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ORIGINAL RESEARCH

Uterine kisspeptin receptor critically regulates epithelial estrogen receptor α transcriptional activity at the time of embryo implantation in a mouse model

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ABSTRACT: Embryo implantation failure is a major cause of infertility in women of reproductive age and a better understanding of uterine factors that regulate implantation is required for developing effective treatments for female infertility. This study investigated the role of the uterine kisspeptin receptor (KISS1R) in the molecular regulation of implantation in a mouse model. To conduct this study, a conditional uterine knockout (KO) of *Kiss1r* was created using the *Pgr-Cre* (progesterone receptor-CRE recombinase) driver. Reproductive profiling revealed that while KO females exhibited normal ovarian function and mated successfully to stud males, they exhibited significantly fewer implantation sites, reduced litter size and increased neonatal mortality demonstrating that uterine KISS1R is required for embryo implantation and a healthy pregnancy. Strikingly, in the uterus of *Kiss1r* KO mice on day 4 (D4) of pregnancy, the day of embryo implantation, KO females exhibited aberrantly elevated epithelial ERα (estrogen receptor α) transcriptional activity. This led to the temporal misexpression of several epithelial genes [*Cftr* (Cystic fibrosis transmembrane conductance regulator), *Aqp5* (aquaporin 5), *Aqp8* (aquaporin 8) and *Cldn7* (claudin 7)] that mediate luminal fluid secretion and luminal opening. As a result, on D4 of pregnancy, the lumen remained open disrupting the final acquisition of endometrial receptivity and likely accounting for the reduction in implantation events. Our data clearly show that uterine KISS1R negatively regulates ERα signaling at the time of implantation, in part by inhibiting ERα overexpression and preventing detrimentally high ERα activity. To date, there are no reports on the regulation of ERα by KISS1R; therefore, this study has uncovered an important and powerful regulator of uterine ERα during early pregnancy.

Key words: kisspeptin receptor / G protein-coupled receptors / receptivity / implantation / infertility / uterus / fluid homeostasis / aquaporin / estrogen receptor α

Introduction

Approximately 15% of clinical pregnancies result in miscarriage (Rai and Regan, 2006). While miscarriages are associated with factors such as embryonic chromosomal abnormalities, with each additional miscarriage endometrial defects increase in prominence as a cause of pregnancy failure. These defects block the acquisition of endometrial receptivity and decidualization, which then impact negatively on embryo implantation. Despite the advances made with assisted reproduction, up to half of all morphologically high-grade embryos, when transferred back into a morphologically normal uterine cavity will fail to implant (Koot *et al.*, 2011, 2016). Recurrent implantation failure

remains a challenge to treat clinically (Bashiri et al., 2018) but, undoubtedly, effective treatment strategies will be developed with a stronger understanding of uterine factors that regulate embryo implantation.

Embryo implantation is a complex process and remains the least understood rate-limiting step in the establishment of a successful pregnancy (Koot et al., 2012; Su and Fazleabas, 2015). This process is tightly regulated by estradiol (E2) and progesterone (P4) and a fine local balance in E2 and P4 signaling via the uterine-based nuclear receptors, estrogen receptor α (ERα) and progesterone receptor A and B (PR), is critical for normal implantation (Hamilton et al., 2017; de Oliveira et al., 2019). Thus, any perturbations in this balance of E2 and P4 signaling can lead to implantation failure and reproductive dysfunction.

Much of our understanding of the acquisition of endometrial receptivity and embryo implantation is from studies conducted on genetically modified mouse models of human infertility. In the mouse, the uterine epithelium on days 1 and 2 (D1 and D2) of pregnancy is under the regulation of E2 where E2 induces epithelial cell proliferation and initiates the acquisition of endometrial receptivity. On D3 and D4, the uterus is under the regulation of P4 where P4 blocks E2 effects on the epithelium and induces epithelial cell differentiation to adopt a secretory phenotype (Cha et al., 2012). Collectively, E2 and P4, acting via their receptors, create an epithelial lining that is receptive to embryo implantation on D4 of pregnancy. In women, the uterus achieves a receptive state in less than a week after ovulation, exhibiting maximum receptivity to implantation between D20 and D24 of the menstrual cycle (Radovick and Babwah, 2019).

In addition to E2 and P4 acting via their receptors, many other factors regulate the acquisition of endometrial receptivity and implantation (early pregnancy events). These factors include the G protein-coupled receptors (GPCRs) (Babwah, 2020). Although several GPCRs are expressed in the uterus during the peri-implantation period, only three uterine GPCRs have been directly identified as regulators of early pregnancy events in the mouse. These are the $G\alpha_s$-coupled leucine-rich repeat-containing GPCR 4 (LGR4) (Mohri et al., 2010; Sone et al., 2013), the $G\alpha_{q/11}$- and $G\alpha_{i/o}$-coupled lysophosphatidic acid receptor 3 (LPAR3) (Diao et al., 2015) and the $G\alpha_{q/11}$-coupled kisspeptin receptor (KISS1R) (Calder et al., 2014; Bhattacharya and Babwah, 2015; León et al., 2016; Radovick and Babwah, 2019). In the mouse, genetic deletion of epithelial *Lgr4* results in persistent epithelial ERα signaling and P4 resistance (Mohri et al., 2010; Sone et al., 2013), while deletion of *Lpar3* results in persistent epithelial PR signaling and the inability to exit the pre-receptive phase of early pregnancy (Diao et al., 2015). In both the *Lgr4* and *Lpar3* knockout (KO) mouse, these aberrations result in implantation failure. Systemic deletion of *Kiss1r* or *Kiss1* (the gene that encodes the KISS1R ligands called kisspeptins) fully prevents implantation and this was shown to be the result of reduced endometrial gland function (Calder et al., 2014; León et al., 2016).

The kisspeptin (KP)/KISS1R signaling system has emerged as an important clinical target (Jayasena et al., 2014; Prague and Dhillo, 2015; Abbara et al., 2020; Chan et al., 2020; Terse et al., 2021) and it is crucial to better understand the role of this system throughout the body. In addition to the uterus, KP and KISS1R are expressed in the hypothalamus, ovary and peripheral metabolic tissues such as the liver, pancreas and adipose tissues (Song et al., 2014; Tolson et al., 2014; Wolfe and Hussain, 2018; Tolson et al., 2020). To date, the KP/KISS1R signaling system is best understood for its role as a potent trigger of GnRH secretion and thereby a major regulator of fertility (de Roux et al., 2003; Seminara et al., 2003; Lapatto et al., 2007; Millar and Babwah, 2015). More recently, it has also been shown to regulate energy homeostasis (Song et al., 2014; Tolson et al., 2014; Wolfe and Hussain, 2018; Tolson et al., 2020) and thereby could affect fertility. Since the studies uncovering potential roles for uterine KISS1R and kisspeptins in regulating embryo implantation in the mouse were conducted in the whole-body KO of *Kiss1r* or *Kiss1* it is plausible that the implantation failure or the severity of the defect in the systemic KOs is also due to their hypogonadal and/or metabolic phenotypes. Given their potential role as major regulators of embryo implantation, establishing that uterine KISS1R regulates embryo implantation, independent of other tissues, is important and requires the use of a uterine KISS1R KO mouse. For this primary reason, this study was conducted.

In this study, we generated the first conditional KO of uterine *Kiss1r* using the well-established progesterone receptor-CRE recombinase (*Pgr-Cre*) driver (Soyal et al., 2005). Reproductive profiling was conducted on the KO females on D4 of pregnancy, the day of embryo implantation, and this revealed that loss of uterine KISS1R led to aberrantly persistent epithelial ERα transcriptional activity. This resulted in the temporal misexpression of several genes that increase fluid secretion into the uterine lumen. Excessive luminal fluids on the day of implantation likely disrupted embryo-endometrial interactions resulting in reduced implantation events and an unhealthy pregnancy. Overall, the data clearly reveal in the mouse that uterine KISS1R facilitates embryo implantation by tightly regulating epithelial ERα transcriptional activity on the day of embryo implantation. To date, there are no reports on the regulation of ERα by KISS1R, therefore our study has uncovered an important and powerful regulator of uterine ERα during early pregnancy.

Materials and methods

Animal husbandry

Animal studies (protocol number: PROTO201702536) were approved by Rutgers University according to guidelines established by the Institutional Animal Care and Use Committee. The mice were maintained under a 12-h light/dark cycle and provided with standard rodent chow and water *ad libitum*. Studies were conducted in 129 and C57 Bl/6 wild-type (WT) mice.

Generation of uterine *Kiss1r*$^{fl/fl}$; *Pgr-Cre*$^{+/-}$ (*Kiss1r*$^{Pgr\text{-}Cre}$-KO) experimental and *Kiss1r*$^{fl/fl}$ littermate control mice

The uterine *Kiss1r* conditional KO (*Kiss1r*$^{Pgr\text{-}Cre}$-KO) was generated in our laboratory by crossing floxed *Kiss1r* (*Kiss1r*$^{fl/fl}$) mice (Novaira et al., 2014) to *Pgr-Cre*$^{+/-}$ (Soyal et al., 2005) mice. F1 males of genotype *Kiss1r*$^{fl/+}$; *Pgr-Cre*$^{+/-}$ were then backcrossed to female *Kiss1r*$^{fl/fl}$ and this generated a segregating population of KO (*Kiss1r*$^{fl/fl}$; *Pgr-Cre*$^{+/-}$) and control (CTRL) (*Kiss1r*$^{fl/fl}$) littermates.

Fertility assessment

Uterine *Kiss1r*$^{Pgr\text{-}Cre}$-KO and CTRL females (8–10 weeks old) were mated to stud males at a ratio of one female to one male. Mating pairs

were housed continuously together for 3 months and the following data were recorded: number of pregnancies, number of pups per/litter and pup mortality rates.

Estrous cyclicity

Estrous staging (proestrus, estrus, metestrus and diestrus) of adult uterine *Kiss1r*$^{Pgr\text{-}Cre}$-KO and CTRL females (8–10 weeks old) was conducted for 2 weeks, as previously described (Caligioni, 2009).

E2 and P4 treatments

WT Bl/6 female mice in proestrus or diestrus were injected s.c. with either peanut oil (100 µl), E2 (100 ng in a final volume of 100 µl) or P4 (1 mg in a final volume of 100 µl) at 3 p.m. and euthanized 24 h later. Uteri were collected and stored in RNA later (Life Technologies Inc., Burlington, ON, Canada.).

Unstimulated pregnancy

Females (8–10 weeks-old) in proestrus were mated to either stud or vasectomized males; the day of mating is defined as D0, while D1 is the day the vaginal copulatory plug is observed. Only females that showed a copulatory plug (evidence of successful mating) on the morning of D1 were studied further. Mice were euthanized between 8 and 9 a.m. on D1, and D4–8 of pregnancy and uteri were collected and rapidly frozen. Based on the visibility of implantation swellings, D6–8 uteri were cut and separated into implantation sites (IS) and inter-implantation sites (IIS). On D4 of pregnancy, the hypothalamus, pituitary, ovary, liver, pancreas, white adipose tissue (WAT), brown adipose tissue (BAT) and kidney were collected.

Oviduct and uterine horn flushing

To determine whether or not the oviduct contained one-cell zygotes or whether the uterine horns contained unimplanted blastocysts, oviducts were isolated on D1 of pregnancy, while the horns were collected on the morning of D4 of pregnancy (the day of mating is defined as D0) and flushed with PBS. Embryos were then examined and counted under the dissecting microscope.

E2, P4 or E2 + P4 regulation of gene expression

Eight-week-old ovariectomized mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and 2 weeks after ovariectomy, mice were injected s.c. with either peanut oil (100 µl), E2 (100 ng in a final volume of 100 µl), P4 (1 mg in a final volume of 100 µl) or E2 (100 ng) + P4 (1 mg) (in a final volume of 200 µl) (Jeong *et al.*, 2010). Twenty-four hours after hormone administration, mice were euthanized and uteri were collected and stored in RNAlater (Life Technologies Inc., Burlington, ON, Canada). E2 and P4 were purchased from Tocris Bioscience (Minneapolis, MN, USA).

Hematoxylin and eosin staining

Mouse uteri were fixed in 4% paraformaldehyde overnight, transferred to 70% ethanol, paraffin embedded and sectioned at 5 µm. Uterine sections were deparaffinized, rehydrated and stained with hematoxylin and eosin (H&E) using standard techniques. Sections were then dehydrated in ethanol, cleared in xylene, overlaid with Permount mounting medium (Fisher Scientific, Ottawa, ON, Canada) and sealed with a coverslip.

Serum E2 and P4 assay

Uterine *Kiss1r*$^{Pgr\text{-}Cre}$-KO and CTRL females were euthanized on D4 of pregnancy, and whole blood was collected by intracardiac puncture and serum was prepared by clotting the whole blood at room temperature. Serum E2 and P4 were measured at The Ligand Assay & Analysis Core at the University of Virginia by ELISA. P4 assay: intra- and inter-assay % coefficients of variation are 6.9 and 10.1%, respectively. E2 assay: intra- and inter-assay % coefficients of variation are 5.7 and 10.2%, respectively.

Quantitative PCR

Gene expression was determined on total RNA prepared from uteri, uterine IS and IIS, hypothalamus, pituitary, ovary, liver, pancreas, WAT, BAT and kidney. Uterine tissues were snap frozen post isolation, later homogenized, and RNA was isolated using the Qiagen RNeasy mini kit according to manufacturer's instructions (Qiagen, Missassauga, ON, Canada). RNA was isolated from cell fractions using TRIzol reagent (Thermo Fisher Scientific, Waltham, MA, USA). cDNA was synthesized using iScript^{TM} Reverse Transcription Supermix from Bio-Rad. Briefly, 1 µg of total RNA was reverse transcribed and diluted 2-fold in water. Five µl of diluted cDNA was used in each quantitative PCR (qPCR) reaction. qPCR was conducted using the iTaq Universal SYBR Green Supermix from Bio-Rad Laboratories (Hercules, CA, USA).

Each qPCR assay was performed in duplicate and conducted a total of three independent times using PrimePCR Assays (Bio-Rad Laboratories): *Aqp5* (aquaporin 5) (Unique Assay ID: qMmuCID0006880), *Aqp8* (aquaporin 8) (Unique Assay ID: qMmuCID0007218), *Aqp11* (aquaporin 11) (Unique Assay ID: qMmuCED0048241), *Cftr* (cystic fibrosis transmembrane conductance regulator) (Unique Assay ID: qMmuCID0025170), *Cldn7* (claudin 7) (Unique Assay ID: qMmuCED0044866), *Esr1* (estrogen receptor 1) (Unique Assay ID: qMmuCED0044294), *Hand2* (heart and neural crest derivatives expressed 2) (Unique Assay ID: qMmuCID0009541), *Ihh* (Indian hedgehog) (Unique Assay ID: qMmuCED0044390), *Il13ra2* (interleukin 13 receptor subunit alpha 2) (Unique Assay ID: qMmuCID0025232), *Kiss1* (kisspeptin 1) (Unique Assay ID: qMmuCED0040677), *Kiss1r* (kisspeptin 1 receptor) (Unique Assay ID: qMmuCID0024269), *Lcn2* (lipocalin) (Unique Assay ID: qMmuCED0045799), *Ltf* (lactoferrin) (Unique Assay ID: qMmuCID0022149), *Npl* (N-acetylneuraminate pyruvate lyase) (Unique Assay ID: qMmuCID0010972), *Pgr* (progesterone receptor) (Unique Assay ID: qMmuCED0047267), *Rpl13a* (ribosomal protein L13a) (Unique Assay ID: qMmuCED0040629), *Scnn1a* (sodium channel non-neuronal 1 α subunit) (Unique Assay ID: qMmuCED0047355) and *Sprr2f* (small proline-rich protein 2F) (Unique Assay ID: qMmuCED0050668). Additional qPCR primers were purchased from OriGene; these were for *Gpr30* (G protein-coupled receptor 30) (NM_029771) and *Esr2* (estrogen receptor 2) (NM_207707). All qPCR assays used in this study exhibited efficiencies between 90 and 110%. Since the assays exhibit similar efficiencies (90-110%), mRNA expression level was calculated as relative expression to the housekeeping gene (*Rpl13a*), using the $2^{-\Delta\Delta Ct}$ method.

SDS-PAGE and western blotting

D4 pregnant uterine *Kiss1r*$^{Pgr\text{-}Cre}$-KO and CTRL uteri tissue was lysed in RIPA buffer containing protease and phosphatase inhibitors, and ERα, PR-A, PR-B, tubulin and vinculin protein levels were assayed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and western blotting, as previously described (Pampillo *et al.*, 2009; Szereszewski *et al.*, 2010; de Oliveira *et al.*, 2019; Parobchak *et al.*, 2020). ERα was detected using a primary rabbit polyclonal antibody (Santa Cruz Biotechnology, Dallas, TX, USA) (HC-20), Cat# sc-543, 1:1000, PR-A and PR-B was detected using a primary rabbit polyclonal antibody (Santa Cruz Biotechnology (HC-190), Cat# sc-7208, 1:1000), tubulin was detected using a primary mouse monoclonal antibody (ABCAM, Cat# ab184613, 1:5000) and vinculin was detected using a primary mouse monoclonal antibody (BioRad, Cat # MCA465GA, 1:1000). The primary antibodies were detected using the following secondary antibodies: Anti-Mouse IgG HRP Linked Antibody (Cell Signaling, Cat # 7076S, 1:2500) and Anti-Rabbit IgG HRP Linked Antibody (Cell Signaling, Cat # 7074S, 1:2500). Blots were developed using an ECL detection system (Thermo Fisher Scientific) and the Bio-Rad ChemiDoc Imaging System. Densitometric analysis of protein bands was performed using the Bio-Rad Image Lab Software.

Immunohistochemistry

D4 pregnant uterine *Kiss1r*$^{Pgr\text{-}Cre}$-KO and CTRL uteri were fixed in 10% formalin overnight, transferred to 70% ethanol, paraffin embedded and sectioned at 5 µm. Uterine sections were deparaffinized, rehydrated and blocked with 5% normal blocking serum (mouse) and then incubated with the following primary antibodies for 1 h at room temperature followed by 48 h at 4°C: AQP5 rabbit polyclonal antibody (ABCAM, Cat # ab78486, 1:75 dilution), CLDN7 rabbit polyclonal antibody (ABCAM, Cat # ab27487, 1:200 dilution) and ERα rabbit polyclonal antibody (Santa Cruz Biotechnology (HC-20), Cat # sc-543, 1:50 dilution). Antigen-bound primary antibodies were detected with the ImmunoCruz rabbit ABC Staining System (Santa Cruz Biotechnology, Inc. Dallas, TX, USA). Stained sections were overlaid with Permount mounting medium (Fisher Scientific, Ottawa, ON, Canada) and sealed with a coverslip. All samples were processed in parallel and treated with 3,3′-diaminobenzidine for an identical period of time. This permitted a direct comparison of the KO and CTRL tissue samples. Experimental conditions were carefully maintained between independent assays and analyses were conducted three independent times.

Image acquisition

For 3,3′-diaminobenzidine-stained tissue sections, slides were scanned using the Aperio ImageScope XT (Leica Biosystems Inc., Buffalo Grove, IL, USA). Sections were then viewed and analyzed using the ImageScope software.

Statistics

The differences between groups were determined using unpaired, two-tailed Student's *t*-test or one-way ANOVA followed by *post hoc* Bonferroni's Multiple Comparison test (GraphPad Prism Software, Inc, La Jolla, CA, USA). All values are expressed as mean ± SEM and a value of $P < 0.05$ was considered statistically significant.

Results

Uterine *Kiss1r* mRNA is expressed in the pregnant mouse

We previously reported the mouse uterus expresses *Kiss1r* mRNA in the epithelium on D4 of pregnancy (the day that endometrial receptivity is achieved and embryo implantation begins) (Calder *et al.*, 2014). To further characterize the expression of uterine *Kiss1r* during early pregnancy, we profiled *Kiss1r* mRNA expression on D1 and D4–8 of pregnancy in the WT mouse (Fig. 1A). Results showed that uterine *Kiss1r* expression, relative to D1 of pregnancy, is significantly upregulated on D4 and D5 (Fig. 1A). On D6–8 of pregnancy, when implantation sites (comprising the decidua and developing embryo) became visible as distinct swellings, they were separated from the inter-implantation sites and both sites were analyzed for *Kiss1r* expression. Results revealed that *Kiss1r* expression was the highest on D6 at the IS, and expression at this site decreased gradually on D7–8 (Fig. 1A). Interestingly, on D6–8, expression at the IIS was low and unchanged, suggesting a role for uterine *Kiss1r* in regulating embryo implantation.

Uterine *Kiss1r* mRNA is an E2/P4-responsive gene

To determine whether *Kiss1r* expression is regulated by E2- and/or P4, expression was determined in the ovariectomized (OVX) WT mouse 24 h following oil, E2 and/or P4 injections. Results showed that uterine *Kiss1r* expression is induced by the combined treatment of E2 and P4 (Fig. 1B). To further confirm that uterine *Kiss1r* mRNA is regulated by both E2 and P4, WT mice in proestrus (high E2:P4 ratio) and diestrus (high P4:E2 levels) were injected with E2 or P4 and uterine *Kiss1r* mRNA expression was quantified 24 hrs later. In proestrus, only exogenous P4 in the background of high endogenous E2 levels increased *Kiss1r* expression (Fig. 1C) while in diestrus, only exogenous E2 in the background of high endogenous P4 levels increased expression (Fig. 1D). Collectively, these findings strengthen the conclusion that uterine *Kiss1r* expression is an E2/P4-responsive gene.

The preimplantation embryo does not modulate uterine *Kiss1r* mRNA expression on D4 of pregnancy

Hatched blastocysts are localized to the uterine horn on the morning of D4 of mouse pregnancy (Deb *et al.*, 2006) and prior to implantation can modulate the expression of some endometrial genes (Das *et al.*, 1994; Passaro *et al.*, 2018, 2019). To determine whether uterine *Kiss1r* expression was influenced by the blastocyst on the morning of D4, mRNA expression level was compared between the pregnant female mouse (mated to an intact male) and the pseudopregnant female (mated to a vasectomized male). The results revealed that in the presence or absence of preimplantation embryos, mRNA expression of uterine *Kiss1r* was similar (Fig. 1E) revealing that the preimplantation embryo does not modulate uterine *Kiss1r* mRNA expression.

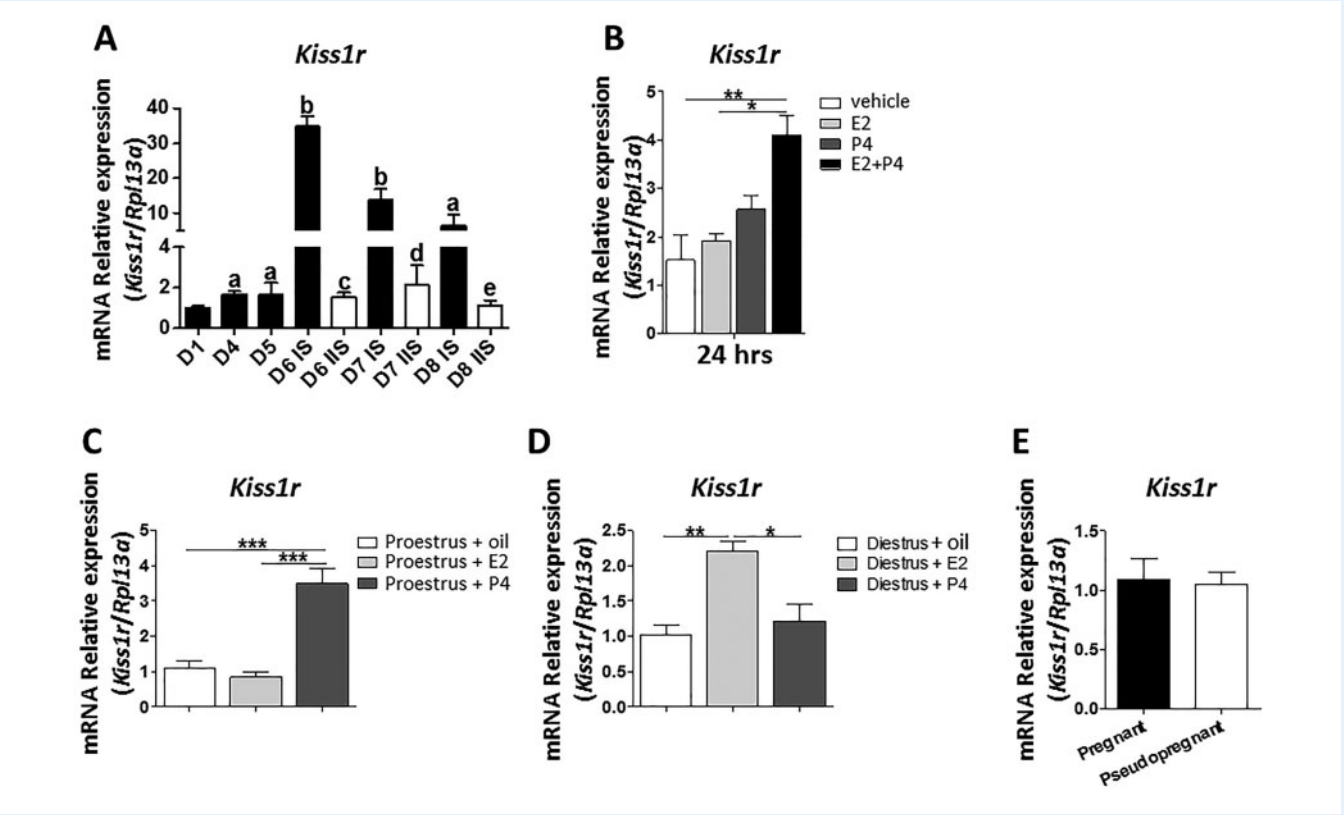

Figure 1. Uterine *Kiss1r* mRNA is an estrogen + progesterone responsive gene that is highly expressed in the peri- and post-implantation period and diminishes in expression with progressive decidualization at the implantation site. *Kiss1r* (kisspeptin receptor) expression was determined by quantitative PCR (qPCR) in the (**A**) pregnant mouse uterus during early pregnancy (day (D) 1–8); a: $P < 0.01$ versus D1; b: $P < 0.001$ versus D1; c: $P < 0.001$ versus D6 IS; d: $P < 0.01$ versus D7 IS; e: $P < 0.01$ versus D8 IS; (**B**) OVX mouse + oil, E2, P4 or E2 + P4 following 24 h of treatment; (**C**) non-pregnant mouse uterus during proestrus + oil, E2 or P4 following 24 h of treatment; (**D**) non-pregnant mouse uterus during diestrus + oil, E2 or P4 following 24 h of treatment; and (**E**) pregnant and pseudo-pregnant mouse uterus on D4 of pregnancy. Statistics (n = 3–6 for all groups): Student's unpaired two-tailed *t*-test and one-way ANOVA with Bonferroni Multiple Comparison *post hoc* test. *$P < 0.05$; **$P < 0.01$; and ***$P < 0.001$. IS, implantation site; IIS, inter-implantation site.

Pgr-Cre triggers efficient ablation of *Kiss1r* in the KO uterus

To establish a role for uterine *Kiss1r* in regulating embryo implantation, *Kiss1r* was conditionally deleted in the uterus by crossing *Kiss1r*$^{fl/fl}$ mice to *Pgr-Cre*$^{+/-}$ mice. Uterine *Kiss1r* mRNA expression level was then quantified by qPCR in pregnant *Kiss1r*$^{Pgr\text{-}Cre}$- KO mice (KO: *Kiss1r*$^{fl/fl}$; *Pgr-Cre*$^{+/-}$) on D4 of pregnancy and this revealed that expression was reduced by about 95% relative to control mice (CTRL: *Kiss1r*$^{fl/fl}$) (Fig. 2A). *Kiss1r* mRNA expression was also quantified along the neuroendocrine-reproductive axis (hypothalamus, pituitary and ovary) and peripheral metabolic tissues (liver, pancreas, WAT, BAT and kidney) on D4 of pregnancy. Results showed that, except for the KO liver, expression was not different between KO and CTRL cells (Supplementary Fig. S1). In the liver, a small but significant increase (about 2-fold) in *Kiss1r* mRNA expression level was observed in the KO but whether this is physiologically relevant is unknown (Supplementary Fig. S1D). Uterine *Kiss1* mRNA was also quantified on D4 of pregnancy and this showed no difference between KO and CTRL females (Fig. 2B).

Ovarian function is unaffected in *Kiss1r*$^{Pgr\text{-}Cre}$-KO females

In the ovary, *Pgr* is expressed in granulosa cells (Breen *et al.*, 2013), while *Kiss1r* is expressed in developing oocytes (Gaytan *et al.*, 2014). This makes it unlikely that ovarian *Kiss1r* expression was deleted in the *Kiss1r*$^{Pgr\text{-}Cre}$-KO mouse, a finding supported by qPCR analysis of *Kiss1r* expression in the KO and CTRL ovaries (Supplementary Fig. S1C). Nevertheless, it was determined whether ovarian function was impaired in KO mice. To do this, estrous cyclicity of KO and CTRL females was quantified and it was found that KO females (n = 8) exhibited a similar number of estrus events as CTRL littermate females (n = 9) (Fig. 2C). Additionally, following mating to WT stud males, based on the number of one-cell embryos recovered from the oviduct on D1 of pregnancy, ovulatory rates were similar between KO (n = 5) and CTRL (n = 5) females (KO = 8.1 ± 1.3 zygotes; CTRL = 9.4 ± 3.2 zygotes). Next, serum E2 and P4 levels on D4 of pregnancy was quantified in KO (n = 3) and CTRL (n = 3) females. Results showed that serum P4 levels (KO mice: 47.1 ± 7.6 ng/ml; CTRL mice: 41.0 ± 12.2 ng/ml) and serum E2 levels (KO mice: 15.2 ± 5.2 ng/ml;

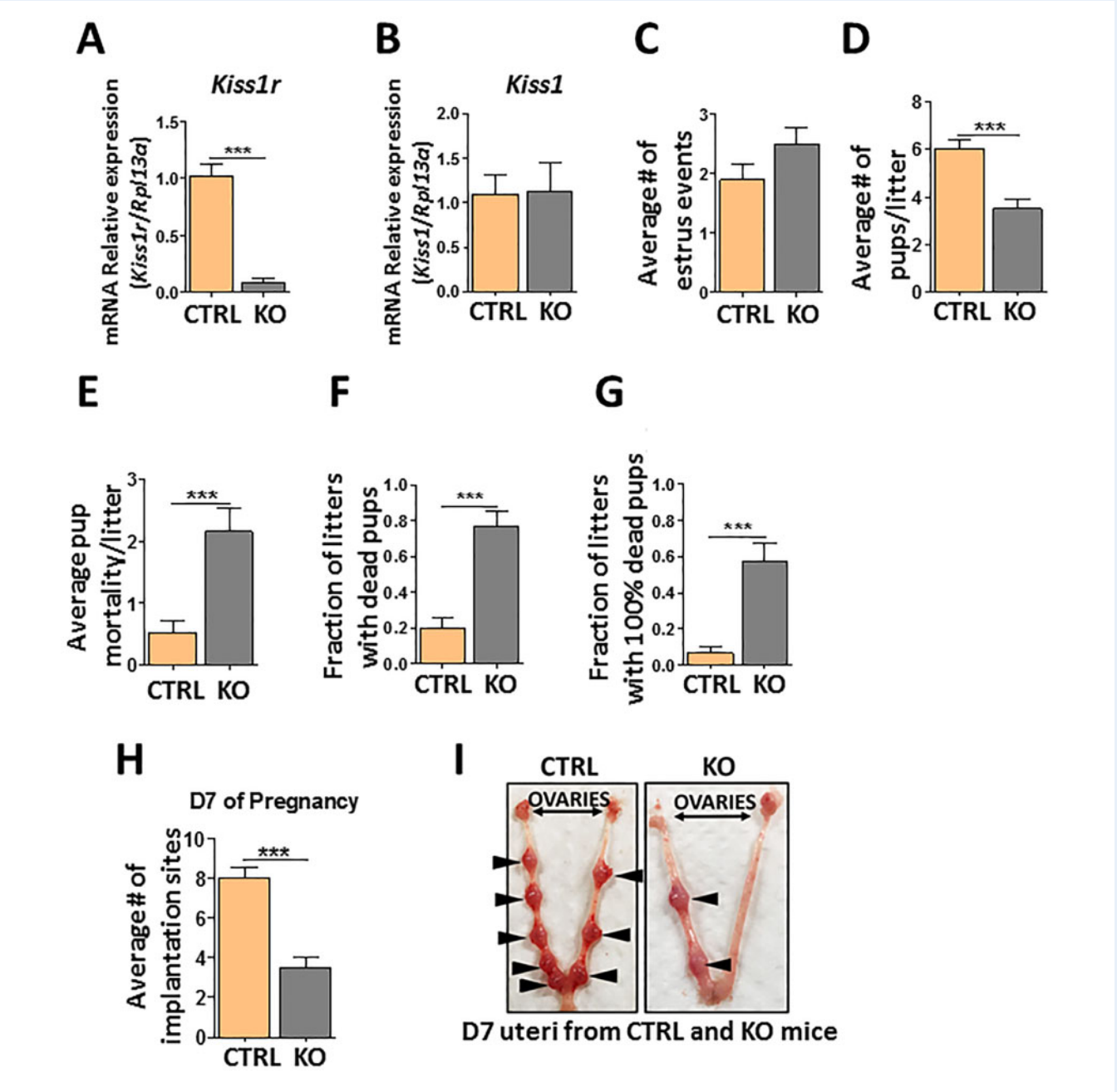

Figure 2. Characterization of the reproductive phenotypes of the uterine *Kiss1r*$^{Pgr\text{-}Cre}$-knockout mouse. (**A**) qPCR analysis of progesterone receptor-CRE recombinase (*Pgr-Cre*) triggering the efficient deletion of uterine *Kiss1r*; (**B**) qPCR analysis of effect on the ligand *Kiss1*; (**C**) ovarian function assessment of average number of estrus events; (**D**) average number of pups/litter from all pregnancies; (**E**) average number of pup mortality/litter from all pregnancies; (**F**) fraction of litters with pup mortality from all pregnancies; (**G**) fraction of litters with 100% pup mortality from all pregnancies; (**H**) average number of implantation sites on D7 of pregnancy; and (**I**) gross anatomy visual of the average number of implantation sites on D7 of pregnancy (representative images shown). Statistics (n = 4–19 for all groups): Student's unpaired two-tailed *t*-test; ***$P < 0.001$. KO, knockout.

CTRL mice: 13.4 ± 4.3 ng/ml) were similar ($p > 0.05$) between KO and CTRL mice. Together, the estrus cyclicity, ovulation capacity and serum E2/P4 data reveal that KO females exhibit normal ovarian function. Therefore, any observed reproductive phenotypes in KO females would be related to the loss of uterine *Kiss1r* expression.

Kiss1r$^{Pgr\text{-}Cre}$-KO females exhibit implantation failure and increased neonatal mortality rates

To determine the effect of a loss of uterine *Kiss1r* expression on pregnancy, KO and CTRL females were subjected to further reproductive profiling. KO and CTRL females underwent a 3-month fertility test

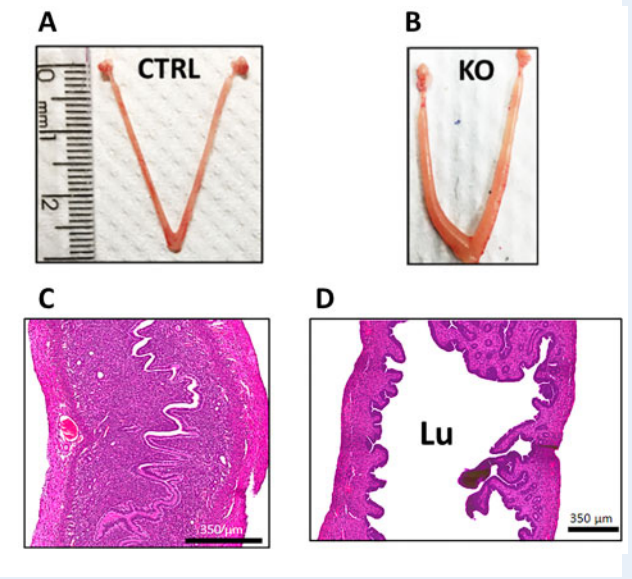

Figure 3. The D4 pregnant *Kiss1r*$^{Pgr\text{-}Cre}$-KO uterus exhibits a defect in luminal closure. (**A, B**) Gross anatomy of D4 pregnant uterus from control (CTRL) and KO females; and (**C, D**) histology of uterine longitudinal section stained with hematoxylin and eosin (H&E). Representative images shown. CTRL (n = 3–8) and KO (n = 3–5). Lu, lumen.

where females were mated continuously to WT stud males. During this period, it was observed that among 12 KO females, seven exhibited one pregnancy, four exhibited two pregnancies and one failed to become pregnant. In striking contrast, among 12 CTRL females, 11 exhibited either three or four pregnancies while only one exhibited a single pregnancy.

Next, it was observed that KO females produced fewer pups per litter (91 pups from 26 litters; 3.5 pups/litter) compared to CTRL females (271 pups from 45 litters; 6.0 pups/litter) (Fig. 2D). In addition to a smaller litter size, litters from KO females exhibited a higher pup mortality rate (56 dead pups among 91 pups; pup mortality rate: 61.5%) compared to CTRL females (23 dead pups among 271 pups; pup mortality rate: 8.5%) (Fig. 2E). Pup mortality was determined on the evening of birth and on the following morning after birth. Further analysis of the pup mortality data revealed that in litters from KO females, 20 out of the 26 litters (76.9%) had at least one dead pup while 15 out of the 26 litters (57.7%) had 100% dead pups. In contrast, among CTRL females, 9 out of the 45 litters (20%) had at least one dead pup (Fig. 2F), while 3 out of the 45 litters (6.7%) had 100% dead pups (Fig. 2G).

Since KO and CTRL females exhibited normal ovarian function and produced a similar number of fertilization-competent oocytes (based on zygote formation), we hypothesized that the smaller litter size that was observed among *Kiss1r*$^{Pgr\text{-}Cre}$-KO females (Fig. 2D) was caused by an implantation defect, as was observed with the whole body *Kiss1* and *Kiss1r* KO mice (Calder *et al.*, 2014). To test this, KO and CTRL females were mated to WT stud males and on the morning of D4 (before implantation begins), uteri horns were flushed and unimplanted blastocysts were quantified. Results showed that the number of blastocysts was similar between KO (n = 5) and CTRL (n = 5) females (KO = 8.0 ± 1.5 blastocysts; CTRL = 7.0 ± 2.2 blastocysts). Next, implantation sites were quantified visually on D7 of pregnancy. Results showed that uteri from KO females (n = 4) had an average of four sites/uterus, while CTRL females (n = 5) had an average of eight sites/uterus (Fig. 2H and I). Overall, the data clearly show that loss of uterine *Kiss1r* results in reduced implantation and an unhealthy pregnancy resulting in a higher pup mortality rate in the neonatal period.

The uterus from *Kiss1r*$^{Pgr\text{-}Cre}$-KO females fails to undergo luminal closure on D4 of pregnancy

To understand the cause of reduced implantation sites in KO females, the uterus was examined on D4 of pregnancy, the day that implantation begins. Gross visual inspection of intact uterine horns revealed that compared to CTRL females, horns from KO females were thicker (Fig. 3A and B). Upon examination of H&E-stained longitudinal sections of the horns, it was observed that the increased thickness of the uterus from KO females was caused by an open lumen (Fig. 3C and D). In mice, the lumen opens on D1 and D2 of pregnancy in an E2-dependent manner but closes on D4 of pregnancy in a P4-dependent manner (de Oliveira *et al.*, 2020).

E2-induced genes in the uterine epithelium are persistently and abnormally expressed on D4 of pregnancy in *Kiss1r*$^{Pgr\text{-}Cre}$-KO mice

To develop a molecular understanding underlying the open lumen on D4 of pregnancy in KO females, RNA Seq analysis of the D4 pregnant uterus from KO (n = 1) and CTRL (n = 1) females was conducted and preliminary findings were verified by qPCR. Results showed that on D4 of pregnancy, there was an abnormal upregulation of E2-induced genes that are highly expressed in the epithelium on D1 and D2 of pregnancy but weakly expressed on D4 (Das *et al.*, 1998; Contreras *et al.*, 2010; Daikoku *et al.*, 2014; de Oliveira *et al.*, 2020). These genes included *Lcn2*, *Ltf*, *Sprr2f*, *Aqp5*, *Aqp8*, *Cftr* and *Cldn7* (Fig. 4A–G). *Aqp5*, *Aqp8*, *Cftr* and *Cldn7* encode protein molecules that mediate water movement across the epithelium into the uterine lumen (luminal secretion) on D1 and D2 of pregnancy. As predicted, based on increased *Aqp5* and *Cldn7* mRNA expression level (Fig. 4E and G), AQP5 and CLDN7 protein levels were visibly increased in the uterine luminal epithelium in KO females, as shown by immunohistochemistry (Fig. 4H and I). Owing to a lack of robust and specific antibodies, increased protein levels of LTF, LCN2 SPRR2F and AQP8 could not be verified. Overall, the data reveal that loss of *Kiss1r* expression results in persistent ERα signaling and an upregulation in the expression of epithelial target genes and proteins that maintain luminal opening on D4 of pregnancy.

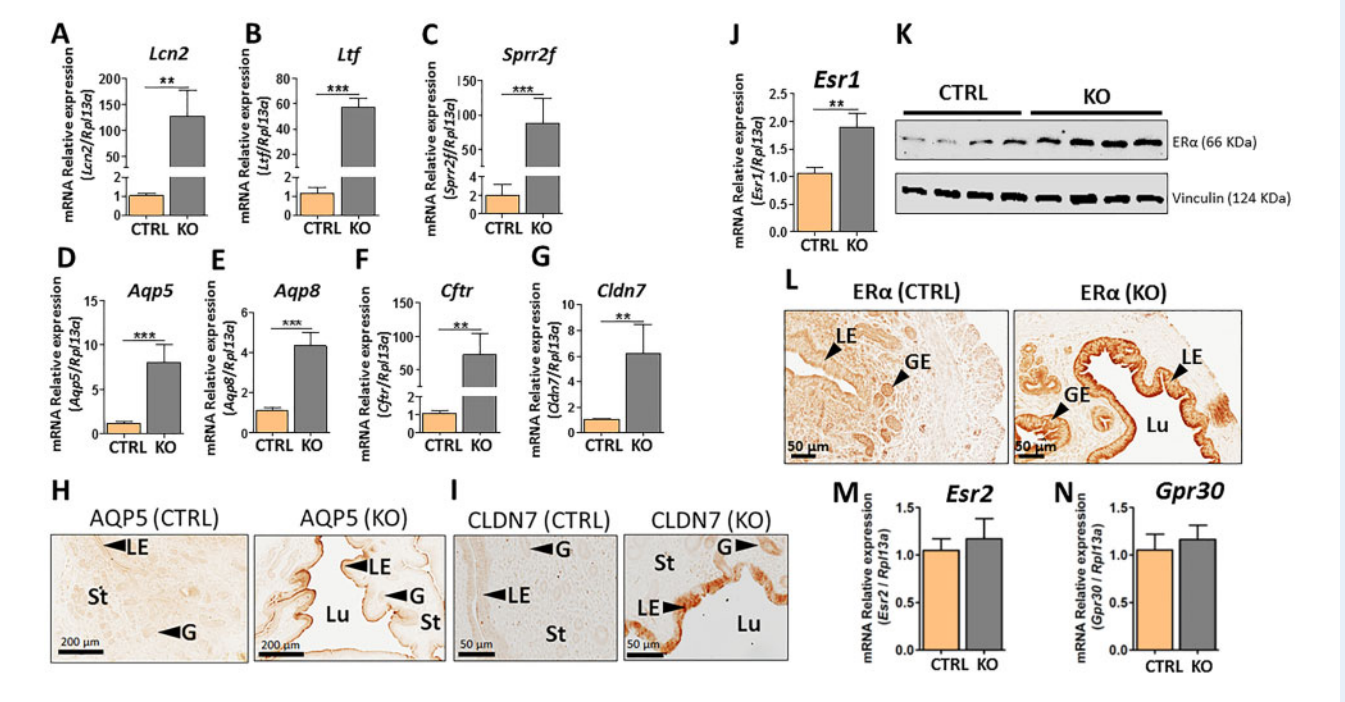

Figure 4. *Kiss1r*$^{Pgr\text{-}Cre}$-KO uterus exhibits increased ERα expression and transcriptional activity on D4 of pregnancy. qPCR analysis of known transcription targets and molecular markers of estrogen receptor (ER)α signaling and regulators of luminal secretion (**A**) *Lcn2* (lipocalin 2), (**B**) *Ltf* (lactoferrin), (**C**) *Sprr2f* (small proline-rich protein 2F) (**D**) *Aqp5* (aquaporin 5) (**E**) *Aqp8* (aquaporin 8) (**F**) *Cftr* (cystic fibrosis transmembrane conductance regulator) and (**G**) *Cldn7* (claudin 7). Representative images of the immunohistochemical analysis of (**H**) AQP5 and (**I**) CLDN7. qPCR analysis of (**J**) *Esr1* (estrogen receptor 1), western blot analysis of (**K**) ERα protein levels (the uncropped blot is shown in Supplementary Information) and (**L**) representative images of the immunohistochemical analysis of ERα in CTRL and KO uteri. qPCR analysis of (**M**) *Esr2* (estrogen receptor 2) and (**N**) *Gpr30* (G protein-coupled receptor 30). Statistics (n = 3–8 per group): Student's unpaired two-tailed *t*-test; $^{**}P < 0.01$ and $^{***}P < 0.001$. G, gland; LE, luminal epithelium; Lu, lumen; St, stroma.

The uterus from *Kiss1r*$^{Pgr\text{-}Cre}$-KO females, relative to CTRL females, exhibits increased epithelial *Esr1* and ERα levels on D4 of pregnancy

To determine whether the persistent D4 expression of E2-induced genes in the epithelium of KO females, relative to the CTRL females, was associated with increased *Esr1* mRNA and ERα levels, *Esr1* and ERα levels were quantified by qPCR and western blotting, respectively. Results revealed that in the uterus from KO females, *Esr1* mRNA levels were increased (Fig. 4J) and this increase was associated with increased ERα protein levels (Fig. 4K) specifically localized to the luminal epithelium (Fig. 4L). This leads us to conclude that the persistent expression of ERα target genes in the epithelium is at least in part the result of increased levels of epithelial ERα. To determine whether the loss of uterine *Kiss1r* also altered the expression of other genes that mediate the effects of E2, the expression of *Esr2* (encodes ERβ) and *Gpr30* (encodes GPR30 or GPER1) was quantified. The data clearly showed that gene expression was similar between KO and CTRL uteri (Fig. 4M and N) revealing a specific relationship between KISS1R signaling and *Esr1* expression.

The uterus from *Kiss1r*$^{Pgr\text{-}Cre}$-KO females exhibits normal PR levels and transcriptional activity on D4 of pregnancy

Luminal closure on D4 of pregnancy is regulated by P4-induced genes such as *Aqp11* and *Scnn1a* [a component of the epithelial sodium channel (ENaC)] (Ruan *et al.*, 2014; de Oliveira *et al.*, 2020). To determine whether the failure of the lumen to undergo closure on D4 was in part caused by reduced PR signaling, we analyzed *Pgr* gene expression, PR levels and PR-target gene expression. Results revealed that loss of uterine *Kiss1r* had no effect on *Pgr* gene expression (Fig. 5A), PR expression (Fig. 5B), or the PR-regulated expression of *Aqp11*, *Scnn1a*, *Hand2*, *Ihh*, *Il13ra2* and *Npl* (Takamoto *et al.*, 2002; Doan and Bany, 2010; Franco *et al.*, 2010, 2012; Xiao *et al.*, 2013; de Oliveira *et al.*, 2019, 2020; Marquardt *et al.*, 2020) (Fig. 5C–H). We therefore conclude that the failure of the lumen to undergo closure on D4 is not caused by diminished PR signaling but by abnormally persistent ERα signaling.

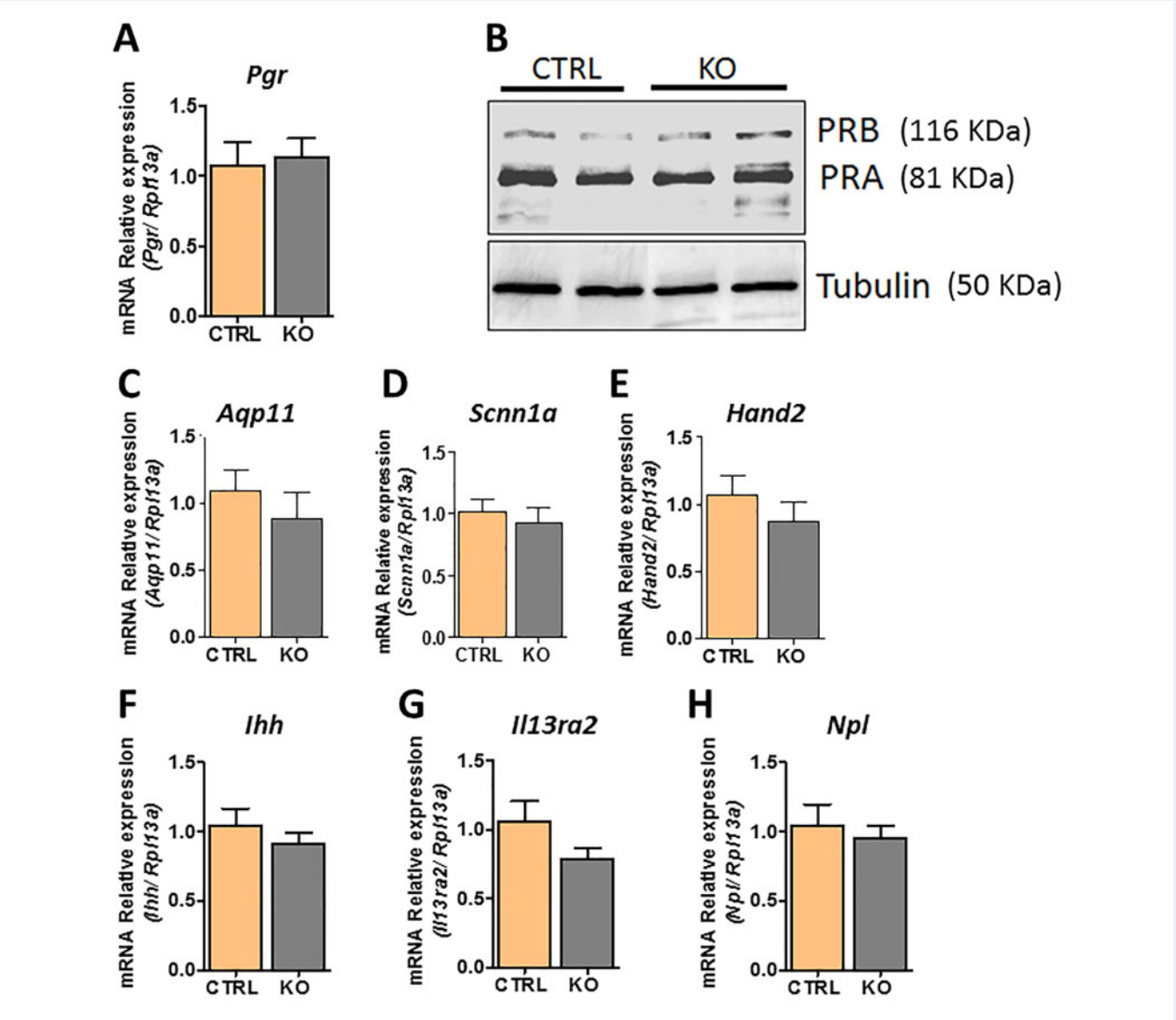

Figure 5. *Kiss1r*$^{Pgr\text{-}Cre}$-KO uterus exhibits normal PR level and transcriptional activity on D4 of pregnancy. qPCR analysis of (**A**) *Pgr* (gene encoding progesterone receptor: PR); Representative image of PR (the PR antibody detects both PRA and PRB isoforms) levels was determined by (**B**) western blot analysis with tubulin serving as the house keeping control (the uncropped blot is shown in Supplementary Information); qPCR analysis of known transcription targets and molecular markers of PR signaling (**C**) *Aqp11* (aquaporin 11) (**D**) *Scnn1a* (sodium channel non-neuronal 1 α subunit), (**E**) *Hand2* (Heart and neural crest derivatives expressed 2), (**F**) *Ihh*, (Indian hedgehog), (**G**) *Il13ra2* (interleukin 13 Receptor Subunit Alpha 2) and (**H**) *Npl* (N-acetylneuraminate pyruvate lyase). Statistics (n = 3–8 per group): Student's unpaired two-tailed *t*-test.

Discussion

The results from this study reveal a significant role for uterine KISS1R in establishing a successful pregnancy by tightly controlling ERα-regulated epithelial gene expression in the peri-implantation period. On D1 and D2 of mouse pregnancy, under the control of preovulatory E2, water is secreted into the lumen resulting in luminal opening (Zhang *et al.*, 2015; de Oliveira *et al.*, 2020). This permits the passage of sperm from the cervix to the oviduct where oocyte fertilization occurs. On D3 and D4 of pregnancy, ERα-regulated epithelial gene expression is downregulated and, under the control of P4, water is absorbed from the lumen resulting in luminal closure (Zhang *et al.*, 2015; de Oliveira *et al.*, 2020). Closure coincides with the entry of the blastocyst into the lumen on the morning of D4 and this facilitates contact between the blastocyst and luminal epithelium, initiating the first step in implantation.

Water secretion into the lumen is driven by Cl^- secretion through the cystic fibrosis transmembrane conductance regulator (CFTR) Cl^- channel along with other Cl^- channels in the apical membrane of luminal epithelial cells. This is coupled to the accumulation of Cl^- via

NKCC ($Na^+/K^+/Cl^-$ cotransporter) and an anion exchanger in the basolateral membrane of the epithelial cells. Collectively, active Cl^- secretion creates an electrochemical gradient that passively drives Na^+ into the lumen via the paracellular pathway. Water then follows, moving through the transcellular pathway via the aquaporins (such as AQP5 and AQP8) (de Oliveira *et al.*, 2019, 2020) and through the paracellular pathway. The paracellular or intercellular pathway represents an important pathway for the movement of water and solutes across the epithelium and is regulated by tight junctions, which comprise cell–cell adhesion proteins such as CLDN7 (Günzel and Yu, 2013).

Water absorption out of the lumen is driven by active Na^+ absorption from the lumen through the epithelial sodium channel (ENaC, comprising three subunits α, β, and γ or δ, β, and γ) in the apical membrane. This is coupled to the Na^+/K^+-ATPase in the basolateral membrane that moves Na^+ out of the cell. Collectively, active Na^+ absorption creates the electrochemical driving force for passive Cl^- transport out of the lumen via the paracellular pathway. Water then follows, moving through the transcellular pathway via the aquaporins (like AQP11) and through the paracellular pathway (Chan *et al.*, 2009).

Our data showed that loss of KISS1R signaling results in persistent ERα-dependent *Cftr*, *Aqp5*/AQP5, *Aqp8* and *Cldn7*/CLDN7 expression on D4 of pregnancy resulting in prolonged water secretion into the lumen and the maintenance of an open lumen when it should be closed. Interestingly, although PR-dependent genes, such as *Aqp11* and *Scnn1a*, were expressed on D4 of pregnancy and should drive water absorption from the lumen, ERα-dependent secretion was greater than absorption, thereby maintaining an open lumen. It is important to note that in the WT uterus, although epithelial ERα transcriptional activity is greatly diminished on D4 relative to D1, epithelial ERα is still expressed on D4 (Ye, 2020). Therefore, in the D4 WT epithelium, we conclude that KISS1R does not block or prevent expression of epithelial ERα, instead it negatively regulates ERα signaling in part by inhibiting its overexpression (i.e. it keeps its expression in check) and preventing detrimentally high ERα activity.

The P4-dependent downregulation of E2-induced epithelial gene expression is a critical step in the final acquisition of endometrial receptivity and embryo implantation, but the underlying mechanisms by which P4 exerts this function are still not well understood. Among the known molecular mediators of this P4-dependent inhibitory function is COUP-TFII (chicken ovalbumin upstream promoter transcription factor II; also known as NR2F2), a member of the nuclear receptor superfamily (Kurihara *et al.*, 2007). Studies have shown that the *Pgr-Cre*-dependent deletion of uterine COUP-TFII in mice was associated with persistent epithelial ERα expression and increased E2 transcriptional activity during the window of receptivity leading to the failure of KO mice to undergo the epithelial remodeling necessary for implantation (Kurihara *et al.*, 2007; Lee *et al.*, 2010; Hantak *et al.*, 2014). It was also shown that the *Pgr-Cre*-dependent deletion of uterine *Gp130* or *Stat3* led to increased estrogenic responses in mice and this disrupted the acquisition of uterine receptivity and blocked implantation (Sun *et al.*, 2013). However, unlike the *Kiss1r*$^{Pgr\text{-}Cre}$-KO and *Couptf2*$^{Pgr\text{-}Cre}$-KO females, persistent E2 signaling in the *Gp130* or *Stat3* conditional KO females was not caused by changes in E2 and P4 levels or expression of their nuclear receptors (Sun *et al.*, 2013). Despite this difference, collectively, these studies reinforce the understanding that the acquisition of endometrial receptivity and successful embryo implantation depend on the fine balance between uterine E2 and P4 signaling, and our study identifies uterine KISS1R as a major regulator of this critical process.

The finding that $G\alpha_{q/11}$-coupled KISS1R negatively regulates *Esr1* mRNA expression and ERα transcriptional activity is consistent with our previous observations that attenuated uterine $G\alpha_{q/11}$ signaling results in persistent ERα signaling on the day of endometrial receptivity, resulting in implantation failure (de Oliveira *et al.*, 2019). However, the mechanisms by which KISS1R exerts this powerful effect are unknown. It is possible that KISS1R activates a signaling pathway that epigenetically regulates *Esr1* expression and thereby ERα cellular levels and activity. For example, KISS1R could regulate the expression and/or the recruitment of histone acetyltransferases to the *Esr1* promoter and thereby epigenetically control *Esr1* expression. Indeed, it was shown that for another GPCR, the $G\alpha_{i/o}$-coupled δ opioid receptor, signaling facilitates the recruitment of histone acetyltransferase p300 to promoter sequences and thereby regulates chromatin modification and gene expression (Kang *et al.*, 2005), opening the possibility that other GPCRs can do the same. This exciting mechanistic possibility requires future investigation.

In women, whether fluid volume regulates luminal opening and closing is not known, but it is reported that at the time of implantation the lumen is closed in women (Richard *et al.*, 2003). In healthy women, luminal secretion and absorption (composition and quantity) change over the menstrual cycle, where larger volumes are observed at the mid-cycle (83–180 µl) than in the mid-luteal phase (5–35 µl) (Casslén, 1986). These natural variations, reflecting changes in E2:P4 ratios (high at mid-cycle and relatively low at the mid-luteal phase), can be disrupted under disease conditions (e.g. endometriosis) or clinical interventions (ovarian stimulation) that are associated with perturbed natural E2:P4 levels.

The abnormal accumulation of fluid in the uterine lumen in women reduces implantation rates (Chien *et al.*, 2002). Evidence suggest that the increase in luminal fluid levels reduces the acquisition of endometrial receptivity and a mechanical disruption in embryo-luminal epithelium contact (Strandell, 2000). Fluid homeostasis is also very important at the glandular epithelium since fluid availability will influence secretion rates and solute concentrations in glandular secretions. The human endometrium expresses the major molecules that regulate water movement (such as AQPs, CFTR and CLDNs) and altered endometrial expression levels of these molecules is associated with sub-fertility in women (Yang *et al.*, 2011; Mikołajczyk *et al.*, 2013; Cui *et al.*, 2018). For example, AQP2 downregulation during controlled ovarian stimulation is associated with impaired endometrial receptivity (Zhang *et al.*, 2016). Furthermore, genes encoding AQP3, CLDNs and CFTR-related family members are recognized regulators of embryo implantation and are included in the endometrial receptivity array used for diagnosing human endometrial receptivity in IVF clinics (Díaz-Gimeno *et al.*, 2011).

Normal embryo implantation is critical for healthy placentation and a successful pregnancy. Not surprisingly, abnormal implantation appears linked to the manifestation of many pregnancy-related complications that arise late in gestation (such as pre-eclampsia and preterm labor). For example, a superficially implanted embryo might result in the shallow invasion of the decidua and spiral arteries by the fetal trophoblasts leading to hypertension and pre-eclampsia (Radovick and Babwah, 2019). Our data show that loss of uterine KISS1R expression disrupts the acquisition of endometrial receptivity and reduces

implantation. Furthermore, among the embryos that do implant, many offspring die in the neonatal period suggesting that their health was compromised during development. These observations are consistent with the idea that implantation was compromised.

The whole body *Kiss1* and *Kiss1r* KO mice exhibit hypogonadotropic hypogonadism and females are anovulatory and thus, infertile (Lapatto *et al.*, 2007). We demonstrated that estradiol/gonadotrophin therapy rescued follicular development and ovulation in the systemic KOs, but the uterus remained unreceptive to implantation events owing to diminished gland function (Calder *et al.*, 2014; León *et al.*, 2016). In the current study, using the conditional uterine *Kiss1r* KO mouse, evidence of diminished gland function was not apparent; instead, we showed that implantation failure is the result of aberrantly elevated uterine *Esr1* expression, ERα levels, persistent epithelial ERα activity and a failure of the lumen to undergo closure on the day of embryo implantation. This contrasts with the systemic Kos, which showed similar uterine *Esr1* expression, and ERα levels compared to their littermate CTRLs (Calder *et al.*, 2014). In the whole-body KOs, ERα transcriptional activity and luminal opening were not assessed. While the reasons for the contrasting mechanisms that underlie implantation failure in the systemic and conditional KOs are not clear, it is in part likely related to the reproductive and metabolic deficits that characterize the systemic KOs. These findings further justify the need for the current study that investigated uterine KISS1R functions in the absence of these deficits.

In conclusion, using the first conditional KO of uterine *Kiss1r*, we now confirm that uterine KISS1R is absolutely required for the acquisition of endometrial receptivity and embryo implantation. This finding warrants a close examination of KISS1 and KISS1R expression in the human endometrium in the peri-implantation period under normal and diseased conditions that disrupt endometrial receptivity and implantation.

Supplementary data

Supplementary data are available at *Molecular Human Reproduction* online.

Data availability

All data generated or analyzed during this study are included in this published article.

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Authors' roles

A.V.B. developed the research study and all authors contributed to the analysis of the data and preparation of the manuscript.

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Conflict of interest

The authors declare that there are no competing financial, personal or professional competing interests.

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