Intestine-specific Disruption of Hypoxia-inducible Factor (HIF)-2α Improves Anemia in Sickle Cell Disease*

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**Background:** Sickle cell disease is an inherited blood disorder resulting in severe anemia.

**Results:** Limiting iron toxicity through disruption of intestinal HIF-2α improves the anemia.

**Conclusion:** Intestinal HIF-2α deficiency improves anemia in sickle cell disease.

**Significance:** Intestinal HIF-2α is a novel therapeutic target in sickle cell disease.

Sickle cell disease (SCD) is caused by genetic defects in the β-globin chain. SCD is a frequently inherited blood disorder, and sickle cell anemia is a common type of hemoglobinopathy. During anemia, the hypoxic response via the transcription factor hypoxia-inducible factor (HIF)-2α is highly activated in the intestine and is essential in iron absorption. Intestinal disruption of HIF-2α protects against tissue iron accumulation in iron overload anemias. However, the role of intestinal HIF-2α in regulating anemia in SCD is currently not known. Here we show that in mouse models of SCD, disruption of intestinal HIF-2α significantly decreased tissue iron accumulation. This was attributed to a decrease in intestinal iron absorptive genes, which were highly induced in a mouse model of SCD. Interestingly, disruption of intestinal HIF-2α led to a robust improvement in anemia with an increase in RBC, hemoglobin, and hematocrit. This was attributed to improvement in RBC survival, hemolysis, and insufficient erythropoiesis, which is evident from a significant decrease in serum bilirubin, reticulocyte counts, and serum erythropoietin following intestinal HIF-2α disruption. These data suggest that targeting intestinal HIF-2α has a significant therapeutic potential in SCD pathophysiology.

Sickle cell disease (SCD)3 is an autosomal recessive hemolytic disorder. SCD is a major public health concern in the United States and in many other countries (1). In the United States, about 100,000 individuals are affected by SCD, the majority being African-American, and worldwide, more than 3,000,000 patients are born every year with this disorder (2). SCD is caused by a single amino acid substitution of valine for glutamic acid in the β-globin chain. The resultant hemoglobin is known as hemoglobin S (HbS). Upon deoxygenation, HbS results in decreased life span and sickling deformity of the RBC membrane (3). SCD presents with hemolytic anemia, tissue iron overload, and episodes of vaso-occlusive events (4–6). At present, hematopoietic stem cell transplantation is the definitive curative approach, but it is associated with significant morbidity and mortality as well as high cost (7). Neonatal screenings, penicillin prophylaxis for infection, and disease-modifying intervention such as hydroxyurea and blood transfusions have been shown to improve disease outcome (8). Blood transfusion is an important component of SCD management that increases oxygen-carrying capacity and replaces sickled RBCs. However, repeated blood transfusions contribute further to the existing iron overload (9) and necessitate iron chelation therapy (10). Cellular iron overload in SCD patients is associated with increased expression of inflammatory markers and mortality (11). Previous work has demonstrated that decreasing intestinal iron absorption either through treatment with hepcidin mimetics or through disruption of intestinal HIF-2α, the major local regulator of iron absorption, led to a decrease in iron overload in β-thalassemia mouse models. Paradoxically, the decrease in iron absorption also improved anemia (12, 13). This has been demonstrated to be through a decrease in iron-induced reactive oxygen species, leading to decreased RBC apoptosis. However, it is not clear whether limiting intestinal iron absorption may have beneficial effects in SCD. The present work demonstrates that disruption of intestinal HIF-2α limits intestinal iron absorption and improves anemia while decreasing systemic iron levels.

**Materials and Methods**

Humanized SCD Mouse Model—The mice utilized had the α- and β-globin genes replaced by fragments of the human α- and sickle β3-globin loci (14). Heterozygote mating of these mice produces homozygous SS SCD mice. Humanized SCD mice were bred and maintained as described previously (14). HPLC hemoglobin electrophoresis was performed on all mice to confirm sickle cell genotype (15). All animal experiments

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1 The abbreviations used are: SCD, sickle cell disease; BMT, bone marrow transplantation; SS, sickle cell; qPCR, quantitative PCR.
were approved by the University of Michigan Committee on Use and Care of Animals.

**Bone Marrow Transplantation (BMT)**—Total body irradiation and BMT were performed as described previously (12), and engraftment of the donor marrow was near 100%. All the mice were sacrificed 6 months following BMT.

**Western Blot Analysis**—Isolation of whole cell extracts from duodenal scrapings, followed by detection of HIF-2α (Novus Biologicals) and GAPDH (Santa Cruz Biotechnology), was performed as mentioned previously (16).

**Iron Staining**—Tissue iron detection was performed in formalin-fixed paraffin-embedded sections stained with Perls’ Prussian blue.

**Tissue Iron Quantitation**—Tissue nonheme iron was quantified as described previously (12).

**Iron and Hematological Analysis**—Serum iron was analyzed using the QuantiChrom iron assay kit (Bioassay Systems, Hayward, CA). The Unit for Laboratory Animal Medicine Pathology Core at The University of Michigan performed complete blood count analysis.

**Histology**—Histologic analysis was done on H&E-stained paraffin sections.

**Statistics**—Results are expressed as means ± S.D. p values were calculated by independent t test, one-way analysis of variance, and two-way analysis of variance.

**Results and Discussion**

A humanized model of SCD was used in our study. Murine adult α- and β-globin genes were replaced in the germ line by fragments of the human α- and sickle βS-globin loci (14). Heterozygous mating of these mice produced homozygous SCD mice, displaying the characteristic severe form of SCD (14). To investigate the role of intestinal HIF-2α in sickle cell disease, Hif2αAIE mice, which have a disruption of HIF-2α specifically in intestinal epithelial cells, and wild type littermates were compared with control mice (Hif2αAIE-SS mice) (16). To further understand the intestinal HIF-2α expression in the small intestine as compared with control mice (Hif2αAIE-SS WT), which was abrogated in the Hif2αAIE-SS mice, demonstrating that intestinal epithelial HIF-2α is activated in SCD. Furthermore, gene expressions of HIF-2α-regulated duodenal iron transporters, DcytB, DMT1, and ferroportin (Fpn1) (19, 20), were significantly induced in the Hif2αAIE-SS mice, and their expressions were reduced to near control levels in the Hif2αAIE-SS group (Fig. 1C). Also, mRNA expression analyses of intestinal glycolytic (glucose transporter 1 (Glut1), pyruvate dehydrogenase kinase isozyme 1 (PDK1), and phosphoglycerate kinase 1(PGK1)), apoptosis-related (Bcl2, Bcl-xL, and Casp1 (caspase 1)), and angiogenic (VEGF) genes were done to evaluate whether intestinal HIF-2α inhibition has a general effect of enterocyte survival and metabolism that could indirectly influence the intestinal iron absorption. Unlike the duodenal iron transporters, the expressions of these genes were not altered in the Hif2αF/F-SS and Hif2αAIE-SS mice as compared with the corresponding control groups i.e. Hif2αAIE-SS mice (Fig. 1C). This is consistent with previous work demonstrating that HIF-2α inhibition predominantly influences the expression of critical iron transporters, whereas no appreciable alterations in the intestinal villi morphology and enterocyte survival are observed (21). In agreement with the gene expression data, the serum and tissue iron parameters also displayed a characteristic iron overload in Hif2αAIE-SS mice, whereas disruption of intestinal HIF-2α abolished the tissue iron overload in the Hif2αAIE-SS mice, establishing the role of HIF-2α in heightened intestinal iron uptake in SCD (Fig. 1D). Transplantation of SS bone marrow to the control group (Hif2αAIE-SS) displayed high serum and spleen iron, although the hepatic iron level was only mildly increased. Intestinal HIF-2α disruption in the SCD model (Hif2αAIE-SS) significantly decreased tissue and serum iron accumulation (Fig. 1D). Consistent with these data, Perls’ staining for iron in the liver and the spleen also showed distinctly increased tissue iron level in the Hif2αAIE-SS (Fig. 1E), which in the Hif2αAIE-SS liver and spleen showed significant improvement (Fig. 1E). Hepatic hepcidin expression in Hif2αAIE-SS WT, Hif2αAIE-SS, and Hif2αAIE-SS mice was significantly reduced as compared with that in Hif2αAIE-SS WT. However, there was no significant difference between the Hif2αAIE-SS and Hif2αAIE-SS groups, demonstrating a significant role of intestinal HIF-2α in developing SCD-associated iron overload (Fig. 1F). Although measurement of hepatic hepcidin transcript remains as the standard method for assessing systemic iron status (22), a recent work describes a competitive ELISA method of hepcidin protein detection that is more sensitive and correlative to the systemic iron status in some cases of iron overload disorders (23). We think that assessment of serum hepcidin by this new method could be more informative for the further assessment of systemic iron dysregulation in SCD. Moreover, the data also provide evidence that non-transfusion iron overload may contribute significantly to tissue iron accumulation in SCD. Despite appreciable differences in tissue iron accumulation, spleen and liver histology were similar in the Hif2αAIE-SS and Hif2αAIE-SS mice. In the liver, there were no signs of cholestasis or fat accumulation; however, vascular occlusions were present. The spleens were enlarged, and occlusions of the splenic cords along with increased fibroconnective tissue were observed (Fig. 1G).

Blood smear analysis shows that Hif2αAIE-SS mice developed sickling of RBC characteristic of typical SCD, whereas no significant difference in sickling was observed between Hif2αAIE-SS and Hif2αAIE-SS mice (Fig. 2A). Hif2αAIE-SS exhibited severe anemia, showing significant reduction in RBC, hemoglobin, and hematocrit values (Fig. 2B). However, Hif2αAIE-SS mice exhibited an improvement in hematological parameters as compared with Hif2αAIE-SS mice. RBC, Hb, and hematocrits, although lower than the normal values, were significantly elevated in Hif2αAIE-SS mice (Fig. 2B). To further understand the mechanism, serum bilirubin was assessed as a marker of hemolysis. Hif2αAIE-SS mice exhibited a significant increase in total and indirect bilirubin, suggesting enhanced hemolysis (Fig. 2C). In the Hif2αAIE-SS mice, there was a reduction in hemolysis as
FIGURE 1. Disruption of intestinal HIF-2α improves SCD-induced tissue iron overload. A–C, Hif2α^{FF} and Hif2α^{AI E} mice were lethally irradiated and transplanted with Hb-WT or Hb-SS bone marrow (A) and analyzed 6 months later for HIF-2α protein expression in the duodenal mucosa (B) and gene expression analysis of duodenal Dcytb, Dmt1, Fpn1, Bcl2, Bcl-xl, Casp1, Glut1, PDK1, PGK1, and VEGF1 (normalized to β-actin) (C), D, serum (μg/dl) and tissue iron content (calculated as μg per gm of wet weight). E, Perls’ iron stain of the spleen and liver. F, gene expression analysis of liver hepcidin (Hepc) normalized to β-actin. G, H&E staining of spleen and liver. Bar graph represents the mean value ± S.D. ****, p < 0.0001; **, p < 0.01; *, p < 0.05; ns, not significant.
evidenced by a decrease in serum bilirubin level, both total and indirect (Fig. 2C). Furthermore, reticulocyte counts and serum erythropoietin levels were high in the *Hif2α*/*H9251* F/F-SS mice, and significantly improved in the *Hif2α*/*H9251* IE-SS, providing further evidence of decreased hemolysis following disruption of intestinal HIF-2α (Fig. 2, D and E). We propose, similar to the benefits of hepcidin overexpression in β-thalassemic iron overload (13), that intestinal HIF-2α disruption in SCD mice reduces the RBC hemoglobinization because of the diminished intestinal iron absorption. As a consequence, there is reduced density of Hb-SS in the individual RBCs, decreasing their vulnerability to deformity and thereby hemolysis. The resultant reduction in hemolysis would thus reduce free heme-induced toxicity and reactive oxygen species formation to ultimately improve both RBC lifespan and anemia.

Improvement in the hematologic and iron parameters of sickle cell anemia following intestine-specific HIF-2α deletion offers a significant promise in therapy for SCD, either alone or in conjunction with the existing regimens. Targeting intestinal HIF-2α may reduce the need of repeated blood transfusions or pharmacologic iron chelation. With a recent discovery of orally bioavailable HIF-2α-specific inhibitors (24), the present data suggest that use of these inhibitors could be a novel and effective treatment strategy for SCD.
REPORT: Intestinal HIF-2α in Sickle Cell Disease


