Maternal prolactin or estrogen signaling in hepatocytes does not regulate iron homeostasis during pregnancy

Iron is essential for maternal and fetal health but the molecular mechanisms ensuring increased iron availability during pregnancy are not well understood. Hepcidin (*HAMP*), the key iron-regulatory hormone, is produced by hepatocytes and functions by blocking iron absorption, recycling and mobilization from stores, effectively decreasing plasma iron levels. During healthy pregnancy, maternal hepcidin decreases starting in the second trimester, and is nearly undetectable by late pregnancy. This decrease in hepcidin allows for greater iron availability for transfer to the fetus. In rodents, if maternal hepcidin is not appropriately suppressed during pregnancy, it can result in fetal anemia, intrauterine growth restriction (IUGR), and even fetal death.[1] In complicated human pregnancies, higher maternal hepcidin was also associated with increased risk of IUGR.[2] The mechanism(s) of pregnancy-related hepcidin suppression are not fully understood. Hormones prolactin and estrogen, which increase throughout gestation,[3] have been previously implicated in hepcidin regulation, although with conflicting conclusions as to the direction of hepcidin change. Prolactin was reported to reduce hepcidin mRNA levels in human hepatic cell line HepG2.[4] In hyperprolactinemic individuals, treatment with prolactin-reducing therapy increased hepcidin levels in one study,[4] but did not affect hepcidin levels in another.[5] The role of estrogens in hepcidin regulation is also unclear. A partial estrogen response element has been reported in the hepcidin promotor region,[6,7] suggesting that estrogens may play a role in regulating hepcidin expression. In non-pregnant women, stimulation of endogenous estrogen production resulted in decreased hepcidin expression.[5] Treatment of HepG2 cells, with 17β-estradiol was reported to either suppress hepcidin,[7] have no effect,[8] or even induce hepcidin.[9] In ovariectomized mice, which have low estrogen levels, hepcidin levels were increased in one study,[6] and decreased in another.[9] Although these studies were not conducted in a physiological setting and do not provide conclusive evidence as to the role of prolactin and estrogen in hepcidin regulation, they raised the possibility that these hormones could play a role in hepcidin suppression in pregnancy.

Here we aimed to determine the role of prolactin and estrogen in hepcidin regulation during pregnancy, a physiologically normal state characterized by a large increase in estrogens and prolactin that coincides with a decrease in hepcidin levels.

All experiments were approved by the Animal Research Committee (ARC-2014-100) at the University of California, Los Angeles (UCLA). Reagents used in this study can be found in *Online Supplementary Table S1*.

The prolactin receptor (PRLR) binds not only prolactin, but also placental lactogen and growth hormone.[10] In the liver, prolactin receptor is predominantly expressed in hepatocytes in humans and rodents,[11] with studies in non-pregnant rats showing that transcript variant 2, the short form of the receptor, is the predominant form.[12] We first characterized the time course of *Prlr* expression in maternal liver during wild-type (WT) mouse pregnancy, and showed it was elevated throughout gestation compared to non-pregnant females ($P<0.001$, one-way ANOVA) (Figure 1A). In term mouse pregnancies (embryonic day 18.5 [E18.5]), *Prlr* was

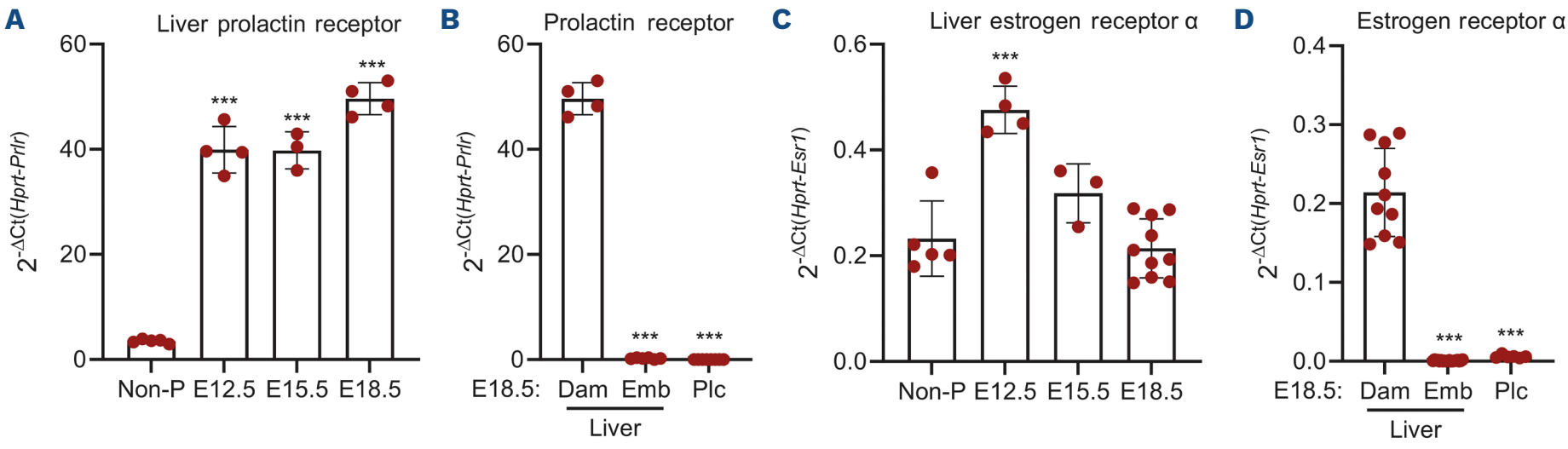

Figure 1. Expression of prolactin receptor and estrogen receptor-α in wild-type mice. (A) Maternal liver *Prlr* mRNA expression over gestation compared to non-pregnant females. (B) *Prlr* mRNA expression in maternal liver, embryo liver and placenta at E18.5. (C) Maternal liver *Esr1* mRNA expression over gestation compared to non-pregnant females. (D) *Esr1* mRNA expression in maternal liver, embryo liver and placenta at E18.5. Statistical differences between groups were determined by one-way ANOVA followed by Dunnett's multiple comparisons test *versus* the Non-P group (***$P<0.001$). *Hprt*: hypoxanthine phosphoribosyltransferase 1; *Prlr*: prolactin receptor; Non-P: non-pregnant; E: embryonic day; Emb: embryo; Plc: placenta; *Esr1*: estrogen receptor α.

highly expressed in maternal livers but not detectable in embryo livers nor placental tissue (Figure 1B). Similar to the non-pregnant state, the short form of PRLR, transcript variant 2, is the predominant form expressed in pregnant livers (*Online Supplementary Figure S1A*).

Estrogen receptors (ER) bind endogenous estrogens including estradiol, estrone and estriol.[13] There are two isoforms of classical ER: ERα (*ESR1*) and ERβ (*ESR2*). ERα is expressed in mammary gland, uterus, ovary, bone, male reproductive organs, prostate, liver, and adipose tissue, while ERβ is expressed in the prostate, bladder, ovary, colon, adipose tissue, and immune system.[13] Non-classical G-protein-coupled estrogen receptor (*GPER*) is not expressed in mouse liver.[14] During mouse pregnancy, liver Esr1 expression increased transiently with mRNA levels highest at E12.5; however, expression returned to non-pregnant levels by term (Figure 1C). Like *Prlr*, *Esr1* expression was detected in maternal livers but not embryo livers nor placentae at E18.5 (Figure 1D). *Esr2* and *Gper* mRNA were not detectable in WT non-pregnant and pregnant livers over gestation (*data not shown*). The time-course of *Hamp* suppression compared to the *Prlr* and *Esr1* expression in maternal liver is summarized in *Online Supplementary Figure S1B*.

Studying the role of estrogens and prolactins during pregnancy is challenging as both hormones are required for pregnancy. Mice with global knockout (KO) of either *Esr1* or *Prlr* are infertile. Thus, to determine the contribution of estrogens and prolactin to pregnancy-related iron regulation, we generated hepatocyte-specific knockouts of either prolactin receptor ($Prlr^{f/f}$;$Alb\text{-}Cre^{+}$) or estrogen receptor alpha ($Esr1^{f/f}$;$Alb\text{-}Cre^{+}$), subsequently abbreviated as PRLR KO and ERα KO. Female PRLR KO and ERα KO were bred with WT or $Esr1^{flox/flox}$ males, respectively, and pregnancies were analyzed at E18.5. The PRLR KO ablates effects on maternal liver of not only prolactin but of placental lactogens as well, which are also elevated during pregnancy.[15] Given the virtual absence of ERβ in the liver, the ERα KO ablates the effects of all endogenous estrogens on maternal liver.

In PRLR KO maternal livers, *Prlr* mRNA expression was decreased 78-fold ($2^{-\Delta Ct(Hprt\text{-}Prlr}$: 52 *vs*. 0.65; $P<0.001$, two-tailed *t* test) (*Online Supplementary Figure S2A*) and PRLR protein was not detected (*Online Supplementary Figure S2B*). In ERα KO pregnant livers, *Esr1* mRNA was decreased 6-fold ($2^{-\Delta Ct(Hprt\text{-}Esr1r)}$: 0.22 *vs*. 0.03; $P<0.001$, two-tailed *t* test) (*Online Supplementary Figure S2C*) and ERα protein was undetectable (*Online Supplementary Figure S2D*). In ERα KO dams, compensatory *Esr2* expression was not observed; in fact,

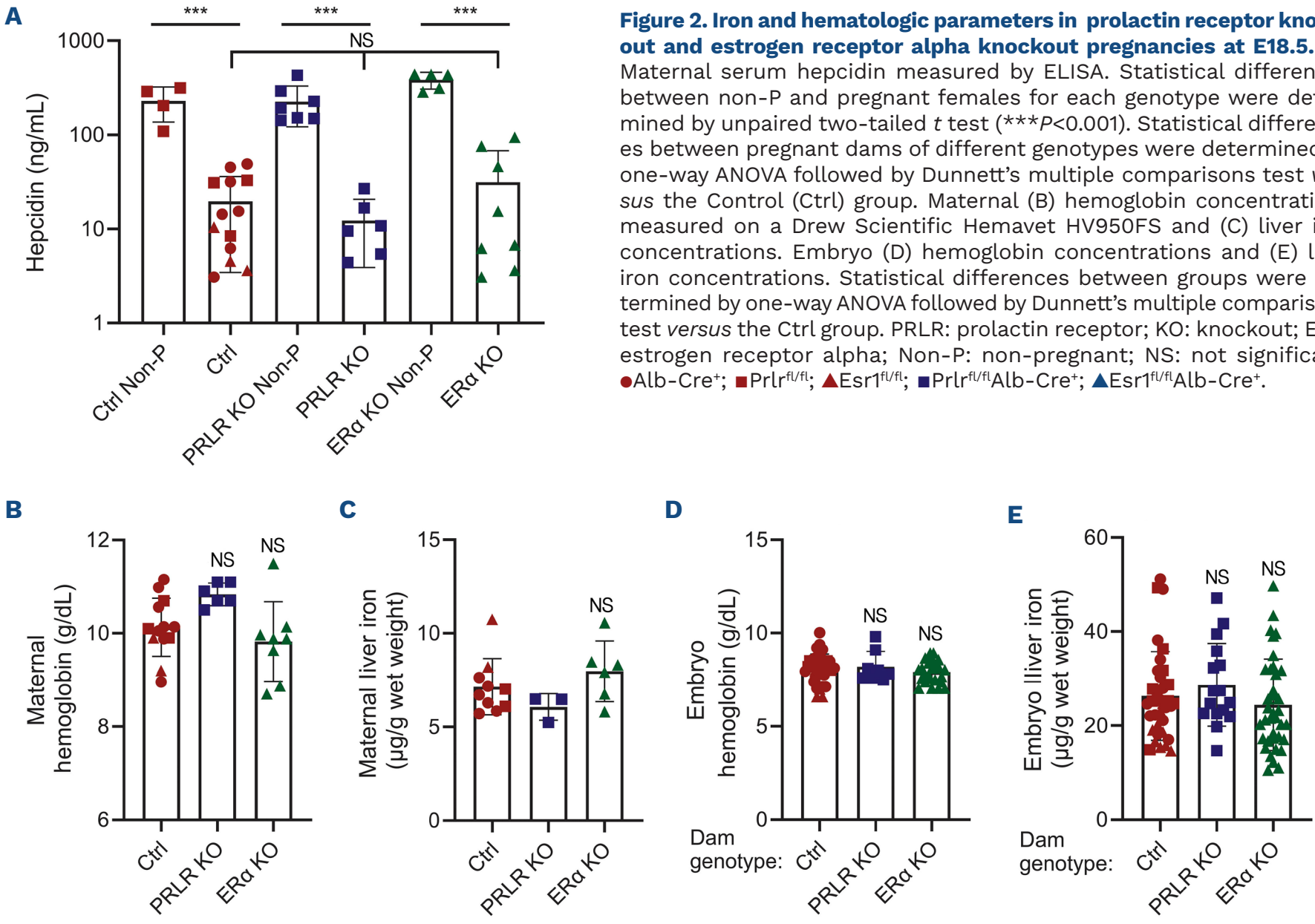

Figure 2. Iron and hematologic parameters in prolactin receptor knockout and estrogen receptor alpha knockout pregnancies at E18.5. (A) Maternal serum hepcidin measured by ELISA. Statistical differences between non-P and pregnant females for each genotype were determined by unpaired two-tailed *t* test (***$P<0.001$). Statistical differences between pregnant dams of different genotypes were determined by one-way ANOVA followed by Dunnett's multiple comparisons test *versus* the Control (Ctrl) group. Maternal (B) hemoglobin concentrations measured on a Drew Scientific Hemavet HV950FS and (C) liver iron concentrations. Embryo (D) hemoglobin concentrations and (E) liver iron concentrations. Statistical differences between groups were determined by one-way ANOVA followed by Dunnett's multiple comparisons test *versus* the Ctrl group. PRLR: prolactin receptor; KO: knockout; ERα: estrogen receptor alpha; Non-P: non-pregnant; NS: not significant. ●$Alb\text{-}Cre^{+}$; ■$Prlr^{fl/fl}$; ▲$Esr1^{fl/fl}$; ■$Prlr^{fl/fl}Alb\text{-}Cre^{+}$; ▲$Esr1^{fl/fl}Alb\text{-}Cre^{+}$.

Table 1. Pregnancy characteristics and outcomes.

Maternal	Control	N	PRLR KO	N	ERα KO	N	*P*
Litter size	7.6±1.9	14	7.7±0.7	6	8.0±1.7	8	0.89
Maternal weight E18.5, gr	35.7±3.0	10	34.1±2.1	6	37.9±3.7	8	0.23
Liver weight, % BW	4.2±0.4	10	4.4±0.7	6	4.3±0.3	8	0.83
Spleen weight, % BW	0.2±0.0	10	0.3±0.0	6	0.3±0.1	8	0.17
Embryo (average/litter)							
Embryo weight, gr	1.1±0.1	6	1.1±0.2	5	1.2±0.1	3	0.83
Embryo length, cm	2.1±0.1	6	2.1±0.2	5	2.2±0.1	3	0.56
Placenta, gr	0.08±0.00	6	0.08±0.01	5	0.07±0.00	3	0.67

Data presented as mean±Standard Deviation. % BW: percent body weight (tissue weight [gr] / pregnant body weight [gr])*100. Statistical analysis by one-way ANOVA. N: number; PRLR: prolactin receptor; ERα: estrogen receptor alpha; KO: knockout; E: embryonic day.

Esr2 expression remained exceedingly low compared to *Esr1* (>3000-fold and >800-fold lower in control [Ctrl] and ERα KO E18.5 whole pregnant livers, respectively) (*Online Supplementary Figure S2E*). Additionally, downstream ER signaling targets,[16] estrogen receptor binding site associated 9 (*Ebag9*) and cathepsin D (*Ctsd*), were both decreased in ERα KO animals, indicating lack of compensatory ERβ signaling (*Online Supplementary Figure S2F, G*).

Pregnancies from PRLR KO and ERα KO dams were viable and litter sizes did not differ from controls (Ctrl: 7.6, PRLR KO: 7.7 and ERα: 8.0; *P*=0.89, one-way ANOVA) (Table 1). Maternal body weight, liver weight and spleen weight were comparable between PRLR KO, ERα KO, and control dams (Table 1). There was no difference in embryo weight, embryo length and placenta weight between control and KO animals (Table 1). Maternal serum hepcidin at E18.5 decreased significantly and to a similar degree in all dam groups (WT, PRLR KO and ERα KO) compared to their non-pregnant counterparts (*P*<0.001 by two-tailed *t* test). Importantly, there was no difference in dam serum hepcidin concentrations at E18.5 between WT, PRLR KO and ERα KO pregnancies (*P*=0.30, one-way ANOVA). These data suggest that maternal hepcidin suppression is not influenced by hormones that bind to PRLR (prolactin and placental lactogens) nor ERα (estradiol, estrone and estriol). To further support our findings, we evaluated hematologic and iron parameters. If hepcidin was inappropriately elevated in our PRLR KO or ERβ KO dams, we would expect to see decreased maternal hemoglobin and increased liver iron concentration. Conversely, if hepcidin was further suppressed by PRLR KO or ERα KO, both hemoglobin and maternal liver iron concentration would be expected to increase. However, maternal hemoglobin (Figure 2B) and liver iron concentrations (Figure 2C) were similar between WT, PRLR KO, and ERα KO pregnancies, suggesting that neither hepatocyte PRLR nor ERα are major regulators of hepcidin expression during pregnancy. Similarly, embryo hemoglobin (Figure 2D) and liver iron concentrations (Figure 2E) were also similar between embryos from WT, PRLR KO and ERα KO pregnancies, further supporting the lack of hepcidin regulation by hepatocyte PRLR and ERα during pregnancy.

In summary, we investigated the role of prolactin and estrogen signaling in hepcidin regulation during pregnancy, a physiological state characterized by a profound increase in these hormones. We demonstrate that hepatocyte PRLR and ERα, which mediate the effect of prolactin, placental lactogens and estrogens on the maternal liver during pregnancy, do not regulate maternal liver hepcidin expression, nor maternal and embryo iron homeostasis or erythropoiesis in pregnancy. In conclusion, prolactin and estrogens are not major physiological regulators of hepcidin, even under conditions where these hormones are highly elevated. The mechanisms by which maternal hepcidin is suppressed during pregnancy remain to be determined.

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Disclosures

TG and EN are scientific co-founders of Intrinsic LifeSciences and Silarus Therapeutics. TG is a consultant for ADARx, Akebia, Pharmacosmos, Chugai, Ionis, Gossamer Bio, Global Blood Therapeutics, American Regent, Disc Medicine, RallyBio, and Rockwell Scientific. EN is a consultant for Chugai, Protagonist, Vifor, RallyBio, Ionis, GSK, Novo Nordisk, AstraZeneca, FibroGen, and Disc Medicine. All of the other authors have no conflicts of interest to disclose.

Contributions

VZ designed and performed experiments, analyzed data, and edited the manuscript. ALF and SMH performed experiments. EN and TG analyzed data and edited the manuscript. VS designed, performed and supervised experiments, analyzed data, and wrote the manuscript.

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Data-sharing statement

Data that support the study are available upon reasonable request to the corresponding author.

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