Effects of maternal iron status on placental and fetal iron homeostasis

Veena Sangkhae, … , Tomas Ganz, Elizabeta Nemeth


Find the latest version:
https://jci.me/127341/pdf
Effects of maternal iron status on placental and fetal iron homeostasis

Veena Sangkhoe1, Allison L Fisher1,2, Shirley Wong1, Mary Dawn Koenig3, Lisa Tussing-Humphreys4,5, Alison Chu6, Melisa Lelić7, Tomas Ganz1 and Elizabeta Nemeth1*

1Center for Iron Disorders, Department of Medicine and 2Molecular, Cellular & Integrative Physiology Graduate Program, David Geffen School of Medicine at UCLA, Los Angeles, California, USA; 3Department of Women, Children and Family Health Science, College of Nursing, 4Division of Academic Internal Medicine, Department of Medicine and 5Institute for Health Research and Policy, University of Illinois at Chicago, Chicago, Illinois, USA; 6Department of Pediatrics, Division of Neonatology and Developmental Biology, David Geffen School of Medicine at UCLA; 7Medical faculty, University of Tuzla, Tuzla, Bosnia and Herzegovina.

*Corresponding author: Elizabeta Nemeth, David Geffen School of Medicine at UCLA. Mailing address: 10833 LeConte Ave, CHS 43-229, Los Angeles, CA 90095. Telephone: (310) 825-2841. Electronic address: ENemeth@mednet.ucla.edu

Author conflicts of interest: T.G. and E.N. are shareholders and scientific advisors of Intrinsic LifeSciences and Silarus Therapeutics, and consultants for Ionis Pharmaceuticals, Protagonist, Keryx Pharmaceuticals, La Jolla Pharma, Vifor, Akebia (TG) and Gilead (TG). Other authors declare no competing financial interests.
ABSTRACT

Iron deficiency is common worldwide and is associated with adverse pregnancy outcomes. The increasing prevalence of indiscriminate iron supplementation during pregnancy also raises concerns about the potential adverse effects of iron excess. We examined how maternal iron status affects the delivery of iron to the placenta and fetus. Using mouse models, we documented maternal homeostatic mechanisms which protect the placenta and fetus from maternal iron excess. We determined that under physiological conditions or in iron deficiency, fetal and placental hepcidin does not regulate fetal iron endowment. With maternal iron deficiency, critical transporters mediating placental iron uptake (transferrin receptor 1, TFR1) and export (ferroportin, FPN) were strongly regulated. In mice, not only was TFR1 increased but FPN was surprisingly decreased to preserve placental iron, in the face of fetal iron deficiency. In human placentas from pregnancies with mild iron deficiency, TFR1 was increased but without a change in FPN. However, induction of more severe iron deficiency in human trophoblast in vitro resulted in the regulation of both TFR1 and FPN, similarly to the mouse model. This placental adaptation prioritizing placental iron is mediated by the iron-regulatory protein 1 and is important for the maintenance of mitochondrial respiration, thus ultimately protecting the fetus from the potentially dire consequences of generalized placental dysfunction.
INTRODUCTION

Iron is essential for a healthy pregnancy, with about 1g of iron needed for expansion of maternal red cell mass, and placental and fetal development (1). Severe iron deficiency and iron deficiency anemia during pregnancy have been associated with increased maternal mortality, perinatal death, preterm birth, low birth weight, impaired immune function and long-term cognitive defects in newborns and infants (2-6). To prevent these adverse outcomes, the World Health Organization currently recommends daily iron supplementation for all pregnant adolescents and adult women (7). On the other side of the spectrum however, consequences of high maternal iron levels in pregnancy are not well understood (8). Excess iron can result in generation of harmful reactive oxygen species that damage proteins, lipids and nucleic acids (9), or increased susceptibility to infection (10). Maternal serum ferritin, a marker of iron stores, has a U-shaped association with adverse pregnancy outcomes (11, 12). However, elevated ferritin is not only a marker of increased iron stores but also inflammation, and the specific contribution of excess iron has not been resolved. Despite its importance, it is poorly understood how maternal and fetal iron homeostasis during pregnancy is regulated, including the relative contribution of the maternal, placental and/or fetal signals.

On the systemic level, iron homeostasis is regulated by the hepatic hormone hepcidin, which controls iron absorption and recycling. Hepcidin is induced when systemic iron levels are high, and downregulates its receptor, ferroportin (FPN), thereby preventing iron export to blood plasma (13). Thus, changes in hepcidin levels can rapidly modulate plasma iron concentration. In human pregnancy, maternal hepcidin is suppressed during the second and third trimesters, which is thought to increase iron availability for placental transfer (1). The mechanism of maternal hepcidin suppression is unknown, as is the effect of maternal iron status on this regulatory circuitry.

Iron endowment of the fetus is entirely dependent on iron transport through the placenta. Iron-transferrin (Fe-Tf) from the maternal circulation is taken up by transferrin receptor 1 (TFR1) located on the apical surface of the syncytiotrophoblast (14). The complex of Fe-Tf and TFR1 is endocytosed, iron is released in the endosome and is eventually exported on the basal side of placental syncytiotrophoblasts through the iron transporter FPN into fetal circulation (15). Both TFR1 and FPN are thought to be critical for iron transport across the placenta, as global deletion of either transporter results in embryonic death (16, 17). Embryonic lethality in TFR1 knockouts occurs before E12.5 (16) and in FPN knockout mice before E9.5 (17). However, the placenta-specific role was only demonstrated for FPN, where preservation of FPN expression in the placenta but not the embryo proper rescues embryonic lethality (17). The regulation of iron transporters in normal pregnancy or in pathologic conditions such as iron deficiency, iron restriction or inflammation has not been extensively explored. It was reported that expression of placental TFR1 increased in response to maternal iron deficiency in humans and animal models (18, 19), and this was interpreted as a compensatory mechanism to ensure iron delivery to the fetus. Our data refine this model and suggest that both TFR1 and FPN are subject to regulation during iron deficiency, particularly in its more severe forms.

On the cellular level, iron homeostasis is regulated by the iron-regulatory proteins (IRP) 1 and 2, which control the stability or translation of mRNAs containing iron-responsive elements (IREs) on their 3’ or 5’ termini respectively (20). During cellular iron deficiency, IRPs bind to IREs, repressing translation of mRNAs encoding proteins involved in cellular iron export (FPN) or storage (ferritin), and stabilizing mRNAs encoding proteins involved in iron uptake (TFR1, DMT1), thus maintaining cellular iron levels in the face of iron deficiency. Double IRP1/IRP2 deficiency is embryonic lethal, whereas the individual IRP knockouts are viable (21), but their role in regulating materno-fetal iron transfer has not been explored.
We studied mouse models of pregnancy over its time-course, as well as human pregnancies and primary human trophoblast in vitro, and examined the mechanisms by which maternal iron status (iron-replete, iron-deficient and iron-loaded) affects the transfer of iron from mother to fetus. We found that maternal hepcidin, through regulating maternal plasma iron concentrations, determines the amount of iron taken up by the placenta, and protects the fetus from iron excess even when the mother is iron-overloaded. However, we were surprised to discover that during severe maternal iron deficiency, the placenta downregulates FPN to maintain its own iron homeostasis, resulting in decreased iron availability for transfer to the fetus. We devised the placental iron deficiency index (PIDI), a ratio of placental FPN and TFR1, as a sensitive and biologically relevant measure of iron deficiency of the maternalfetal unit. We present evidence that placental iron homeostasis is mediated by the IRE/IRP system, to maintain placental function including functional preservation of its mitochondrial electron transport chain.

RESULTS
Effect of maternal iron status on hepcidin and iron availability during pregnancy

In humans, the iron regulatory hormone hepcidin is suppressed during the second and third trimesters of pregnancy (22); however, the mechanisms driving hepcidin suppression remain unknown. To determine if maternal hepcidin suppression is a consequence of changes in maternal iron status, we assessed suppression in iron-deficient, iron-replete and iron-loaded wild-type (WT) C57BL/6 dams. Females between 6 and 8 weeks of age were fed either a standard chow diet (185 ppm iron), a low iron diet (4 ppm iron) 2 weeks prior to and throughout the pregnancy, or were injected with 20 mg iron dextran at time of mating (Figure 1A). Pregnant animals were analyzed at embryonic day (E) 12.5, E15.5 and E18.5 (gestation in C57BL/6 mice is ~19 days). Females that were subjected to the same iron regimen but did not get pregnant were used as controls, and were euthanized on the same day as the pregnant dams. In non-pregnant females, hepcidin (Hamp) mRNA and protein (Figure 1B and 1C) changed as expected depending on their iron status: iron deficiency lowered hepcidin and iron loading increased hepcidin levels compared to the iron-replete group (serum hepcidin 19, 83 and 346 ng/mL; P<0.001 deficient vs replete and P<0.001 replete vs loaded by one-way ANOVA and Holm-Sidak method for multiple comparisons). Compared to non-pregnant females of the same iron status, pregnant females in each group had decreased hepcidin at all time points examined (E12.5-E18.5), replicating the changes observed in human pregnancy (23). Decreased hepcidin was not caused by hemodilution of pregnancy as equivalent changes were observed for both the liver mRNA and serum protein (Figure 1B and 1C). Importantly, hepcidin suppression was preserved under all maternal iron states: E18.5 serum hepcidin was reduced compared to non-pregnant controls to 12% in iron-deficient, 11% in iron-replete and 42% in iron-overload conditions (Figure 1C). However, maternal hepcidin was still relatively higher in iron-loaded compared to iron-replete pregnancies suggesting that iron loading partially counteracts the suppressive pregnancy signal.

Liver non-heme iron concentrations gradually decreased from E12.5 to E18.5 compared to values in non-pregnant control mice, indicating that pregnancy induces iron mobilization from stores (Figure 1D). We surmise that lowering of hepcidin precedes liver iron mobilization as at the earliest time point examined - E12.5 - hepcidin mRNA and protein in iron-replete animals were already nearly-maximally suppressed (92% of maximal serum hepcidin suppression), whereas the decrease in liver iron compared to non-pregnant levels was not yet maximally suppressed at E12.5 (51% of maximal liver iron suppression) (middle panels Figure 1B-D). More importantly, as a result of decreased hepcidin and iron mobilization from stores, serum iron concentration was maintained throughout pregnancy in iron-replete dams (Figure 1E, middle panel), despite intense utilization of iron for fetal growth during this period. Maintenance of
serum iron levels at E18.5 despite low liver stores may be possible because of the relatively high iron content in standard mouse chow.

Unlike iron-replete mothers, both iron-deficient and iron-overloaded mothers were hypoferremic between E12.5 and E18.5 (Figure 1E). This was expected in the iron-deficient group, as both iron stores and dietary iron content was low and obviously insufficient to maintain serum iron, even with profoundly decreased hepcidin. Surprisingly though, when mothers were iron-loaded, serum iron was lower compared to non-pregnant animals (P<0.001 by one-way ANOVA), and also compared to iron-replete pregnant animals (P=0.028 for pregnant iron-replete vs iron-loaded by two-way ANOVA) (Figure 1E). This is almost certainly due to the relatively higher level of maternal hepcidin at E12.5-E18.5, limiting the release of excess iron from stores (Figure 1B and 1C, right panel). Changes in maternal transferrin saturation (Supplemental Figure 1) paralleled those in serum iron levels.

In iron-replete and iron-loaded pregnant animals, hemoglobin (Hb) concentration and red blood cell (RBC) count were lower compared to non-pregnant levels as expected (Figure 1F and Supplemental Table 1), because of pregnancy-associated plasma volume expansion. In the iron-deficient group, despite a decrease in hepcidin to nearly undetectable levels, mothers developed frank iron-deficiency anemia, with decreased Hb (diet, P<0.001), RBC count (diet, P=0.002), mean corpuscular volume (MCV) (diet, P<0.001) and mean corpuscular hemoglobin (MCH) (diet, P=0.016 for iron-deficient v iron-replete by two-way ANOVA) (Figure 1F and Supplemental Table 1).

**Maternal regulation of iron bioavailability prevents embryo iron overload but does not protect the embryo from iron deficiency**

To determine the consequences of varied maternal iron status for the embryo and placenta, we measured embryo hematological and iron parameters from pregnancies at E18.5 in Figure 1. Embryos from iron-deficient mothers were severely iron-deficient as reflected by their decreased serum and liver iron concentrations (Figure 1G and 1H), as well as brain and whole embryo iron (Supplemental Figure 2A, B). Furthermore, embryo anemia was much more severe than that of their mothers (Hb in Figure 1I; complete CBC in Supplemental Table 2). Thus, maternal adaptations to iron deficiency were not sufficient to protect embryos from severe iron deficiency anemia. Embryos from iron-loaded pregnancies, however, had serum iron, liver iron and hematological parameters similar to embryos from control iron-replete pregnancies (Figure 1 G-I). These data suggest that maternal iron-regulatory mechanisms including hepcidin protect embryos from iron overload even when mothers are iron-loaded.

Similar results were observed when iron deficiency and iron overload were caused by alternative approaches: feeding an iron-deficient diet starting at mating (short-term) rather than 2 weeks prior to mating (long-term), or feeding females a high iron diet (10,000 ppm) starting 2 weeks prior to pregnancy instead of injection with iron dextran at mating. Short-term iron-deficient diet resulted in similar changes in maternal, fetal and placental parameters to longer-term iron deficiency (Supplemental Figure 3, Supplemental Tables 3 and 4), with somewhat less impaired maternal hematocrit and MCV. Iron-loading through a high iron diet efficiently increased maternal liver iron compared to iron-replete animals at E18.5 (P=0.006; t-test on ranks iron-replete v 10K ppm). Despite maternal iron loading, serum iron concentrations at E18.5 were not higher than the iron-replete group (P=0.709; two-tailed t-test iron-replete v 10K ppm) presumably because of the relatively increased maternal hepcidin. Comparing the two iron-loading models, however, dietary iron loading resulted in higher maternal serum iron. This in turn resulted in increased placental iron content and mildly increased fetal liver iron, although fetal liver iron content remained comparable to iron-replete fetuses (P=0.981; two-tailed t-test iron-replete v 10Kppm) (Supplemental Figure 3B, F, H). This suggests that the mode of iron administration during pregnancy could result in subtle differences in iron bioavailability.
When maternal iron availability is low in mice, placental iron homeostasis is prioritized despite fetal iron deficiency

Iron delivery to the fetus is wholly dependent on transfer from the maternal circulation through the placenta, yet factors affecting this transfer are not fully understood. To evaluate the effect of maternal iron status on placental iron content and transport, we analyzed placentas from pregnancies in Figure 1 at E12.5, E15.5 and E18.5. Placental non-heme iron in the iron-loaded group was only slightly increased at E18.5 compared to the iron-replete group (Figure 1J). The moderation of fetal overload is likely a consequence of the relatively low serum iron in maternal circulation, maintained by maternal hepcidin. In iron-deficient pregnancies, placenta non-heme iron concentration was comparable to other groups at E12.5 and E15.5, and was mildly decreased at E18.5 compared to iron-replete or iron-loaded pregnancies (Figure 1J), with no change in placental weight (Figure 1K). The decrease in placenta iron concentration at E18.5 was significantly less than the iron decrease in fetal livers or fetal hemoglobin (~2-fold in the placenta compared to a 10-fold decrease in fetal livers and 3.6-fold decrease in fetal hemoglobin). Furthermore, placental heme iron content measured at E18.5 was not different between iron-deficient and iron-replete pregnancy, whereas fetal liver heme iron at E18.5 was significantly lower in the iron-deficient group (Supplemental Figure 2C, D). Our data suggest that the placenta prioritizes its own iron homeostasis despite fetal iron deficiency.

The principal transporters mediating cellular iron uptake and efflux - TFR1 and FPN - are abundantly expressed in human and mouse syncytiotrophoblast (Figure 2A, B). We measured TFR1 and FPN proteins in placentas at E12.5, E15.5 and E18.5 from iron-deficient, iron-replete and iron-loaded mouse pregnancies (Figure 2C, D). Protein levels of both transporters increased with gestational age, presumably to meet the increasing iron needs of the developing embryo. TFR1 expression was maximal by E15.5 whereas FPN expression was maximal at E18.5 (Figure 2C, D). Interestingly, TFR1 expression was not strongly affected by maternal iron status. During maternal iron deficiency, TFR1 protein was moderately increased only at E15.5 compared to the iron-replete group (Figure 2C), but was not significantly higher at the other two time-points (E12.5 and E18.5). TFR1 was not different in iron-loaded compared to the iron-replete group at any time-point examined. Surprisingly, maternal iron deficiency resulted in significantly decreased FPN protein expression at all time-points (Figure 2D), with no difference observed in FPN levels between iron-replete and iron-loaded groups. Decreased FPN during iron deficiency could compromise iron delivery to the fetus while maintaining placental iron content. This is consistent with our observation that maternal iron deficiency had a more profound impact on fetal iron homeostasis than on the placenta: fetuses had dramatically decreased hemoglobin as well as decreased non-heme and heme iron content in fetal liver, whereas placental non-heme and heme iron were much less affected or unaffected (Supplemental Figure 2C, D). Regulation of placental iron transporters during iron deficiency suggests the existence of placental iron-sensing mechanisms, which maintain placental iron homeostasis to the detriment of fetal iron endowment. Interestingly, unlike TFR1 and FPN, placental ferritin expression did not increase with gestational age (Supplemental Figure 4), and was more responsive to iron loading than ferroportin (at E18.5). This could be related to ferritin expression in multiple placental cell types (24), unlike FPN which is primarily expressed in syncytiotrophoblast.

We devised the placental iron deficiency index (PIDI) as a ratio of placental FPN to placental TFR1 protein expression. Decrease in FPN or increase in TFR1 or changes in both transporters would lead to lower PIDI, which would be indicative of placental sensing of iron deficiency and a greater risk of fetal iron deficiency. PIDI was lower in iron-deficient pregnancies compared to iron-replete pregnancies at all three time points measured (E12.5, 15.5 and 18.5, Figure 2E) reflecting a consistent placenta response whereby iron is prioritized to the placenta resulting in decreased fetal iron endowment. We argue that using the ratio of FPN to TFR1 in the same placental sample is a superior approach for assessing placental iron handling
than evaluating single transporter levels, as it minimizes the variability in single transporter expression related to placental sampling inconsistencies, which is of particular concern in human placental analysis.

Correlation between placental non-heme iron levels and PIDI was marginal at earlier pregnancy time-points (E12.5 R=0.1335; E15.5 R=0.3284, Figure 2F, G). This is expected considering that placental iron levels were maintained in the constant range (Figure 1J) by the alterations in placental iron transporters (Figure 2C, D). A stronger positive correlation between placental non-heme iron and PIDI was observed at E18.5 (R=0.610, Figure 2H), where greater placental iron deficiency resulted in lower PIDI, suggesting decreased iron transfer to the embryo.

To formally test the effect of placental TFR1 and FPN changes on placental iron transfer during maternal iron deficiency in mice, we measured iron transport using a stable Fe isotope. At E17.5, iron-replete and iron-deficient WT C57BL/6J dams received a single intravenous injection of 5 μg iron as $^{58}$Fe-Tf. Placentas and fetal livers were harvested 6 h following treatment and total iron content (nonheme+heme) in each tissue was measured by inductively coupled plasma-mass spectrometry (ICP-MS) (Figure 3A). Injection of $^{58}$Fe-Tf did not affect placental TfR1 nor FPN expression: in iron-deficient pregnancies, placenta FPN remained significantly lower than in iron-replete pregnancies (Figure 3B). Both $^{56}$Fe and $^{58}$Fe were measured in the placenta and fetal liver. The $^{56}$Fe isotope has the highest natural abundance and provides information on the long-term iron distribution between the placenta and fetal liver.

The short-term $^{58}$Fe transport snapshot demonstrated that $^{58}$Fe-Tf was taken up by the placenta and $^{58}$Fe was transferred to the fetus as it was detectable in the fetal liver. $^{58}$Fe did not reach the threshold of detectability when whole embryos were analyzed. In placentas, no statistically significant difference was observed in the amount of $^{58}$Fe retained in iron-deficient vs iron-replete pregnancies, although there was a trend toward ~10 ng lower $^{58}$Fe in iron-deficient placentas (Figure 3C). In fetal livers, total $^{58}$Fe content was significantly lower in iron-deficient pregnancy, with an average of ~30 ng lower $^{58}$Fe than in iron-replete pregnancy (Figure 3D). These data demonstrate that during iron deficiency decreased placental FPN results in less iron transport to the fetus. Interestingly, measurements of total $^{56}$Fe, which reflects chronic iron transport and handling, showed much greater differences between placental and fetal iron content than $^{58}$Fe. There was no statistically significant difference in total $^{56}$Fe (heme+nonheme) between placentas from iron-deficient and iron-replete pregnancies (16.2±13.2 μg and 19.9±16.1 μg, respectively) (Figure 3E). However, fetal liver total $^{56}$Fe was 68% lower when mothers were iron-deficient (4.9 μg for iron-replete compared to 1.6 μg for iron-deficient) (Figure 3F). This substantial decrease in fetal liver total iron content despite the stable placental iron content is consistent with results obtained for non-heme iron in our previous experiments. The more profound difference observed in $^{56}$Fe content than $^{58}$Fe could be related to the timing of $^{58}$Fe injection, which was near the end of pregnancy, when the placenta is already fully formed and has acquired most of the iron it needs for its function, and therefore is more likely to have sufficient iron for transfer to the fetus.

**Regulation of placental iron transporters in human pregnancy**

To examine alterations in placental TFR1 and FPN in human pregnancy with varying maternal iron status, we obtained human placental samples at delivery from 39 American women with uncomplicated pregnancies and iron status ranging from normal to moderate iron deficiency. Unlike in our mouse models, iron deficiency in these mothers was not severe enough to cause anemia. TFR1 and FPN levels were quantified by western blotting and PIDI calculated for each sample by dividing normalized FPN with TFR1. Data were grouped by maternal serum ferritin levels, with 10 ng/ml ferritin used as a cut-off for iron deficiency. Serum ferritin was assessed in the same women both at 32-34 weeks gestation and at delivery.
Regardless of the timing of ferritin assessment (32-34 weeks or at delivery), at the protein level, placental TFR1 was mildly but significantly increased in the <10 compared to >10 ng/ml ferritin group, consistent with placental sensing of iron deficiency, although the TFRC mRNA differences were not statistically significant (Fig 4A-D). We also assessed levels of regnase-1 (Reg1), a recently described TFRC mRNA-targeting ribonuclease (25) and found no difference in REG1 mRNA expression between the <10 and >10 ng/ml ferritin groups (Supplemental Figure 5A, B). FPN protein levels remained unchanged between <10 and >10 ng/ml ferritin groups (Figure 4E-F). PIDI, the ratio of placental FPN to TFR1, was significantly lower in the <10 ng/ml ferritin group than in the >10 ng/ml group (Figure 4G, H). Placental non-heme iron concentrations were not significantly different in the two groups of women (Figure 4I, J), reflecting maintenance of placental iron homeostasis. PIDI appeared to be a superior indicator of even mild iron restriction during pregnancy than other parameters commonly measured to assess pregnancy iron status including maternal Hb, cord blood Hb and cord blood ferritin which did not differ between the <10 and >10 ng/ml ferritin groups (Figure 4K-O).

FPN1 mRNA includes isoforms that contain 5'-IRE or not (26, 27). Only the 5'IRES-containing isoform is regulated by intracellular iron levels, as its translation is repressed during iron deficiency. In the human placenta, the 5'IRES-containing isoform is the predominant FPN mRNA form (Supplemental Figure 5C), over 10,000 times more abundant than the isoform lacking the 5'IRES. Therefore, FPN translation would be expected to be inhibited by placental iron deficiency. The lack of FPN protein change between <10 and >10 ng/ml ferritin groups is likely attributable to the relatively mild iron deficiency experienced by the placentas in our maternal cohort. To determine if more severe iron deficiency affects FPN protein levels, we analyzed freshly isolated primary human trophoblast cells grown in DMEM/10%FBS with the addition of iron chelator desferoxamine (DFO), apo-transferrin or holo-transferrin for 24 h. Both DFO and apo-TF treatment decreased FPN protein levels compared to the holo-TF condition (Figure 4P), in addition to increasing TFR1 and decreasing ferritin levels. We thus surmise that the initial response of the human placenta to mild iron deficiency is to increase TFR1, and that FPN decrease only occurs with more severe placental iron deficiency. The mechanism of this differential sensitivity to intracellular iron levels remains to be determined.

Placental IRP1 regulates placental iron transporters in response to changes in maternal iron status

To understand the mechanisms involved in regulating placental iron transporter expression in response to changes in maternal iron status, we used the mouse pregnancy model to analyze the mRNA expression of transferrin receptor (Tfrc) and Fpn, and their known regulators, as well as several other transporters and molecules potentially involved in iron homeostasis. We detected little change in placental Tfrc mRNA concentration (Supplemental Figure 6A). In control iron-replete pregnancies, there was no difference in Tfrc mRNA concentration between E12.5 and E18.5 suggesting that TFR1 has already reached maximal expression by E12.5. Similar to TFR1 protein results, maternal iron status had small effects on Tfrc which were significant only at E18.5 (iron-deficient v iron-loaded, P=0.005, iron-replete v iron-loaded: P=0.005; two-way ANOVA comparison within E18.5). We also found no difference in placental Reg1 mRNA levels either related to gestational age or maternal iron status (Supplemental Figure 6B). To assess Fpn mRNA expression, we analyzed two known transcript variants of FPN, Fpn1A and 1B (27). Similarly to the human placenta, we found that the iron-regulated, IRE-containing Fpn1A transcript was the predominant isoform in the mouse placenta (Supplemental Figure 6C and D). Fpn1A increased with gestational age (Supplemental Figure 6C), but was unaffected by maternal iron status (diet P=0.099, gestation P<0.001; two-way ANOVA). Fpn1B expression was uniformly low regardless of the gestational age or maternal iron status. No strong iron-dependent difference was observed in placental mRNA expression of iron
transporter divalent metal transporter 1 (Dmt1), homeostatic iron regulator (Hfe), ferrooxidase zyklopen ( HepH1), heme transporter feline leukemia virus subgroup C receptor-related protein 1 (Flvcr1), or inflammatory markers interleukin-6 (II-6) and serum amyloid A1 (Saa1) (Supplemental Figure 6E-J).

The reciprocal change in TFR1 and FPN proteins in response to maternal iron status is consistent with their regulation by the IRE/IRP system, which has been reported to function in human placentas (28, 29). Global single IRP1 or IRP2 KO mice are viable, whereas double IRP1/2 KO embryos die in utero at E6.5 (30). We measured total IRP activity by an electrophoretic mobility shift assay (EMSA) and found significantly increased IRP binding in placentas during iron-deficiency (Figure 5A), consistent with decreased FPN protein in the same pregnancies, and strongly suggesting that placental iron transporters are regulated by the IRP/IRE system. We were unable to detect any supershift following addition of IRP2 antibody (data not shown) suggesting IRP1 as the predominant regulator of placental IRE-containing mRNAs. To test the contribution of placental IRP1 to the regulation of placental FPN and TFR1 during maternal iron deficiency, we mated heterozygous IRP1 mice to generate placentas and embryos lacking IRP1 or not. Pregnant dams were placed on a 4 ppm iron diet at E7.5 and placentas collected at E18.5 (Figure 5B). Loss of IRP1 resulted in loss of placental FPN regulation; FPN protein was significantly higher during maternal iron deficiency in Irp1+/− placentas compared to Irp1+/+ (Figure 5C). Interestingly, levels of TFR1 protein or mRNA were not different between Irp1+/+ and Irp1+/− placentas (Figure 5C, D) indicating that Tfrc mRNA is stable even in the absence of IRP1, possibly because of the very low expression of the ribonuclease regnase-1 (Figure 5E). We also evaluated whether in IRP1 deficiency, IRP2 may be increased as a compensatory mechanism and stabilize Tfrc mRNA. However, we did not detect any IRP2 activity by EMSA in Irp1+/− placentas (Figure 5F). Interestingly, there were no detectable differences between Irp1+/+ and Irp1+/− E18.5 embryos in placental or fetal liver iron concentration (Figure 5G, H), likely because of very low iron availability within the entire materno-placental-fetal unit. At the time of harvest, Irp1+/− dams were severely iron-deficient (average maternal liver iron 9.6 µg/g wet weight, n=5), anemic (average Hb 8.58 g/dL) and hypoferremic (average serum iron 9.7 µM). Additionally, there could be a survival advantage for Irp1+/− compared to Irp1+/+ embryos as we did not observe expected Mendelian ratios for embryos from Irp1+/− x Irp1+/− matings when dams were placed on an iron-deficient diet (WT: 16%, HET: 51%, KO: 33%); differences however, did not reach statistical significance (P=0.281; Chi-square test, two-tailed P-value). If so, fetal liver iron data for Irp1+/− embryos may be skewed as animals with very low iron levels may not survive. Importantly, however, in the absence of IRP1 regulation (Irp1−/−), placentas had higher PIDI (ratio of FPN to TFR1 in each placenta) compared to Irp+/+ placentas, confirming that IRP1 is the major regulator of placental iron transporters in response to maternal iron deficiency (Figure 5I).

Placental and fetal hepcidin do not regulate fetal iron endowment

During iron deficiency, local hepcidin regulation was reported to be important for cardiac function (31). In the heart, hepcidin protein was paradoxically increased by iron deficiency even though its mRNA expression was decreased. If hepcidin protein in the placenta or embryo was also unexpectedly stabilized during iron deficiency, this could account for decreased FPN protein levels in the placenta. Additionally, placental FPN localizes to the basolateral membrane of syncytiotrophoblasts (14) and thus could be subject to regulation by embryo hepcidin. In fact, in embryos that overexpress hepcidin either because of hepcidin transgene insertion or Tmprss6 deficiency, placental FPN is downregulated resulting in severe fetal iron deficiency (32, 33). However, embryo hepcidin is low in normal mouse pregnancies (32, 33). To test whether the placental response during iron deficiency may be subject to regulation by placental or embryo hepcidin, we bred heterozygous hepcidin mice, placed mothers on either iron-replete or iron-deficient diet one week prior to mating, generating iron-replete or iron-deficient Hamp+/+, Hamp−/− or Hamp−/− placentas and embryos. As expected, low iron diet elicited maternal iron deficiency and anemia.
(Supplemental Figure 7A-F), as well as fetal iron deficiency (Figure 6A, B). However, within the same diet group, there was no difference in fetal liver iron or serum iron between Hamp^{+/+}, Hamp^{-/-} or Hamp^{+/--} fetal genotypes. Placental iron concentration did not differ between iron-replete and iron-deficient groups, nor between different Hamp genotypes (Figure 6C). Placental hepcidin mRNA expression was not different between different diet groups nor between WT and heterozygous placentas (Figure 6D). Placental Tfrc mRNA was mildly increased and FPN protein significantly decreased in iron-deficient group as expected, but again with no difference observed between different Hamp genotypes (Figure 6E, F). The results indicate that loss of placental and embryo hepcidin has no effect on placental or fetal liver iron status in iron-replete or iron-deficient pregnancies.

We also measured expression of microRNA miR-485-3p (34), which was reported to be induced by cellular iron deficiency and mediate suppression of FPN via a post-transcriptional mechanism. However, we found no difference in mouse miR-485-3p expression between iron-deficient and iron-replete pregnancies (Supplemental Figure 8).

Iron is necessary for trophoblast function

The placenta is a highly metabolically active tissue, consuming approximately 40% of total uterine oxygen uptake (35). This large energy consumption is primarily used for mitochondrial ATP synthesis to support protein production, nutrient transport and fetal metabolite waste transport (36). To assess the consequences of iron-deficiency for the placenta, we measured the concentrations of electron transport chain (ETC) complexes I-V in E18.5 placentas from iron-replete and iron-deficient pregnancies. We found no differences in complex expression (Supplemental Figure 9A). In vivo, however, the placenta is protected from large changes in iron status by its homeostatic response during iron deficiency, i.e. increased iron import through TFR1 and decreased export through FPN. Therefore, we isolated trophoblasts from term human placentas (primary human trophoblasts, PHTs) and cultured them for 24 h under a range of iron conditions. Iron chelator DFO was used to iron-deplete the cells, holo-TF to iron-load them, and apo-TF as a baseline control. As shown in Figure 4P, iron deficiency increased TFR1 and decreased FPN and ferritin. As in mouse placentas, we detected no change in expression of OXPHOS complexes following iron treatments (Figure 7A), likely because of protective redistribution of intracellular iron at this time-point. We next performed respirometry assays to measure how trophoblast iron levels affect mitochondrial respiration. Iron depletion decreased all the measured bioenergetics parameters of respiration compared to apo-Tf and holo-Tf treatments, with significantly lower basal, ATP-linked, maximal and spare capacity OCR in iron-depleted cells (Figure 7B, C). The reduction in maximal OCR suggests that iron-deficiency may perturb the placenta’s ability to generate energy for its synthetic and transport functions. Following inhibition of ATP synthase by oligomycin, PHTs compensated by shifting to glycolysis under all iron conditions (Figure 7D), demonstrating that the cells remained viable. Comparison of baseline OCR and ECAR (Figure 7E) confirmed that iron depletion decreased OCR but not ECAR. The uniformly low ECAR indicated that mitochondrial respiration rather than glycolysis is the predominant form of energy production in trophoblasts.

As an alternative trophoblast model, we used BeWo cells, a placental cell line derived from a human choriocarcinoma often used to study placental transport, including that of iron (37). Like PHTs, BeWo cells were treated with DFO, apo-Tf or holo-Tf for 24hr. DFO treatment efficiently iron-depleted cells, with a 70% percent average reduction in cellular ferritin levels (Supplemental Figure 9B, C), and upregulation of TFR1 (Supplemental Figure 9B, D), whereas FPN was undetectable in all groups. Importantly, expression of all ETC complexes decreased in the iron-depleted group compared to apo-Tf or holo-Tf
treated cells (Supplemental Figure 9E, F) suggesting that severe placental iron-deficiency could impair the machinery of cellular oxidative phosphorylation.

Taken together, our data support the hypothesis that severe placental iron deficiency could compromise critical determinants of placental function, providing an evolutionary rationale for the prioritization of placental iron retention in iron-deficient pregnancy.

DISCUSSION

Although the importance of iron for healthy pregnancy is well recognized, how iron transfer from mother to the fetus is regulated during pregnancy is not well understood. To determine how the maternal-placental-fetal unit responds to changes in maternal iron status, we comprehensively evaluated mechanisms in mouse models, human pregnancies and isolated trophoblast. In mouse models, we confirmed that maternal iron-regulatory hormone hepcidin is suppressed during pregnancy, consistent with previous reports in humans (38). However, the mechanism of suppression is not yet known. We show that maternal hepcidin is regulated by maternal iron status during pregnancy, with iron-loaded mothers having higher hepcidin than iron-replete or iron-deficient mothers. However, within each group, hepcidin was still suppressed compared to non-pregnant females, revealing a strong effect by a pregnancy-associated factor. Furthermore, reductions of both hepcidin protein and mRNA levels confirm that maternal hepcidin suppression is not a consequence of hemodilution. Additional studies are required to determine the exact mechanism(s) of maternal hepcidin suppression during pregnancy.

Embryo hepcidin overexpression in mice, as in transgenic hepcidin embryos or Tmprss6-deficient embryos, was reported to regulate placental FPN. However, in normal murine pregnancy, embryo hepcidin expression is very low (32, 33). In our studies, iron endowment of the fetus was unaffected by genetic ablation of fetal and placental hepcidin in iron-replete or iron-deficient pregnancy, indicating that embryonic or placental hepcidin is too low to regulate iron transfer across the placenta under these conditions.

Importantly, our animal models showed that during iron-replete pregnancy, homeostatic maternal adaptations - hepcidin suppression and iron mobilization from stores - maintained constant maternal serum iron levels throughout the pregnancy, despite increased iron utilization in advanced pregnancy. In iron-loaded mothers, maternal hepcidin was relatively increased, preventing release of excess iron into the maternal circulation, and protecting embryos from iron overload, with only a small increase in placental iron.

In contrast to embryonic protection from maternal iron-overload, maternal adaptations to iron deficiency, including the profound suppression of hepcidin, were not sufficient to protect the embryos from iron deficiency anemia. We were surprised to uncover a placental response which prioritized placental iron retention despite fetal iron deficiency. In the face of maternal iron deficiency, the murine placenta strongly decreased the levels of iron exporter FPN, resulting in decreased iron transport to the fetus. Although this ensured that the placenta developed only relatively mild iron deficiency, it was to the severe detriment of fetal iron content, with fetuses developing profound iron deficiency anemia as reflected by their low hemoglobin and liver stores. In contrast to our mouse models that were stressed with severe iron deficiency, in human pregnancies that were only modestly iron-deficient, TFR1 protein increased but FPN did not change. Because human pregnancies with severe maternal iron deficiency are rare in settings where we can obtain fresh placentas, we simulated the condition by exposing human trophoblast to a more severe iron deficiency in vitro. In agreement with the mouse model, we observed a decrease in FPN and
ferritin.

We propose the placental iron deficiency index (PIDI), a ratio of placental iron exporter and importer (FPN/TFR1), as an indicator of the fetal exposure to iron deficiency in utero. Utilizing the ratio would minimize the effect of regional differences in human sample collection. TFR1 and FPN expression is restricted to the syncytiotrophoblast; if placental tissue collection includes an area with low or no expression of transporters, this could be erroneously interpreted as a "regulated" low expression of an individual transporter, whereas a ratio of the two transporters would account for the sampling variability.

Using mouse models, we also determined the mechanism underlying the altered expression of placental iron transporters following maternal iron deficiency. We demonstrated that placental IRP1 plays a critical role in sensing placental iron levels and consequent modulation of iron transporters, and that 1rp1−/− placentas failed to decrease FPN protein levels in the face of maternal iron deficiency.

The ability of the placenta to retain iron for its needs prior to transport to the fetus is consistent with previous observations that when oxygen supply to the uterus is limited, the majority of the oxygen is consumed by the placenta and oxygen transfer to the fetus is adversely affected (39). Placental retention of iron during severe iron deficiency would preserve oxidative phosphorylation to supply necessary ATP for placental protein synthesis and transport functions (36). Indeed, we show that severe iron deficiency in primary human placental trophoblast impaired mitochondrial respiration whereas in BeWo cell line it even decreased the levels of all five ETC complexes. Unlike in BeWo cells, we did not observe ETC complex decrease in primary trophoblast at the 24h time-point, and speculate that the differences in the sensitivity of these two cell types to iron deficiency reflect the variability of protective cellular mechanisms against this stress. We argue that prioritization of placenta iron retention in response to iron deficiency may have an evolutionary benefit: it would preserve placental iron levels even during iron deficiency so that all iron-dependent placental functions are protected, thus indirectly benefiting the fetus overall despite diminishing fetal iron availability.

Prioritization of placenta iron acquisition over iron transport to the fetus suggests that fetuses are unable to compensate for maternal iron deficiency by increasing placental iron transfer. A common misconception is that the fetus is a "perfect parasite", able to acquire adequate iron irrespective of the mother's iron status (40). However, consistent with our data, several human and macaque studies confirmed that neonatal iron stores are compromised when the mother is iron-deficient or anemic (41-47). Furthermore, in agreement with our model, maternal iron deficiency anemia resulted in higher placenta/newborn weight ratio (48, 49). Our observation underscores the importance of detecting and treating iron-deficient pregnancies.

One of the current challenges, recognized by the U.S. Preventative Services Task Force (50), is the ability to accurately identify iron deficiency in pregnant women to inform whether iron supplementation is necessary. Currently, the most common laboratory measure is maternal Hb, where low Hb is presumed to be a result of iron deficiency; however, distinguishing actual iron deficiency anemia from Hb changes related to hemodilution remains a challenge (50). Lack of correlation between maternal Hb and PIDI in our human study suggests that Hb is not an adequate indicator of maternal iron deficiency nor the risk of fetal iron deficiency. Serum ferritin is another measure used for assessment of maternal iron status; although often a good indicator, this value can be confounded by infection or inflammation. In our human pregnancies, which were not inflamed, maternal serum ferritin <10 ng/mL was an accurate indicator of the risk of fetal iron deficiency as determined by PIDI. Although hepcidin and ferritin are both acute phase reactants that respond to infection and inflammation, some studies have suggested that hepcidin is a better indicator of iron status in pregnant women than ferritin (51, 52). However, we found no correlation between PIDI and maternal hepcidin.

Neonatal iron deficiency has been linked to numerous neurobehavioral effects (4). Most recently, an
association was reported between maternal anemia diagnosed earlier in pregnancy (≤30 weeks) and increased offspring risk of autism spectrum disorder, attention-deficit/hyperactivity disorder, and intellectual disability (6). Therefore, it is important that neonatal iron deficiency be accurately quantitated. A commonly used indicator of neonatal iron deficiency is cord blood Hb or cord blood ferritin. However, previous studies indicated that during fetal iron deficiency, iron is preferentially used for hemoglobin synthesis, sacrificing neonatal brain iron endowment (53). Thus, low Hb may only manifest in the most severe forms of iron deficiency. Additionally, in our human study, the lack of correlation between cord blood Hb or ferritin and PIDI indicates that cord blood Hb and ferritin may not be sensitive indicators of neonatal iron deficiency. Therefore, we propose that PIDI may be a superior indicator of iron deficiency in utero. As it can only be measured after birth, PIDI cannot be used to guide iron supplementation during human pregnancy, but may be used for future research studies to identify better markers of neonatal iron deficiency. Our study also shows that in the absence of infection or inflammation, maternal serum ferritin below 10 ng/ml may also signal the risk of fetal iron deficiency.

Our study demonstrates how the healthy maternal-placental-fetal unit handles iron. Maternal hepcidin is suppressed allowing for increased absorption from the diet and release of iron from stores to maintain serum iron concentrations. This allows for optimal transfer of iron from the maternal circulation through the placenta, via TFR1 and FPN, to the fetus where iron is then used for erythropoiesis and any excess is stored in the fetal liver. In states of iron-overload, maternal hepcidin is elevated and prevents overloading of the embryo. During iron-deficiency, however, the placental homeostatic response retains iron within the placenta and protects the metabolically active placenta from severe iron deficiency and consequently decreased oxidative phosphorylation. Although this occurs at the cost of fetal iron deficiency, it may ultimately protect the fetus from more severe adverse effects of broader placental dysfunction.
MATERIALS AND METHODS

**Mice** - C57BL/6J mice were obtained from Jackson Laboratory or bred in our facility. *Irp1*−/− mice on the C57BL/6 background were kindly provided by Dr. Tracey Rouault (54). Hepcidin knock-out mice (*Hamp*−/−) were originally provided to our laboratory by Dr. Sophie Vaulont (55) and backcrossed by us onto the C57BL/6 background. Unless otherwise specified, mice received a standard diet (PicoLab® Rodent Diet 20, 5053 Irradiated, 185 ppm iron) and were fed at libitum. Iron-deficient diet (4 ppm) (TD.80396) and iron-loaded diet (10,000 ppm) (TD.08043) was purchased from Envigo Tekland Diets.

To test the effects of maternal iron status, C57BL/6J females were maintained on standard diet (iron-replete), placed on iron-deficient diet 2 weeks prior to mating and throughout gestation (iron-deficient) or received a single intraperitoneal injection of 20 mg iron dextran (Sigma, D8517) at mating (iron-loaded). For alternative iron deficiency and loading strategies, C57BL/6J females were placed on iron-deficient diet at mating and throughout gestation (short-term iron-deficient) or placed on a 10,000 ppm diet 2 weeks prior to mating and throughout gestation (10K ppm). Pregnant females were euthanized at the indicated gestational age and non-pregnant female controls were euthanized on the same day.

To test the contribution of IRP1 to the maintenance of placental iron homeostasis, *Irp1*+/− females were mated with *Irp1*−/− males. Pregnant females were placed on an iron-deficient diet at E7.5 until E18.5 when they were euthanized and tissues were collected for analysis.

To determine the contribution of local placental and embryo hepcidin, *Hamp*+/− females were maintained on an iron-replete diet or placed on an iron-deficient diet 1 week prior to mating and throughout gestation. *Hamp*−/− females were mated with *Hamp*+/− males maintained on an iron-diet and euthanized at E18.5.

**Human subjects** - 43 pregnant women we recruited in their late 2nd or early 3rd trimester between August 2015 and October 2017 at the UIC Center for Women’s Health (UI Health). Women were eligible if: pre-pregnancy body mass index (BMI)>18.5 kg/m²; naturally-conceived, singleton pregnancy, 26–33 gestational weeks, obtaining prenatal care and planning to deliver at UI Health; 17-45 years of age; parity 0-3; able to read and write English; gaining the minimum amount of weight based on pre-pregnancy BMI from 23-24 gestational weeks onward based on the Institute of Medicine recommendations, and had access to a phone to alert research staff when admitted to UI Health for labor and delivery. Women were ineligible if: live birth or other pregnancy (including ectopic and molar pregnancies) in the previous 12 months; autoimmune disorder; gestational diabetes mellitus or previously diagnosed diabetes; current or previous premature rupture of membranes or chorioamnionitis; previous spontaneous premature birth; current bacterial or viral infection; receiving steroid or anti-inflammatory treatment; previous bariatric surgery; malabsorptive disease; current hyperemesis; hematologic disorder (i.e., sickle cell disease, sickle cell trait or hemochromatosis); tobacco use in the past 3 months; current alcohol consumption; and current illicit drug use.

One blood sample was collected at 32-34 gestational weeks. Women were asked to fast for 1.5 hours and refrain from all vitamin and mineral supplements for 48h prior to the visit. Upon admission to the UI Health Labor and Delivery (at 38.9 +/- 1.4 weeks), an additional maternal blood sample was obtained. Placentae were processed within 30 minutes of delivery. The placenta was visually divided into quadrants, a core sampled from each quadrant, sectioned into smaller pieces, and pieces from all four cores combined to provide a representative sample of the tissue. Aliquots were either snap-frozen or RNAlater (Ambion) added.
Cell culture – Primary human trophoblasts (PHTs) were prepared from normal term placentas using a modified trypsin-deoxyribonuclease-dispase/Percoll method (56, 57). PHTs were cultured in DMEM+10% fetal bovine serum (FBS). BeWo (ATCC CCL-98) were cultured in Ham’s F-12K nutrient mix+10% FBS. For in vitro iron-depletion and iron-loading experiments, PHTs were seeded at 1x10^5/well and BeWo cells at 1x10^5/well in a 6-well collagen coated plate (Corning BioCoat), allowed to attach overnight and treated with either 100 μM desferoxamine (SIGMA, D9533), 100 μM apo-transferrin (Celliance, 4452-01) or 100 μM holo-transferrin (Millipore, 4455-01) for 24h.

Complete blood counts (CBCs) – Mouse CBCs were performed on a Hemavet 950FS hematology system (Drew Scientific). Human hemoglobin was determined using an Abbott Hemocue point-of-care monitor (Abbott Park, IL).

Iron measurements – Serum, liver and placenta non-heme iron concentrations were measured using a previously described method (58)(Sekisui Diagnostics, 157-30). Human maternal and neonatal cord serum iron and serum ferritin were measured at Quest Diagnostics (Wood Dale, IL). Unsaturated iron binding capacity (UIBC) was measured using a Pointe Scientific assay (I7504). Transferrin saturation % = (serum iron/TIBC)*100.

56Fe isotope preparation and sample analysis – 56Fe (93% enrichment) was purchased from Trace Sciences International, dissolved in 12N HCl to form H2+56FeCl2, and H2O2 was added to oxidize the H2+56FeCl2 to H2O+56FeCl3. 56FeCl3 was incubated with nitritolriacetate (NTA) at a molar ratio of 1:5 in 20 mM NaHCO3 for 5 min at room temperature to form 58Fe-NTA. Human apotransferrin (Tf) (Celliance #4452-01) was dissolved in Tf-loading buffer (0.1M HEPES, pH 7.5; 0.15M NaCl) and incubated with 56Fe-NTA at a molar ratio of 1:2 for 2.5h at room temperature to form 58Fe-Tf. To remove unbound 58FeCl3, the 58Fe-Tf mixture was centrifuged in an Amicon Ultra Filter 30K at 2500xg for 15 min and washed with Tf-loading buffer and saline. Each pregnant mouse received a retro-orbital injection of 3.5 mg human 58Fe-Tf (equivalent to 5 μg of 58Fe) at E17.5. Tissues were collected 6h post-injection. Placentas and fetal livers were processed for nonheme iron as previously described (58) and supernatant submitted for ICP-MS analysis of 56Fe and 58Fe. The remaining iron (mostly heme) was extracted from tissue pellets by digestion in HNO3 supplemented with H2O2, samples were heated up to 200°C for 15 min followed by dilution of samples with 2% HNO3, and samples analyzed by ICP-MS. The sum of nonheme and heme iron was presented as total iron content. For 58Fe measurements, natural abundance (0.28% of total Fe) was subtracted from measured 58Fe values.

Serum hepcidin – Mouse serum hepcidin was measured using a sandwich ELISA by Amgen (59).

qPCR - RNA was prepared using TRizol (Invitrogen), and cDNA synthesized using iScript cDNA Synthesis Kit (Bio-Rad). qPCR was performed with SsoAdvanced SYBR Green Supermix (Bio-Rad) (primers listed in Supplemental Table 5). Samples were run in duplicate on a CFXconnect qPCR instrument (Bio-Rad). MiR-485-3p was measured using a Qiagen miScript Primer Assay (#MS00006335).

Immunofluorescence – Formalin-fixed paraffin-embedded 5 μm sections of mouse and human placentas were used. Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 9.0) was used for antigen retrieval, and sections heated to 96°C for 10 min. Primary antibodies (Supplemental Table 6) were incubated overnight at room temperature in a humidified chamber, secondary antibodies for 1 h at room temperature. Images were captured using a Zeiss LSM 700 Confocal Microscope.

Western blot – Tissues were lysed by homogenization in RIPA buffer and cells by addition of RIPA (Santa Cruz Biotechnology, sc-24948), and lysates cleared by centrifugation at 17,000xg for 15min at 4°C. Protein
was quantified using a BCA Assay (ThermoFisher Pierce, 23225). Samples for FPN and OXPHOS were prepared in Laemmli sample buffer without reducing agent and samples were not boiled. For all other proteins, samples were prepared in Laemmli buffer with DTT and incubated at 100°C for 5 min. Samples were resolved on Bio-Rad 4–20% TGX™ gels or Any kD TGX™ gels for OXPHOS, electroblotted onto nitrocellulose (Trans-Blot Turbo system, Bio-rad) and imaged with ChemiDoc XRS+ (Bio-Rad). Primary and secondary antibodies are listed in Supplemental Table 6. Membranes were stripped using 0.2N NaOH for 10 min at room temperature and re-probed for loading controls. Quantitation was performed using Image Lab™ Software (Bio-Rad Version 5.2.1).

Placental iron deficiency index (PIDI) calculation: Because mouse FPN and TFR1 were analyzed on separate western blots due to the need for different handling of the samples, we first normalized each of the proteins to their β-actin loading control, then generated PIDI as the ratio of normalized FPN to normalized TFR1. In human placenta, because several electrophoresis gels had to be used to analyze the large number of samples, TFR1, FPN and β-actin values were normalized to the median for each membrane to account for intermembrane variability, and then values were divided by normalized β-actin values to account for any loading variation. Normalized FPN values were then divided by normalized TFR1 values to generate PIDI.

**EMSA** - The direct interaction between placental IRP1/2 and IREs was determined using a LightShift™ Chemiluminescent RNA EMSA Kit (ThermoFisher 20158). 4 µg of whole placenta tissue lysate was used for each reaction, samples were then resolved on a pre-cast 6% DNA retardation gel (ThermoFisher EC6365BOX), transferred onto a Biodyne B nylon membrane (ThermoFisher 77016) using a Trans-Blot Turbo Transfer System (Bio-Rad) at 200 mA for 30 min. Transferred RNA was crosslinked onto the membrane using the auto crosslink function on a Stratagene Stratalinker UV Crosslinker 2400. RNA detection was performed using a Chemiluminescent Nucleic Acid Detection Module (ThermoFisher 89880) and membranes imaged on a ChemiDoc XRS+.

**Respirometry Assays** – The oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were measured using the Seahorse XFe96 flux analyzer (Agilent). PHTs (40,000 or 60,000/well) were plated into a Seahorse XF96 cell culture plate (Agilent 101085-004) coated with collagen (SIGMA 125-50). PHTs were cultured for 3 hrs to allow attachment, washed three times with PBS and cultured in DMEM +1%FBS supplemented with 100 µM DFO, 100 µM apo-Tf or 100 µM holo-Tf for 24 hrs. At the time of assay, treatment medium was removed and replaced with the appropriate pre-warmed Seahorse XF Assay Medium (Agilent), DMEM containing 10 mM glucose and 4 mM glutamine. Cell were then subjected to a Mitochondria stress test™ (Agilent). Oxygen consumption measurements were made approximately every 5 minutes under basal conditions, after the addition of 2.5 µM oligomycin, 1 µM carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) and 2 µM antimycin A and rotenone. OCR and ECAR measurements were normalized to number of cells per well following the assay. Contribution from non-mitochondrial respiration as measured following antimycin A and rotenone injection was subtracted from all raw OCR values. Basal OCRs: the last measurement prior to the injection of oligomycin. ATP-linked respiration: difference between oligomycin resistant OCR and basal respiration. Maximal respiration: the highest value after the addition of FCCP. Spare capacity: difference between maximal FCCP and basal OCR. ECAR measurements were performed in parallel. Basal ECARs: the last measurement prior to the injection of oligomycin. Post-oligomycin maximum ECAR was the calculated average of four measurements taken after oligomycin injection.

**Data presentation and statistical analysis** – Box plots: The upper portion of the box plot indicates the 75th percentile and the bottom indicates the 25th percentile, whiskers above the box indicate the 90th
percentile and below the 10th percentile, individual points represent outliers. The solid line within the box indicates the median and dashed line the mean. Statistical analysis was performed using SigmaPlot scientific graphing and data analysis software (Systat Software). Participants provided written informed consent. All study procedures were approved by the UIC Institutional Review Board (#2015-0353).
AUTHOR CONTRIBUTIONS

V.S. designed and performed experiments, analyzed data and wrote the manuscript. A.L.F. performed experiments and assisted with data interpretation. S.W. assisted with experiments. M.D.K. and L.T.-H. enrolled and collected human subject data and samples and edited the manuscript. A.C. participated in studies using primary human trophoblast, and edited the manuscript. M.L. assisted with human placental studies and method development, and edited the manuscript. T.G. and E.N. conceived the project, analyzed data and wrote the manuscript.

ACKNOWLEDGEMENTS

The authors thank: Dr. Deliang Zhang and Dr. Tracey Rouault for kindly providing us with \textit{lrp1} knock-out mice; the UCLA Translational Pathology Core laboratory for histology processing, the UCLA Broad Stem Cell Center Microscopy Core for providing access and training to confocal microscopes, Dr. Linsey Stiles and Brandon Desousa at the UCLA Mitochondria and Metabolism Core for running the respirometry assays, UCLA ICP-MS core facility within the UC Center for Environmental Implications of Nanotechnology, Tiffany Coon and Dr. Yoel Sadovsky at the Magee-Womens Research Institute for their assistance with primary trophoblast isolation, and Victoria Gabayan and Erika Valore for their assistance with methods development.

Sources of support: The Executive Advisory Board of the Iris Cantor-UCLA Women’s Health Center and NCATS UCLA CTSI Grant Number UL1TR000124 (to EN), pilot award (to VS), and NIH Ruth L. Kirschstein National Research Service Award T32-5T32HL072752-13 (to VS); and Robert Wood Johnson Foundation, Nurse Faculty Scholars Award #72117 (to MDK) and UIC College of Nursing Dean's Fund (to MDK and LTH).
REFERENCES

8. Taylor CL, and Brannon PM. Introduction to workshop on iron screening and supplementation in iron-replete pregnant women and young children. The American journal of clinical nutrition. 2017;106(Suppl 6):1547S-54S.


44. Lubach GR, and Coe CL. Preconception maternal iron status is a risk factor for iron deficiency in infant rhesus monkeys (Macaca mulatta). *J Nutr.* 2006;136(9):2345-9.


Figure 1: Maternal hepcidin and serum iron determine embryo and placental iron status. Iron status of wild-type C57BL/6 females was altered using diet or iron dextran injections. (A) Adult females were fed ad libitum standard chow (185 ppm iron) or low iron diet (4 ppm iron) 2 weeks prior to and throughout the pregnancy, or were injected with 20 mg iron dextran at time of mating. Pregnant females were analyzed at E12.5, 15.5 and 18.5. Non-pregnant (Non-P) females were subjected to an equivalent iron treatment. (B-F) Maternal measurements: (B) Hepcidin (Hamp) mRNA. (C) Serum hepcidin. (D) Liver non-heme iron. (E) Serum iron concentrations. (F) Hemoglobin concentration. Statistical differences between groups was determined by one-way ANOVA for normally distributed values or one-way ANOVA on ranks otherwise (indicated by * following P-value). (G-I) Embryo measurements at E18.5: (G) Serum iron. (H) Liver non-heme iron. (I) Hemoglobin concentration. (J) Placental non-heme iron levels at E12.5, E15.5 and E18.5. (K) Placental weight at E15.5 and E18.5 (we did not obtain whole placentas at E12.5). Statistical differences between groups was determined by one-way ANOVA for normally distributed values followed by Holm-Sidak method for multiple comparisons versus iron-replete control group (***P<0.001) or one-way ANOVA on ranks followed by Dunn’s method for multiple comparisons versus iron-replete control group (#P<0.05). Animal numbers in each group are indicated in each graph.
Figure 2: Placental iron transporters respond to changes in maternal iron status. Immunofluorescence staining of human (A) and mouse (B) placentas for TFR1 (red) and FPN (green) at 100X magnification, nuclei are blue, M: maternal circulation, F: fetal circulation. Mouse placentas from Figure 1 were analyzed by western blotting to determine protein concentration of TFR1 (C) and FPN (D). β-Actin was used as a loading control. Four representative placentas are shown in western blots, and a total of 8 placentas (from 3-4 different dams per group) were used for quantitation. Data are presented as mean +/- SE. Statistical differences between groups were determined by one-way ANOVA for normally distributed values followed by Holm-Sidak method for multiple comparisons versus iron-replete control group. (E) Placental iron deficiency index (PIDI) is the ratio of placental FPN protein expression to placental TFR1 protein expression, and reflects iron export to the fetus relative to iron import into placenta from the maternal circulation. Statistical differences were determined using two-tailed Student’s t-test. (F-H) Correlation of non-heme iron and PIDI at E12.5, E15.5 and E18.5.
Figure 3: $^{58}$Fe transport across the placenta. (A) WT C57BL/6 females were maintained on standard chow diet or placed on an iron-deficient diet 2 weeks prior to mating and maintained on an iron-deficient diet throughout gestation. At E17.5, dams received a single intravenous injection of $^{58}$Fe-Tf. Dams were sacrificed 6 hours post-injection (p.i.) and placenta and embryo tissue collected and analyzed by ICP-MS. (B) Placental TFR1 and FPN protein expression was assessed by western blotting. Quantitation of protein relative to β-actin is presented below western blots. Total $^{56}$Fe content in placentas (C) and fetal livers (D). Total $^{56}$Fe content in placentas (E) and fetal livers (F). (B-F) Statistical analysis was performed by two-tailed Student's t-test for normally distributed values and Mann Whitney rank sum test otherwise (denoted by an asterisk after P-value).
Figure 4: Placental response to maternal iron deficiency in human pregnancy. Placentas from uncomplicated human pregnancies were analyzed by western blotting to determine protein expression of TFR1 and FPN, normalized to β-actin. qPCR was used to determine TFRC mRNA expression, normalized to HPRT. (A, B) TFR1 protein levels, (C, D) TFRC mRNA levels, and (E, F) FPN protein levels according to maternal ferritin at week 32-34 or at delivery. (G, H) Placental iron deficiency index (PIDI) was calculated as the ratio of placental FPN to TFR1 protein expression, with lower PIDI reflecting pregnancies at increased risk of fetal iron deficiency. PIDI was lower in pregnant women with serum ferritin below 10 ng/ml than in those with ferritin above 10 ng/ml, regardless of whether ferritin was measured at 32-24 weeks of pregnancy or at delivery. No difference between <10 and >10 ng/ml ferritin groups was observed for placental non-heme iron concentrations (I, J), maternal Hgb (K), cord blood Hgb (L, M), or cord blood ferritin (N, O). (P) Primary human trophoblasts (PHTs) were treated with 100 μM DFO, apo-Tf or holo-Tf for 24h. TFR1, FPN, ferritin heavy chain (HC) and β-actin were assessed by western blotting. Statistical differences between groups was determined by two-tailed Student’s t-test; asterisks indicate Mann-Whitney Rank Sum Test for non-normally distributed values.
Figure 5: IRP1 mediates placental iron homeostatic responses during maternal iron deficiency. (A) Activity of IRP1/2 in placentas from iron-deficient, -replete and -loaded pregnancies were analyzed by EMSA. Statistical differences between groups was determined by one-way ANOVA on ranks followed by Dunn’s method for multiple comparisons versus iron-replete control group (#P<0.05). (B) lrp1+/- females were mated with lrp1+/- males, pregnant females placed on an iron-deficient diet starting at E7.5 until E18.5, placentas and embryos were harvested at E18.5. (C) Placental TFR1 and FPN protein expression was assessed by western blotting. Quantitation of protein relative to β-actin is presented below western blots. (D) Placental Tfrc mRNA expression. (E) Placental Reg1 mRNA expression. (F) IRP-IRE binding as determined by EMSA in lrp1+/- and lrp1+/- placentas. (G) PMID in lrp1+/- and lrp1+/- placentas. Placental (H) and fetal liver (I) non-heme iron concentration. (C-I) Statistical analysis was performed by two-tailed Student’s t-test for normally distributed values and Mann Whitney rank sum test otherwise (denoted by an asterisk after P-value).
Figure 6: Placental and embryonic hepcidin do not regulate placental iron homeostasis under either iron-replete or iron-deficient conditions. *Hamp*^+/-^ females were mated with *Hamp*^+/-^ males. Females were placed on iron-replete or iron-deficient diet 1 week prior to mating. Placentas and embryos were analyzed at E18.5. Fetal liver iron (A) and serum iron concentrations (B). Placental iron concentration (C), *Hamp* expression (D), *Tfr1* expression (E), and FPN protein levels (F). Animal numbers are indicated in each graph. Statistical differences between groups were determined by two-way ANOVA. Listed P-value is for variation by diet. There were no statistical differences between genotypes for any of the measured parameters. Non-normally distributed values are indicated by *.
**Figure 7: Iron deficiency impairs oxidative phosphorylation in primary human trophoblasts.** Primary human trophoblasts (PHTs) were treated with 100 μM DFO, apo-Tf or holo-Tf for 24h. (A) Western blotting of OXPHOS complexes I-V. β-actin was used as a loading control. (B) Mitochondrial respiration under basal conditions, following injection of oligomycin, the uncoupler carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) or electron transport inhibitors antimycin A and rotenone. (C) Quantitation of basal respiration, ATP-linked respiration, maximal respiratory capacity and spare respiratory capacity normalized to total cells/well. Statistical differences between groups were determined by one-way ANOVA for normally distributed values followed by Holm-Sidak method for multiple comparisons versus iron-replete control group (**P<0.001) or one-way ANOVA on ranks followed by Dunn’s method for multiple comparisons versus iron-replete control group (#P<0.05). n=6 technical replicates. (D) Extracellular acidification rate (ECAR). (E) Basal OCR versus basal ECAR.