Therapeutic effects of induced pluripotent stem cells in chimeric mice with β-thalassemia

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ABSTRACT

Although β-thalassemia is one of the most common human genetic diseases, there is still no effective treatment other than bone marrow transplantation. Induced pluripotent stem cells have been considered good candidates for the future repair or replacement of malfunctioning organs. As a basis for developing transgenic induced pluripotent stem cell therapies for thalassemia, β^{654} induced pluripotent stem cells from a β^{654}-thalassemia mouse transduced with the normal human β-globin gene, and the induced pluripotent stem cells with an erythroid-expressing reporter *GFP* were used to produce chimeric mice. Using these chimera models, we investigated changes in various pathological indices including hematologic parameters and tissue pathology. Our data showed that when the chimerism of β^{654} induced pluripotent stem cells with the normal human β-globin gene in β^{654} mice is over 30%, the pathology of anemia appeared to be reversed, while chimerism ranging from 8% to 16% provided little improvement in the typical β-thalassemia phenotype. Effective alleviation of thalassemia-related phenotypes was observed when chimerism with the induced pluripotent stem cells owning the erythroid-expressing reporter *GFP* in β^{654} mouse was greater than 10%. Thus, 10% or more expression of the exogenous normal β-globin gene reduces the degree of anemia in our β-thalassemia mouse model, whereas treatment with β^{654} induced pluripotent stem cells which had the normal human β-globin gene had stable therapeutic effects but in a more dose-dependent manner.

Introduction

The thalassemias are a collection of congenital hemolytic anemias caused by aberrant globin-chain production, with at least 60,000 severely affected subjects born worldwide every year. In particular, there are over 40,000 annual births for β-thalassemias, and about 50% occur in Southeast Asia, as estimated by the World Health Organization.[1] Over the last decades, the birth rate of affected children in some areas of Middle Eastern, Southern and Southeast Asia has continued to increase.[2] Prenatal diagnosis may effectively reduce the number of births with this genetic disease; however, this is not possible in some developing countries. Babies with the severe forms of thalassemia can now survive but require much medical intervention, resulting in a rising global healthcare burden.[3]

In China, β^{654} thalassemia is the most common form, and represents approximately one-fifth of total β-thalassemia cases. It is caused by a C→T substitution at nucleotide 654 of intron 2 (β IVS–2–654) in the β-globin gene, leading to an incorrectly spliced RNA isoform that produces trace amounts of a poorly functioning polypeptide.[4,5] The resulting imbalance of α-globin with β-globin results in the precipitation of excess free α-chains around the erythrocyte membrane,[4] leading to intramedullary destruction of the erythroid precursors, and a syndrome of ineffective erythropoiesis.[6,7] As a result, these patients are presented with severe anemia, with abnormal hematologic indices, including lowered red blood cells (RBC), hemoglobin (Hgb) and mean corpuscular hemoglobin concentration (MCHC), etc. Splenomegaly and hepatomegaly related to significant extramedullary hematopoiesis (EMH), as well as iron deposits in multi-organs are typically observed.

Previous attempts to correct the underlying molecular mechanisms have been reported. For example, the transduction of an extra copy of the normal human β-globin gene into allogenic hematopoietic stem cells was first demonstrated by May *et al.*[8,9] The transgenic human β–globin approach, combined with RNAi and anti-sense RNA against α-globin leading to partial reversion of anemia phenotypes, was also verified in mouse model systems.[10,11] However, these treatment options still largely fall into the domain of basic research. In clinical therapy, β-thalassemia patients mostly rely on lifelong blood transfusions combined with iron chelation.[12,13] Recently, therapeutic treatment using splenectomy has been introduced to decrease blood consumption and iron intake.[14,15] Hematopoietic stem cell or cord blood transplantation are also used as a strategy for partially relieving the symptoms.[16-18] However, resources for finding matched bone marrow donors are limited. All of the aforementioned therapies are expensive and can be painful procedures. With the development of stem cell induction techniques,[19] induced pluripotent stem cells (iPSCs) potentially represent an excellent approach to treat genetic hematopathies after birth.[20] Herein, we used

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chimeric murine models to evaluate the *in vivo* efficacy, safety, and stability of treatment with β^{hu}-β^{654} iPSCs (β^{654} mice transduced with the human β-globin gene), and compared this to treatment with non-transgenic HG-iPSCs.

Methods

The β^{654} mice[21] and HG mice were used to generate β^{654} iPSCs and HG-iPSCs carrying defective or wild-type β-globin genes, respectively. Following the generation of β^{654} iPSCs derived from tail tip fibroblasts (TTF) of β^{654} mice with methods previously described,[22] a wild-type human β-globin gene was introduced into the β^{654} iPSCs using a lentiviral vector (hBG) (resulting in β^{hu}-β^{654} iPSCs). β^{hu}-β^{654} iPSCs or *HG*-iPSCs were then injected into the blastocytes of wild-type (WT) or β^{654} mice. These blastocytes were transferred into the uteri of 2.5 days post coitus (dpc) pseudo-pregnant WT mice (ICR strain), which gave birth to chimeric pups at approximately 20 dpc.[23] Expression of human β-globin was detected by RT-PCR and Western blot analysis. Hematologic parameters were determined using the Hematology Analyzer (KX-21, Sysmex, Japan). The peripheral blood smears and bone marrow slides were stained with Wright-Giemsa (BASO BA4017, Zhuhai, China) or brilliant tar blue (BASO BA4003, Zhuhai, China). The tissue sections, including spleens and livers, were stained with hematoxylin-eosin (BASO BA4025, Zhuhai, China) or Pearl's Prussian blue (BASO BA4089B, Zhuhai, China). Quantitative PCR was performed using primers for *Tfr1, Twsg1*, and *Gdf15* to analyze EMH in the spleens, and with *Bmp6* primers to evaluate iron accumulation in the livers. All primer pairs are listed in *Online Supplementary Table S1*. The mouse serum soluble transferrin receptor (*sTfR*) level was determined with ELISA (AMEKO, Shanghai, China).

Additional study methods and analyses are provided in the *Online Supplementary Appendix.*

The strain of wild-type (WT) mice used in this study was ICR. In transgenic *HS23-GFP* mice (HG), the erythroid-expressing GFP was driven by the human β-globin promoter. The study used β^{654} thalassemia mice with a copy of the human β^{654} globin gene[21] (*see Online Supplementary Appendix for details*). β^{654} iPSCs were derived from the tail tip fibroblasts (TTF) of a β^{654} mouse. β^{654} iPSCs were transduced with a wild-type human β-globin gene. HG-iPSCs were derived from the TTF of an HG mouse.

Chimeras generated from injection of β^{hu}-β^{654} iPSCs into blastocysts of WT mice produced β^{hu}-β^{654}↔WT mice. Chimeras generated from injection of β^{hu}-β^{654} iPSCs into blastocysts of β^{654} mice produced β^{hu}-β^{654}↔β^{654} mice.

β^{hu}-β^{654}↔β^{654H} represents chimerism over 30%; β^{hu}-β^{654}↔β^{654L} represents chimerism below 30%. Chimeras generated from blastocyst injection of *HG*-iPSCs into blastocysts of β^{654} mice produced *HG*↔β^{654} mice. Chimeras generated from blastocyst injection of *HG*-iPSCs into blastocysts of WT mice produced *HG*↔WT mice.

Results

Production of chimeric mice

Production of β^{654} iPSCs and *HG*-iPSCs was confirmed by PCR analysis of transgene expression (*Online Supplementary Appendix* and *Online Supplementary Figure S1*). After the β^{654} iPSCs were transduced with a human β-globin gene, five β^{654} iPS cell lines were screened using PCR for long terminal repeat (LTR) sequence in vector, three of which were shown to be positive for β^{hu}-β^{654} iPSCs (*Online Supplementary Figure S2*). A β^{hu}-β^{654} iPS cell line with only a single copy of exogenous normal human β-globin was used as the source of donor cells for producing chimeras that generated β^{hu}-β^{654}↔β^{654} and β^{hu}-β^{654}↔WT (see Figure 1A for methods, Figures 1B for results and *Online Supplementary Table S2*). HG↔β^{654} chimeras were also generated as controls (Figure 1C).

Chimerism ranged from 7% to 92% in β^{hu}-β^{654}↔WT mice, and from 6% to 84% in β^{hu}-β^{654}↔β^{654} mice. These iPSCs can differentiate into multiple tissue types with varying efficiency (Figure 1D and E). All β^{hu}-β^{654} iPSC chimeras had stable transgene expression of the human β-globin mRNA (Figure 2A) and protein (Figure 2B).

Improvement of hematologic indices in chimeras

Erythrocytes from two different sources co-existed in the same blood smears of HG↔β^{654} mice (Figure 2C), indicating that the iPSCs had differentiated into erythrocytes in the chimeric mice. HG-iPSCs could differentiate into GFP erythrocytes in HG↔β^{654} mice (Figure 2D), and the GFP expressing erythrocytes derived from HG-iPSCs had normal morphology, with biconcave-discoid shape and even pigmentation, while the cells without *GFP* expression (originated from β^{654} blastocysts) had morphology typical of β-thalassemia (Figure 2C). As expected, β^{hu}-β^{654} iPSCs can differentiate into blood cells effectively in β^{hu}-β^{654} iPSC chimeras, the mRNA and protein levels of exogenous human β-globin were shown to increase with increasing percentage of chimerism (Figure 2A, B and E). The ratio of human normal *versus* abnormal splicing β-globin at the mRNA level increased (Figure 2E). The percentage of reticulocytes in blood declined in β^{hu}-β^{654}↔β^{654} mice (Figure 2F), and the excessive hematopoiesis was effectively controlled. The ratio of murine α-globin *versus* exogenous human β-globin and the ratio of murine α-globin *versus* murine β-globin decreased with increasing per-

Table 1. Hematologic analyses.

Group	N	RBC (×10^{12}/L)	Hgb (g/L)	Hct %	MCV (fL)	MCH (pg)	MCHC (g/L)
WT	35	10.64±0.56*	158.90±7.43*	50.04±2.17*	47.98±2.68*	15.38±0.87*	322.50±5.21*
β^{hu}-β^{654}↔*WT*	28	10.58±0.49*	156.73±6.77*	50.02±2.17*	46.77±2.78*	15.18±0.78*	321.00±5.56*
β^{hu}-β^{654}↔β^{654}	12	9.39±0.93[a]	126.42±10.89*	42.88±3.47*	45.17±3.36*	14.57±0.74*	317.50±7.01 *
HG↔β^{654}	15	9.04±1.09*	123.13±11.91*	41.15±3.17*	45.12±3.90*	14.71±1.34*	314.41±11.41[a]
β^{654}	31	8.50 ±1.25	104.77±11.78	34.77±3.90	39.85±2.46	12.09±1.03	303.31±12.86

*Values represent mean ± SD; RBC: red blood cell; Hgb: hemoglobin; Hct: hematocrit; MCV: mean corpuscular volume; MCH: mean corpuscular hemoglobin; MCHC: mean corpuscular hemoglobin concentration. Statistically significant differences, WT, β^{hu}-β^{654}↔WT, β^{hu}-β^{654}↔β^{654}, HG↔β^{654} compared with β^{654} group: *P<0.01; a: P<0.05.*

centage of chimerism (Figure 2B and 2G). These results indicated that the exogenous human β-globin played an important role in balancing the ratio of α-globin and β-globin.

The β^{hu}-β^{654}↔β^{654} and HG↔β^{654} chimeric mice exhibited elevated levels of RBC, Hgb, MCV, and MCHC (Table 1), compared to the β^{654} mice, indicating that the microcytic hypochromic anemia was ameliorated in these chimeras.

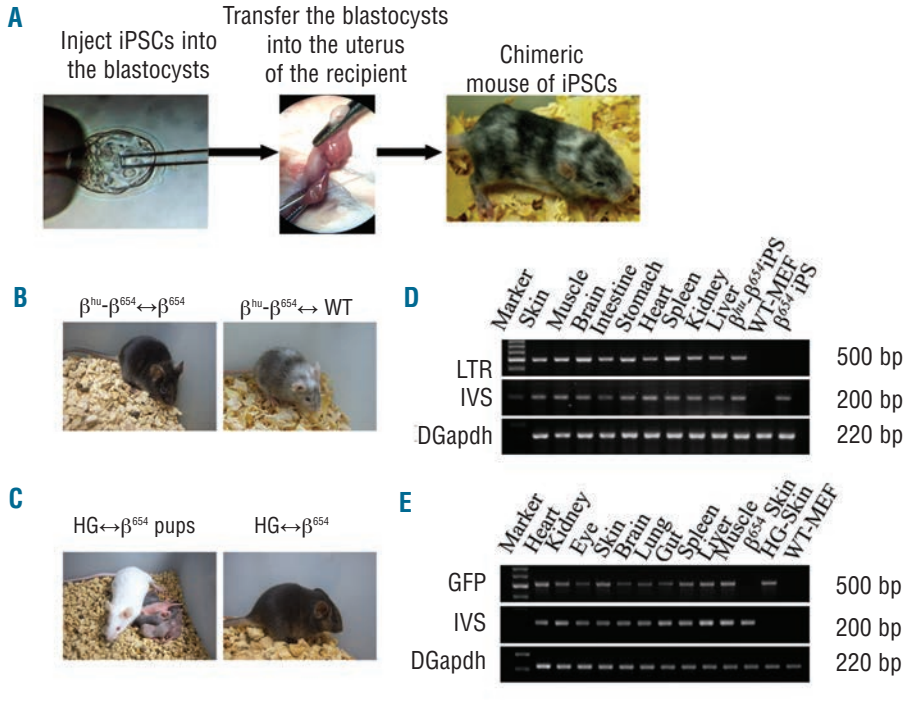

Figure 1. Generation of chimeric mice and tissue engraftment of iPSCs. (A) Steps for generating chimeras using iPSCs. After approximately 20 dpc, the chimeric pups were born. (B) Chimeric mice were generated by injecting β^{hu}-β^{654} iPSCs into the WT blastocysts (right) or β^{654}blastocysts (left). (C) HG↔β^{654}chimeric mice were derived from the HG-iPSCs and β^{654} blastocysts. *HG*↔β^{654} pups on the left and *HG*↔β^{654} mice on the right. (D) Genomic DNA from various tissues of β^{hu}-β^{654}↔β^{654} mice was assayed for the presence of transgenic hBG (LTR) by PCR. (E) Genomic DNA from various tissues of *HG*↔β^{654} mice was assayed for the presence of *GFP* and IVS by PCR.

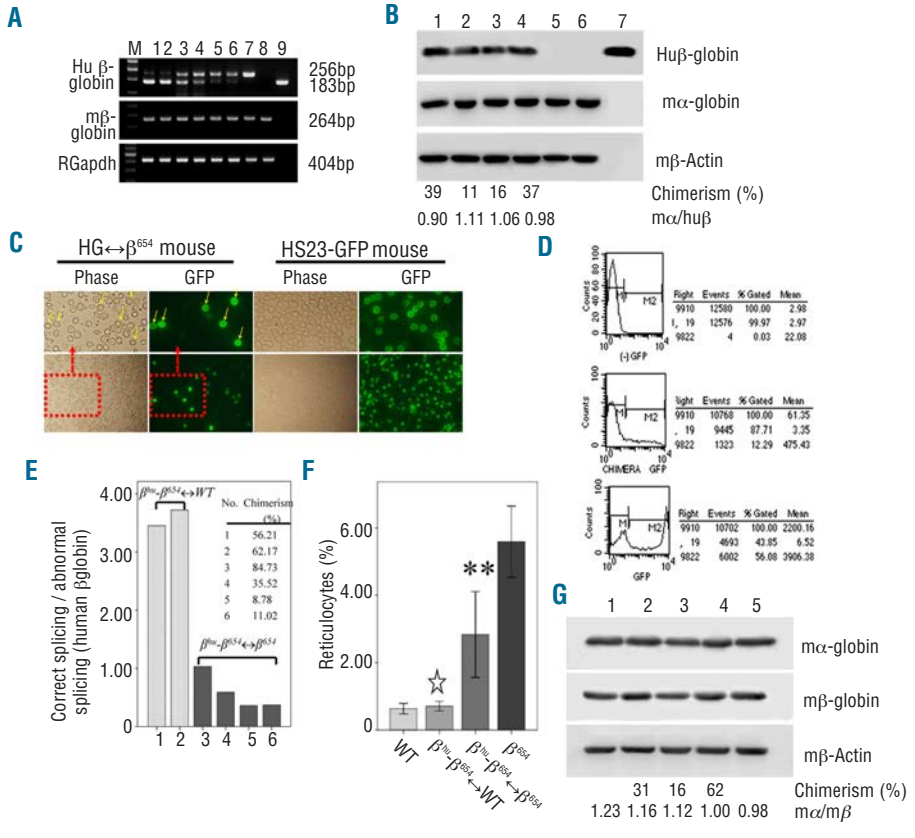

Figure 2. Differentiation of stem cells into hematopoietic cells expressing β-globin. (A) Human β-globin and murine β-globin RNA was detected in blood cells by RT-PCR assays. 183bp band: correct splicing; 256bp band: abnormal splicing. 1-2: β^{hu}-β^{654}↔WT mice; 3-6: β^{hu}-β^{654}↔β^{654} mice with different chimerism; 7: β^{654} mouse; 8: WT mouse; 9: human. (B) Western blot analysis of human β-globin and murine α-globin expression. 1: β^{hu}-β^{654}↔WT mouse; 2-4: β^{hu}-β^{654}↔β^{654} mice with different chimerism; 5: β^{654} mouse; 6: WT mouse; 7: human β–globin. (C) *HG*-iPSC-derivative cells with erythrocyte morphology *in vivo* (yellow arrows). (D) The percentage of erythrocytes expressing *GFP* was determined by flow cytometry analysis. (-)GFP: WT mouse, CHIMERA: HG↔β^{654} mouse, GFP: *HG* transgenic mouse. (E) The ration of correct splicing globin and abnormal splicing globin varied with different levels of chimerism. 1-6 samples were the same as (A). (F) The percentage of reticulocytes was determined by counting stained cells and total cells in the blood smears. (☆ difference from WT mice $P>0.05$; ** difference from β^{654} mice $P<0.01$). WT: ICR mice, n=24; β^{hu}-β^{654}↔WT mice, n=24; β^{hu}-β^{654}↔β^{654} mice, n=12; β^{654} mice, n=23. (G) Western blot analysis of murine α-globin and β-globin expression. 1-5: β^{654}, β^{hu}-β^{654}↔β^{654}, *HG*↔β^{654}, β^{hu}-β^{654}↔WT and WT mice in sequence.

Various mice with different levels of chimerism were selected to monitor pathological changes. The β^{hu}-β^{654}↔WT mice with high chimerism displayed the same characteristics as the WT mice in blood smears, bone marrow slides and reticulocyte numbers, whereas β^{hu}-β^{654}↔β^{654} mice with higher levels of chimerism (> 30%) showed fewer poikilocytes, target cells, and reticulocytes in their blood smears, and a smaller proportion of nucleated cells were found in their bone marrow, when compared to mice with lower levels of chimerism (<30%) (*Online Supplementary Figure S3A-C*). Thus, the hematopoietic disorder of β^{hu}-β^{654}↔β^{654} mice appeared to be improved when the chimerism reached a certain proportion (Figure 3A-C). On the other hand, disease symptoms such as fewer reticulocytes, diseased anisocytosis, poikilocytosis and number of target cells were reduced in HG↔β^{654} mice regardless of the level of chimerism (Figure 3A-C).

sTfR (soluble transferrin receptor) expression can sometimes be used to indicate levels of compensatory erythropoiesis, as its serum level rises when erythropoiesis increases in the cases of autoimmune hemolytic anemia, hereditary spherocytosis, and thalassemia.[24-29] The *sTfR* expression levels are found to be normal in β^{hu}-β^{654}↔WT mice, and decreased in β^{hu}-β^{654}↔β^{654} mice from their abnormally high level (*Online Supplementary Figure S4A*), suggesting improved erythropoiesis in the chimeras.

Histopathological changes in spleens and livers of chimeras

The histopathology of spleens in β^{654} mice displayed a significant expansion of red pulp, a dense occupation of nucleated erythroid precursors, and a relative decrease in white pulp, and the marginal zones were obscured by a large number of nucleated RBCs (Figure 3D and E). However, in the β^{hu}-β^{654}↔β^{654} mice and the HG↔β^{654} mice, the amount of red pulp was considerably decreased and the marginal zones became visible (Figure 3D and E); the state of the splenomegaly was also improved (Figure 4A-C).

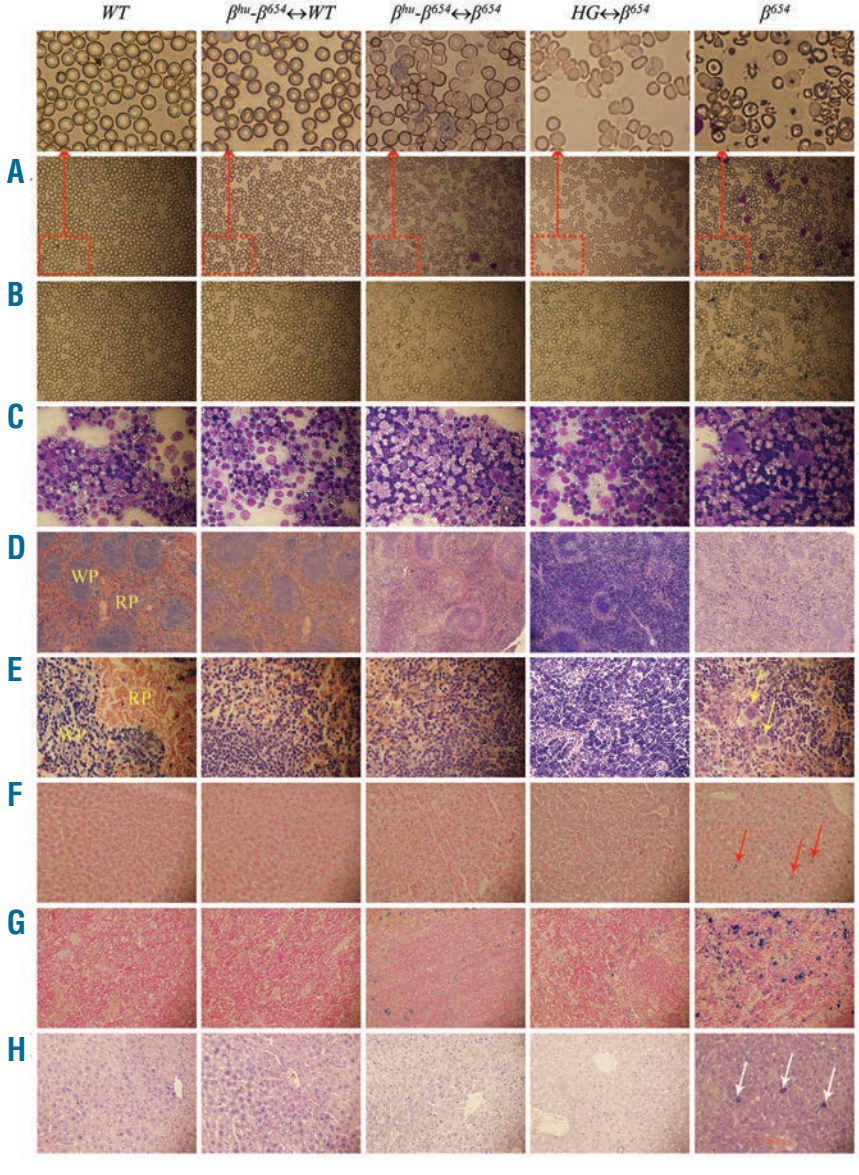

Figure 3. Tissue pathologies in chimeric mice. (A) Blood smears were stained with Wright-Giemsa (magnification 400×). (B) Blood smears were stained with brilliant tar blue for counting reticulocytes (400x). (C) Bone marrow slides were stained with Wright-Giemsa (400x). (D) The spleen sections were stained with Hematoxylin-eosin (40x). (E) The spleen sections were stained with Hematoxylin-eosin (400x). Nucleated erythroid precursors and megakaryocytes (yellow arrows) are rare in the chimeric spleens. (F) Ferrocyanide iron staining of liver samples (400x). Blue staining (red arrows) indicates iron accumulation. (G) Ferrocyanide iron staining of spleen samples (400x). (H) The liver sections were stained with Hematoxylin-eosin (400x). Almost no erythroid precursors (white arrows) were found in the sinusoids of chimeric liver. β^{hu}-β^{654}↔WT mouse, β^{hu}-β^{654}↔β^{654} mouse, and *HG*↔β^{654} mouse had 85%, 37% and 16% chimerism, respectively.

The levels of hemosiderin in the spleens and the livers of these chimeras were examined. β^{hu}-β^{654}↔β^{654} mice and HG↔β^{654} mice had less iron accumulation in their livers and spleens compared to β^{654} mice (Figure 3F and G). *Bmp6* RNA levels are known to increase with increasing levels of iron overload,[30-32] and the expression levels of *Bmp6* in β^{hu}-β^{654}↔β^{654} mice was found to decrease by approximately 67% compared to that of β^{654} mice (*Online Supplementary Figure S4B*). This also confirmed that the hemosiderin was reduced in β^{hu}-β^{654}↔β^{654} mice.

Extramedullary hematopoiesis (EMH) was examined in the chimeric models. The regression of EMH was assessed by morphological examination of spleens and livers from 10-week old chimeras. The number of nucleated erythroid precursors in red pulp was reduced (Figure 3D and E), other immature hematopoietic cells such as megakaryocytes (Figure 3E, yellow arrows) in spleens and erythroid precursors (Figure 3H, white arrow) in livers were also much reduced in the chimeras.

To further characterize changes in the level of EMH, selected relevant genes were analyzed in detail. *Twsg1, Gdf15*, and *Tfr1* are markers of ineffective erythropoiesis,[33] and these markers had higher expression in the spleens of β^{654} mice, especially *Tfr1* with 30-fold higher expression compared to WT mice, while expression levels were lower in β^{hu}-β^{654}↔β^{654} chimeric mice (Figure 4D-F). These molecular results, combined with the reduced numbers of nucleated erythroid precursors in spleens of β^{hu}-β^{654}↔β^{654} chimeric mice (Figure 3E), implied that the degree of EMH declined.

Effect of different chimerism levels on thalassemia phenotypes

The above results illustrated that anemia symptoms were ameliorated in β^{hu}-β^{654}↔β^{654} mice. To determine whether pathology reversal is influenced by the level of chimerism, β^{hu}-β^{654}↔β^{654} mice were separated into two groups, β^{hu}-β^{654}↔β^{654L} (8-16% chimerism) and β^{hu}-β^{654}↔β^{654H} (31-37% chimerism).

The β^{hu}-β^{654}↔β^{654H} group had a higher ratio of normal *versus* abnormally spliced β-globin mRNA expression compared to the β^{hu}-β^{654}↔β^{654L} group (*Online Supplementary Figure S5*). Different blood indices, including RBC, hemoglobin and reticulocytes, showed that the quantity of exogenous human β-globin in the β^{hu}-β^{654}↔β^{654L} group did not slow the progress of thalassemia effectively. When chimerism surpassed 30% in the β^{hu}-β^{654}↔β^{654H} group, the indices were improved (*Online Supplementary Figure S6A-*

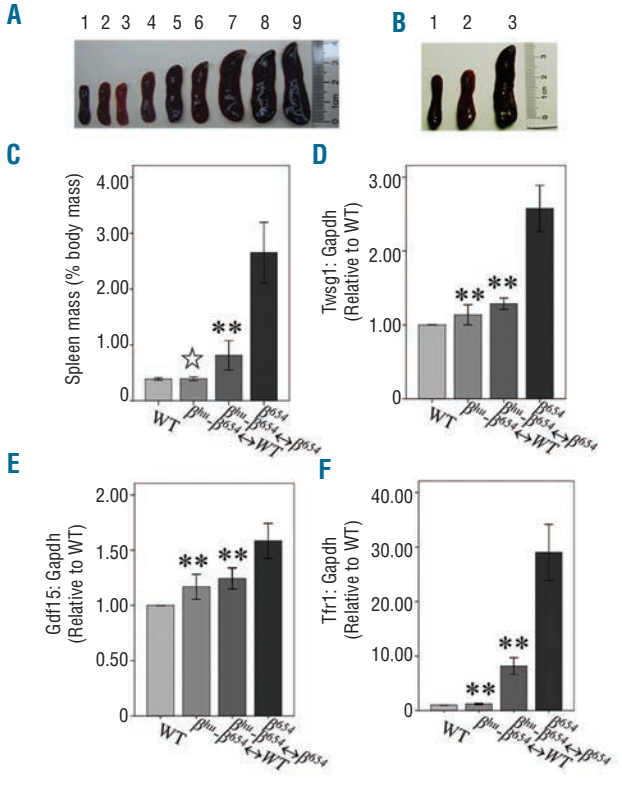

Figure 4. Analyses of abnormal hematopoiesis. (A) Spleens of β^{hu}-β^{654}↔β^{654} mice. 1: WT mouse; 2-3: β^{hu}-β^{654}↔WT mice; 4-6: β^{hu}-β^{654}↔β^{654} mice; 7-9: β^{654} mice. (B) Spleens of *HG*↔β^{654} mice. 1: WT mouse; 2: *HG*↔β^{654} mouse; 3: β^{654} mouse. (C) Spleen mass (as % body mass), ☆ difference from WT mice *P*>0.05; ** difference from β^{654} mice *P*<0.01. (D-F) *Twsg1, Gdf15* and *Tfr1* RNA levels relative to *Gapdh* levels in spleen samples were measured by RT-qPCR from WT, chimeras and β^{654} mice. **difference from β^{654} mice *P*<0.01. WT: ICR mice, n=24; β^{hu}-β^{654}↔WT mice, n=24; β^{hu}-β^{654}↔β^{654} mice, n=12; β^{654} mice, n=23.

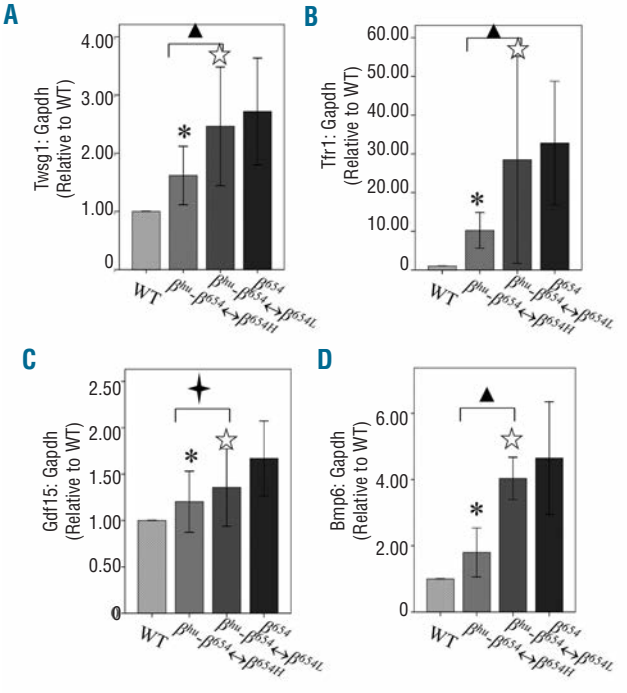

Figure 5. RNA expression markers for ineffective erythropoiesis and iron accumulation were measured by RT-qPCR. (A-C) *Twsg1*, *Tfr1* and *Gdf15* RNA levels relative to *Gapdh* levels in spleen samples. (D) *Bmp6* RNA levels relative to *Gapdh* levels in liver samples. WT: ICR mice, n=5; β^{hu}-β^{654}↔β^{654H} mice with 31-37% chimerism, n=3; $\beta^{hu}\beta^{654}$↔β^{654L} mice with 8-16% chimerism, n=3; β^{654}: β^{654} mice, n=5. (A-D: difference from β^{654} mice, **P*<0.05, ☆ *P*>0.05; β^{hu}-β^{654}↔β^{654L} *vs.* β^{hu}-β^{654}↔β^{654H}, ▲ *P*<0.05, ✦ *P*>0.05).

C), illustrating better control of hemolytic anemia than in the β^{hu}-β^{654}↔β^{654L} group. With the destruction of erythrocytes in the rich blood organs reduced in the β^{hu}-β^{654}↔β^{654H} group, lesions in related organs were controlled effectively, and splenomegaly in β^{hu}-β^{654}↔β^{654H} mice was reduced to a greater degree than in β^{hu}-β^{654}↔β^{654L} mice (P<0.05) (*Online Supplementary Figure S6D*).

The degree of EMH in β^{hu}-β^{654}↔β^{654L} and β^{hu}-β^{654}↔β^{654H} groups at the molecular level was further analyzed. *Twsg1* and *Tfr1* expression levels were lower in the β^{hu}-β^{654}↔β^{654H} group (P<0.05) (Figure 5A and B), but *Gdf15* levels showed no statistically significant differences (P>0.05) (Figure 5C). This suggests gradual improvements of EMH with increasing levels of iPSC chimerism, with highest chimerism producing full control of spleen EMH in the β^{hu}-β^{654}↔β^{654H} group. *Bmp6* was again used as a marker to determine the amount of hemosiderin in spleens. Lower *Bmp6* expression in spleens from the β^{hu}-β^{654}↔β^{654H} group compared to β^{hu}-β^{654}↔β^{654L} confirmed that iron deposition was alleviated effectively (Figure 5D) when chimerism surpassed 30%.

Collectively, these measures indicated more effective erythropoiesis and effective relief of β-thalassemia symptoms when β^{hu}-β^{654}iPSC chimerism exceeded 30% in β^{hu}-β^{654}↔β^{654} mice.

Discussion

In 2007, one year after the first successful induction of pluripotent stem cells from adult cells, Hanna *et al*. demonstrated that iPSCs could be used therapeutically in treating sickle cell anemia in a mouse model.[20] We have previously generated viable mice from iPSCs via tetraploid compensation,[34] confirming the totipotency of iPSCs. These results support the strategy of using iPSCs from normal donors, or corrected iPSCs from the patient's own differentiated cells, for potential treatment of a variety of genetic diseases.

Successful treatment of β-thalassemia based on the transplantation of genetically modified autologous hematopoietic cells using lentiviral vectors set the stage for its use in clinical applications,[35] and Cavazzano *et al*. reported one effective case in their Phase I/II clinical trial study.[36] However, several recently published reports showed that these approaches remain inefficient.[8,37-39] Therapies using iPSCs combined with a transgene (or transgenes) can potentially cure thalassemia in the future, but issues including efficacy, safety and stability of the cells must be resolved.

In current clinical practice, a state of mixed chimerism of host- and donor-derived cells is detected early after transplantation and often progresses towards complete chimerism after hematopoietic stem cell transplantation (HSCT) for thalassemia, but some patients would remain in a mixed chimeric state for a long period of time after HSCT state.[40-44] This implies that thalassemia can be relieved with appropriate doses of hematopoietic stem cells. Marco *et al*. found that most of the erythrocytes detected were derived from small proportions of donor-engrafted cells in the allogeneic HSCT patients.[44] Thus, it is important to study the dose-effect of stem cells/iPSCs in treating these diseases. As stem cells (including iPSCs) in chimeras were shown to display sustainable structural and functional integrity, our iPSC-chimeric model provides a unique platform to study the *in vivo* physiology and therapeutic effect of iPSCs following one life course.[45-47] The iPSCs used could avoid immunological rejection, and this is valuable for studying the dose-thresholds of iPSCs needed for treating thalassemia under normal physiological state without immunological suppression. In our study, we used the classic approach of gene therapy to transfer normal human β-globin into β^{654} iPSCs and then used these modified β^{hu}-β^{654} iPSCs to generate chimeras; chimeras of *HG*-iPSCs were also generated (*Online Supplementary Table S2*). In these chimeras, the hematologic parameters and the pathological phenotype of β^{654} thalassemia were improved in β^{hu}-β^{654}↔β^{654} mice and HG↔β^{654}mice, including increased RBC, Hgb, and MCHC, reduced iron deposition, alleviated splenomegaly and disappearance of EMH. The therapeutic effect was further verified at the molecular level, with three markers of ineffective erythropoiesis (*Twsg1, Gdf15* and *Tfr1*) and a marker of iron deposition (*Bmp6*), all reduced in these chimeras.

Subsequently, mice with different levels of chimerism were used to analyze the therapeutic dose-threshold of β^{hu}-β^{654}iPSCs and HG-iPSCs. In *HG*↔β^{654} mice, the effective dose of *HG* iPSCs was more than 10%, suggesting that if normal β-globin increased 22%, thalassemia could be ameliorated effectively. However, the β^{654} gene existed in β^{hu}-β^{654} iPSCs, so greater numbers of these cells were needed to treat thalassemia. What level of chimerism for β^{hu}-β^{654} iPSCs in β^{hu}-β^{654}↔β^{654} mice could effectively treat thalassemia? Our study showed that at levels less than 30%, the β^{hu}-β^{654}↔β^{654} chimeras still had the thalassemia phenotype; however, when the chimerism exceeded 30%, anemia was controlled effectively in these chimeras.

Our results showed that the pathology of thalassemia was alleviated by β^{hu}-β^{654} iPSCs and *HG*-iPSCs, and no other abnormal symptoms occurred in these chimeras. The therapeutic effect seemed stable. Although this model system might not be translated directly into human therapy, it is a very important animal model for treatment of genetic diseases. It provides vital information for the development of prenatal and postnatal iPSC therapies in these patients. Our previous work demonstrated the feasibility of generating chimera (exceeding 30% of chimerism) through intrauterine transplantation of hematopoietic stem cells.[48] This could provide a model system for prenatal therapy using iPSCs to treat thalassemia fetuses. On the other hand, effective post-natal therapy with autologous modified iPSCs may require engineered expression of normal β-globin and chimerism levels that exceed 20%.

The results of our study provide useful base-line data for developing clinical iPSC treatments for β^{654} thalassemia, the most common form of β-thalassemia in China. β-thalassemia has different genotypes, which may or may not be correlated to the degree of the disease severity.[49] For example, patients with IVSI-6 mutation of β-globin in the homozygous state have slightly increased HbF and are not transfusion dependent;[50] but Gringras *et al*. found that the patient compound heterozygous β^{0}/β^{++} with C-T substitution at nucleotide position -101 in the promoter region of the β-globin started transfusion as early as the age of four years.[49] These clinical treatments according to different types of β-thalassemia inferred that effective therapeutic dose of iPSCs should be carefully developed and studied. In the future, other types of β-thalassemia will be used for the study of effective iPSC therapies with or without genetic

modification of patients' iPSCs, and this will no doubt have a positive impact on the use of iPSCs in clinical applications.

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