A novel transgenic mouse model produced from lentiviral germline integration for the study of β-thalassemia gene therapy

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ABSTRACT

Background
β-thalassemia is one of the most common genetic diseases in the world and requires extensive therapy. Lentiviral-mediated gene therapy has been successfully exploited in the treatment of β-thalassemia and showed promise in clinical application. Using a human β-globin transgenic mouse line in a β-thalassemia diseased model generated with a lentiviral-mediated approach, we investigate the stable therapeutic effect on a common thalassemia syndrome.

Design and Methods
Human β-globin gene lentiviral vector was constructed, followed by subzonal microinjection into single-cell embryos of $\beta^{IVS\text{-}2\text{-}654}$-thalassemia mice to generate a transgenic line. Human β-globin gene expression was examined with RT-PCR, Western-blotting and ELISA. The hematologic parameters and tissue pathology were investigated over time in founder mice and their offspring.

Results
Transgenic mice with stable expression of the lentivirus carrying human β-globin gene were obtained. A marked improvement in red blood cell indices and a dramatic reduction in red blood cell anisocytosis, poikilocytosis and target cells were observed. Nucleated cell proportion was greatly decreased in bone marrow, and splenomegaly with extramedullary hematopoiesis was ameliorated. Iron deposition in liver was also reduced. There was a two-fold increase in the survival rate of the $\beta^{IVS\text{-}2\text{-}654}$ mice carrying human β-globin transgene. Significantly, the germline integration of the lentiviral construct was obtained and stable hematologic phenotype correction was observed over the next two generations of the transgenic mice.

Conclusions
The generation of human β-globin transgenic mice in a $\beta^{IVS\text{-}2\text{-}654}$-thalassemia mouse mediated with lentiviral vectors provides a useful model and offers an attractive means to investigate the transgenic stable therapeutic effect in β-thalassemia.

Key words: β-thalassemia, transgenic mouse, gene therapy, lentiviral vector, subzonal injection.

Citation: Li W, Xie S, Guo X, Gong X, Wang S, Lin D, Zhang J, Ren Z, Huang S, Zeng F, and Zeng Y. A novel transgenic mouse model produced from lentiviral germline integration for the study of β-thalassemia gene therapy. Haematologica 2008 Mar; 93(3):356-362. doi: 10.3324/haematol.12010

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Acknowledgments: we would like to thank Dr. Jiaying Liu for the help in hematologic analysis and Dr. Qingxue Wang for discussion and preparation of the manuscript.

Funding: the work was supported by grants from National Basic Research Program ("973 Program") of China (No. 2004 CB518806), National Natural Science Foundation of China (No. 30571777) and Shanghai Leading Academic Discipline Project (Project Number: B204).

Manuscript received July 24, 2007. Manuscript accepted October 31, 2007

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Introduction

β-thalassemia is one of the most common monogenetic disorders in the world. Globally, there are an estimated 80 million carriers.[1] Severe β-thalassemia is characterized by markedly ineffective erythropoiesis and severe anemia, and patients usually need lifelong blood transfusion. In addition to increased iron absorption, transfusion therapy can lead to progressive iron accumulation and tissue damage in multiple organs.[2] Currently, the most definitive and effective treatment of the disease is believed to be hematopoietic stem cell transplantation.[3,4] However, allogenic bone marrow transplantation is not an option for the majority of patients without a histocompatible donor. As a result, for the last two decades, treatment of β-thalassemia and hemoglobinopathies (sickle cell anemia) by transplantation of genetically modified autologous hematopoietic stem cells or embryonic stem cells has been considered an important treatment strategy, although the many technical difficulties have made progress slow.[5-7]

In 2000, May *et al.* first reported the alleviation of β-thalassemic symptoms in a mouse model after engraftment of bone marrow cells stably transduced with a lentiviral vector (TNS9) carrying a large fragment of the human β-globin gene.[8] Long-term improvement of clinical symptoms of human β-thalassemia and sickle cell anemia was also reported by other groups using these lentiviral vectors.[9-11]

C→T substitution at position IVS-2 nt 654 ($\beta^{IVS\text{-}2\text{-}654}$) in the human β-globin gene is one of the most common β-thalassemia alleles in the Chinese.[12] Patients with $\beta^{IVS\text{-}2\text{-}654}$ mutation lead to abnormally spliced β-globin mRNA with only an approximately 15% normal β-globin gene expression.[13] We report the generation of a human β-globin transgenic mouse in a $\beta^{IVS\text{-}2\text{-}654}$-thalassemia diseased model mediated by lentiviral vector to investigate the stable therapeutic effect on this common thalassemia syndrome.

Design and Methods

Lentiviral vector construction and production

Human β-globin gene fragment (from –1671 to +1570) was amplified from the human genomic DNA by PCR with specific primers that contained the restriction enzyme *Xba* I and *Kpn* I cutting sites (Forward: 5'-TGCTCTAGAGCTCCAGATAGCCATAGAAGAACC-3'-*Xba* I,

Reverse: 5'-GGGGTACCGCGAGCTTAGTGATACTTGT-3'- *Kpn* I, the cutting sites are underlined). The amplified fragment was digested with *Xba* I and *Kpn* I, and then cloned into the corresponding sites of lentiviral vector FUGW.[14] Lentiviral human β-globin gene vector (LBG) is schematically shown in Figure 1A. The most important features of the lentivirus are the CMV enhancer substituted for the U3 region of the 5' LTR (pCL configuration) to maximize expression of viral RNA genomes, the human β-globin promoter from –1671 to +1 to provide erythroid specificity, and a deletion in the U3 region of the 3' LTR to render the 5' LTR of the integrated provirus transcriptionally inactive. The β-globin locus control region (LCR) was omitted because incorporation of hypersensitive (HS) elements was known to render oncoretroviral vectors unstable during passage.[15]

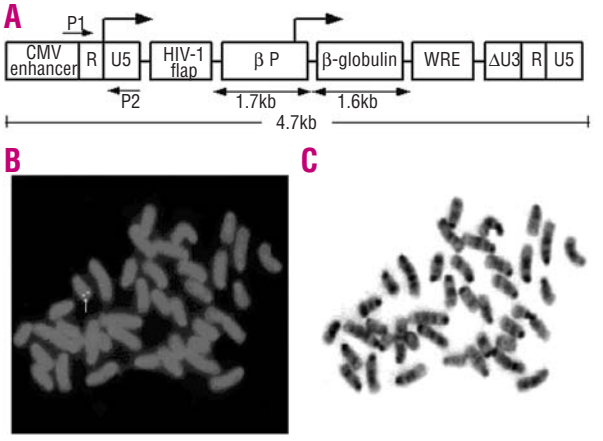

Figure 1. Diagram of the human β-globin lentiviral vector (LBG) and FISH analysis of the integration in mouse chromosome. (A) Diagram shows the lentiviral vector construct used to generate transgenic mice. βP: human β-globin promoter spanning from –1671 to +1; β-globin: human β-globin gene from +1 to +1570, WRE: woodchuck hepatitis virus posttranscriptional regulatory element; ΔU3 denotes a deletion in the U3 region of the 3'LTR that renders the 5'LTR of the integrated provirus transcriptionally inactive. P1 and P2: primers for identification of lentiviral integration in transgenic mice. (B) FISH analysis of the integration of lentivral vector in mouse chromosome. The arrow indicates the integration site. (C) shows the corresponding G-banding result.

The vector LBG was co-transfected with ΔR8.9 and VSVG into human embryonic kidney cells 293T. The viral supernatants were collected at 72 hrs after transfection and concentrated by low-speed filtration using 0.2 μm polyethersulfone filter (Hyclone) followed by ultracentrifugation (26,000 rpm, 4°C for 90 min). The viral was concentrated 1,000-fold from viral supernatants. The crude viral suspension was then quantified by measuring levels of the *gag* protein, p24 in viral stocks by using the HIV-1 p24 Core Profile ELISA kit from PerkinElmer Life and Analytical Sciences (Boston, MA). A viral aliquot containing 1 pg of p24 *gag* protein equals 1-10 infectious units (U). Several viral preparations were pooled and the final concentration was approximately 2×10^8U/mL.

Mouse strain

$\beta^{IVS\text{-}2\text{-}654}$ *thalassemia mice.* The $\beta^{IVS\text{-}2\text{-}654}$-thalassemia mice were obtained from the Jackson Laboratory (JAX). This heterozygote ($Hbb^{th\text{-}4}/Hbb^{+}$) carries one wild type murine β-major globin allele and one defective human $\beta^{IVS\text{-}2\text{-}654}$ allele associated with aberrant splicing due to C→T substitution at nt654 of intron 2, characterized by a moder-

ate form of β-thalassemia (anemia, splenomegaly, abnormal hematologic indices).[16] The $\beta^{IVS\text{-}2\text{-}654}$-thalassemia mouse strain used in this study was reviewed and approved by the Review Board of Shanghai Children's Hospital.

Generation of transgenic mice.[14] The $\beta^{IVS\text{-}2\text{-}654}$-thalassemia male mice were mated to superovulated wild type females. Single-cell embryos were collected and injected with LBG viral particles in the perivitelline space (subzonal microinjection). Each embryo was injected with 5×10^{-4}μL of viral suspension (10^8 U/mL), then immediately implanted in the oviduct of pseudo-pregnant wild type mice and allowed to develop to full term to provide the founder transgenic mice (F0). The production of F1 and F2 generations was respectively achieved by crossing founder (F0) or F1 with wild type mice (Hbb^+/ Hbb^+).

DNA analysis

After the pups weaned (approximately 3 weeks after birth), mouse genomic DNA was isolated from the tail tissue. PCR was then performed to determine lentiviral integration, the allele of human $\beta^{IVS\text{-}2\text{-}654}$ and murine β^{major} with primer pair 1 (5'-GACTTACAAGGCAGCTGTAG-3' and 5'-GTACAGTCCGGATGCAGCTC-3'), primer pair 2 (5'-AGTGATAATTTCTGGGTTAAGGT-3' and 5'-AGGGCCTAGCTTGGACTCAG-3') and primer pair 3 (5'-AGGCAGCTCACAAGAAGAAG-3' and 5'-TGGAGACTGCTCCCTAGAAT-3') respectively. Reaction was performed in a total volume of 25 μL mixture including 2.5 μL of 10×PCR buffer, 2 μL of $MgCl_2$ (25 mM), 2 μL of dNTP (each 2.5 mM), 0.6 μL of each primer pair (10 pmol/μL each primer), 0.2 μL of Taq enzyme (5 U/μL), adding ddH_2O to 25 μL for 30 cycles: denaturing at 94°C for 45 secs., annealing at 60°C for 45 secs., and extension at 72°C for 60 sec.

FISH analysis

The mouse spleen cells were used for FISH analysis according to the methods previously described.[17] To visualize lentiviral vector integration, the corresponding vectors were labeled with the DIG-Nick Translation Kit (Roche, Germany) according to the manufacture's protocol. FISH signals were examined with a Leica DM RXA2 fluorescent microscope.

Human β-globin mRNA analysis

Total RNA was isolated from the murine fresh peripheral blood using an RNA Extraction Kit (U-gene) according to the manufacturer's instructions. After RT reaction, the correctly spliced human β-globin transcripts were amplified with specific primers (5'-CCTTTGGGGATCT-GTCCACTCCTGA-3' and 5'-CAGCACGTTGC-CCAGGAGCC-3') for 30 cycles in a PCR machine (Eppendorf): denaturating at 94°C for 45 secs., annealing and extension at 70°C for 60 secs..

Human β-globin protein analysis

Fresh peripheral blood from murine tail vein was collected and then lysated. Proteins were separated by 12% SDS-PAGE and then transferred to nylon membrane using the electronic transfer method. Primary human β-globin monoclonal antibody (H00003043-M01, Abnova, 1:1,000 diluted) was used for hybridization at 4°C for 2 hours. After the primary incubation had been completed, the secondary hybridization was performed using peroxidase conjugated goat anti-mouse IgG (Rockland, 1:1,000 diluted) at 4°C for another 2 hrs.. Hb bands were visualized by DAB staining. ELISA was also used to quantitatively assess human β-globin contents as previously described.[18]

Hematologic analysis

Mouse peripheral blood smears were prepared using 1-2 μL of blood samples collected in heparinized microhematocrit tubes, air dried and stained with Wright-Giemsa. Whole blood samples from mice starting from 6 weeks of age were collected in 40 μL microhematocrit tubes containing 2 μL of 0.5 M EDTA (pH 8.0). The RBC count, hemoglobin (Hb), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and reticulocyte counts for each sample were determined using the Hematology Analyzer (KX-21, Sysmex) equipped with software to analyze murine cells.

Histopathology

Transgenic mice and age-matched controls (> 1 year old) were used for tissue pathology analysis. Small pieces of livers and spleens were embedded in paraffin wax, cut with a LEICA RM 2135, and then mounted onto glass slides. The tissue sections were stained with hematoxylin-eosin and subsequently examined by light microscopy. Liver tissue sections were also stained with Pearl's Prussian blue to investigate the iron accumulation. Bone marrow smears were stained with Wright-Giemsa staining to calculate the proportion of nucleated cells.

Statistical analysis

The χ^2 test was used to determine difference in Table 1, and the One Way ANOVA was used to analysis the data in Table 2. Analytical data were processed using SPSS 11.0 software.

Table 1. Elevated survival rate of $\beta^{IVS\text{-}2\text{-}654}$ mice carrying human β-globin transgene.

Mouse	$\beta^{IVS\text{-}2\text{-}654}$ survival (β^{tg-}/β^{tg+})	Total	Rate (%)
F1	13 (6/7)	26	50.0*
F2	17 (8/9)	32	53.1*
Controls	186	695	26.8

*F1 and F2 were produced by the cross of β^{tg}-$Hbb^{th\text{-}4}$/Hbb^+ with wild type mice. Controls produced by mating $Hbb^{th\text{-}4}$/Hbb^+ to wild type ones; β^{tg-}: no integration of human β–globin gene; β^{tg+}: positive integration of human β–globin gene; *Statistically significant difference from controls; p<0.01*

Results

Efficient generation of transgenic mice using lentiviral vectors by subzonal microinjection

LBG virus particles were microinjected subzonally into 125 single-cell embryos and subsequently implanted into the oviducts of pseudo-pregnant female mice. Twenty-three (18.4%) embryos developed to full-term. Eight (34.7%) live-born mice were shown to be positive for LBG integration by PCR analysis.

The birth rate did not differ greatly from those generated by pronuclear injection with similar vectors in our laboratory (18.4% *vs.* 12.5%). However, the rate of transgene integration seems to be significantly higher in the live-born pups when using the subzonal microinjection method (34.7% *vs.* 8.0%) (Supplementary Table 1).

Germline integration of the lentiviral construct

The live-born mice showed four genotypes according to the PCR results: *Hbb*$^{+}$/*Hbb*$^{+}$ (wild type), *Hbb*$^{th\text{-}4}$/*Hbb*$^{+}$ (β$^{IVS\text{-}2\text{-}654}$ thalassemia), β^{hu}-*Hbb*$^{+}$/*Hbb*$^{+}$ (human β-globin transgenic), and β^{hu}-*Hbb*$^{th\text{-}4}$/*Hbb*$^{+}$ (human β-globin transgenic mouse with β$^{IVS\text{-}2\text{-}654}$ thalassemia). Stable integration of the lentiviral β-globin transgene vector into genome of the founders and transmission to their progenies were observed (Figure 2A) by PCR analysis and confirmed by fluorescence *in situ* hybridization (FISH) analysis (Figure 1B).

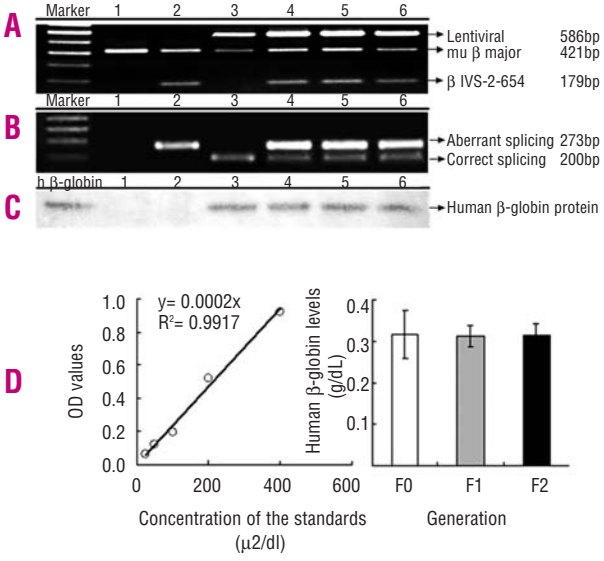

Figure 2. Mouse genotypes identified by PCR and the expression of human β-globin in LBG transgenic mice. (A) Genotypes of the mice are determined by PCR (2% agarose gel). (B) Human β-globin mRNA analysis, correctly spliced human β-globin mRNA (200bp of the amplified fragment) produced in lentiviral integrated transgenic mice β^{hu}-Hbb^{+}/Hbb^{+} and β^{hu}-Hbb$^{th\text{-}4}$/Hbb^{+}mouse, whereas only aberrant splicing (273bp of the amplified fragment) produced in Hbb$^{th\text{-}4}$/Hbb^{+} mouse. (C) Western-blotting analysis, human β-globin protein is shown in LBG transgenic mice. 1: Hbb^{+}/Hbb^{+} (wild type mouse); 2: Hbb$^{th\text{-}4}$/Hbb^{+} (β$^{IVS\text{-}2\text{-}654}$ thalassemia mouse); 3: β^{hu}-Hbb^{+}/Hbb^{+} (human β-globin transgenic mouse); 4-6: β^{hu}-Hbb$^{th\text{-}4}$/Hbb^{+} (human β-globin transgenic mouse with β$^{IVS\text{-}2\text{-}654}$ thalassemia) F0, F1, F2 generation respectively. (D) Quantitative analysis of human β-globin by ELISA in 3 generations of β^{hu}-Hbb$^{th\text{-}4}$/Hbb^{+} mice. F0: 0.31±0.05 g/dL (n=3); F1: 0.31±0.02 g/dL (n=4); F2: 0.31±0.03 g/dL (n=3). (*Mean*±SD).

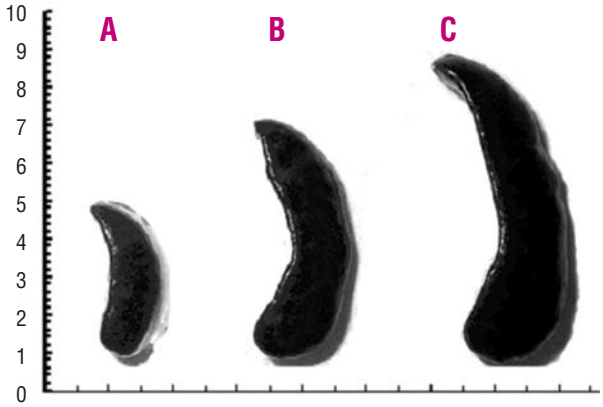

Figure 3. Spleen weight and size are decreased in β^{hu}-Hbb$^{th\text{-}4}$/Hbb^{+} mice. (A) Hbb^{+}/Hbb^{+} (99±34mg, n=5). (B) β^{hu}-Hbb$^{th\text{-}4}$/Hbb^{+} (251±54 mg, n=4). (C) Hbb$^{th\text{-}4}$/Hbb^{+} (455±93 mg, n=5).

Elevated survival rate of the β$^{IVS\text{-}2\text{-}654}$ mice carrying human β-globin transgene

Mendelian inheritance suggests a 50% heterozygote rate in the offspring of a cross between heterozygous β$^{IVS\text{-}2\text{-}654}$ thalassemia (*Hbb*$^{th\text{-}4}$/*Hbb*$^{+}$) and wild type mice (*Hbb*$^{+}$/*Hbb*$^{+}$). However, only 26.8% of the weaned pups appeared to carry the β$^{IVS\text{-}2\text{-}654}$allele, indicating a reduced survival rate of *Hbb*$^{th\text{-}4}$/*Hbb*$^{+}$ mice. Interestingly, a much increased survival rate of 50.0% was observed in F1, and 53.1% in F2 generation of β$^{IVS\text{-}2\text{-}654}$ thalassemia mice carrying the human β-globin transgene crossed to wild type mice, suggesting a positive therapeutic effect of the human β-globin transgene (Table 1).

Stable expression of human β-globin gene in transgenic mice

RT-PCR was performed to analyze the expression of human β-globin gene in LBG transgenic mice. We found that the correctly spliced transcript (200bp of the amplified fragment) was present in wild type and β$^{IVS\text{-}2\text{-}654}$ thalassemia mice that acquired a human β-globin transgene (β^{hu}-*Hbb*$^{+}$/*Hbb*$^{+}$ and β^{hu}-*Hbb*$^{th\text{-}4}$/*Hbb*$^{+}$ respectively), as well as in their F1 and F2 offspring (Figure 2B). Western blotting analysis showed the presence of human β-globin in β^{hu}-*Hbb*$^{th\text{-}4}$/*Hbb*$^{+}$ founders and their progeny (Figure 2C). Human β-globin content in β^{hu}-*Hbb*$^{th\text{-}4}$/*Hbb*$^{+}$ founder mice was approximately 0.31 g/dL as detected by ELISA (Figure 2D). Similarly, human β-globin expression was also detected in F1 and F2 generations and sustained a stable level.

Correction of hematologic parameters and improvement of RBC morphology in LBG integrated thalassemia mice

The β^{hu}-*Hbb*$^{th\text{-}4}$/*Hbb*$^{+}$ mice with stable expression of human β-globin protein also showed marked improve-

Table 2. Hematologic parameters in β^{hu}-$Hbb^{th\text{-}4}$/Hbb^{+} mice.

Group	N	RBC ($x10^{12}$/L)	Hb (g/dL)	MCV (fL)	MCH (pg)	P&T (%)	Retic (%)
1	16	9.1±0.6*	14.3±0.5*	52.5±2.1*	15.9±0.9*	N/A*	1.32±0.22*
2	5	7.6±0.9*	10.3±1.5*	48.5±2.2*	13.5±0.3*	35.8±3.2*	11.95±1.48*
3	6	7.5±1.1*	10.0±1.5*	47.2±2.0*	13.3±0.4*	36.3±2.4*	11.66±0.78*
4	3	8.3±0.9*	11.7±1.4*	50.9±2.6*	14.0±0.2*	34.5±2.3*	11.29±0.76*
5	10	6.6±0.2*	8.9±0.3	44.5±1.2*	13.3±0.1*	57.7±3.5*	14.71±0.88*

*Values represent mean±SD. Groups represent: 1: Hbb^{+}/Hbb^{+}; 2: β^{hu}-$Hbb^{th\text{-}4}/Hbb^{+}$ (F0); 3: β^{hu}-$Hbb^{th\text{-}4}/Hbb^{+}$ (F1); 4: β^{hu}-$Hbb^{th\text{-}4}/Hbb^{+}$ (F2); 5: $Hbb^{th\text{-}4}/Hbb^{+}$. Hb: hemoglobin; RBC: red blood cell; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; P&T: poikilocyte and target cell; Retic: reticulocyte. *Statistically significant difference from group 5; $p<0.01$.*

ments in red blood cell indices. Compared with $Hbb^{th\text{-}4}/Hbb^{+}$ mice, a significant elevation occurred in red blood cell number (7.5-8.3 *vs.* 6.6×10^{12}/L, $p<0.01$), Hb concentration (10.0-11.7 *vs.* 8.9 g/dL, $p<0.01$), MCV (47.2-50.9 *vs.* 44.5 fL, $p<0.01$). In addition, a dramatic reduction in reticulocyte numbers from 14.71% to 11.29-11.95% was also found (Table 2). Furthermore, morphological observation of blood smears revealed a marked decrease in red blood cell anisocytosis, poikilocytosis and target cells from 57.7% to 34.5-36.3% (Table 2, Figure 4A).

Pathological changes in spleen, liver and bone marrow of β^{hu}-$Hbb^{th\text{-}4}/Hbb^{+}$ mice

To further determine the positive therapeutic effect of stable human β-globin expression on hematopoiesis, we investigated the extent of splenomegaly and extramedullary hematopoiesis (EMH) in β^{hu}-$Hbb^{th\text{-}4}/Hbb^{+}$ and age-matched $Hbb^{th\text{-}4}/Hbb^{+}$ control mice. Spleen weight and size in $Hbb^{th\text{-}4}/Hbb^{+}$ control mice were much heavier and larger than those of β^{hu}-$Hbb^{th\text{-}4}/Hbb^{+}$ mice (Figure 3, Supplementary Table 2).

The regression of EMH was corroborated by morphological examination of spleens and livers from one-year-old transgenic mice and age-matched controls. The histopathology of spleens in $Hbb^{th\text{-}4}/Hbb^{+}$ mice showed a significant expansion of red pulp and a dense occupation of nucleated erythroid precursors. There was a relative decrease in white pulp and the marginal zones were obscured by a large number of nucleated RBCs. In β^{hu}-$Hbb^{th\text{-}4}/Hbb^{+}$ mice, the amount of red pulp was considerably decreased and the numbers of nucleated erythroid precursors in red pulp were reduced (Figure 4B). Other immature hematopoietic cells such as mega-karyocytes were present in red pulp of $Hbb^{th\text{-}4}/Hbb^{+}$ mice, but much less frequent in β^{hu}-$Hbb^{th\text{-}4}/Hbb^{+}$ mice. The hemosiderin was also obvious in the spleens of $Hbb^{th\text{-}4}/Hbb^{+}$ mice, while it was rarely observed in β^{hu}-$Hbb^{th\text{-}4}/Hbb^{+}$ mice (Figure 4C). Compared with $Hbb^{th\text{-}4}/Hbb^{+}$ mice, β^{hu}-$Hbb^{th\text{-}4}/Hbb^{+}$ mice showed fewer foci of intrasinusoidal EMH (Figure 4D) and lower levels of iron accumulation in the livers (Figure 4E). The proportion of nucleated cells in bone marrow was also considerably decreased in β^{hu}-$Hbb^{th\text{-}4}/Hbb^{+}$ mice, indicating the improvement in abnormal bone marrow proliferation (Figure 4F).

Discussion

Successful treatment of β-thalassemia using lentiviral vectors may lead to a promise of clinical application.[19] Previous studies in lentiviral-mediated gene therapy for β-thalassemia were mostly based on the transplantation of genetically modified autologous hematopoietic cells (HSC) in mouse models.[9-11] However, the efficacy of the transgene and the stability of the transgene expression mediated by lentiviral vectors require further investigation. In this study, we generated a human β-globin transgenic mouse line in a β-thalassemia ($\beta^{IVS\text{-}2\text{-}654}$) diseased model with a lentiviral-mediated vector, and investigated the effectiveness, inheribility and the positive outcome of this therapeutic approach on a common thalassemia syndrome.

Lentiviral transgenesis by subzonal microinjection was used in the current study. The results indicated that it was more efficient than pronuclear injection. This agrees with the studies by Lois *et al.* and Hofmann *et al.*[14,20] Compared with the pronuclear injection performed in our laboratory, subzonal microinjection can obtain approximately 4 times more transgenic mice. Therefore, subzonal microinjection mediated by lentivirus is a simple and effective method for the generation of transgenic mice.

In this study, a mouse line carrying both normal human β-globin and $\beta^{IVS\text{-}2\text{-}654}$ genes (β^{hu}-$Hbb^{th\text{-}4}/Hbb^{+}$ mouse) was produced and subsequently observed in detail. Our results showed a stable integration of lentiviral-human β-globin gene vector (LBG) in the genome of the transgenic founders as well as F1 and F2 offspring. Furthermore, a stable level of human β-globin expression was also observed in three generations (Supplementary Figure 7), indicating germline inheritance of the lentiviral construct and stable expression of the human β-globin transgene. In this study, human β-globin expression was obviously identified in β^{hu}-$Hbb^{th\text{-}4}/Hbb^{+}$ mice and 0.31g/dL of human β-globin protein was examined in the peripheral blood. Such a stable expression of human β-globin transgene may result in an increase in hemoglobin concentration (Table 2) as well as an improvement in erythropoiesis and thalassemic phenotype in $\beta^{IVS\text{-}2\text{-}654}$ mice.

Therapeutic efficacy was also evaluated by investigating the survival rates of β-thalassemia mice in weaned pups.

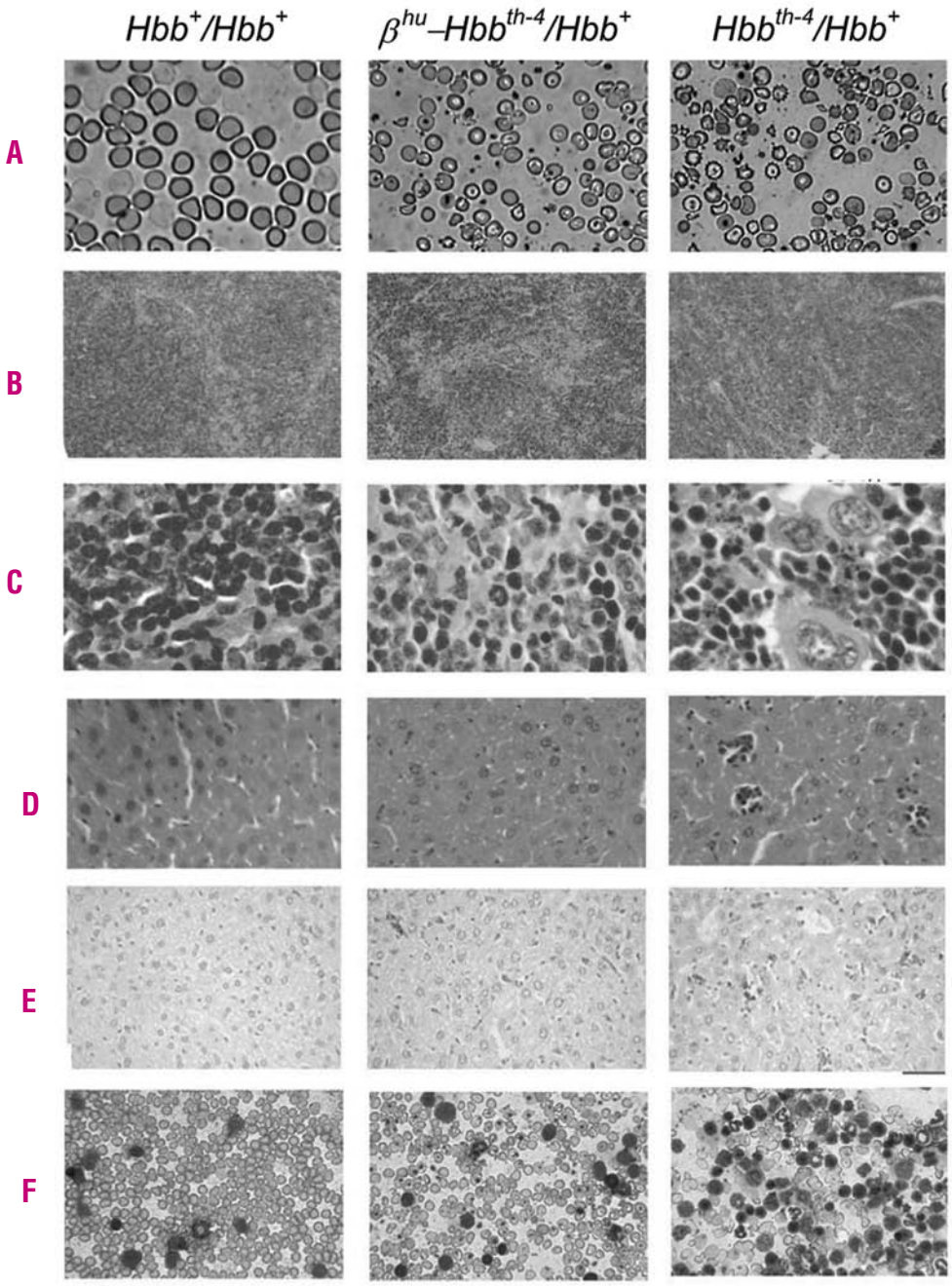

Figure 4. Improvement of RBC morphology and pathological changes in β^{hu}-*Hbb*$^{th-4}$/*Hbb*$^{+}$ mice. A. Blood smears with Wright-Giemsa staining show the marked reduction in RBC anisocytosis, poikilocytosis and target cells (original magnification, ×400). B. The pictures show the lesser expansion of red pulp and the distinct marginal occurred in spleen (HE staining, ×40). C. It is rare to find nucleated erythroid precursors and mega-karyocytes in the spleen (HE staining, ×400). D. Few erythroid precursors appear in the sinusoids of the liver (HE staining, ×400). (E) Much less iron accumulation is identified in the livers (Ferrocyanide iron staining, ×400). F. The proportion of the nucleated cells considerably decreased in bone marrow (Wright-Giemsa stainning, ×400).

As described in this study, a much higher survival rate (nearly 50%) in $\beta^{IVS\text{-}2\text{-}654}$ pups carrying human β-globin transgene was observed. Obviously, the increased survival rate was due to a positive therapeutic effect and the production of human β-globin partly corrected the defect in thalassemic mice. Nevertheless, cross-breeding will be carried out to expand the numbers of transgenic mice so that we can further analyze the long-term survival of mice which had lentiviral-mediated gene therapy.

Safety issues concerning lentiviral-mediated gene therapy must be considered.[21] One of the concerns about lentiviral vectors is the possibility of insertional activation of cellular oncogenes by random integration of the vector provirus into the host genome. Recently, scientists developed SIN vectors to reduce the risk of insertion mutagenesis which makes lentiviral vector even safer.[22] It is likely

that only one or a few copies may integrate the genome of each embryo by subzonal microinjection. Therefore, the risk of insertional mutagenesis for one embryo will be far lower than when transducing lentiviral-mediated transgene to millions of hematopoietic stem cells that will be infused in one mouse. In addition, the mouse model described in this study can be useful to analyze the absence of tissue-dependent toxicity or long-term toxicity of transgenes carried by lentiviral vectors.

In conclusion, we believe that generation of human β-globin transgenic mice using lentiviral vectors could be useful and informative for the pre-clinical assessment of lentiviral-mediated gene therapy in thalassemia and could also be exploited in other diseases.

Authorship and Disclosures

WL designed and performed most of the experiment, analyzed the data and drafted the manuscript; SX designed and analyzed the data; XG, XG, SW, DL and JZ participated in different parts of the experiment. ZR, SH, FZ and YZ drafted and revised the manuscript critically for important content; SH, FZ and YZ also conceived and designed the project; FZ and YZ approved the final version of the manuscript to be published. The authors reported no potential conflicts of interest.

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