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In iron-deficient mouse pregnancies, erythroferrone contributes to iron mobilization for embryo erythropoiesis

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Abstract

Erythroferrone (ERFE) is an erythroblast-secreted regulator of iron metabolism. The production of ERFE increases during stress erythropoiesis, leading to decreased hepcidin expression and mobilization of iron. Pregnancy requires a substantial increase in iron availability to sustain maternal erythropoietic expansion and fetal development and is commonly affected by iron deficiency. To define the role of ERFE during iron-replete or iron-deficient pregnancy, we utilized mouse models expressing a range of ERFE levels: transgenic mice overexpressing ERFE (TG), wild-type (WT) and ERFE knockout (KO) mice. We altered maternal iron status using diets with low or standard iron content and performed analysis at E18.5.

Iron deficiency increased maternal ERFE in WT pregnancy. Comparing different maternal genotypes, ERFE TG dams had lower hepcidin relative to their liver iron load but similar hematological parameters to WT dams on either diet. In ERFE KO dams, most hematologic and iron parameters were comparable to WT, but MCV was decreased under both iron conditions. Similar to dams, TG embryos had lower hepcidin on both diets, but their hematologic parameters did not differ from those of WT embryos. ERFE KO embryos had lower MCV than WT embryos on both diets. The effect was exacerbated under iron-deficient conditions where ERFE KO embryos had higher hepcidin, lower Hb and Hct and lower brain iron concentration compared to WT embryos, indicative of iron restriction.

Thus, under iron-deficient conditions, maternal and embryo ERFE facilitate iron mobilization for embryonic erythropoiesis.

Visual abstract text

Iron is necessary for a healthy pregnancy. We studied the role of the iron-regulatory hormone erythroferrone (ERFE) in pregnancy by comparing outcomes in mice with varying levels of ERFE. When dams were iron-deficient, embryo ERFE ensured embryo hepcidin was suppressed to facilitate iron delivery to the brain and for production of red blood cells to carry oxygen to tissues.
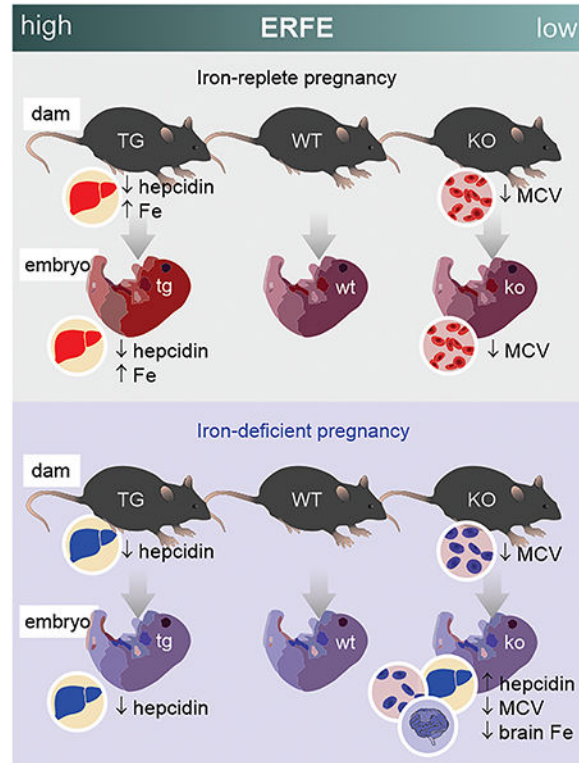
Keywords

hepcidin; erythroferrone; pregnancy; iron deficiency

INTRODUCTION

Iron is an essential micronutrient required for a variety of metabolic and cellular processes ranging from mitochondrial function to hemoglobin synthesis. Iron deficiency (ID) is the most prevalent micronutrient deficiency in the world and the most common nutrient deficiency in the United States[1]. During pregnancy, iron demands increase because of maternal erythropoietic expansion, placental growth, and fetal development[2]. Maternal iron deficiency can result in iron deficiency anemia (IDA), which has been associated with adverse pregnancy outcomes including preterm delivery, low birthweight, impaired immune function, and long-term neurocognitive and behavioral consequences[3–7]. A greater understanding of iron regulation across gestation is needed to improve assessment of women at risk of iron deficiency and to optimize treatment approaches.

Iron homeostasis is maintained by the hormone hepcidin. Hepcidin, a peptide produced primarily in the liver, occludes ferroportin (FPN), the only known mammalian cellular iron exporter, and triggers its proteolysis. Loss of ferroportin activity decreases plasma iron levels by blocking dietary iron absorption by enterocytes and the release of iron stores from macrophages and hepatocytes[8–10]. Consequently, physiological suppression of maternal hepcidin observed during pregnancy acts to increase dietary iron absorption and mobilization of iron from stores to support increased iron needs in pregnancy[11].

Expansion of maternal red blood cells and de novo synthesis of fetal red blood cells during pregnancy resembles the increased erythroid output of stress erythropoiesis in other settings. Moreover, ID and IDA are prevalent during pregnancy, further stressing maternal and fetal erythropoiesis. Coordination of iron supply during stress erythropoiesis is achieved by the interplay between the hormones erythropoietin (EPO), erythroferrone (ERFE) and hepcidin (HAMP)[12, 13]. ERFE was first identified in 2014 as an erythropoietic regulator of hepcidin[12]. Produced by erythroid precursors in response to EPO stimulation, ERFE sequesters bone morphogenetic proteins (BMPs) and inhibits their signaling, thus reducing BMP-stimulated hepcidin production by hepatocytes[12, 14]. In both humans and animal models, ERFE production increases in conditions associated with accelerated erythropoietic activity including after phlebotomy or EPO administration, in IDA or anemias of ineffective erythropoiesis[15]. By suppressing hepcidin, ERFE enhances iron delivery into the circulation to support increased erythrocyte production[15]. Because ERFE and iron loading have opposing effects on the BMP pathway signaling, ERFE alters the relationship between liver iron concentration and hepcidin expression.

Much less is known about the regulation of ERFE production in pregnancy and its role in pregnancy iron homeostasis, including when IDA is present. In normal human pregnancy, maternal ERFE was detected at midgestation and delivery, correlated with erythropoietic drive, and was higher in anemic pregnancies[16]. ERFE was also detected in cord blood at delivery and correlated with fetal soluble transferrin receptor, ferritin and hemoglobin[17]. Cord blood ERFE was higher than maternal ERFE, suggesting that fetal ERFE may also play a role in modulating fetal iron homeostasis and erythropoiesis[17]. In complicated human pregnancy, specifically in spontaneous abortions, maternal serum ERFE levels were reduced compared to normal pregnancies[18]; although, causation is unknown. These studies suggest that ERFE may play a role in pregnancy iron homeostasis, particularly in pregnancies affected by IDA.

To define the contribution of maternal, embryo and placental ERFE on maternal and fetal iron homeostasis and erythropoiesis during pregnancy, we used mouse models with a range of ERFE concentrations: *Erfe*-overexpressing transgenic (TG), wild-type (WT) and *Erfe* knockout (KO) mice and performed a comprehensive analysis of iron and hematological parameters in dams, placentae and embryos, both in the setting of iron-replete as well as iron-deficient pregnancy.

MATERIALS AND METHODS

Mice

Experiments were conducted in accordance with guidelines by the Animal Research Committee and were approved by the University of California, Los Angeles (UCLA). ERFE transgenic (*Erfe* TG) mice were generated in our lab[19] on a C57BL/6N background and backcrossed onto a C57BL/6 background. ERFE knock-out (*Erfe* KO/*Fam123b* KO) were originally obtained from the UC Davis Mutant Mouse Resource & Research Center and maintained in our lab on a C57BL/6 background. *Mice* were fed ad libitum a purified iron-replete diet (100ppm, TD.200065) or iron-deficient diet (4ppm, TD.80396) from Envigo Teklad. Experimental animals were euthanized by isoflurane inhalation.

Erfe KO mice (*Fam132b*$^{-/-}$) genotyping was performed via PCR using ear punch DNA isolated with the Gentra Puregene Core Kit A (Qiagen). The PCR reaction was run using GoTaq G2 Green Master Mix (Promega). Genotyping primers and product sizes are listed in Supplemental Table 2. PCR products were visualized on 1.5% agarose gels with DNA Safestain (Lamda Biotech, C138). PCR conditions for genotyping: *Erfe* Tg: 1 cycle of 94°C for 3 min; 30 cycles of 94°C for 30s, 60°C for 30s, and 72°C for 1 min; and 1 cycle of 72°C for 2 min. *Erfe* KO: 1 cycle of 95°C for 3 min; 30 cycles of 95°C for 30s, 60°C for 30s, and 72°C for 30s; and 1 cycle of 72°C for 5 min.

Quantitative PCR

Tissue samples were homogenized in TRIzol reagent in accordance with the manufacturer's instructions (Invitrogen). Total RNA was isolated by chloroform extraction and cDNA was subsequently synthesized using the iScript cDNA Synthesis Kit (Bio-Rad). Quantitative real-time PCR was then performed using SsoAdvanced SYBR Green Supermix (Bio-Rad) on a CFX96 Connect Real-Time PCR Detection System (Bio-Rad). All samples were run in duplicate and normalized to *Hprt*. Primer sequences and product sizes are listed in Supplemental Table 3.

Western blot

Mouse tissue lysates were obtained by homogenizing tissues in the RIPA buffer lysis system, (Santa Cruz Biotechnology, sc-24948), tissue lysate homogenate was cleared by centrifugation at 21,000 *x g* for 15 min at 4°C. Protein concentration was determined using a BCA Protein Assay Kit (ThermoFisher Pierce™ #23227), resolved on either 4–20% Mini-PROTEAN TGX gels or Criterion™ TGX 26-well gels (Bio-Rad), electroblotted onto nitrocellulose membrane using a Trans-Blot Turbo system (Bio-Rad) and visualized on a ChemiDoc XRS+ (Bio-Rad). Samples were run on two blots: samples for FPN were prepared in Laemmli buffer without reducing agent and not boiled; samples for TFR1 and Ferritin heavy chain were prepared in Laemmli buffer with 0.1M DTT and incubated at 100°C for 5 min. β-Actin was used as a loading control for each blot. Quantitation with normalization to β-Actin was performed using Image Lab Software, version 6.1.0 (Bio-Rad). Antibodies: rat anti-mouse monoclonal 1C7 ferroportin antibody (kindly provided by Amgen), donkey pAb to rat IgG HRP (ab102265, AbCam), mouse monoclonal transferrin receptor antibody (H68.4, ThermoFisher), rabbit anti-human ferritin heavy chain

mAb (clone D1D4) (4393S, Cell Signaling), monoclonal anti-β-Actin-peroxidase (A3854, Sigma), anti-mouse IgG HRP (367076S, Cell Signaling), and anti-rabbit IgG HRP (7074S, Cell Signaling).

Non-Heme Iron Concentration Measurements

Non-heme iron concentrations were measured as previously described[20] using acid treatment followed by colorimetric assay for iron quantification (Iron-SL 157–30, Sekisui Diagnostics). Serum was obtained from maternal and fetal mouse clotted whole blood by centrifugation at 3,300 g for 10 minutes. For embryo tissue iron measurements, approximately 90% of the embryo liver, half of each placenta, and the entire brain were used for the assay. The carcasses used for iron measurements were exsanguinated with liver and brain removed.

Complete Blood Counts (CBCs)

Blood was drawn from euthanized mice by cardiac puncture into K_2EDTA-containing tubes. Blood counts were performed on a Hemavet 950FS hematology system (Drew Scientific).

Enzyme-linked immunosorbent assays (ELISA)

Serum hepcidin was measured as previously described[12]. Serum ERFE was measured by ELISA according to the manufacturer's instructions (Intrinsic Lifesciences, ERF-200). Serum EPO was measured using a mouse EPO quantikine ELISA kit according to the manufacturer's instructions (R&D Systems, MEP00B).

Statistical analysis

Statistical analysis was performed using the statistical package included with GraphPad Prism 9. A Q or P value less than 0.05 was considered statistically significant. Statistical differences between predetermined groups were determined using two-tailed, unpaired Student's t test for normally distributed values; Mann-Whitney U test for non-normally distributed values; one-way ANOVA followed by the Holm-Sidak method for multiple comparisons for normally distributed values; one-way ANOVA on ranks followed by Dunn's multiple comparison test for non-normally distributed values; or two-way ANOVA followed by two-stage set-up method of Benjamini, Krieger and Yekutieli with false discovery rate (FDR) set at 5% to correct for multiple comparisons (analysis reported as Q value). To test associations, Person's correlations were used. Specific statistical tests used are noted in figure legends.

RESULTS

Erythroferrone expression is induced during iron-deficient pregnancy

We assessed changes in serum ERFE concentrations in iron-replete and iron-depleted wild-type (WT) non-pregnant females and pregnant dams at embryonic day (E)18.5 (term in mice). To iron-deplete pregnant dams, non-pregnant females were placed on purified iron-deficient (4 ppm iron) diet for at least 2 weeks prior to timed mating and throughout pregnancy, while iron-replete animals were maintained on purified iron-replete (100 ppm

iron) diet. During physiological adaptions to pregnancy, mice (like humans) develop decreased hemoglobin concentrations and depleted iron stores compared to non-pregnant animals (Figure 1A–C). For this reason, we did not use the shorthand terms "anemia" or "iron deficiency" to denote differences between pregnant and non-pregnant females, but rather only to denote outcomes where hemoglobin concentrations or key iron measurements were lower in iron-deficient compared to control pregnancy.

Pregnant females fed iron-deficient diet developed anemia compared to those on iron-replete diet (Hb: 8.8±1.2 v 10.6±0.6 g/dL, Q<0.001) (Figure 1C). Consistent with anemia, serum EPO was elevated in dams on iron-deficient vs -replete diet (5.4±5.5 vs. 0.4±0.2 pg/mL, Q=0.019) (Figure 1D). Serum ERFE levels in non-pregnant females were barely detectable (Figure 1E), regardless of diet. At E18.5, in iron-replete pregnancies, there was a non-significant increase in serum ERFE compared to non-pregnant controls (0.004±0.004 v 0.2±0.2 ng/mL, Q=0.525) (Figure 1E). However, on iron-deficient diet, maternal serum ERFE levels were significantly elevated compared to non-pregnant controls (3.1±3.3 v 0.01±0.01 ng/mL, Q=0.020) and compared to iron-replete E18.5 dams (3.1±3.3 v 0.2±0.2 ng/mL, Q=0.020) (Figure 1E). We observed a significant positive correlation between maternal serum ERFE and EPO at E18.5 (R^2 = 0.475, P<0.01, Pearson's correlation) (Figure 1F).

We next assessed maternal *Erfe* mRNA expression in the bone marrow (BM) and spleen. *Erfe* mRNA levels in both organs mirrored serum ERFE levels, with *Erfe* significantly increased during iron-deficient compared to iron-replete pregnancy (Figure 1G) (- Ct BM: -4.1±1.2 v -6.2±1.3, Q=0.002; Spleen: -4.5±0.08 v -7.1±2.0, Q=0.005). To evaluate changes in erythropoietic response, we measured the expression of the erythroid marker glycophorin A (*Gypa*). Extramedullary erythropoiesis was expanded during pregnancy regardless of dietary iron content, demonstrated by similarly increased splenic *Gypa* in both groups (Figure 1H, right); however, in the bone marrow, erythropoiesis decreased on iron-deficient diet (Figure 1H, left). Nevertheless, *Erfe* expression per erythroid cell increased in both bone marrow and spleen during iron-deficient compared to iron-replete pregnancy, as reflected by *Erfe* mRNA expression relative to *Gypa*, likely as a result of higher EPO levels (- Ct BM: -9.2±0.2 v -5.1±0.6, Q<0.001; Spleen: -7.9±1.5 v -5.5±0.8, Q=0.003) (Figure 1I). Induction of *Erfe* mRNA expression by iron deficiency was also observed at earlier gestational time points (E12.5 and E15.5) (Supplemental Figure 1).

Serum hepcidin and liver *Hamp* in pregnant dams (Figure 1J and Supplemental Figure 2A) were reduced compared to their respective non-pregnant controls. Comparing hepcidin between pregnant females on different diets, iron deficiency further reduced serum hepcidin (17.2±17.0 v 2.5±1.7 ng/mL) but the difference was not statistically significant after correction for multiple comparisons (Q=0.217). Overall, serum hepcidin inversely correlated with erythroferrone concentrations (R^2 = 0.667; P<0.001 Pearson's correlation) (Figure 1K). We only analyzed pregnancy outcomes at the end of gestation, and it remains to be examined whether the relationship between ERFE, hepcidin and erythropoiesis is similar in earlier stages of pregnancy.

The effects of altered ERFE levels during pregnancy on maternal iron and hematological parameters

To determine the role of ERFE during pregnancy, we utilized genetic mouse models expressing varying levels of ERFE: *Erfe* transgenic (TG)[19], WT and knock-out (KO) mice[12]. As before, female mice between 6 and 10 weeks of age were fed a formulated iron-replete (100ppm) or iron-deficient (4ppm) diet 2 weeks prior to timed mating and throughout pregnancy. Pregnant animals were analyzed at term (E18.5). Maternal characteristics and embryo outcomes are presented in Supplemental Table 1. Maternal age, weeks on diet, and number of embryos per litter were not different between groups (Supplemental Table 1). However, maternal weight (including embryos) at term was lower for all iron-deficient dams, and particularly for ERFE KOs (WT vs KO on 4ppm, P=0.032 by t-test). We also observed increased in-utero embryo death in iron-deficient pregnancies (Supplemental Table 1).

In transgenic dams, serum ERFE (Figure 2A) as well as bone marrow and splenic *Erfe* expression (Figure 2B–C) were elevated compared with WT dams. However, these parameters were not further affected by diet. In WT dams, as described before (Figure 1E), iron-deficient diet induced maternal ERFE expression (Figure 2A–C). In KO animals, serum ERFE and tissue *Erfe* expression was not reliably detected beyond levels associated with assay background (Figure 2A–C).

For dams, the experimental setup was used to address if ERFE genotype or diet significantly affected iron or hematological parameters. Two distinct pre-determined questions were asked: (1) in the absence of ERFE, do dams develop iron restriction and anemia because of an inability to suppress hepcidin? And (2) can overexpression of ERFE in dams protect against severe iron deficiency or iron-deficiency anemia? Consistent with the expected effect of increased ERFE expression, iron-replete ERFE TG dams had increased liver iron stores relative to WT controls (43.7 v 9.2 μg/g, P=0.026, Mann-Whitney test) (Figure 2D). However, when ERFE TG dams were iron-depleted, liver iron levels were similar to those of WT dams (Figure 2D). ERFE deficiency did not affect liver iron stores as liver iron in KOs remained comparable to WT controls within groups on iron-replete and iron-deficient diets (Figure 2D). Maternal spleen iron and serum iron were affected by maternal iron status but not ERFE genotype (Figure 2E and 2F). Liver hepcidin expression in iron-replete dams was similar between all ERFE genotypes (Figure 2G–H) but hepcidin expression relative to liver iron load was significantly lower in TG dams (Supplemental Figure 2B), consistent with previous data[19]. In the iron-deficient diet group, where TG iron overload was precluded, *Hamp* expression was significantly decreased in ERFE TG dams compared to WT (- Ct -1.2 to 1.2, P=0.025 t-test) (Figure 2G). Serum hepcidin was likewise decreased (2.8±3.2 v 4.8±4.1 ng/mL), although the difference did not reach statistical significance due to the overall low concentrations (Figure 2H). Conversely, hepcidin expression in iron-deficient ERFE KO dams was similar to WT controls (Figure 2G–H).

Differences in maternal hematological parameters were largely driven by diet-induced changes in iron status; all dams on iron-deficient diet had decreased red blood cell (RBC) count, hemoglobin (Hb), hematocrit (Hct) and mean corpuscular volume (MCV) (Figure 2I–L). These hematological parameters were unchanged between ERFE genotypes (Figure

2I–K), except decreased MCV in ERFE KO dams compared to WT (Figure 2L), observed on both iron-deficient and -replete diets. MCV is a sensitive indicator of iron restriction over the lifespan of erythrocytes; thus, these data suggest the absence of ERFE results in mild maternal iron restriction.

Our data suggest that under physiological conditions, ERFE only modestly contributes to iron mobilization for maternal erythropoiesis.

Embryo ERFE promotes iron mobilization for embryo erythropoiesis during iron-deficient pregnancy

Iron and hematological parameters were measured in E18.5 embryos from mothers in Figure 2. In the text and figures, capital letters (TG, WT and KO) refer to dam *Erfe* genotype while lowercase letters (tg, wt and ko) refer to embryo *Erfe* genotype. ERFE TG females (heterozygous for the transgene) were bred with WT males to generate both tg and wt embryos, yielding TG-tg and TG-wt mother-embryo genotype combinations. For the wild-type group, WT females were bred with WT males generating wt embryos (WT-wt). For the ERFE knockout group, KO dams were bred with KO males generating ko embryos (KO-ko). We considered breeding heterozygous KO females with heterozygous KO males to generate HET-ko and HET-wt mother-embryo combinations to evaluate the effect of embryonic ERFE knockout separately from the maternal. However, as the phenotype in KO-ko group was relatively modest, we concluded that the use of additional animals to identify potential intermediate phenotypes was not justified.

Embryo liver *Erfe* mRNA (Figure 3A) and embryo serum ERFE (Figure 3B) levels reflected the embryo *Erfe* genotype: very high in tg, moderate in wt, and undetectable in ko embryos. Maternal ERFE does not appreciably cross the placenta into the embryo circulation as wt embryos from TG vs WT mothers had comparable serum ERFE concentrations. In the WT-wt iron-replete group, embryo serum ERFE was not significantly higher than maternal serum ERFE (dam: 0.20±0.20 ng/mL; embryo 0.36±0.25 ng/mL; P=0.192, t-test) (Supplemental Figure 1D). The effect of iron deficiency on embryo ERFE was small: wt embryos had only marginally higher ERFE production in the iron-deficient compared to iron-replete condition with statistical significance detected only with the mRNA measurement (serum ERFE: 1.8-fold increase, P=0.083 t-test, *Erfe* mRNA: 1.4-fold increase, P=0.027 t-test) (Figure 3A and 3B). ERFE genotype did not affect embryo weight (Supplemental Figure 3A).

To understand the effects of embryo ERFE on embryo iron homeostasis, embryo hepcidin and iron parameters were assessed. The experimental setup was used to address two questions: (1) can overexpression of ERFE in embryos facilitate increased iron endowment? To address this question tg and wt embryos from TG dams were compared since TG dams are iron loaded and maternal iron status determines embryo iron endowment [21]. And (2) are ERFE ko embryos iron-restricted compared to WT embryos due to an inability to suppress hepcidin? To address this question ko embryos from KO dams were compared to wt embryos from WT dams.

ERFE tg embryos on either iron-replete or iron-deficient diet had significantly decreased liver *Hamp* (Figure 3C) compared to wt littermates (replete: - Ct -3.0±3.8 v 1.3±2.0, P=0.001 Mann-Whitney test; deficient: - Ct -5.1±1.3 to -3.0±1.0, P<0.001 t-test). The large variance is attributable to one outlier litter from a TG dam. Serum hepcidin, which was measured in fewer embryos, was likewise variable in iron-replete tg and wt embryos from TG dams and was undetectable in iron-deficient condition (Figure 3D), although the difference did not reach statistical significance. Despite differences in embryo hepcidin expression, embryo liver, brain and carcass iron were similar in TG-tg and TG-wt embryos under both iron conditions (Figure 3E–F and Supplemental Figure 3B). Similarly, embryo hematological parameters were comparable between tg and wt embryos from both iron-replete and iron-deficient pregnancies (Figure 3G–J). These data suggest that supraphysiological embryo ERFE can suppress embryo hepcidin; however, this reduction in hepcidin beyond already low wt hepcidin levels did not increase fetal iron endowment nor did it affect fetal hematopoiesis.

ERFE ko embryos were derived from KO mothers; however, because maternal iron status of KO dams was comparable to that WT dams (Figure 2D–K), this allowed the detection of embryo ERFE effects independently of maternal iron effects. In pregnancies on iron-replete diet, wt and ko embryos had comparable liver *Hamp* mRNA, serum hepcidin and embryo liver iron (Figure 3C–E). However, in pregnancies on iron-deficient diet, ko embryos had increased liver *Hamp* and serum hepcidin (Figure 3C, D). These data suggest that embryo ERFE contributes to suppressing embryo hepcidin during iron deficiency. Interestingly, liver iron concentrations were not different between ERFE ko and wt embryos from iron-deficient pregnancies (Figure 3E), but embryo brain iron concentration was decreased in ko embryos compared to wt controls (Figure 3F). Carcass iron, which was measured after embryos were exsanguinated and livers and brains removed, was higher in ERFE ko compared to wt embryos (Supplemental Figure 3B). This retention of iron in carcass tissue is suggestive of iron restriction, although we did not determine which specific cell types accumulated iron. These data indicate that during iron deficiency, embryo ERFE promotes mobilization of iron in the embryo, including for incorporation in the developing brain.

In ERFE wt and ko embryos from iron-replete dams, RBC, Hb and Hct were similar (Figures 3G–I), but MCV was lower in ko compared to wt embryos (Figure 3J). When these pregnancies were stressed by dietary iron deficiency (note the scale differences between the iron-replete and iron-deficient diet groups in Figure 3), ERFE ko embryos had decreased Hb, Hct and MCV compared to wt embryos (Figure 3H–J). In summary, absence of ERFE resulted in redistribution of iron within the embryo with decreased brain iron and erythropoiesis and increased carcass iron, likely a consequence of elevated embryo hepcidin. Thus, these data suggest that embryo ERFE promotes mobilization of iron for erythropoiesis and utilization in the brain. However, comparison of maternal-embryo combinations with HET-ko vs HET-wt, and KO-het vs KO-ko genotypes would be required to establish the precise contribution of embryonic vs maternal ERFE effects to the erythroid changes in the embryo.

The placenta produces little ERFE and its iron homeostasis is not appreciably affected by embryo or maternal ERFE

Erfe expression in wt placentae at E18.5 was very low, more than 60-fold lower than in the fetal liver or maternal bone marrow and was unaffected by iron status (Figure 4A). Overall, placentae were the same size (Figure 4B) and placental iron concentrations were lower in the in groups where dams were fed an iron-deficient rather than an iron-replete diet (Figure 4C). In embryos from dams fed an iron-replete diet, placental iron concentrations were slightly lower in ERFE ko compared to wt embryos (Figure 4C). This difference cannot be explained by changes in placental iron flux as iron importer TFR1, iron exporter FPN and iron storage protein ferritin were comparable between genotypes (Figure 4D–G). In addition, iron endowment of iron-replete wt and ko embryos (Figure 3E) and PIDI (Figure 4H, placental iron deficiency index, the ratio of placental FPN to TFR1[21]), were not different. One possibility is that KO dams developed transient iron restriction at some point during pregnancy, resulting in decreased delivery of iron to the placenta. In iron-deficient pregnancies, like in our previous reports [11, 21], placental FPN (Figure 4D and E) and PIDI (Figure 4H) were decreased, but were not further affected by ERFE genotype. These data suggest that embryo ERFE plays a minimal role in placental iron transport.

DISCUSSION

In mouse models, we explored the role of ERFE in maternal and embryonic iron-associated and hematological adaptations to pregnancy, with or without iron deficiency. We evaluated the effects of iron-deficient versus iron-replete diets on ERFE production in wild-type animals, and the effects of genetic manipulations of *Erfe* in the dam or the embryo on iron homeostasis and erythropoiesis in pregnancy. We concluded that ERFE has an adaptive role in iron-deficient but not iron-replete pregnancy, and primarily in the embryo and not the mother.

ERFE regulation in pregnancy—

In WT animals, circulating maternal ERFE as well as maternal bone marrow and spleen *Erfe* mRNA were increased in iron-deficient but not iron-replete pregnancies at term. Interestingly, earlier during pregnancy, at E12.5, *Erfe* was elevated in maternal bone marrow in both iron-replete and iron-deficient groups, perhaps reflecting maternal red blood cell expansion [22] in the second trimester. Overall, serum ERFE and EPO positively correlated in WT dams at E18.5, similar to what was reported in human pregnancy [16], suggesting that EPO is the main regulator of ERFE levels during pregnancy. The correlation has a well-established mechanistic basis as previous studies have documented that EPO induces ERFE via the JAK-STAT5 pathway[23]. We found no evidence that ERFE is independently regulated by pregnancy itself.

The effect of ERFE on maternal iron and hematology—

Only supraphysiological maternal ERFE levels modulated maternal iron stores: TG dams had increased liver iron stores at term under iron-replete conditions; this increase is likely a consequence of both iron-loading pre-pregnancy[19] as well as elevated iron absorption over gestation. Regarding maternal hematological parameters, only KO dams had changes

in erythropoiesis: MCV was mildly decreased in both iron-replete and iron-deficient KO dams. This decrease in MCV was also observed in ERFE ko embryos and is a well-established marker of iron-restricted erythropoiesis caused by hepcidin excess[24, 25]. In previous studies of non-pregnant mice, ERFE KO also had lower MCV compared to WT during recovery from phlebotomy[12]. Although a direct effect of ERFE on MCV remains possible, we know of no mechanism or independent evidence for such an effect. Consistent with our previous characterization of non-pregnant ERFE TG mice[19], hepcidin levels in iron-replete TG pregnant dams were inadequate relative to their iron load; however, ERFE-mediated hepcidin suppression was most evident in iron-deficient TG dams since in these mice the reactive upregulation of hepcidin by dietary iron-loading was prevented by an essentially iron-free diet. Overall, we conclude that maternal ERFE does not play a major role during pregnancy, and may only have a minor contribution to iron mobilization for maternal erythropoiesis.

The effect of ERFE on embryo iron and hematology—

Our results indicate that embryo ERFE also does not play a major role in regulating embryo iron homeostasis under iron-replete conditions, but it does under iron-deficient conditions.

Embryo ERFE is only derived from embryonic sources without any contribution from maternal serum, considering that wt embryos from TG and WT dams had similar serum ERFE levels. Total embryo iron endowment was not dependent on embryo ERFE but rather on maternal iron loading (it was higher in embryos from iron-loaded TG dams), consistent with our previous evidence[21] that maternal iron status is a key determinant of embryo iron endowment. However, iron redistribution *within* the embryo was regulated by embryo ERFE, particularly under iron-deficient conditions.

Our results show that embryo ERFE can function to suppress embryo hepcidin, as tg embryos had lower hepcidin than wt (under either iron-replete or iron-deficient conditions), and ko embryos had somewhat elevated embryo hepcidin (under iron-deficient conditions). Interestingly, only this elevation of hepcidin under iron-deficient conditions had functional consequences: it resulted in iron restriction which manifested as worse anemia and decreased embryo brain iron. Decreased brain iron could have serious negative effects on neurodevelopment[26]. ERFE regulation of embryo hepcidin did not result in significantly altered placental iron transporter expression nor changes in embryo liver iron stores, in agreement with our previous report[21] that fetal hepcidin does not play a physiologic role in regulating iron transport across the placenta. However, during iron-deficient pregnancy, embryo ERFE does regulate embryo iron distribution and may play a role in ensuring optimal iron distribution to tissues.

Conclusion—

In iron-deficient mouse pregnancy, embryo ERFE promotes embryo iron mobilization for erythropoiesis and brain iron accretion.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

ACKNOWLEDGEMENTS

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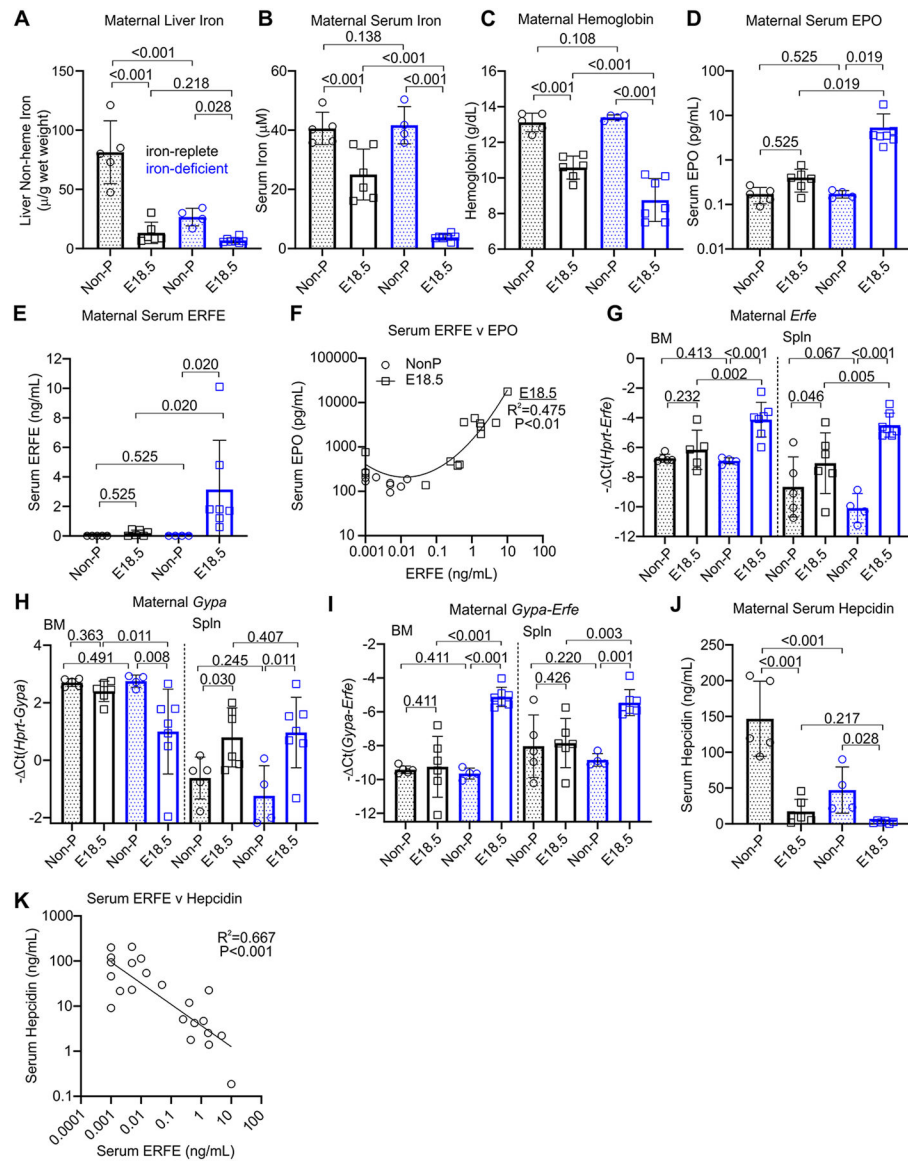
Figure 1: Maternal ERFE, iron and erythropoiesis in iron-replete and iron-deficient mouse pregnancy.

Wild-type (WT) C57BL/6 female mice were fed an iron-replete (100ppm) diet or iron-deficient (4ppm) diet and set up for timed pregnancy with WT C57BL/6 male mice. Pregnant females were analyzed at E18.5 (term for mice). Non-pregnant females (Non-P) were subjected to an equivalent iron diet regimen. Maternal (**A**) liver iron, (**B**) serum iron, (**C**) hemoglobin concentration, (**D**) serum EPO and (**E**) serum ERFE were quantified. (**F**) Correlation between serum EPO and serum ERFE in non-pregnant (circles) and pregnant animals (squares); Pearson's correlation and regression line is displayed for E18.5 pregnant animals only. Maternal bone marrow (BM) and spleen measurements of (**G**) *Erfe* and (**H**) *Gypa* normalized to *Hprt*, and (**I**) *Erfe* normalized to *Gypa*. (**J**) Serum hepcidin was also quantified. (**A-E, G-J**) Statistical differences between groups were determined by Two-way ANOVA followed by two-stage set-up method of Benjamini, Krieger and Yekutieli test to correct for multiple comparisons by controlling the False Discovery Rate which was set to

0.05, Q-values (p-value that has been adjusted for the False Discovery Rate) are presented. (**K**) Correlation between serum ERFE and serum hepcidin for all samples. Pearson's correlation was used to test the association between serum ERFE and serum hepcidin. Black: iron-replete; Blue: iron-deficient; Non-P: non-pregnant; E: embryonic day; BM: bone marrow; Spln: spleen; EPO: erythropoietin; ERFE: erythroferrone; HPRT: hypoxanthine phosphoribosyltransferase 1; GYPA: glycophorin A.
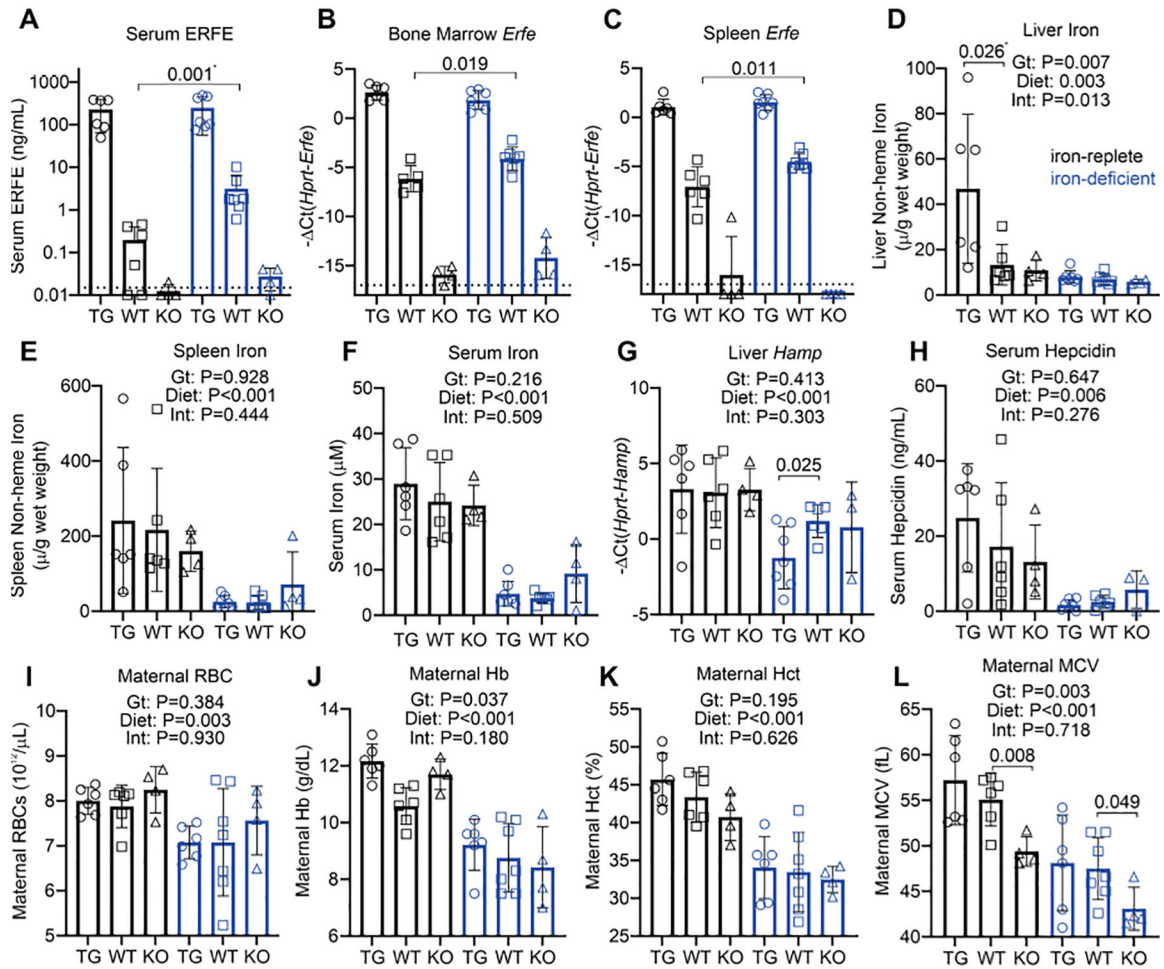
Figure 2. Maternal iron and hematological parameters in ERFE transgenic, wild-type and ERFE knockout mice during iron-replete and iron-deficient pregnancy.
Female mice expressing varying levels of ERFE were placed on iron-replete or iron-deficient diet, mated and dams analyzed at E18.5. Maternal (**A**) serum ERFE, (**B**) bone marrow *Erfe* mRNA and (**C**) spleen *Erfe* mRNA were measured. Dotted lines indicate assay limit of detection. (**A-C**) Statistical differences between groups were determined by Student's t-test for normally distributed values or Mann-Whitney U test for non-normally distributed values (denoted by * after *P* value). Iron concentrations were measured in maternal (**D**) liver, (**E**) spleen and (**F**) serum. Hepcidin was assessed in (**G**) liver by mRNA and (**H**) serum. Maternal hematological parameters were also assessed: (**I**) RBCs, (**J**) Hb, (**K**) Hct and (**L**) MCV. (**D-L**) Statistical differences were determined by two-way ANOVA. For pre-determined analyses comparing two groups (**D, G, L**), Student's t-test for normally distributed values or Mann-Whitney U test for non-normally distributed values (denoted by * after *P* value) were used to determine statistical differences. Black: iron-replete; Blue: iron-deficient; TG: transgenic; WT: wild-type; KO: knockout; ERFE: erythroferrone; Gt: genotype; Int: interaction; RBC: red blood cell; Hb: hemoglobin; Hct: hematocrit; MCV: mean corpuscular volume.
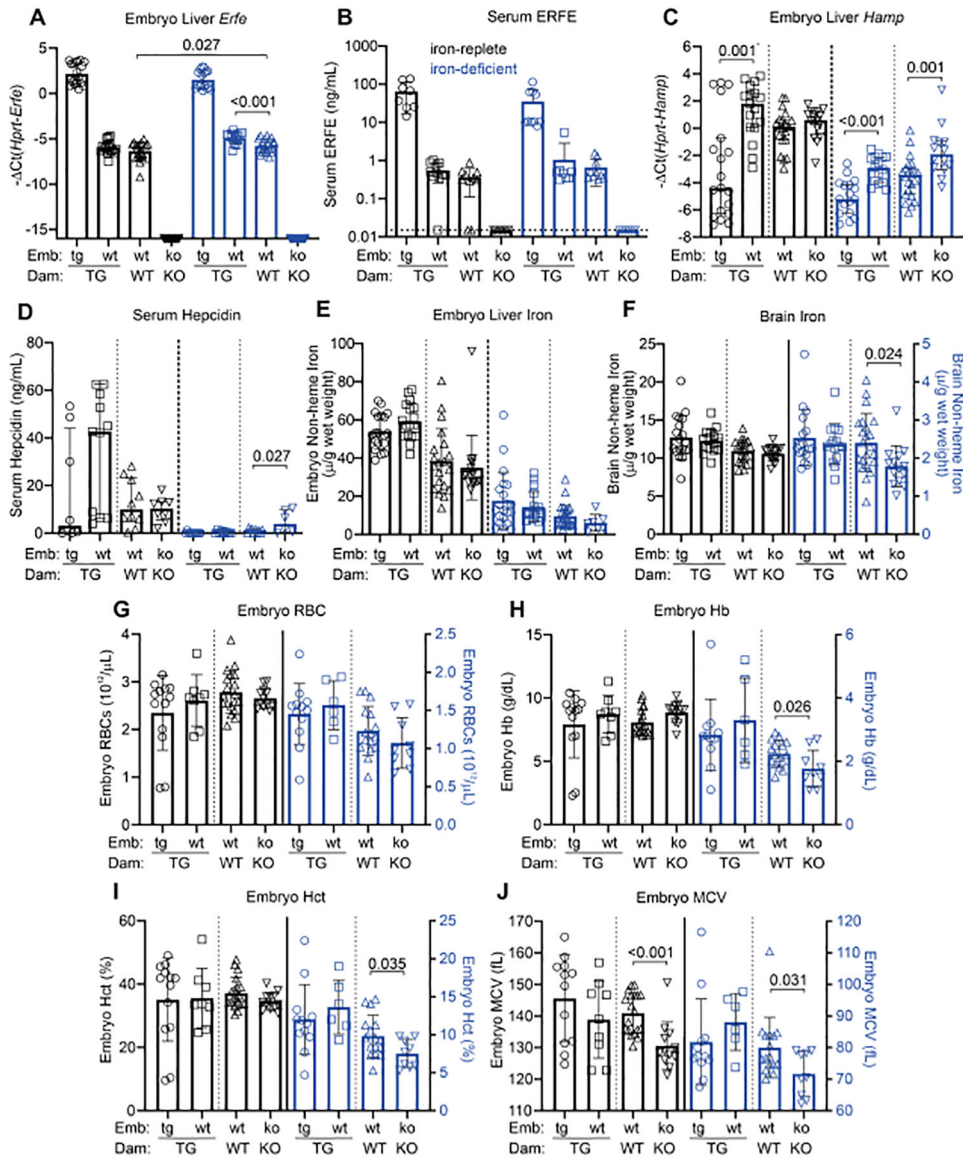
Figure 3. Iron and hematological parameters of E18.5 embryos with varying ERFE levels from iron-replete and iron-deficient pregnancy.

Embryos from dams in Figure 2 were analyzed. In graphs, capital letters (TG, WT and KO) denote dam *Erfe* genotype while lowercase letters (tg, wt and ko) denote embryo *Erfe* genotype. Embryo (**A**) liver *Erfe* mRNA, (**B**) serum ERFE, (**C**) liver hepcidin mRNA, (**D**) serum hepcidin, (**E**) liver iron concentrations, (**F**) brain iron concentrations, (**G**) RBC, (**H**) Hb, (**I**) Hct and (**J**) MCV were assessed at E18.5. For **A-B**, individual Student's t-tests or Mann-Whitney U tests were used to answer pre-determined questions: (1) comparing WT-wt iron-replete v iron-deficient animals to determine ERFE regulation in response to maternal iron-deficiency and (2) comparing TG-wt v WT-wt under iron-replete or iron-deficient conditions to determine if maternal ERFE overexpression and associated iron status affected embryo ERFE regulation. Only statistically significant comparisons are presented. For pre-determined analyses comparing two groups (**C-J**), Student's t-test for normally distributed values or Mann-Whitney U test for non-normally distributed values

(denoted by * after *P* value) were used to determine statistical differences. Black: iron-replete; Blue: iron-deficient; Emb: embryo; ERFE: erythroferrone; HPRT: Hypoxanthine phosphoribosyltransferase 1; HAMP: hepcidin; RBC: red blood cell; Hb: hemoglobin; Hct: hematocrit; MCV: mean corpuscular volume.
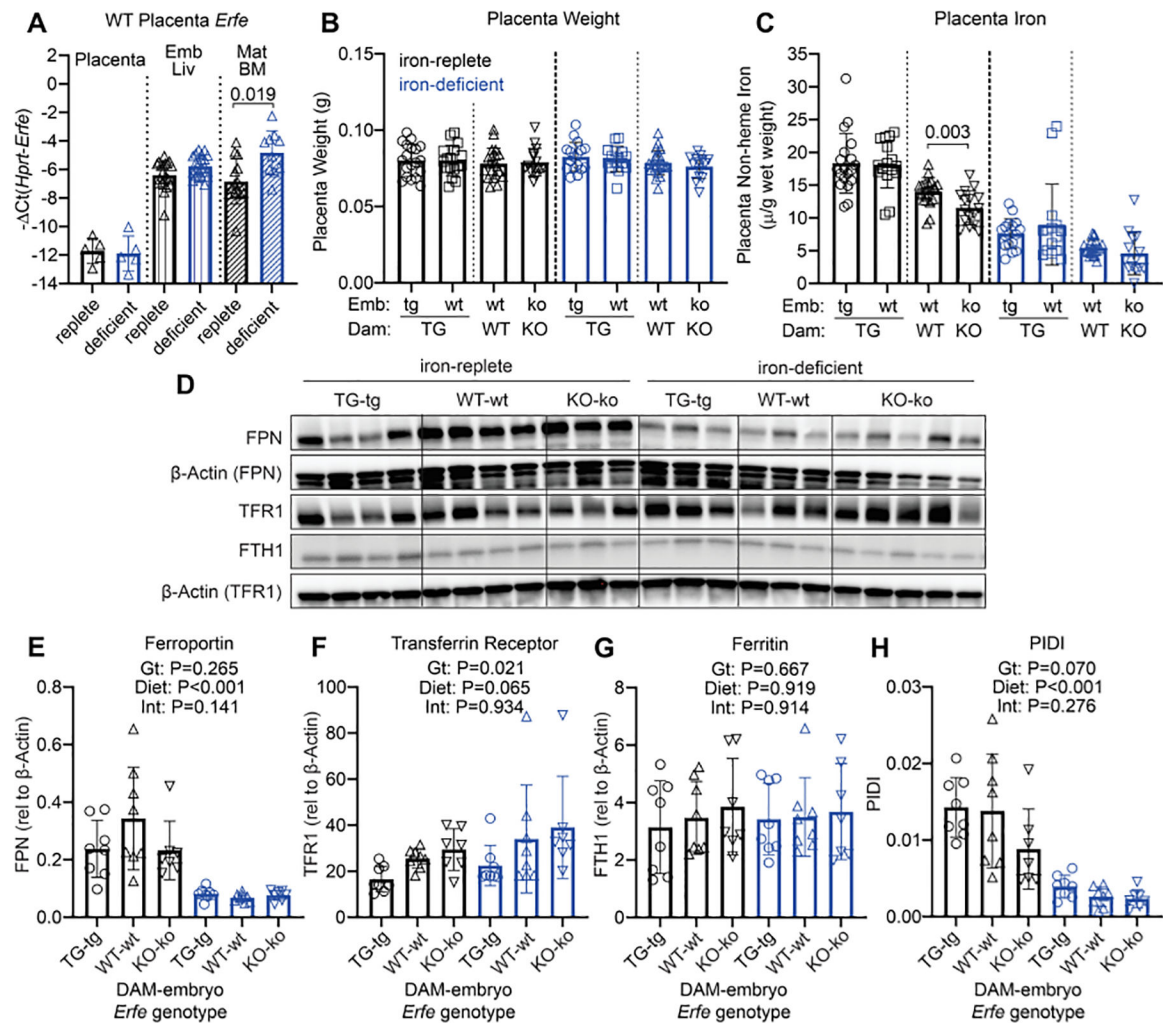
Figure 4. Placental erythroferrone and iron parameters.

(**A**) *Erfe* mRNA expression was measured in WT placentas and compared to *Erfe* levels in embryo liver and maternal bone marrow. Embryo liver and maternal bone marrow *Erfe* levels were replicated from Figure 3A and 2B, respectively for ease of comparison. (**B**) Placental weight and (**C**) placental iron concentration from varying ERFE genotypes at 18.5. (**A-C**) Statistical differences between groups were determined by Student's t-test for normally distributed values or Mann-Whitney U test for non-normally distributed values (denoted by * after *P* value) (**D-G**) Iron exporter FPN, iron importer TFR1 and iron storage protein FTH1 were measured by Western blotting and quantified by densitometry. (**H**) Placental iron deficiency index, the ratio of placental FPN to TFR1, was determined. (**D-H**) Statistical differences between groups were determined by two-way ANOVA. Black: iron-replete; Blue: iron-deficient; Emb: embryo; Liv: liver; Mat: maternal; ERFE: erythroferrone; HPRT: Hypoxanthine phosphoribosyltransferase 1; TG: transgenic; WT: wild-type; KO: knockout; FPN: ferroportin; TFR1: transferrin receptor; FTH1: ferritin; PIDI: placental iron deficiency index Gt: genotype; Int: interaction.