Liver receptor homolog-1 is essential for pregnancy

Cong Zhang1,2, Michael J Large3, Raj Duggavathi4, Francesco J DeMayo3, John P Lydon3, Kristina Schoonjans5, Ertug Kovanci6 & Bruce D Murphy1

Successful pregnancy requires coordination of an array of signals and factors from multiple tissues. One such element, liver receptor homolog-1 (Lrh-1), is an orphan nuclear receptor that regulates metabolism and hormone synthesis. It is strongly expressed in granulosa cells of ovarian follicles and in the corpus luteum of rodents and humans. Germline ablation of Nr5a2 (also called Lrh-1), the gene coding for Lrh-1, in mice is embryonically lethal at gastrulation.

To explore the role of Lrh-1 in gestation, we bred mice carrying a fusion gene encoding the progesterone receptor fused to Cre recombinase (Pgr-Cre mice) to mice with exons 3 and 4 of the Nr5a2 gene flanked byloxP sites (Nr5a2fl/fl). The resultant conditional knockout (cKO) mice (Nr5a2fl/fl; PgrCre+/−) were uniformly infertile. Adult cKO females had a higher frequency and duration of estrus cycles compared to control (CON) littermates, suggesting successful ovulation. Hormone replacement permitted embryo implantation but was followed by gestational failure with impaired endometrial decidualization, compromised placental formation, fetal growth retardation and fetal death. Lrh-1 is also expressed in the mouse and human endometrium, and in a primary culture of human endometrial stromal cells, reduction of NR5A2 transcript abundance by RNA interference abrogated decidualization. These findings show that Lrh-1 is necessary for maintenance of the corpus luteum, for promotion of decidualization and for formation of the placenta. It therefore has multiple, indispensible roles in establishing and sustaining pregnancy.

To explore the role of Lrh-1 in gestation, we bred mice carrying a fusion gene encoding the progesterone receptor fused to Cre recombinase (Pgr-Cre mice) to mice with exons 3 and 4 of the Nr5a2 gene flanked byloxP sites (Nr5a2fl/fl). The resultant conditional knockout (cKO) mice (Nr5a2fl/fl; PgrCre+/−) were uniformly infertile. Adult cKO females had a higher frequency and duration of estrus cycles compared to control (CON) littermates, suggesting successful ovulation (Fig. 1a). Gonadotropin stimulation of immature CON and cKO mice indicated no impairment of ovulation in the cKO strain (Fig. 1b).

To establish whether abnormal fertilization or embryogenesis contributed to sterility of cKO mice, we collected eggs and embryos from oviducts and uteri of adult CON and cKO mice at intervals after mating (Fig. 2a). We found no differences in the yield of unfertilized eggs, zygotes or two-cell embryos between CON and cKO mice.
On the fourth day post coitum (d.p.c. 4), nearly all recovered CON embryos were blastocysts, whereas the majority in the cKO strain were morulae (Supplementary Table 1). We restored timely progression of cKO embryos to blastocysts on d.p.c. 4 by implanting progesterone-releasing pellets on d.p.c. 1 (cKO+P4, Supplementary Table 1). Next, we assessed the viability of embryos from cKO mice: we found that transplantation of zygotes on d.p.c. 1 from cKO and CON mice into oviducts of CON mice resulted in no difference in frequency of parturition or in numbers of offspring (Fig. 2b).

Although implantation failed in cKO mice, it was rescued by progesterone administration (Fig. 2c,d). Nevertheless, implanted embryos in progesterone-treated cKO (cKO+P4) mice were improperly spaced at d.p.c. 5 (Fig. 2d, right), and by d.p.c. 8 there was embryo crowding (Fig. 2e, left) and variation in size of the uterine enlargements (Fig. 2d,e). By d.p.c. 12, we saw crowding and further loss of uniformity in uterine swellings (Fig. 2e, middle, as compared with 2f, middle). Fetal size (Fig. 2g) and mean weight of implantation sites, fetuses and placentas were lower in cKO+P4 than in CON mice by d.p.c. 12 (Fig. 2h). By d.p.c. 17, fetoplacental units in individual cKO+P4 mice were of markedly different sizes (Fig. 2f, right), and the number of sites with fetuses was lower compared to CON mice (Fig. 2h). Successful parturition occurred only rarely (in 1 out of 22 cKO+P4 females).

Ablation implantation in cKO+P4 mice raised the question of whether Lrh-1 is expressed in the uterus. We found Nr5a2 transcripts (Fig. 3a) and an Lrh-1 protein signal (Fig. 3b) in the epithelial and stromal compartments of the endometrium from CON mice at d.p.c. 3 to d.p.c. 5. The signal persisted in the endometrial component of the placenta through gestation in CON mice (Supplementary Fig. 3a,b). In cKO mice, uterine Nr5a2 mRNA declined progressively in abundance from d.p.c. 3, becoming nearly undetectable by d.p.c. 5 (Fig. 3a). Lrh-1 protein was less abundant in endometrial stroma of cKO mice relative to CON mice at d.p.c. 4 and disappeared more slowly from the endometrial epithelium (Fig. 3c). We did not detect Lrh-1 in the endometrial stroma at any sampling times during the aberrant pregnancies of progesterone-treated cKO mice (Supplementary Fig. 3c).

Comparison of uterus from cKO+P4 mice with their CON counterparts on d.p.c. 5 and implantation sites (sacs fetus) at d.p.c. 12 revealed that expression of genes essential for successful embryo implantation and placentation was compromised and not restored by progesterone replacement; these genes included Hoxa10, Wnt4, Wnt5a, Ihh and Bmp2 (Fig. 3d,e) and Pparδ, Hbegf and Cyp19a1 (Supplementary Fig. 4a–c). At d.p.c. 5, transcript abundance of a number of other genes tested was lower in the uteri of cKO+P4 than in CON mice, but was restored or modestly increased by progesterone; these genes included Plts2, Pla2g4a, Vegfa, Pparg, Arg2, Mkia67 and Er2 (Supplementary Fig. 3d–j). Transcript abundance for other implantation genes, Lif, Vegfb and Er1, was not different in the uteri from cKO compared to CON mice. Multiple disruptions were not unexpected, as Lrh-1 targets numerous ovarian genes. We conclude that although implantation failure in cKO mice is caused by luteal insufficiency, irregularities in expression of key endometrial genes persist in spite of progesterone replacement, resulting in aberrant gestation.

To confirm that pregnancy failure in these mice is of uterine provenance, we reciprocally transferred single ovaries from CON or cKO mice to ovarian bursae of ovarioctomized CON or cKO mice, which were then mated to fertile males. Mice receiving ovaries from cKO mice, supplemented with progesterone to compensate for luteal deficiency, had serum progesterone concentrations similar to those of mice receiving a single ovary from CON mice (43.5 ± 8.7 ng ml⁻¹ and 33.5 ± 18.4 ng ml⁻¹, respectively). Unilateral implantation and pregnancy ensued in uteri of CON mice receiving either ovaries from CON or cKO+P4 mice (Fig. 3f), and viable offspring were born to all but a few mice (Fig. 3g). When ovaries from CON mice were transplanted to cKO mice, implantation occurred, but gestation failed.
with a pathological signature similar to that seen in cKO+P4 mice mated to CON males, including crowding, differential fetoplacental size and fetal death (Fig. 3h). A single litter was born to a CON ovary–cKO uterus mouse, but no neonates survived to 12 h of life (Fig. 3g). Genotyping of dead fetuses and live-born pups confirmed that the offspring were derived from the transplanted ovary. These results clearly implicate uterine dysfunction as the genesis of gestation failure in cKO mice.

In the endothelial-chorial placenta, endometrial stromal cells differentiate into large, secretory and often polyploid cells in a process known as decidualization. Ovarian progesterone is a prerequisite for this process. Both the embryo and artificial stimuli can induce a decidual reaction in mice on d.p.c. 5, whereby a new gene expression pattern overwrites the stromal palimpsest, first by proliferation, then by terminal differentiation of these cells.

To further explore the role of Lrh-1 in uterine function, we induced artificial decidualization by intraluminal injection of oil into uteri of pseudopregnant CON, cKO and cKO+P4 mice. The pronounced decidual response in pseudopregnant CON mice was lacking in the cKO uterus and was much reduced in the cKO+P4 mice, in spite of the progesterone replacement (Fig. 4a,b). Likewise, the robust stromal cell proliferation found in CON mice on d.p.c. 4–5 was absent in cKO mice and only modestly compensated in cKO+P4 mice (Supplementary Fig. 5a). Key genes required for decidualization, including Hoxa10, necessary for proliferation, and Wnt4 (refs. 9,11,14), were lower in the cKO+P4 uterus relative to the CON uterus at both d.p.c. 5 and 12 (Fig. 3d,e), whereas Ptgs2 (refs. 9,10) appeared to be expressed appropriately at the implantation site (Supplementary Fig. 5b–d).

Small embryos (Fig. 2g), smaller implantation sites and lower placental weight in cKO+P4 compared to CON mice (Fig. 2h) indicated that placental formation was compromised in the cKO+P4 strain. Placental depth in cKO mice was half of that in CON mice at d.p.c. 12, owing to reductions in the maternal stroma, vascularization and the placental spongiotrophoblast layer (Fig. 4c–e). In contrast to lenticular placentas from CON mice, placentas from cKO+P4 mice were markedly dome shaped (Fig. 4e). Circulating progesterone did not differ between CON and cKO+P4 mice through day 12 of gestation but was marginally lower in cKO+P4 mice at day 17 (Supplementary Fig. 5e), presumably owing to decreases in placental steroid synthetic components (Supplementary Fig. 5f,g). Supplementation of cKO+P4 mice with a second progesterone pellet from d.p.c. 10 to d.p.c. 17 elevated circulating progesterone concentrations at d.p.c. 17 to 103.4 ± 7.1 ng ml⁻¹, well above those found in cKO mice bearing single implants and in untreated CON mice (Supplementary Fig. 5e), but did not result in liveborn mice.
We then explored whether LRH-1 was present in the human endometrium during the menstrual cycle. We detected the NR5A2 transcript and the LRH-1 nuclear signal in both the proliferative and secretory phases of the menstrual cycle (Fig. 4f,g) and found the transcripts for markers of decidualization, PRL and IGFBP1 (ref. 15), in the expected greater abundance during the secretory phase (Fig. 4g).

Proper decidualization is crucial to successful gestation in humans16, and human uterine stromal fibroblasts undergo decidual reprogramming that shares multiple gene expression patterns with mouse decidualization12,17. We depleted NR5A2 mRNA by RNA interference in primary culture of human endometrial stromal cells18 (Fig. 4h), thereby decreasing the abundance of decidual markers19,20, including PRL and IGFBP1 (Fig. 4c) and KLF9, HAND2 and FOXO1 (Supplementary Fig. 6a–c). Notably, RNA interference-induced reduction of NR5A2 mRNA abrogated the expression of WNT4 (Fig. 4h), which encodes an indispensable mediator of human decidualization23 and is disrupted in both early and late gestation (Fig. 3d,e) in cKO+P4 mice. Chromatin immunoprecipitation (ChIP) results demonstrated that WNT4 is a direct target of LRH-1 (Supplementary Fig. 6d), thereby confirming the existence of a mechanistic link by which LRH-1 drives decidualization in human stromal cells. The apparent depletion of NR5A2 mRNA as decidualization progresses in vivo (Fig. 4g) and the observed decline in vitro (Fig. 4h) are consistent with studies showing declining steroid receptor mRNA abundance that accompanies decidualization23, in spite of increased tissue sensitivity to the nuclear receptors. Waning expression is attributed to their sumoylation23, a post-translational modification that regulates the activity of LRH-1 (ref. 23).

We then examined the dynamics of LRH-1 regulation of decidualization in a human endometrial stromal cell line24 in vitro. Immunoocytochemistry revealed nuclear expression of LRH-1 (ref. 25) (Supplementary Fig. 7a), and NR5A2 transcript abundance was upregulated during in vitro decidualization (by a mean of 346%), as were the abundances of PRL (427%) and IGFBP1 (465%) transcripts (Supplementary Fig. 7b), further demonstrating the role of LRH-1 in regulation of human stromal cell decidualization. In contrast to what was seen in the primary decidual cells, NR5A2 mRNA abundance in the stromal cell line did not decline as decidualization progressed, presumably because the cells were immortalized24 and thus not terminally differentiated.

Overall, these findings show that maternal expression of Lrh-1 is required for two aspects of gestation: luteal function and placentation formation. In the cKO mouse, correction of luteal deficiency by progesterone replacement rescues implantation and permits continuation of pregnancy, but placental development is compromised, resulting in fetal death, illustrating the crucial role of Lrh-1 in the uterus. The effects are first manifest early during the peri-implantation period. Postimplantation gestational failure in progesterone-supplemented cKO mice has multiple causes. Lrh-1 deficiency in gestation results in a "ripple effect", whereby improperly achieved pre- and peri-implantation...
Figure 4 LRH-1 is crucial for decidualization of both the mouse and human endometrial stroma. (a) Oil-induced deciduoma formation at d.p.c. 8 in pseudopregnant CON mice, cKO and cKO+P4 mice. Scale bars, 10 mm. (b) Ratio of oil-treated–to–control uterine horn weights (means ± s.e.m.) at d.p.c. 8 after decidual stimulus at d.p.c. 4. (c,d) Histological comparison of placental sections at d.p.c. 12 from CON (e) and cKO+P4 (d) mice. (La, placental labyrinth; Sp, spongy zone; St, maternal stroma; M, myometrium.) Scale bars, 100 µm. (e) Macroscopic comparison of placentas dissected from CON and cKO+P4 mice at d.p.c. 17. Scale bar, 10 mm. (f) Nuclear localization of LRH-1 in the proliferative and secretory human endometrium. Scale bar, 10 µm. (g) Abundance of transcripts for NR5A2 and the decidual markers PRL and IGFBP1 in human endometrium during the proliferative and secretory phases of the menstrual cycle. MCF-7 breast cancer cells were used as a positive control for NR5A2 transcript expression. Data are expressed as means ± s.e.m.; asterisks indicate significant differences at *P < 0.05. (h) Quantitative PCR analysis to determine the degree of siRNA-mediated knockdown (initiated on day 0) of NR5A2 transcripts in primary cultures of human endometrial stromal cells undergoing decidualization in vitro and to determine the abundance of decidual marker genes NR5A2, PRL, WNT4 and IGFBP1 in human stromal cells in vitro. Data are expressed as means ± s.e.m.; asterisks indicate significant differences (*P < 0.05) between control and siRNA-mediated knockdown at each sampling time.

events such as spacing and decidualization are propagated as placental anomalies through pregnancy14. Further, LRH-1 is essential for human decidualization, which, in itself, is necessary for successful pregnancy. Depletion of NR5A2 mRNA abrogates expression of genes known to be indispensable for differentiation of uterine stromal cells26,27. Among these is WNT4 (ref. 21), which we have shown by ChIP to be target of LRH-1, demonstrating a mechanism by which this protein regulates placental formation.

METHODS
Methods and any associated references are available in the online version of the paper.

ACKNOWLEDGMENTS
This study was funded by OPG 11018 from the Canadian Institutes of Health Research to B.D.M. C.Z. was supported by the National Natural Science Foundation of China (31172040) and Shandong Natural Science Foundation (ZR2011CM047). K.S. was supported by grants from the École Polytechnique Fédérale de Lausanne, the Swiss National Science Foundation (SNF) and the Swiss Cancer League, J.P.L. by US National Institutes of Health (NIH) R01CA77530 and F.J.D. by NIH U54 HD07495-31. M.J.L. is supported by NIH ST32HD007165. We are grateful to V. Roussel for preparation of the figures, M. Dobias for hormone analyses, S. Ruiz Orduna for human endometrial stromal cell line experiments, J. Fenelon and K. Bertolin for aid with transcript studies, L. Lian for aid with oilviduct transfer, X. Tang for statistical analyses, L. Giudice for coordinating acquisition of human endometrium from the NIH–University of California at San Francisco (UCSF) Endometrium Tissue Bank and J. Auwerx for Nr5a2 floxed (Nr5a2fl/fl) mice.

AUTHOR CONTRIBUTIONS
C.Z., R.D., F.J.D. and B.D.M. planned mouse experiments, which were carried out by C.Z. and B.D.M. C.Z., R.D., F.J.D. and B.D.M. wrote the manuscript. F.J.D., E.K. and B.D.M. planned mouse experiments, which were carried out by C.Z. and B.D.M. C.Z., R.D., F.J.D. and B.D.M. wrote the manuscript. F.J.D., E.K. and B.D.M. provided mouse models and edited the manuscript.

COMPETING FINANCIAL INTERESTS
The authors declare no competing financial interests.

Reprints and permissions information is available online at http://www.nature.com/reprints/index.html.

1. Lee, Y.K. & Moore, D.D. Liver receptor homolog-1, an emerging metabolic modulator. Front. Biosci. 13, 5950–5958 (2008).
2. Hinshelwood, M.M. et al. Expression of LRH-1 and SF-1 in the mouse ovary: localization in different cells correlates with differing function. Mol. Cell Endocrinol. 207, 39–45 (2003).
3. Labelle-Dumais, C., Jacob-Wagner, M., Pare, J.F., Belanger, L. & Dufort, D. Nuclear receptor NR5A2 is required for proper primitive streak morphogenesis. Dev. Dyn. 235, 3359–3369 (2006).
4. Duggavathi, R. et al. Liver receptor homolog 1 is essential for ovulation. Genes Dev. 22, 1871–1876 (2008).
5. Soyal, S.M. et al. Cre-mediated recombination in cell lineages that express the progesterone receptor. Genesis 41, 58–66 (2005).
6. Coste, A. et al. LRH-1-mediated glucocorticoid synthesis in enterocytes protects against inflammatory bowel disease. Proc. Natl. Acad. Sci. USA 104, 13096–13103 (2007).
7. Miranda-Jiménez, L.M., Binelli, M., Bertolin, K., Pelletier, R.M. & Murphy, B.D. Scavenger receptor-B1 and luteal function in mice. J. Lipid Res. 51, 2362–2371 (2010).
8. Choi, J.H., Ishida, M., Matsuwaki, T., Yamanouchi, K. & Nishihara, M. Involvement of 20α-hydroxysteroid dehydrogenase in the maintenance of pregnancy in mice. J. Reprod. Dev. 54, 408–412 (2008).
9. Das, S.K. Regional development of uterine decidualization: molecular signaling by Hoxa-10. Mol. Reprod. Dev. 77, 387–396 (2010).
10. Wang, H., Xie, H. & Dey, S.K. Endocannabinoid signaling directs periimplantation events. AAPS J. 8, E425–E432 (2006).
11. Das, S.K. Cell cycle regulatory control for uterine stromal cell decidualization in implantation. Reproduction 137, 889–899 (2009).
12. Ramathal, C.Y., Bagchi, I.C., Taylor, R.N. & Bagchi, M.K. Endometrial decidualization: of mice and men. Semin. Reprod. Med. 28, 17–26 (2010).
13. Lim, H. et al. Multiple female reproductive failures in cyclooxygenase 2-deficient mice. Cell 91, 197–208 (1997).
14. Cha, J., Sun, X. & Dey, S.K. Mechanisms of implantation: strategies for successful pregnancy. Nat. Med. 18, 1754–1767 (2012).
15. Dunn, C.L., Kelly, R.W. & Critchley, H.O. Decidualization of the human endometrial stromal cell: an enigmatic transformation. Reprod. Biomed. Online 7, 151–161 (2003).
16. Cartwright, J.E., Fraser, R., Leslie, K., Wallace, A.E. & James, J.L. Remodelling at the maternal-fetal interface: relevance to human pregnancy disorders. Reproduction 140, 803–813 (2010).
17. Lynch, V.J., Brayer, K., Gellersen, B. & Wagner, G.P. HoxA-11 and FOXO1A cooperate to regulate decidual prolactin expression: towards inferring the core transcriptional regulators of decidual genes. PLoS ONE 4, e6845 (2009).
18. Tang, M., Naidu, D., Hearing, P., Handewiger, S. & Tabibzadeh, S. LEFTY, a member of the transforming growth factor-β superfamily, inhibits uterine stromal cell differentiation: a novel autocrine role. Endocrinology 151, 1320–1330 (2010).
19. Buzzio, O.L., Lu, Z., Miller, C.D., Unterman, T.G. & Kim, J.J. FOXO1A differentially regulates genes of decidualization. Endocrinology 147, 3870–3876 (2006).
20. Huyen, D.V. & Bany, B.M. Evidence for a conserved function of heart and neural crest derivatives expressed transcript 2 in mouse and human decidualization. Reproduction 142, 353–368 (2011).
21. Li, Q. et al. WNT4 acts downstream of BMP2 and functions via β-catenin signaling pathway to regulate human endometrial stromal cell differentiation. Endocrinology 154, 446–457 (2013).
22. Cloke, B. et al. The androgen and progesterone receptors regulate distinct gene networks and cellular functions in decidualizing endometrium. Endocrinology 149, 4452–4474 (2008).
23. Chalkiadaki, A. & Talianidis, I. SUMO-dependent compartmentalization in promyelocytic leukemia protein nuclear bodies prevents the access of LRH-1 to chromatin. Mol. Cell Biol. 25, 5095–5105 (2005).
24. Krikun, G. et al. A novel immortalized human endometrial stromal cell line with normal progestational response. Endocrinology 145, 2291–2296 (2004).
25. Yang, F.M. et al. Liver receptor homolog-1 localization in the nuclear body is regulated by sumoylation and cAMP signaling in rat granulosa cells. FEBS J. 276, 425–436 (2009).
26. Franco, H.L. et al. WNT4 is a key regulator of normal postnatal uterine development and progesterone signaling during embryo implantation and decidualization in the mouse. PNAS 108, 1176–1187 (2011).
27. Li, Q. et al. Bone morphogenetic protein 2 functions via a conserved signaling pathway involving Wnt4 to regulate uterine decidualization in the mouse and the human. J. Biol. Chem. 282, 31725–31732 (2007).
ONLINE METHODS

Mice and treatments. Experiments were approved by the Comité d'éthique de l'utilisation des animaux, Université de Montréal. C57BL/6 background mutant mice were maintained on 14 h:10 h light:dark cycle and provided food and water ad libitum. The mice carrying loxp-flanked (floxed) Nr5a2 (Nr5a2m200 mice), generated by J. Auwerx, served as controls (CON). They were crossed with Pgr-Cre (PgrCre+/−) mice to produce Nr5a2 conditional knockout (cKO) mice. Immature females (aged 21–25 d) were superovulated with 5 IU equine chorionic gonadotropin (Intervet Canada) followed by 5 IU human chorionic gonadotropin (hCG, Intervet) 48 h later. Ovulated eggs from oviducts were enumerated 16 h after hCG injection. Ovaries were snap frozen and stored at −80 °C or embedded in paraffin for histology. Vaginal smears were taken daily (09:00) to determine estrous stages. For breeding assays, eight cKO and eight CON females aged 6 weeks were housed with reproducively proven C57BL/6 males for 6 months (two females per male) and observed daily, and parturition dates and litter sizes recorded. For analysis of gestation, adult (aged >60 d) female CON and cKO mice were bred. The day that copulatory plugs were found was designated day 1 post coitum (d.p.c. 1). Mice were killed at stages of gestation as dictated by experimental objectives. To establish frequency of mating, CON and cKO mice were housed with vasectomized males, and plugs, where present, were determined daily.

Vasectomies were performed by removing 1 cm of vasa deferentia from males anesthetized by injection of ketamine (100 mg per kg body weight, Bioniche Animal Health) and xylazine (10 mg per kg body weight, Bayer)). Sterility was confirmed by housing vasectomized mice with fertile females and observing absence of sperm in copulatory plugs.

In cKO females mated with fertile males, a 2-cm silastic implant (inner diameter 3.35 mm, outer diameter 4.65 mm, Dow Corning) containing progesterone (Sigma Chemical) was implanted subcutaneously on d.p.c. 1 at 17:00. Implants were removed d.p.c. 17 (17:00) to allow parturition. To test whether pregnant females required further progesterone, another implant was placed in some mice on d.p.c. 10 (09:00) and both were removed at d.p.c. 17 (17:00).

Deciduomas were induced in CON and cKO females mated to vasectomized males. On d.p.c. 1, cKO females received progesterone implants. On the afternoon of d.p.c. 4 (15:00), 50 µl sesame oil was infused intraluminally in one uterine horn. On d.p.c. 8 (17:00), mice were killed and uterine weights of infused and noninfused horns were recorded.

Incipient implantation sites on d.p.c. 5 were identified by an intravenous injection of 1% (wt/vol) Evans Blue dye (100 µl, 1% in 1× PBS, Sigma). Injected mice were killed 3 min later, and uteri removed for examination.

To investigate preimplantation development, mouse oviducts and uteri were flushed with PBS on d.p.c. 1–4, and embryos recovered. After implantation, uteri of pregnant females were dissected at 15:00 on d.p.c. 8, 12 and 17, and isolated implantation sites were weighed individually, then dissected to isolate embryos and placenta. Crown-rump lengths were measured by Vernier calipers.

To assess quality of embryos, pseudopregnant recipients were generated by mating vasectomized CON males. Embryos on d.p.c. 1 (09:00) were obtained from bred cKO or CON donors by flushing oviducts. Pseudopregnant recipients were anesthetized, and yzogotes (15 per female) were transferred to oviducts on d.p.c. 1. Recipients were observed once daily through time of expected parturition.

Ovary transplants were performed as described23. Briefly, females were anesthetized, the ovarian fat pad with the ovary exteriorized, the bursa opened, and ovaries removed. The excised ovaries were held briefly in M2 medium (Sigma) and then grafted back into the bursal cavity of recipients. After a 3-week recovery, the mice were mated with CON male mice. Samples from each gestation were genotyped to confirm that products of conception were derived from the transplanted ovary.

Human endometrial stromal cells: decidualization, transfection with siRNA and hormone treatment. Human uterine tissues were acquired from the National Institutes of Health University of California at San Francisco Endometrial Tissue and DNA Bank, approved by the UCSC Institutional Review Board, and collected and archived after informed consent was obtained25. For primary culture of stromal tissue, histologically normal endometrial samples from the proliferative phase were collected by biopsy from individuals of reproductive age (aged 18–45 years) with normal menstrual cycles. The experimental protocol was approved by the Institutional Review Board of Baylor College of Medicine (Houston, Texas). All subjects gave written informed consent. Samples were collected at room temperature and transported on ice in HBSS containing 1% antibiotic-antimycotic (Invitrogen) and processed for the endometrial stromal cell isolation26. Tissue samples were washed with DMEM-F12 (Invitrogen) containing 1% antibiotic-antimycotic and minced to <1 mm³. Tissues were incubated for 1.5 h at 37 °C in DMEM-F12 containing 0.25% (wt/vol) collagenase and 0.05% DNase I (Sigma). After enzymatic digestion, stromal cells were separated from epithelial aggregates using a 40-µm nylon cell strainer (BD Biosciences). Fibrates were washed twice and plated in DMEM-F12 medium containing 10% FBS and 1% antibiotic-antimycotic. Cells were seeded in 6-well plates at a concentration of 1.5 × 10⁵ cells per well and cultured to confluence of approximately 50%, then transfected with four sequences of Nr5a2 siRNA (ON-TARGET plus SMART Pool, L-003430-00) or with a pool of nontargeting sequences (ON-TARGET plus Non-Targeting Pool, D-00180-10–20, both from Dharmacon) using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instructions. The day of cell plating and siRNA treatment was designated day 0. All decidualization experiments were conducted in opti-MEM medium (Invitrogen) containing 2% charcoal-stripped FBS and 1% antibiotic-antimycotic. On day 2, decidualization was induced by treatment with 1 µM medroxyprogesterone acetate, 10 nM E2 and 50 µM dibutyryl cAMP (all from Sigma-Aldrich) every 48 h25.

The human endometrial stromal cell line immortalized by retroviral transfection of human telomerase (ATCC CRL-4003) was cultured as described by Krikun et al. In vitro decidualization was induced when cells achieved 80–85% confluence by incubation with 1 µM medroxyprogesterone acetate and 0.3 mM dibutyryl cAMP for 7–9 d.

Chromatin immunoprecipitation. Human endometrial stromal cells were seeded in 15 cm² dishes, grown to approximately 75% confluence and decidualized induced. On day 3, 1% formaldehyde was added to cross-link chromatin and DNA. The ChIP was performed using the ChIP-IT Express Chromatin Immunoprecipitation Kit (Active Motif) according to manufacturer’s instructions. Shearing of chromatin was achieved by sonication and LRH-1 was immunoprecipitated with 3 µg antibody (SC-25389, Santa Cruz Biotechnology). Enrichment was evaluated by qPCR for ChIP using Sybr-Green technology (Applied Biosystems) using sequence specific primers (WNT4: F, TCCCGACGGTTCAAGTTAAAGA, R, GGTCTTTTCTTCACCCATACCTA; Nef: F, AGCATGACGCTATAGGACCTA, R, TGTTGATGGGAGGCCAGAG). Results were normalized to input.

Hormone analysis. Mice were anesthetized using isoflurane (Baxter) and blood collected by cardiac puncture. Progesterone and estradiol-17β concentrations were analyzed in a single radioimmunoassay27 for each. Intra-assay coefficients of variation were 7.76% for progesterone and 4.66% for estradiol-17β.

Label microdissection. Frozen ovaries were sectioned at 25 µm onto polyethylene naphtalene (PEN) membrane–covered glass slides (PALM Microalaser Technologies), stained with toluidine blue, dehydrated in 70% and 100% ethanol and warmed for 30 min at 37 °C. Corpora lutea were dissected by laser microdissection (LMD) (Leica AS LMD) and collected into extraction buffer from PicoPure RNA isolation kit (Arcturus).

Immunofluorescence and immunohistochemistry. Ovaries were cryosectioned at 8 µm, fixed in acetone at −20 °C for 10 min and blocked in IgG from the same animal species of the primary antibody for 1 h at room temperature. Cryosections were incubated at 4 °C overnight with primary antibodies to Lrh-1 (Santa Cruz SC-21132) and Cyp11a1 (Abcam, ab78416-100) at dilution 1:400. Slides were incubated with 4 ′,6-diamidino-2-phenylindole (DAPI, Roche Applied Science) for 5 min at room temperature. Digital images were captured using an Olympus confocal microscope (Olympus FV1000) or a Leica fluorescence microscope. For immunohistochemistry of Lrh-1, paraffin sections of mouse uteri were incubated with the Santa Cruz antibody SC-21132.
at 1:100, and paraffin sections of human uterine tissue incubated with R&D Systems PP-H325-00 mouse monoclonal antibodies at 1:50. Biotinylated secondary antibodies were used (Santa Cruz SC-2011, 1:200 dilution for mouse tissue, Abcam18293 for human tissue) and sections were incubated with diaminobenzidine (DAB)-peroxidase substrate (Santa Cruz) counterstained with hematoxylin, dehydrated, mounted and digitally photographed.

5-Ethynyl-2′-deoxyuridine incorporation assay. Females (four of each genotype) mated to fertile CON males were injected with 5-ethynyl-2′-deoxyuridine (EdU) (20 mg per kg body weight) on the afternoon of d.p.c. 4 (17:00). Uteri were harvested 2 h after injection. Incorporated EdU was detected using a Click-it Imaging Kit (Invitrogen) according to manufacturer’s instructions. EdU-positive cells were counted at ×40 in four fields per section, and four individual sections were quantified per specimen.

RNA isolation and qPCR. Total RNA was isolated from uteri or placentas using Trizol reagent (Invitrogen) from three or more mice and reverse transcribed with the SuperScript First-Strand Synthesis System (Invitrogen). For corpora lutea obtained by LMD, 1 µg RNA was used, and for uteri or placentas, 3 µg. RT-PCR was performed in an ABI Prism 7300 instrument with SYBR green PCR master mix (Applied Biosystems) with general PCR conditions (3 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C) to amplify the product. Melting-curve analyses verified product identity. Samples were run in triplicate and were expressed relative to β-actin in the same sample. Data were normalized to a calibrator sample using ΔΔCt method with correction for amplification efficiency by LinRegPCR 11.0 software (Academic Medical Center, Amsterdam).

Statistical analyses. Data are expressed as means ± s.e.m., and all experiments were repeated at least three times. Data were analyzed using the Student’s t-test and one-way analysis of variance, followed by Tukey’s post hoc multiple-range test. Values were considered significant if P < 0.05.

28. Shaw, J.M. & Trounson, A.O. Ovarian tissue transplantation and cryopreservation. Application to maintenance and recovery of transgenic and inbred mouse lines. Methods Mol. Biol. 180, 229–251 (2002).
29. Sheldon, E. et al. Biobanking human endometrial tissue and blood specimens: standard operating procedure and importance to reproductive biology research and diagnostic development. Fertil. Steril. 95, 2120–2122 (2011).
30. Markoff, E., Zeitler, P., Peleg, S. & Handwerger, S. Characterization of the synthesis and release of prolactin by an enriched fraction of human decidual cells. J. Clin. Endocrinol. Metab. 56, 962–968 (1983).
31. Kessler, C.A., Schroeder, J.K., Brar, A.K. & Handwerger, S. Transcription factor ETS1 is critical for human uterine decidualization. Mol. Hum. Reprod. 12, 71–76 (2006).