blood advances

TO THE EDITOR:

Single-cell transcriptomics reveals heterocellular γ-globin gene expression in Aγδβ-thalassemia

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Around birth, red blood cell (RBC) β-like globin gene expression shifts from γ-globin (*HBG1* and *HBG2*, hereafter *HBG*) to adjacent β-globin (*HBB*), resulting in a switch from fetal hemoglobin (HbF, α2γ2) to adult hemoglobin (HbA, α2β2). Consequently, anemias caused by mutations in *HBB*, such as sickle cell disease (SCD) and β-thalassemia, become symptomatic postnatally. In hereditary persistence of fetal hemoglobin (HPFH), rare genetic variants in the β-like globin gene cluster lead to high levels of postnatal *HBG* expression and HbF that can reduce or eliminate the pathologies of coinherited β-hemoglobinopathies.[1,2] Several promising therapeutic strategies use genome engineering to create HPFH-like mutations in hematopoietic stem cells of patients with SCD or β-thalassemia, thereby reactivating HbF in RBC progeny.[3] The efficacy of such therapies depends on the proportion of individual RBCs expressing therapeutic levels of HbF.[4]

Some forms of HPFH are caused by kilobase-scale deletions downstream of *HBG2* (Gγ-globin) and often include *HBG1* (Aγ-globin), *HBD* (δ-globin) and *HBB* (Figure 1A).[3] Similar but distinct deletions cause related conditions termed Aγδβ-thalassemia or δβ-thalassemia, which are associated with mild microcytic, hypochromic anemia, elevated HbF, and partial amelioration of β-hemoglobinopathies. Seemingly subtle differences in HbF induction caused by different deletions can produce different clinical consequences. For example, individuals with compound heterozygosity for the 85 kb "Black HPFH1" deletion (HPFH1) and the sickle hemoglobin (HbS) allele have RBC HbF levels of 28.7% to 36.0% (average 32.6%) and are usually asymptomatic.[5] In contrast, individuals with HbS and the 35.8 kb "Black $(Aγδβ)^0$-thalassemia" deletion (Aγδβ-thal) express 21.8% to 34.0% HbF (average 26.6%) and experience clinical symptoms, typically on the milder end of the disease spectrum.[6] RBCs from individuals with HbS/HPFH1 or HbS/Aγδβ-thal are reported to express HbF in a homogeneous (ie, pancellular) pattern, according to immunostaining with anti-HbF antibody (F-cells),[5,6] a sensitive but nonquantitative assay. We performed single-cell RNA sequencing (scRNA-seq) of reticulocytes to better define and compare the patterns of γ-globin gene expression in individuals with HbS/HPFH1 or HbS/Aγδβ-thal.

We studied 3 siblings with HbS/HPFH1 and 2 unrelated individuals with HbS/Aγδβ-thal (Table 1). Individuals with HbS/HPFH1 experienced no SCD symptoms. Their hemoglobin and reticulocyte levels were normal. Participant HbS/Aγδβ-thal.1 had mild anemia (average Hb 11.4 g/dL) and experienced multiple episodes of acute chest syndrome and vaso-occlusive pain. Hydroxyurea therapy was initiated but adherence was low and there was no hematological or clinical response. Participant HbS/Aγδβ-thal.2 was hospitalized once for acute chest syndrome but had no history of pain episodes. During well clinic visits, sickle-shaped RBCs were observed intermittently on blood smears from both individuals

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Single-cell RNA sequencing data have been deposited in the National Center for Biotechnology Information Gene Expression Omnibus database under accession number GSE280004.

The full-text version of this article contains a data supplement.

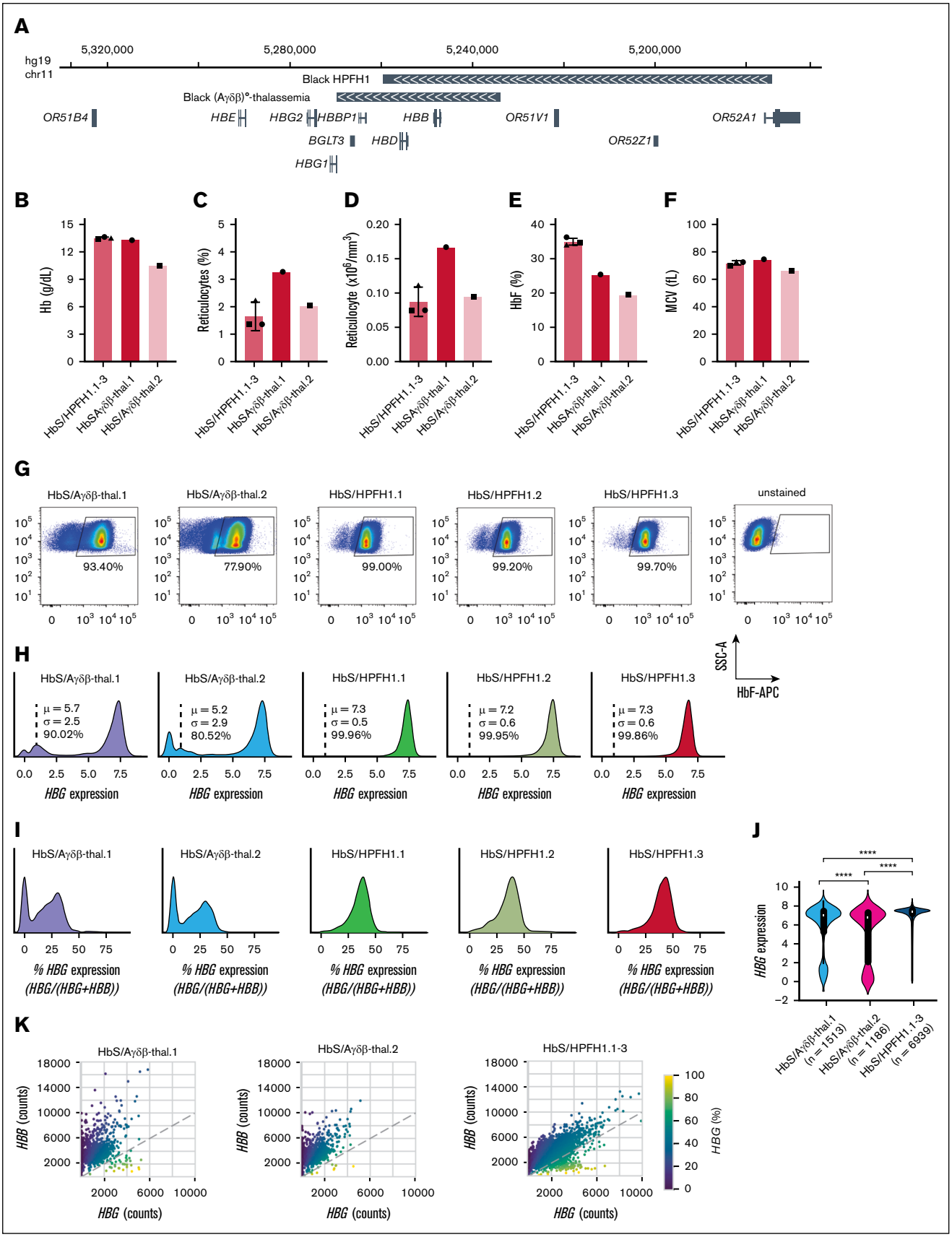
A
hg19
chr11
5,320,000
5,280,000
5,240,000
5,200,000
Black HPFH1
Black (Aγδβ)°-thalassemia
OR51B4
HBE
HBG2
HBBP1
HBB
OR51V1
OR52A1
BGLT3
HBD
OR52Z1
HBG1
B
Hb (g/dL)
C
Reticulocytes (%)
D
Reticulocyte (x10^6/mm^3)
E
HbF (%)
F
MCV (fL)
HbS/HPFH1.1-3
HbSAγδβ-thal.1
HbS/Aγδβ-thal.2
G
HbS/Aγδβ-thal.1
HbS/Aγδβ-thal.2
HbS/HPFH1.1
HbS/HPFH1.2
HbS/HPFH1.3
unstained
93.40%
77.90%
99.00%
99.20%
99.70%
H
SSC-A
HbF-APC
μ = 5.7
σ = 2.5
90.02%
μ = 5.2
σ = 2.9
80.52%
μ = 7.3
σ = 0.5
99.96%
μ = 7.2
σ = 0.6
99.95%
μ = 7.3
σ = 0.6
99.86%
HBG expression
I
% HBG expression
(HBG/(HBG+HBB))
J
HBG expression
****
HbS/Aγδβ-thal.1 (n = 1513)
HbS/Aγδβ-thal.2 (n = 1186)
HbS/HPFH1.1-3 (n = 6939)
K
HBB (counts)
HBG (counts)
HBG (%)

Figure 1.

Table 1. Clinical characteristics of participants with HbS/Aγδβ-thal or HbS/HPFH1

Characteristics	HbS/Aγδβ-thal.1	HbS/Aγδβ-thal.2	HbS/HPFH1.1	HbS/HPFH1.2	HbS/HPFH1.3
Age at last visit, y	15	11	7	15	9
Gender	M	F	F	M	F
α-Thalassemia status	$-\alpha^{3.7}\alpha^{+}/\alpha^{+}\alpha^{+}$	$\alpha^{+}\alpha^{+}/\alpha^{+}\alpha^{+}$	$\alpha^{+}\alpha^{+}/\alpha^{+}\alpha^{+}$	$\alpha^{+}\alpha^{+}/\alpha^{+}\alpha^{+}$	$\alpha^{+}\alpha^{+}/\alpha^{+}\alpha^{+}$
Hemoglobin, g/dL	11.4 (1.0); n = 16	10.7 (0.4); n = 22	13.3 (0.7); n = 4	13.3 (0.3); n = 10	12.6 (0.7); n = 6
MCV, fL	74.9 (2.6); n = 16	67.7 (1.2); n = 22	74.1 (1.01); n = 4	74.7 (2.2); n = 10	70.3 (1.4); n = 6
MCHC, g/dL	34.3 (1.03); n = 16	34 (0.7); n = 22	34.9 (0.4); n = 4	35.2 (1.0); n = 10	34.3 (0.9); n = 6
ARC, × 10^6/mm^3	0.134 (0.02); n = 13	0.105 (0.01); n = 14	0.069 (0.01); n = 4	0.07 (0.01); n = 5	0.10 (0.01); n = 6
HbF percentage	28.6 (1.4)	25.4 (1.8)	40.5 (0.3)	41.2 (2.0)	39.9 (1.7)
F cell percentage	86.2%; n = 2	90.5%; n = 1	Not done	Not done	Not done
Sickled RBCs on blood smear	Yes, on some well clinic visits	Yes, on some well clinic visits	No	No	No
Total bilirubin, mg%	1.5 (0.7); n = 14	0.7 (0.1); n = 14	0.2 (0.06); n = 3	0.3 (0.05); n = 9	0.23 (0.05); n = 4
Hydroxyurea therapy	Started at age 14 years; low adherence	No	No	No	No
Transcranial doppler ultrasound velocity	Normal	Normal	Not done	Not done	Not done
Acute chest syndrome	3	1	0	0	0
Vaso-occlusive crisis	14	0	0	0	0
Blood transfusions	None	None	None	None	None

Laboratory values are reported as mean (standard deviation). The number of values (n) obtained from well visits from age 3 years, to the last follow-up visit is shown. Vaso-occlusive crisis refers to acute pain episodes requiring hospitalization. α-Thalassemia status $\alpha^{3.7}$ indicates a single deletion of 3.7 kb in the *HBA* locus. HbS/HPFH1.1-3 are full siblings.
ARC, absolute reticulocyte count; F, female; M, male; MCV, mean corpuscular volume; MCHC, mean corpuscular hemoglobin concentration.

with HbS/Aγδβ-thal but not from those with HbS/HPFH1. After age 3 years, HbF levels averaged 40.54 ± 1.56% and 27.98 ± 2.10% for individuals with HbS/HPFH1 and HbS/Aγδβ-thal, respectively. F-cell fractions determined in a commercial laboratory for individuals HbS/Aγδβ-thal.1 and HbS/Aγδβ-thal.2 were 86.2% and 90.5%, respectively.

At the time of blood collection for scRNA-seq, hemoglobin levels were normal for all 3 HbS/HPFH1 participants and participant HbS/Aγδβ-thal.1, and mildly reduced for participant HbS/Aγδβ-thal.2 (10.5 g/dL) (Figure 1B). The reticulocyte count was mildly elevated in participant HbS/Aγδβ-thal.1 (3.27%; 0.17 × 10^6/mm^3) and normal in all others (Figure 1C-D). HbF levels in hemolysates were 25.4% for participant HbS/Aγδβ-thal.1, 19.4% for participant HbS/Aγδβ-thal.2, and 35.0 ± 1.1% for HbS/HPFH1 participants 1 to 3 (Figure 1E). Mean corpuscular volume was mildly reduced for participant HbS/Aγδβ-thal.2 (Figure 1F). RBC F-cell fractions were 93.4% for participant HbS/Aγδβ-thal.1, 77.9% for HbS/Aγδβ-thal.2, and 99 ± 0.36% for participants HbS/HPFH1.1 to 3 (Figure 1G).

Reticulocytes were purified by fluorescence-activated cell sorting (supplemental Figure 1), and scRNA-seq was performed on at least 1000 reticulocytes from each individual. We classified transcripts mapping to *HBG1* or *HBG2* as *HBG*, and those mapping to *HBA1* or *HBA2* as *HBA*. We observed distinct distributions of *HBG* expression in individual reticulocytes from the 2 patients with HbS/Aγδβ-thal compared with those with HbS/HPFH1 (Figure 1H-K). Specifically, HbS/HPFH1 reticulocytes exhibited relatively high *HBG* levels in a narrow range, whereas *HBG* expression was more variable in HbS/Aγδβ-thal reticulocytes, with more cells expressing low or no *HBG* messenger RNA. During progressive hypoxia in a microfluidic platform,[7] individual RBCs from participants with HbS/Aγδβ-thal sickled more readily than those from individuals HbS/HPFH1.1 to 3 (supplemental Figure 2A-C).[7] In addition, incubation in 2% oxygen caused extensive sickling of RBCs from individuals with HbS/Aγδβ-thal, whereas RBCs from individuals with HbS/HPFH1 were relatively resistant to the same treatment (supplemental Figure 2D-E).

We also observed differences between the 2 individuals with HbS/Aγδβ-thal (Table 1). Presence of the $\alpha^{3.7}$ deletion may contribute to the higher hemoglobin level observed in participant HbS/Aγδβ-thal.1.[8] Despite having slightly higher levels of F-cells and *HBG* expression, this individual exhibited more episodes of pain and acute chest syndrome than participant HbS/Aγδβ-thal.2.

Figure 1. γ-Globin expression is more heterogeneous in primary reticulocytes from HbS/Aγδβ-thal than HbS/HPFH1. (A) Genomic map representing the extended β-globin locus and the regions deleted in HPFH1 and Aγδβ-thal. (B) Blood hemoglobin levels, (C) % reticulocytes, (D) absolute reticulocyte counts, (E) HbF levels in RBC lysates, determined by ion exchange high-performance liquid chromatography, and (F) mean corpuscular volume determined at the time of whole blood collection for scRNA-seq analysis. (G) F-cell plots from each participant with the indicated F-cell-positive fraction. (H) scRNA-seq analysis of reticulocytes. The x-axis shows log-normalized *HBG* (*HBG1* + *HBG2*) expression in individual reticulocytes from each participant. Histograms show the distribution of expression levels, with the mean (μ), standard deviation (σ), and percentage of reticulocytes expressing *HBG* above a normalized value of 1 indicated. A vertical dashed line marks this threshold. (I) Distribution of %*HBG* expression (*HBG*/[*HBG*+*HBB*]) in samples from panel H. (J) Analysis of reticulocyte scRNA-seq showing *HBG* expression in individuals with the indicated genotypes. Box and whisker plots show minimum, maximum, median, and interquartile ranges. Violin plots show the distribution of individual data points. Kruskal-Wallis with Dunn's multiple-comparison test, ****$P < .0001$. (K) Scatterplot analysis of scRNA-seq showing *HBB* (y-axis) and *HBG* (x-axis) read counts in reticulocytes from each participant with HbS/Aγδβ-thal or pooled participants with HbS/HPFH1.1 to 3. Symbol colors are based on the %*HBG* heat map. Dashed line represents 50% *HBG*. APC, allophycocyanin; MCV, mean corpuscular volume; SSC-A, side scatter area.

These findings reflect the heterogeneity of SCD, which is influenced by many genetic, social, and environmental factors in addition to HbF levels.[9-12]

At least 1 γ-globin gene and its promoter remain intact in deletional HPFH, δβ-thal, or Aγδβ-thal. In these cases, induction of γ-globin is thought to occur through loss of repressive regulatory elements, juxtaposition of distal enhancers, and/or deletion of the *HBB* promoter, which competes with *HBG* genes for interaction with the locus control region, a powerful upstream enhancer (supplemental Figure 3).[13-17] Most likely, distinct combinations of regulatory perturbations account for differences in the patterns of γ-globin gene activation in HPFH1 vs Aγδβ-thal. The large differences in γ-globin expression between genetically identical erythroid cells in Aγδβ-thal represents position-effect variegation, which causes mosaic phenotypes by repositioning normally expressed euchromatic genes near a region of heterochromatin.[17,18] The genetic alterations responsible for position effect variegation in Aγδβ-thal may involve loss or rearrangement of chromosomal boundary regions that prevent spread of heterochromatin into actively transcribed DNA.[19]

Overall, our data support the concept that the proportion of erythroid cells with sufficient levels of HbF to inhibit HbS polymerization is more important for alleviating SCD than the F-cell percentage or the concentration of HbF in the hemolysate.[20] The heterogeneity of *HBG* expression in HbS/Aγδβ-thal indicates that a substantial proportion of F-cells detected by immunostaining express subtherapeutic levels of HbF. In contrast, HbS/HPFH1 F-cells express high-level *HBG* more homogeneously. This important difference cannot be fully appreciated by F-cell determination, which is highly sensitive but has a limited dynamic range for detecting clinically meaningful differences in *HBG* expression. Our study is limited by the small number of patients examined. Therefore, future studies are required to confirm our findings in individuals with HbS/Aγδβ-thal or HbS/HPFH1. In addition, scRNA-seq analysis of reticulocytes may be useful for analyzing the effects of genome engineering strategies or drugs to induce HbF in patients with β-hemoglobinopathies. It will also be useful to correlate reticulocyte scRNA-seq analysis with experimental protocols for accurate quantification of HbF protein expression in individual mature RBCs.[21]

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