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Ancestral β-globin gene haplotypes modify β-thalassemia severity in a mouse model

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Abstract:

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ABSTRACT

β-Thalassemia is a prevalent anemia caused by mutations in the *HBB* (β-globin) gene. We show that the severity of β-thalassemia in the frequently studied $Hbb^{th3/+}$ mouse model is influenced by ancestral β-globin gene (*Hbb*) haplotypes that differ in common strains.

TEXT

Mutations in the *HBB* gene cause β-thalassemia by inhibiting the production of the β-globin subunit of adult hemoglobin (HbA, $\alpha 2\beta 2$)[1]. Consequently, excess free α-globin forms cytotoxic precipitates that cause maturation delay and apoptosis of erythroid precursors (ineffective erythropoiesis, IE) and shortened lifespan of circulating red blood cells (RBC) (hemolysis). The severity of β-thalassemia varies according to the causal *HBB* mutation and modifier alleles that influence the ratio of α-globin to β-like globins (mainly β- and γ-), which form HbA or fetal hemoglobin (HbF, $\alpha 2\gamma 2$), respectively. Patients with transfusion-dependent β-thalassemia (TDT), also called β-thalassemia major, express little or no β-like globin and require regular red blood cell (RBC) transfusions. Patients with non-transfusion-dependent (NTD) β-thalassemia, also called β-thalassemia intermedia, express residual β-globin and/or γ-globin and require transfusions sporadically. Both TDT and NTD β-thalassemia are common disorders that cause substantial morbidity and premature mortality.

Mouse models for β-thalassemia are helpful for elucidating disease pathophysiology and developing new therapies[2]. These models were created by generating targeted mutations in the mouse β-globin (*Hbb*) genes. While humans have a single *HBB* gene on each chromosome, mice express β-globin from two tandem, ancestrally duplicated *Hbb* genes[3-5]. Common inbred strains of mice used for laboratory research harbor one of two different *Hbb* haplotypes, termed single and diffuse (supplemental Figure 1A). The *Hbb* single haplotype, normally found in

C57BL/6 mice, contains *Hbb-bs* and *Hbb-bt*, which encode identical β-globin proteins. The diffuse haplotype, found in 129 and BALB/c strains, contains *Hbb-b1* and *Hbb-b2,* which encode proteins that differ from each other and from β-globin single (supplemental Figure 1B-C). The *Hbb-b1* and *Hbb-b2* genes are also referred to as *Hbb-major* (β^{maj}*)* and *Hbb-minor* (β^{min}*)* because they generate approximately 80% and 20% of RBC β-globin protein, respectively[6].

The commonly studied $Hbb^{th3/+}$ mouse harbors a heterozygous 20 kb deletion that eliminates both *Hbb* genes in cis (supplemental Figure 1A), resulting in β-globin haploinsufficiency with a phenotype that resembles human NTD β-thalassemia[7]. Commercially available $Hbb^{th3/+}$ mice are hybrids of C57BL/6 and 129 strains, which harbor *Hbb* single and diffuse haplotypes, respectively (Jackson Laboratories #003253; B6;129-Hbb-b1tm1Unc Hbb-b2tm1Unc/J). Thus, this β-thalassemic strain is hemizygous for a wild-type *Hbb* haplotype, which can be single or diffuse. Considering that the severity of α-thalassemia and normal RBC traits in mice are both influenced by these *Hbb* haplotypes[8,9], we tested their effects on β-thalassemic phenotypes by generating C57BL/6 $Hbb^{th3/+}$ strains that were congenic for hemizygous *Hbb* single or diffuse haplotypes (supplemental Methods and supplemental Figure 1A).

Compared to $Hbb^{th3/+}$ single mice, $Hbb^{th3/+}$ diffuse mice exhibited improved RBC indices, including a 15% increase in RBC count ($P < 0.0001$), a 14% increase in hematocrit, a 30% reduction in reticulocyte count ($P < 0.0001$) and a 17% reduction in the red cell distribution width (RDW, $P < 0.0001$) (Figure 1A, supplemental Table 1). In contrast, these indices were not significantly different between wild-type ($Hbb^{+/+}$) congenic C57BL/6 mice with *Hbb* single or diffuse haplotypes (supplemental Table 1). Biotin labeling studies revealed circulating RBC half-lives of 5.9 days for $Hbb^{th3/+}$ single mice and 9.9 days for $Hbb^{th3/+}$ diffuse mice, compared to approximately 20 days for wild-type $Hbb^{+/+}$ mice that were homozygous for either haplotype

(Figure 1B). Compared to *Hbb*$^{th3/+}$ single mice, *Hbb*$^{th3/+}$ diffuse mice exhibited reduced spleen-to-body weight ratios (Figure 1C), improved maturation of erythroid precursors (Figure 1D, supplemental Figure 2A) and reduced erythroid hyperplasia in spleen and bone marrow (Figure 1E, supplemental Figure 2B-C, supplemental Table 2). Thus, the *Hbb* diffuse haplotype is associated with reductions in hemolysis and ineffective erythropoiesis in *Hbb*$^{th3/+}$ mice compared to *Hbb*$^{th3/+}$ mice with *Hbb* single haplotype.

Biochemical fractionation followed by triton acid urea gel electrophoresis revealed a 60% increase of insoluble α-globin in RBC lysates from *Hbb*$^{th3/+}$ single mice compared to *Hbb*$^{th3/+}$ diffuse mice ($P < 0.001$) (Figure 2A). Similarly, transmission electron microscopy with quantitative image analysis revealed a 100% increase of α-globin precipitates in reticulocytes from *Hbb*$^{th3/+}$ single mice compared to *Hbb*$^{th3/+}$ diffuse mice ($P < 0.01$) (Figure 2B). These findings indicate that the β-globin to α-globin imbalance in *Hbb*$^{th3/+}$ mice is increased with the *Hbb* single haplotype compared to the *Hbb* diffuse haplotype. This imbalance could result from reduced stability of the β-globin single protein or a reduction in its expression compared to β-globin diffuse.

All mouse β-globins contain a conserved cysteine at position β93, similar to human β-globin protein. Diffuse β-globins also contain a reactive cysteine at β13, which may protect against hemoglobin oxidation and denaturation under oxidizing conditions, such as β-thalassemia[10]. Additionally, the *Hbb* genes are expressed at slightly higher levels in mouse strains harboring the diffuse haplotype compared to those with *Hbb* single[9]. In agreement, RNA-sequencing (RNA-seq) analysis revealed greater *Hbb/Hba* mRNA ratios in Ter119^{+} erythroblasts from the spleen or bone marrow of wild-type (*Hbb*$^{+/+}$) and β-thalassemic (*Hbb*$^{th3/+}$) mice with the diffuse *Hbb* haplotype, as compared to those with *Hbb* single (Figure 2C). Similarly, *Hbb/Hba* mRNA

ratios were higher in flow cytometry-purified, developmental stage-matched EryB ($Ter119^{+}/CD71^{high}/FSC^{low}$) erythroblasts and circulating reticulocytes from $Hbb^{th3/+}$ diffuse mice compared to those from $Hbb^{th3/+}$ single mice (Figure 2D). We detected similar differences in the ratios of intron-containing RNAs (Figure 2E), suggesting that *Hbb* single genes are transcribed at lower rates than *Hbb* diffuse genes.

In summary, we generated and compared C57BL/6 β-thalassemic $Hbb^{th3/+}$ mice that were congenic for hemizygous *Hbb* single or diffuse haplotypes and showed that these are genetic modifiers of β-thalassemia. The *Hbb* single haplotype, normally present in the C57BL/6 strain, is associated with more severe β-thalassemia, at least in part due to its reduced mRNA expression, resulting in increased levels of free α-globin. Additionally, cysteine at position 14 of diffuse β-globins, which is not present in single β-globin (supplemental Figure 1B), may confer protection against the denaturing effects of β-thalassemia-associated oxidant stress[10]. Although humans do not harbor homologs for mouse *Hbb* single and diffuse haplotypes, our findings are consistent with the general concept that genetic variants, both linked and unlinked to the *HBB* locus, modify the severity of β-thalassemia[11,12].

From a practical perspective, commercially available $Hbb^{th3/+}$ β-thalassemic mice are of mixed C57BL/6 and 129 backgrounds and therefore hemizygous for either the *Hbb* single or diffuse haplotype, with reduced hemolysis and ineffective erythropoiesis occurring with the latter. Because this variability is likely to complicate the interpretation of experimental perturbations, most studies will be improved by utilizing $Hbb^{th3/+}$ mice with the same *Hbb* haplotype. Either haplotype may have distinct advantages or disadvantages, depending on experimental design and objectives. For example, *Hbb* single is associated with more severe β-thalassemia, which may enhance the sensitivity for testing new drugs or other therapeutic interventions in adult

animals. On the other hand, the single haplotype is more likely to reduce the perinatal survival of *Hbb*$^{th3/+}$ mice that harbor deleterious modifier alleles introduced during genetic screens (see reference 13, for example). Regardless of which *Hbb* haplotype is present, it is most important to control for this variable during studies of β-thalassemic mice.

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Authorship Contributions

C.L., J.K., E.K. and M.J.W conceived the project and designed and interpreted experiments. C.L, J.K., A.G.F, T.M. X.Q. and H-M.H. performed experiments. L.P. analyzed RNA-seq data. K.M., and R.T. assisted with animal breeding. H.S. performed histopathological analysis. C.L., J.K., H-M.H., and M.J.W., wrote the manuscript.

Conflict-of-interest disclosures

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Figure legends

Figure 1. Reduced hemolysis and ineffective erythropoiesis in *Hbb*$^{th3/+}$ mice with the *Hbb* diffuse haplotype. (**A**) Erythroid indices (y-axis) according to genotype (x-axis) in 8 week-old mice. *Hbb*$^{th3/+}$ single (sing), n = 15; *Hbb*$^{th3/+}$ diffuse (diff), n = 15. RBC, red blood cell number; Hb, hemoglobin; Retic, reticulocyte; RDW, red cell distribution width. (**B**) Circulating RBC survival determined after biotin labeling. Graph shows mean values ± SD for 7 mice each with the indicated genotypes. Calculated RBC half-life in days (d) are shown in parentheses. Differences between *Hbb*$^{th3/+}$ sing and *Hbb*$^{th3/+}$ diff mice were significant at all time points between days 2 and 14, with a false discovery rate of 0.05 by the Benjamini and Hochberg method. (**C**) Representative spleens from mice of the indicated genotypes. The scale bar represents 1 cm. Graph on right shows spleen-to-body weight ratios. n = 10 mice for each genotype. **(D)** Developmental stage distribution of erythroblasts in spleen (left panel) and bone marrow (right panel) determined by flow cytometry for surface antigens and forward scatter (FSC). Ery.A (Ter119^{high}CD71^{high}FSC^{high}), Ery.B (Ter119^{high}CD71^{high}FSC^{low}) and Ery.C (Ter119^{high}CD71^{low}FSC^{low}) represent increasingly mature erythroblast populations[14]. Representative flow cytometry plots are shown in supplemental Figure 2A. *Hbb*$^{th3/+}$ sing, n = 5 or 6; *Hbb*$^{th3/+}$ diff, n = 5 or 6. **(E)** Semiquantitative evaluation of splenic and bone marrow erythropoiesis in hematoxylin and eosin (H&E) stained sections (supplemental Figure 2B) assigned by a blinded, board-certified veterinary pathologist (H.S.). Graph shows the levels of erythroid precursors on a 6-point scale (arbitrary units), with 0 being the lowest. n = 6-8 mice for each genotype. All graphs show data as mean value ± SD. Bar graphs were analyzed by 2-sample Student's *t*-test. ****P< 0.0001; ***P* < 0.01; **P* < 0.05; ns: not significant.

Figure 2. Reduced free α-globin in *Hbb*$^{th3/+}$ mice with the *Hbb* diffuse haplotype. (**A**) Soluble and insoluble globins in RBCs determined by biochemical fractionation and triton-acid (TAU) gel electrophoresis. Representative TAU gel images are shown in the left panels and the results of multiple experiments are summarized in the graph on the right. The y-axes represent relative staining intensities of insoluble α-globin on TAU gels, measured by automated image analysis and expressed in arbitrary units. *Hbb*$^{th3/+}$ sing, n = 5; *Hbb*$^{th3/+}$ diff, n = 5-6. (**B**) Transmission electron microscopy of flow cytometry-purified reticulocytes showing electron-dense α-globin inclusions. Representative micrographs are shown in the two left panels. The scale bars represent 2 µm. The graph on the right shows the results of quantitative automated image analysis of multiple mice with relative levels of α-globin inclusions indicated on the Y-axis using an arbitrary scale. *Hbb*$^{th3/+}$ sing, n = 6; *Hbb*$^{th3/+}$ diff, n = 5. (**C)** *Hbb/Hba* mRNA ratios in Ter119^{+} erythroblasts from bone marrow (BM, left) or spleen (right) of mice with the indicated genotypes, determined by RNA-sequencing (RNA-seq) analysis. Five mice were assessed per genotype. **(D)** *Hbb/Hba* mRNA ratios in EryA and EryB erythroblasts isolated from bone marrow and reticulocytes (retic) isolated from peripheral blood, determined by RNA-seq analysis. Four mice were assessed per genotype. (**E**) Intronic *Hbb/Hba* RNA ratios in the samples described for panel D. All graphs show data as mean value ± SD. ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$ ns: not significant.

Figure 1

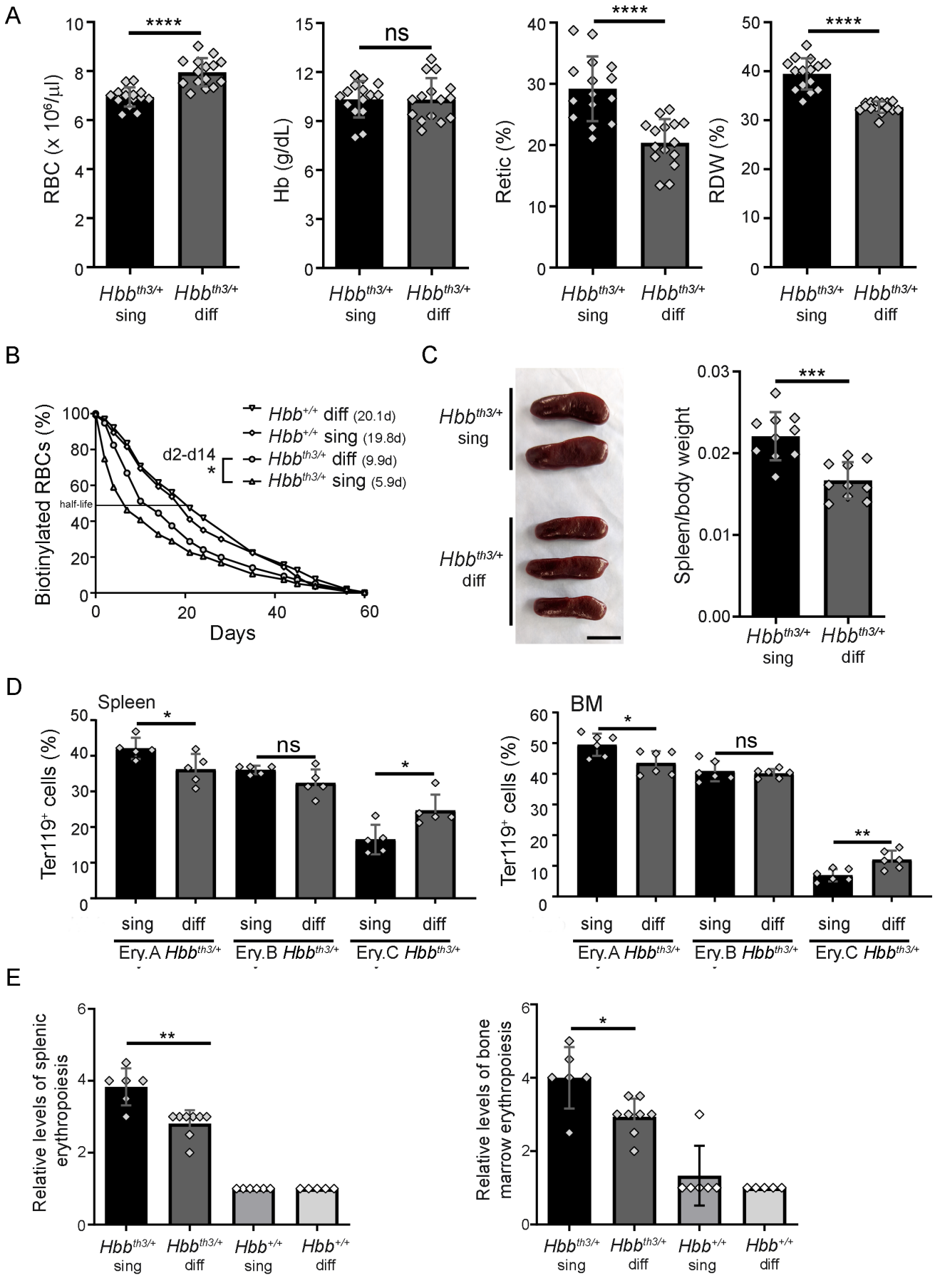

Figure 2