

Forkhead box a2 (FOXA2) is essential for uterine function and fertility

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Establishment of pregnancy is a critical event, and failure of embryo implantation and stromal decidualization in the uterus contribute to significant numbers of pregnancy losses in women. Glands of the uterus are essential for establishment of pregnancy in mice and likely in humans. Forkhead box a2 (FOXA2) is a transcription factor expressed specifically in the glands of the uterus and is a critical regulator of postnatal uterine gland differentiation in mice. In this study, we conditionally deleted FOXA2 in the adult mouse uterus using the lactotransferrin Cre (*Ltf-Cre*) model and in the neonatal mouse uterus using the progesterone receptor Cre (*Pgr-Cre*) model. The uteri of adult FOXA2-deleted mice were morphologically normal and contained glands, whereas the uteri of neonatal FOXA2-deleted mice were completely aglandular. Notably, adult FOXA2-deleted mice are completely infertile because of defects in blastocyst implantation and stromal cell decidualization. Leukemia inhibitory factor (LIF), a critical implantation factor of uterine gland origin, was not expressed during early pregnancy in adult FOXA2-deleted mice. Intriguingly, i.p. injections of LIF initiated blastocyst implantation in the uteri of both gland-containing and glandless adult FOXA2-deleted mice. Although pregnancy was rescued by LIF and was maintained to term in uterine gland-containing adult FOXA2-deleted mice, pregnancy failed by day 10 in neonatal FOXA2-deleted mice lacking uterine glands. These studies reveal a previously unrecognized role for FOXA2 in regulation of adult uterine function and fertility and provide original evidence that uterine glands and, by inference, their secretions play important roles in blastocyst implantation and stromal cell decidualization.

uterus | gland | FOXA2 | pregnancy | LIF

The uterus is comprised of two tissue compartments, the endometrium and the myometrium. The endometrium contains three cell types, luminal epithelium (LE), glandular epithelium (GE), and stroma. In mice, the uterus becomes receptive to blastocyst implantation on gestational day (GD) 4 (with the observation of a postcoital vaginal plug designated GD 0.5); it is prereceptive on GD 1–3, and by the afternoon of GD 5 it becomes nonreceptive (refractory) (1–3). Dynamic changes in ovarian estrogen and progesterone production act via the uterus to regulate uterine receptivity, blastocyst implantation, and stromal cell decidualization necessary for the establishment of pregnancy (4–6). The implantation process, which is initiated by the attachment of the blastocyst trophoblast to the receptive LE, occurs before or right after midnight in the evening of GD 4 and becomes more prominent by the morning of GD 5. By GD 6, the trophoblast begins to contact directly stromal cells that then begin to differentiate into decidual cells, which are required for successful pregnancy because they regulate placental development and local maternal immune responses (7, 8).

The infertility observed in *leukemia inhibitory factor* (*Lif*)-null mice and uterine gland-knockout (UGKO) mice and sheep established the importance of the uterine glands for blastocyst implantation and pregnancy success (9–15). Recently, forkhead box a2 (FOXA2) was demonstrated to be essential for differentiation and development of glands in the neonatal mouse uterus (12). Forkhead box transcription factors play important roles in regulating the ex-

pression of genes involved in cell growth, proliferation, differentiation, and longevity in a number of different organs (16, 17). FOXA2 is expressed specifically in the GE of the neonatal and adult uterus in mice (11, 12, 18) and also is expressed in the GE of the human uterus (19). Null mutation of *Foxa2* is embryonic lethal in mice (20, 21), because it is required for the development of endoderm-derived organs (17, 22). Subsequently, *Foxa2* was conditionally deleted in the uterus after birth in neonatal mice using the progesterone receptor Cre (*Pgr^{Cre}*) mouse (23). The uteri of those neonatal *Foxa2* conditional mutant mice lacked glands, and the adult mice were infertile because of defects in blastocyst implantation (11, 12). The uteri of aglandular *Foxa2*-deleted (*Pgr^{Cre/+Foxa2^{fl/fl}}*) mice lacked *Lif*, which is a gland-derived implantation factor induced by estrogen from the ovary on GD 4 (24, 25) and also is expressed in the subluminal stroma at the implantation site on GD 5 (26). LIF is critical for embryo implantation (15, 25) and has pleiotropic effects on the LE and stroma to regulate uterine receptivity and perhaps stromal cell decidualization (24, 27).

Here we present findings that conditional deletion of FOXA2 after puberty in the adult uterus, using *lactotransferrin Cre* (*Ltf^{Cre}*) and floxed *Foxa2* (*Foxa2^{fl/fl}*) mice, results in complete infertility. Although the uteri of *Ltf^{Cre/+Foxa2^{fl/fl}}* mice are histologically normal and contain glands, *Lif* is not expressed in during early pregnancy, and blastocysts do not attach to the LE and implant. The stromal cells of *Ltf^{Cre/+Foxa2^{fl/fl}}* uteri also fail to decidualize. Remarkably, i.p. injections of LIF on GD 4 stimulate blastocyst implantation in the uterus of both gland-containing *Ltf^{Cre/+Foxa2^{fl/fl}}* and glandless *Pgr^{Cre/+Foxa2^{fl/fl}}* mice. Although pregnancy is maintained to term in LIF-replaced *Ltf^{Cre/+Foxa2^{fl/fl}}* mice, pregnancy is lost by GD 9.5 in LIF-replaced *Pgr^{Cre/+Foxa2^{fl/fl}}* mice. Taken together, these findings provide clear *in vivo* evidence that FOXA2 regulates *Lif* expression in the uterus for blastocyst implantation and that uterine glands play

Significance

These studies define biological roles for Forkhead box a2 (FOXA2) and glands of the uterus in female reproduction and fertility. FOXA2 is a critical regulator of uterine gland development in the neonate as well as of differentiated gland function in the adult uterus. Our findings provide clear *in vivo* evidence that FOXA2 regulates uterine expression of Leukemia inhibitory factor (*Lif*) for blastocyst implantation and that uterine glands play active roles in stromal cell decidualization and placental development. These findings support the idea that pregnancy loss and complications in women may have their origin in uterine gland dysfunction.

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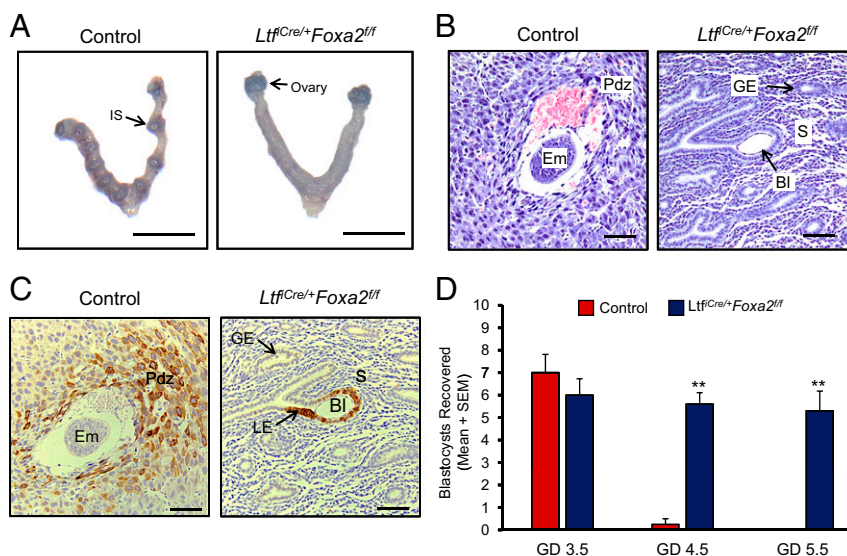


Fig. 1. Conditional deletion of FOXA2 in the adult uterus compromises embryo implantation. (A) Embryo implantation sites (IS) were not observed on GD 5.5 in *Ltf^{Cre/+}Foxa2^{fl/fl}* mice ($n = 5$ per genotype). (Scale bars, 1 cm.) (B) Histology of longitudinal sections of GD 5.5 uteri. Bl, blastocyst; Em, embryo; GE, glandular epithelium; PdZ, primary decidua; S, stroma. (Scale bars, 75 μ m.) (C) Immunolocalization of PTGS2 in longitudinal sections of GD 5.5 uteri. (Scale bars, 75 μ m.) (D) Blastocysts recovered by uterine flush of control and *Ltf^{Cre/+}Foxa2^{fl/fl}* mice at 1600 h on GD 3.5, 4.5, and 5.5 ($n = 4$ mice per genotype). ** $P < 0.01$.

an active role in postimplantation stromal cell decidualization and placental development critical for the establishment of pregnancy.

Results

Mice Lacking FOXA2 in the Adult Uterus Are Infertile. To understand the biological role of FOXA2 in function of the adult uterus, mice with a conditional ablation of *Foxa2* in the adult uterus were generated by crossing *Ltf^{Cre}* mice (28) with *Foxa2^{fl/fl}* mice (29). In the uterus of *Ltf^{Cre}* mice, Cre expression and activity are restricted to the LE and GE and are initiated during puberty (28). In the resulting *Ltf^{Cre/+}Foxa2^{fl/fl}* mice, FOXA2 was undetectable in the adult uterus (Fig. S1A). No obvious histoarchitectural differences were observed in the female reproductive tract of *Ltf^{Cre/+}Foxa2^{fl/fl}* mice (Fig. S1A). In fact, FOXA2 was not deleted by *Ltf^{Cre}* in the epithelia of either the oviduct or cervix, and FOXA2 was not detected in the ovary or vagina (Fig. S1B). Importantly, the uteri of *Ltf^{Cre/+}Foxa2^{fl/fl}* mice contained glands in the endometrium that were normal in morphology and number (Fig. S1A).

Control (*Ltf^{+/+}Foxa2^{fl/fl}*) and *Ltf^{Cre/+}Foxa2^{fl/fl}* female mice displayed normal mating behavior indicated by the presence of copulatory plugs in the vagina following mating to an intact or vasectomized male. Compared with control mice, *Ltf^{Cre/+}Foxa2^{fl/fl}* mice were completely infertile in a 6-mo breeding trial (Table S1). *Ltf^{Cre/+}Foxa2^{fl/fl}* mice displayed no visible implantation sites on GD 5.5 when mated to males of proven fertility (Fig. 1A). In control mice, the implanting embryos were surrounded by decidualizing stromal cells (Fig. 1B), evidenced by the up-regulation of prostaglandin synthase 2 (PTGS2) expression in the primary decidua zone (Fig. 1C). Consistent with the lack of noticeable implantation sites (Fig. 1A), the uteri of *Ltf^{Cre/+}Foxa2^{fl/fl}* mice on GD 5.5 contained nonimplanted blastocysts (Fig. 1B). The trophoblast was in apposition with an intact LE adjacent to stroma that lacked morphological signs of transformation to polyploid decidual cells (Fig. 1B). Although PTGS2 was up-regulated in the LE adjacent to the blastocyst, none of the underlying stromal cells expressed PTGS2 (Fig. 1C). By GD 4.5, blastocysts in control mice are firmly attached to the LE and can no longer be flushed from the uterus (Fig. 1D). In contrast, blastocysts could still be recovered in the uterine flush from *Ltf^{Cre/+}Foxa2^{fl/fl}* mice on

GDs 4.5 and 5.5 (Fig. 1D). Thus, blastocysts fail to attach and adhere to the LE and initiate stromal cell decidualization in adult FOXA2-deficient uteri.

The decidual response of the *Ltf^{Cre/+}Foxa2^{fl/fl}* mouse uterus was determined using an artificial decidual formation model (30). The uteri of control mice displayed a robust decidual response (Fig. 2A), as evidenced by the increased size and wet weight of the decidual response formed in the stimulated left uterine horn compared with the unstimulated right uterine horn ($P < 0.01$) (Fig. 2A–C). However, the uteri of *Ltf^{Cre/+}Foxa2^{fl/fl}* mice failed to form a decidua (Fig. 2A–C), and the wet weight of the stimulated horn was not different from that of the unstimulated horn ($P > 0.10$) (Fig. 2B). The transformation of stromal cells to rounded polyploid decidual cells was observed in the stimulated uterine horn of control mice but not *Ltf^{Cre/+}Foxa2^{fl/fl}* mice (Fig. 2C). In control mice, FOXA2 was observed in the GE of the unstimulated uterine horn as well as in the GE of the endometrium interposed between the decidua and myometrium of the stimulated uterine horn (Fig. 2C). Expression of the decidualization markers bone morphogenetic protein 2 (*Bmp2*), *Bmp8a*, prolactin family 8 subfamily a member 2 (*Prl8a2*), and wingless-type MMTV integration site family member 4 (*Wnt4*) increased in the stimulated uterine horn compared with the unstimulated uterine horn in control mice ($P < 0.01$) but not in *Ltf^{Cre/+}Foxa2^{fl/fl}* mice ($P > 0.10$) (Fig. 2D). These results support the idea that the infertility of *Ltf^{Cre/+}Foxa2^{fl/fl}* mice is caused by defects in uterine receptivity and blastocyst implantation and also by a defect in stromal cell decidualization.

LIF Is Not Expressed in the Glands of the FOXA2-Deficient Uterus.

Successful implantation requires both a competent embryo and a receptive uterus (2, 3). Uterine receptivity in mice is regulated mainly by the ovarian steroid hormones estrogen and progesterone acting via their cognate nuclear receptors, estrogen receptor alpha (ESR1) and progesterone receptor (PGR), respectively (31, 32). Spatiotemporal patterns of immunoreactive ESR1, phosphorylated ESR1, and PGR were not different in the LE, GE, and stroma of uteri obtained from control and *Ltf^{Cre/+}Foxa2^{fl/fl}* mice on GD 2.5, 3.5, and 4.5 (Fig. 3A and Fig. S2). Of note, ESR1 and phosphorylated ESR1 were abundant in the GE of both control and *Ltf^{Cre/+}Foxa2^{fl/fl}* uteri on GD 3.5 (Fig. 3A

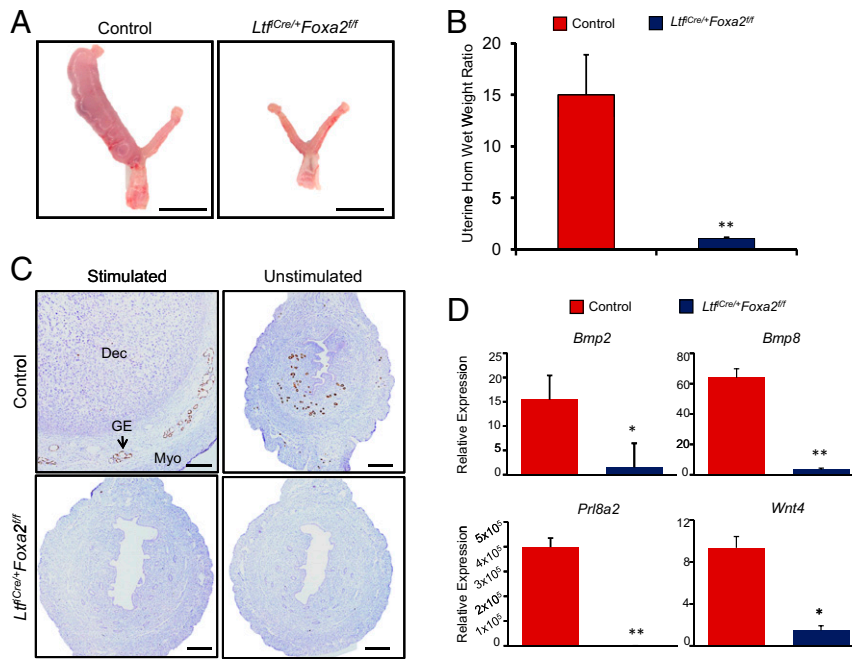


Fig. 2. Uterine decidualization is defective in adult FOXA2-deficient mice. (A) Artificial decidualization in control and *Ltf^{Cre/+}Foxa2^{fl/fl}* mice ($n = 5$ mice per genotype). Mice were bred to a vasectomized male, received oil stimulus into the lumen of the left uterine horn on day 4.5, and were assessed on day 9.5. (Scale bars, 1 cm.) (B) Wet weight ratio of the stimulated to unstimulated uterine horn. $**P < 0.001$. (C) Histology and immunolocalization of FOXA2 in cross-sections of the stimulated and unstimulated uterine horns. Note the presence of decidualized stromal cells and FOXA2⁺ glands in the stimulated and unstimulated uterine horns of control mice. Dec, deciduoma; GE, glandular epithelium; Myo, myometrium. (Scale bars, 250 μ m.) (D) Quantification of decidualization marker genes. Data are presented as fold change in the stimulated horn relative to the unstimulated uterine horn. $*P < 0.05$, $**P < 0.01$.

and Fig. S2). Consistent with the lack of differences in steroid receptor expression, the abundance of antiadhesive mucin 1 (MUC1) in the uterine LE and the cell proliferation marker Ki-67 in the stroma were not different in control and *Ltf^{Cre/+}Foxa2^{fl/fl}* uteri (Fig. 3A and Fig. S2). The expression of other studied uterine receptivity- and implantation-related genes [*Muc1*, *Bmp2*, Indian hedgehog (*Ihh*), Msh homeobox 1 (*Msx1*), *Ptgs2*, *Wnt4*, and *Wnt7a*] was not different in the uteri of *Ltf^{Cre/+}Foxa2^{fl/fl}* mice ($P > 0.10$) (Fig. 3B and Fig. S3).

Although the *Ltf^{Cre/+}Foxa2^{fl/fl}* mice contain glands in their uteri, a number of GE-specific genes were not expressed, including chemokine C-X-C motif ligand 15 (*Cxcl15*), *Lif*, protease serine 29 (*Prss29*), serine peptidase inhibitor Kazal type 3 (*Spink3*), transthyretin receptor (*Ttr*), and WAP four-disulfide core domain 3 (*Wfdc3*) during the peri-implantation period (Fig. 3B and Fig. S3).

LIF Elicits Embryo Implantation in FOXA2-Deficient Mice. LIF is essential for blastocyst implantation in mice and is transiently expressed in the GE of the uterus on GD 4 in response to “nidatory” estrogen from the ovary (15, 24, 25). The i.p. administration of LIF in *Lif*-null mice rescued embryo implantation (15, 24). Here, LIF rescue of implantation was conducted using *Ltf^{Cre/+}Foxa2^{fl/fl}* and *Pgr^{Cre/+}Foxa2^{fl/fl}* mice (Table 1). Female mice received two i.p. injections of vehicle or 10 μ g recombinant mouse LIF on GD 3.5, and embryo implantation sites were evaluated on GD 5.5 or 9.5 (Fig. 4A). As expected, implantation was observed in control mice but not in *Ltf^{Cre/+}Foxa2^{fl/fl}* mice receiving vehicle on GD 3.5. Remarkably, implantation sites were observed both in LIF-replaced *Ltf^{Cre/+}Foxa2^{fl/fl}* uteri that contain glands and in *Pgr^{Cre/+}Foxa2^{fl/fl}* uteri that lack glands (Fig. 4B) (12, 18). On GD 5.5, the number of implantation sites was not different between control and LIF-replaced *Pgr^{Cre/+}Foxa2^{fl/fl}* and *Ltf^{Cre/+}Foxa2^{fl/fl}* mice ($P > 0.10$) (Table 2). All implantation sites contained

an embryo with a FOXA2⁺ endoderm and surrounded by PTGS2⁺ decidualizing stromal cells (Fig. 4C and Fig. S4).

Although embryo implantation and stromal cell decidualization clearly occurred in the uteri of LIF-replaced FOXA2-deficient mice, the expression of decidual marker genes was altered in these mice (Fig. 4D). Expression of *Alpl* and *Ptgs2* was increased ($P < 0.05$), and *Bmp2*, *Fstl1*, *Prl8a2*, and *Wnt4* were decreased ($P < 0.01$) in *Ltf^{Cre/+}Foxa2^{fl/fl}* mice relative to control mice. Although *Hoxa10* expression was increased ($P < 0.05$) in the implantation sites of *Pgr^{Cre/+}Foxa2^{fl/fl}* mice, the expression of *Alpl*, *Bmp2*, *Bmp8a*, *Fstl1*, *Prl8a2*, and *Wnt4* was considerably lower ($P < 0.01$) than in control mice. As expected, expression of the FOXA2-regulated and GE-specific genes (*Foxa2*, *Prss29*, *Spink3*, *Ttr*, and *Wfdc3*) was substantially decreased ($P < 0.01$) in the uteri of *Ltf^{Cre/+}Foxa2^{fl/fl}* and *Pgr^{Cre/+}Foxa2^{fl/fl}* mice compared with control mice (Fig. 4E).

Uterine Glands Are Essential for Pregnancy Establishment. Although LIF injections on GD 3.5 elicited embryo implantation in FOXA2-deficient mice, pregnancies were maintained only to GD 9.5 in *Ltf^{Cre/+}Foxa2^{fl/fl}* mice, because implantation sites were no longer observed in the glandless uteri of LIF-replaced *Pgr^{Cre/+}Foxa2^{fl/fl}* mice on GD 9.5 (Fig. 5A). No blastocysts were found in the uteri of *Pgr^{Cre/+}Foxa2^{fl/fl}* mice on GD 9.5, indicating that embryos were resorbed between GD 5.5 and 9.5. The number of implantation sites on GD 9.5 was not different in control and LIF-replaced *Ltf^{Cre/+}Foxa2^{fl/fl}* mice ($P < 0.10$) (Table 2), and all contained a developing fetus, placenta, and decidua (Fig. 5B). The placentae contained trophoblast giant cells (Tgc) and a developing labyrinth placenta. Although no overt morphological differences were observed in the secondary decidual zone, the expression of several decidual marker genes (*Alpl*, *Bmp2*, *Bmp8a*, *Prl8a2*, and *Wnt4*) was lower in the implantation sites from *Ltf^{Cre/+}Foxa2^{fl/fl}* mice than from control mice on GD 9.5 ($P < 0.05$) (Fig. 5C).

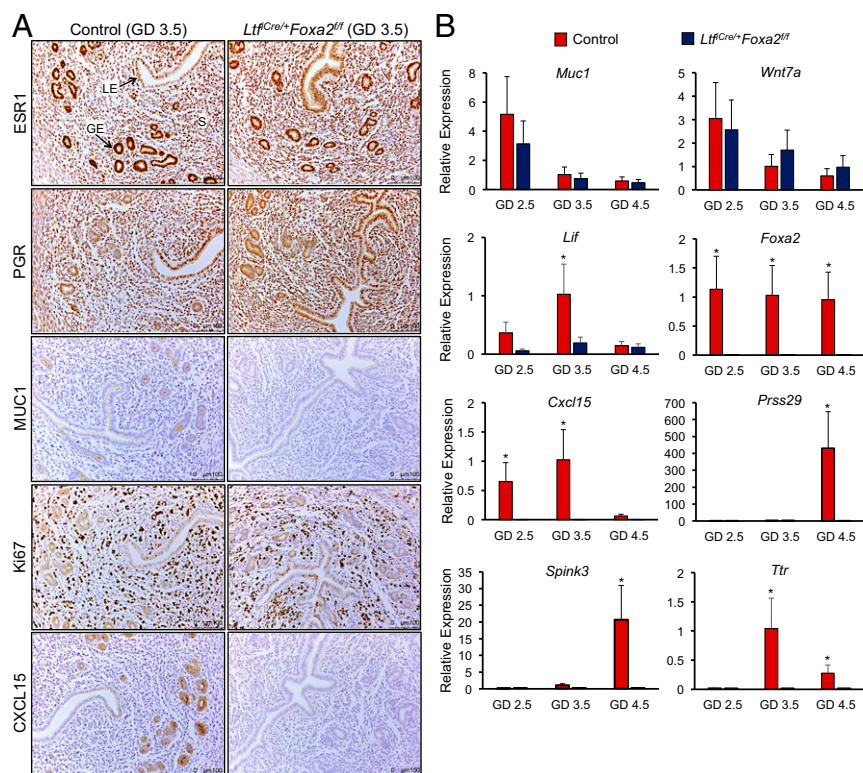


Fig. 3. *Lif* and several gland-specific genes are deficient in adult FOXA2-deficient uteri. (A) Immunolocalization of ESR1, PGR, MUC1, the Ki-67 cell-proliferation marker, and CXCL15 in the uteri of control and *Ltj^{Cre/+}Foxa2^{fl/fl}* mice on GD 3.5 ($n = 4$ mice per genotype). GE, glandular epithelium; LE, luminal epithelium; S, stroma. (Scale bars, 100 μ m.) (B) Quantification of uterine receptivity and gland-specific genes in the uteri of control and *Ltj^{Cre/+}Foxa2^{fl/fl}* mice on GD 2.5, 3.5, and 4.5 ($n = 4$ mice of each genotype per day). * $P < 0.05$.

Immunostaining for TROMA-1, a pan trophoblast cell marker that detects cytokeratin 8, revealed that the placentae of LIF-replaced *Ltj^{Cre/+}Foxa2^{fl/fl}* mice were morphologically different from those of control mice (Fig. 6A). The placentae of control mice contained one or two layers of Tgc (Figs. 5B and 6A). In contrast, an increased number of Tgc layers were apparent in the placentae of LIF-replaced *Ltj^{Cre/+}Foxa2^{fl/fl}* mice. The expression of placental marker genes was altered in LIF-replaced *Ltj^{Cre/+}Foxa2^{fl/fl}* mice compared with control mice (Fig. 6B). Genes expressed in the trophoblast (Cited2 and Esrb), extraembryonic ectoderm (Ascl2), ectoplacental cone (Tpbpa), and Tgc (Ctsq, Hand1, and Imfa) were reduced in the implantation sites of LIF-replaced *Ltj^{Cre/+}Foxa2^{fl/fl}* mice ($P < 0.05$). In contrast, the expression of other genes expressed in the trophoblast (Cdx2, Eomes, Gcm1) did not differ between control and LIF-replaced *Ltj^{Cre/+}Foxa2^{fl/fl}* mice ($P > 0.10$). Despite differences in decidual gene expression and placental morphology and gene expression, successful pregnancies were achieved in *Ltj^{Cre/+}Foxa2^{fl/fl}* mice that were supplemented with i.p. injections of LIF on GD 3.5. The number of pups delivered at term did not differ in control mice (7.0 ± 1.1 pups, $n = 4$) and LIF-replaced *Ltj^{Cre/+}Foxa2^{fl/fl}* mice (7.4 ± 0.7 pups, $n = 5$) ($P > 0.10$), nor were differences in gestation length and pup survivability to weaning observed in the control and LIF-replaced *Ltj^{Cre/+}Foxa2^{fl/fl}* mice ($P > 0.10$).

Discussion

A finding of these studies is that FOXA2 is a critical regulator of uterine gland function, embryo implantation, and pregnancy establishment. The present study deleted FOXA2 in the uteri of adult mice using the *Ltj^{Cre}* mouse model (28). Although adult FOXA2-deficient uteri were histologically normal and contained

uterine glands, *Ltj^{Cre/+}Foxa2^{fl/fl}* mice presented complete infertility. Blastocyst implantation was defective in *Ltj^{Cre/+}Foxa2^{fl/fl}* mice, because embryos could still be recovered from the uterus on GD 4.5 and 5.5. Those uteri contained unimplanted blastocysts in which the trophoblast was in clear apposition to an intact LE with no evidence of stromal cell decidualization. Similarly, mice lacking uterine glands do not express *Lif* and exhibit a defect in blastocyst implantation (12, 33–38). Thus, uterine glands and, by inference, their secretions do not influence preimplantation embryo growth and development but are required for implantation and successful establishment and maintenance of pregnancy.

Successful implantation requires a competent blastocyst and a receptive uterus (2, 3, 6). Attainment of uterine receptivity involves cell-specific actions of the steroid hormones estrogen and progesterone via their nuclear receptors (4, 5, 31, 32). With the exception of *Lif*, spatiotemporal expression patterns of steroid receptor and uterine receptivity genes were not altered in the uteri of *Ltj^{Cre/+}Foxa2^{fl/fl}* mice. In mice, estrogen from the ovaries

Table 1. Summary of mouse phenotypes

Genotype	Uterine glands	Gland Foxa2	Uterine		Deciduoma formation
			<i>Lif</i> GD 3.5	Implantation GD 5.5	
Control	+	+	+	+	+
<i>Pg^{Cre/+}Foxa2^{fl/fl}</i>	–	–	–	–	–
<i>Ltj^{Cre/+}Foxa2^{fl/fl}</i>	+	–	–	–	–

Plus and minus signs indicate presence or absence, respectively.

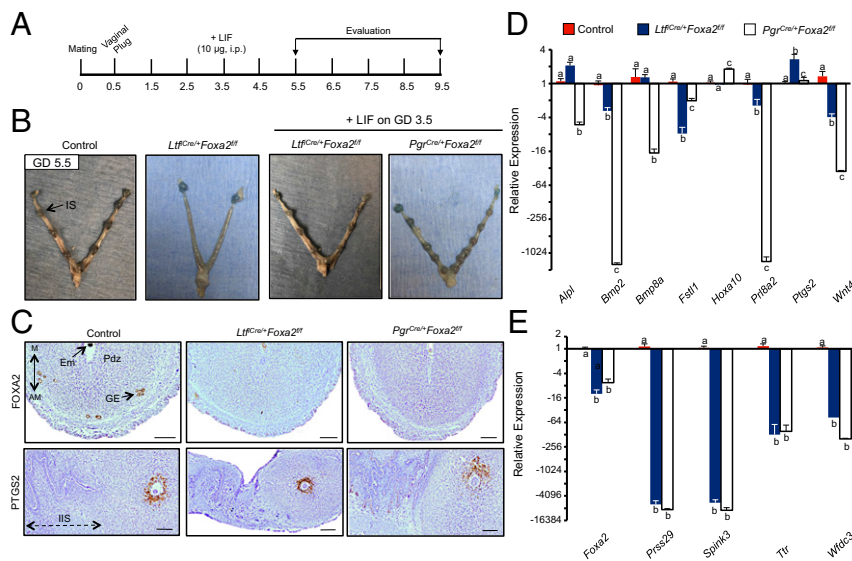


Fig. 4. Lif rescues embryo implantation in FOXA2-deficient mice. (A) Experimental design. Control, gland-containing *Ltf^{Cre/+}Foxa2^{fl/fl}*, and glandless *Pgr^{Cre/+}Foxa2^{fl/fl}* mice received i.p. injections of either vehicle or recombinant mouse LIF on GD 3.5 and were evaluated on GD 5.5 and 9.5 ($n = 5$ mice of each genotype per day). (B) Embryo implantation sites (IS) were observed on GD 5.5 in vehicle-treated control mice and in Lif-replaced *Ltf^{Cre/+}Foxa2^{fl/fl}* and *Pgr^{Cre/+}Foxa2^{fl/fl}* mice but not in vehicle-treated *Ltf^{Cre/+}Foxa2^{fl/fl}* mice. (C) Histology and immunolocalization of FOXA2 in cross-sections and PTGS2 in longitudinal sections of the uteri from GD 5.5 vehicle-treated control and LIF-replaced *Ltf^{Cre/+}Foxa2^{fl/fl}* and *Pgr^{Cre/+}Foxa2^{fl/fl}* mice. (Scale bars, 250 μ m.) (D) Quantification of decidualization marker genes in the GD 5.5 implantation sites of vehicle-treated control and LIF-replaced *Ltf^{Cre/+}Foxa2^{fl/fl}* and *Pgr^{Cre/+}Foxa2^{fl/fl}* mice. Letters indicate differences between genotypes ($P < 0.05$). (E) Quantification of uterine gland-expressed genes in the implantation sites from control and LIF-replaced *Ltf^{Cre/+}Foxa2^{fl/fl}* and *Pgr^{Cre/+}Foxa2^{fl/fl}* mice. Data are presented as fold change in the stimulated uterine horn compared with the unstimulated uterine horn.

on GD 3.5 induces the expression of *Lif* in the glands of the uterus (24). *Lif*-null and uterine-glandless mice (*Pgr^{Cre}* mice with conditional deletion of *Foxa2*, *Ctnnb1*, *Lef1*, *Wnt4*, *Wnt5a*, or *Wnt7a* and also mice with progesterone-induced uterine gland knockout, PUGKO) all exhibit deficiencies in peri-implantation *Lif* expression and defects in blastocyst implantation (12, 33–37). Of note, blastocysts from UGKO mice are implantation competent when transferred into normal uteri (38). Here, i.p. injections of LIF into GD 3.5 FOXA2-deficient mice with or without uterine glands elicited blastocyst implantation but rescued pregnancy only in gland-containing *Ltf^{Cre/+}Foxa2^{fl/fl}* mice. Given that LIF could not be detected in the uterine luminal fluid of wild-type mice by either mass spectrometry or ELISA (38), LIF may be preferentially secreted in a basolateral manner from the glands and impact uterine receptivity for blastocyst implantation as well as stromal cell decidualization (38).

Another key finding here is that FOXA2 is essential for adult uterine function and particularly for *Lif* expression by the uterine glands of mice on GD 3.5. The lack of *Lif* and other GE-specific genes (*Cxcl15*, *Prss29*, *Spink3*, *Ttr*, and *Wfdc3*) in the *Ltf^{Cre/+}Foxa2^{fl/fl}* uterus demonstrates the substantial influence of FOXA2 on the GE-specific transcriptome (18). *Lif* expression in the uterine glands definitively requires ESR1 (39), but ESR1 expression was not affected by the loss of FOXA2 in the uteri of adult mice in the present study. The molecular mechanism underlying the regulatory effects of FOXA2 and ESR1 on *Lif* expression in the uterine glands is not well defined. Both FOXA1 and FOXA2 are pioneer transcription factors that can mimic linker histone, bind directly to compacted chromatin, decompact chromatin, and reposition nucleosomes, thereby providing the opportunity for other transcription factors to associate with chromatin and positively or negatively influence the transcriptional program of cells (40, 41). In breast cancer and non-breast cancer cells, FOXA1 is a major determinant of ESR1 activity and estrogen responses (42). Both FOXA1 and FOXA2 are pioneer transcription factors with essential roles in the develop-

ment of and estrogen signaling in the liver (43). In breast cancer cells, FOXA factors and ESR1 are frequently bound to adjacent cis-regulatory elements in the genome, and recruitment of ESR1 to those binding sites is dependent on FOXA factors (42, 44). Of note, the majority of uterine gland-specific genes (*Cxcl15*, *Prss29*, *Spink3*, *Ttr*, and *Wfdc3*) studied here are bound by FOXA2 in ChIP-seq analyses of GD 3.5 mouse uteri (18). Expression of *Lif* induction in the uterine glands requires ESR1 (45), and ChIP-seq analysis of the mouse uterus revealed ESR1 binding to the coding and 3' UTR regions of the *Lif* gene (46). However, the *Lif* gene and its upstream promoter-enhancer region are not bound by FOXA2 in available ChIP-seq analyses of the mouse uterus (18, 39). Indeed, FOXA1 renders ESR1 functional in breast cancer cells by mediating ESR1 binding and transcriptional activity and maintaining chromatin structure and accessibility (42, 47). Thus, *Lif* gene activation by ligand-activated ESR1 is postulated to involve FOXA2 pioneer transcription activity (48, 49). Future studies should focus on the molecular mechanism by which FOXA2 programs the GE transcriptome and influences steroid hormone responses of uterine glands, particularly because FOXA2 is expressed in the glands of the human uterus, is involved in

Table 2. Effects of i.p. LIF injections on embryo implantation

Genotype*	GD 5.5 implantation sites		GD 9.5 implantation sites	
	n^{\dagger}	Mean (range)	n^{\dagger}	Mean (range)
Control	5	8.4 ^a (6–11)	6	7.6 ^a (5–9)
<i>Pg^{Cre/+}Foxa2^{fl/fl}</i>	5	7.6 ^a (4–11)	5	0 ^b (0)
<i>Ltf^{Cre/+}Foxa2^{fl/fl}</i>	4	9.8 ^a (9, 10)	4	8.2 ^a (7–11)

Superscript letters indicate differences between genotypes, $P < 0.05$.

*Control mice received i.p. injections of vehicle on GD 3.5; *Foxa2*-deficient mice received i.p. injections of recombinant mouse LIF on GD 3.5.

[†]Number of mice per genotype evaluated for implantation sites.

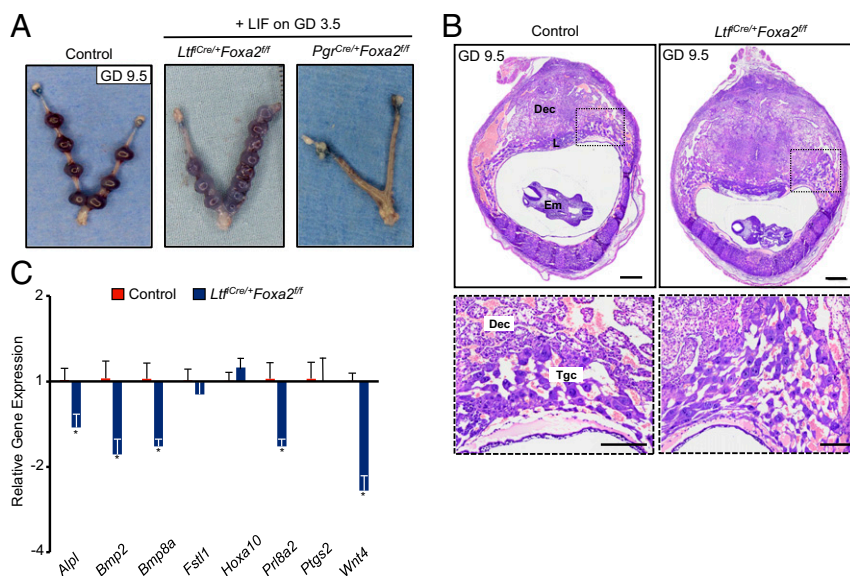


Fig. 5. Uterine glands are essential for pregnancy establishment in mice. Control gland-containing *Ltf^{Cre/+}Foxa2^{fl/fl}* and glandless *Pgr^{Cre/+}Foxa2^{fl/fl}* mice received i.p. injections of either vehicle or recombinant mouse LIF on GD 3.5 and were evaluated on GD 9.5 ($n = 5$ mice per genotype). (A) Embryo implantation sites were observed on GD 9.5 in vehicle-treated control mice and LIF-replaced *Ltf^{Cre/+}Foxa2^{fl/fl}* mice but not in *Pgr^{Cre/+}Foxa2^{fl/fl}* mice. (B) Histology of cross-sections from GD 9.5 implantation sites from vehicle-treated control and LIF-replaced *Ltf^{Cre/+}Foxa2^{fl/fl}* mice. Dec, decidua; Em, embryo; L, labyrinth; Tgc, trophoblast giant cell. (Scale bar, 250 μ m.) (C) Quantification of decidualization marker genes in the GD 9.5 implantation sites of vehicle-treated control and LIF-replaced *Ltf^{Cre/+}Foxa2^{fl/fl}* mice. * $P < 0.05$.

endometrial hyperplasia (19), is frequently mutated and inactivated in endometrioid endometrial cancer (50), and is down-regulated in ectopic endometrium in women with endometriosis (51).

Similar to mice that lack uterine glands (11, 12), the uteri of adult *Ltf^{Cre/+}Foxa2^{fl/fl}* mice also possessed a defect in the differentiation of stromal cells into decidual cells. PTGS2 is a rate-limiting enzyme involved in prostaglandin synthesis, is up-regulated in the LE adjacent to the blastocyst trophoblast and then in decidualizing stromal cells, and is required for implantation and placentation in mice (52, 53). Here, PTGS2 expression was induced in the LE adjacent to the blastocyst in adult FOXA2-deficient mice but not in the surrounding stroma, which was also observed in *Lif*-null and PUGKO *Pgr^{Cre/+}Foxa2^{fl/fl}* UGKO mice (11, 26). The uteri of *Ltf^{Cre/+}Foxa2^{fl/fl}* mice exhibited no decidualization response. Similarly, decidualization was defective in PUGKO mice (11, 12). LIF is important for uterine decidualization in mice (24); however the administration of LIF into the uterine lumen only partially rescued decidualization in glandless *Pgr^{Cre/+}Foxa2^{fl/fl}* mice and had no effect on decidualization in PUGKO mice (11, 12). In contrast, intrauterine injection LIF can replace estrogen in inducing both implantation and decidualization in hormonally prepared ovariectomized wild-type mice that have glands in their uteri (11, 24). Thus, uterine glands are fundamentally required for stromal cell decidualization in mice. The present study clearly demonstrates that FOXA2⁺ uterine glands are present in the endometrium surrounding the decidual cells on GD 5.5 and 9.5. Moreover, those uterine glands remain active and express a number of genes encoding secreted factors such as PRSS28, PRSS29, SPINK3, and WFDC3. The biological roles of those secreted factors in the uterus have not been established using mouse genetic models. Although *Spink3* is expressed only in the glands of the mouse uterus, SPINK3 protein was found in the LE and decidual cells as well as in uterine glands (54). That finding suggests that SPINK3 is secreted in a basolateral manner, as found for LIF and many other proteins secreted by polarized epithelia (55). These results support the idea that uterine glands secrete LIF and other factors that govern stromal cell decidualization (56). The different FOXA2-deficient mouse models that contain

and lack uterine glands will be useful for discovering the GE-derived factors involved in stromal cell decidualization.

In mice, LIF was among the first factors discovered to be necessary for uterine receptivity and implantation (15, 25). In *Lif*-null mice, a single i.p. injection of LIF induced embryo implantation and rescued pregnancy (24). Here, we found that i.p. injections of LIF on GD 3.5 also induce embryo implantation in the uteri of FOXA2-deficient mice regardless of whether they possess glands in their uteri. On GD 5.5, the implantation sites appeared normal in LIF-replaced, FOXA2-deficient mice based on gross morphology (decidual swelling), histology (polyploid decidual cells), and PTGS2 expression in the primary decidual zone. However, pregnancy was maintained only in the LIF-replaced *Ltf^{Cre/+}Foxa2^{fl/fl}* mice that contained uterine glands. Further, the decidual cells in the LIF-replaced glandless *Pgr^{Cre/+}Foxa2^{fl/fl}* mice on GD 5.5 were clearly abnormal based on decidual marker gene analysis; *Alpl*, *Bmp2*, *Bmp8a*, *Prl8a2*, and *Wnt4* were substantially lower in the implantation sites in these mice than in control and LIF-replaced *Ltf^{Cre/+}Foxa2^{fl/fl}* mice. BMP2 and WNT4 are key regulators of stromal cell decidualization (34, 57), and ALPL, BMP8A, and PRL8A2 are expressed only by fully differentiated decidual cells (57–59). Of note, complete embryo resorption occurred by GD 9.5 in LIF-replaced glandless *Pgr^{Cre/+}Foxa2^{fl/fl}* mice, likely because of inadequate formation of the decidua (7).

Although pregnancy was maintained to term in LIF-replaced *Ltf^{Cre/+}Foxa2^{fl/fl}* mice, distinct differences were present in the conceptus and decidua. On GD 5.5 and 9.5, the expression of several decidual marker genes (*Alpl*, *Bmp2*, *Bmp8a*, *Prl8a2*, and *Wnt4*) was reduced in the implantation sites of LIF-replaced *Ltf^{Cre/+}Foxa2^{fl/fl}* mice but not to the extent observed in LIF-replaced *Pgr^{Cre/+}Foxa2^{fl/fl}* mice. Further, alterations in the outer layer of Tgc was apparent in the placentae of LIF-replaced *Ltf^{Cre/+}Foxa2^{fl/fl}* mice on GD 9.5, and the expression of trophoblast marker genes (*Ascl2*, *Cited2*, *Ctsq*, *Esrrb*, *Hand1*, *Imfa*, and *Tpbbpa*) was reduced relative to control mice. Thus, the perturbations in decidual and placental gene expression were apparently not sufficient to affect pregnancy outcome negatively. Collectively, the results here strongly support the hypothesis that

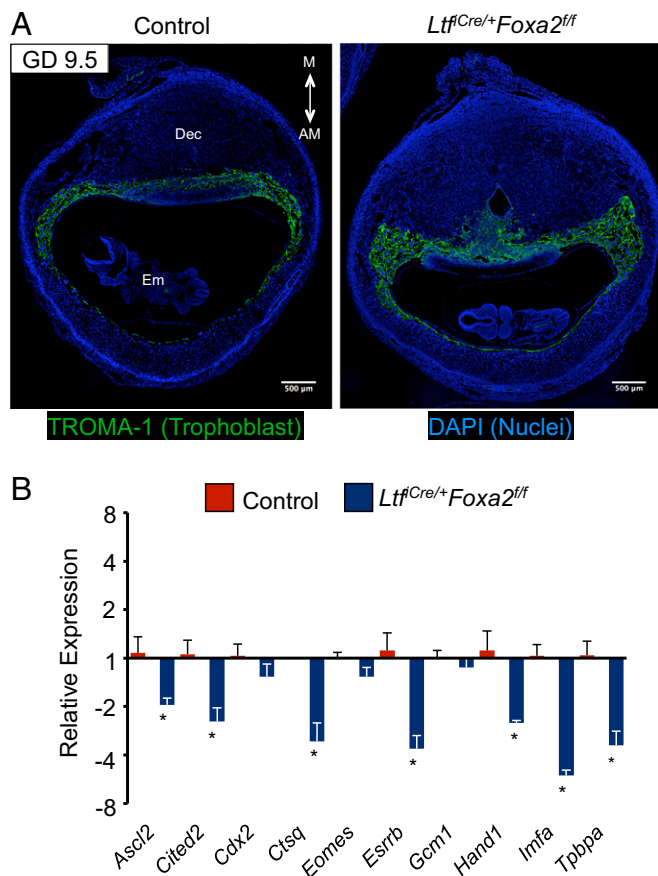


Fig. 6. Effects of FOXA2 deficiency on placental morphology and gene expression. Control mice received i.p. injections of vehicle, and gland-containing $Ltf^{Cre/+}Foxa2^{fl/fl}$ mice received recombinant mouse LIF on GD 3.5 and were evaluated on GD 9.5 ($n = 5$ mice per genotype). (A) Immunolocalization of the pan trophoblast marker TROMA-1 (cytokeratin 8) in cross-sections of GD 9.5 implantation sites from vehicle-treated control and LIF-replaced $Ltf^{Cre/+}Foxa2^{fl/fl}$ mice. AM, antimesometrial; Dec, decidua; Em, embryo; M, mesometrial. (Scale bars, 500 μ m.) (B) Quantification of placenta marker genes in the GD 9.5 implantation sites of vehicle-treated control and LIF-replaced $Ltf^{Cre/+}Foxa2^{fl/fl}$ mice. * $P < 0.05$.

genes expressed in the glands of the uterus are required for stromal cell decidualization and for the placental development necessary for the establishment and maintenance of pregnancy. Comparison of pregnancy outcomes in the two different LIF-replaced FOXA2-deficient mouse models support the ideas that (i) FOXA2 has a primary role in regulating *Lif* expression in the uterine glands; (ii) FOXA2-independent genes in the glands have a primary role in regulating stromal cell decidualization; and (iii) FOXA2-dependent genes in the glands are not requisite for but do influence stromal cell decidualization and placental growth and development. Indeed, FOXA2⁺ glands are present throughout the first half of pregnancy in the endometrium surrounding the implantation sites and adjacent to the decidua, but little is known about what they express and secrete in either mice or humans.

Emerging evidence over the last decade in humans points to roles for uterine glands and their secretory products in uterine receptivity, blastocyst implantation, and postimplantation conceptus (embryo/fetus and associated extraembryonic membranes or placenta) growth and development during the first trimester (56, 60, 61). Although for many decades the secretions of glands have been hypothesized to play an essential role in sustaining the conceptus before implantation (62, 63), their potential role in

postimplantation development of the embryo and placenta in humans was largely ignored until recently (60, 64). The human conceptus undertakes interstitial implantation beginning around day 7 postfertilization that is completed by day 10–12 (60, 65). As the mantle of syncytiotrophoblast enlarges at the implantation site, it encircles and erodes into the lumen of the uterine glands. Consequently, connections between the glands and developing intervillous space of the placenta can be observed as early as day 17 postfertilization and persist throughout the first trimester (66). Similar to the invasion of spiral arteries, uterine glands are also occupied by extravillous trophoblast cells termed “endoglandular trophoblasts” (67–70). They have been speculated to provide gland-derived nutrition to the embryo before the establishment of functional uteroplacental circulation at the end of the first trimester. Histologically, the GE cells present in and near the decidua, termed “decidual uterine glands,” appear highly active during early pregnancy and resemble those of the early secretory phase of the cycle (66, 71, 72). Thus, uterine glands continue to function during the first trimester and deliver their contents into the intervillous space where they may play important biological roles in placental development. Indeed, circumstantial evidence suggests that deficient glandular activity may be a causative factor in early pregnancy failure in humans (63, 73).

In human pregnancy, decidualization extends to the basal endometrium and is a critical regulator of placental development and trophoblast invasion (74). Recent evidence suggests that paracrine cross-talk between the glands and stroma may be needed for optimal stromal cell decidualization (75). A number of in vitro studies have found that locally and temporally produced products of the endometrium, such as cytokines and growth factors (LIF, IL-11, relaxin, prostaglandins, and activin A), progress or enhance progesterone-induced decidualization of endometrial stromal cells from humans and/or mice (76, 77). Determination of factors produced by uterine glands and their potential interactions with stromal cells, decidual cells, and trophoblast cells is an important area for future studies in the human. In summary, it is increasingly clear that uterine glands are important for blastocyst implantation and also for stromal cell decidualization and placental development after implantation. Thus, uterine gland dysfunction may lead to pregnancy loss and complications such as miscarriage, preeclampsia, and fetal growth retardation (64, 78). A more comprehensive understanding of uterine gland biology is expected to improve pregnancy success in humans.

Materials and Methods

Animals. All animal procedures were approved by the Institutional Animal Care and Use Committee of Washington State University or the University of Missouri and were conducted according to NIH *Guide for the Care and Use of Laboratory Animals* (79). Floxed *Foxa2* mice (29) were crossed with *Pgr^{Cre}* (23) or *Ltf^{Cre}* (28) mice to generate conditional knockout animals. Floxed *Foxa2* mice were obtained from The Jackson Laboratory (stock no. 022620). *Ltf^{Cre}* mice were generously provided by T. Daikoku and S. K. Dey of the Cincinnati Children’s Hospital Medical Center, Cincinnati, OH and also were obtained from the Jackson Laboratory (stock no. 026030). Gestational time points were obtained by the mating of wild-type CD-1 mice with 8- to 10-wk-old females; the day that a vaginal plug was observed was considered day 0.5 of gestation. For rescue of implantation in FOXA2-deficient uteri, mice received 10- μ g i.p. injections of recombinant mouse LIF (BioLegend) at 1000 h and 1800 h on GD 3.5. Implantation sites on GD 4.5 were visualized by i.v. injection of 1% Evans blue dye (Sigma-Aldrich Corp.) into the tail vein 5 min before necropsy. Uterine tissues were flash-frozen or fixed with 4% paraformaldehyde at necropsy.

Fertility Trial. Control and FOXA2-deficient 8-wk-old female mice were housed individually and continuously with proven-fertile CD-1 male mice. Mating was confirmed by presence of a vaginal plug. Fertility was assessed by monitoring litter frequency and size for 6 mo.

Artificial Decidua-Formation Assay. Female control ($n = 5$) or $Lt^{iCre/+}Foxa2^{fl/fl}$ ($n = 5$) mice were mated to a vasectomized male to induce pseudopregnancy. To stimulate decidua formation, 30 μ L of sesame oil was instilled into the left uterine horn using a 25-gauge needle at 0700 h on GD 4.5. The right uterine horn was not stimulated and served as a control. Mice were necropsied on day 9.5. Gross histology of the uterus and weights of the stimulated and nonstimulated uterine horn were determined. A portion of each uterine horn was fixed for histology or snap-frozen in liquid nitrogen and stored at -80°C for RNA analyses.

RNA Extraction and Real-Time PCR. Total RNA was isolated from uteri using a standard TRIzol-based protocol. To eliminate genomic DNA contamination, extracted RNA was treated with DNase I and purified using an RNeasy MinElute Cleanup Kit (Qiagen). Total RNA (1 μ g) from each sample was reverse transcribed in a total reaction volume of 20 μ L using iScript RT Supermix (Bio-Rad). Real-time PCR was performed using a CFX384 Touch Real-Time System with SsoAdvanced Universal SYBR Green Supermix (Bio-Rad) using Bio-Rad PrimePCR primers (Table S2) and previously described methods (37). *Mus musculus Gapdh* and *Rplp0* were used as the reference genes.

Histology, Immunohistochemistry, and Immunofluorescence Analyses. Immunolocalization studies were performed using previously described methods (80). Briefly, fixed uteri were sectioned (5 μ m), mounted on slides, deparaffinized, and rehydrated in a graded alcohol series. Antigen retrieval was performed by incubating sections for 10 min in boiling 10 mM citrate buffer (pH 6.0). Sections were blocked with 10% (vol/vol) normal serum in PBS

(pH 7.2) and were incubated with an antibody (Table S3) to FOXA2 (1:1,000; LS-C 138006; LifeSpan Biosciences), MUC1 (1:300 dilution; NB120-15481; Novus Biologicals), PGR (1:400 dilution; A0098; DAKO), ESR1 (1:500 dilution; sc-542; Santa Cruz Biotechnology), phospho(Ser118)-ESR1 (1:700 dilution; ab31477; Abcam), Ki-67 (1:1,000 dilution; ab15580; Abcam), PTGS2 (1:250 dilution; 160106; Cayman Chemicals), or TROMA-1 (1:50 dilution; University of Iowa Developmental Studies Hybridoma Bank) overnight at 4°C . Sections were washed in PBS and incubated with 5 μ g/mL biotinylated secondary antibody (Vector Laboratories, Inc.) for 1 h at 37°C . Immunoreactive protein was visualized using a Vectastain ABC kit (Vector Laboratories, Inc.) and diaminobenzidine tetrahydrochloride as the chromagen. Sections were lightly counterstained with hematoxylin before coverslips were affixed with Permount. Immunofluorescence was performed using Alexa 488-conjugated secondary antibody (Thermo Fischer Scientific), and nuclei were counterstained with using VECTASHIELD mounting medium containing DAPI to visualize nuclei (Vector Laboratories, Inc.).

Statistics. All quantitative data were subjected to least-squares ANOVA using the general linear models procedures of the Statistical Analysis System (SAS Institute) to determine the effects of genotype. In all analyses, error terms used in tests of significance were identified according to the expectation of the mean squares for error. Significance ($P < 0.05$) was determined by probability differences of least-squares means.

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