Placental heme receptor LRP1 correlates with the heme exporter FLVCR1 and neonatal iron status

Chang Cao, Eva K Pressman¹, Elizabeth M Cooper¹, Ronnie Guillet¹, Mark Westerman² and Kimberly O O'Brien

Division of Nutritional Sciences, Cornell University, 230 Savage Hall, Ithaca, New York 14853, USA, ¹School of Medicine, University of Rochester, Rochester, New York, USA and ²Intrinsic LifeSciences, La Jolla, California, USA

Correspondence should be addressed to K O O'Brien; Email: koo4@cornell.edu

Abstract

LDL receptor-related protein 1 (LRP1) is a transmembrane receptor highly expressed in human placenta. It was recently found to be the receptor for heme and its plasma-binding protein hemopexin (Hx) and is integral to systemic heme clearance. Little is known about systemic concentrations of Hx during pregnancy and whether maternal Hx and placental LRP1 contributes to fetal iron (Fe) homeostasis during pregnancy. We hypothesized that placental LRP1 would be upregulated in maternal/neonatal Fe insufficiency and would be related to maternal circulating Hx. Placental LRP1 expression was assessed in 57 pregnant adolescents (14–18 years) in relationship with maternal and cord blood Fe status indicators (hemoglobin (Hb), serum ferritin, transferrin receptor), the Fe regulatory hormone hepcidin and serum Hx. Hx at mid-gestation correlated positively with Hb at mid-gestation ($r=0.35$, $P=0.02$) and Hx at delivery correlated positively with cord hepcidin ($r=0.37$, $P=0.005$). Placental LRP1 protein expression was significantly higher in women who exhibited greater decreases in serum Hx from mid-gestation to term ($r=0.28$, $P=0.04$). Significant associations were also found between placental LRP1 protein with cord hepcidin ($r=-0.29$, $P=0.03$) and placental heme exporter feline leukemia virus C receptor 1 ($r=0.34$, $P=0.03$). Our data are consistent with a role for placental heme Fe utilization in supporting fetal Fe demands.

Reproduction (2014) **148** 295–302

Introduction

The placenta serves as the sole conduit for the transfer of maternal nutrients to the fetus across pregnancy. It is believed that most, if not all, of the iron (Fe) delivered to the placenta is supplied as non-heme Fe. It is unclear whether plasma heme obtained from either intravascular red blood cell catabolism (Tolosano *et al.* 2010), or from macrophage heme export (Yang *et al.* 2010), might also be utilized to support fetal Fe demands.

Hemopexin (Hx) is a plasma protein with high affinity for heme and contributes to systemic Fe homeostasis by delivery heme to the liver for storage (Tolosano *et al.* 2010). Decreases in serum Hx reflect a discharge of heme into the circulation and Hx is commonly measured along with the plasma hemoglobin (Hb) scavenger protein, haptoglobin (Hp), to assess the severity of intravascular hemolysis (Delanghe & Langlois 2001). Few data are available on Hx concentrations in pregnant women and it is unclear whether Hx is related to Fe status.

The LDL receptor-related protein 1 (LRP1) is a transmembrane protein expressed in a variety of cell types including macrophages, hepatocytes, and neurons (Moestrup *et al.* 1992). Of interest, LRP1 is highly

expressed in human placenta (Jensen *et al.* 1988, 1989) and has been suggested to play a role in placental lipid transport (Gafvels *et al.* 1992). Recently, Hvidberg *et al.* (2005) identified LRP1 as the receptor for the Hx–heme complex and revealed a novel role of LRP1 in systemic heme recycling. These authors suggested that the high expression of LRP1 in the human placenta may enable Fe recovered from maternal circulating heme to be delivered to the fetus (Hvidberg *et al.* 2005), but no studies have examined this hypothesis.

Fe efflux from the placenta to the fetus is thought to be mediated by the only known mammalian cellular non-heme Fe exporter, ferroportin (FPN; Donovan *et al.* 2000, McKie *et al.* 2000). Consistent with its role in Fe export, FPN is localized to the basolateral side of human placental syncytiotrophoblast (Bastin *et al.* 2006). FPN is under tight control by the hormone hepcidin, which binds and induces degradation of FPN protein and thus limits cellular Fe export. Whether this FPN–hepcidin model in macrophages applies to the human placenta and whether placental FPN is related to expression of heme Fe transporters remain unknown.

The objectives of this study were i) to evaluate placental expression of the heme transporter LRP1 and the Fe export protein FPN in pregnant adolescents

delivering at term and ii) to investigate the relationships between placental LRP1 and FPN with placental heme Fe exporter feline leukemia virus C receptor 1 (FLVCR1), maternal and neonatal Fe status, and changes in maternal Hx across gestation.

Materials and methods

Subjects

Pregnant adolescents in this study were enrolled in a larger prospective study addressing the relationship between maternal and fetal mineral status. All participants were recruited from the Rochester Adolescent Maternity Program in Rochester, NY, USA between 2007 and 2011. Characteristics of this study cohort have been described elsewhere (Young *et al.* 2012, 2012a) and data on placental expression of Fe and vitamin D-related proteins have been published (Young *et al.* 2010, Jaacks *et al.* 2011, O'Brien *et al.* 2014). The Institutional Review Boards at Cornell University and the University of Rochester approved all study procedures and written informed consent was obtained from all participants. A maternal blood sample was taken at mid-gestation (~26 weeks) and at delivery (~40 weeks) at which time cord blood and placental tissue were also obtained. Placental tissue used in this study was from a subset ($n=57$) of the 113 teens who delivered term infants.

Biochemical analysis

Blood Hb was measured using a Cell Dyn 4000 hematology analyzer (Abbott Laboratories). The serum was separated and stored at -80°C before analysis. The levels of serum Hx and Hp were determined by sandwich ELISAs from Genway Biotech (San Diego, CA, USA) and ALPCO Diagnostics (Salem, NH, USA) respectively. Serum Hp lower than the normal range (0.3–2.0 g/l) was considered indicative of hemolysis (Delanghe & Langlois 2001). Serum ferritin (SF) and serum-soluble transferrin receptor (sTfR) were measured using commercially available ELISA kits (Ramco Laboratories, Inc., Stafford, TX, USA). Total body iron (TBI) was calculated from SF and sTfR concentrations using the equation developed by Cook *et al.* (2003): $\text{TBI (mg/kg)} = -(\log_{10} (\text{sTfR/SF}) - 2.8229)/0.1207$. Maternal iron deficiency (ID) was defined as either $\text{SF} < 12 \mu\text{g/l}$, $\text{sTfR} > 8.5 \text{ mg/l}$, or $\text{TBI} < 0 \text{ mg/kg}$ (Akesson *et al.* 1998, Mei *et al.* 2011). As the Hb distribution for African Americans is shifted to the left of that for the Caucasians, we adjusted the Hb cutoffs downward by 0.8 g/dl to define anemia in African American teens as recommended by IOM for black populations (Institute of Medicine 1993). Specifically, anemia in whites was defined as $\text{Hb} < 10.5 \text{ g/dl}$ in the second trimester and $\text{Hb} < 11.0 \text{ g/dl}$ in the third trimester and anemia in blacks was defined as $\text{Hb} < 9.7 \text{ g/dl}$ in the second and Hb

$< 10.2 \text{ g/dl}$ in the third trimester. Neonatal anemia was defined when cord blood Hb was $< 13.0 \text{ g/dl}$ (Brugnara 2009). C-reactive protein (CRP) was measured using Immulite 2000 immunoassay system (Seimens Diagnostics, Los Angeles, CA, USA). Serum hepcidin was measured using a competitive ELISA specific for the mature peptide (Intrinsic LifeSciences, La Jolla, CA, USA). The lower limit for detection of this assay is $5 \mu\text{g/l}$. Serum hepcidin concentration below the limit of detection was given a value of $2.5 \mu\text{g/l}$ for data analysis purposes as previously described for this variable (Young *et al.* 2012b).

Placental sample collection

The placentas were collected immediately after delivery. Placental weight and dimensions were recorded. Multiple (4–5) placental samples were collected from different quadrants of the placenta, the maternal and fetal membranes removed, and the samples then each cut into quarters and then randomly distributed into aliquots. These aliquots were either flash frozen (for western blotting analyses) or placed in RNeasy Lysis Buffer (Qiagen, Austin, TX, USA) and kept at -80°C . The placental protein lysates were prepared as described previously (Young *et al.* 2010, Jaacks *et al.* 2011) and protein concentrations of lysates were determined using Bio-Rad dye reagent (Bio-Rad). The lysates were diluted in SDS-PAGE sample buffer and stored at -80°C until analysis.

Placental tissue Fe content

Total tissue Fe content of the placental tissue was determined by atomic absorption spectrophotometry as detailed before (Jaacks *et al.* 2011) and expressed as $\mu\text{g Fe/g}$ placental dry weight.

Real-time quantitative RT-PCR (qRT-PCR)

The expression of placental *LRP1* mRNA was quantified in 42 placentas with quality RNA. Total RNA was extracted from the placental tissue samples using the RNeasy Microarray Tissue Mini Kit (Qiagen). The extracted RNA was quantified and verified for integrity by the Experion automated electrophoresis system (Bio-Rad). The purity of RNA was checked by the absorbance ratios at 260 and 280 nm on a Nanodrop spectrophotometer (Thermo Scientific, Wilmington, DE, USA). A total of $1 \mu\text{g}$ RNA was reverse-transcribed into cDNA with a transcriptor cDNA synthesis kit (Roche Applied Sciences). All qRT-PCRs were set up in a $10 \mu\text{l}$ reaction mixture, containing $2 \mu\text{l}$ of the cDNA template, $5 \mu\text{l}$ of SYBR Green I Master reaction mix, and $0.7 \mu\text{M}$ of primers, and run in triplicate in 384-well plates on a LightCycler 480 instrument (Roche Applied Sciences).

The cycling conditions included an initial denaturation step at 95 °C for 5 min, followed by 45 cycles at 95 °C for 10 s, annealing at 60 °C for 10 s, and extension at 72 °C for 10 s. Specificity of amplifications was verified by melt curve analysis after PCR cycles. A single peak was consistently observed in all samples. The relative expression of *LRP1* mRNA was normalized to β -actin and compared with that obtained from a control placenta sample using the $2^{-\Delta\Delta C_t}$ method using the following equations: $\Delta C_t = C_t (LRP1) - C_t (\beta\text{-actin})$; $\Delta\Delta C_t = \Delta C_t (\text{sample}) - \Delta C_t (\text{control placenta})$; and fold change = $2^{-\Delta\Delta C_t}$. The primers were designed using the ProbeFinder software from Roche Applied Science using the NCBI sequence ID (NM_002332 for *LRP1* and NM_001101 for β -actin) and purchased from Integrated DNA Technologies (Coralville, IA, USA). The sequences of the primer pairs were as follows: *LRP1*, forward: 5'-GAT GAG ACA CAC GCC AATC TG-3', reverse: 5'-CGG CAC TGG AAC TCA TCA-3'; β -actin, forward: 5'-CCA ACC GCG AGA AGA TGA-3', reverse: 5'-CCA GAG GCG TAC AGG GAT AG-3'.

Western blotting analysis

Western blotting analysis was carried out to determine placental protein expression of LRP1 and FPN. Placental lysates were separated by SDS-PAGE using a triple-wide electrophoresis unit (CBS Scientific, Del Mar, CA, USA) and electro-transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were blocked in Odyssey Blocking Buffer (Li-Cor, Lincoln, NE, USA) for 1 h and incubated overnight at 4 °C with antibodies against the following targets: mouse anti-LRP1 (1:3000 dilution; ab28320, Abcam, Cambridge, MA, USA), rabbit anti-FPN (1:500 dilution; MTP-11A, Alpha Diagnostics, San Antonio, TX, USA), and β -actin (rabbit anti β -actin: 1:5000 dilution: Abcam or mouse anti β -actin: 1:2000 dilution: Santa Cruz Biotechnology). The blots were then incubated with appropriate fluorescence-coupled secondary antibodies (Li-Cor) for 1 h at room temperature. After washing, the fluorescence of the protein bands was quantified by with the Odyssey IR imaging system (Li-Cor). A band at 85 kDa corresponding to the LRP1 β -chain was observed in human placenta and rat liver (Supplementary Figure 1, see section on supplementary data given at the end of this article). A band of a lower molecular weight was evident in the placental lysates and may represent differential phosphorylation of the LRP1 β -chain (May *et al.* 2003), as similar patterns have been observed in human placenta (Kristensen *et al.* 1990). The combined fluorescence of the upper and lower bands and that of the upper band was highly correlated ($r=0.96$) thus the latter was used for data analysis. The use of combined signal of the upper and lower bands did not change the study findings. A control placenta sample was loaded in the left, middle, and right part of the gel, and the CV for

the three samples was 2.6%, indicating minimal influence of the sample location on band intensity. A representative image of FPN western blotting analysis is shown in Supplementary Figure 2.

Detailed western blotting methods for the determination of placental FLVCR1 expression in this cohort were previously published (Jaacks *et al.* 2011). Existing data on placental FLVCR1 were used in this study to explore its relationship with placental LRP1 and FPN.

Statistical analysis

Non-normally distributed variables were transformed to achieve normality before data analysis. Paired *t*-tests were used to compare serum Fe status indicators and Hx between mid-gestation and delivery. Pearson's correlations were used to examine associations between the expression of placental LRP1, FPN, and FLVCR1, and serum Fe status indicators. Multivariate analysis was used to study associations while controlling for possible confounders. Multiple linear regression was used to model predictors of placental LRP1 expression. *P* values <0.05 were considered statistically significant. Data were reported as the mean $\pm$ s.d.s unless otherwise stated.

Results

Subject characteristics

General characteristics of the study participants are given in Table 1. Average age at enrollment was 17 years and the majority of adolescents were African American and non-Hispanic. Approximately, 19.3% of the adolescents were obese before pregnancy and 61.4% gained more than the IOM gestational weight gain recommendations. Of the 57 adolescents, five delivered by Cesarean section. Eleven infants were born small for gestational age and two were large for gestational age.

Maternal Fe status and Hx concentrations across pregnancy

The suboptimal Fe status in this adolescent cohort was evident (Table 2). The prevalence of anemia was 4.3% at mid-gestation and increased significantly to 10.2% at term. Serum Hx decreased significantly by 9.9% from mid-gestation to delivery ($P=0.005$). Mid-gestation Hx was positively associated with maternal Hb ($r=0.35$, $P=0.02$) and CRP ($r=0.30$, $P=0.03$) at mid-gestation and with maternal hepcidin ($r=0.29$, $P=0.03$) at delivery. Delivery Hx correlated with maternal hepcidin ($r=0.28$, $P=0.04$) at mid-gestation.

Both mid-gestation Hx ($r=0.50$, $P=0.002$) and delivery Hx ($r=0.52$, $P=0.002$) correlated strongly with cord Hb and remained positive predictors of cord Hb after controlling for maternal Hb at the respective

Table 1 Characteristics of study participants ($n=57$).

Characteristics	Mean $\pm$ s.d. (range)
Age at enrollment (years)	17.1 $\pm$ 1.1 (14.0 to 18.7)
Gestational age at mid-gestation blood draw (weeks)	25.8 $\pm$ 4.0 (20.1 to 37.2)
Gestational age at delivery (weeks)	40.0 $\pm$ 1.0 (37.9 to 41.7)
Parity ≥ 1 (%)	11
Pre-pregnancy BMI (kg/m^2)	25.1 $\pm$ 6.0 (17.2 to 43.5)
Gestational weight gain (kg)	17.6 $\pm$ 7.7 (−2.1 to 43.2)
Race (%)	
African American	61
Caucasian	37
Native American	2
Ethnicity (%)	
Hispanic	25
Non-Hispanic	75
Delivery mode (%)	
Vaginal delivery	91
Cesarean section	9
Placental weight (kg)	0.61 $\pm$ 0.11 (0.35 to 0.86)
Birth weight (kg)	3.30 $\pm$ 0.45 (2.50 to 4.71)
Infant gender (%)	
Male	60
Female	40

blood sampling point. Higher maternal Hx at delivery was related to better neonatal Fe status, as evidenced by its association with high cord hepcidin ($r=0.37$, $P=0.005$) and low cord sTfR ($r=-0.27$, $P=0.046$). There was a nonsignificant positive correlation between the decrease in Hx from mid-gestation to delivery and the time elapsed between the two blood sampling points ($r=0.26$, $P=0.06$). Greater decreases in Hx across gestation were observed in women with lower mid-gestation hepcidin ($r=-0.27$, $P=0.046$) and in neonates with lower hepcidin ($r=-0.30$, $P=0.03$). These relationships remained significant after controlling for time interval between the mid-gestation and delivery measures. Using all the variables obtained, a multivariate model that included weeks elapsed between the biochemical assessments made at mid-gestation and delivery, maternal hepcidin at mid-gestation, and maternal TBI at delivery was found to capture 23.3% of the variation in changes in Hx from mid-gestation to delivery ($P=0.004$).

Serum Hp was assessed as an additional indicator of hemolysis in maternal mid-gestation blood samples. Serum Hp did not correlate with Fe status indexes in the maternal or neonatal circulation. Four of the 57 teens had low Hp levels (<0.3 g/l) suggestive of hemolysis, but there were no differences in Fe status indicators or hepcidin between these four teens and the rest of the cohort. Correlation analysis revealed significant positive associations between serum Hp with serum CRP ($r=0.31$, $P=0.02$) and Hx ($r=0.33$, $P=0.01$) at mid-gestation.

Neonatal Fe status and Hx concentrations

Neonatal hematological data are presented in Table 2. Nearly 1/3 of neonates were anemic at birth (cord Hb <13 g/dl). Neonatal serum Hx was 74% lower than ($P<0.0001$), and moderately correlated with maternal Hx at mid-gestation ($r=0.29$, $P=0.046$) but not at delivery. Cord Hx was not related to cord Fe status indicators or hepcidin.

Associations between placental LRP1 and FPN with Fe status and Hx concentrations

The expression of LRP1 and FPN in the placenta was not related to maternal race, ethnicity, gestational age at delivery, delivery mode (vaginal delivery, Cesarean section), placental weight, or infant weight.

Placental LRP1 protein expression correlated positively with suboptimal neonatal Fe status as indicated by low cord hepcidin ($r=-0.29$, $P=0.03$; Fig. 1A) and high cord sTfR ($r=0.36$, $P=0.006$; Fig. 1B). Greater decreases in Hx from mid-gestation to delivery were associated with higher placental LRP1 protein expression ($r=0.28$, $P=0.04$; Fig. 1C). In the 41 adolescents with data on placental FLVCR1 protein, there was a positive correlation between placental LRP1 and FLVCR1 ($r=0.34$, $P=0.03$; Fig. 1D). Placental LRP1 mRNA was not significantly correlated with LRP1 protein ($P=0.14$). Greater LRP1 mRNA tended to be associated with higher placental weight ($r=0.28$, $P=0.08$) and birth weight ($r=0.26$, $P=0.09$). None of the maternal or

Table 2 Iron status indicators in 57 pregnant adolescents and their neonates^a.

Variable	Mid-gestation	Delivery	Cord blood	P value ^b
Hb (g/dl)	11.4 $\pm$ 0.8 (47)*	11.8 $\pm$ 1.3 (49)*	13.7 $\pm$ 2.7 (36) [†]	<0.0001
SF ($\mu\text{g/l}$)	22.2 $\pm$ 19.1 (57)*	22.6 $\pm$ 12.9 (57)*	133.0 $\pm$ 79.4 (57) [†]	<0.0001
Serum sTfR (mg/l)	5.6 $\pm$ 5.9 (57)*	5.4 $\pm$ 2.7 (57)*	8.3 $\pm$ 2.5 (57) [†]	<0.0001
TBI (mg/kg)	3.2 $\pm$ 3.8 (57)*	3.6 $\pm$ 3.6 (57)*	8.0 $\pm$ 2.6 (57) [†]	<0.0001
Serum hepcidin ($\mu\text{g/l}$)	28.0 $\pm$ 19.9 (57)*	36.2 $\pm$ 28.4 (57)*	131.8 $\pm$ 88.8 (57) [†]	<0.0001
Serum Hx (g/l)	1.0 $\pm$ 0.2 (57)*	0.9 $\pm$ 0.3 (54) [†]	0.3 $\pm$ 0.2 (47) [†]	<0.0001
Serum Hp (g/l)	0.9 $\pm$ 0.5 (57)	ND	ND	
Serum CRP (mg/l)	6.6 $\pm$ 6.9 (55)*	ND	2.2 $\pm$ 10.8 (44) [†]	<0.0001

Hb, hemoglobin; SF, serum ferritin; serum sTfR, serum soluble transferrin receptor; TBI, total body iron; Hx, hemopexin; Hp, haptoglobin; CRP, C-reactive protein; ND, not determined. Sample sizes are different due to missing assay (Hb) or sample availability (serum Hx and CRP). Values with different subscripts (*, [†]) are significantly different.

^aData are presented as mean $\pm$ s.d. (sample size). ^bIndicators were compared between groups using a linear regression model with Group (mid-gestation, delivery, and cord) as a fixed effect and Subject as a random effect.

Figure 1 Significant correlates of placental LRP1 protein expression in 57 healthy pregnant adolescents. Placental LRP1 protein expression was assessed as a ratio between LRP1 and β -actin and log-transformed as required for regression analysis. Placental LRP1 protein expression increased in neonates with high Fe demand as indicated by its inverse correlation with cord hepcidin (A) and positive correlation with cord sTfR (B). Placental LRP1 protein expression correlated positively with the decrease in Hx from mid-gestation to term (C) and with placental protein expression of the heme exporter FLVCR1 (D).

neonatal Fe status variables were significantly associated with placental *LRP1* mRNA.

Placental FPN protein expression was insignificantly negatively correlated with maternal serum hepcidin ($r = -0.23$, $P = 0.08$) and TBI at mid-gestation ($r = -0.23$, $P = 0.09$). No significant relationship was found between placental FPN and neonatal hepcidin or other Fe status indicators. Similarly, there were no significant relationships between placental FPN and neonatal hepcidin when data were examined in adolescents who were ID or non-ID. Placental Fe content was positively correlated with placental FPN protein expression ($r = 0.37$, $P = 0.01$). Neither LRP1 nor FLVCR1 was correlated with FPN protein expression in the placenta.

Discussion

During late pregnancy, Fe flux across the placenta reaches a maximum of 3–8 mg/day (Viteri 1994), a magnitude several times higher than the amount absorbed from the diet. The high Fe transport capacity of the placenta is partly explained by the abundant presence of TfR on the syncytiotrophoblast (Loh *et al.* 1980) and it is believed that transferrin-bound inorganic Fe is the predominant form of Fe utilized by the placenta to support fetal requirements (van Dijk 1988, McArdle *et al.* 2011). Recently, attention has been focused on the presence of multiple heme transport proteins in the human placenta (Cao & O'Brien 2013), which suggests

that this organ may be an alternate site of heme clearance and/or utilization. In a group of healthy pregnant adolescents, we assessed maternal and neonatal serum Hx's relationship with placental expression of the heme-Hx receptor LRP1, the heme exporter FLVCR1, and the non-heme Fe exporter FPN. We documented for the first time a significant positive correlation between the two heme transporters LRP1 and FLVCR1 in the human placenta. In addition, placental LRP1 protein expression was higher in neonates with suboptimal Fe status and in women who experienced greater decreases in serum Hx from mid-gestation to delivery. Finally, we report novel associations between maternal Hx and neonatal Hb and hepcidin, adding to the biological plausibility of a role for plasma heme in maintenance of Fe homeostasis during pregnancy.

Circulating Hx and liver LRP1 facilitate plasma heme clearance and protect against heme-associated oxidative damage. The importance of Hx in the resolution of hemolysis is evident from knockout studies showing that mice lacking Hx are more sensitive to both induced hemolysis (Tolosano *et al.* 1999) and heme overload (Vinci *et al.* 2008). In addition to systemic heme scavenging, Hx and LRP1 may mediate localized heme clearance in the brain during cerebral hemorrhage (Li *et al.* 2009). Functional analyses have demonstrated the Hx–heme binding activity of LRP1 purified from human placenta (Hvidberg *et al.* 2005), but few studies have examined the potential role of placenta as a site of

Figure 2 Proposed pathway of heme Fe utilization in the placenta. Maternal plasma heme may be sourced from heme export from macrophages, oxidation of hemoglobin released during intravascular hemolysis or dietary heme intake. Free heme binds with hemopexin and the complex is taken up by the placental syncytiotrophoblast via LRP1-mediated endocytosis. Upon lysosomal degradation of hemopexin, free heme may be either i) exported into the maternal circulation by FLVCR1 or ii) converted to inorganic Fe by heme oxygenase in the endoplasmic reticulum and exported to the fetal circulation by FPN. The figure was modified from the schematics depicting the LRP1-mediated heme uptake pathway by Hvidberg *et al.* (2005).

Hx–heme clearance. In our study, placental protein expression of LRP1 was positively associated with that of FLVCR1, a plasma membrane heme exporter essential for erythroid cell survival and macrophage heme Fe recycling (Keel *et al.* 2008). It is possible that placental heme uptake (mediated by LRP1) and heme export (mediated by FLVCR1) are coordinately regulated to allow for sequestration of circulating heme while preventing accumulation of intracellular heme as depicted in Fig. 2.

Little is known about the cellular regulation of LRP1 expression by systemic Fe status. Studies on mouse hepatoma cells have found that Fe deprivation doubled the number of LRP1 and increased heme uptake, indicating that LRP1 may be negatively regulated by Fe availability (Smith & Ledford 1988). Consistent with this reciprocal Fe regulation of LRP1 in the liver, we found that high placental LRP1 was associated with suboptimal neonatal Fe status, which may represent a compensatory mechanism to increase placental heme uptake during Fe insufficiency in support of fetal demands.

Knockout experiments and localization studies provide convincing evidence that FPN is the protein that mediates Fe exit from placenta to the fetal circulation (Donovan *et al.* 2000, 2005, Bastin *et al.* 2006). At this time, it is unclear whether levels of FPN protein in the placenta are controlled by hepcidin in a similar way to that found in macrophages. Our study, and previous research in rodents (Gambling *et al.* 2001, 2009, Cornock *et al.* 2013) and humans (Li *et al.* 2008), suggests that placental FPN protein is not regulated by maternal or neonatal Fe status. These data support the notion that FPN-mediated Fe export may not be the rate-limiting step for placental Fe transfer (Gambling *et al.* 2001). However, it still may be possible that changes in FPN subcellular location may inhibit Fe export without increased proteolytic degradation.

The Hx concentrations observed in maternal and neonatal circulation were within the ranges previously established for healthy adults (Delanghe & Langlois 2001) and term infants (Kanakoudi *et al.* 1995). Similar to previous reports (Muller-Eberhard *et al.* 1968, Gitlin & Biasucci 1969), cord Hx concentrations were 74% lower than those observed in maternal circulation, a phenomenon that has been suggested to be a consequence of low fetal Hx production (Lundh *et al.* 1970).

Kinetic studies demonstrated that extracellular Hx enhances heme export from human macrophages, supporting an emerging role of Hx in systemic heme trafficking (Yang *et al.* 2010). It has been suggested that the high serum concentration of Hx, which is comparable with that of the non-heme Fe transporter transferrin, is supportive of the importance of Hx in macrophage heme recycling under physiological conditions (Keel *et al.* 2008). Consistent with a role of Hx in systemic Fe balance in pregnancy, we observed associations between maternal Hx with cord Hb and hepcidin.

Few studies have examined possible relationships between Hx and Fe status. In diabetic patients and healthy volunteers ($n=213$), serum transferrin was a weak predictor of serum Hx while serum tri-acylglycerol and the diabetic state accounted for the majority of the variation (Van Camphenout *et al.* 2006). It is possible that the positive association between maternal Hx with maternal and cord Hb is representative of a greater capacity of maternal heme recycling and transport in support of fetal erythropoiesis.

The major strength of this study is the concurrent measurement of placental expression of LRP1 and FLVCR1 proteins with serum Hx in a group of pregnant adolescents with well-characterized Fe status. Due to the observational nature of this study, we could not determine cellular processes underlying the associations between LRP1, Hx, and Fe status, and mechanistic studies are needed to assess the heme transport activity of placental LRP1 and determine whether this process is responsive to changes in maternal/neonatal Fe status.

In summary, this study extends the current discussion of heme utilization to the placenta by documenting a relationship between placental LRP1 with maternal and neonatal Fe status in pregnant adolescents with term delivery. Because of the observational nature of this study, further research is needed to elucidate the mechanisms underlying these associations and examine the significance of placental heme scavenging in supporting fetal Fe demands.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-14-0053>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This work was supported by the National Research Initiative or Agriculture and Food Research Initiative of the USDA National Institute of Food and Agriculture, award numbers 2005-35200-15218 and 2008-01857-05171. Additional support was provided by the National Institute of Diabetes and Digestive and Kidney Diseases of the National Institutes of Health under award number T32DK007158. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

Author contribution statement

C Cao and K O O'Brien designed and performed the research, analyzed the data, and wrote the manuscript; E K Pressman,

E M Cooper, and R Guillet conducted the research and assisted with participant recruitment. M Westerman carried out hepcidin analyses.

Acknowledgements

The authors thank the Highland Hospital staff and the URM Midwifery Group for their clinical assistance, Allison W McIntyre and Hannah Stillings for their assistance in subject recruitment, and the pregnant adolescents for their participation. They are grateful to Dr Janis L Abkowitz from the University of Washington for the gift of FLVCR1 antibody.

References

- Akesson A, Bjellerup P, Berglund M, Bremme K & Vahter M 1998 Serum transferrin receptor: a specific marker of iron deficiency in pregnancy. *American Journal of Clinical Nutrition* **68** 1241–1246.
- Bastin J, Drakesmith H, Rees M, Sargent I & Townsend A 2006 Localisation of proteins of iron metabolism in the human placenta and liver. *British Journal of Haematology* **134** 532–543. (doi:10.1111/j.1365-2141.2006.06216.x)
- Brugnara C 2009 Reference values in infancy and childhood. In *Nathan & Oski's Hematology of Infancy and Childhood*, edn 7, pp 1769–1796. Eds Fisher DE, Ginsburg D, Look AT, Lux SE, Nathan DG, and Orkin SH. Philadelphia: Saunders/Elsevier.
- Cao C & O'Brien KO 2013 Pregnancy and iron homeostasis: an update. *Nutrition Reviews* **71** 35–51. (doi:10.1111/j.1753-4887.2012.00550.x)
- Cook JD, Flowers CH & Skikne BS 2003 The quantitative assessment of body iron. *Blood* **101** 3359–3364. (doi:10.1182/blood-2002-10-3071)
- Cornock R, Gambling L, Langley-Evans SC, McArdle HJ & McMullen S 2013 The effect of feeding a low iron diet prior to and during gestation on fetal and maternal iron homeostasis in two strains of rat. *Reproductive Biology and Endocrinology* **11** 32. (doi:10.1186/1477-7827-11-32)
- Delanghe JR & Langlois MR 2001 Hemopexin: a review of biological aspects and the role in laboratory medicine. *Clinica Chimica Acta* **312** 13–23. (doi:10.1016/S0009-8981(01)00586-1)
- van Dijk JP 1988 Regulatory aspects of placental iron transfer – a comparative study. *Placenta* **9** 215–226. (doi:10.1016/0143-4004(88)90018-5)
- Donovan A, Brownlie A, Zhou Y, Shepard J, Pratt SJ, Moynihan J, Paw BH, Drejer A, Barut B, Zapata A *et al.* 2000 Positional cloning of zebrafish ferroportin1 identifies a conserved vertebrate iron exporter. *Nature* **403** 776–781. (doi:10.1038/35001596)
- Donovan A, Lima CA, Pinkus JL, Pinkus GS, Zon LI, Robine S & Andrews NC 2005 The iron exporter ferroportin/Slc40a1 is essential for iron homeostasis. *Cell Metabolism* **1** 191–200. (doi:10.1016/j.cmet.2005.01.003)
- Gafvels ME, Coukos G, Sayegh R, Coutifaris C, Strickland DK & Strauss JF III 1992 Regulated expression of the trophoblast α 2-macroglobulin receptor/low density lipoprotein receptor-related protein. Differentiation and cAMP modulate protein and mRNA levels. *Journal of Biological Chemistry* **267** 21230–21234.
- Gambling L, Danzeisen R, Gair S, Lea RG, Charania Z, Solanky N, Joory KD, Srai SK & McArdle HJ 2001 Effect of iron deficiency on placental transfer of iron and expression of iron transport proteins *in vivo* and *in vitro*. *Biochemical Journal* **356** 883–889. (doi:10.1042/0264-6021.3560883)
- Gambling L, Czopek A, Andersen HS, Holtrop G, Srai SK, Krejpcio Z & McArdle HJ 2009 Fetal iron status regulates maternal iron metabolism during pregnancy in the rat. *American Journal of Physiology. Regulatory, Integrative and Comparative Physiology* **296** R1063–R1070. (doi:10.1152/ajpregu.90793.2008)
- Gitlin D & Biasucci A 1969 Development of gamma G, gamma A, gamma M, beta IC-beta IA, C 1 esterase inhibitor, ceruloplasmin, transferrin, hemopexin, haptoglobin, fibrinogen, plasminogen, alpha 1-antitrypsin, orosomucoid, beta-lipoprotein, alpha 2-macroglobulin, and prealbumin in the human conceptus. *Journal of Clinical Investigation* **48** 1433–1446. (doi:10.1172/JCI106109)
- Hvidberg V, Maniecki MB, Jacobsen C, Hojrup P, Moller HJ & Moestrup SK 2005 Identification of the receptor scavenging hemopexin-heme complexes. *Blood* **106** 2572–2579. (doi:10.1182/blood-2005-03-1185)
- Institute of Medicine 1993 Recommended guidelines for preventing and treating iron deficiency anemia in pregnant women. Iron deficiency anemia: a synthesis of current scientific knowledge and U.S. recommendations for prevention and treatment. In *Iron Deficiency Anemia: Recommended Guidelines for Prevention, Detection and Management Among U.S. Children and Women of Childbearing Age*, pp 24–25. Eds Earl R and Woteki CE. Washington, DC: National Academies Press.
- Jaacks LM, Young MF, Essley BV, McNanley TJ, Cooper EM, Pressman EK, McIntyre AW, Orlando MS, Abkowitz JL, Guillet R *et al.* 2011 Placental expression of the heme transporter, feline leukemia virus subgroup C receptor, is related to maternal iron status in pregnant adolescents. *Journal of Nutrition* **141** 1267–1272. (doi:10.3945/jn.110.135798)
- Jensen PH, Moestrup SK, Sottrup-Jensen L, Petersen CM & Gliemann J 1988 Receptors for α 2-macroglobulin- and pregnancy zone protein-proteinase complexes in the human placental syncytiotrophoblast. *Placenta* **9** 463–477. (doi:10.1016/0143-4004(88)90019-7)
- Jensen PH, Moestrup SK & Gliemann J 1989 Purification of the human placental α 2-macroglobulin receptor. *FEBS Letters* **255** 275–280. (doi:10.1016/0014-5793(89)81105-6)
- Kanakoudi F, Drossou V, Tzimouli V, Diamanti E, Konstantinidis T, Germanis A & Kremenopoulos G 1995 Serum concentrations of 10 acute-phase proteins in healthy term and preterm infants from birth to age 6 months. *Clinical Chemistry* **41** 605–608.
- Keel SB, Doty RT, Yang Z, Quigley JG, Chen J, Knoblaugh S, Kingsley PD, De Domenico I, Vaughn MB, Kaplan J *et al.* 2008 A heme export protein is required for red blood cell differentiation and iron homeostasis. *Science* **319** 825–828. (doi:10.1126/science.1151133)
- Kristensen T, Moestrup SK, Gliemann J, Bendtsen L, Sand O & Sottrup-Jensen L 1990 Evidence that the newly cloned low-density-lipoprotein receptor related protein (LRP) is the α 2-macroglobulin receptor. *FEBS Letters* **276** 151–155. (doi:10.1016/0014-5793(90)80530-V)
- Li YQ, Yan H & Bai B 2008 Change in iron transporter expression in human term placenta with different maternal iron status. *European Journal of Obstetrics, Gynecology, and Reproductive Biology* **140** 48–54. (doi:10.1016/j.ejogrb.2008.02.012)
- Li RC, Saleem S, Zhen G, Cao W, Zhuang H, Lee J, Smith A, Altruda F, Tolosano E & Dore S 2009 Heme-hemopexin complex attenuates neuronal cell death and stroke damage. *Journal of Cerebral Blood Flow and Metabolism* **29** 953–964. (doi:10.1038/jcbfm.2009.19)
- Loh TT, Higuchi DA, van Bockxmeer FM, Smith CH & Brown EB 1980 Transferrin receptors on the human placental microvillous membrane. *Journal of Clinical Investigation* **65** 1182–1191. (doi:10.1172/JCI109773)
- Lundh B, Oski FA & Gardner FH 1970 Plasma hemopexin and haptoglobin in hemolytic diseases of the newborn. *Acta Paediatrica Scandinavica* **59** 121–126. (doi:10.1111/j.1651-2227.1970.tb08975.x)
- May P, Bock HH, Nimpf J & Herz J 2003 Differential glycosylation regulates processing of lipoprotein receptors by gamma-secretase. *Journal of Biological Chemistry* **278** 37386–37392. (doi:10.1074/jbc.M305858200)
- McArdle HJ, Lang C, Hayes H & Gambling L 2011 Role of the placenta in regulation of fetal iron status. *Nutrition Reviews* **69** (Suppl 1) S17–S22. (doi:10.1111/j.1753-4887.2011.00428.x)
- McKie AT, Marciani P, Rolfs A, Brennan K, Wehr K, Barrow D, Miret S, Bomford A, Peters TJ, Farzaneh F *et al.* 2000 A novel duodenal iron-regulated transporter, IREG1, implicated in the basolateral transfer of iron to the circulation. *Molecular Cell* **5** 299–309. (doi:10.1016/S1097-2765(00)80425-6)
- Mei Z, Cogswell ME, Looker AC, Pfeiffer CM, Cusick SE, Lacher DA & Grummer-Strawn LM 2011 Assessment of iron status in US pregnant women from the National Health and Nutrition Examination Survey (NHANES), 1999–2006. *American Journal of Clinical Nutrition* **93** 1312–1320. (doi:10.3945/ajcn.110.007195)
- Moestrup SK, Gliemann J & Pallesen G 1992 Distribution of the α 2-macroglobulin receptor/low density lipoprotein receptor-related protein in human tissues. *Cell and Tissue Research* **269** 375–382. (doi:10.1007/BF00353892)
- Muller-Eberhard U, Javid J, Liem HH, Hanstein A & Hanna M 1968 Plasma concentrations of hemopexin, haptoglobin and heme in patients with various hemolytic diseases. *Blood* **32** 811–815.

- O'Brien KO, Li S, Cao C, Kent T, Young BV, Queenan R, Pressman EK & Cooper EM 2014 Placental cyp27b1 and cyp24a1 expression in human placental tissue and their association with maternal and neonatal calcitropic hormones. *Journal of Clinical Endocrinology and Metabolism* **99** 1348–1356. (doi:10.1210/jc.2013-1366)
- Smith A & Ledford BE 1988 Expression of the haemopexin-transport system in cultured mouse hepatoma cells. Links between haemopexin and iron metabolism. *Biochemical Journal* **256** 941–950.
- Tolosano E, Hirsch E, Patrucco E, Camaschella C, Navone R, Silengo L & Altruda F 1999 Defective recovery and severe renal damage after acute hemolysis in hemopexin-deficient mice. *Blood* **94** 3906–3914.
- Tolosano E, Fagoonee S, Morello N, Vinchi F & Fiorito V 2010 Heme scavenging and the other facets of hemopexin. *Antioxidants & Redox Signaling* **12** 305–320. (doi:10.1089/ars.2009.2787)
- Van Campenhout A, Van Campenhout C, Lagrou AR, Abrams P, Moorkens G, Van Gaal L & Manuel-y-Keenoy B 2006 Impact of diabetes mellitus on the relationships between iron-, inflammatory- and oxidative stress status. *Diabetes/Metabolism Research and Reviews* **22** 444–454. (doi:10.1002/dmrr.635)
- Vinchi F, Gastaldi S, Silengo L, Altruda F & Tolosano E 2008 Hemopexin prevents endothelial damage and liver congestion in a mouse model of heme overload. *American Journal of Pathology* **173** 289–299. (doi:10.2353/ajpath.2008.071130)
- Viteri FE 1994 The concurrences of iron deficiency and anemia in pregnancy. In *Nutrient Regulation During Pregnancy, Lactation, and Infant Growth*, pp 127–139. Eds Allen L, King J, Lönnnerdal B. New York: Plenum Press.
- Yang Z, Philips JD, Doty RT, Giraudi P, Ostrow JD, Tiribelli C, Smith A & Abkowitz JL 2010 Kinetics and specificity of feline leukemia virus subgroup C receptor (FLVCR) export function and its dependence on hemopexin. *Journal of Biological Chemistry* **285** 28874–28882. (doi:10.1074/jbc.M110.119131)
- Young MF, Pressman E, Foehr ML, McNanley T, Cooper E, Guillet R, Orlando M, McIntyre AW, Lafond J & O'Brien KO 2010 Impact of maternal and neonatal iron status on placental transferrin receptor expression in pregnant adolescents. *Placenta* **31** 1010–1014. (doi:10.1016/j.placenta.2010.08.009)
- Young BE, McNanley TJ, Cooper EM, McIntyre AW, Witter F, Harris ZL & O'Brien KO 2012 Vitamin D insufficiency is prevalent and vitamin D is inversely associated with PTH and calcitriol in pregnant adolescents. *Journal of Bone and Mineral Research* **27** 177–186. (doi:10.1002/jbmr.526)
- Young BE, McNanley TJ, Cooper EM, McIntyre AW, Witter F, Harris ZL & O'Brien KO 2012a Maternal vitamin D status and calcium intake interact to affect fetal skeletal growth *in utero* in pregnant adolescents. *American Journal of Clinical Nutrition* **95** 1103–1112. (doi:10.3945/ajcn.111.023861)
- Young MF, Griffin I, Pressman E, McIntyre AW, Cooper E, McNanley T, Harris ZL, Westerman M & O'Brien KO 2012b Maternal hepcidin is associated with placental transfer of iron derived from dietary heme and nonheme sources. *Journal of Nutrition* **142** 33–39. (doi:10.3945/jn.111.145961)

Received 31 January 2014

First decision 5 March 2014

Revised manuscript received 27 May 2014

Accepted 19 June 2014