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

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SUPPLEMENTARY APPENDIX

Therapeutic effects of induced pluripotent stem cells in chimeric mice with beta-Thalassemia

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Online Supplementary Methods

Animals

The β^{654} thalassemia mice (β^{654} mice) were obtained from the Jackson Laboratory. These heterozygotic mice carry one wild-type murine β-major globin allele and one defective human βIVS-2-654 allele associated with aberrant splicing due to C→T substitution at nucleotide 654 of intron 2, characterized by a moderate form of β-thalassemia (anemia, splenomegaly, abnormal hematologic indices)[1].

Transgenic *HG* mice were generated by our laboratory using Kunmin white mice transduced with a construct of erythroid expressing *GFP*[2]. The offspring of a transgenic male mouse mated with a C57 female mouse were used as the source of fibroblasts; the fur of this offspring was gray, thus the iPSCs derived from the offspring's tip tail fibroblasts encode pigmented fur, and allow us to easily distinguish the chimeras by coat color.

The wild type (*WT*) ICR mice were purchased from Shanghai Slaccas, and were

used for blastocyst collection and as foster mothers.

The mouse cage room lighting was kept on a 14-hour light, 10-hour dark cycle. All animal experiments were approved by the Review Board of Shanghai Children's Hospital.

Tail tip fibroblasts (TTF) culture

The tail tips (about 1cm) of a three week-old β^{654} mouse and a *HG* mouse were cut in fine pieces and rinsed with PBS (GIBCO 15140, CA, USA) containing 2% Pen/Strep (GIBCO 15140, CA, USA), followed by incubation in FP medium changed every 2 days. After incubation for about 10 days, fibroblasts were subcultured at ratio of 1:3. FP medium: 15% FBS (HyClone SH30084.03, Victoria, Australia), 1% Pen/Strep (GIBCO 15140, CA, USA), DMEM (GIBCO 11965, CA, USA).

iPSCs induction

According to the protocol we previously established[3], TTF of a β^{654} mouse and a *HG* mouse were transfected using the 4 reprogramming factors in pMXs retroviral vectors, then changed to iPSCs culture medium after 2 days, and passaged as iPSCs until obvious clones formed (*Online Supplementary Figure S1A*). iPSCs culture medium included 15% FCS (PAA A15-108, CA, USA), 1% non-essential amino acid (GIBCO 11140, CA, USA), 1% Pen/Strep (GIBCO 15140, CA, USA), 4mM L-glutamine (GIBCO 25030, CA, USA), 0.1mM β-mercaptoethanol (GIBCO 0091, CA, USA), 1000U/ml LIF (Millipore ESG1107, Billerica, MA, USA) and DMEM (GIBCO 11965, CA, USA).

hBG transfected β^{654} iPSCs

The hBG vector[4] (Online Supplementary Figure S2A) with its two conventional helpers, ΔR8.9 and VSVG were co-transfected into 293T cells. Then the viral supernatants were collected at 72 hours. β^{654} iPSCs were then transfected by hBG pseudovirus overnight. After being cultured for 3 days, single iPS clones were picked. PCR was performed with LTR primer pairs to choose the hBG transduced β^{654} iPS cell (β^{hu}-β^{654} iPSCs) lines.

Copy number quantification of exogenous human β-globin gene

Quantitative real-time PCR was performed to determine the transgenic copy number with primer pairs for LTR. *Gapdh* was used as internal control. The copy number of reference standard was set as one. Copy numbers of the exogenous human β-globin were analyzed according to Kindich et al[5].

Production of chimeric mice

15-20 β^{hu}-β^{654} iPSCs or *HG*-iPSCs were injected into the *WT* blastocysts or β^{654} blastocysts (*WT*♂×*WT*♀ or β^{654}♂×*WT*♀). The injected blastocysts were cultured until blastocoeles were formed in a 40-ul droplet of KSOM+AA (Millipore, MR-107-D, Billerica, MA, USA) covered with mineral oil in a humidified atmosphere of 5% CO_2 at 37°C. The blastocysts were then transferred into the uteri of 2.5-dpc pseudo pregnant *WT* female mice. After about 17 days, chimeric pups were born. All procedures followed the protocol of Nagy[6].

Genotyping of the β^{654}iPSCs and chimeras

Genomic DNA of the chimeric pups was isolated from the tail tip tissues after weaning. PCR was performed to identify the allele of human β^{654} with IVS primer pairs, *Gapdh* as the internal control gene, using 2× PCR Master Mix kit (TIANGEN KT201-01, Beijing, China). *GFP* was detected with *GFP* primer pair, the allele of human $\beta^{IVS\text{-}2\text{-}654}$ was detected with IVS primer pair. PCR was performed for 32 cycles, with denaturing at 94ºC for 45 sec, annealing at 60ºC for 45 sec, and extension at 72ºC for 60 sec. All primers used in this study can be found in *Online Supplementary Table S1*.

Analysis of chimerism

To determine the levels of chimerism for β^{hu}-β^{654} iPSCs in recipient mice, real time PCR was performed with SYBR Premix (Takara DRR041, Japan, Dalian, China) using primer pairs for *Q-LTR* (a sequence that only exists in the β^{hu}-β^{654} iPSCs and not in the β^{654} mice or *WT* mice). *Q-Gapdh* was used as internal control. Real time PCR were performed as follows: 95ºC for 30 sec, then 40 cycles of a two-step protocol with 94ºC for 5 sec followed by 60ºC for 30 sec. The ΔCt data were collected and calculated by the software in the AB 2900HT fluorescence ratio PCR instrument.

The levels of chimerism of *HG*-iPSCs was determined as above, using primer pairs for Q-*GFP;* and the *β-actin* gene was used for internal control.

Flow cytometry analysis

Peripheral blood of *HG↔WT* mice was collected in heparinized microhematocrit tubes. Samples were washed 3 times with PBS, and flow cytometry analysis was performed by BD FACS Calibur (San Diego, CA, USA).

Effect of human β-globin expression by RT-PCR

Total RNA was extracted from the fresh peripheral blood of chimeric mice for RT-PCR. The spliced human *β*-globin transcripts were amplified with Hu*β* primer pairs for 30 cycles, denatured at 94^{o}C for 45 sec, and annealed at 60^{o}C for 45 sec, followed by extension at 72^{o}C for 60 sec.

Western blot

Fresh peripheral blood from mouse eye vein was collected and lysed. Proteins were separated by 12% SDS-PAGE and then transferred to polyvinylidene fluoride (PVDF, Sigma P7794, St. Louis, MO, USA) membrane using electronic transfer apparatus. Human blood was used as a positive control. Primary human *β*-globin (Hb) monoclonal antibody (Santa Cruz sc-130320, CA, USA), murine hemoglobin α ploYclonal antibody (Proteintech 14537-1-AP, Chicago, USA), or murine hemoglobin *β* ploYclonal antibody (Santa Cruz sc-31116, CA, USA) were used to specifically detect different kinds of globin. The antibody was hybridized at 4^{o}C overnight. The secondary hybridization was performed using peroxidase-conjugated goat

anti-mouse IgG (Santa Cruz sc-3791, CA, USA) at room temperature for 1 hour; Hb bands were visualized by DAB staining. The mean gray values of western blots were determined by Image J software.

Hematologic analysis

Mouse peripheral blood smears and bone marrow slides were stained with Wright-Giemsa (BASO, BA4017, Zhuhai, China). Whole blood samples from mice were collected starting from 6 weeks of age. The RBC count, hemoglobin (Hgb), hematocrit (Hct), mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) for each sample were determined using the Hematology Analyzer (KX-21, Sysmex, Japan) equipped with software to analyze murine cells, see Table 1. Reticulocyte counts were performed using brilliant tar blue stain (BASO BA4003, Zhuhai, China).

Histopathology analysis of chimeric mice

Chimeric mice and age-matched controls (> 8 weeks 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 (BASO BA4025, Zhuhai, China); liver and heart tissue sections were also stained with Pearl's Prussian blue (BASO BA4089B, Zhuhai, China) to investigate the iron accumulation. Bone marrow slides were stained with Wright-Giemsa (BASO BA4017, Zhuhai, China).

Quantitative PCR

Quantitative PCR was used to analyze molecular phenotypes of β^{654} thalassemia, i.e. iron accumulation and extramededullary hematopoiesis. Total RNA was extracted from livers and spleens. Primers for *Tfr1, Twsg1,* and *Gdf15* were used to analyze the extramedullary hematopoiesis in spleens. *Bmp6* primers were used to evaluate the iron accumulation in livers. Two-stage real time PCR was performed to determine the level of iron accumulation and extramedullary hematopoiesis. Step 1, pre-denaturing: 95°C for 30 sec; Step 2 for 40 cycles: 94°C for 5 sec, then 60°C for 30 sec.

ELISA analysis

Blood samples were collected, centrifuged at 2,000 rpm for 10 min, and 40 ul serum was aspirated for *sTfR* ELISA analysis using *sTfR* ELISA kit (AMEKO, Shanghai, China).

Statistical analysis

The $\chi 2$ test was used to determine the significance of difference in *Online Supplementary Table S2*, and one-way ANOVA was used to analyze the data in Table1 and the quantitative PCR data. Analytical data were processed using SPSS 17.0 software.

The sequences of all PCR primers described below and used for this study are

provided in *Online Supplementary Table S1*.

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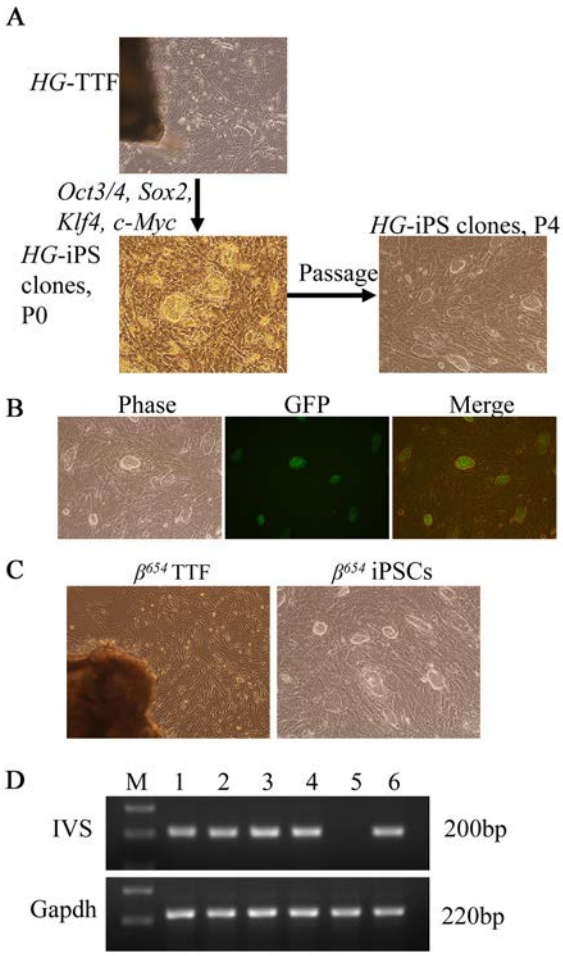
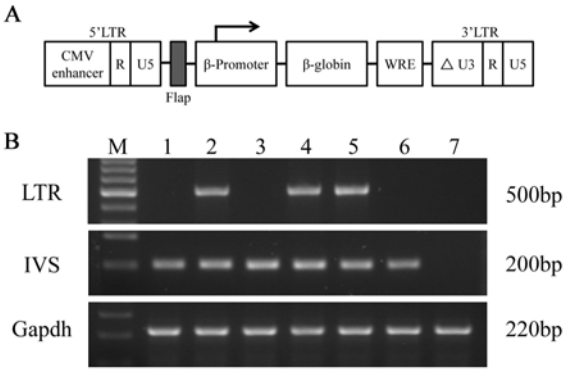
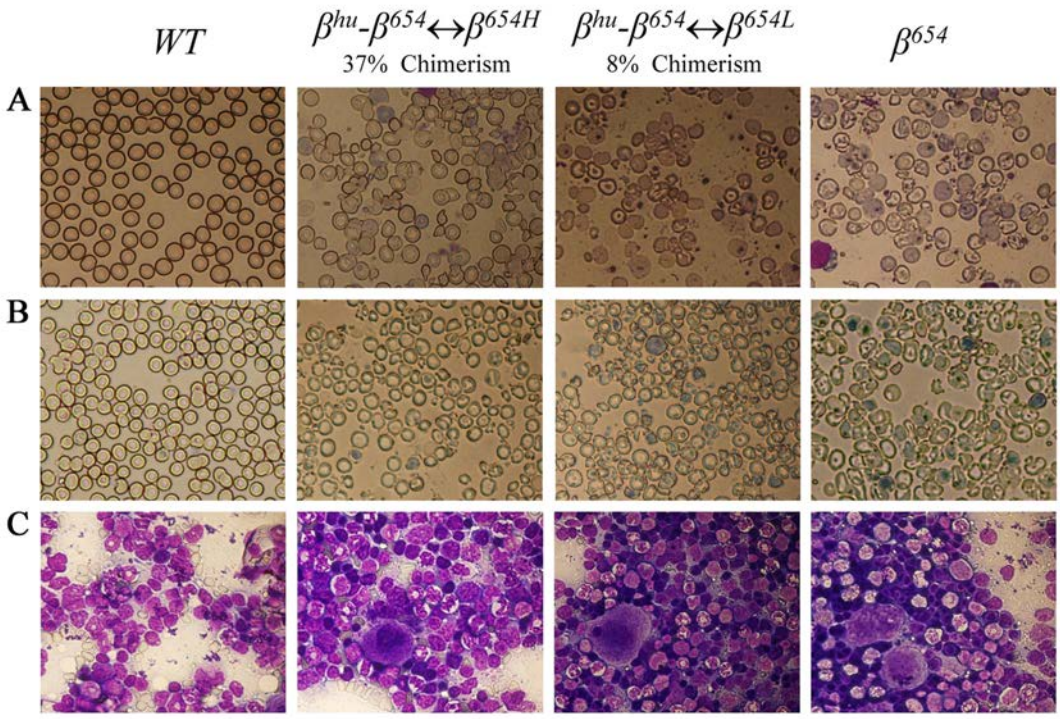


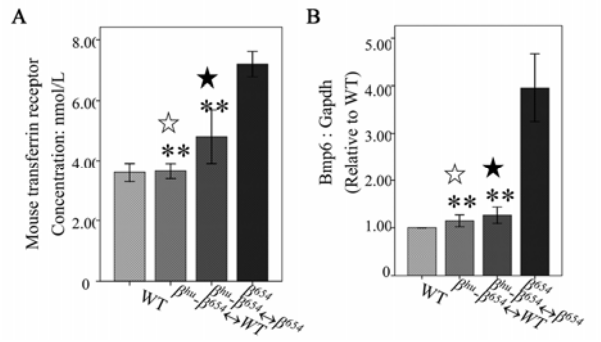
Online Supplementary Figure S1. Production of *HG*-iPSCs and β^{654} iPSCs. (A) The primary tail tip fibroblasts (TTF) of an *HG* mouse were used to generate *HG*-iPSCs using the method previously described[3]. (B) *GFP* was expressed in *HG*-iPSCs. (C) β^{654} iPSCs derived from the TTF of β^{654} thalassemia mouse. The left panel is β^{654} TTF, the right panel is β^{654} iPS clones. (D) PCR results from genomic DNA determined the genotype of β^{654} iPSCs. 1-4: β^{654} iPS cell lines; 5: *WT* MEF; 6: β^{654} TTF.



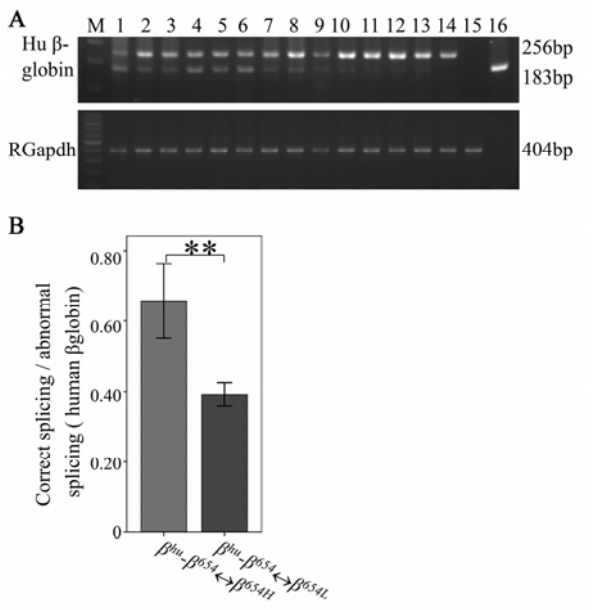
Online Supplementary Figure S2. Diagram of the human *β*–globin lentiviral vector (hBG) and identification of transduced β^{654} iPS cell lines. (A) The lentiviral vector is designed to express normal human *β*-globin with physiological regulation of transcription. *β*-Promoter: human *β*-globin promoter. *β*-globin: human *β*-globin gene. WRE: woodchuck hepatitis virus posttranscriptional regulatory element; ΔU3 denotes a deletion in the U3 region of the 3' long terminal repeat (LTR) that renders the 5' LTR of the integrated provirus transcriptionally inactive. (B) PCR assays of β^{654} iPSCs' genomic DNA to detect vector LTR and human *β*-globin gene intervening sequence (IVS). 1-5: 5 β^{654} iPS cell lines (2, 4 and 5 are β^{hu}-β^{654} iPS cell lines), 6: β^{654} TTF, 7: *WT* MEF.



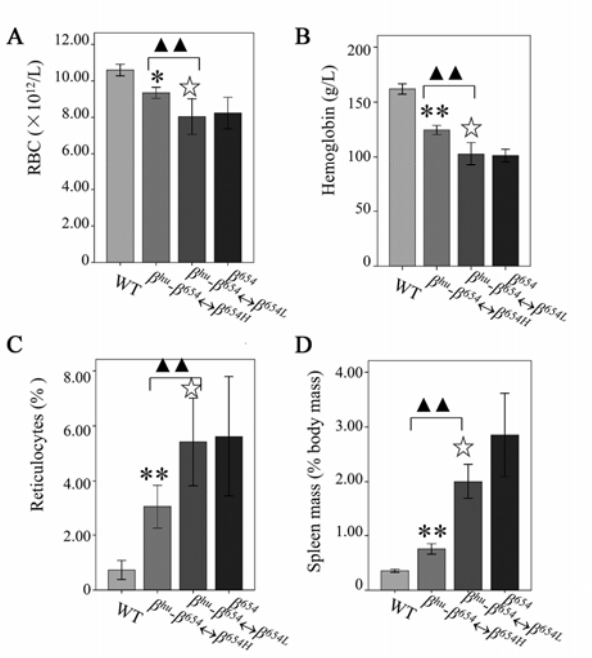
Online Supplementary Figure S3. Hemograms and bone marrow smears for animals with different levels of chimerism. (A) Blood smears were stained with Wright-Giemsa (magnification 1000×). (B) Blood smears were stained with brilliant tar blue for counting reticulocytes (1000×). (C) Bone marrow slides were stained with Wright-Giemsa (1000×).



Online Supplementary Figure S4. Altered iron accumulation in chimeras. (A) Mouse serum soluble transferrin receptor concentration was measured by ELISA assay. (B) *Bmp6* RNA levels relative to *Gapdh* levels in liver samples were measured by RT-qPCR. (☆difference from *WT* mice P>0.05; ★difference from *WT* mice P<0.01; **difference from β^{654} mice P<0.01). *WT* 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.



Online Supplementary Figure S5. The ratio of normal globin and abnormally spliced globin in β^{hu}-β^{654}↔β^{654} mice. (A) Human β-globin RNA was detected in blood cells by RT-PCR assays. 1-13: β^{hu}-β^{654}↔β^{654} mice; 1: chimerim=72%; 2-8: β^{hu}-β^{654}↔β^{654H} , chimerim 31-37%; 9-13: β^{hu}-β^{654}↔β^{654L}, chimerim 8-16%; 14: β^{654} mouse; 15: *WT* mouse; 16: human. 183bp band: correct splicing; 256bp band: abnormal splicing. (B) The ratio of correctly to abnormally spliced globin is significantly different between the β^{hu}-β^{654}↔β^{654L} group and the β^{hu}-β^{654}↔β^{654H} group (** P<0.01).



Online Supplementary Figure S6. Pathological parameters in β^{hu}-β^{654}↔β^{654} mice with different levels of chimerism. (A) RBC counts; (B) hemoglobin levels; (C) reticulocytes (% of blood cell population) and (D) Spleen mass (as % body mass) were measured about 10 weeks after birth. *WT*: ICR mice, n=10; β^{hu}-β^{654}↔β^{654H} mice with 31-37% chimerism, n=7; β^{hu}-β^{654}↔β^{654L} mice with 8-16% chimerism, n=5; β^{654}: β^{654} mice, n=10. (A-D: difference from β^{654} mice **P<0.01, *P<0.05, ☆P>0.05; β^{hu}-β^{654}↔β^{654L} vs. β^{hu}-β^{654}↔β^{654H}, ▲▲P<0.01)

Online Supplementary Table S1. PCR primers used in the study.

Primer ID	Sequence	PCR product length
Gapdh-F	5'-TGGGCAAGGTCATCCCAGAGC-3'	400bp
Gapdh-R	5'-CTTGCTCAGTGTCCTTGCTGGGGT-3'	(DNA PCR)
Q-LTR-F	5'- AGACCAGATCTGAGCCTGGGAGC-3'	109bp
Q-LTR-R	5'-ACACAACAGACGGGCACACACT-3'	
Q-Gapdh-F	5'- AATGGAGGGAACCCTGGAACAGA-3'	220bp
Q-Gapdh-R	5'- TACCCAGCCGTGCTACTGACA-3'	
Gdf15-F	5'- AGCCGAGAGGACTCGAACTCAG-3'	106bp
Gdf15-R	5'- GGTTGACGCGGAGTAGCAGCT-3'	
Tfr1-F	5'- GGAAGACTCTGCTTTGCAGCTAT-3'	72bp
Tfr1-R	5'- GCCCAGGTAGCCACTCATGA-3'	
Twsg1-F	5'- TTACAGCGACACCCCGCCCA-3'	238bp
Twsg1-R	5'- GGGGAAGGGGGCGTGGACAT-3'	
Bmp6-F	5'- GCGGTGACGGCTGCTGAGTT-3'	281bp
Bmp6-R	5'-GCACGGGGGTTGACGTGGAG-3'	
LTR-F	5'-TGCTAGAGATTTTCCACACTG-3'	500bp
LTR-R	5'-TGGAAGGGCTAATTCACTC-3'	
IVS-F	5'-CCTTTGGGGATCTGTCCACTCCTGA-3'	200bp
IVS-R	5'-AGCGTCCCATAGACTCACCCTGA-3'	
mHba-F	5'- GGGTCACGGCAAGAAGGT-3'	233bp
mHba-R	5'- TGCTCACAGAGGCAAGGAAT-3'	
mHbb-F	5'- TGTGGGGAAAGGTGAACTCC-3'	264bp
mHbb-R	5'- TCTCAGGATCCACATGCAGC-3'	
Huβ-F	5'-CTCGGTGCCTTTAGTGATGG-3'	183bp correct
Huβ-R	5'-AGCCTGCACTGGTGGGGTGAA-3'	256bp abnormal
R-Gapdh-F	5'- GGCTGTGGGCAAGGTCATCCC-3'	404bp
R-Gapdh-R	5'-TTGCTCAGTGTCCTTGCTGGGG-3'	
GFP-F	5'-TGACCTACGGCGTGCAGTGCTT-3'	500bp
GFP-R	5'-TCGTCCATGCCGAGAGTGATCC-3'	DNA
Q-GFP-F	5'-GTCCGCCCTGAGCAAAGA-3'	54bp
Q-GFP-R	5'-TCCAGCAGGACCATGTGATC-3'	(DNA PCR)
β-Actin-F	5'-GTCACGCACGATTTCCCTCT-3'	56bp
β-Actin-R	5'-TGACCGAGCGTGGCTACA-3'	(DNA PCR)

Q prefix: used for quantitative PCR; R prefix: used for reverse transcription PCR; F suffix: forward member of primer pair; R suffix: reverse member of primer pair.

Online Supplementary Table S2. Generation of chimeric mice.

iPSCs	Strain	Receptor	Number of Chimeric embryostransferred	Total pups	Stillborn	Alive	Chimeric mice (%)
β^{hu}-β^{654}	C57BL/6J×	WT♂×WT♀	234	58	7	51	32 (62.7%) *
	DBA/2	β^{654}♂×WT♀	810	172	58	114	60[a] (52.6%) **
HG	WT×DBA2	WT♂×WT♀	108	40	6	34	18 (52.9%)
		β^{654}♂×WT♀	381	117	23	94	52[b] (55.3%)

a :Of 60 total chimeras, only 28 were β^{hu}-β^{654}↔β^{654} mice, and the other 32 chimeras were not derived from β^{654} blastocysts. b: Of 52 total chimeras, only 23 were *HG*↔β^{654} mice, and the other 29 chimeras were not derived from β^{654} blastocysts. * difference from *HG*-iPSCs, $P<0.01$; ** difference from *HG*-iPSCs, $P>0.05$