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Quantitating Iron Transport across the Mouse Placenta *In Vivo* using Nonradioactive Iron Isotopes

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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].

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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, 6, 7, 8, 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 more 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. Dissolve ^{58} Fe in 12 N HCl at 50 µL of HCl/mg of ^{58} Fe.
    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.

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

2. Oxidize any remaining $Fe^{(II)}$ Cl2 to generate the $Fe^{(III)}$ Cl3 solution.
    1. Warm up the ^{58} Fe/HCl solution to 60 °C with the cap off to facilitate oxidation.
    2. Add 1 µL of 35% H_2O_2 per 50 µL of ^{58} Fe/HCl solution to further facilitate oxidation.

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

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

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. Prepare 500 mM NTA in 1 N NaOH.
    2. Prepare 5x transferrin-loading buffer (0.5 M HEPES, pH 7.5; 0.75 M NaCl).
    3. Prepare 1 M $NaHCO_3$ in ultrapure H_2O.
    4. To a 15 mL conical tube, add 150 µL of 100 mM $^{58}Fe^{(III)}$ Cl3 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.
    5. Incubate the mixture for 5 min at room temperature.

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

    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.
    4. Incubate for 2.5 h at room temperature.

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

    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.
    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.

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

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

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

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

    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.

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

    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

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

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

    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).

2. Anesthetize a pregnant mouse using isoflurane.

    1. Use an isoflurane regulator with a chamber.

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

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

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

3. Slowly and carefully inject the ^{58}Fe-Tf solution into the retro-orbital sinus.
4. Allow the mouse to recover from anesthesia; do not leave the animal unattended until it has regained sufficient consciousness to maintain sternal recumbency.
5. Six hours post injection, euthanize E17.5 pregnant females by isoflurane overdose.
    1. Pin the feet down with needles for stabilization.
    2. Perform a cardiac puncture to exsanguinate the mouse as a form of secondary euthanasia.
6. Collect the placentae and embryo livers.
    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.
    2. Carefully cut through the uterus and amniotic sac without disturbing the fetus and placenta.
    3. Peel back the amniotic sac and remove the fetus and placenta.
    4. Cut the umbilical cord.
    5. Blot the fetus and placenta on a clean task wipe to remove the excess amniotic fluid.
    6. Record the weights of the whole placentae.
    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.
    8. To collect embryo livers, sacrifice the embryo: use a razor blade to rapidly decapitate the embryo.
    9. Pin down the embryo for stabilization, leaving the abdomen exposed.
    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.
    11. Use forceps to remove the fetal liver.
    12. Record the weights of the whole embryo livers.

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.

7. Store the tissues indefinitely at −80 °C.

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

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

    1. Thaw placental halves and whole fetal livers, and weigh placental halves (see step 3.6.12 for recording fetal liver weights).
    2. Add 400 μL of protein precipitation solution (0.53 N HCl, 5.3% TCA).
    3. Homogenize the tissue using an electric homogenizer.
    4. Incubate the samples at 100 °C for 1 h.
    5. Cool the samples in room temperature water for 2 min.
    6. Open the caps to release pressure, then close the tubes again.
    7. Centrifuge at 17,000 × *g* for 10 min at room temperature to pellet tissue debris.
    8. Carefully transfer the supernatant to a new labeled tube.
    9. Send samples off for ICP-MS analysis.

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.

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

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

1. Subtract the natural abundance of ^{58} Fe (0.28% of total Fe) from the measured ^{58} Fe values.
2. Calculate total nonheme ^{58} Fe.
    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.
    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.
3. Calculate total heme ^{58} Fe.
    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.
    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.
4. Sum the calculated nonheme and heme ^{58} Fe values to determine the total iron content for each tissue.

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.

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.

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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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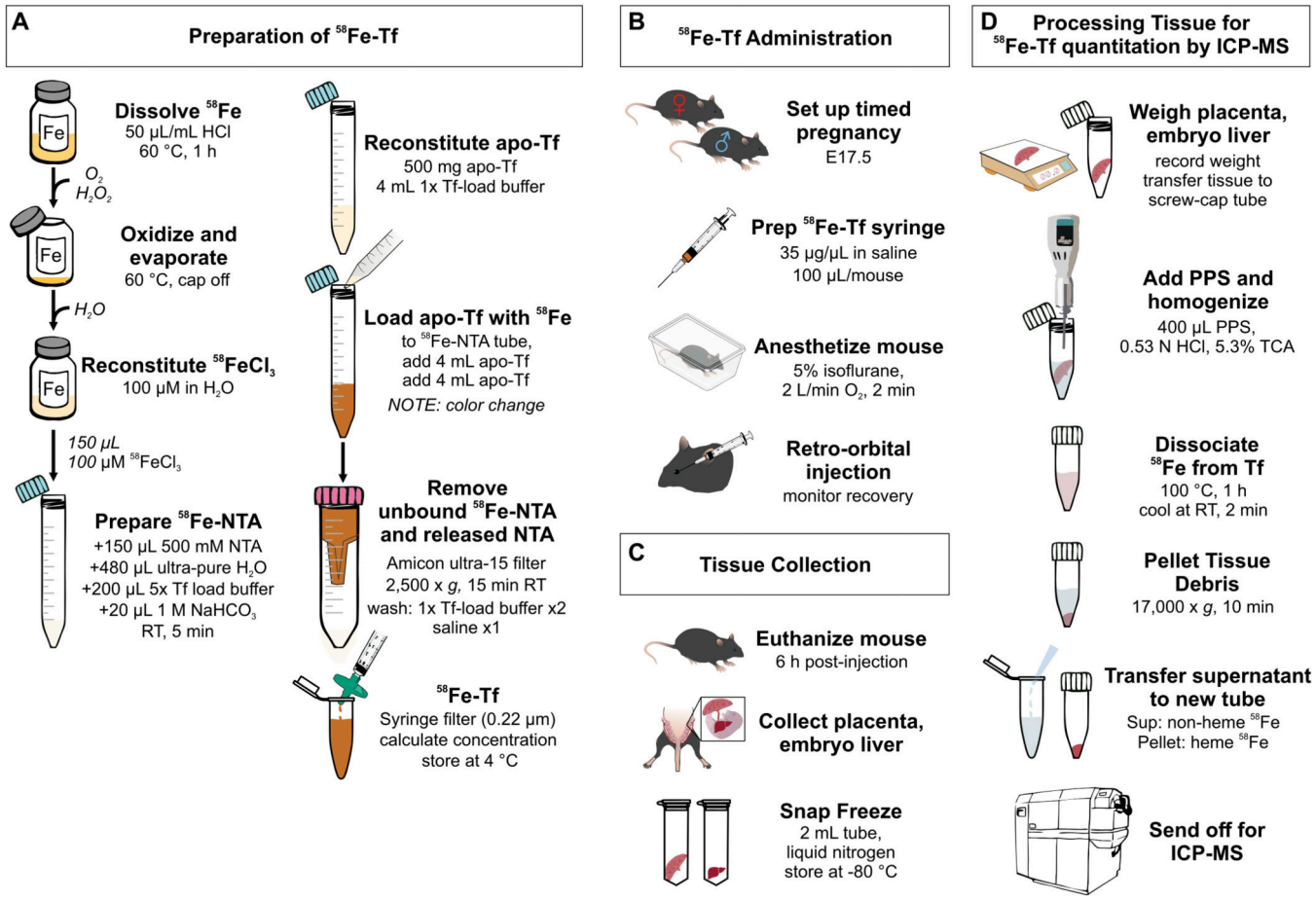

Figure 1: Visual summary of steps in the protocol.

(**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.

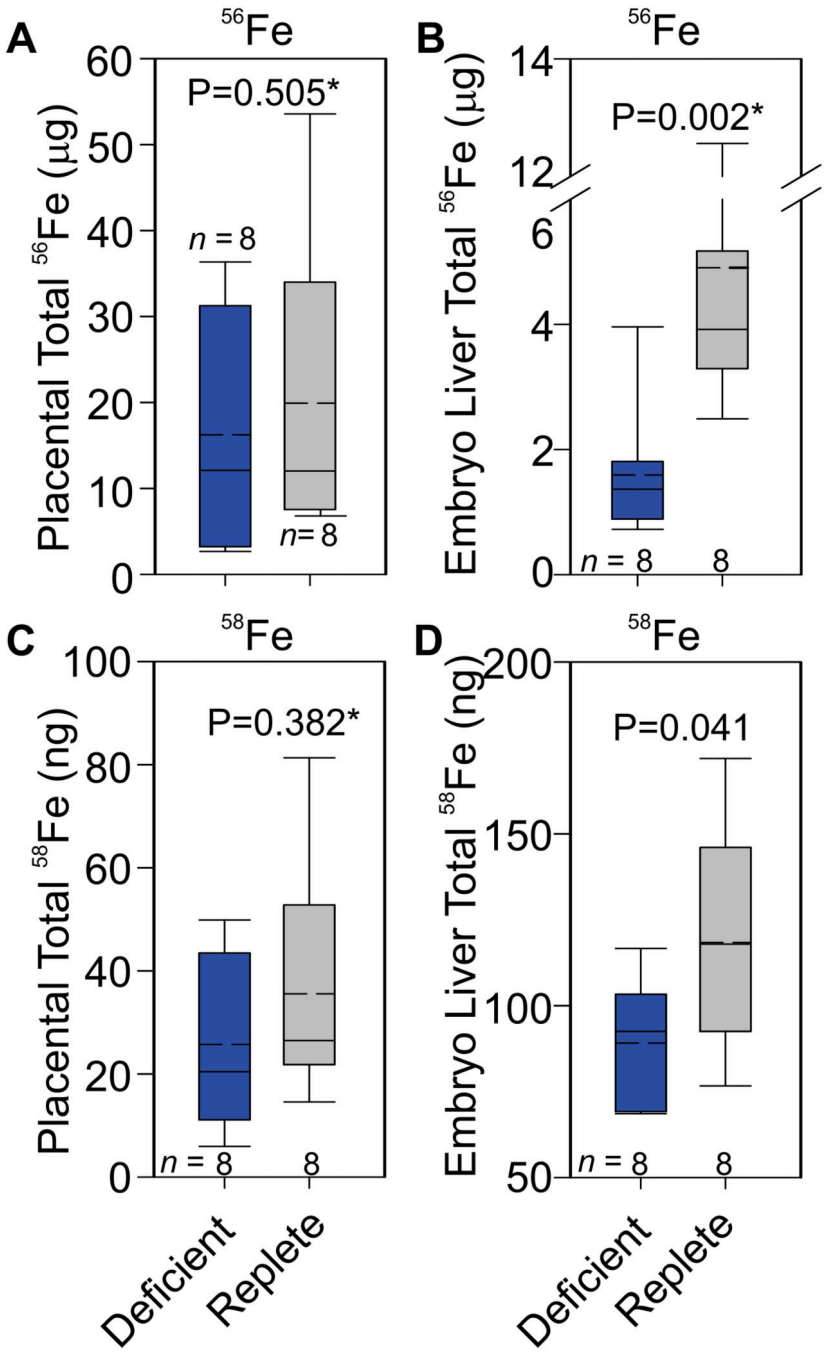

Figure 2: ^{56} Fe and ^{58} Fe transport across the placenta in iron-deficient or iron-replete pregnancies.

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.

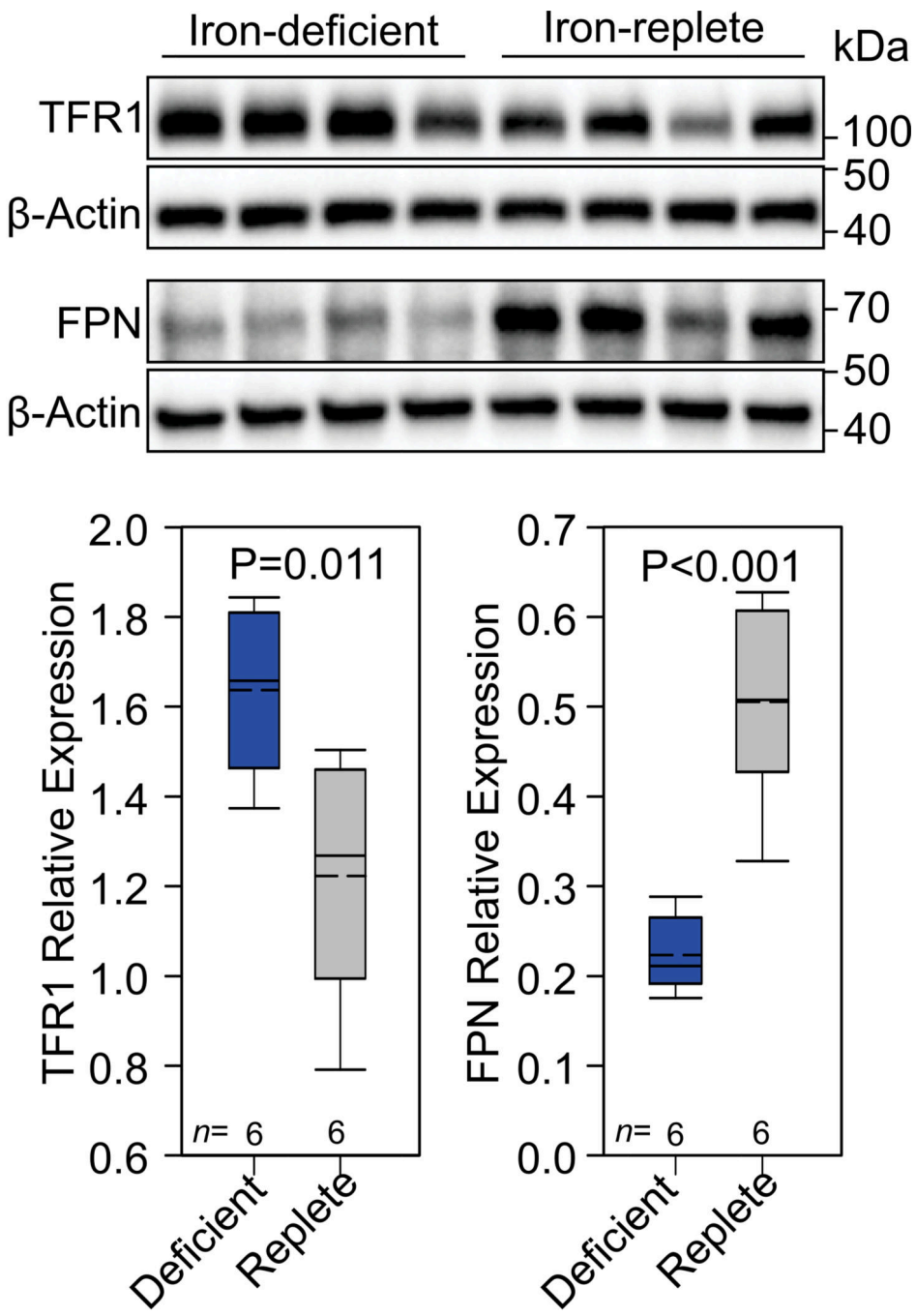

Figure 3: Placental TFR1 and FPN levels.

(**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.

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	112.9	145.6	0.5	5664.6
			4617.4	78.6	91.6	1.0	4709.0

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	
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

Abbreviations: ppb = parts per billion; stdev = standard deviation; ICP-MS = inductively coupled plasma mass spectrometry.