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Corresponding Author:	Veena Sangkhae, Ph.D. University of California Los Angeles Los Angeles, California UNITED STATES
Corresponding Author's Institution:	University of California Los Angeles
Corresponding Author E-Mail:	vsangkhae@mednet.ucla.edu
Order of Authors:	Veena Sangkhae, Ph.D.
	Elizabeta Nemeth
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TITLE:

Quantitating Iron Transport Across the Mouse Placenta *In Vivo* Using Nonradioactive Iron Isotopes

AUTHORS AND AFFILIATIONS:
Veena Sangkhae, Elizabeta Nemeth

Center for Iron Disorders, David Geffen School of Medicine, University of California, Los Angeles, CA, USA

Email address of co-author:
Elizabeta Nemeth (ENemeth@mednet.ucla.edu)

Corresponding author:
Veena Sangkhae (VSangkhae@mednet.ucla.edu)

SUMMARY:
This article demonstrates how to prepare and administer transferrin-bound nonradioactive isotopic iron for studies of iron transport in mouse pregnancy. The approach for quantifying isotopic iron in fetoplacental compartments is also described.

ABSTRACT:
iron is essential for maternal and fetal health during pregnancy, with approximately 1 g of iron needed in humans to sustain a healthy pregnancy. Fetal iron endowment is entirely dependent on iron transfer across the placenta, and perturbations of this transfer can lead to adverse pregnancy outcomes. In mice, measurement of iron fluxes across the placenta traditionally relied on radioactive iron isotopes, a highly sensitive but burdensome approach. Stable iron isotopes (^{57}Fe and ^{58}Fe) offer a nonradioactive alternative for use in human pregnancy studies.

Under physiological conditions, transferrin-bound iron is the predominant form of iron taken up by the placenta. Thus, ^{58}Fe-transferrin was prepared and injected intravenously in pregnant dams to directly assess placental iron transport and bypass maternal intestinal iron absorption as a confounding variable. Isotopic iron was quantitated in the placenta and mouse embryonic tissues by inductively coupled plasma mass spectrometry (ICP-MS). These methods can also be employed in other animal model systems of physiology or disease to quantify *in vivo* iron dynamics.

INTRODUCTION:
Iron is critical for various metabolic processes, including growth and development, energy production, and oxygen transport[1]. Maintenance of iron homeostasis is a dynamic, coordinated process. Iron is absorbed from food in the duodenum and transported around the body in the circulation bound to the iron transport protein transferrin (Tf). It is utilized by every cell for enzymatic processes, incorporated into hemoglobin in nascent erythrocytes, and recycled from

aged erythrocytes by macrophages. Iron is stored in the liver when in excess and lost from the body through hemorrhage or cell sloughing. The amount of iron in circulation is the result of the balance between the consumption and the supply of iron, the latter being tightly regulated by the hepatic hormone hepcidin (HAMP), the central regulator of iron homeostasis[1]. Hepcidin functions to limit iron bioavailability in blood by occluding or inducing ubiquitination and degrading the iron exporter ferroportin (FPN)[2]. Reduction in functional FPN leads to decreased dietary iron absorption, iron sequestration in the liver, and decreased iron recycling from macrophages[1].

Hepcidin is regulated by iron status, inflammation, erythropoietic drive, and pregnancy (reviewed in [3]). Given that iron homeostasis is highly dynamic, it is important to understand and measure the total iron pool and iron distribution and turnover. Animal studies traditionally relied on radioactive iron isotopes, a highly sensitive yet burdensome approach to measure iron dynamics. However, in more recent studies, including the study presented here[4], nonradioactive, stable iron isotopes (^{58}Fe) are utilized to measure iron transport during pregnancy[5-9]. Stable isotopes are valuable tools for studying nutrient metabolism (reviewed in [10]). The use of stable iron isotopes in human studies demonstrated that i) iron absorption increases toward the end of gestation[5,6], ii) transfer of dietary iron to the fetus is dependent on maternal iron status[7], iii) maternally ingested heme iron is more readily incorporated by the fetus than nonheme iron[8], and iv) iron transfer to the fetus is negatively correlated with maternal hepcidin levels[8,9]. These experiments measured iron isotopes in sera or their incorporation into RBCs; however, measurement of iron incorporated into RBCs alone may underestimate true iron absorption[9]. In the current study, both heme and nonheme iron are measured in tissues.

During pregnancy, iron is required to support the expansion of maternal red blood cell volume and for transfer across the placenta to support the growth and development of the fetus[11]. Fetal iron endowment is wholly dependent on iron transport across the placenta. During human[12] and rodent[4,13] pregnancy, hepcidin levels dramatically decrease, increasing plasma iron availability for transfer to the fetus.

The fundamentals of placental iron transport were initially characterized in the 1950s–70s using radioactive tracers (^{59}Fe and ^{55}Fe). These studies determined that iron transport across the placenta is unidirectional[14,15] and that diferric transferrin is a major source of iron for the placenta and fetus[16,17]. The current understanding of placental iron transport is complete, although some key iron transporters and regulatory mechanisms remain unknown. Mouse models have been essential for understanding iron regulation and transport[18] because the key transporters and mechanisms are remarkably similar. Both human and mouse placentae are hemochorial, that is, maternal blood is in direct contact with the fetal chorion[19]. However, there are some notable structural differences.

The syncytiotrophoblast is the placental cell layer that separates the maternal and fetal circulation and actively transports iron and other nutrients[20]. In humans, the syncytiotrophoblast is a single layer of fused cells. In contrast, the mouse placenta consists of two syncytiotrophoblast layers[21], Syn-I and Syn-II. However, gap junctions at the interface of Syn-I and Syn-II allow the

diffusion of nutrients between layers[22,23]. Thus, these layers function as a single syncytial layer similar to the human syncytiotrophoblast. Additional similarities and differences between human and mouse placentae are reviewed by Rossant and Cross[21]. Placental iron transport is triggered by the binding of iron-Tf from maternal blood to the transferrin receptor (TfR1) localized on the apical side of the syncytiotrophoblast[24]. This interaction induces iron-Tf/TfR1 internalization via clathrin-mediated endocytosis[25]. Iron is then released from Tf in the acidic endosome[26], reduced to ferrous iron by an undetermined ferrireductase, and exported from the endosome to the cytoplasm by a yet-to-be determined transporter. How iron is chaperoned within the syncytiotrophoblast also remains to be described. Iron is eventually transported to the fetal side by the iron exporter, FPN, localized on the basal or fetal-facing surface of the syncytiotrophoblast (reviewed in[27]).

To understand how physiological and pathological regulation of TfR1, FPN, and hepcidin affects placental iron transport, stable iron isotopes were utilized to quantitate iron transport from the maternal circulation to the placenta and embryo *in vivo*[4]. This paper presents the methods for preparing and administering isotopic iron-transferrin to pregnant mice, processing of tissues for ICP-MS, and calculating iron concentrations in tissues. The use of stable iron isotopes *in vivo* can be adapted to investigate iron regulation and distribution in different animal models to investigate physiologic and pathologic iron regulation.

PROTOCOL:

All animal protocols and experimental procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California Los Angeles.

1. Preparation of ^{58}Fe-Tf

NOTE: The protocol uses ^{58}Fe; however, an identical protocol can be used for ^{57}Fe. Either isotope can be used and disposed of as a standard iron chemical without additional precautions.

1.1. Dissolve ^{58}Fe in 12 N HCl at 50 µL of HCl/mg of ^{58}Fe.

1.1.1. Add HCl to the metal in the glass vial supplied by the vendor, and replace the cap loosely. To dissolve the iron, warm the ^{58}Fe/HCl solution to 60 °C for 1 h. If still not dissolved, leave the solution overnight at room temperature in the fume hood to dissolve.

NOTE: Dissolved ^{58}Fe/HCl solution is yellowish-orange in color.

$$Fe_3O_{4(s)} + 8HCl_{(aq)} \rightarrow Fe^{(II)}Cl_{2(aq)} + 2Fe^{(III)}Cl_{3(aq)} + 4H_2O$$

1.2. Oxidize any remaining $Fe^{(II)}Cl_2$ to generate the $Fe^{(III)}Cl_3$ solution.

1.2.1. Warm up the ^{58}Fe/HCl solution to 60 °C with the cap off to facilitate oxidation.

1.2.2. Add 1 µL of 35% H_2O_2 per 50 µL of ^{58}Fe/HCl solution to further facilitate oxidation.

$4Fe^{(II)}Cl_{2(aq)} + O_2 + 4HCl \rightarrow 4Fe^{(III)}Cl_{3(aq)} + 2H_2O$

1.3. Prepare the ferric chloride ($^{58}Fe^{(III)}Cl_3$) solution.

1.3.1. Leave the ferric chloride solution in the hood at 60 °C with the cap off to evaporate the sample.

NOTE: Evaporation may take between one and several days.

1.3.2. Reconstitute $^{58}Fe^{(III)}Cl_3$ to 100 mM with ultrapure H_2O, and calculate the amount of ultrapure H_2O required based on the initial metal weight used in step 1.1 (molecular weight of $^{58}Fe^{(III)}Cl_3$ is 162.2).

1.4. Prepare $^{58}Fe^{(III)}$-nitrilotriacetate (NTA) by incubating $^{58}Fe^{(III)}Cl_3$ with NTA at a 1:5 molar ratio in the presence of 20 mM $NaHCO_3$.

1.4.1. Prepare 500 mM NTA in 1 N NaOH.

1.4.2. Prepare 5x transferrin-loading buffer (0.5 M HEPES, pH 7.5; 0.75 M NaCl).

1.4.3. Prepare 1 M $NaHCO_3$ in ultrapure H_2O.

1.4.4. To a 15 mL conical tube, add 150 µL of 100 mM $^{58}Fe^{(III)}Cl_3$ solution (from step 1.3.2), 150 µL of 500 mM NTA prepared in 1 N NaOH, 480 µL of ultrapure H_2O, 200 µL of 5x transferrin loading buffer, and 20 µL of 1 M $NaHCO_3$ solution.

1.4.5. Incubate the mixture for 5 min at room temperature.

1.5. Load apo-Tf with $^{58}Fe^{(III)}$-NTA to form ^{58}Fe-Tf.

NOTE: This protocol was adapted from McCarthy and Kosman[28].

1.5.1. Dissolve 500 mg of apo-Tf in 4 mL of 1x Tf-loading buffer.

1.5.2. To the 15 mL conical tube in step 1.4.4 containing 1 mL of the $^{58}Fe^{(III)}$-NTA solution, add 4 mL of apo-Tf solution.

NOTE: This is a 3:1 molar ratio of ^{58}Fe-NTA with apo-Tf. Each Tf contains 2 Fe binding sites; excess ^{58}Fe-NTA was added to ensure that Tf was fully loaded.

1.5.3. To allow maximal loading of ^{58}Fe-NTA onto apo-Tf, check that the solution is at pH 7.5, and adjust the pH, if necessary, with $NaHCO_3$ or HCl.

1.5.4. Incubate for 2.5 h at room temperature.

1.6. Remove excess unbound $^{58}Fe^{(III)}$-NTA and released NTA.

1.6.1. Transfer the ^{58}Fe-Tf solution to a molecular weight cutoff column (30 kDa cutoff) and centrifuge at 2,500 × *g* for 15 min at room temperature.

1.6.2. Wash the column with 10 mL of 1x transferrin-loading buffer and centrifuge at 2,500 × *g* for 15 min at room temperature. Repeat the wash and centrifugation, perform a saline wash with 10 mL of saline, and centrifuge at 2,500 × *g* for 15 min at room temperature.

1.7. Calculate the concentration of ^{58}Fe-Tf.

NOTE: Due to the addition of excess ^{58}Fe in step 1.5.2, assume that all transferrin is diferric. As 500 mg of apo-Tf was used, ~500 mg ^{58}Fe-Tf was produced in step 1.5.4.

1.7.1. Measure the volume recovered from centrifugation after the saline wash in step 1.6.2.

1.7.2. Divide 500 mg by the volume recovered to determine the concentration (in mg/mL) of the ^{58}Fe-Tf solution.

1.8. Sterilize the ^{58}Fe-Tf solution using a 0.22 µm syringe filter; store at 4 °C until ready to use.

NOTE: ^{58}Fe-Tf solution was used between 1 to 4 weeks post preparation.

2. Set up timed mouse pregnancies

2.1. Use 6- to 8-week-old female mice. Place animals on a low-iron diet (4 ppm iron) or standard chow (185 ppm iron) for 2 weeks prior to mating and maintain animals on the respective diets throughout pregnancy.

2.2. *Option 01*: Confirm pregnancy by weight gain at E7.5.

2.2.1. Set up multiple breeding cages. For each cage, combine 2 females with 1 male overnight; the following day when animals are separated is considered embryonic day (E)0.5. Weigh females at E7.5 to determine if pregnant. Mate males again with females that did not gain weight.

NOTE: In WT C57BL/6, a weight gain of 1 g at E7.5 is a good indicator of pregnancy. This method ensures that implantation occurred within a specific 16 h timeframe, allowing for synchronous treatment of all animals that became pregnant during the same mating period.

2.3. *Option 02*: Confirm pregnancy by plug checks.

2.3.1. Combine 2 females with 1 male and perform daily plug checks to determine if copulation has occurred.

NOTE: This method may result in staggered pregnancies, and the presence of a plug does not guarantee pregnancy.

3. Administer ^{58}Fe-Tf intravenously to E17.5 pregnant mice.

3.1. Prepare ^{58}Fe-Tf from step 1.8 for injection.

3.1.1. Prepare ^{58}Fe-Tf solution at 35 mg/mL in saline; inject 100 µL per mouse.

3.1.2. Fill an insulin syringe with 100 µL of the ^{58}Fe-Tf solution.

NOTE: Each dose contains 3.5 mg of human ^{58}Fe-Tf (5 µg of ^{58}Fe).

3.2. Anesthetize a pregnant mouse using isoflurane.

3.2.1. Use an isoflurane regulator with a chamber.

3.2.2. Use the following settings: 5% isoflurane, 2 L/mL of O_2, 2 min.

3.2.3. Confirm the mouse is anesthetized by looking for lack of response to a toe pinch.

3.2.4. Apply eye lubricant to the surface of the eye and place the mouse on a heating pad.

3.3. Slowly and carefully inject the ^{58}Fe-Tf solution into the retro-orbital sinus.

3.4. Allow the mouse to recover from anesthesia; do not leave the animal unattended until it has regained sufficient consciousness to maintain sternal recumbency.

3.5. Six hours post injection, euthanize E17.5 pregnant females by isoflurane overdose.

3.5.1. Pin the feet down with needles for stabilization.

3.5.2. Perform a cardiac puncture to exsanguinate the mouse as a form of secondary euthanasia.

3.6. Collect the placentae and embryo livers.

3.6.1. Using sterile forceps and dissection scissors, carefully remove the uterus from the pregnant mouse. Cut off a placental fetal-placental unit, which comprises a single fetus and placenta in the amniotic sac surrounded by a portion of the uterus.

3.6.2. Carefully cut through the uterus and amniotic sac without disturbing the fetus and placenta.

3.6.3. Peel back the amniotic sac and remove the fetus and placenta.

3.6.4. Cut the umbilical cord.

3.6.5. Blot the fetus and placenta on a clean task wipe to remove the excess amniotic fluid.

3.6.6. Record the weights of the whole placentae.

3.6.7. Cut each placenta in half with a razor blade, place each half in a 2.0 mL tube, and snap-freeze in liquid nitrogen.

NOTE: Because ^{58}Fe does not require special handling precautions and disposal, one-half of the placentae can be used for ^{58}Fe measurement and the other half for any other analyses, including quantitation of transferrin receptor (TFR1) and ferroportin (FPN) expression by western blotting and qPCR.

3.6.8. To collect embryo livers, sacrifice the embryo: use a razor blade to rapidly decapitate the embryo.

3.6.9. Pin down the embryo for stabilization, leaving the abdomen exposed.

3.6.10. Using dissection scissors, make a small incision where the umbilical cord was attached, insert one end of the dissection scissors into the incision, and perform a median plane cut toward the coronal plane about ¼ inch. Then, perform transverse plane cuts to expose the fetal liver.

3.6.11. Use forceps to remove the fetal liver.

3.6.12. Record the weights of the whole embryo livers.

3.6.13. Place the whole embryo livers in 2 mL tubes and snap-freeze them in liquid nitrogen.

NOTE: Alternatively, only a portion of the embryo liver can be used for ^{58}Fe measurement if additional analyses are desired. Using 2.0 mL tubes allows for better tissue homogenization than 1.5 mL tubes.

3.7. Store the tissues indefinitely at -80 °C.

4. Process tissues for quantitative iron analysis by ICP-MS.

4.1. Process the placentae and fetal livers for the quantitation of **nonheme iron**.

4.1.1. Thaw placental halves and whole fetal livers, and weigh placental halves (see step 3.6.12 for recording fetal liver weights).

4.1.2. Add 400 µL of protein precipitation solution (0.53 N HCl, 5.3% TCA).

4.1.3. Homogenize the tissue using an electric homogenizer.

4.1.4. Incubate the samples at 100 °C for 1 h.

4.1.5. Cool the samples in room temperature water for 2 min.

4.1.6. Open the caps to release pressure, then close the tubes again.

4.1.7. Centrifuge at 17,000 × *g* for 10 min at room temperature to pellet tissue debris.

4.1.8. Carefully transfer the supernatant to a new labeled tube.

4.1.9. Send samples off for ICP-MS analysis.

4.2. Process the placentae and fetal livers for the quantitation of **heme-iron.**

NOTE: Following extraction of nonheme iron in step 1, the iron remaining in the pellet is predominantly heme.

4.2.1. Record the weight of each pellet from step 4.1.7.

4.2.2. Digest the pellets in 10 mL of concentrated 70% HNO_3 supplemented with 1 mL of 30% H_2O_2

NOTE: Consult with the ICP-MS core or center to optimize the volume of HNO_3 for specific studies; the volume will partly be dependent on sample weight.

4.2.3. Heat the samples to 200 °C for 15 min.

4.2.4. Send the samples off for ICP-MS analysis.

NOTE: If distinguishing between heme and nonheme iron sources is not required and only total iron is measured, whole tissue can be digested in HNO_3 as the first step.

5. Data analysis

NOTE: Data from ICP-MS has been provided as ^{56}Fe and ^{58}Fe concentrations in ng/mL or mg, ppb (**Table 1**). ^{56}Fe is the most abundant iron isotope in nature, and its measurement reflects iron accumulation in the placenta/embryo over the entire pregnancy, whereas ^{58}Fe measurement

reflects iron that was transferred during 6 h after injection.

5.1. Subtract the natural abundance of ^{58}Fe (0.28% of total Fe) from the measured ^{58}Fe values.

5.2. Calculate total nonheme ^{58}Fe.

5.2.1. Calculate embryo liver total nonheme iron (ng) by first multiplying the iron concentration (ng/mL) calculated in step 5.1 by the volume (mL) during initial processing in step 4.1.2 to estimate total ^{58}Fe.

5.2.2. Calculate the amount of iron in the whole placenta by taking the total weight of the placenta measured in step 3.6.6 and dividing it by the weight of the placenta processed in step 4.1.1. Multiply this value by the total nonheme iron (ng) calculated in step 5.2.1 to obtain the total nonheme ^{58}Fe content of the placenta.

5.3. Calculate total heme ^{58}Fe.

5.3.1. Calculate total heme ^{58}Fe by first multiplying the iron concentration (ng/mg) calculated in step 5.1 by the weight of the pellet (in mg) measured in step 4.2.1.

5.3.2. Then, divide the total weight of the placenta measured in step 3.5.1 by the weight of the placenta pellet measured in step 4.2.1. Multiply this value by the total heme iron (ng) calculated in step 5.3.1 to obtain total heme ^{58}Fe content of the placenta.

5.4. Sum the calculated nonheme and heme ^{58}Fe values to determine the total iron content for each tissue.

[Place Figure 1 here]

REPRESENTATIVE RESULTS:

An earlier study using stable iron isotopes to measure iron transport demonstrated that maternal iron deficiency resulted in the downregulation of the placenta iron exporter, FPN[4]. FPN is the only known mammalian iron exporter, and the absence of FPN during development results in embryonic death before E9.5[29]. To determine whether the observed decrease in FPN expression translated functionally to decreased placental iron transport, ^{58}Fe-Tf was injected intravenously into pregnant dams, and iron in the placenta and the embryo was quantified in the presence of maternal iron deficiency.

To understand how placental iron transport is affected by maternal iron status, iron deficiency was modeled in mice[4]. Female C57BL/6 mice were placed on a low-iron diet (4 ppm iron) or standard chow (185 ppm iron) for 2 weeks prior to and throughout pregnancy. This dietary regimen results in lower maternal liver nonheme iron and serum iron and hemoglobin at E12.5, E15.5, and E18.5 compared to animals on a standard diet[4]. At E18.5, embryos from iron-deficient mothers had lower liver iron and were hypoferremic and anemic than embryos from iron-replete

mothers. Three pregnant mice were used in each of the iron-replete and iron-deficient groups, and 2–3 placentae were used from each pregnant mouse for analysis.

To quantitate placental iron transport, ^{58}Fe-transferrin was prepared and injected intravenously in pregnant dams and ^{58}Fe measured in the placenta and fetal liver by ICP-MS, as described in the protocol and illustrated in **Figure 1**. Prior to sending nonheme iron samples out for ICP-MS analysis, total nonheme iron levels were independently quantified via a ferene method described previously[30]. Nonheme iron concentrations measured by the ferene versus ICP-MS methods were highly significantly correlated in all tissues measured (R^2 = 0.94, P < 0.0001, n = 36). Representative results from ICP-MS quantitation of iron isotopes are presented in **Table 1**. Total ^{58}Fe was calculated as described in step 5 of the protocol. Data are presented as total rather than heme or nonheme iron (**Figure 2A-D**) because the aim was to quantitate total iron transferred into the placenta and total iron transferred to the embryo from the placenta.

On average, 21% of the administered ^{58}Fe dose was recovered in the placenta, embryo liver, and embryo serum combined. The ^{56}Fe measurement provides insight into the long-term iron transfer in the placenta and embryo liver throughout pregnancy. The total placental ^{56}Fe was similar in the iron-deficient and -replete groups (**Figure 2A**), whereas the total embryo liver iron was decreased in the iron-deficient group (**Figure 2B**). This was expected based on the observed decrease in placental FPN in the iron-deficient group[4], which would result in iron retention in the placenta at the expense of the embryo. Total ^{58}Fe provides a snapshot of short-term iron transport. In this study, similar to ^{56}Fe, placental ^{58}Fe was similar in both the iron-deficient and -replete groups (**Figure 2C**), and embryo liver ^{58}Fe was decreased in the iron-deficient group (**Figure 2D**). These data indicate that during iron-deficient pregnancy, the downregulation of placental FPN results in decreased iron transport to the embryo, leading to cumulative differences in iron content in the placenta and embryo.

It is important to consider the dose of iron administered as it could lead to unintended changes in hepcidin concentration or iron transporter expression[31]. It was demonstrated that maternal iron deficiency caused a decrease in placental FPN[4]. To determine if Fe-Tf injection affected this regulation, placenta FPN was measured 6 h post injection by western blot. The iron dose of 5 µg was insufficient to alter placental FPN regulation by maternal iron deficiency (**Figure 3**).

In summary, this method was used to demonstrate that physiological regulation of placental FPN during maternal iron deficiency results in decreased iron transport across the placenta *in vivo*. Stable iron isotopes provide a sensitive and quantifiable alternative to radioactivity for the measurement of iron transport and distribution, allowing the simultaneous use of tissues for additional analyses.

FIGURE AND TABLE LEGENDS:

Figure 1: Visual summary of steps in the protocol. (**A**) Preparation of ^{58}Fe-transferrin. (**B**) *In vivo* administration of ^{58}Fe-transferrin. (**C**) Tissue collection and storage. (**D**) Processing of the placenta and embryo liver for quantitation of metal species by ICP-MS. Abbreviations: Fe = iron;

NTA = nitrilotriacetic acid; Tf = transferrin; PPS = protein precipitation solution; Sup = supernatant; TCA = trichloroacetic acid; ICP-MS = inductively coupled plasma mass spectrometry.

Figure 2: ^{56}Fe and ^{58}Fe transport across the placenta in iron-deficient or iron-replete pregnancies. Total ^{56}Fe in the placenta (**A**) and embryo liver (**B**). Total ^{58}Fe in the placenta (**C**) and fetal liver (**D**). Statistical analysis was performed using a 2-tailed Student's *t*-test for normally distributed values and otherwise by Mann-Whitney *U* rank-sum test (denoted by an asterisk after the P-value). The number of animals is indicated in the x-axes of the box and whisker 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 those below the box indicate the 10th percentile. The solid line within the box indicates the median and the dashed line the mean. Statistical analysis was performed using scientific graphing and data analysis software. This figure has been modified from[4]. Abbreviation: Fe = iron.

Figure 3: Placental TFR1 and FPN levels. (**A**) TFR1 and FPN expression was assessed by western blot in iron-deficient and -replete placentae 6 h post treatment of mothers with ^{58}Fe-Tf. (**B**) Protein expression was quantitated and presented as protein expression relative to β-actin. Statistical analysis was performed using a 2-tailed Student's *t*-test for normally distributed values. The number of animals is indicated in the x-axes of the box and whisker 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 those below the box indicate the 10th percentile. The solid line within the box indicates the median and the dashed line the mean. Statistical analysis was performed using scientific graphing and data analysis software. This figure has been modified from[4]. Abbreviations: TFR1 = transferrin receptor; FPN = ferroportin.

Table 1: Representative results from ICP-MS quantitation of ^{56}Fe and ^{58}Fe in placentae and embryo livers. Abbreviations: ppb = parts per billion; stdev = standard deviation; ICP-MS = inductively coupled plasma mass spectrometry.

DISCUSSION:

Iron is important for many biological processes, and its movement and distribution within the body are highly dynamic and regulated. Stable iron isotopes provide a consistent and convenient alternative to radioactive isotopes for the assessment of the dynamics of iron homeostasis. A critical step in the protocol is keeping track of all the tissue weights and volumes. Iron is an element and therefore cannot be synthesized nor broken down. Thus, if all weights and volumes are carefully logged, all the iron within the system can be accounted for by calculation. As described, this method can be used to distinguish between heme and nonheme iron sources. However, if this distinction between iron forms is not necessary and only total iron is measured, the protocol can be simplified by treating tissue only with concentrated HNO_3 as described in protocol step 4.2. It is important to note that if tissues are not perfused before analysis, especially highly vascular tissues such as the placenta, the presence of blood may result in the overestimation of tissue heme iron content.

Transferrin-bound iron was selected for the study as it is the major source of iron taken up by the placenta[16,17]. Global knockdown of *TFR1* in mice resulted in embryonic lethality before E12.5, suggesting that transferrin-bound iron is critical for development. It is possible that other iron species, such as ferritin and nontransferrin bound iron (NTBI), also contribute to fetal iron endowment to a lesser extent. However, the contribution of these alternative iron species was not assessed. In the future, stable isotopes could be used to determine the contribution of different iron sources to development and embryo iron endowment.

The aim of the study was to determine the effects of changes in maternal iron status on placental iron transport. However, decreased hepcidin during iron deficiency results in elevated enterocyte FPN levels and enhanced iron transport into the circulation[1]. Thus, in iron-deficient dams, iron absorption from the diet would have been inherently increased and confounded interpretation of results if ^{58}Fe was administered orally. Thus, intravenous administration of ^{58}Fe-Tf was selected as it bypasses iron regulation at the level of intestinal absorption. A dose of 5 µg of ^{58}Fe/mouse was selected based on serum iron concentrations of iron-replete E18.5 pregnant dams. In wild-type C57BL/6 E18.5 pregnant dams, serum iron concentrations range from 10 to 50 µM[4]. A pregnant E18.5 mouse is expected to have approximately 2 mL of total blood volume[32]. Thus, the total amount of iron in the circulation of iron-replete pregnant dams ranges from 1.1 to 5.6 µg. Thus, 5 µg of ^{58}Fe/mouse is equivalent to physiological concentrations observed in iron-replete animals.

A limitation of ICP-MS detection of ^{58}Fe is the isobaric interference from ^{58}Ni. Endogenous Ni concentrations in the mouse placenta are 0.04 ± 0.02 µg/g wet weight[33]. An average E18.5 mouse placenta weighs 0.080 g; therefore, the total amount of Ni is approximately 3.2 ng. The natural abundance of ^{58}Ni is 68%; thus, the amount of ^{58}Ni in the mouse placenta is ~2.2 ng, which is approximately 10-fold lower than the detected ^{58}Fe levels. In the embryo, Ni concentrations are even lower at 0.01 ± 0.01 µg/g wet weight[33]. The average E18.5 mouse embryo weighs 1 g; thus, the total amount of Ni in a normal mouse embryo is approximately 10 ng. Assuming all the embryo Ni is found in the embryo liver, these levels are still 10-fold lower than the ^{58}Fe concentrations and nearly 1,000-fold lower than the total embryo liver iron content. Given the lower abundance of Ni in these mouse tissues, ^{58}Ni interference was not factored in this study.

An additional consideration is the assay's limit of detection. The limit of detection in this study was 250 pg/mL ^{58}Fe. However, this limit can be altered to detect even lower concentrations of ^{58}Fe if dilution of tissues is reduced at the tissue processing step (protocol step 4.1.2 and **Figure 1D**) or via modifications at the ICP-MS core facility. When ^{58}Fe was measured in the entire embryo, its levels were undetected as the ^{58}Fe concentration was below the limit of detection. However, ^{58}Fe was detected in the embryo liver, which is the primary iron storage organ. It is possible that the administration of a larger dose of ^{58}Fe would have allowed detection of ^{58}Fe even in the whole embryo. However, a relatively small amount of ^{58}Fe was used to avoid iron-loading of the placenta, which could trigger feedback mechanisms and alter the expression of iron transporters. In this model, which utilized wild-type C57BL/6 mice, embryo liver iron was measured as a reflection of total placental iron transport, as embryo liver iron concentration is proportional to the whole embryo iron concentration[4]. However, in mouse models where iron

distribution is altered[34], embryo liver iron alone may not accurately represent total placental iron transport. In such cases, it may be necessary to measure iron incorporated into the entire embryo or the erythrocyte compartment. Additionally, variations in experimental time points will also require further optimization and measurement of iron in various fetal compartments. This stable isotope tracing approach was utilized to quantify iron transport during mouse pregnancy. The methodology is easily adaptable to study iron transport in nonpregnant mice and other animal models.

ACKNOWLEDGMENTS:

The authors acknowledge the use of the ICP-MS facility within the UC Center for Environmental Implications of Nanotechnology in CNSI at UCLA for their assistance with optimizing the protocol for ^{58}Fe measurements. The study was supported by the NIH National Institute of Diabetes and Digestive and Kidney Diseases (NIDDK) (K01DK127004, to VS) and NIH National Institute of Child Health and Human Development (NICHD) (R01HD096863, to EN).

DISCLOSURES:

EN is a scientific co-founder of Intrinsic LifeSciences and Silarus Pharma and a consultant for Protagonist, Vifor, RallyBio, Ionis, Shield Therapeutics, and Disc Medicine. VS declares no conflicts.

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Figure 1

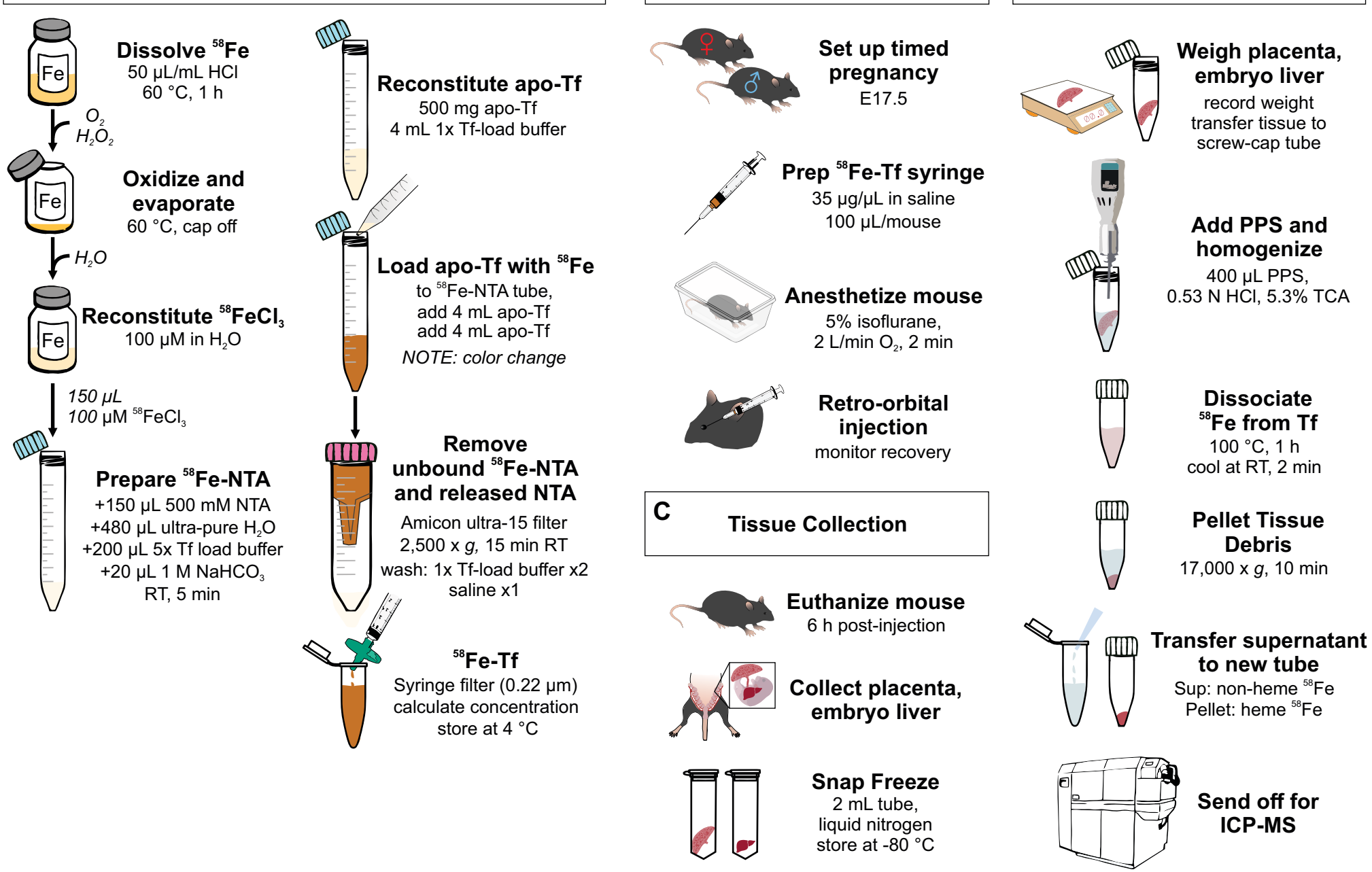

Figure 2

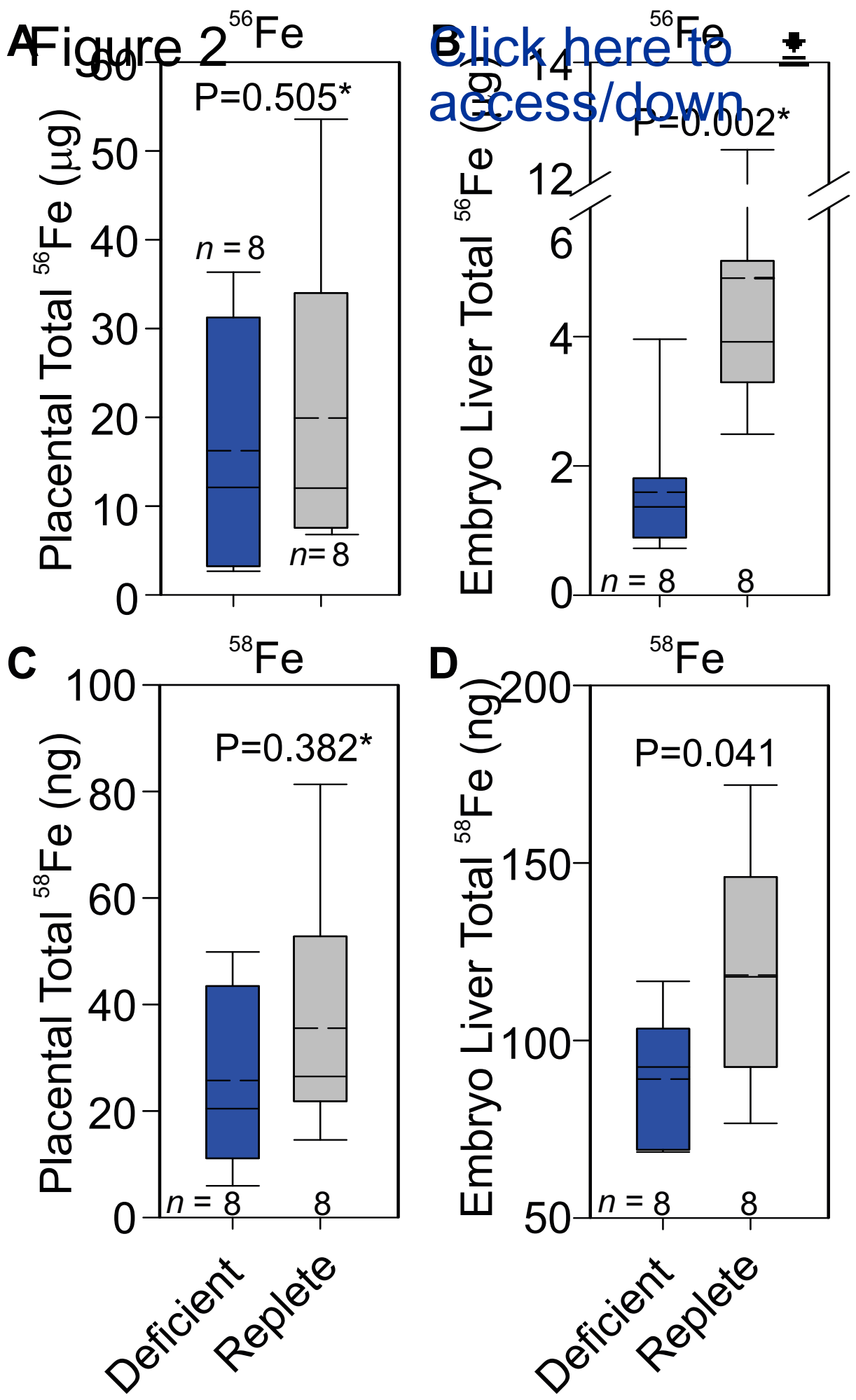

Figure 3

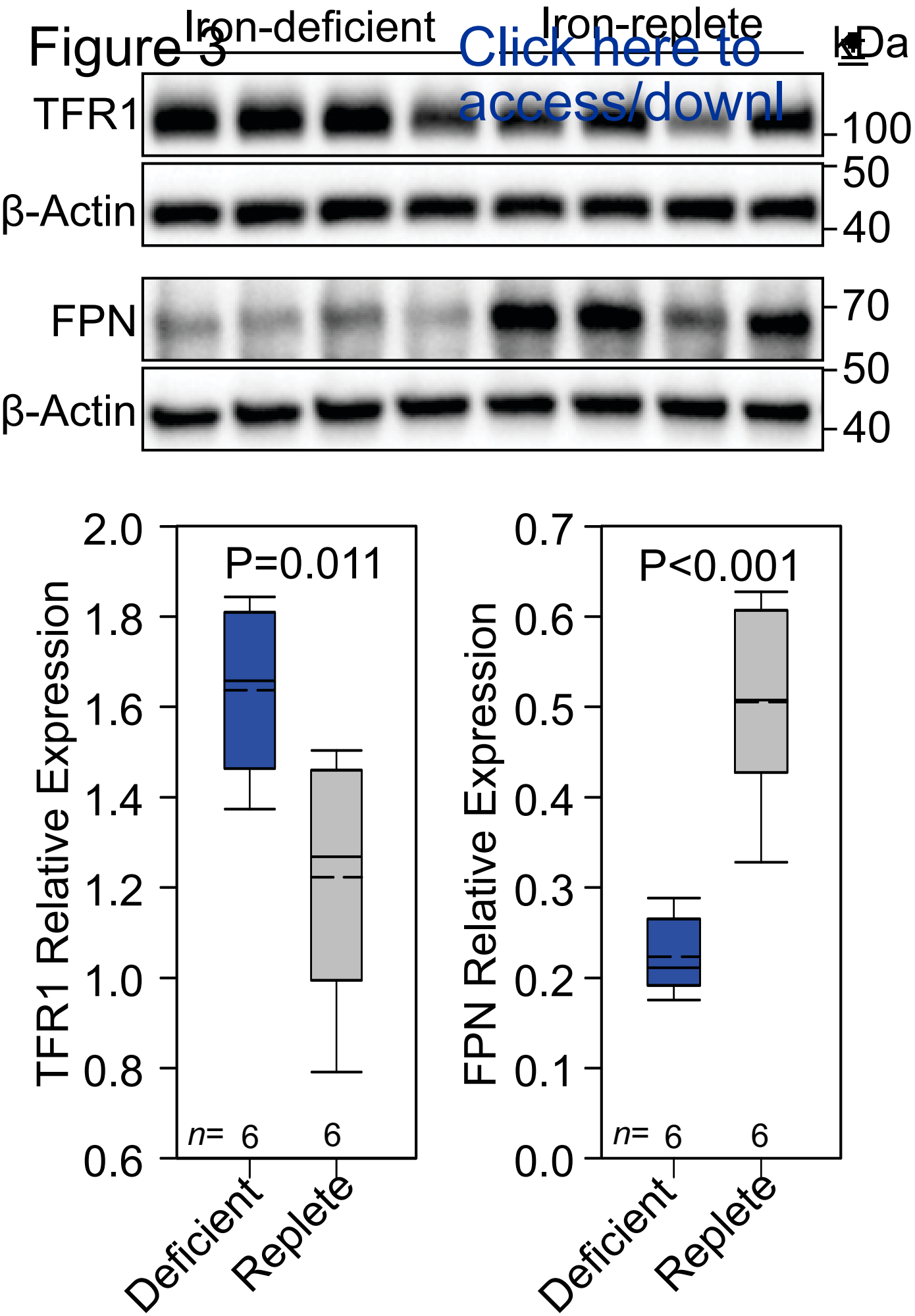

Table 1

Table 1

Sample			^{56}Fe		^{58}Fe		Total Fe
			Concentration [ng/mL or mg, ppb]		Concentration [ng/mL or mg, ppb]		Sum of Isotopes [ng/mL or mg]
			Average*	stdev	Average*	stdev	
Nonheme iron	Placenta	iron-deficient	729.7	17.7	2.5	0.5	732.2
			704.9	6.2	3.8	0.1	708.8
			649.8	3.8	0.0	0.0	649.8
			799.2	4.6	3.8	0.2	803.0
		iron-replete	1919.1	5.3	11.0	0.2	1930.1
			1610.0	26.8	11.7	0.6	1621.7
			1925.5	39.0	14.0	0.3	1939.5
			2551.6	16.1	8.3	0.4	2559.9
Heme	Placenta	iron-deficient	253.8	1.8	1.1	0.0	254.9
			32.9	0.4	0.3	0.0	33.2
			337.7	5.1	1.4	0.0	339.1
			402.3	5.3	1.7	0.0	404.0
		iron-replete	123.5	1.3	0.6	0.0	124.0
			75.7	1.3	0.4	0.0	76.1
			441.9	3.0	1.9	0.0	443.8
			250.4	1.1	1.1	0.0	251.5
Nonheme iron	Embryo Liver	iron-deficient	361.6	8.3	31.9	1.0	393.5
			652.4	3.4	61.7	0.3	714.1
			411.9	10.7	43.1	0.8	455.0
			631.1	7.5	62.8	0.2	693.9
		iron-replete	7657.5	129.3	226.4	2.2	7883.8
			3820.2	69.5	119.4	3.4	3939.6
			5519.0	112.9	145.6	0.5	5664.6
			4617.4	78.6	91.6	1.0	4709.0
Heme	Embryo Liver	iron-deficient	44.5	0.3	1.6	0.0	46.0
			31.0	0.4	2.9	0.0	34.0
			11.8	0.2	1.1	0.0	12.9
			42.3	0.1	3.2	0.0	45.5
		iron-replete	54.3	1.4	2.1	0.0	56.4
			31.9	0.8	1.3	0.1	33.2
			59.4	0.6	2.2	0.0	61.6
			66.7	0.6	2.1	0.0	68.8

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Table of Materials

JoVE_Materials Sangkhae Nemeth resub02.xls

Thank you for the constructive comments. Below are our point-by-point responses in blue.

Editorial comments:

Editorial Changes

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Done

2. Please provide suitable citations for the following lines: 47-50, 60-67.

Appropriate citations have been added to the manuscript

3. Please write all gene names in capital and italicize the gene names in the manuscript as well as the figures.

Done

4. Table 1: Please denote 56Fe and 58Fe using proper convetion i.e., 56Fe and 58Fe. Please define all abbreiviatiosn and symbols used in the tabel in the legends.

Table 1 has been updated using proper convention for 56Fe and 58Fe. Abbreviations and symbols in tables and legends have been defined.

5. Please do not number the NOTES and equations in the protocol.

Numbering of notes and equations has been removed

6. Please use h, min, s, for hour, minute, second.

Abbreviations have been updated throughout the manuscript and figures

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.

All text in the protocol is now in imperative tense

8. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

All personal pronouns have been removed from the manuscript

9. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

Step 1.3.3: How was the 100 mM concentration determined?

This was step was elaborated on as follows, it is now step 1.3.2:

> “1.3.2. Reconstitute 58Fe(III)Cl3 to 100mM with ultra-pure H2O, calculate the amount of ultra-pure H2O required based on the initial metal weight used in step 1.1 (Mw of 58Fe(III)Cl3 is 162.2)”

Step 1.6.2: Was each wash followed by a centrifugation step? If yes, please include this and mention the centrifugation conditions and duration.

Each wash was followed by a centrifugation step, this has been included along with conditions and duration

> “1.6.2. Wash column with 10 mL 1X transferrin-loading buffer and centrifuge at 2,500 x g for 15 minutes at room temperature, repeat wash and centrifugation, then perform a saline wash with 10mL saline and centrifuge at 2,500 x g for 15 minutes at room temperature”

Step 3.2: Please mention how proper anesthetization is confirmed. Please specify the use of vet ointment on eyes to prevent dryness while under anesthesia.

The following statements have been added:

> 3.2.3. Confirm animal is anesthetized by performing a toe pinch; if animal is appropriately anesthetized, it will not respond
>
> 3.2.4. Apply eye lubricant to the surface of the eye and place the animal on a heating pad

Step 3.4: Please specify that the animal is not left unattended until it has regained sufficient consciousness to maintain sternal recumbency.

Statement has been added

Step 3.5: Is it E7.5 or E17.5? Please check. How was the placenta and embryo liver collected? Please describe all the associated steps.

It is indeed E17.5. How the placenta and embryos livers were collected has been added to the protocol:

"3.5. 6 hours post-injection, euthanize E17.5 pregnant females by isoflurane overdose

3.5.1. Pin feet down with needles for stabilization

3.5.2. Perform a cardiac puncture to exsanguinate as a form of secondary euthanasia

3.6. Collect placentas and embryo livers

3.6.1. Using sterile forceps and dissection scissors carefully remove the uterus from the pregnant mouse

3.6.2. Cut off a placental fetal-placental unit, which is comprised of a single fetus and placenta in the amniotic sac surrounded by a portion of the uterus

3.6.3. Carefully cut through the uterus and amniotic sac without disturbing the fetus and placenta

3.6.4. Peel back the amniotic sac and remove the fetus and placenta

3.6.5. Cut the umbilical cord

3.6.6. Blot the fetus and placenta on a clean task wipe to remove excess amniotic fluid

3.6.7. Record weights of whole placentae

3.6.8. Cut each placenta in half with a razor blade, place each half into a 2.0 mL tube and snap freeze in liquid nitrogen

NOTE: because 58Fe does not require special handling precautions and disposal, one half of placentae can be used for 58Fe measurement, and the other half for any other analyses, including quantitation of transferrin receptor (TFR1) and ferroportin (FPN) expression by Western blotting and qPCR

3.6.9. To collect embryo livers, sacrifice the embryo: use a razorblade to rapidly decapitate the embryo

3.6.10. Pin down embryo for stabilization, leaving abdomen exposed

3.6.11. Using dissection scissors, make a small incision where the umbilical cord was attached, insert one end of the dissection scissors into the incision and perform a median plane cut toward the coronal plane about ¼ inch, then perform transverse plane cuts to expose the fetal liver

3.6.12. Use forceps to remove the fetal liver

3.6.13. Record weights of whole embryo livers"

Step 4.2.2: How much HNO3 and H2O2 was added? Was this volume dependent on sample weight? If yes, please specify.

Volumes were specific to our study:

"4.2.2. Digest pellets in 10mL concentrated 70% HNO3 supplemented with 1mL of 30%

NOTE: Consult with the ICP-MS core or center to optimize the volume of HNO3 for specific studies, volume will in part be dependent on sample weight"

10. Please include a single line space between all the steps, sub steps, and notes.

Line spacing has been corrected as requested

11. Figure 1 : Please use h for hour in the figure.

This has been corrected in Figure 1

12. Figure 2 and 3: Please remove commercial software names from the legend. These can be refernced in the table of materials.

Commercial software names have been removed from the legend and are now referenced in the table of materials.

13. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

The explicit copyright permission has been uploaded to my Editorial Manager account. The figures are also cited as requested.

14. Please do not abbreviate the journal titles in the reference section.

We downloaded the JoVe EndNote output style; however, the journal names are still automatically abbreviated. Please advise.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In order to follow and thoroughly analyze iron distribution in the body, especially with regard to homeostatic regulation of placental iron metabolism and regulatory mechanisms, well-validated experimental setups must be established. This study presents a well-described experimental setup to quantify placental iron transport with a non-radioactive method that allows distinguishing already small changes in iron metabolism. I am sure this study finds interest in readers of JOVE and would recommend accepting the manuscript after small changes.

Abstract and Introduction:

- As it is stated in the abstract (Line 23) and the introduction part (line 48) the stable isotopes Fe57 and Fe58 were already used to study iron homeostasis in human pregnancy studies. Please provide the respective references in line 48. Moreover, it would be valuable to either summarize hitherto analysis with these isotopes in the introduction and/or contextualize the findings of this study with the literature in the discussion part.

We agree that the previous studies using stable iron isotopes needed to be cited. We unfortunately overlooked this, appropriate citations have been added to line 48. We also elaborated on the studies themselves:

"Stable isotopes in general are a valuable tool to study nutrient metabolism (reviewed in [10]). The use of stable iron isotopes in human studies demonstrated that iron absorption increases toward the end of gestation[5,6], that transfer of dietary iron to the fetus is dependent on maternal iron status[7], that maternally ingested heme iron is more readily incorporated by the fetus than non-heme iron[8], and that iron transfer to the fetus is negatively correlated with maternal hepcidin levels[8,9]. These experiments measured iron isotopes in sera or incorporation into RBCs; however, measurement of iron incorporated into RBCs alone may underestimate true iron absorption[9]. In the current study, both heme and non-heme iron are measured in tissues."

- Line 35: I would suggest to also name the main transport protein in the blood

As per your suggestion, we have altered Line 35 to the following:

"Iron is absorbed from the diet in duodenum, transported around the body in the circulation bound to iron transport protein transferrin (Tf)…"

Representative results:
- The results part needs to be a little bit better structured. For example, It is not clear whether the information given in lines 232-233 refers to the present study or to reference 7. if it is the former, then it is better not to mention the main result right at the beginning of the results section, but to present the individual results first. However, if a reference is made at this point to an earlier study (source 7), this should also be stated more clearly.

We apologize that the statement was unclear, we have clarified that lines 232-233 refer to the previous study mentioned. The altered statement is as follows:

"In an earlier study using stable iron isotopes to measure iron transport, we demonstrated that maternal iron deficiency resulted in downregulation of the placenta iron exporter, FPN[4]"

- Although it is not part of the protocol, it would be important to understand the representative results and certainly interesting for the reader to know how iron deficiency was achieved and controlled in the animal model.

Thank you for the suggestion, we have added the information regarding how iron deficiency was achieved in our animal model. The following statement was added:

"To understand how placental iron transport is affected by maternal iron status, we modeled iron deficiency in mice[4]. Female C57BL/6 mice were placed on a low iron diet (4ppm iron) or standard chow (185ppm iron) for 2 weeks prior to and throughout pregnancy. This dietary regimen results in lower maternal liver non-heme iron, serum iron and hemoglobin at E12.5, E15.5 and E18.5 compared to animals on standard iron diet[4]. At E18.5, embryos from iron deficient mothers had lower liver iron and were hypoferremic and anemic compared to embryos from iron-replete mothers."

Discussion:
- The discussion lacks reference to the previous use of isotopes in the literature or radioactive analyses previously applied to study similar processes. If the latter exceeds the scope of the paper, a reference to review articles would still be good.

We have now extensively referenced within the introduction the literature on the use of isotopes in pregnancy. We would prefer not to repeat the same statements in the discussion.

Reviewer #2:
Manuscript Summary:
The manuscript by Sangkhae and Nemeth describes a non-radioactive method for examining the transfer of iron across the placenta. This has traditionally been assayed using radioisotopes, so a detailed protocol using stable isotopes of iron will be useful to those who, for various reasons, are unable to use radioactivity. The procedure is very detailed and will allow the reader to replicate all

aspects of the protocol. Below I have listed two potential limitations of the procedure that I feel the authors should discuss so that readers are aware of them.

Major Concerns:
1. The authors state that this is a method for quantitating the iron transport across the placenta (title and various other places in the manuscript). However, they only measure the amount of iron deposited in the liver, which represents only a portion of the total iron transferred. In most cases, it can be assumed that this is a reflection of total placental iron transfer, however, there will be certain situations where this will not be the case. For example, total body iron levels are normal in newborn DMT1 knockout mice when compared to WT mice, but they are anemic and have higher liver iron stores (JCI, 115:1258, 2005). This suggests that, while the placental iron transfer is unimpeded by a lack of DMT1, the transferred iron is preferentially taken up by the liver rather than the RBC compartment. In these mice, measuring liver iron levels only, as outlined in the current manuscript, would likely overestimate placental iron transfer. The authors should discuss this potential limitation and, although likely to occur with specific mouse models only, should explain that the method described is only valid if the distribution of iron in the fetus is normal.

We agree with the reviewer and have added the following statement to the discussion:

"In this model, which utilized wild-type C57BL/6 mice, embryo liver iron was measured as a reflection of total placental iron transport, as embryo liver iron concentration is proportional to the whole embryo iron concentration[4]. However, in mouse models where iron distribution is altered[33], embryo liver iron alone may not be an accurate representation of total placental iron transport. In such cases, it may be necessary to measure iron incorporated into the entire embryo or the erythrocyte compartment. Additionally, variations in experimental time points will also require further optimization and measurement of iron in various fetal compartments."

2. When measuring total heme iron in the placenta using 56Fe, the authors use tissue that has not been perfused to remove RBCs. As heme iron is relatively low in tissue when compared to RBCs, any blood remaining in the placenta will lead to an overestimation of tissue heme iron, especially as the placenta is a highly vascularized organ. Perfusing the placenta from both the maternal and fetal sides would be very difficult to include in the protocol, so I would be satisfied if the authors acknowledged in the manuscript that contamination with blood may compromise placental heme iron measurements.

We agree that perfusing the placenta would be ideal since the heme in RBCs confounds the total iron measurement. We added an acknowledgement that blood contamination in tissues may compromise the resulting iron measurements. We have added the following statement to the discussion:

"It is important to consider that prior to analysis, if tissues are not perfused, especially highly vascular tissues such as the placenta, presence of blood may result in overestimation of tissue heme iron content."

Reviewer #3:
Manuscript Summary:
The manuscript under review provides a protocol for tracing iron uptake across the murine placenta.

Given the importance of iron to the developing fetus, the prevalence of iron deficiency in pregnancy across the globe, and the large areas of iron metabolism in the placenta that are still not clearly understood, this is a very important area of work. The protocol, therefore, is a welcome addition to the field, and the use of 58Fe, which is not radioactive but also stable and not abundant naturally further enhances the relevance. A few relatively minor concerns are listed below.

Minor Concerns:
* The introduction regarding iron transport across the placental cell barriers should discuss the unique architecture of the mouse placenta.

We have added some information in the introduction regarding similarities between human and mouse placenta as follows, this includes a citation of a review that addresses the topic in greater detail:

"Mouse models have been essential for our understanding of iron regulation and transport[18], since the key transporters and mechanisms are remarkably similar. Both human and mouse placentae are hemochorial, meaning that maternal blood is in direct contact with the fetal chorion[19]. However, there are some notable structural differences. The syncytiotrophoblast is the placental cell layer that separates the maternal and fetal circulation and actively transports iron and other nutrients[20]. In humans, the syncytiotrophoblast is a single layer of fused cells. In contrast, the mouse placenta is comprised of two syncytiotrophoblast layers[21], Syn-I and Syn-II; however, gap junctions at the interface of Syn-I and Syn-II allow for diffusion of nutrients between layers[22,23], thus these layers function as a single syncytial layer similar to human syncytiotrophoblast. Additional similarities and differences between human and mouse placentae are reviewed by Rossant and Cross[21]."

* How stable is the 58Fe-Tf solution, i.e. how far in advance can it be prepared?

We used 58Fe-Tf solution between one to four weeks after preparation and added this statement to protocol line 1.8. Commercial suppliers such as Sigma, R&D and others state that holo-transferrin solution is stable at 4C for at least 1-4 weeks.

* Given that larger embryo/placental units are more likely to have a higher total 58Fe, it seems it would be more informative to evaluate the data on a per mg basis, rather than using these data to calculate total 58Fe.

We presented the data as total iron transferred because that is the best reflection of placental iron transport. If we present data on a per mg basis, in larger embryos that would underestimate the total iron that was transferred across the placenta.

* How was the dose of 58Fe administered to the dams determined? There is mention, correctly, of giving a small amount in order to avoid feedback mechanisms within the placenta, but it is unclear how it was determined that the protocol represents the optimal dose.

To address the selection of dose, we have added the following to the discussion section:

A dose of 5µg ^{58}Fe/mouse was selected based on serum iron concentrations of iron replete E18.5 pregnant dams. Serum iron concentrations in wild-type C57BL/6 E18.5 pregnant dams ranges from 10-50uM[4]. A pregnant E18.5 mouse is expected to have approximately 2mL total blood volume[32]. This

means the total amount of iron in the circulation of iron-replete pregnant dams ranges from 1.1-5.6µg. Thus, 5µg ^{58}Fe/mouse is equivalent to physiological concentrations observed in iron replete animals.

Reviewer #4:

Manuscript summary:

This manuscript describes in detail the use of stable iron isotope (58Fe) in studying fetal iron transport in mice. Stable iron isotope technique is commonly used to study iron absorption in humans. This study provides novel and helpful data that extend its application to iron kinetics in animals specifically in the context of pregnancy. The manuscript is very well written, protocol well illustrated. However, I have a few questions about the methodology and application:

1. The gold standard for kinetics study in animals is using radiotracers. It is accurate, allows for the determination of tracer distribution in multiple organs, does not require the use of a large amount of tissue or estimation of tissue iron content, which could be a significant source of error. For a method paper, I think it is important to validate the results against the gold standard method (or alternatives), especially when considering the sources of errors and uncertainties introduced in the new method.

Although we agree that the gold standard is using radiotracers, a direct comparison of our method to the radioactive method is beyond the scope of our manuscript.

2. Another consideration for isotope studies is the dose and the % recovery of dose in tissues. How does 5ug Fe compare to total serum Fe in mice? The blood volume in pregnant animals at late gestation E17.5 is considerably greater than in normal mice. It would be informative to include a section explaining the choice of dose and make suggestions for applications in non-pregnant animals. How much of the dose administered is recovered in the tissues measured?

Although plasma volume in C57BL/6 increases by 27% (Kulandavelu et al 2006 PMID: 16636199) at E17.5, all our calculations were based on previous iron measurements in pregnant mice, not non-pregnant mice.

To address the selection of dose, we have added the following to the discussion section:

A dose of 5µg ^{58}Fe/mouse was selected based on serum iron concentrations of iron replete E18.5 pregnant dams. Serum iron concentrations in wild-type C57BL/6 E18.5 pregnant dams ranges from 10-50uM[4]. A pregnant E18.5 mouse is expected to have approximately 2mL total blood volume[32]. This means the total amount of iron in the circulation of iron-replete pregnant dams ranges from 1.1-5.6µg. Thus, 5µg ^{58}Fe/mouse is equivalent to physiological concentrations observed in iron replete animals.

The amount of ^{58}Fe in the placenta, embryo liver and embryo serum was equivalent to 21% of the injected dose. We also added this point to the manuscript.

3. How many pregnant mice were used in the study? How many placentas from each mouse were used for isotope measurement?

Thank you for pointing out this information was missing in the manuscript, it has been added to the 'representative results' section as follows:

"3 pregnant mice were used per group, iron-replete and iron-deficient, and 2-3 placentas were used from each pregnant mouse for analysis."

4. The authors gave reasons in the discussion why they did not correct for nickel in the 58Fe measurement. While I agree that the naturally occurring 58Ni in tissues is small, the primary source of nickel contamination is from tubes, pipettes, and containers during sample transfer and preparation. Ni inference for 58Fe measurement can be severe in tissues that do not have high iron content (i.e., large red cell volume). Was Ni measured in the experiment?

No, Ni was not measured in the experiment.

5. Are there differences in 58Fe enrichment of heme and non-heme iron fractions between deficient and replete groups?

The difference between deficient and replete groups was greater in non-heme iron compartment than heme iron compartment for embryo liver; however, because this difference is an issue of cellular iron regulation rather than total placental iron transfer, we are showing total iron levels.

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