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A-two to the rescue

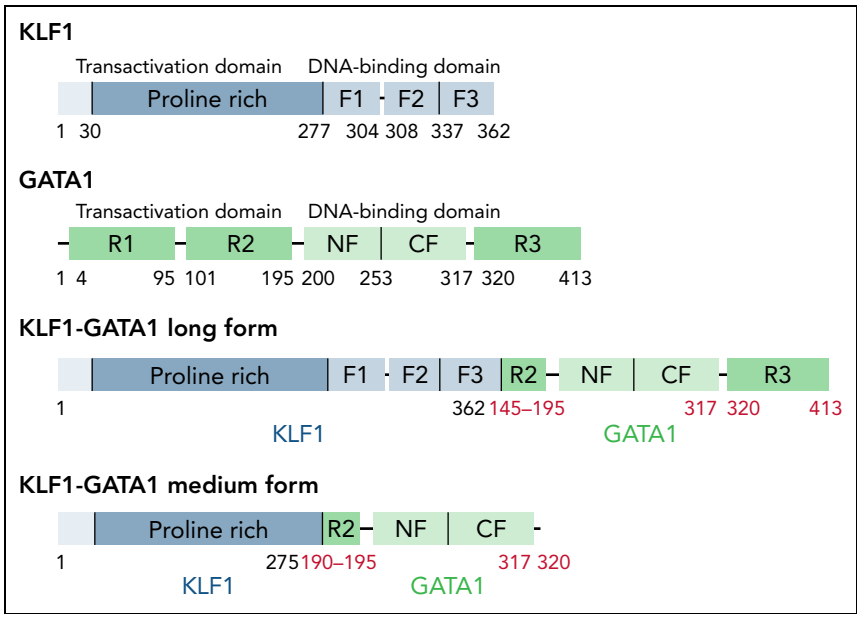
Abdullah Kutlar | Medical College of Georgia

In this issue of *Blood*, Zhu et al[1] report on a novel gene therapy approach, enhancing the expression of the δ-globin chain of human minor adult hemoglobin, HbA_2 (α2δ2), to treat β-hemoglobinopathies.

In adult erythroid cells, δ-globin gene expression is ~1/40th of the β-globin of adult HbA. HbA_2 functions normally as an oxygen carrier and has normal stability, but these properties are physiologically inconsequential due to the low concentration of HbA_2 in adult red blood cells (RBCs).[2] The δ-globin gene is transcribed at a much lower rate compared with β-globin, because its promoter lacks the KLF1-binding motif, CACCC box. It has been known for decades that HbA_2 has potent anti-sickling properties, similar to HbF; this anti-sickling effect is largely due to the differences in amino acid residues between β- and δ-chains, specifically Thr87Gln and Glu22Ala in δ-chains, which inhibit deoxy-HbS polymerization by blocking lateral and axial contacts.[3] It has not been possible to take advantage of this property of HbA_2 because of its low level of expression (2.5%-3.0%) in RBCs. Several groups have developed approaches to activate δ-globin transcription. These have included generation of a KLF1-binding motif by creating a CACCC box in the δ-globin promoter by site-directed mutagenesis[4-6]; this has been tested in mouse models of β-thalassemia[7] and sickle cell disease,[8] with amelioration of the phenotype in both cases.

Zhu et al report an alternative approach to upregulation of δ-globin expression. They previously showed that δ-globin expression was upregulated twofold to fourfold in K562 cells and in normal bone marrow $CD34^+$ cells by different lengths (ie, short, medium, and long) of a fusion protein between the DNA-binding domain of the erythroid transcription factor GATA1 and the *trans*-activating domain of KLF1.[9] In the current study, the investigators extended these observations to erythroid cultures derived from $CD34^+$ cells from sickle cell patients transduced with medium and long KLF1-GATA1 fusion constructs (see figure), as well as Berkeley SCD mice transplanted with mouse hematopoietic stem cells transduced with KLF1-GATA1 fusion constructs. The investigators observed that the fusion proteins had no impact on erythroid differentiation, proliferation, or enucleation. In $CD34^+$ cell–derived erythroid cultures, the δ-globin expression was increased 2.3-fold by medium and 4.3-fold by long KLF1-GATA1 fusion proteins. Hypoxia-induced sickling decreased in erythroid cells derived from sickle $CD34^+$ cell cultures. The percentage of anti-sickling hemoglobins (HbA_2 + HbF) increased to 19.7% and 14.4% in erythroid cells transduced with medium and long fusions, respectively. Similar observations were made in Berk sickle cell mice transplanted with mouse hematopoietic stem cells transduced with medium and long fusion constructs. Hematologic improvement was more pronounced in mice transplanted with the medium-length KLF1-GATA1 construct (Hb 11.8 g/dL vs 6.48 g/dL in mock mice, and 7.84 g/dL in mice transplanted with the long construct), likely resulting from higher expression of HbA_2 (18.8%) in this group, compared with 11.5% in mice transduced with the long fusion. In addition, there was significant improvement in the phenotype of mice transplanted with the medium-length fusion construct, including a substantial reduction in spleen size, improvement in splenic architecture, improvement in iron deposition in the liver and kidneys, and increase in urine concentrating ability.

Taken together, these data provide proof of principle that upregulation of HbA_2 expression can provide significant amelioration of the phenotype in β-hemoglobinopathies by reducing the globin chain imbalance in β-thalassemia syndromes and by inhibition of deoxy HbS polymerization in sickle cell disease. The data provided are consistent with significant "disease modification," not "cure." The question remains whether the expression of anti-sickling hemoglobins (HbA_2 + HbF) can be further increased, with pancellular distribution among RBCs, closer to a "cure." The authors also suggest that an advantage of their approach is the use of non-myeloablative conditioning, with resultant relatively low donor chimerism, and selection of "non-sickling" cells. It



Schematic diagram of the structure of KLF1, GATA1, and KLF1-GATA1 fusion constructs. F1, F2, and F3 represent three finger domains of KLF1; CF and NF represent C- and N-fingers of GATA1; and R1, R2, and R3 represent three regions of the transactivation domain of GATA1. See the complete Figure 1 in the article by Zhu et al that begins on page 2276.

remains to be seen whether this level of chimerism and HbA_2 expression is sustainable long term. Nevertheless, the data presented, especially the results in Berk mice, support the consideration of a clinical trial.

Conflict-of-interest disclosure: A.K. declares no competing financial interests. ■

REFERENCES

1. Zhu J, Li H, Aerbajinai W, et al. Kruppel-like factor 1–GATA1 fusion protein improves the sickle cell disease phenotype in mice both in vitro and in vivo. *Blood*. 2022;140(21):2276-2289.
2. Steinberg MH, Adams JG III. Hemoglobin A_2: origin, evolution, and aftermath. *Blood*. 1991;78(9):2165-2177.
3. Nagel RL, Bookchin RM, Johnson J, et al. Structural bases of the inhibitory effects of hemoglobin F and hemoglobin A_2 on the polymerization of hemoglobin S. *Proc Natl Acad Sci USA*. 1979;76(2):670-672.
4. Donze D, Jeancake PH, Townes TM. Activation of delta-globin gene expression by erythroid Krupple-like factor: a potential approach for gene therapy of sickle cell disease. *Blood*. 1996;88(10):4051-4057.
5. Ristaldi MS, Casula S, Porcu S, Marongiu MF, Pirastu M, Cao A. Activation of the delta-globin gene by the beta-globin gene CACCC motif. *Blood Cells Mol Dis*. 1999;25(3-4):193-209.
6. Tang DC, Rodgers GP. Activation of the human delta-globin gene promoter in primary adult erythroid cells. *Br J Haematol*. 1998;103(3):835-838.
7. Manchinu MF, Marongiu MF, Poddie D, et al. In vivo activation of the human δ-globin gene: the therapeutic potential in β-thalassemic mice. *Haematologica*. 2014;99(1):76-84.
8. Porcu S, Simbula M, Marongiu MF, et al. Delta-globin gene expression improves sickle cell disease in a humanised mouse model. *Br J Haematol*. 2021;193(6):1228-1237.
9. Zhu J, Chin K, Aerbajinai W, Trainor C, Gao P, Rodgers GP. Recombinant erythroid Kruppel-like factor fused to GATA1 up-regulates delta- and gamma-globin expression in erythroid cells. *Blood*. 2011;117(11):3045-3052.

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THROMBOSIS AND HEMOSTASIS

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Platelets: out of shape and misbehaving

Robert Flaumenhaft | BIDMC/Harvard Medical School

The most prominent morphological feature of platelets is their unusual discoid shape from which they derive their name. But why do platelets have such a unique design? In this issue of *Blood*, Kimmerlin et al[1] show that knockout of α4A and β1 tubulin results in mice with spherical platelets that have marked defects in hemostasis and thrombosis.

In 1882, Giulio Bizzozero was evaluating flowing blood in the mesenteric vessels of rabbits and guinea pigs when he first observed a new type of tiny cell that he described as "a very thin platelet, disc-shaped, with parallel surfaces."[2] In 1965, with the advent of electron microscopy, Behnke demonstrated a peripheral microtubule bundle in human and rat platelets and proposed that this structure gave platelets the platelet discoid shape first described by Bizzozero.[3] This microtubule structure was subsequently termed the marginal band. Microtubules that make up the marginal band consist of heterodimers of α- and β-tubulin subunits aligned head to tail to form protofilaments.[4,5] These protofilaments laterally associate, enabling the formation of hollow rigid tubular structures. There are many isoforms of α- and β-tubulins in platelets, with α4-tubulin and β1-tubulin (whose expression is restricted to hematopoietic lineages) being among those most highly expressed.[6] In the resting platelet, this marginal band is a submembranous ring of approximately 9 μm and 8 to 12 microtubules that circumscribes the platelet periphery and encircles all platelet organelles.

Despite detailed knowledge of marginal band composition and dynamics, the reason platelets are discoid has remained a mystery. One popular idea is that platelets evolved a flat shape to improve their ability to form tight aggregates by providing an extended surface for platelet-platelet interactions. This idea was bolstered by the identification of a patient with a bleeding disorder whose platelets were spherical and lacked a marginal band.[7] Studies of β1-tubulin knockout mice, however, raised doubts about this premise. These mice showed reduced microtubule content and spherical platelets but nonetheless demonstrated normal bleeding times and normal adhesion under shear.[8] The fact that the β1-tubulin knockout platelets retained a thin marginal band and adopted a somewhat elliptical shape kept open the question of whether platelet discoid shape promotes platelet adhesion.

The generation and extensive phenotyping of the α4A- and β1-tubulin double knockout mouse by Kimmerlin et al now address this issue. The investigators show that the double mutant has reduced platelet counts and increased platelet volumes. Erythrocytes and leukocytes appeared to be unaffected. A strength of these experiments is that the investigators evaluated single α4A-tubulin and β1-tubulin knockouts as well as wild-type and double knockout mice. Nearly all the double knockout platelets (97%) lacked discoid shape and 73% were spherical, whereas in β1-tubulin knockouts 68% lacked discoid shape and 24% were spherical. Peritoneal bleeding was observed in double knockout mice, but not in the other genotypes. Consistent with a profound bleeding abnormality, tail snip assays showed continued bleeding after 30 minutes in double knockout mice, whereas the majority of mice of the other genotypes achieved hemostasis. To rule out the possibility that enhanced bleed resulted from thrombocytopenia observed in the double knockout animals, mice were infused with romiplostim to increase their platelet counts. Enhancement of platelet counts did not reverse the bleeding phenotype. Defective hemostatic function correlated with impaired ability to form thrombi in vivo. Only a thin layer of platelets accumulated following exposure of carotid arteries to ferric chloride ($FeCl_3$) in double knockout mice. In contrast, other mutants and wild-type mice demonstrated arterial occlusion under the same conditions.