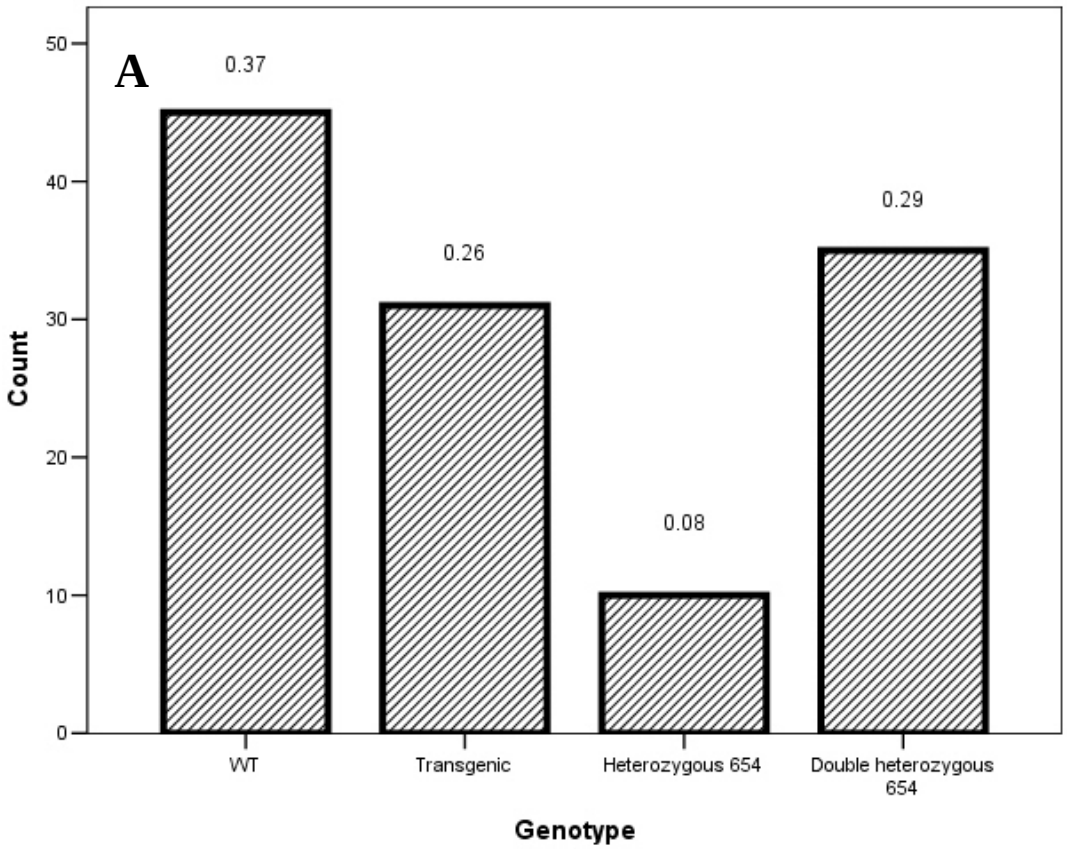


Figure 24 A) Genotypic frequency from a breeding scheme to generate the double heterozygous βIVSII-654 mice. Bar chart represents the number and genotype frequency of offspring acquired from the breeding of heterozygous βIVSII-654 mice with hemizygous HbE transgenic mice. Genotype frequency of wild type, transgenic, heterozygous βIVSII-654 and double heterozygous βIVSII-654 equal to 0.37, 0.26, 0.08 and 0.29 respectively.

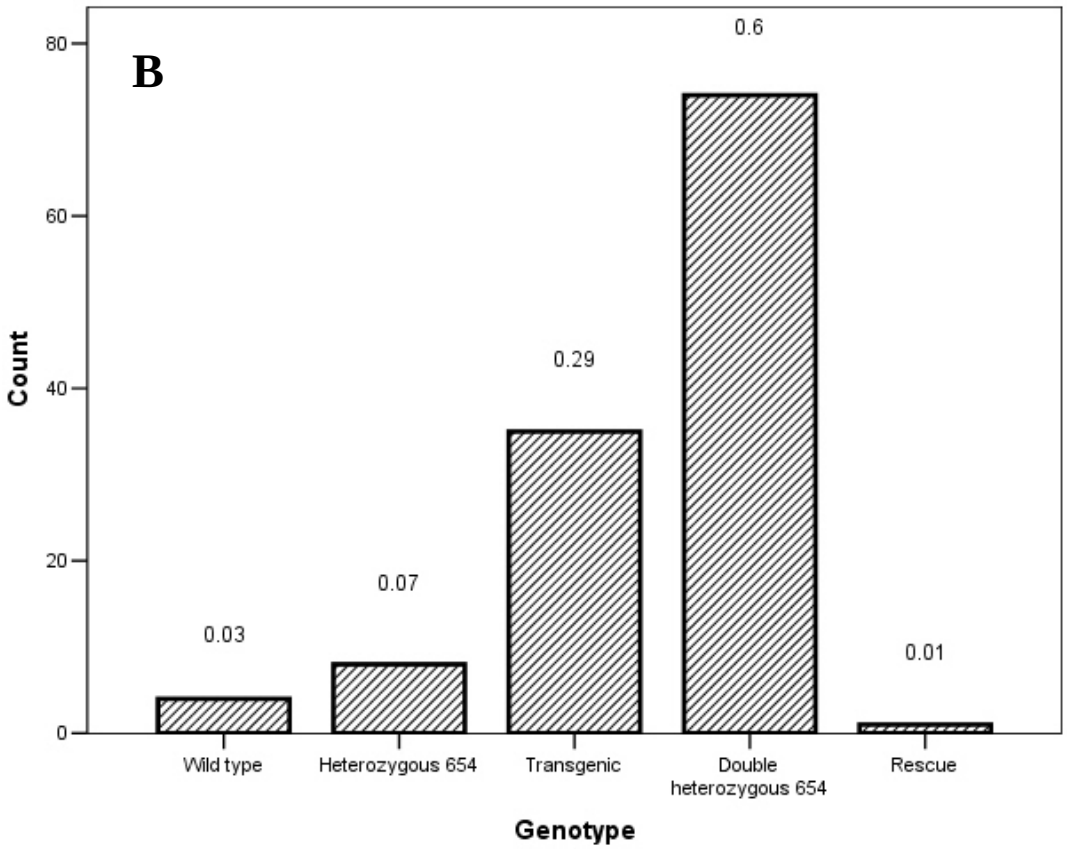


Figure 24 B) Genotypic frequency from a breeding scheme to generate the rescued mice, using double heterozygous βIVSII-654 mice as parents. Bar chart represents the number and genotype frequency of offspring obtained from wild type, transgenic, heterozygous βIVSII-654, double heterozygous βIVSII-654 and rescued mice equal to 0.03, 0.29, 0.07, 0.6 and 0.01 respectively.

2. Characterization of Thalassemic Mice

2.1 Identification of mice genotypes by multiplex PCR

Multiplex PCR is the initial technique to identify the mouse genotype generated from every step of the breeding scheme. The primer pairs LUG1A/LUG2A detected 447-bp product from human β-globin gene; HPRT-FW/HPRT-RW detected 315-bp product of HPRT minigene in β-knockout and β-knock in constructs, and Mouse β-FW/ Mouse β-RW detected the 260-bp product from mouse β-globin gene. This technique identified genotypes of the wild type, transgenic HbE, heterozygous β^{mouse} -knockout and rescued mice according to the pattern of PCR products (Figure 25). One band of PCR product (260 bp) from mouse primer represents the wild type, two bands (260 bp and 315 bp) from mouse and HPRT primers represented heterozygous β^{mouse} -knockout mice, two bands (260 bp and 447 bp) from mouse and LUG primers represented transgenic mice, and the rescued mice produced two bands (315 bp and 447 bp) from HPRT and LUG primer. Nevertheless, genotypes of heterozygous βIVSII-654, double heterozygous βIVSII-654 and double heterozygous β^{mouse} knockout mice were unable to be identified by multiplex PCR because they all had the same PCR products (240, 315 and 447) from three primers (Fig 25, lane 4-6). Cellulose acetate electrophoresis was used to distinguish heterozygous βIVSII-654 from double heterozygous βIVSII-654 mice and the techniques of allele specific PCR was used to classify double heterozygous βIVSII-654 from double heterozygous β^{mouse} knockout mice.

2.2 Identification of heterozygous βIVSII-654 mice and double heterozygous βIVSII-654 mice by cellulose acetate electrophoresis

Human β^E-globin protein expressed from an integrated β^E locus transgene can be bound with mouse α-globin protein to form the chimeric hemoglobin hetero-tetramer (α_2^m/β_2^{hE}) that can be found in double heterozygous mice, both in heterozygous βIVSII-654 and heterozygous β-knockout background (Fig. 27). Whereas cellulose acetate electrophoresis showed only one band of mouse Hb (α_2^m/β_2^m) from heterozygous βIVSII-654 and heterozygous β-knockout (Fig. 31). Therefore the heterozygous βIVSII-654 and heterozygous β-knockout can be distinguished from double heterozygous with HbE transgene because of the absence of chimeric hemoglobin hetero-tetramer in the former genotype.

2.3 Characterization of double heterozygous β-knockout and double heterozygous βIVSII-654 mice.

Double heterozygous β-knockout and double heterozygous βIVSII-654 mice could not be identified by multiplex PCR and cellulose acetate electrophoresis techniques (Fig. 27) as both genotypes produced the same patterns of PCR products and hemoglobin typing comprising of mouse and chimeric hemoglobins (Fig. 27). Some categories of breeding scheme as shown in Figure 19, the two genotypes can be occurred and the offspring were mixed up. Or in the unexpected accident, the mixing up of the two genotypes might happen in a handling and housing mice. Identification of these two genotypes was necessary in order to get the correct genotype for further study.

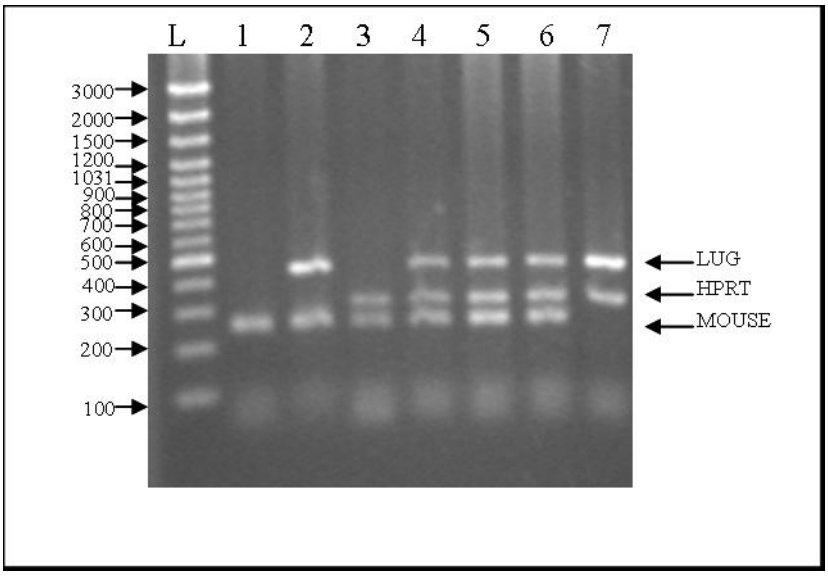


Figure 25 2% Agarose gel electrophoresis represented a genotyping of β-thalassemic mice by multiplex PCR, multiplex PCR using three primer pairs specific to the human β-globin gene (447-bp product), the mouse β-globin gene (260-bp product) and HPRT cassette (315-bp product) were used to identify mice genotypes. The letter L represented 100 bp ladder markers, lane 1: wild type mice, lane 2: hemizygous transgenic mice, lane 3: heterozygous β-knock out mice, lane 4: heterozygous β IVSII-654 knock in mice, lane 5: double heterozygous β IVSII-654 knock in mice, lane 6: double heterozygous β-knock out mice, lane 7: Rescued mice.

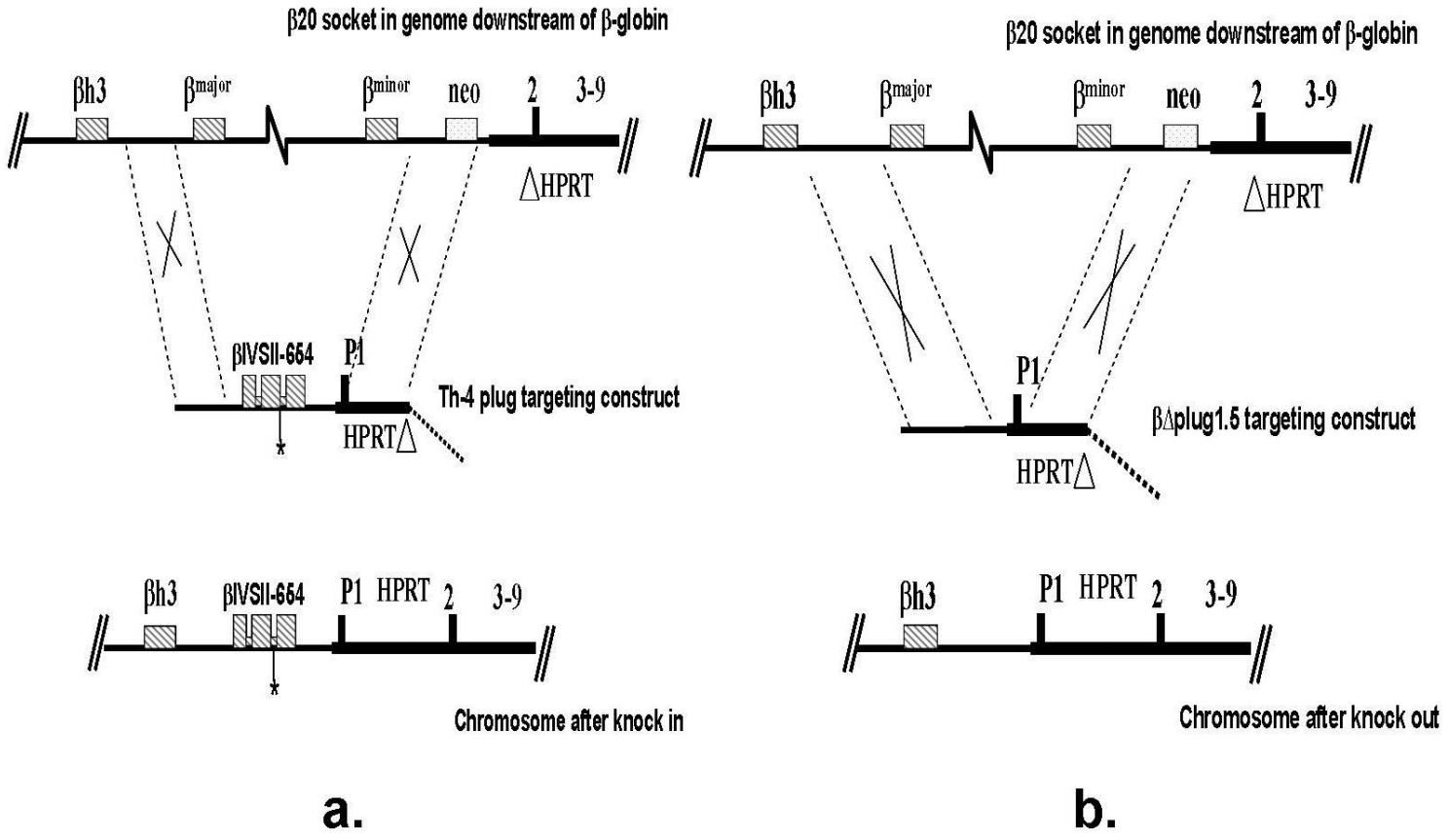


Figure 26 Replacement of the murine adult β globin gene on chromosome 7 in socket-containing embryonic stem cell (β_{20}) by the th-4 plug and $\beta\Delta plug_{1.5}$ targeting construct. The socket-containing chromosome, the linearized plug targeting construct for deletion and the chromosome obtained after deletion are shown. Recombination (indicated by Xs) between a target locus and plug targeting construct, yielding a chromosome that contains human βIVSII-654 gene (a) and non-functional portion of HPRT gene; HPRTΔ (b) in place of mouse β major and βminor gene. The exons and introns of gene are represented as boxes and thick line. The position of $IVSII_{-654}$ mutation shown with asterisk and promoter (P) and exon 1-9 of *HPRT* are marked.

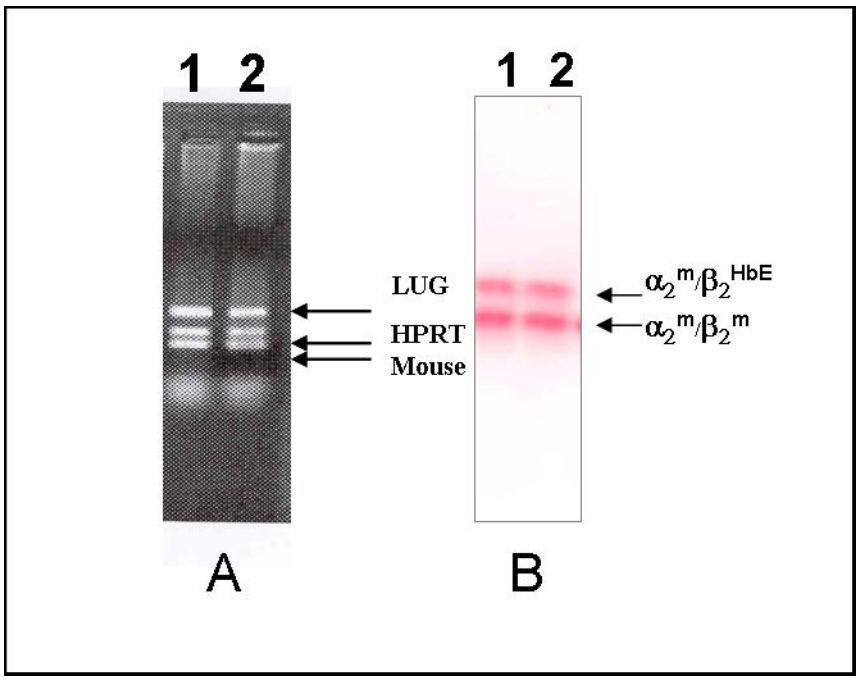


Figure 27 Genotyping of double heterozygous βIVSII-654 and double heterozygous β-knock out mice. The mice were identified with three primers for multiplex PCR (A) and cellulose acetate electrophoresis (B). Lane 1 represented double heterozygous βIVSII-654 mice, lane 2 represented double heterozygous β-knock out mice. α_2^m/β_2^{HbE} represented the chimeric hemoglobin heterotetramer comprised of mouse α- and human β^E-globin chains. α_2^m/β_2^m represented normal mouse hemoglobin.

2.3.1 Identification of double heterozygous βIVSII-654 and double heterozygous β-knock out mice by allele specific PCR

Allele specific PCR was used for separation the double heterozygous βIVSII-654 from double heterozygous β-knock out mice. This technique was based on specific bases designing at 3'-end of primer (Newton *et al*., 1989). Allele specific primers comprise of normal primer, specified to normal human and mutant primer which specified to human βIVSII-654 C→T mutation (Old *et al*., 2001). PCR products generated from allele specific primers were of the same size of 829 bp (Figure 28). To test the specificity of normal and mutant primers, mouse's tail genomic DNA sample from heterozygous βIVSII-654 mice were used. These mice carried human βIVSII-654 C→T mutation sequence from a th-4 plug knock-in construct. The hemizygous HbE transgenic mice, which contained a normal human βIVSII-654 sequence form an integrated β^E locus transgene on mouse chromosome 2, was also used as normal control (Jamsai *et al*., 2004). So, DNA from mouse carrying HbE transgene produced PCR product from the normal primer and the mouse carrying human βIVSII-654 C→T mutation gave PCR product from mutant primer (Figure 28, left).

In the double heterozygous βIVSII-654 mice, PCR product was obtained from both the normal and mutant primers (figure 28, right). The former was from an integrated β^E- locus transgene. The latter was 829 bp PCR product from mutant primer, which amplified human βIVSII-654 C→T mutation sequence from a th-4 plug knock-in construct. Whereas double heterozygous β-knockout mice generated only PCR product from normal primer, which amplified normal human βIVSII-654 sequence from β^E locus transgene.

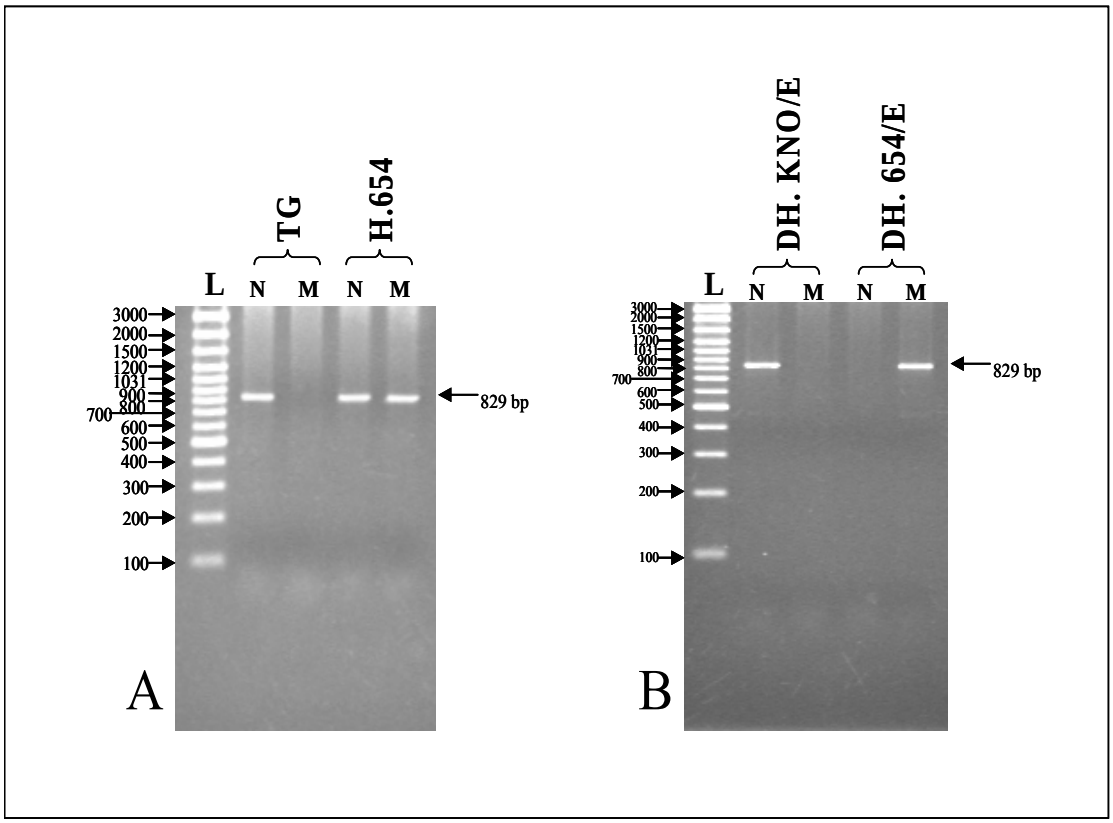


Figure 28 The 2% agarose gel electrophoresis, A) represented the specific-test of normal (N) and mutant (M) primer to genotype HbE transgenic (TG) and heterozygous βIVSII-654 mice (H.654) that both genotype carrying normal and mutant of human βIVSII-654 sequence, respectively. B) Double heterozygous β-knock out mice (DH. KNO/E) generated 829 bp PCR product (indicated by arrow) from normal primer and double heterozygous βIVSII-654 mice(DH.654/E) generated two bands of PCR product from normal and mutant primer. Mouse's tail DNA sample from each genotype were diluted to 44 ng/μl (approximated by OD_{260}), for preventing non-specific binding. The letter L represented 100 bp ladder markers.

3. Levels of Human β^E-Globin in Different Genotypes of HbE Transgenic Mice

Expression of the human β^E-globin gene in different genotypes of transgenic mice were examined by cellulose acetate electrophoresis (Fig. 29) and polyacrylamide urea gel electrophoresis (Fig. 30). Cellulose acetate electrophoresis was performed on blood sample of HbE transgenic, double heterozygote, and rescued mice to examine chimeric hemoglobin (α_2^m/β_2^{HbE}) (Fig. 29). All transgenic mice carried 4 copies of human β^E-globin gene excepted rescued mice which presumed carried 8 copies human β^E-globin gene (see the discussion of hematologic analysis, p. 109). The result show that, level of chimeric hemoglobin increased inversely to the number of mouse β-globin gene. Thereby the level of chimeric hemoglobin in the transgenic was less than that of double heterozygote and rescued mice, 1.5-3%, 26-33% and 100% respectively. The rescue mice showed the presence of 100% chimeric hemoglobin tetramer indicating the complete absence of mouse β-globin chains due to the complete loss of mouse β-globin genes. The result was confirmed by tritron x-100 urea gel electrophoresis (Fig. 30). Adult mice with normal genotype expressed two major globin chains, α- and β-globin chains would form heterotetrameric structure (mouse α_2/mouse β_2) (Fig. 30, lane5). Whereas only mouse α- and human β^E-globin gene expressed in rescued mice (Fig. 30, lane3).

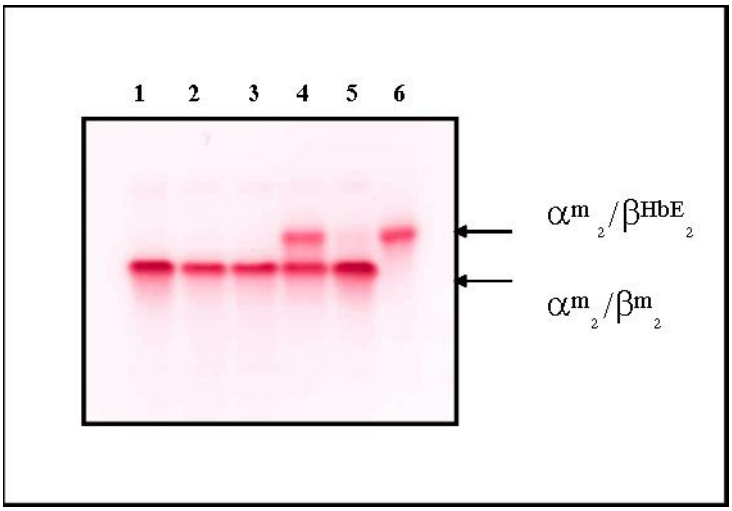


Figure 29 Hemoglobin typing of transgenic mice. Cellulose acetate electrophoresis was performed from blood samples of HbE transgene on normal background (transgenic), on heterozygous background (double heterozygote) and homozygous background (rescued) mice. Lane 1: wild type mouse, lane 2: heterozygous β-knock out mouse, lane 3: heterozygous βIVSII-654 mouse, lane 4: double heterozygous βIVSII-654 mouse, lane 5: hemizygous transgenic mouse, lane 6: rescued mouse.

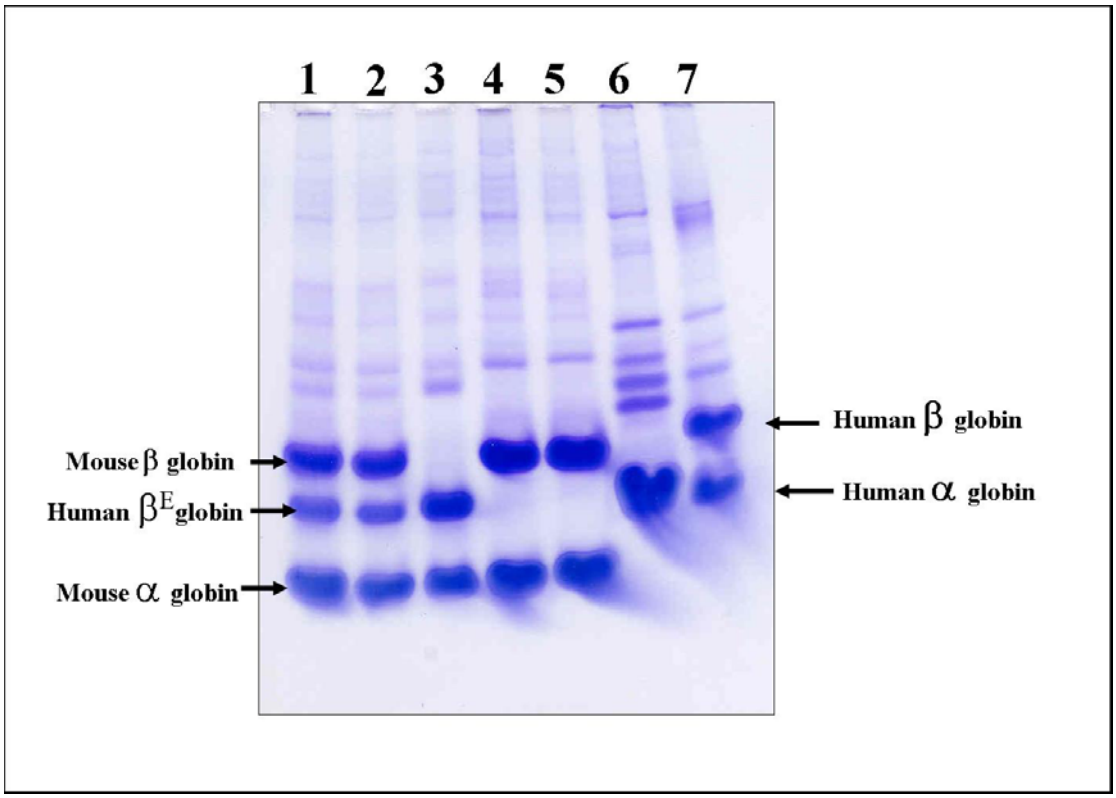


Figure 30 Analysis of globin chains in thalassemic mice by 12 % polyacrylamide urea gel electrophoresis. Globin chains were separated by urea gel electrophoresis of wild type, thalassemic mice, HbE transgene on heterozygous background (double heterozygote), on homozygous background (rescued), and human normal and β^E homozygote. Lane 1: double heterozygous βIVSII-654 knockin mice, lane 2: double heterozygous β-knockout mice, lane 3: rescued mice, lane 4: heterozygous βIVSII-654 knockin mice, lane 5: wild type mice, lane 6: human HbE homozygote, lane 7: human normal

4. Hematologic Analysis

Hematologic study was performed of 2-3 months old mice. Blood samples were generally collected by cardiac puncture (Fig. 20a), except the retro-orbital sinus puncture was used for the valuable rescued mice (Figure 20b). Morphologic studies of red blood cells led to the comparison of anemia in each mouse genotypes.

4.1 Hemizygous transgenic mice

Morphologic study revealed that RBCs of transgenic mice were normorcytic, normochromic (Fig. 31B), which was similar to the wild type mice (Fig. 31A). The value of hemoglobin concentration, MCH, MCHC and MCV (Fig. 32-35, Table 5), which resembled the wild type, indicated that there was no sign of anemia in this genotype.

4.2 Heterozygous βIVSII-654 knockin mice

This genotype showed a microcytic hypochromic anemia, marked anisocytosis, poikilocytosis and target cell in the peripheral blood smear (Fig. 31C). Markedly decreased in hemoglobin concentration, hematocrit, MCV, MCH and red blood cell count as well increased reticulocyte count and RDW were demonstrated (Fig. 32-35).

4.3 Double heterozygous βIVSII-654 knockin mice

The expression of human β^E-globin transgene can compensate for the loss to one side of an allele locus of mouse β-globin gene. Blood smear from this genotype revealed normorcytic, normochromic RBCs (Fig. 31D).

The hematologic parameter indicated that RBCs were normal (Fig. 32-35). Normal erythropoiesis was indicated from the normal reticulocyte count (Fig. 35H).

4.4 Rescued mice

Although these mice had the normal hemoglobin level, they had lower MCV, MCH, MCHC, and increased RDW (Fig. 33D, 34 and 35G). Morphologic study revealed that red blood cell were hypochromic, anisocytosis (Fig 31E). In addition to the hematologic parameter, the rescued mice gained an active erythropoiesis from the significant increase of red blood cell count (10.18 $x10^6$ cells/μl) (Fig. 32A) and hematocrit (43.8%) (Fig. 33C). The high value of reticulocyte indicated that the rescued mice had more red cells turnover (Fig. 35H). From breeding scheme in Figures 16-19, both rescued mice have the chance to take HbE locus transgene, 4 or 8 copies, by the law of Independent Assortment. Furthermore, we could presume transgene copies number of rescued mice by comparing to hematological profile, since HbE transgene copies number effected to the level of anemia (see the discussion of hematologic analysis, p. 109).

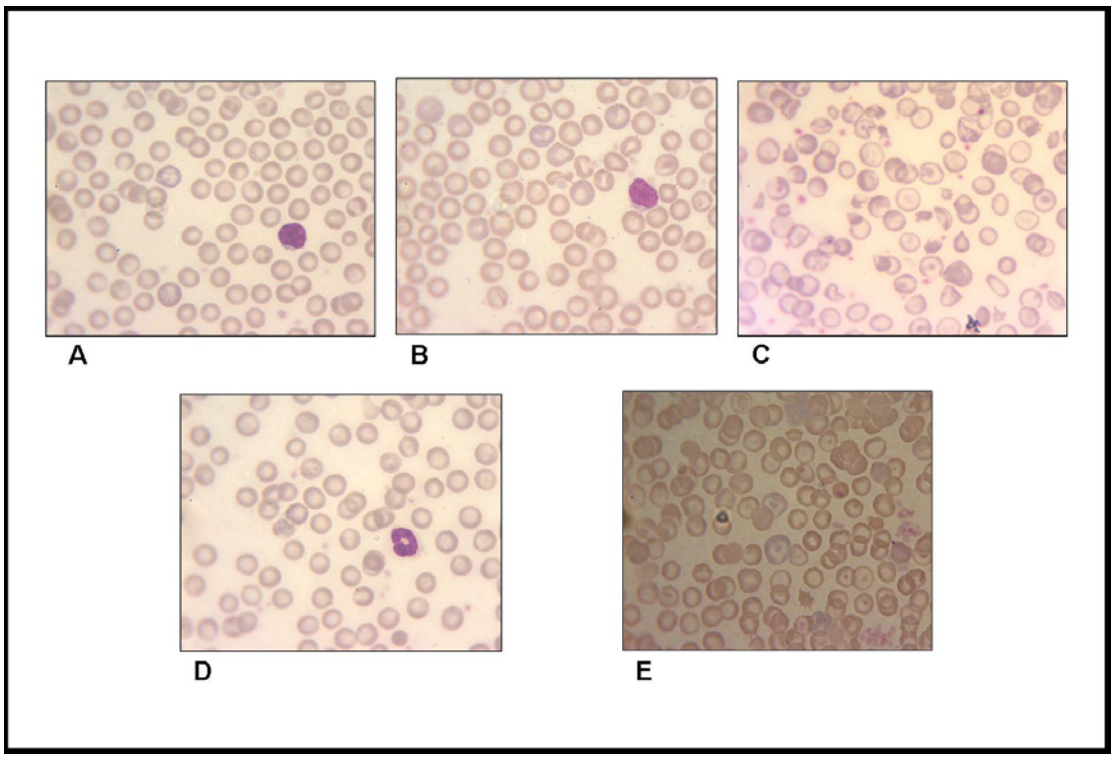
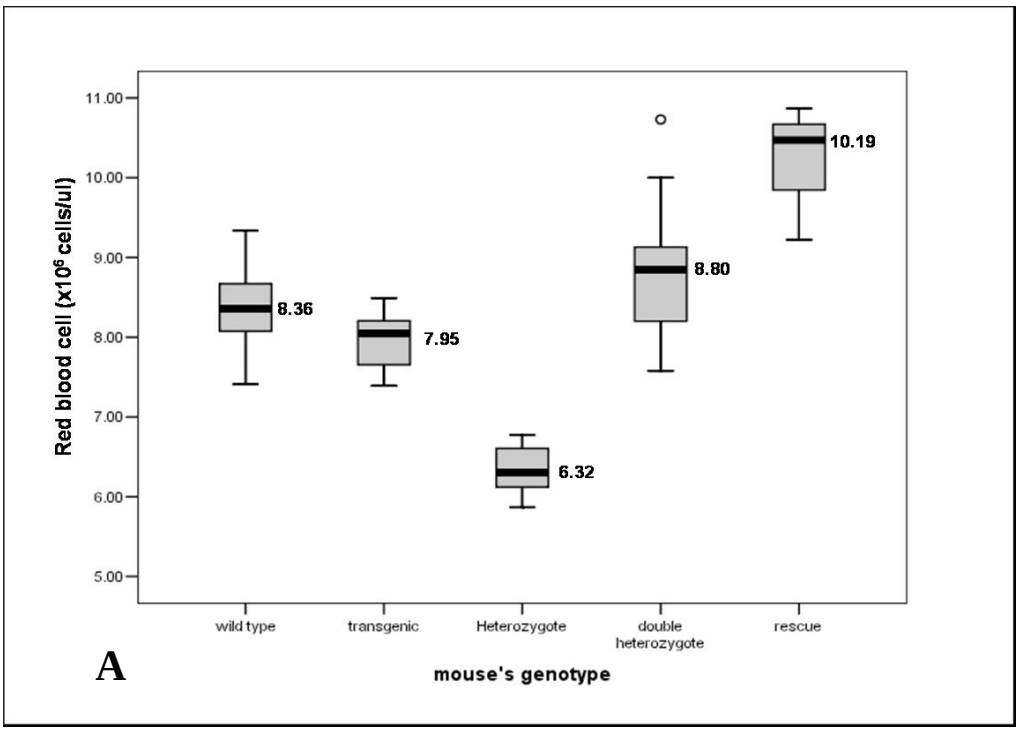


Figure 31 Peripheral blood smears of thalassemic mice. Morphologic study from peripheral blood smear of wild type (A), hemizygous 4 copies of transgenic (B), heterozygous βIVSII-654 knock-in (C), double heterozygous βIVSII-654 knock-in (carried 4 copies of transgene) (D), rescued mice (carried 8 copies of transgene) (E).



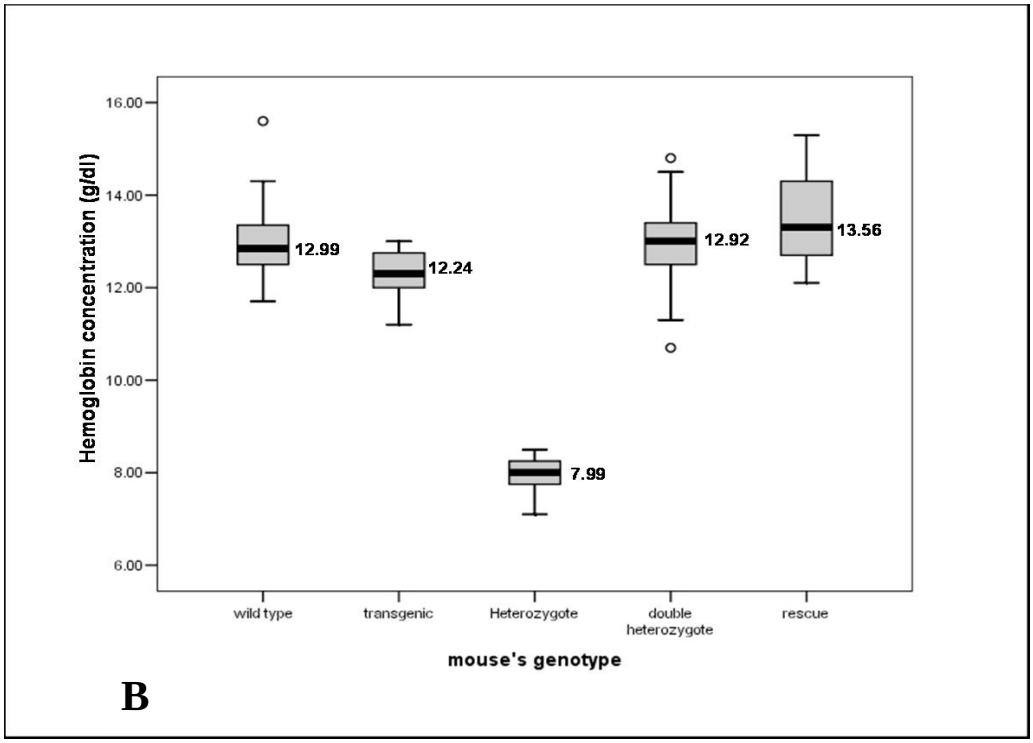


Figure 32 The comparative diagram of RBC count (A) and hemoglobin concentration (B) from wild type and transgenic mice of various genotypes.

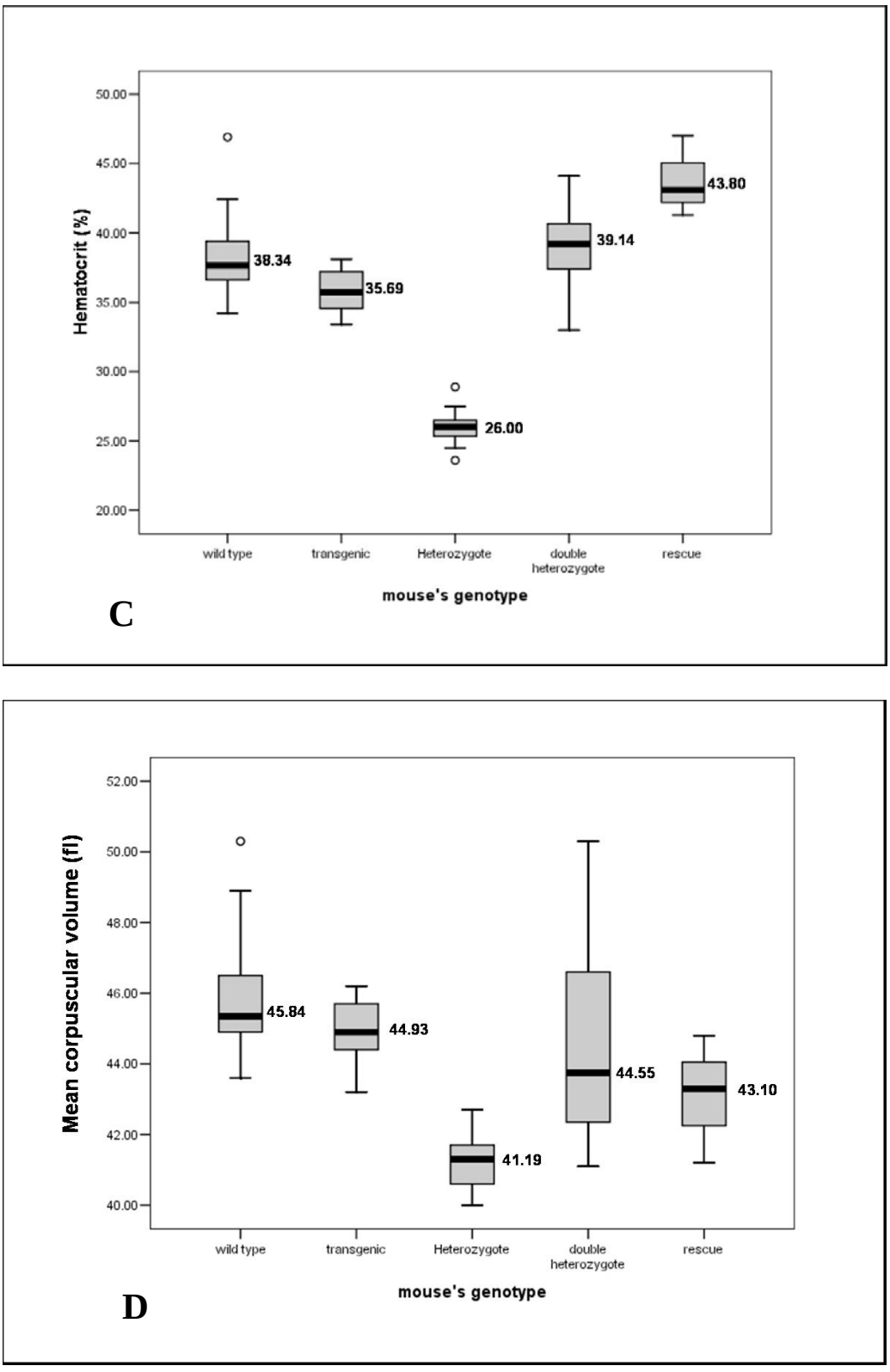
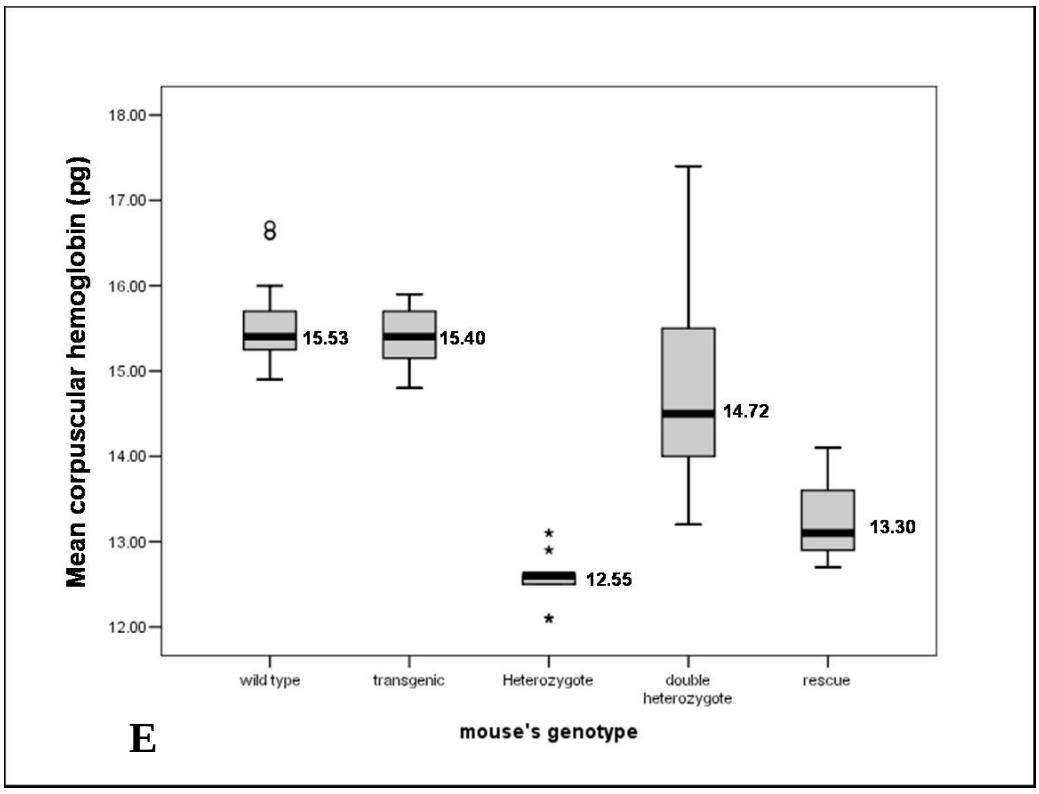


Figure 33 The comparative diagram of hematocrit (C) and mean corpuscular volume (D) from wild type and transgenic mice of various genotypes.



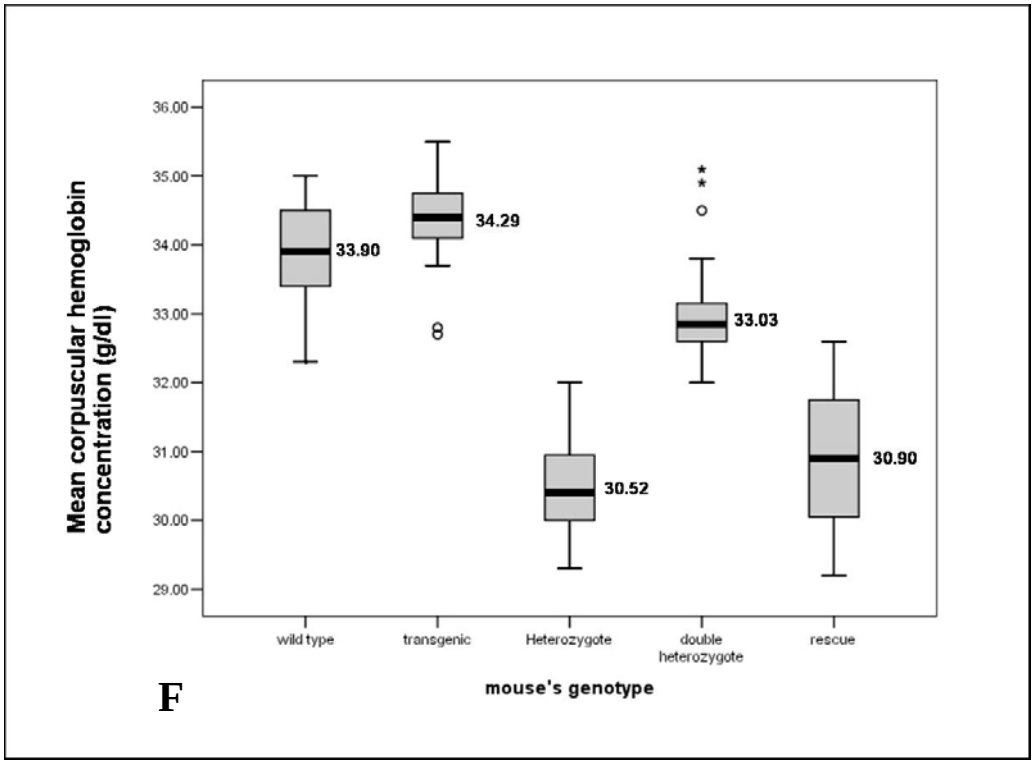
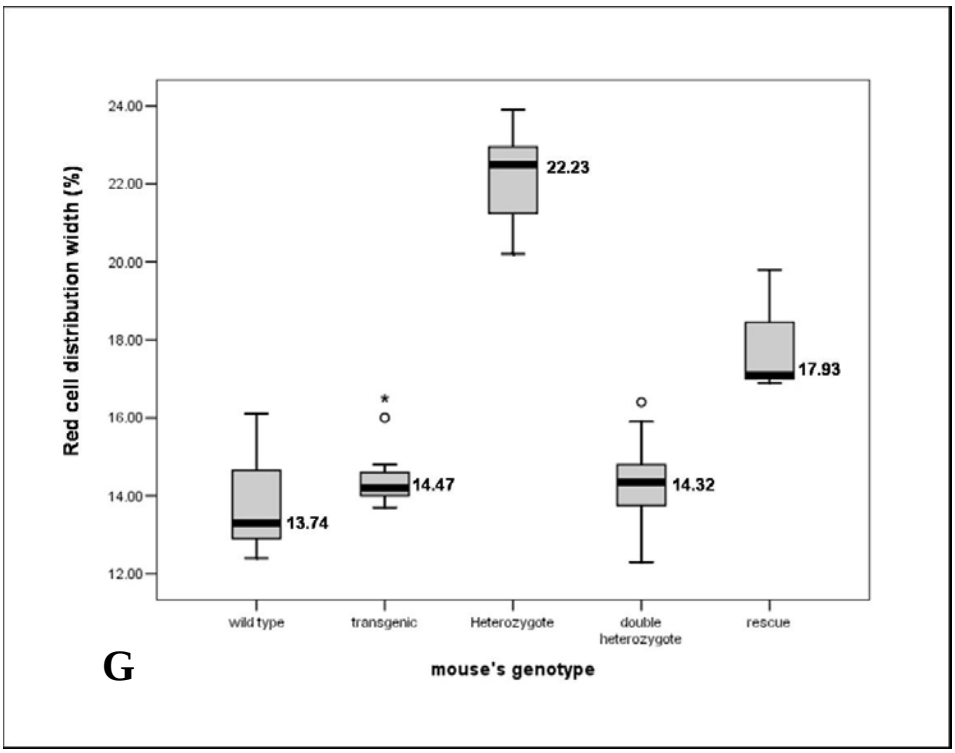


Figure 34 The comparative diagram of mean corpuscular hemoglobin (E) and mean corpuscular hemoglobin concentration (F) from wild type and transgenic mice of various genotypes.



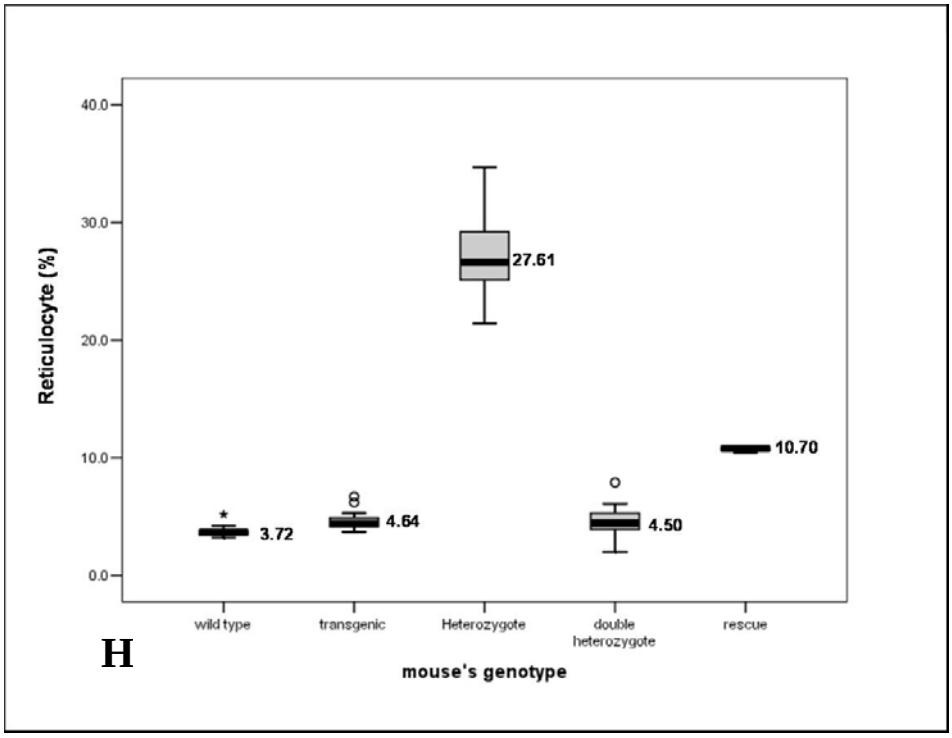


Figure 35. The comparative diagram of red cell distribution width (G) and reticulocyte (H) from wild type and transgenic miceof various genotypes.

Table 5. Hematologic study in thalassemic and various genotypes of HbE transgenic mice

Mouse's genotype		Red blood cell	Hemoglobin concentration	Hematocrit	Mean corpuscular volume	Mean corpuscular hemoglobin	Mean corpuscular hemoglobin concentration	Red cell distribution width	Reticulocyte
Wild type	N	24	24	24	24	24	24	24	18
	Mean	*8.3617*	*12.9958*	*38.3417*	*45.8375*	*15.5333*	*33.9000*	*13.7417*	*3.717*
	Std. Deviation	±.48162	±.83898	±2.83931	±1.50761	±.50705	±.69219	±.98991	±.4409
	Variance	.232	.704	8.062	2.273	.257	.479	.980	.194
	Maximum	9.34	15.60	46.90	50.30	16.70	35.00	16.10	5.2
	Minimum	7.41	11.70	34.20	43.60	14.90	32.30	12.40	3.2
Transgenic 4 copies	N	15	15	15	15	15	15	15	15
	Mean	*7.9467*	*12.2400*	*35.6867*	*44.9267*	*15.4000*	*34.2867*	*14.4733*	*4.647*
	Std. Deviation	±.35660	±.56036	±1.57746	±.84131	±.35254	±.77078	±.79144	±.8601
	Variance	.127	.314	2.488	.708	.124	.594	.626	.740
	Maximum	8.49	13.00	38.10	46.20	15.90	35.50	16.50	6.7
	Minimum	7.39	11.20	33.40	43.20	14.80	32.70	13.70	3.7
Heterozygous IVSII-654	N	11	11	11	11	11	11	11	11
	Mean	*6.3182*	*7.9455*	*26.0091*	*41.1909*	*12.5545*	*30.5182*	*22.2273*	*27.609*
	Std. Deviation	±.30205	±.40587	±1.42580	±.83241	±.29108	±.75342	±1.16111	±4.0404
	Variance	.091	.165	2.033	.693	.085	.568	1.348	16.325
	Maximum	6.77	8.50	28.90	42.70	13.10	32.00	23.90	34.7
	Minimum	5.87	7.10	23.60	40.00	12.10	29.30	20.20	21.4
Double heterozygous IVSII-654 (4 copies transgene)	N	32	32	32	32	32	32	32	30
	Mean	*8.8081*	*12.9250*	*39.1438*	*44.5469*	*14.7219*	*33.0281*	*14.3219*	*4.490*
	Std. Deviation	±.72031	±.87805	±2.71815	±2.55103	±.98921	±.71808	±.89361	±1.2369
	Variance	.519	.771	7.388	6.508	.979	.516	.799	1.530
	Maximum	10.73	14.80	44.10	50.30	17.40	35.10	16.40	7.9
	Minimum	7.58	10.70	33.00	41.10	13.20	32.00	12.30	2.0
Rescued[1] (8 copies HbE transgene)	N	3	3	3	3	3	3	3	3
	Mean	*10.1867*	*13.5667*	*43.8000*	*43.1000*	*13.3000*	*30.9000*	*17.9333*	*10.700*
	Std. Deviation	±.86072	±1.61658	±2.91376	±1.80831	±.72111	±1.70000	±1.61967	±.2646
	Variance	.741	2.613	8.490	3.270	.520	2.890	2.623	.070
	Maximum	10.87	15.30	47.00	44.80	14.10	32.60	19.80	10.9
	Minimum	9.22	12.10	41.30	41.20	12.70	29.20	16.90	10.4

1 Rescued mice represented homozygousβIVSII-654 knocks -in / HbE mice and hemizygous βIVSII-654 knock-in / β^{mouse} –knock out / HbE mice, all data were analyzed by Advia 120.

5 RBC Survival Study

Red cell survival studying was performed by labeling RBCs with biotin via tail vein injection. The number of biotinylated RBCs was determined by flow cytometer (Fig. 36).The results revealed that the wild type had red blood cell life span of 60 days and half life ($T_{1/2}$) for 25 days (Fig. 37). Heterozygous βIVSII-654 knockin mice had short half life ($T_{1/2}$) of red blood cells for 15 days (Fig. 38), which confirmed the abnormal red blood cells morphology. Hemizygous transgenic mice which carry 4 copies of HbE transgene had RBC half life 25.5 days (Fig. 37), whereas double heterozygous βIVSII-654 knockin mice, which carry 4 copies of HbE transgene, had RBC half life of 20 days (Fig. 38). Decrease RBC half life was observed in the rescued mice of both genotype (homozygous βIVSII-654 knockin / HbE mice and compound heterozygous βIVSII-654 knockin / β^{mouse} –knockout / HbE mice), with a half life of RBCs at 14 days (Fig. 39), which is similar to the value found in the symptomatic heterozygous βIVSII-654 knockin mice.

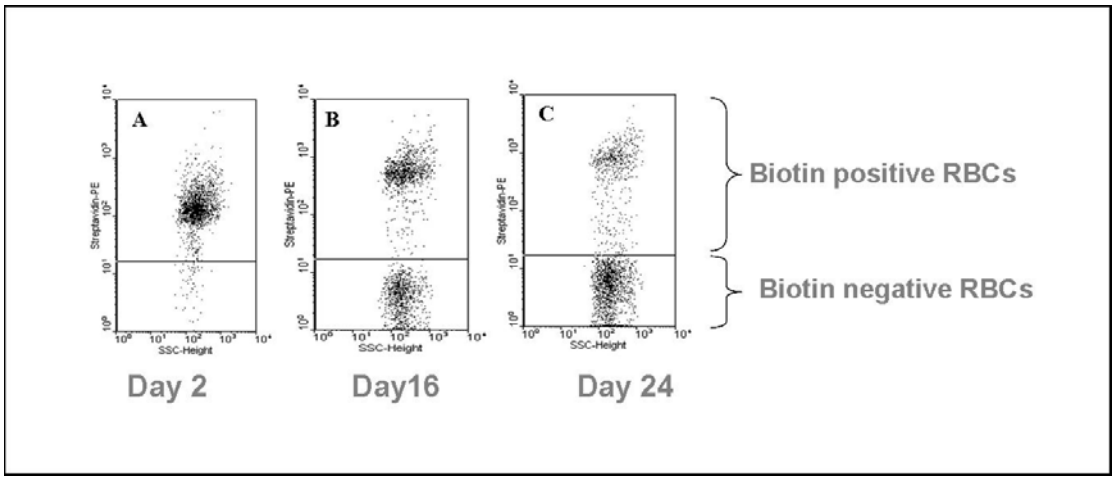


Figure 36 Flow cytometer analyzing of RBC survival in thalassemic mice. Dot plot of the decreased biotin positive RBCs population, detected with phycoerythrin-conjugated streptavidin, compare with the increasing time indicated the age of red blood cells of each genotype.

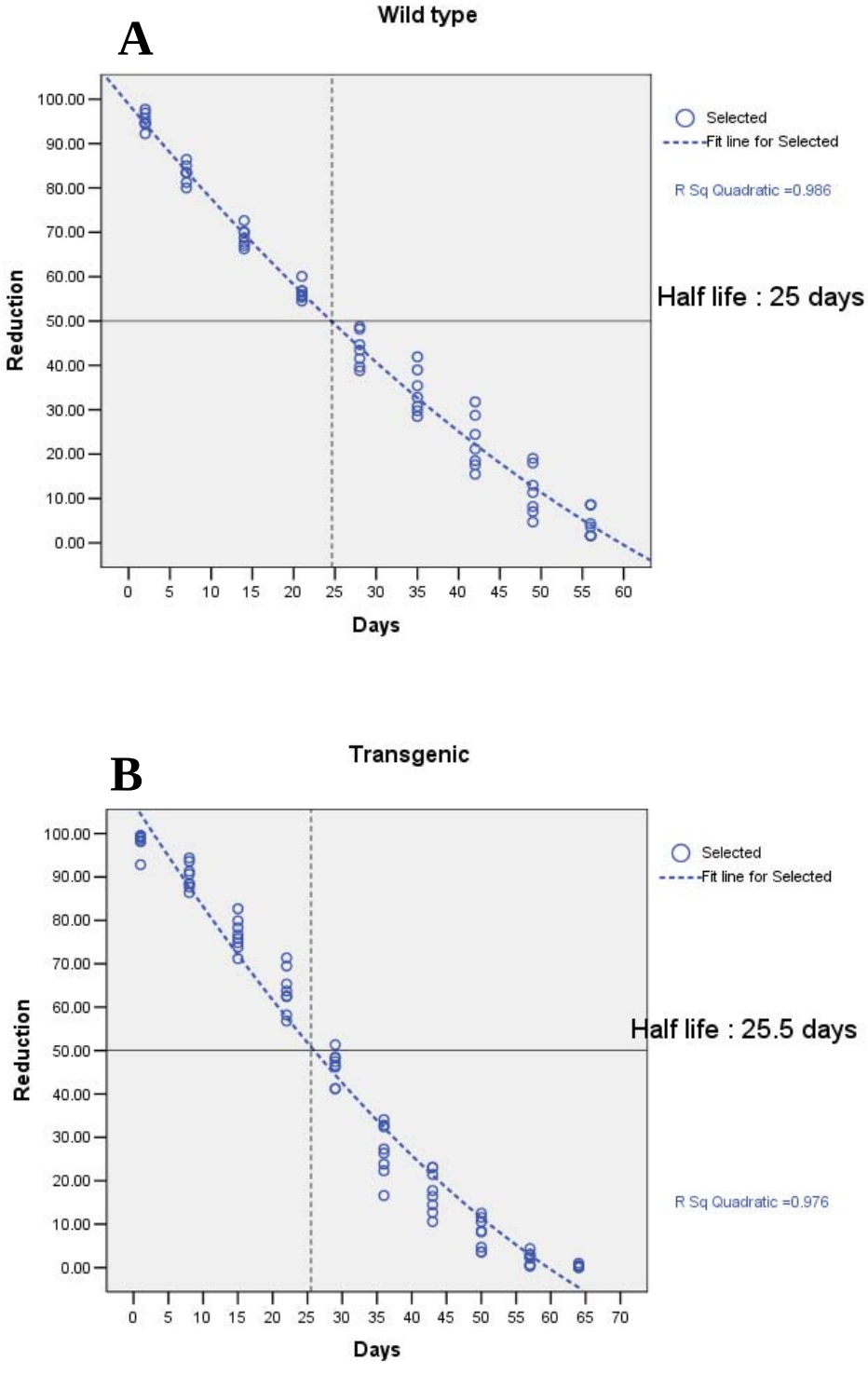


Figure 37 Red cell survival curves and the reduction of biotinylated red blood cells. Wild type mice ($\beta^{mouse}/\beta^{mouse}$) present 25 days (A) and a hemizygous transgenic mice (4 copies of HbE locus transgene on wild type background) present 25.5 days of a mean red blood cell half life ($T_{1/2}$) (B). The dot line was marked to identify the $T_{1/2}$ value.

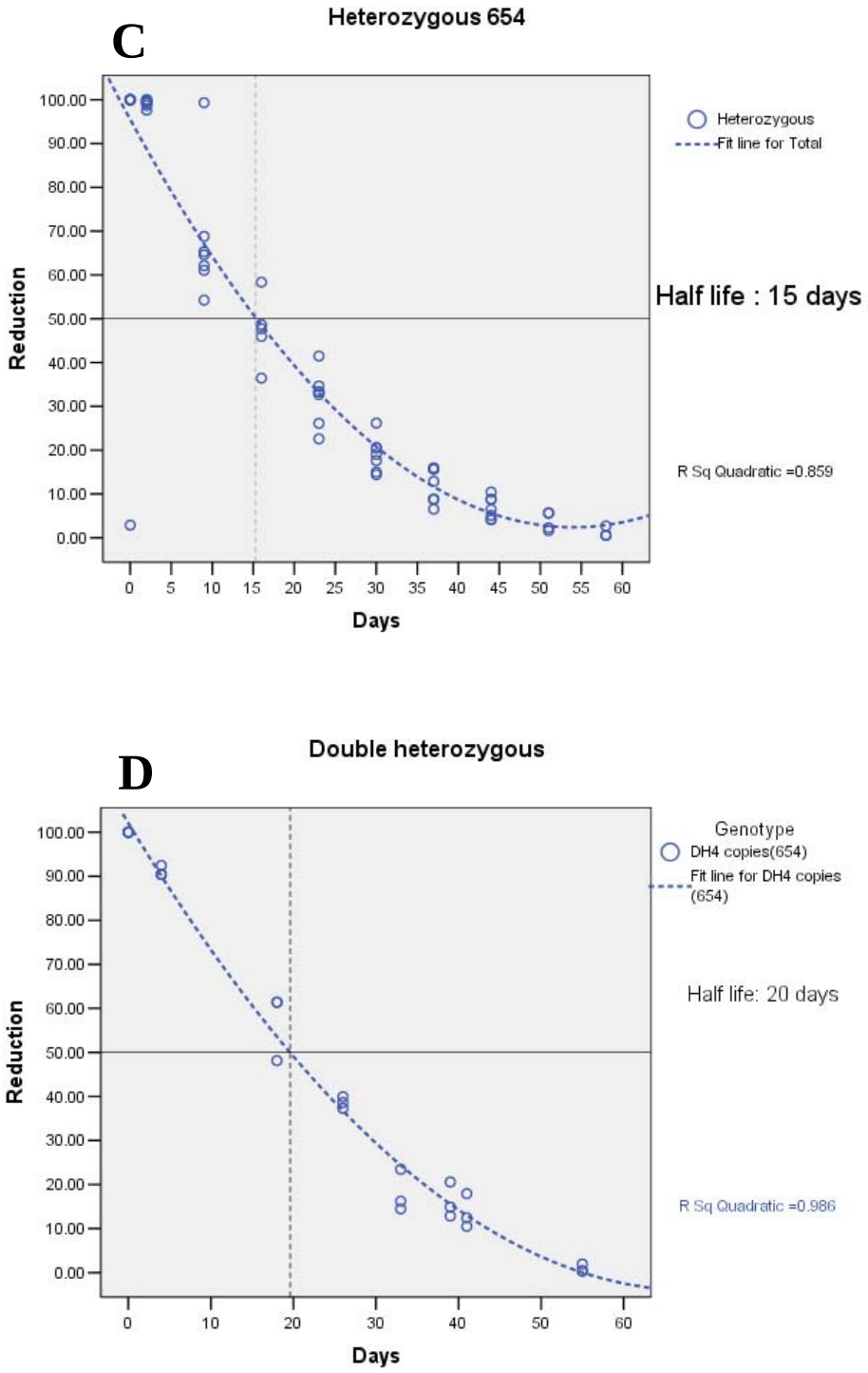


Figure 38 Red cell survival curves and the reduction of biotinylated red blood cells. Heterozygous βIVSII-654 knockin ($\beta^{mouse}/\beta^{knockin\ 654}$) mice present 15 days (C) and double heterozygous βIVSII-654 knockin mice (4 copies of HbE locus transgene heterozygous background) present 20 days of a mean red blood cell half life ($T_{1/2}$) (D). The dot line was marked to identify the $T_{1/2}$ value.

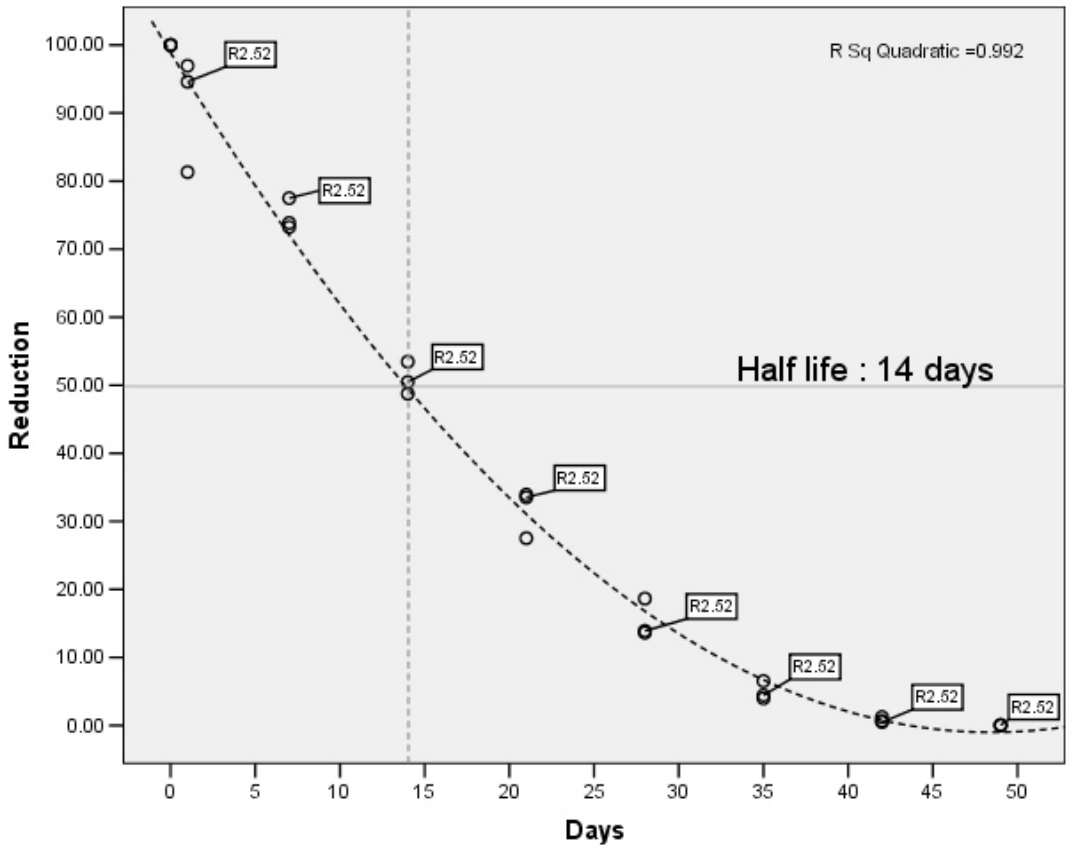


Figure 39 Red cell survival curves and the reduction of biotinylated red blood cells. Rescued mice of both genotypes: (1) homozygous βIVSII-654 knockin with 8 copies of HbE transgene mice (label with the letter "R2.52"), (2) compound heterozygous βIVSII-654 knockin / β^{mouse} –knockout on 8 copies of HbE transgene mice (non-labeled). Both genotypes present 14 days of a mean red blood cell half life ($T_{1/2}$). The dot line was marked to identify the $T_{1/2}$ value.

DISCUSSION

1. Breeding Scheme to Generate the Rescued Mice

The principle of β^0-thalassemic mice model in this study was βIVSII-654 mice, being generated by replacement of 21 kb regions encompassing the mouse β^{major} and β^{minor} gene with 5.7 kb DNA fragment of human β-globin gene containing IVSII-654 splicing mutation (Lewis *et al*., 1998). The second β-thalassemic model was β-knockout mice; this model carried a 20 kb deletion encompassing the entire mouse β^{major} and β^{minor} gene (Yang *et al*., 1995). Both models showed classic signs of β-thalassemia in the heterozygote and displayed embryonic lethality in the homozygous form, due to the requirement of mouse β-globin gene expression during fetal development. However, such models did not facilitate the study of expression of human gene, and there may be significant difference in the regulation of human and mouse genes. So the HbE transgenic mice, which contains human β^E cluster 185 kb-long genomic fragment carrying the HbE mutation, have been established (Jamsai *et al*., 2004). The objective of creating rescued mice, by breeding the heterozygous β-thalassemic mice with HbE transgenic mice through both breeding schemes (Fig.16-19) was to mimic the main feature of β^0-thalassemia/ HbE in patients.

Both breeding schemes to produce rescued mice consisted of two steps. The first step was creating double heterozygous β^0-thalassemic mice (HbE transgene on heterozygous β^0-thalassemic background) as in Figures 16 and 19. The genotypic frequency of heterozygous β^0-thalassemic mice was 0.08 (Fig. 23) which was not follow the law of Segregation and Independent Assortment. The βIVSII-654 mice have only one mouse β-globin gene locus and another was replaced by human βIVSII-654 gene. This made the newborn suffering

from a lack of β-globin production, and caused parts of newborn heterozygous βIVSII-654 mice die at the first day of life. However, HbE transgene can compensate the absence of one mouse β-globin gene locus. Expression of β^E-globin protein as in double heterozygous βIVSII-654 mice (hemizygous HbE transgene on heterozygous βIVSII-654 background) can be detected (Fig. 29 lane4 and Fig. 30 lane 1). This made the genotype frequency of newborn double heterozygous to 0.25, which is closed to the value predicted by the law of Segregation and Independent Assortment.

The second step of both breeding scheme was to produce the rescued mice (Fig.17 and 19). As shown in Figure 24, the genotype frequency of the rescued mice was only 0.01. The low number of the surviving rescued mice could be explained from the pattern of human γ-globin genes expression in transgenic mice which differed substantially from expression in human development. Previous studies in transgenic mice carrying the normal human β-globin locus (on a YAC) showed that human γ-globin genes expression was relatively high until day 12-14 of gestation. Thereafter, the level of γ-globin at mRNA and protein levels decreased to 1-5% at birth and was undetected in mature mice (Gaensler *et al.,* 1993 and Porcu *et al.,* 1997). Thus some of the rescued mice can not survive prenatally because they could not compensate for the loss of endogenous β-globin chains by the low level of human γ-globin chains while human β^E-globin gene expressed at the low level. Thus the rescued mice carrying 8 copies of HbE transgene had more opportunity to survive. This was the reason to design the breeding plan by using the original mouse strains that carry 4 copies of HbE locus transgene.

2. Characterization of Thalassemic Mice

Genotyping of transgenic mice is established in maintaining transgenic mice colony and producing rescued mice. Multiplex PCR was used to genotyping β-thalassemic mice. However the multiplex PCR technique can not separate heterozygous βIVSII-654 from double heterozygous βIVSII-654 mice because of the presence of th-4 plug construct in both genotypes. The-4 plug construct is composed of HPRT minigene and human βIVSII-654 globin gene, which can be detected by HPRT and LUG primer. In addition, HbE transgene in double heterozygous βIVSII-654 mice can be also detected by LUG primer. Therefore cellulose acetate electrophoresis was used to distinguish heterozygous βIVSII-654 from double heterozygous βIVSII-654 mice as no chimeric hemoglobin heterotetramer in the former genotype (fig. 31 lane3).

Double heterozygous β-knockout and double heterozygous βIVSII-654 mice could not be identified by multiplex PCR and cellulose acetate electrophoresis techniques (Fig. 25 and 27). This is because the heterozygous β-knockout and heterozygous βIVSII-654 mice originated from the same ES cell line (β20) (Lewis *et al*., 1989),which contained a neomycin resistance (*neo*) and partial *HPRT* gene (Δ*HPRT*) downstream of the murine β major and β minor globin gene on chromosome 7 (Fig. 26). The th-4 plug targeting construct (Fig. 26a) was used for replacing the 21-kb of mouse genomic DNA containing the murine β major and β minor globin gene with a 5.7-kb DNA fragment containing the human βIVSII-654 splicing mutation gene. The βΔplug1.5 delete both the murine β major, β minor and *neo* gene while completing the HPRT mini gene (Yang *et al*., 1998) (Fig.26b). Two targeted ES cell lines were used generate heterozygous βIVSII-654 and heterozygous β-knockout mice by injection into the host blastocysts. The breeding of both

heterozygotes with hemizygous transgenic mice generated double heterozygous mice, could express both normal and chimeric hemoglobin (Fig. 27). To distinguish the two genotypes of double heterozygote mice, allele specific PCR were employed. This technique identified human βIVSII-654 mutation in double heterozygous βIVSII-654 mice, but not from double heterozygous β-knockout mice. In the allele specific PCR, the concentration of mouse tail genomic DNA sample was very important for the ability of binding normal and mutant primers to bind a correct DNA sequence. This was because both primers differed only one base in the 3'-end. The concentration of mouse tail genomic DNA must be diluted to 1/100-1/500 (approximately 0.2-1.0 ng of DNA sample) to prevent the non-specific binding to an incorrect sequence.

3. Levels of Human β^E-Globin Expression in Difference Genotype of HbE Transgenic Mice

The level of chimeric hemoglobin was increased while the number of mouse β-globin gene decreased, presumable reflected the decreased competition between the human β^E and mouse β-globin chains to bind to the excess mouse α -globin chains. The level of chimeric hemoglobin in hemizygous transgenic mice carried HbE transgene on a normal background, was 1.5-3% of the mouse hemoglobin tetramer. This hemoglobin increased 10-fold to 26-36% of the mouse hemoglobin tetramer in double heterozygous mice (Jamsai *et al*., 2004: p 105-106), and to 100% in the rescued mice (Figure 29 and 30,).

4. Hematological Analysis

Hemizygous transgenic mice which carry the human β^E-locus transgene of 4 copies integrated in chromosome 2. It expressed chimeric hemoglobin

(α_2^m/β_2^{HbE}) for only 1.5-3%. This abnormal hemoglobin molecule, in the minute amount, was not harmful the red cells. Morphologic study revealed that RBCs of transgenic mice was similar to the wild type mice (Fig. 31B), and hematologic parameter indicated that no sign of anemia in this genotype.

Heterozygous βIVSII-654 knocks in mice ($\beta^{IVSII-654}/\beta^{m+}$), where β^{m+} represents the wild type β-globin locus from strain C57BL/6, produce reduced amount of mouse hemoglobin (α_2^m/β_2^m) and no human β-globin chain due to knockin βIVSII-654 splicing mutation gene (Lewis 1998). Morphologic study revealed that the RBCs of this genotype were thalassemia phenotype (Fig 31C, 32-35). The increase in reticulocyte count in indicated an increased red blood cell synthesis and turnover. However, the decrease in red blood cell count (Fig. 32) suggest that the excess α-globin chains and their degraded products which are the source of the oxidative damage, lead to RBC destruction. This was different from the phenotype in human β-thalassemia heterozygotes who usually have a red blood cell count the same as in normal individuals or little higher than normal.

In double heterozygous βIVSII-654 knockin mice (hemizygous 4 copies of HbE transgene on heterozygous βIVSII-654 knock-in background) expressed chimeric hemoglobin ~26-36%(α_2^m/β_2^{HbE}), HbE locus transgene expressing β^E-globin chains to 10 fold compared with hemizygous transgenic. It was because of mouse β-globin (major, minor) gene got loss to one side of allele locus, increased proportion of chimeric hemoglobin presumably reflected the decreased competition between the human β^E and mouse β-globin chains for binding to the excess mouse α-globin chains. Thereby the property of red blood cells improved which was demonstrated from the high value of red blood cell count, hematocrit, hemoglobin concentration and a decreased reticulocyte

(Fig. 32-35). Furthermore, the morphology of red blood cells from blood smear was similar to wild type mice (Fig. 31D).

Rescued mice (homozygous βIVSII-654 knockin/HbE mice and compound heterozygous βIVSII-654 knockin / β^{mouse} –knockout / HbE mice) from both breeding plan (Fig. 16-19) reveled an active erythropoiesis from the significant increase of red blood cell count (10.18 $x10^6$ cells/μl) (Fig. 32A) and hematocrit (43.8%) (Fig. 33C). In addition the high value of reticulocyte indicated that rescued mice had more red cells turnover (Fig. 35H). On the other hand, hemoglobin concentration was not so much different from the wild type mice (13.56 g/dl in the rescued and 12.99 in the wild type) (Fig. 32B), resulting in the decreased MCH, MCHC (Fig. 34), and the presence of hypochromic red cells and target cells in peripheral blood smear (Fig. 31E). Furthermore, *Wannasuphaphol, B.,* compared hematologic data of rescued mice carrying 4 and 8 copies of HbE locus transgene (Wannasuphaphol, 2005). He found that rescued mice carrying 4 copies had severe anemia (Hb ~ 7.8 g/dl), whereas, rescued mice carrying 8 copies had the hemoglobin concentration resembled to the wild type (Hb~ 17.7 g/dl in rescue and 16.3 g/dl in wild type, analyzed by Advia [120]). Thus, in this study the rescued mice of both genotypes, from breeding schemes in figure 16-19, had taken of 8 copies HbE transgene. This means that they were inherited each set of 4 copies β^E transgene from each parent, and were in the homozygote state for HbE.

5 RBC Survival Study

The hematologic data from the various genotypes of β-thalassemic mice revealed the abnormal red blood cells in consequence of the loss of mouse β-globin gene. The abnormality of the red blood cells was improved when HbE locus transgene was introduced to β-thalassemic mice as in double

heterozygous and rescued mice. However, the reticulocyte count of rescued mice, 10.7% was much higher than that of wild type mice, 3.72% (Fig. 35H) which did not in accordance with the other hematologic parameter. The increasing reticulocyte indicated that there was an active erythropoiesis, while the decreasing number of red cells indicated that there was also more red blood cells destruction. To confirm the increasing red blood cells destruction in the β-thalassemic mice, RBC survival of difference transgenic mice genotype were examined. The survival of red blood cells was studied by labeling RBC with biotin, which was injected into mouse tail vein (Fig. 21C). After biotin injection for 4 hours, all of red blood cells were labeled, blood samples were collected every week from mouse tail vein. The decreasing biotin positive red blood cells were detected with phycoerythrin-conjugated streptavidin via flow cytometry (Fig. 36).

Red cell survival study revealed that the wild type had the red blood cell survival for 60 days and half life ($T_{1/2}$) for 25 days (Fig. 37). Heterozygous βIVSII-654 knockin mice had short half life ($T_{1/2}$) of red blood cells for 14 days (Fig. 38). This can be explained from the fact that the excess α-globin chains and their degraded products are a source of oxidative damage and can lead to RBC destruction. Red cell survival study in the mice carried HbE transgene (transgenic, double heterozygote and rescue) is more interesting. The results revealed the inversed relation between red cell survival and the level of chimeric hemoglobin (α_2^m/β_2^{HbE}). The increasing level of chimeric hemoglobin (α_2^m/β_2^{HbE}) decreased the age of red blood cells correlated to the value of reticulocytes, which was clearly increased in rescued mice (Fig. 35H). The mentioned phenomenon could assume that the assemble of mouse α-globin and human β^E-globin proteins appeared to be an unstable heterotetramer hemoglobin, which was proved by DCIP dye test. The abnormal globin chains (β^E-globin chains) from unstable hemoglobin (taken from peripheral blood of

the rescued mice) would precipitate in the *invitro* test tube. In addition the rescued mice had higher number of red blood cell count whereas hemoglobin concentration was similar to the wild type. This means that certain numbers of red cells were destroyed, which confirmed by the short RBC half life.

CONCLUSION

Mouse model of β-thalassemia/HbE have been generated by the two steps breeding. The first breeding was to produce mice carrying HbE transgene on the heterozygous β^0-thalassemic background (double heterozygous mice) by breeding hemizygous transgenic with heterozygous β^0-thalassemic mice (Fig. 16 and Fig. 18).The second breeding was to produce the mice carrying HbE transgene on homozygous β^0-thalassemic background (rescued mice), which was performed by breeding together the two double heterozygous mice (Fig. 17 and Fig. 19). These rescued mice had no mouse β-globin gene but can survived by expression of human β^E transgene, for mimics the main feature of β^0-thalassemia/ HbE in patients. Genotype identification is important to maintain each mouse's genotype colony. Moreover, accurate genotyping is needed when breeding double heterozygous β-knockout with double heterozygous βIVSII-654 mice or from unexpected accident in a handling and housing mice. Multiplex PCR detecting mouse β-globin, human β-globin and HPRT gene was used routinely. However, this technique can not distinguish between double heterozygous β-knockout and double heterozygous βIVSII-654 mice. So the technique of allele specific PCR were applied for solving the problems and preventing the incorrect strain of the next offspring.

After birth rescued mice that are the final product of breeding plan have a chance of survival at the low of percentage (Fig.26), because of the low level of human γ-globin chains, decreasing 1-5% at birth, and the low level of expressing human β^E-globin protein from β^E-locus transgene. Therefore the survival chance of rescued mice depends on the copies number of β^E-locus transgene.

Human β^E-locus transgene can improve balance of globin chains synthesis in β-thalassemic mice by increasing expression of β^E-globin protein compensate mouse globin genes that were deleted resulted in improved red blood cells to resembling the wild type mice. These results can be observed in double heterozygous βIVSII-654 mice (HbE transgene 4 copies on heterozygous βIVSII-654 knockin background). However the relation between the level of HbE transgene and red cell survival has been found. In rescued mice which express 100% chimeric hemoglobin (α_2^m/β_2^{HbE}), the survival of red blood cells was reducing to 14 days (wild type~ 25 days) which according to the increasing of reticulocyte (~10.70%). But the cause of red blood cell destruction in rescued mice (8 copies of HbE locus transgene inherited from parents) do not result from imbalance globin chains synthesis as in heterozygous βIVSII-654 knockin mice, that being confirmed from blood smear, red blood cell count, hematocrit and hemoglobin concentration that resembles to wild type mice. Red blood cells destruction in rescued mice may result from human β^E-globin protein, which mildly oxidatively unstable (Rees *et al*., 1996; Suthipark *et al*., 1987) binds with mouse α-globin chains to form unstable chimeric hemoglobin. This unstable hemoglobin has susceptibility to oxidative attack as in β-thalassemia/ HbE patients. The presumption of unstable chimeric hemoglobin leads to oxidative damage and RBCs destruction which is supported by thesis work of *Wannasuphaphol, B.,* in 2005. This identified that high level of reactive oxygen species in erythrocyte of rescued mice, detected with flow cytometer-based DCF assay.

In addition it is found that rescued mice have massively of erythropoiesis, same as β-thalassemia/ HbE patients. This might come from effectiveness of high RBCs destruction. Anyhow, from the studying of crystallography of chimeric hemoglobin (α_2^m/β_2^{HbE}) by *Kongsaeree, P.* in 2005, saying that the cause of massively of erythropoiesis might come from high

oxygen affinity of this chimeric hemoglobin, stimulated erythropoietin in rescued mice.

Therefore rescued mice of both genotypes (homozygous βIVSII-654 knockin / HbE mice and compound heterozygous βIVSII-654 knockin / β^{mouse} –knockout / HbE mice) might be used for model of β^0-thalassemia/ HbE to understand the mechanism of pathophysiology, such as ineffective erythropoiesis, and to develop the novel treatment for thalassemia patients.

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APPENDIX

Preparation of Reagents

1. Reagent for Tritron X-100 Acid urea Gel Electrophoresis

1.1 Solution A

- Acrylamide	30 g
- Bis-acrylamide	0.2 g
- Distilled water	50 ml

1.2 Solution B

- 8 M Urea	228 ml
- Glacial acetic acid	15 ml
- Triton X-100	6 ml

1.3 Sample buffer

Urea	3.9 g
Glacial acetic acid	0.83 g
Mercaptoethanol	0.83 g
DW add to	10 ml

1.4 Sample preparing

Hemolysate	10 μl
Sample buffer	90 μ

Mix for 5 min at room temperature

1.5 Staining solution: 0.06% Coomassie Brilliant Blue

Methanol	150 ml
Distilled water	315 ml
Glacial acetic acid	35 ml
Coomassie Brilliant Blue (R250)	0.3 g

1.6 Destaining solution

Methanol	150 ml
Distilled water	315 ml
Glacial acetic acid	35 ml

1.7 Other

Electrophoresis buffer: 5% Glacial acetic acid
1% Agarose
15% Ammonium persulphate

2. Reagent for Cellulose Acetate Electrophoresis

2.1 Cellulose acetate buffer: tris-EDTA-boric acid buffer; pH 8.6

2.2 Ponceaus S stain

2.3 Destaining solution: 5% Glacial acetic acid

2.4 Dehydrating agent: absolute methanol

2.5 Clearing solution: Glacial acetic acid : absolute methanol (30:70, v/v)

3. Reagent for Flow Cytometry

3.1 HEPES buffered saline for mouse (HBSM)

First, 2.38 g HEPES and 9.64 g sodium chloride were weighed and put in a clean one-liter beaker. About 850 ml distilled water was added and the solution was stirred on a magnetic stirrer. The pH of the solution was adjusted to a value of 7.4 with 1 N NaOH. After adjusted pH, the buffered solution was filled up with the distilled water to 1,000 ml in volumetric flask. The solution with 10 mM HEPES and 165 mM NaCl was transferred to a new one-liter Duran bottle and autoclaved.

3.2 Phycoerythrine-streptavidin staining

HEPES buffer	100 μl
Working Streptavidin-PE	1 μl
Mouse whole blood	1 μl

4. Reagent for Mouse Tail Genomic DNA Extraction

4.1 Tail lysis buffer

1 M Tris pH 8.0	2.5 ml
0.5 M EDTA	10 ml
add distilled water to adjust volume up to 47 ml	
10% SDS	2.5 ml (last one)

4.2 Mouse tail digesting solution

Lysis buffer	0.5 ml
20 mg/ml Proteinase K	5 µl
Tip of mouse tail	1-2 cm

Self-digest at 55 ^{0}C for 3 hours.

5. **The quantitative colorimetric determination of hemoglobin concentration in hemolysate for polyacrylamide urea gel electrophoresis.**

Drabkin's Reagent is used for the quantitative, colorimetric determination of hemoglobin concentration in whole blood at 540 nm. A colorimetric cyanmethemoglobin method was proposed where total hemoglobin at alkaline pH is rapidly converted to the cyanoderivative. The absorbance of the cyanoderivative is determined at 540 nm. The method was simplified by combining the separate reactants, alkaline ferricyanide and cyanide, into a single reagent.

This procedure is based on the oxidation of hemoglobin and its derivatives to methemoglobin in the presence of alkaline potassium ferricyanide. Methemoglobin reacts with potassium cyanide to form cyanmethemoglobin, which has maximum absorption at 540 nm. The color intensity measured at 540 nm is proportional to thetotal hemoglobin concentration.

5.1 Reagents and Equipment Required

- Drabkin solution (pH 7.0-7.4)

Potassium ferricyanide	200 mg
Potassium cyanide	50 mg
Potassium dihydrogen phosphate	140 mg
Triton x-100	1 mg
Adjust to 1 litter with distilled water	

- Hemoglobin standard, methemoglobin equivalent to 18 g hemoglobin per 100 ml blood (Sigma), for preparation of standard curve
- Spectrophotometer capable of measuring absorbance at 540 nm
- Cuvets
- Test tubes
- Pipetting devices for the accurate delivery of volumes required for the assays

5.2 Calibration Curve

a) Prepare a dilute 1:250 hemolysate samples by adding 10 µl of the hemolysate to 2.5 ml of the Drabkin's Solution

b). Prepare working standards by pipetting and mixing thoroughly the solutions indicated below.

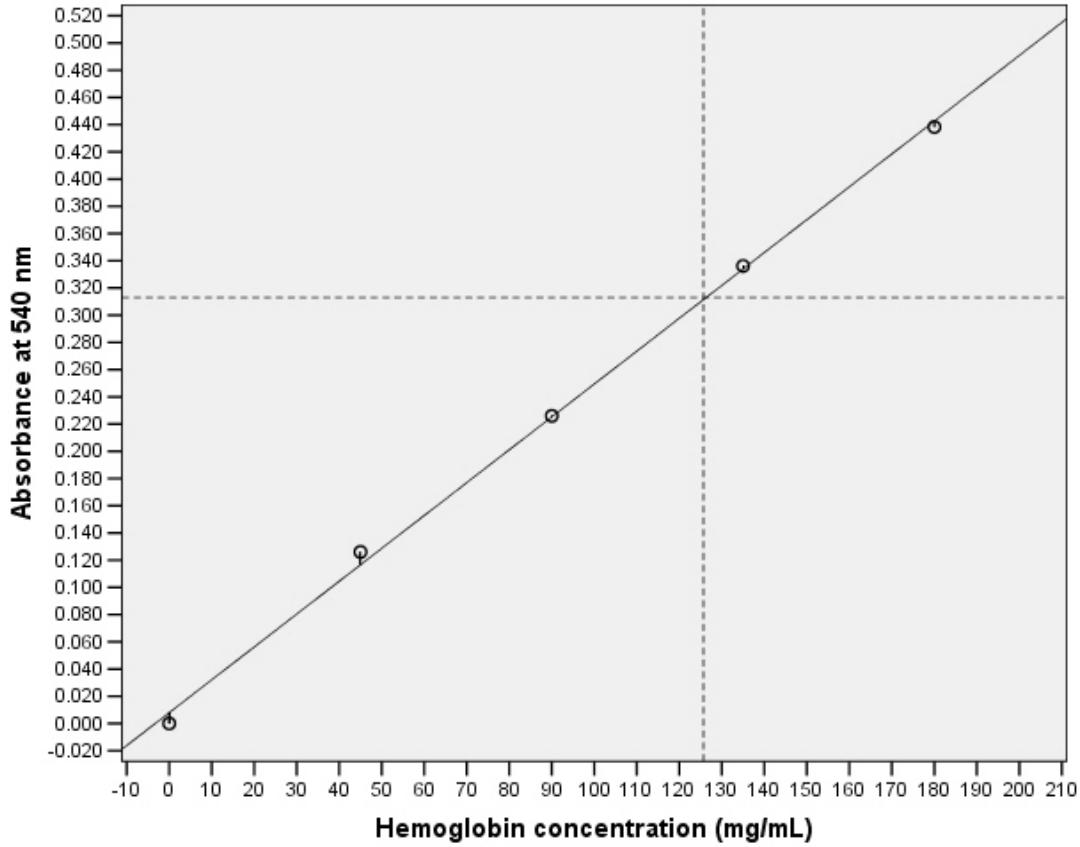
Standard tube No.	Hemoglobin standard (ml)	Drabkin Solution (ml)	Hemoglobin Concentration (mg/ml)
1	0.5	1.5	45
2	1.0	1.0	90
3	1.5	0.5	135
4	2.0	0.0	180
5	0.0	2	Blank

c) Read absorbance of Tubes 2-4 versus Tube 1 as the reference at 540 nm.

d) Record the absorbance values.

e) Plot a calibration curve of absorbance values versus the diluted hemolysate sample (1:250). The curve is linear, passing through the origin.

5.3 Analysis of hemolysate sample



Appendix figure 1 Standard hemoglobin curve for determine hemoglobin concentration in a 1:250 diluted hemolysate sample. The total hemoglobin of hemolysate sample was 31.25 g/ml.