Final Report

Project Title Effects of iron chelators on the expression of iron transport machineries in the duodenum of iron-loaded thalassemic mouse models

By Patarabutr Masaratana

May 2016

Contract No MRG5680059

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This project granted by the Thailand Research Fund

Abstract

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Project Period : June 2013 – May 2016

Executive summary:

Background: Thalassemia, one of the global health problems, is caused by mutations of genes encoding adult globin chains resulting in reduced globin synthesis and imbalance between α and non-α globin chains in erythroid cells. One of the major life-threatening complications of thalassemia is iron overload caused by increased iron absorption and/or blood transfusion. Iron chelators have therefore been used to reduce tissue iron deposition. In thalassemia, the expression of iron regulatory peptide, hepcidin, is affected by several regulators including iron loading and ineffective erythropoiesis. However, the expression of iron transport machineries in the duodenum is not only regulated by systemic signal through hepcidin but also by cellular iron status. It is noteworthy that iron status of enterocytes as well as the response of duodenal iron transport machineries to iron chelation and iron loading have not been fully elucidated.

Materials and methods: Male wild type C57BL/6J and th3/+ (BKO; thalassemia mouse model) mice aged 7 weeks old were given intramuscular injection of 5 mg iron dextran for 2 consecutive days. After 2-week equilibration, the mice were administered deferoxamine (DFO) intraperitoneally or deferiprone (DFP; L1) orally for 14 days. Saline injection was used as control. The mice were sacrificed 24 hours after the last dose of the iron chelator. Liver non-heme iron levels were spectrophotometrically measured by ferrozine-based assay. The expression of hepcidin and iron transport machineries was studied by real-time PCR.

Results and discussion: Thalassemia phenotype of BKO mice was confirmed by complete blood count and spleen non-heme iron measurement, however, only substantial changes in liver iron status and hepcidin mRNA expression were noted. Increased mRNA expression of duodenal iron transport machineries, Dcytb and hephaestin, were also observed. Interestingly, wild type and BKO mice displayed differential responses to iron dextran injection. Remarkably increased iron levels were found in liver, spleen and serum of wild type mice, whereas, only spleen non-heme iron levels were affected in BKO mice. Further studies on the expression of iron transporters in duodenum, liver and spleen demonstrated significantly suppressed Dcyb and hephaestin mRNA levels in the duodenum of BKO mice upon iron dextran administration which might be responsible for the lack of iron accumulation in the serum and liver of these mice. In the present study, iron chelation regimens failed to reduce iron loading in both phenotypes. Indeed, serum iron and spleen non-heme iron levels were increased in BKO mice treated with iron chelators suggesting that inadequate chelation might potentially aggravate iron loading in some compartments. However, possible mechanisms underlying such responses remain to be elucidated as iron transporter mRNA expression and duodenal iron status were comparable across all groups. It is speculative that these mechanisms are hepcidin-independent as hepcidin mRNA expression was unaffected by parenteral iron loading or iron chelation.

Keywords : thalassemia, iron, ferroportin, iron chelation, iron overload

บทคัดย่อ

รหัสโครงการ : MRG5680059

ชื่อโครงการ : โครงการศึกษาอิทธิพลของยาขับเหล็กต่อการแสดงออกของกลไกในการดูดซึมธาตุเหล็กในลำไส้เล็กส่วนต้นของหนูทดลองธาลัสซีเมียที่มีธาตุเหล็กเกิน

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บทสรุป:

ข้อมูลพื้นฐาน: ธาลัสซีเมียเป็นความผิดปกติที่เกิดจากการกลายพันธุ์ของยีนที่ควบคุมการแสดงออกของสายโกลบินของผู้ใหญ่ ส่งผลให้ร่างกายสังเคราะห์โกลบินได้ลดลง จนนำไปสู่ความไม่สมดุลระหว่างอัลฟ่าโกลบินและน็อนอัลฟ่าโกลบินในเซลล์เม็ดเลือดแดงอ่อน ภาวะเหล็กเกินเป็นภาวะแทรกซ้อนที่เป็นอันตรายถึงแก่ชีวิตที่สำคัญประการหนึ่งในผู้ป่วยธาลัสซีเมีย โดยร่างกายจะได้รับเหล็กเกินผ่านการดูดซึมเหล็กที่เพิ่มขึ้นและ/หรือการได้รับเลือด ซึ่งในทางเวชปฏิบัติได้มีการให้ยาขับเหล็กแก่ผู้ป่วยเพื่อลดปริมาณเหล็กที่สะสมในเนื้อเยื่อต่างๆ อนึ่งในผู้ป่วยธาลัสซีเมีย การแสดงออกของเฮ็บซิดิน ซึ่งเป็นโปรตีนที่มีบทบาทหลักในการรักษาสมดุลธาตุเหล็กจะอยู่ภายใต้อิทธิพลของปัจจัยต่างๆหลายปัจจัยรวมถึง ภาวะเหล็กเกิน และ ineffective erythropoiesis อย่างไรก็ตามการแสดงออกของโมเลกุลที่ทำหน้าที่ในการขนส่งธาตุเหล็กที่ลำไส้เล็กส่วนต้นยังอาจถูกควบคุมโดยปริมาณธาตุเหล็กในเซลล์ซึ่งเป็นปัจจัยเฉพาะที่นอกเหนือไปจากเฮ็บซิดินซึ่งเป็นปัจจัยในระดับร่างกาย โดยการศึกษาที่ผ่านมายังขาดข้อมูลเกี่ยวกับสถานะของธาตุเหล็กในเซลล์เยื่อบุลำไส้เล็กส่วนต้น ตลอดจนการตอบสนองของกลไกในการดูดซึมธาตุเหล็กต่อภาวะเหล็กเกิน หรือการได้รับยาขับเหล็กแต่อย่างใด

การดำเนินการทดลอง: ในการศึกษานี้ หนูทดลอง wild type สายพันธุ์ C57BL/6J และหนูธาลัสซีเมีย *th3/+* (BKO) เพศชาย อายุ 7 สัปดาห์ได้รับการฉีดเหล็กในรูปของสารละลาย iron dextran ทางกล้ามเนื้อ วันละ 5 มิลลิกรัม เป็นเวลา 2 วันติดต่อกัน โดยกลุ่มควบคุมได้รับสารละลาย saline ในปริมาตรที่เท่ากัน สองสัปดาห์หลังได้รับเหล็ก หนูทดลองจะได้รับยาขับเหล็ก deferoxamine (DFO) เข้าสู่ช่องท้อง หรือ deferiprone (DFP; L1) ทางปาก ทุกวันเป็นเวลา 14 วัน โดยมีการเก็บตัวอย่างเลือดและเนื้อเยื่อที่ 24 ชั่วโมงหลังการให้ยาขับเหล็กครั้งสุดท้ายเพื่อวิเคราะห์ปริมาณธาตุเหล็ก นอกจากนี้ยังมีการศึกษาการแสดงออกของเฮบซิดินและโมเลกุลที่ทำหน้าที่ในการขนส่งธาตุเหล็กด้วยวิธี real-time PCR

ผลการทดลอง และการวิเคราะห์ผลการศึกษา: ฟีโนไทป์ของธาลัสซีเมียในหนูทดลองได้รับการยืนยันโดยการวิเคราะห์ complete blood count และปริมาณ non-heme iron ในม้าม อย่างไรก็ตาม พบว่าหนูทดลองธาลัสซีเมียมีระดับการแสดงออกของเฮ็บซิดินและปริมาณ non-heme iron ในตับเพิ่มขึ้นจากหนูทดลองปกติเพียงเล็กน้อย นอกจากนี้ลำไส้เล็กส่วนต้นของหนูดังกล่าวยังมีการแสดงออกที่ระดับอาร์เอ็นเอของ Dcytb และ hephaestin ซึ่งมีบทบาทร่วมในการดูดซึมธาตุเหล็กเพิ่มขึ้นอีกด้วย อนึ่งหนูทดลองธาลัสซีเมียและหนูทดลองปกติมีการตอบสนองต่อการได้รับเหล็กในรูป iron dextran ที่แตกต่างกัน โดยการบริหารเหล็กในหนูทดลองปกติส่งผลให้ปริมาณธาตุเหล็กในซีรั่ม ตลอดจน non-heme iron ในตับและม้ามเพิ่มสูงขึ้นอย่างมีนัยสำคัญ ในขณะที่หนูทดลองธาลัสซีเมียจะมีการเพิ่มขึ้นเฉพาะของ non-heme iron ในม้ามเท่านั้น จากการศึกษาการแสดงออกของโมเลกุลที่มีบทบาทในการขนส่งธาตุเหล็กในลำไส้เล็กส่วนต้น, ตับ และม้าม พบว่าหนูธาลัสซีเมียที่ได้รับ iron dextran มีระดับอาร์เอ็นเอของ Dcyb และ hephaestin ในลำไส้เล็กส่วนต้นลดน้อยลง ซึ่งอาจเป็นสาเหตุให้หนูกลุ่มดังกล่าวไม่ได้มีปริมาณธาตุเหล็กในซีรั่มและตับเพิ่มขึ้นแม้จะได้รับเหล็กในรูป iron dextran ก็ตาม ทั้งนี้มาตรการในการบริหารยาขับเหล็กในการศึกษานี้ไม่สามารถลดปริมาณเหล็กที่เกินในหนูทดลองทั้งหนูปกติและหนูธาลัสซีเมียได้ นอกจากนี้หนูธาลัสซีเมียยังมีระดับธาตุเหล็กในซีรั่มและปริมาณ non-heme iron ในม้ามเพิ่มสูงขึ้นหลังได้รับยาขับเหล็กอีกด้วย ข้อมูลดังกล่าวบ่งชี้ว่าการได้รับยาขับเหล็กในขนาดและระยะเวลาที่มีประสิทธิภาพไม่เพียงพอในการลดปริมาณเหล็กที่เกินในร่างกาย อาจส่งผลให้มีธาตุเหล็กสะสมเพิ่มมากขึ้นในบาง compartment ของร่างกาย สำหรับกลไกที่เป็นสาเหตุของการตอบสนองดังกล่าวในหนูธาลัสซีเมียนั้นยังคงต้องได้รับการศึกษาต่อไปในอนาคต เนื่องจากไม่พบว่าการได้รับยาขับเหล็กมีผลต่อปริมาณเหล็กในลำไส้เล็กส่วนต้นหรือการแสดงออกในระดับอาร์เอ็นเอของโมเลกุลที่มีบทบาทในการขนส่งธาตุเหล็กแต่อย่างใด ทั้งนี้ในเบื้องต้นคาดว่ากลไกดังกล่าวไม่ต้องอาศัยเฮ็บซิดินเนื่องจากหนูทดลองทุกกลุ่มมีระดับการแสดงออกของเฮ็บซิดินไม่แตกต่างกัน

คำหลัก : ธาลัสซีเมีย, ธาตุเหล็ก, ferroportin, ยาขับเหล็ก, ภาวะเหล็กเกิน

Objective

1. To determine iron status and the expression of iron transport machineries in enterocytes under thalassemic conditions
2. To explore effects of different iron chelators, DFO and DFP, on the expression of iron-related molecules in the duodenum of *th3/+* mice
3. To study the response of duodenal iron regulatory mechanisms and hepcidin to parenteral iron loading and/or iron chelation under thalassemic conditions

Research methodology

Mice

Wild type C57BL/6J (WT) and *th3/+* (BKO) mice aged 7 weeks old were obtained from the Institute of Science and Technology for Research and Development, Mahidol University, Thailand. The mice were given water and rodent chow *ad libitum.* The temperature and humidity control were maintained at 25±2°C and 60±5%, respectively, with 12 hour light/dark cycle. The mice were intraperitoneally injected with iron dextran at the dosage of 5 mg iron once daily, for 2 consecutive days. After 2-week equilibration, the mice were treated with intraperitoneal DFO (125 mg/kg) or oral DFP (L1) (80 mg/kg) once daily for 14 days (n = 5-6 per group). Saline injection was used as control. The mice were sacrificed 24 hours after the last dose of the iron chelator by exsanguination. Blood samples were collected by cardiac puncture. Parts of the blood samples were sent for complete blood count. Serum samples were collected from the remaining blood samples by centrifugation. Duodenum, liver and spleen samples were collected and snapped frozen. Parts of the tissue samples were also fixed in formaldehyde for subsequent histological studies. The experiments protocols were approved by Mahidol University Animal Care and Use Committee (MU-ACUC).

Measurement of hematological parameters and serum iron

Complete blood count was performed in whole blood samples by Veterinary Technology Laboratory Diagnostic Service, Veterinary Technology, Kasetsart University, Thailand in order to explore hematological parameters. Serum iron was measured with a liquid ferrozine-based iron reagent (BioAssay Systems).

RNA extraction and complementary DNA (cDNA) synthesis

RNA was extracted from tissues using using TRIzol reagent (Invitrogen). The quality and quantity of the RNA was examined using a Nanodrop spectrophotometer. Complementary DNA was synthesized using commercial cDNA synthesis kit according to the manufacturer's protocol.

Quantitative real-time PCR

Quantitative real-time PCR was performed using SYBGR and CFX96 real-time PCR machine (Bio-Rad). Messenger RNA expression was normalized to mRNA expression of beta-actin as a housekeeping gene. The expression of the mRNA was presented as a fold change compared to control group using Livak's method (1).

Protein extraction and Western blot analysis

Membrane proteins were extracted as previously described (2). Protein quantification was performed using Lowry assay-based DC protein assay system (Bio-Rad) according to the manufacturer's protocol. Western blot analysis was performed using standard protocol. Anti-mouse MTP1 antibody (Alpha Diagnostic) and anti-actin antibody (Sigma Aldrich) were utilized to detect ferroportin and actin, respectively. Blot densitometry was obtained using ImageJ software (National Institutes of Health).

Tissue non-heme iron measurement

Tissue non-heme iron levels were determined by a modification of the method of Foy *et al* (3) as described by Simpson and Peters (4).

Histology

Tissue samples were fixed with 3.7% formaldehyde. The samples were gradually dehydrated through 70% ethanol, absolute ethanol and xylene, respectively, before embedding into paraffin blocks. Five-micron thin sections were prepared and stained with hematoxylin and eosin stain or Prussian blue stain for conventional histopathological study and qualitative iron determination, respectively. Slides were inspected and images were captured with a conventional light microscope.

Statistical analysis

Data are presented as mean ± SEM. Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test. A *P* value less than 0.05 was considered as significant. All statistical analyses were performed using Graphpad Prism 4 software (GraphPad Software Inc).

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Results

Determination of iron status

Liver non-heme iron measurement demonstrated a trend toward increased non-heme iron levels in BKO compared to WT mice under control condition, however, the difference was not statistically significant (figure 1). Notably, iron dextran injection resulted in dramatic increases in liver non-heme iron levels in both wild type and BKO mice. However, iron chelator administration had no effect on liver non-heme iron levels. Spleen non-heme iron measurement revealed a significant increase in non-heme iron levels in BKO compared to WT mice under control condition (figure 2). Interestingly, iron dextran injection was associated with increased spleen non-heme iron levels only in WT mice. In contrast, iron chelation (either by DFO or L1) significantly increased spleen non-heme iron levels in BKO mice but had no effect in WT mice.

In addition, the mRNA levels of transferrin receptor 1 (TfR1) were measured to explore tissue iron status according to the reciprocal relationship between TfR1 mRNA expression and cellular iron levels. In accordance with non-heme iron levels, TfR1 mRNA expression in the liver suggested for tissue iron loading upon iron dextran administration while liver iron status was not affected by phenotype and iron chelation (figure 3). Duodenenal TfR1 mRNA expression revealed no significant difference between any groups suggesting that duodenal iron status was not altered by thalassemia, iron loading or iron chelation (figure 4).

Histopathological study (Prussian blue staining) of liver, spleen and duodenum samples also supports the findings of tissue non-heme iron measurement and TfR1 mRNA expression (figure 5-7).

Measurement of hematological and iron parameters

Red blood cell parameters acquired from complete blood count revealed hypochromic, microcytic anemia in BKO mice compared to wild type mice thus confirming thalassemic phenotype in BKO mice (table 1). None of red blood cell parameters in BKO mice was affected by iron dextran or iron chelator administration.

Serum iron measurement showed that wild type and BKO mice in our study had comparable serum iron levels (figure 8). Interestingly, iron dextran administration significantly increased serum iron levels only in wild type mice. On the contrary, iron chelation, particularly DFO, was associated with significant increase in serum iron levels in BKO mice but had no effect in wild type mice.

The expression of hepcidin and its upstream regulators

The iron regulatory hormone, hepcidin, mRNA expression in the liver was determined by real-time PCR. As shown in figure 9, borderline effects of phenotype and iron treatment on hepcidin mRNA levels were noted, however, statistical significance was not reached. Additionally, hepcidin expression was not affected by iron chelation.

The expression of potential erythroid regulators; GDF15, TWSG1 and erythroferrone (ERFE), was also explored in the liver and spleen. In agreement with previous studies, murine GDF15 mRNA expression in both liver and spleen was unaffected in thalassemia, iron loading or iron chelation (figure 10, 13). In contrast, the expression of TWSG1 and ERFE appear to have tissue-specific responses. In the liver, TWSG1 expression did not differ between wild type and BKO mice. Iron loading significantly suppressed TWSG1 mRNA expression only in BKO mice (figure 11). On contrast, splenic TWSG1 mRNA levels were significantly higher in BKO compared to wild type mice but not altered by iron loading or chelation (figure 14). Similarly, liver ERFE mRNA expression was not affected by thalassemia, iron loading or iron chelation (figure 12) whereas splenic ERFE mRNA levels were significantly higher in BKO compared to wild type mice (figure 15).

The mRNA expression of major iron transport machineries in the duodenum

Real-time PCR revealed significant induction of Dcytb and hephaestin mRNA expression in the duodenum of BKO compared to WT mice (figure 16-17). Additionally, significant suppression of Dcytb and hephaestin mRNA expression by iron dextran treatment was detected in BKO mice. In contrast, no effect of phenotype, iron treatment and iron chelation on ferroportin and DMT1 mRNA expression was found (figure 18-20).

The mRNA expression of major iron transport machineries in the liver and spleen

Ferroportin mRNA levels in the liver and spleen were significantly higher in BKO compared to WT mice but no effect of iron loading or iron chelation was noted (figure 21-22). No effect of phenotype, iron treatment and iron chelation on liver and splenic DMT1 mRNA expression was found (figure 23-24).

Ferroportin protein expression

Western blot analysis was performed to explore ferroportin protein expression in the spleen and liver. As shown in figure 25, splenic ferroportin protein expression appears to be unaltered by thalassemia, iron loading or iron chelation although a marginally increased ferroportin protein levels was observed in L1-treated BKO mice.

In the liver, ferroportin protein expression appeared to be unaffected by thalassemia or parenteral iron loading although a trend toward reduced ferroportin protein levels can be observed in BKO mice as compared to wild type mice (figure 26). Interestingly, either DFO or L1 administration was associated with suppressed ferroportin protein expression particularly in wild type mice.

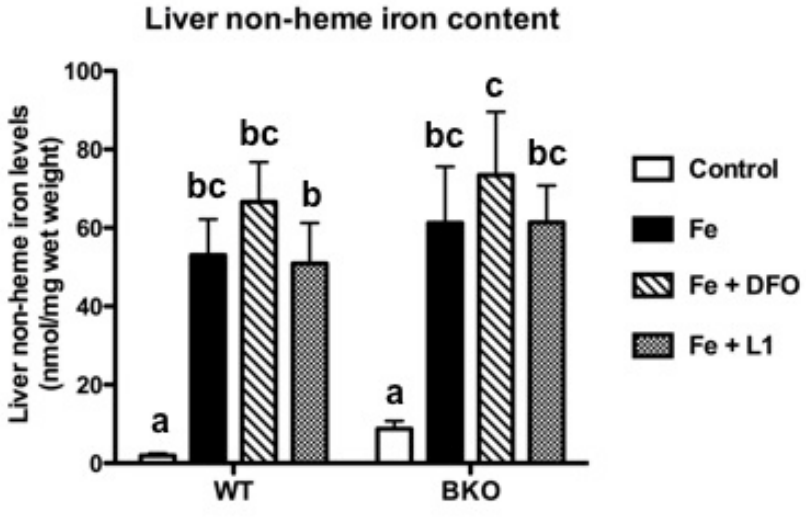

Figure 1 Liver non-heme iron levels in wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Data are presented as mean ± SEM (n = 5-6 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test. Means with different letters are statically different.

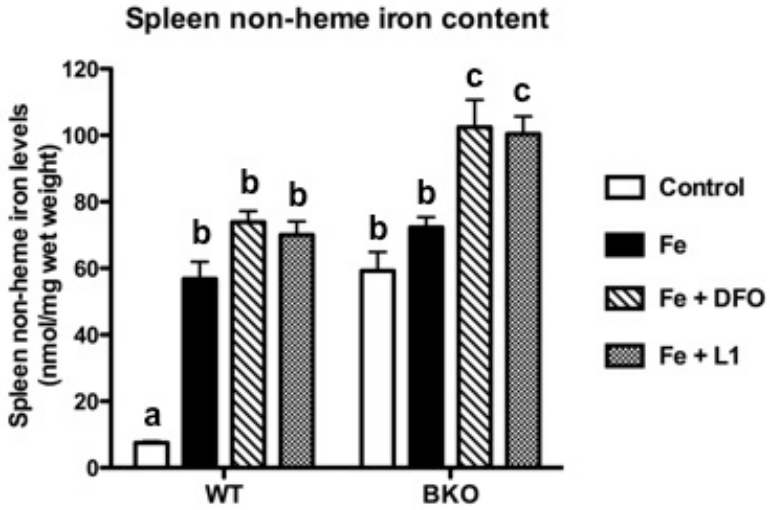

Figure 2 Spleen non-heme iron levels in wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Data are presented as mean ± SEM (n = 5-6 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test. Means with different letters are statically different.

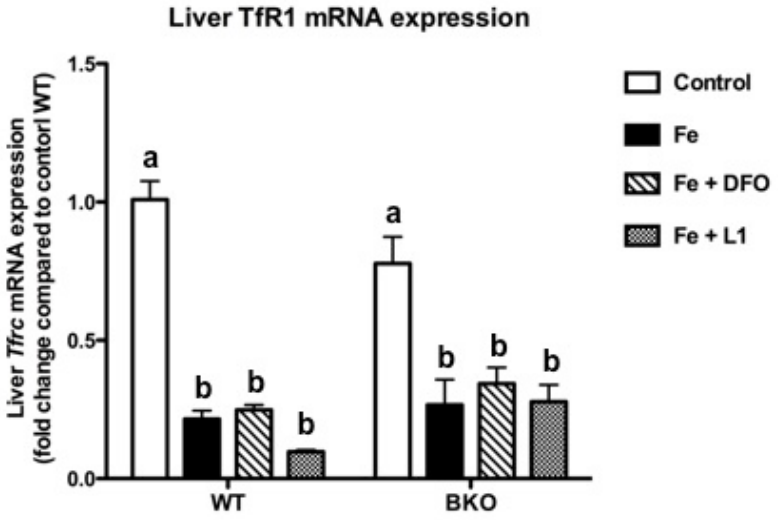

Figure 3 Liver transferrin receptor 1 (TfR1) mRNA expression in wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Gene expression was normalized with the expression of beta-actin (*Actb*) and reported as fold change compared to untreated wild type. Data are presented as mean ± SEM (n = 5-6 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test. Means with different letters are statically different.

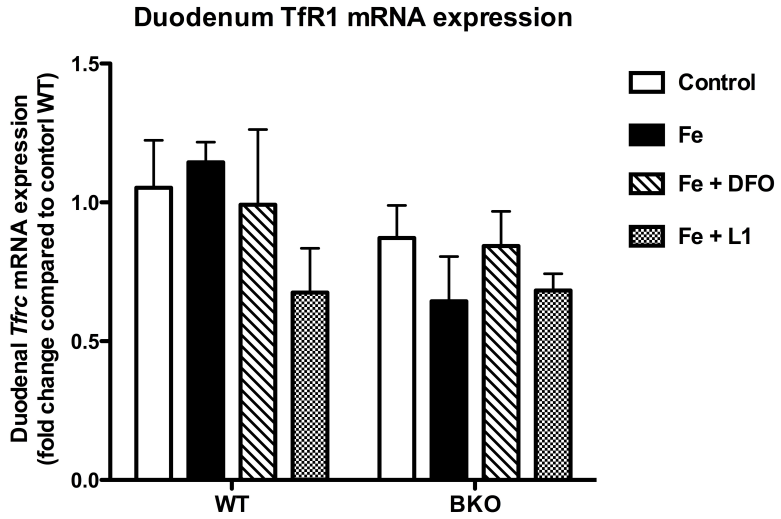

Figure 4 Duodenal transferrin receptor 1 (TfR1) mRNA expression in wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Gene expression was normalized with the expression of beta-actin (*Actb*) and reported as fold change compared to untreated wild type. Data are presented as mean ± SEM (n = 5-6 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test. Means with different letters are statically different.

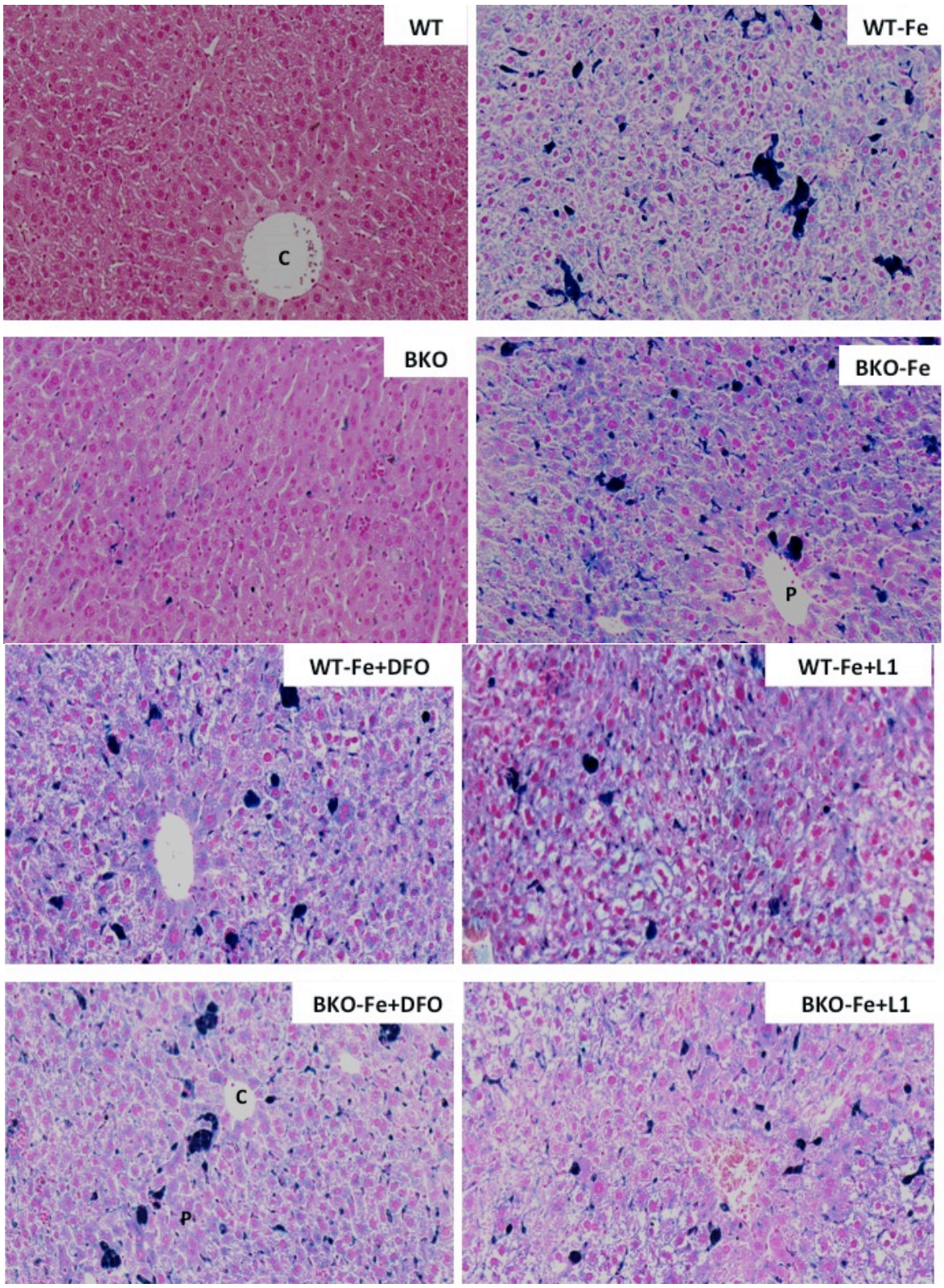

Figure 5 Prussian blue staining of representative paraffin-embedded liver sections from wild type (WT) and *th3/+* (BKO) mice treated with 10-mg iron through intraperitoneal iron dextran injection (Fe) followed by 125mg/kg deferoxamine (DFO) or 80 mg/kg deferiprone (L1) for 14 days. (x100 magnification, P and C indicate portal vein and central vein, respectively)

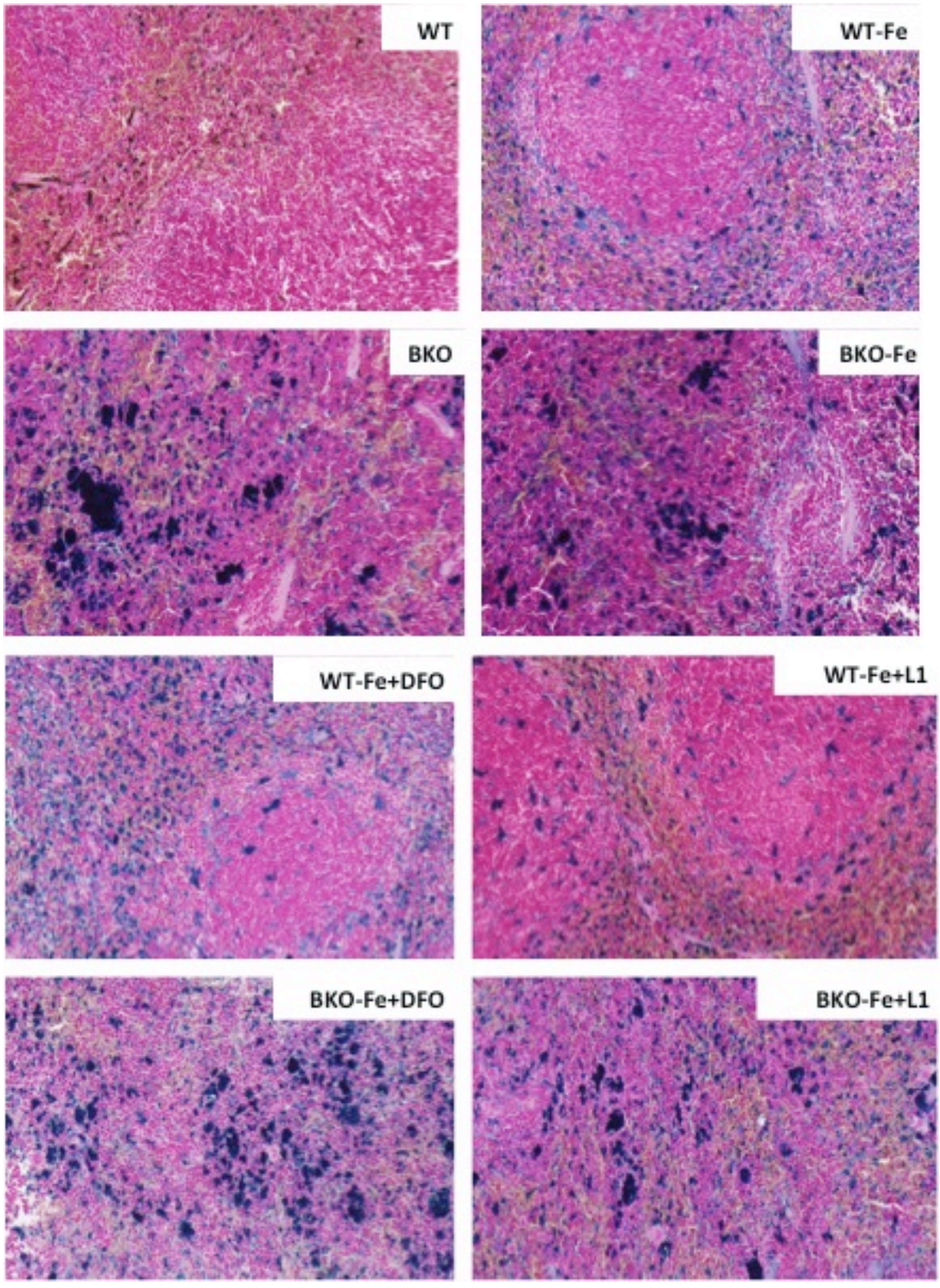

Figure 6 Prussian blue staining of representative paraffin-embedded spleen sections from wild type (WT) and *th3/+* (BKO) mice treated with 10-mg iron through intraperitoneal iron dextran injection (Fe) followed by 125mg/kg deferoxamine (DFO) or 80 mg/kg deferiprone (L1) for 14 days. (x100 magnification)

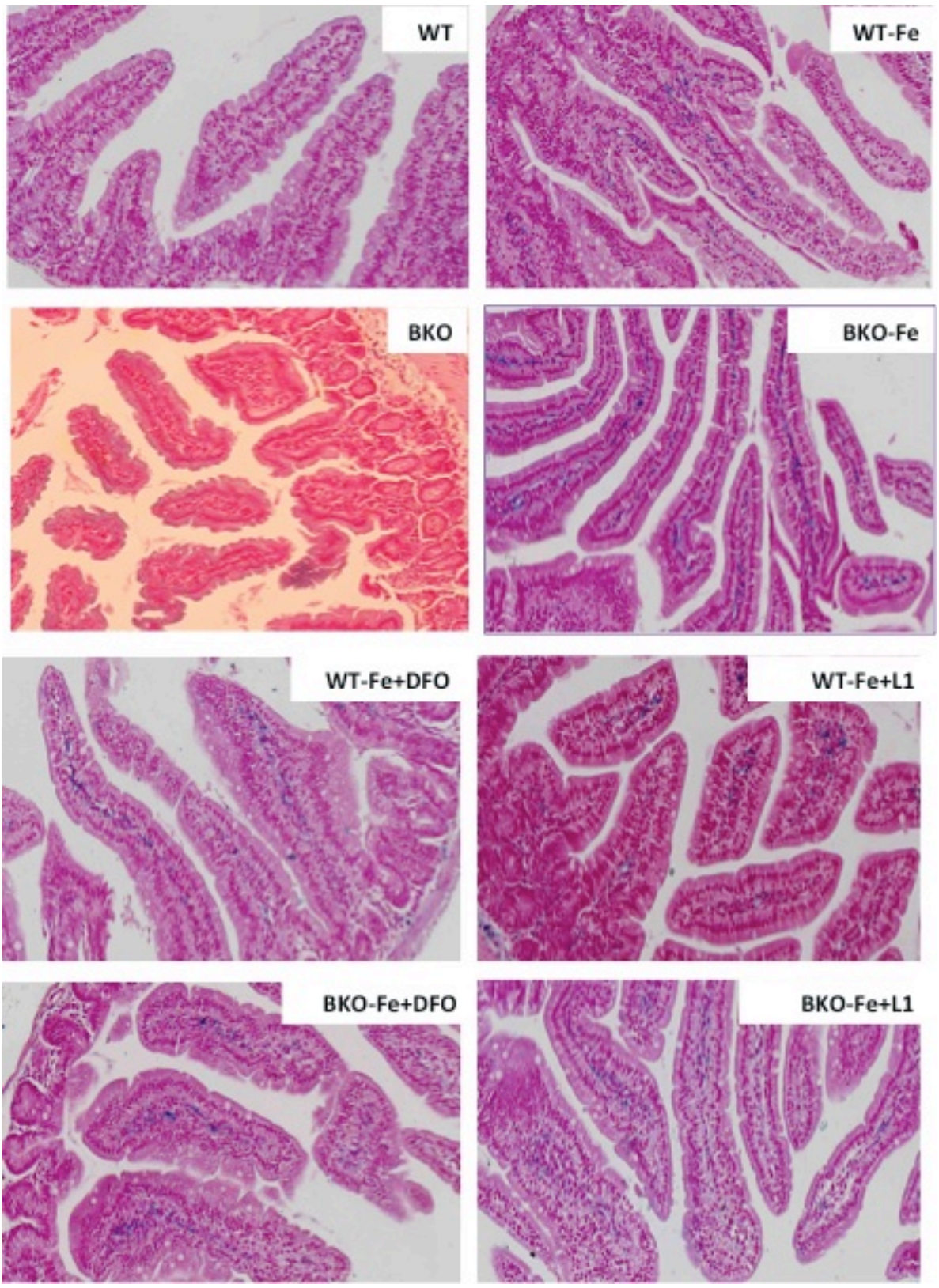

Figure 7 Prussian blue staining of representative paraffin-embedded duodenal sections from wild type (WT) and *th3/+* (BKO) mice treated with 10-mg iron through intraperitoneal iron dextran injection (Fe) followed by 125mg/kg deferoxamine (DFO) or 80 mg/kg deferiprone (L1) for 14 days. (x100 magnification)

Table 1 Red blood cell parameters of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Data are presented as mean ± SD (n = 5-6 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test.

	RBC ($x 10^6$cells/µL)	Hb (g/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	RDW (%)
Wild type							
- Control	8.91 ± 0.78	14.38 ± 1.45	44.66 ± 3.13	50.16 ± 1.05	16.12 ± 0.33	32.14 ± 1.17	12.88 ± 0.11
- Iron	6.25 ± 1.42	10.36 ± 2.45	33.82 ± 8.12	54.00 ± 1.37	16.56 ± 0.30	30.66 ± 0.23	14.92 ± 0.44
- Iron + DFO	5.87 ± 2.05	9.44 ± 3.48	31.14 ± 11.37	52.70 ± 1.75	15.94 ± 0.05	30.26 ± 0.50	13.70 ± 0.40
- Iron + L1	6.54 ± 1.62	10.73 ± 2.57	34.78 ± 8.97	53.02 ± 1.04	16.48 ± 0.60	31.07 ± 1.28	15.00 ± 1.30
BKO							
- Control	4.68 ± 1.48**	5.68 ± 2.00***	19.18 ± 5.67***	41.40 ± 4.19**	12.24 ± 2.80*	29.34 ± 3.99	27.28 ± 7.34**
- Iron	3.70 ± 1.64***	4.40 ± 1.61***	16.04 ± 6.25***	44.34 ± 5.93	12.38 ± 2.48*	27.84 ± 2.74	28.10 ± 8.55***
- Iron + DFO	4.49 ± 2.31**	5.60 ± 3.79***	20.40 ± 11.45**	45.08 ± 4.67	11.74 ± 2.55**	25.90 ± 3.41**	28.62 ± 7.58***
- Iron + L1	5.80 ± 0.99	6.30 ± 1.07***	24.26 ± 3.66**	41.96 ± 1.75**	10.86 ± 0.33***	25.96 ± 1.16**	32.42 ± 2.51***

* $P < 0.5$, ** $P < 0.01$, *** $P < 0.001$ compared to control wild type

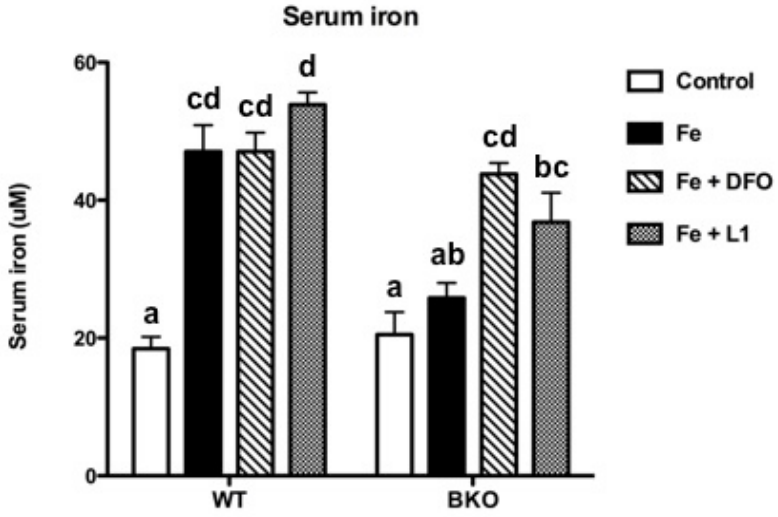

Figure 8 Serum iron of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Data are presented as mean ± SEM (n = 5 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test. Means with different letters are statically different.

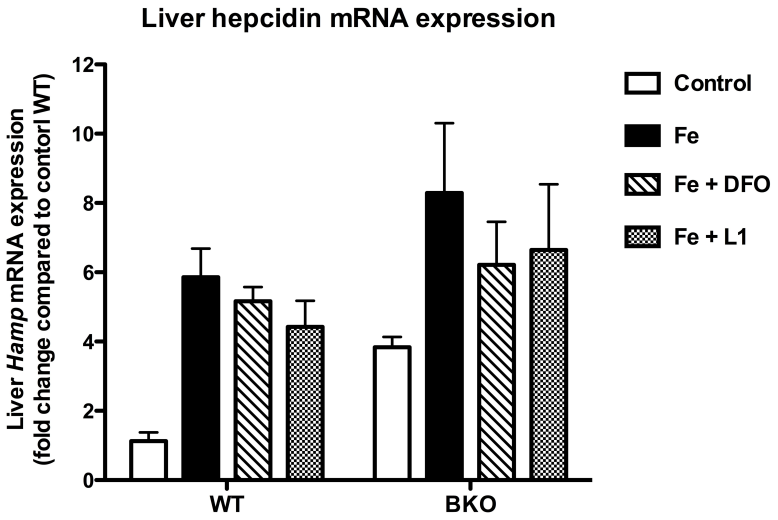

Figure 9 Liver hepcidin mRNA expression in wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Gene expression was normalized with the expression of beta-actin (*Actb*) and reported as fold change compared to untreated wild type. Data are presented as mean ± SEM (n = 4-6 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test.

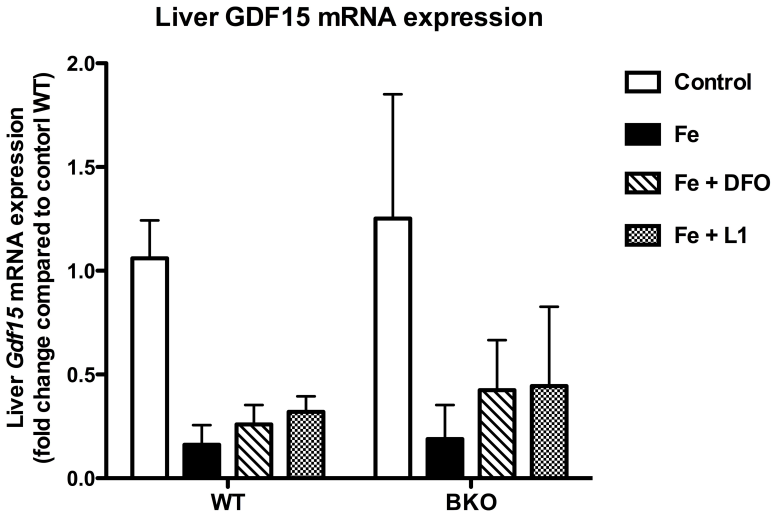

Figure 10 Growth differentiation factor 15 (GDF15) mRNA expression in the liver of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Gene expression was normalized with the expression of beta-actin (*Actb*) and reported as fold change compared to untreated wild type. Data are presented as mean ± SEM (n = 5-6 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test.

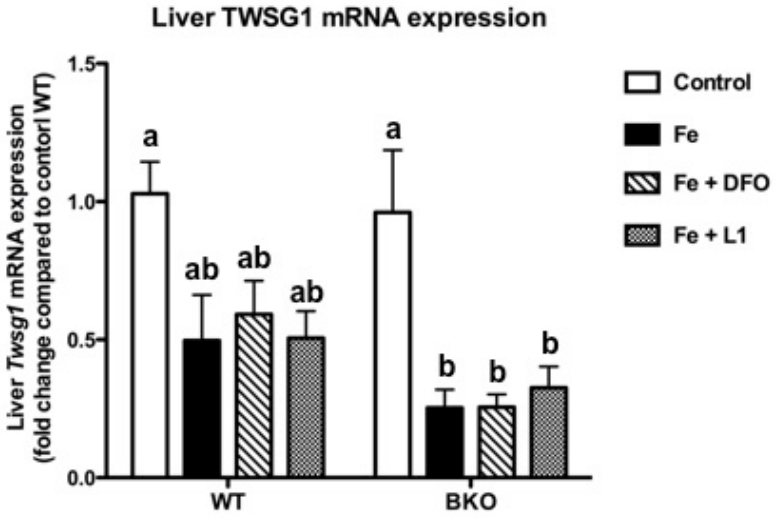

Figure 11 Twisted gastrulation 1 (TWSG1) mRNA expression in the liver of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Gene expression was normalized with the expression of beta-actin (*Actb*) and reported as fold change compared to untreated wild type. Data are presented as mean ± SEM (n = 4-6 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test. Means with different letters are statically different.

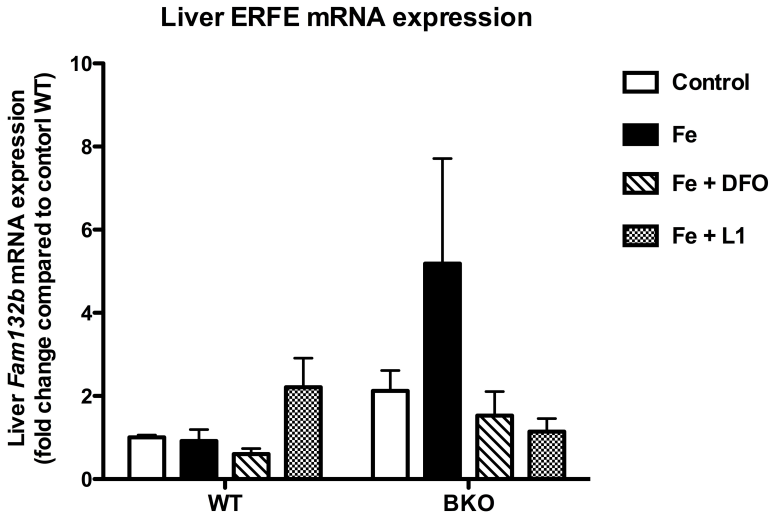

Figure 12 Erythroferrone (ERFE) mRNA expression in the liver of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Gene expression was normalized with the expression of beta-actin (*Actb*) and reported as fold change compared to untreated wild type. Data are presented as mean ± SEM (n = 4-6 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test.

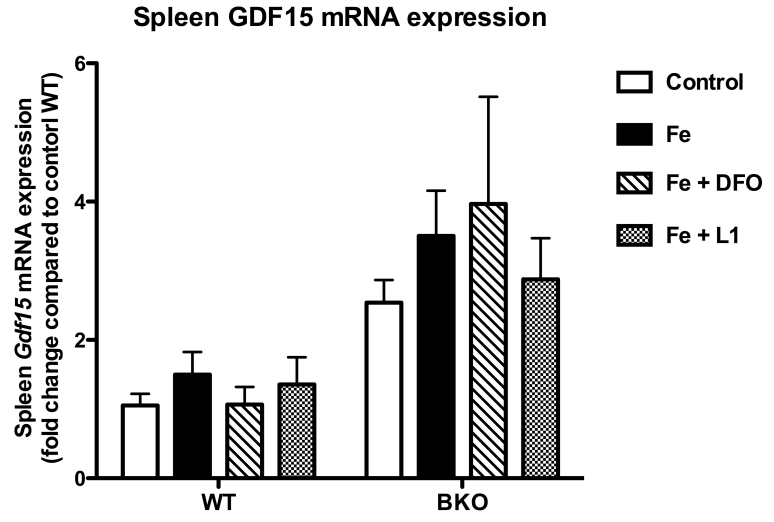

Figure 13 Growth differentiation factor 15 (GDF15) mRNA expression in the spleen of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Gene expression was normalized with the expression of beta-actin (*Actb*) and reported as fold change compared to untreated wild type. Data are presented as mean ± SEM (n = 4-6 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test.

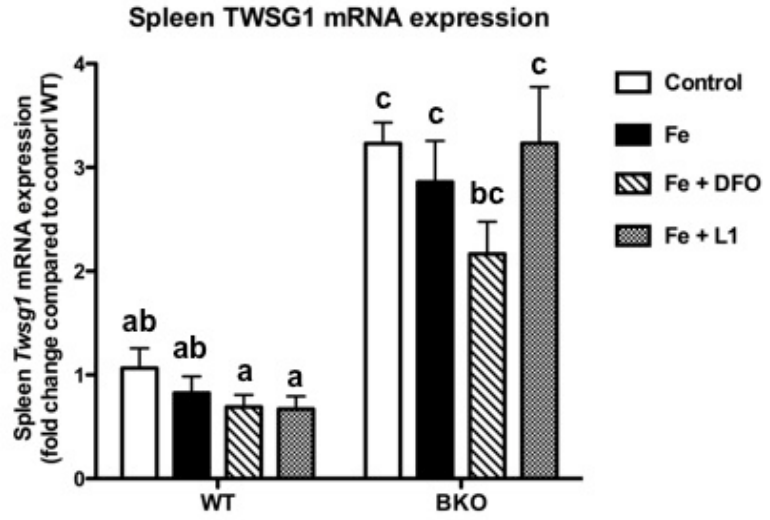

Figure 14 Twisted gastrulation 1 (TWSG1) mRNA expression in the spleen of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Gene expression was normalized with the expression of beta-actin (*Actb*) and reported as fold change compared to untreated wild type. Data are presented as mean ± SEM (n = 5-6 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test. Means with different letters are statically different.

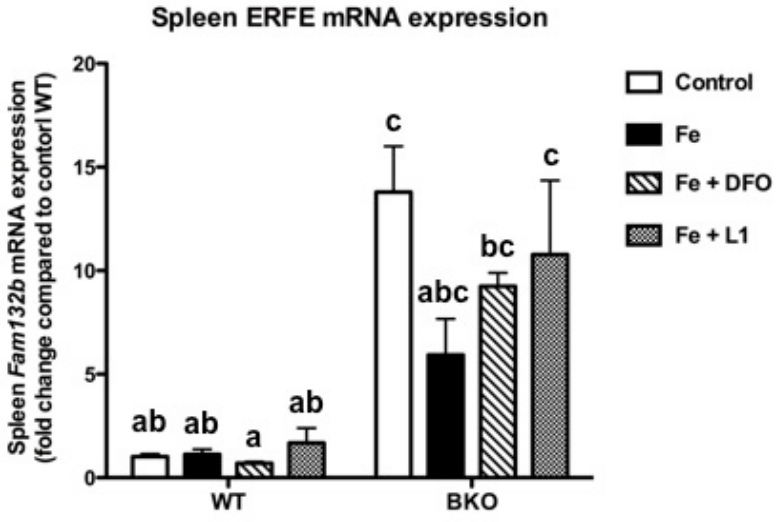

Figure 15 Erythroferrone (ERFE) mRNA expression in the spleen of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Gene expression was normalized with the expression of beta-actin (*Actb*) and reported as fold change compared to untreated wild type. Data are presented as mean ± SEM (n = 4-6 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test.

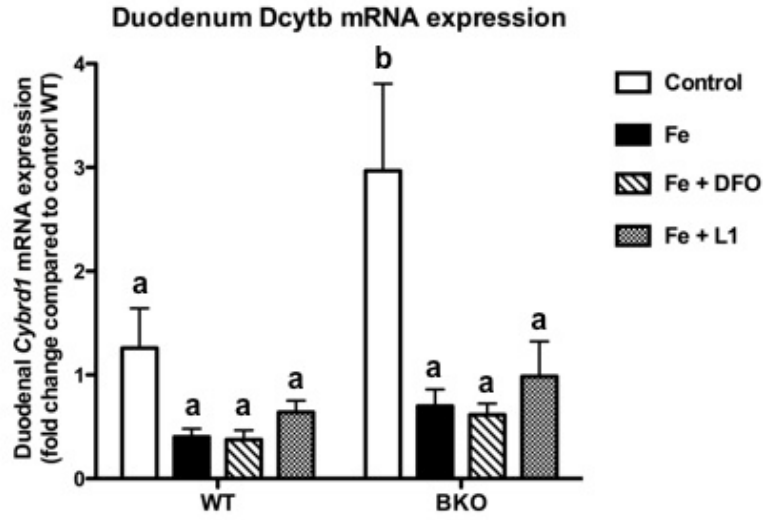

Figure 16 Duodenal cytochrome b (Dcytb) mRNA expression in the duodenum of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Gene expression was normalized with the expression of beta-actin (*Actb*) and reported as fold change compared to untreated wild type. Data are presented as mean ± SEM (n = 4-5 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test. Means with different letters are statically different.

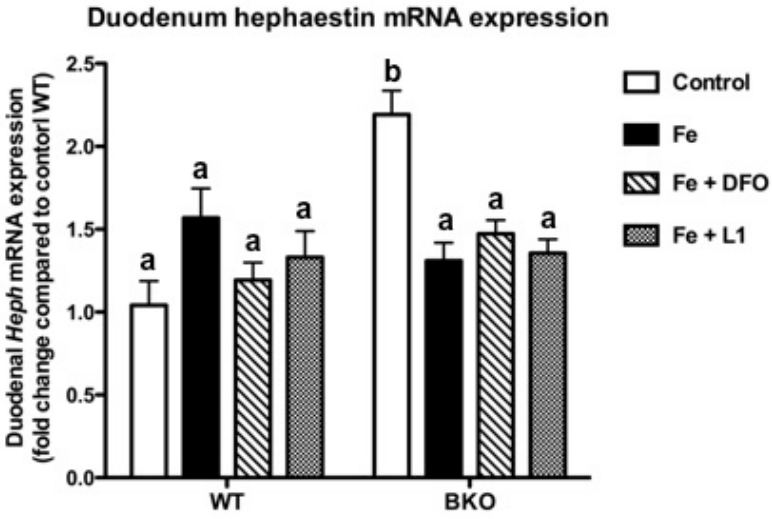

Figure 17 Hephaestin mRNA expression in the duodenum of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Gene expression was normalized with the expression of beta-actin (*Actb*) and reported as fold change compared to untreated wild type. Data are presented as mean ± SEM (n = 4-5 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test. Means with different letters are statically different.

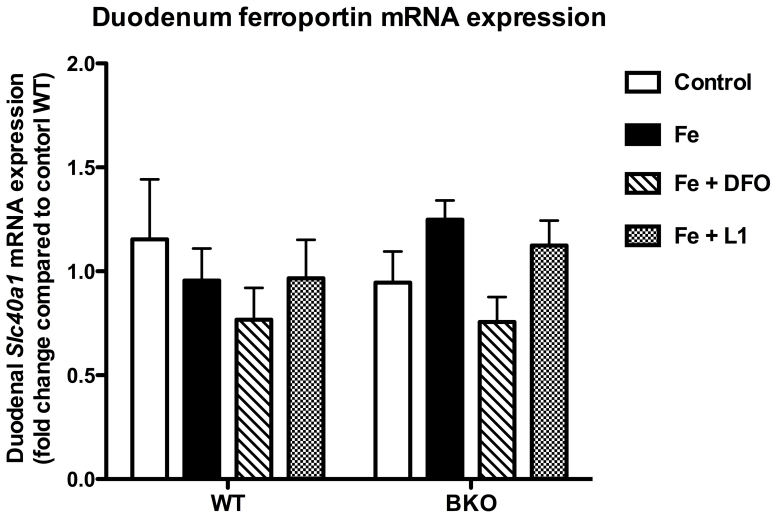

Figure 18 Ferroportin mRNA expression in the duodenum of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Gene expression was normalized with the expression of beta-actin (*Actb*) and reported as fold change compared to untreated wild type. Data are presented as mean ± SEM (n = 4-5 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test.

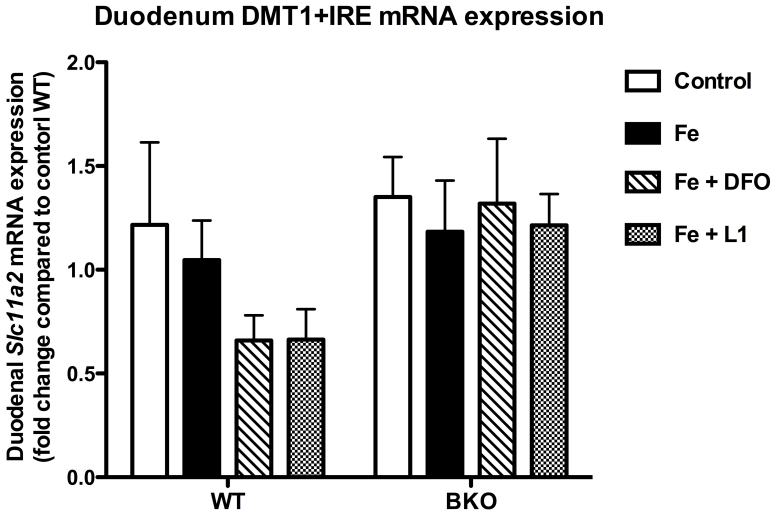

Figure 19 Divalent metal transporter 1, IRE-containing isoform, (DMT1+IRE) mRNA expression in the duodenum of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Gene expression was normalized with the expression of beta-actin (*Actb*) and reported as fold change compared to untreated wild type. Data are presented as mean ± SEM (n = 4-5 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test.

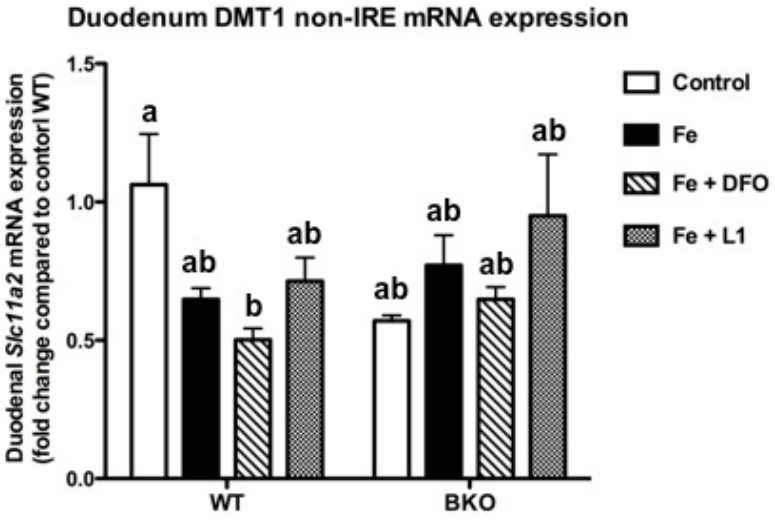

Figure 20 Divalent metal transporter 1, non IRE-containing isoform, mRNA expression in the duodenum of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Gene expression was normalized with the expression of beta-actin (*Actb*) and reported as fold change compared to untreated wild type. Data are presented as mean ± SEM (n = 4-5 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test. Means with different letters are statically different.

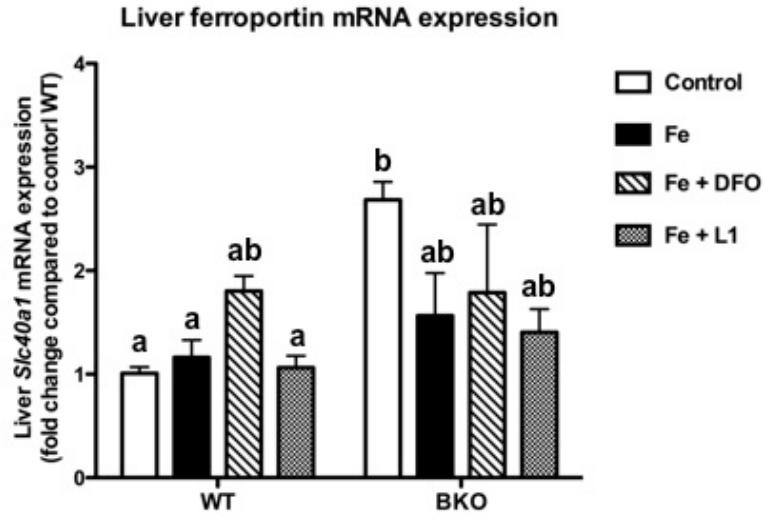

Figure 21 Ferroportin mRNA expression in the liver of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Gene expression was normalized with the expression of beta-actin (*Actb*) and reported as fold change compared to untreated wild type. Data are presented as mean ± SEM (n = 4-5 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test. Means with different letters are statically different.

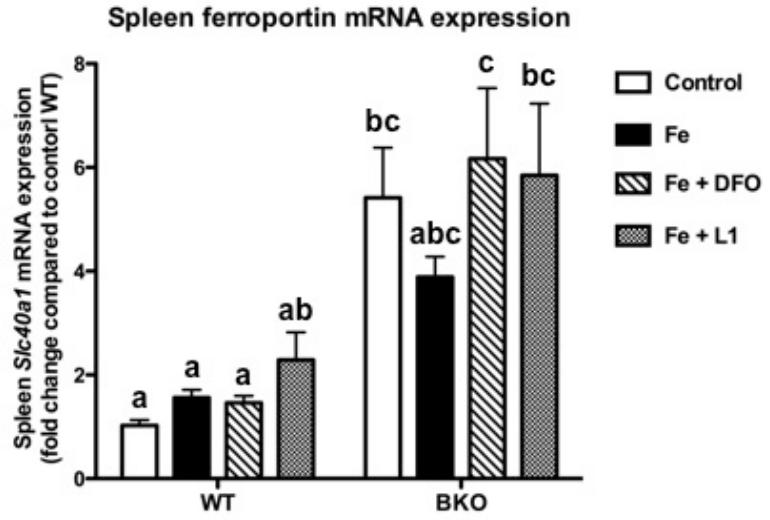

Figure 22 Ferroportin mRNA expression in the spleen of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Gene expression was normalized with the expression of beta-actin (*Actb*) and reported as fold change compared to untreated wild type. Data are presented as mean ± SEM (n = 4-5 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test. Means with different letters are statically different.

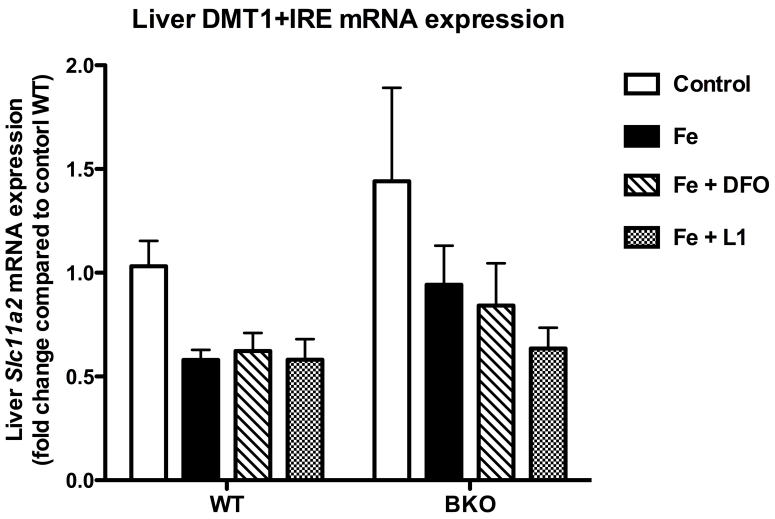

Figure 23 Divalent metal transporter 1, IRE-containing isoform, (DMT1+IRE) mRNA expression in the liver of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Gene expression was normalized with the expression of beta-actin (*Actb*) and reported as fold change compared to untreated wild type. Data are presented as mean ± SEM (n = 4-5 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test.

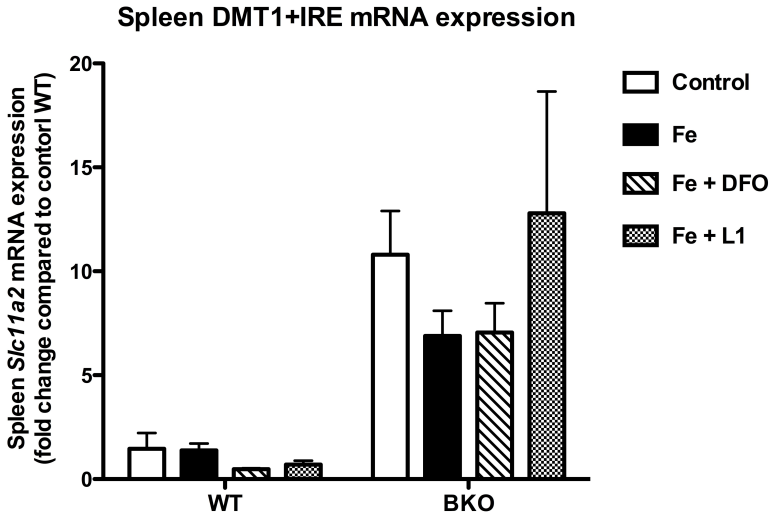

Figure 24 Divalent metal transporter 1, IRE-containing isoform, (DMT1+IRE) mRNA expression in the spleen of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution. Gene expression was normalized with the expression of beta-actin (*Actb*) and reported as fold change compared to untreated wild type. Data are presented as mean ± SEM (n = 4-5 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test.

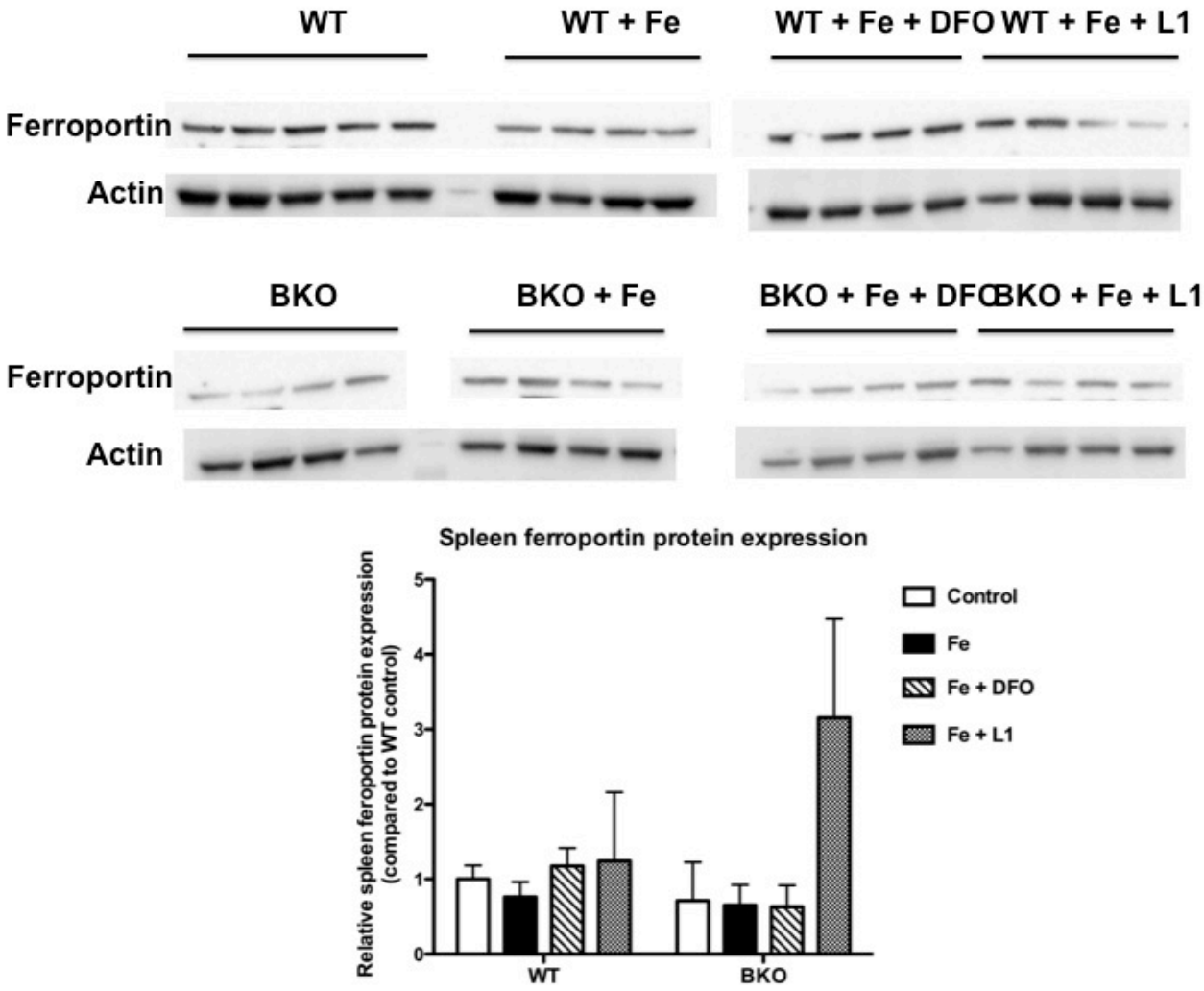

Figure 25 Ferroportin protein expression in the spleen of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution was determined by Western blot analysis. Band intensity was quantified by densitometry using ImageJ software. Ferroportin expression was normalized to the expression of β-actin and presented as relative expression compared to WT control. Data are presented as mean ± SEM (n = 4-5 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test.

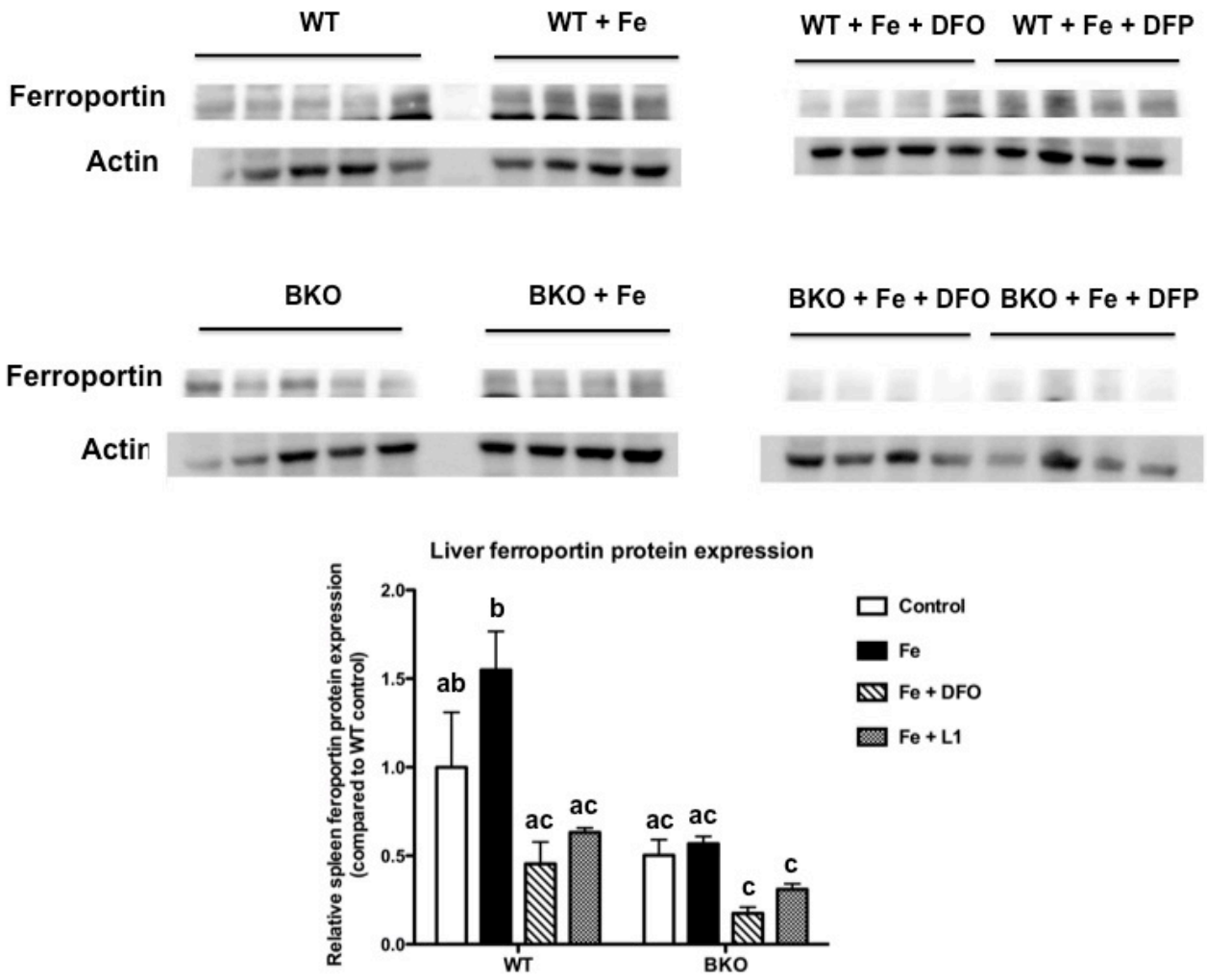

Figure 26 Ferroportin protein expression in the liver of wild type (WT) and thalassemia (BKO) mice treated with 10 mg iron dextran (Fe) followed by 14 doses of DFO (125 mg/kg body weight), L1 (80 mg/kg body weight) or equal volume of saline solution was determined by Western blot analysis. Band intensity was quantified by densitometry using ImageJ software. Ferroportin expression was normalized to the expression of β-actin and presented as relative expression compared to WT control. Data are presented as mean ± SEM (n = 4 per group). Statistical analysis was performed by one-way ANOVA with Tukey's multiple comparison test. Means with different letters are statically different.

Conclusion and Discussion

In the present study, thalassemia phenotype was confirmed through complete blood count and histopathological study. Parenteral iron loading in both wild type and BKO mice was indicated by parenchymal iron accumulation in the liver as evidenced through non-heme iron measurement, Prussian blue staining and TfR1 mRNA expression. However, tissue iron status in the liver was unaltered by iron chelation suggesting that our chelation regimen was insufficient to alleviate iron loading in this organ. It is noteworthy that duodenal iron status as determined by TfR1 mRNA expression was similar between wild type and BKO mice. Moreover, this parameter was also unaffected by iron treatment or iron chelation. Notably, parenteral iron administration resulted in a significant increase in serum iron and spleen non-heme iron levels only in wild type mice. According to the aforementioned data, iron dextran injection resulted in iron accumulation in all compartments (serum, liver and spleen) in wild type mice whereas only liver was affected in BKO mice. Interestingly, iron chelation, either by DFO or L1, significantly increased serum iron and spleen non-heme iron levels in BKO mice suggesting that inadequate iron chelation not only failed to reduce the extent of iron overload but could also aggravated such condition in these mice. These findings demonstrate different ferrokinetics in response to iron loading or iron chelation between wild type and BKO mice.

The expression of iron regulatory hormone, hepcidin, was explored to delineate such differential responses. No significant difference in hepcidin mRNA expression was noted across all groups suggesting that the mechanisms underlying these difference ferrokinetics are hepcidin-independent. It is also possible that the lack of statistical significance of hepcidin expression could be due to small sample size and/or large individual variation. Notably, Twsg1 and Erfe, upstream hepcidin regulators, were upregulated in the spleen on BKO mice which are in accordance with previous reports. Although these potential erythroid regulators were markedly induced, hepcidin expression was not significantly altered indicating that Twsg1 or Erfe are not the ultimate hepcidin regulator which can override store regulators as previously proposed. Indeed, hepcidin regulation should reflect net effects from signals of different regulators depending on the strength of each particular signals.

The expression of iron transporters in three key tissues (duodenum, liver and spleen) was determined. Under control condition, duodenal mRNA expression of Dcytb and hephaestin was significantly induced in BKO mice. Notably, iron dextran injection significantly suppressed Dcytb and hephaestin mRNA expression in BKO mice corresponding to the unchanged serum iron levels in these mice. Therefore, differential duodenal iron absorption should be responsible for phenotype-specific responses to iron loading in our study. However, it is not responsible for the response to iron chelation as none of duodenal iron transporter mRNA expression was affected by DFO or L1. The mRNA expression of

DMT1, an iron uptake molecule, in the liver and spleen was unaffected by thalassemia phenotype, iron loading and iron chelation. Interestingly, decreased ferroportin protein expression was noted upon iron chelator administration despite unaltered mRNA expression suggesting that ferroportin was regulated through post-transcriptional and/or post-translational mechanism(s) in hepcidin-independent manner. However, ferroportin expression was not responsible for the differential response to iron chelation between wild type and BKO mice as no change in ferroportin protein levels was noted in the spleen despite splenic iron accumulation in BKO mice treated with iron chelator. Therefore, the mechanism(s) underlying phenotype-specific response to iron chelation remains to be elucidated.

In conclusion, the present study underscores differential iron homeostasis in response to parenteral iron loading and iron chelation between wild type and thalassemia mice. Such differential responses should be mediated through tissue-specific mechanisms which are hepcidin-independent. Furthermore, our results suggest that inadequate iron chelation in thalassemic condition might result in increased iron retention in the spleen and higher serum iron levels through yet unknown mechanisms.

Appendix

Output (Acknowledge the Thailand Research Fund)

8.1 International Journal Publication

N/A

8.2 Application

N/A

8.3 Others e.g. national journal publication, proceeding, international conference, book chapter, patent

- P. Masaratana, C. Sanyear, W. Eamsaard, S. Svasti, S. Fucharoen. Effects of parenteral iron loading and/or iron chelation on the expression of duodenal iron transport machineries in thalassemia mice. The 6th Congress of the International Bioiron Society. September 6-10, 2015