SOX17 regulates uterine epithelial-stromal cross-talk acting via a distal enhancer upstream of Ihh

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Mammalian pregnancy depends on the ability of the uterus to support embryo implantation. Previous studies reveal the Sox17 gene as a downstream target of the Pgr-Gata2-dependent transcription network that directs genomic actions in the uterine endometrium receptive for embryo implantation. Here, we report that ablating Sox17 in the uterine epithelium impairs leukemia inhibitory factor (LIF) and Indian hedgehog homolog (IHH) signaling, leading to failure of embryo implantation. In vivo deletion of the SOX17-binding region 19 kb upstream of the Ihh locus by CRISPR-Cas technology reduces Ihh expression specifically in the uterus and alters proper endometrial epithelial-stromal interactions, thereby impairing pregnancy. This SOX17-binding interval is also bound by GATA2, FOXA2, and PGR. This cluster of transcription factor binding is common in 737 uterine genes and may represent a key regulatory element essential for uterine epithelial gene expression.

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The steroid hormone progesterone (P4) acts via its cognate receptor PGR to regulate uterine receptivity for embryo implantation. PGR regulates uterine epithelial cell proliferation, the epithelial acceptance of embryo invasion, and decidual differentiation of stromal cells in support of embryo development. Indian hedgehog (encoded by \(Ihh\)) expressed in the uterine epithelium is under the control of P4 via PGR as well as its signaling modifier GATA2, contributing to uterine compartmental cross-talk required for epithelial–stromal interaction and facilitation of a successful pregnancy. Identification of factors modulating the activity of the PGR is critical in identifying the molecular mechanisms regulating fertility. Recent studies using a genome-wide transcriptomic approach have identified SOX17 as a regulator of PGR action in the uterine epithelium.

SOX17 (encoded by \(Sox17\)) is a transcription factor belonging to the family of sex-determining region Y-related high-mobility group box, namely SOX. The mouse and human genomes each encode for 20 Sox genes, many of which (\(Sox2\), \(Sox9\), \(Sox17\), \(Sox4\), and \(Sox18\)) are key determinants of cell identities and regulate the capacity to reprogram the cell fates by pioneering the epigenetic remodeling of the genome. To fulfill such a role, SOX transcription factors can specifically bind and bend DNA with angles ranging from 60° to 70°.

Results

Mice lacking SOX17 in the uterine epithelium are infertile. The temporal and spatial expression of SOX17 in the uterine compartments throughout the preimplantation period, Gestation Day (GD) 0.5 to 4.5, were assayed by immunohistochemistry in the pregnant wildtype female mice (Supplementary Fig. 1a). GD 0.5 corresponds to the morning of the presence of the postcoital plug. Staining for SOX17 protein was visible in both luminal epithelium (LE) and glandular epithelium (GE). By GD 1.5, expression of SOX17 in both LE and GE increased and remained constant through the GD 4.5. SOX17-positive cells were also detected in a minority of cells in the stromal compartment (Supplementary Fig. 1a).

The physiological role of SOX17 in the function of the adult uterus was investigated by breeding mice with a conditional allele of \(Sox17\) (\(Sox17^{f/f}\)) mice with either \(Pgr^{Cre}\) or \(Ltf^{Cre}\) mice to ablate \(Sox17\) in PGR-positive cells in all compartments of the uterus (\(Sox17^{d/d}\)) or specifically in the uterine epithelium (\(Sox17^{d/d}\)), respectively. In addition to being uterine epithelial-specific, gene ablation in the \(Ltf^{Cre}\) mouse is initiated at puberty. Analysis by quantitative real-time PCR (qRT-PCR) showed reduced \(Sox17\) mRNA (\(P < 0.01\), analysis of variance (ANOVA) with Tukey's post hoc test) in the whole uteri of both \(Sox17^{f/f}\) and \(Sox17^{d/d}\) female mice as compared with the \(Sox17^{+/+}\) uteri (Supplementary Fig. 1g). Immunohistochemical analysis showed that SOX17 levels were undetectable in the uterine epithelia of adult female \(Sox17^{d/d}\) and \(Sox17^{d/d}\) mice (Supplementary Fig. 1b), whereas SOX17 remained expressed in the uterine stromal compartment. These \(Sox17^{+}\) positive cells exhibit isocitcin B4 reactivity, indicative of an endothelial identity (Supplementary Fig. 1c). Also, no differences in SOX17 expression were detected in uteri between \(Sox17^{+/+}\) and \(Pgr^{Cre}\)/mice at GD 3.5 (Supplementary Fig. 1d).

\(Sox17^{+/+}\) and \(Sox17^{d/d}\) female mice were infertile. Although controls, \(Sox17^{+/+}\) mice gave birth to 48.2 ± 3.6 pups per female over a 6-month period with an average of 8.0 ± 0.6 pups per litter (\(n = 4\)), neither \(Sox17^{d/d}\) nor \(Sox17^{f/f}\) female mice gave birth to any offspring during this time. In order to determine the cause of the infertility, \(Sox17^{d/d}\) and \(Sox17^{d/d}\) female mice were assayed for embryo implantation, uterine stroma decidualization, and epithelial proliferation. Mice were killed at GD5.5 and the uteri were examined for the presence of embryo implantation sites. As shown in Fig. 1a (top panel), whereas \(Sox12^{d/d}\) showed normal embryo implantation sites, no visible implantation sites were detected in either \(Sox17^{d/d}\) or \(Sox17^{d/d}\) female mice. Superoovulation demonstrated that ovulation in these mice was normal and the implantation defect was owing to the embryos failing to attach to the endometrial epithelium at GD 4.5 (Supplementary Fig. 1e,f). This indicated that the implantation failure was not due to the absence of embryos but rather a failure of these embryos to attach and implant in the uterus.

In addition to the impairment of embryo implantation, the ability of the endometrial stroma cells to undergo an artificially induced decidualization was assayed. The uteri of \(Sox17^{d/d}\) mice displayed a robust decidual response, as evidenced by the increased size and wet weight of the deciduoma formed in the stimulated left uterine horn (\(P < 0.01\), ANOVA with Tukey's post hoc test) as compared with the unstimulated horn (\(P < 0.01\), ANOVA with Tukey's post hoc test) (Fig. 1a, bottom panel). However, the uteri of \(Sox17^{d/d}\) and \(Sox17^{d/d}\) mice failed to form a decidua (Fig. 1a, bottom panel), and the wet weight of the stimulated horn were not different from that of the unstimulated horn (\(P > 0.01\), ANOVA with Tukey's post hoc test) (Fig. 1b).

SOX17 controls epithelial proliferation and differentiation. During the preimplantation period, the uterus initially proliferates in response to the ovulatory estrogen (E2) surge and as P4 levels rise, the PGR inhibits the E2 induced proliferation. Analysis of uterine epithelial cell proliferation, as measured by Ki67 staining, shows that at GD 3.5 \(Sox17^{d/d}\) mice exhibited minimal Ki67 staining (H-score; 2.86 ± 2.00), whereas both \(Sox17^{d/d}\) and \(Sox17^{+/+}\) mice showed normal uterine epithelial characteristics. Cistromic and transcriptomic analyses combined with in vivo editing show that SOX17 regulates \(Ihh\) expression by acting on an enhancer region 19 kb distal to the \(Ihh\) promoter. Analysis of the elements binding this enhancer region suggests a general cassette of regulatory elements comprised of transcription factors for the regulation of uterine epithelial gene expression.
uterine epithelial cell differentiation. Sox17<sup>dd</sup> uteri exhibited a reduction in the number of uterine glands as compared with Sox17<sup>eed</sup> and Sox17<sup>ff</sup> mice (Fig. 1d). We next analyzed the expression of two uterine endometrial gland specific genes, Foxa2 and Lif. FOXA2 transcription factor is a critical regulator of postnatal uterine gland development. Leukemia inhibitory factor (LIF) is a cytokine expressed in the uterine glands and is a critical regulator of embryo implantation. Expression of FOXA2 was undetectable in the GE of both the Sox17<sup>eed</sup> and Sox17<sup>dd</sup> mouse uteri as compared with the Sox17<sup>ff</sup> mouse uteri (Fig. 1c, row 2). As shown in Supplementary Fig. 1f, both Foxa2 and Lif mRNA were significantly reduced in the Sox17<sup>eed</sup> and Sox17<sup>dd</sup> mouse uteri as compared to the Sox17<sup>ff</sup> uteri isolated from GD 3.5 mice. It has been shown that loss of Foxa2 prior to pubertal onset results in infertility due to loss of uterine glands, whereas loss of Foxa2 in the adult results in maintenance of the uterine gland morphology but still loss of fertility due to lack of the GE expression of the cytokine LIF. Therefore, as with FOXA2, Sox17 is critical for neonatal uterine gland development, and adult uterine gland function.

Previous work has demonstrated that loss of uterine glands results in implantation defects and that the implantation defect can be rescued by administration of recombinant LIF, rLIF, to pregnant mice. In order to determine whether the implantation defect in mice with loss of Sox17 was solely owing to a glandular defect and loss of LIF, we attempted to rescue the implantation defect by administration of rLIF. In this experiment, mice with deletion of Foxa2 using the PgrCre mouse served as a control as this mouse has been shown to have an implantation defect, which could be rescued by rLIF. Intraperitoneal injections of mouse rLIF at GD 3.5 did not rescue embryo implantation in Sox17<sup>eed</sup> mice as compared with the positive

**Fig. 1** Phenotype of conditional deletion of Sox17 in the adult mouse uterus. a Embryo implantation sites (top panel) and artificial deciduoma formation (bottom panel) in Sox17<sup>ff</sup> (control), Sox17<sup>eed</sup> (LtfiCreSox17<sup>ff</sup>), and Sox17<sup>dd</sup> (PgrCreSox17<sup>ff</sup>) female mice (n = 5). Scale bars: 10 mm. IS, implantation site. b Ratio of decidual to control horn weight in Sox17<sup>ff</sup> (black dots), Sox17<sup>eed</sup> (blue squares), and Sox17<sup>dd</sup> (pink triangles) mice (n = 5). c Immunohistochemical staining of Ki67 (row 1), FOXA2 (row 2), TRP63 (row 3) in the 2-month old uteri of Sox17<sup>ff</sup>, Sox17<sup>eed</sup>, and Sox17<sup>dd</sup> female mice at GD 3.5 (n = 3). Row 4 is TRP63 staining in the 8-month old uteri (n = 3). Scale bars: 100 µm. S, stroma; LE, luminal epithelium; GE, glandular epithelium. d Number of endometrial glands in the 2-month old uteri from Sox17<sup>ff</sup> (black dots), Sox17<sup>eed</sup> (blue squares), and Sox17<sup>dd</sup> (pink triangles) mice at GD 3.5 (n = 3 mice × 2 sections per mice = 6). Different superscript letters denote significant (P < 0.05, ANOVA with Tukey’s post hoc test) differences. Data are presented as means ± S.E.M.
controls (Sox17<sup>f/f</sup> mice receiving vehicle; and Foxa2<sup>d/d</sup> mice receiving mouse rLIF) and the negative control (Sox17<sup>d/d</sup> mice receiving vehicle) (Supplementary Fig. 1i). This demonstrates that although LIF expression is dependent on SOX17, other pathways regulated by SOX17 in the GE are critical for implantation in the mouse uterus.

Analysis of the uterine epithelial morphology showed that in lieu of the simple columnar luminal epithelial layer observed in the Sox17<sup>f/f</sup> uteri, the Sox17<sup>d/d</sup> uteri displayed a second cell layer at 2-month old. This epithelium showed basal cells staining positive for TRP63<sup>34</sup>. The uterine epithelium in the Sox17<sup>d/d</sup> female mice did not demonstrate this stratification and TRP63-positive cells were only detected sporadically along the uterine epithilia in 2-month old female mice (Fig. 1c, row 3). Quantification by qRT-PCR showed that Trp63 mRNA was the highest (P < 0.05, ANOVA with Tukey’s post hoc test) in Sox17<sup>d/d</sup> uteri, and still high (P < 0.05, ANOVA with Tukey’s post hoc test) in Sox17<sup>f/f</sup> uteri as compared with Trp63 expression in Sox17<sup>f/f</sup> uteri (Supplementary Fig. 1g). Owing to the different timing of recombination events between Pgr<sup>Cre</sup> and Lif<sup>Cre</sup>, further morphological analysis and TRP63 staining were conducted in the uteri of 8-month old mice. Both Sox17<sup>d/d</sup> and Sox17<sup>f/f</sup> uteri showed similar phenotype, the following functional analysis of the role of Sox17 was conducted only using the Sox17<sup>d/d</sup> mice.

SOX17 regulates uterine E2 and P4 signaling. As uterine function is regulated by ovarian steroid hormones<sup>35–38</sup>, the effect of Sox17 ablation on E2 and P4 signaling was investigated. Immunohistochemical analyses of the uteri of GD 3.5, 2-month old Sox17<sup>d/d</sup> mice showed increases in total (ESR1) and phosphorylated (pESR1) estrogen receptor in both epithelial and stromal cells (Fig. 2a). Concomitantly, as a downstream target of ESR1, mRNA levels of Lif and Greb1 were elevated, but Lif mRNA was downregulated in Sox17<sup>d/d</sup> uteri; and no difference in Lif mRNA level was detected between Sox17<sup>f/f</sup> and Sox17<sup>d/d</sup> uteri (Fig. 2b). PGR levels are reduced in the epithelial cells, and are lower in the stromal cells owing to lack of induction during the preimplantation period. The downstream target of PGR, Areg and Ihh were also downregulated in the Sox17<sup>d/d</sup> uteri (Fig. 2b).

 Sox17 ablation impairs the Indian hedgehog signaling pathway. Noting that IHH is a major mediator of PGR signaling in the mouse uterus<sup>9</sup> and significantly reduced in Sox17-ablated uteri, we further investigated the effector downstream of IHH signaling pathways for uterine epithelial–stromal signaling (Fig. 3). Immunohistochemical analyses of the uteri of 2-month old Sox17<sup>d/d</sup> mice at GD 3.5 showed decreases in the receptors of IHH, i.e., PTCH1 and PTCH2, mainly in uterine stromal cells, as well as reduction of downstream mediators, COUP-TFI (NR2F2) and HAND2<sup>39</sup> in the stromal cells beneath the uterine epithelium (Fig. 3a). Consequently, elevated phosphorylated-FRS2 (pFRS2) was detected in the uterine epithelium of Sox17<sup>d/d</sup> mice (Fig. 3a). qRT-PCR showed a similar pattern of gene expression for of Ptch1, Nr2f2, Hand2 as well as Fgf9 and Fgf12 in the Sox17<sup>d/d</sup> uteri at GD 3.5 (Fig. 3b).

SOX17 regulates the uterine transcriptome for receptivity. To further identify the underlying molecular mechanism for the recurrent implantation failure phenotype observed in the Sox17<sup>d/d</sup> mice, an RNA microarray analysis was conducted on whole uterine tissue harvested at GD 3.5 of natural pregnancy. A total of 1595 genes were dysregulated at GD 3.5 with 760 genes upregulated and 835 genes downregulated (Supplementary Data 1). Among dysregulated genes, 125 genes were directly associated with uterine receptivity and implantation as shown in the heatmap (Fig. 4a), including E2-responsive genes (e.g., Clda3, Fad10, Greb1, Iqg1bp5, Wnt4, Lif, Muc1, Spry2), P4-responsive genes (e.g., Fosl2, Il6, Mxa1, Mxs2, Mxs3, Osmr, Runx1, Stat3), LE-specific genes (Cldn7, Alox12e, Calb1, Ch5, Cnn3, Cobl, Cst1, Hko, Hand1, Jam2, Arge), GE-specific genes (Calca, Cbs, Foxa2, Ier3, Ilirf, Lif, Prss28, Prss29, Spry3), hedgehog signaling-associated genes (Ihh, Dhh, Glipr2, Ptc1, Ptc2, Fgf12), Pan-uterine epithelial-associated genes (e.g., Btg2, Cdl4, Gata1, Lif, Muc1, Muc16, Mti, Mt2, Sox17, Spry2), and other uterine receptivity- and implantation-related genes (Foxo1, Notch1, Notch3). The expression in the murine endometrium of some of these genes was validated by qRT-PCR (Supplementary Fig. 4). The top-enriched Ingenuity Pathway annotations for the aberrantly regulated gene list (1595 genes) included (1) reproductive system development, function, and abnormalities; (2) cardiovascular system development and function; (3) cancer and dermatological diseases; (4) inflammatory response; and (5) molecular transport (Fig. 4b). The entire list of functional annotations can be found as Supplementary Data 2. Of interest, pathways associated with endometriosis (affected), implantation (deactivated), epithelial cancer (activated), inflammation (activated) as well as transport and secretion of molecules (deactivated) were affected. The pathways altered are involved in developmental cell proliferation and implantation, which are all important for embryo implantation.

Next, we performed a correlation search to determine which available data sets in the Illumina BaseSpace Correlation Engine database showed similarity to the gene expression profile of Sox17<sup>d/d</sup> in the uterus during the window of receptivity. The top two data sets that share similar gene expression patterns with SOX17-regulated transcriptome at GD 3.5 were (1) FOXA2-regulated transcriptome and (2) ARID1A-regulated transcriptome (Fig. 4c). The Sox17<sup>d/d</sup> and Foxa2<sup>d/d</sup> transcriptomes share 482 affected genes with 212 genes upregulated and 235 genes downregulated (Supplementary Data 3). This finding further supports our hypothesis that the loss of gland epithelial functions in the Sox17<sup>d/d</sup> mice is likely through the Foxa2-mediated pathway. The Sox17<sup>d/d</sup> and Arid1a<sup>d/d</sup> transcriptomes shared 344 common dysregulated genes with 106 genes upregulated and 178 genes downregulated (Supplementary Data 4). Although Arid1a mRNA level did not changed in the Sox17 KO microarray, immunohistochemical analyses showed the reduction of ARID1A in the Sox17<sup>d/d</sup> uteri as compared with Sox17<sup>f/f</sup> uteri at GD 3.5 (Fig. 4d, row 1), which could be owing to the posttranslational regulation. However, no difference in SOX17 expression was detected between Arid1a-ablated and wildtype uteri at GD 3.5 (Fig. 4d, row 2), suggesting a hierarchical role of SOX17 in regulation of ARID1A that is critical for uterine fertility and is remarkably lower in the endometrium from women with endometriosis.<sup>40</sup>

The SOX17 signaling pathway is present in human endometria. To investigate SOX17 role in human endometrial function, we analyzed its expression pattern in endometrial tissue from women with and without endometriosis at proliferative and secretory phases (n = 7 per phase per disease). SOX17 was highly expressed in the endometrial epithelia of women without endometriosis at the proliferative (H-score: 288.00 ± 8.25) and secretory phases (H-score 278.57 ± 9.01), However, its expression was significantly...
decreased in the endometrial epithelia of women with endometriosis at the proliferative (H-score; 119.29 ± 38.83) as well as secretory phases (H-score 48.57 ± 12.94) compared with controls (Fig. 5a, b). This demonstrates that the uterine epithelium expression of SOX17 is conserved between mouse and human, and in the human endometrium SOX17 expression is impacted by endometriosis. Next, the correlation between SOX17 and IHH were examined in the human endometrium. SOX17 and IHH mRNA levels were positively correlated in human endometrium from two independent cohorts, GSE4888 and GSE58144 (Fig. 5c), supporting a conservation of the expression between mouse and human. When comparing genes altered in the Sox17-ablated uteri at GD 3.5 with human endometrial receptivity biomarkers41–43, 31 human orthologs were identified (Fig. 5d). During the window of receptivity, 23 of these genes were downregulated in Sox17-ablated uteri but upregulated in receptive human endometrium; and eight genes were upregulated in Sox17-ablated uteri but downregulated in human endometrium, suggesting the role of SOX17 in human and mouse endometrium is conserved.

SOX17 and major uterine regulators show genome co-occupancy. As Sox17 ablation affected uterine epithelial differentiation, it is difficult to discern which gene expression changes are due to direct transcriptional regulation of gene expression or the consequences of epithelial cell differentiation. To define which genes are potentially regulated by SOX17 at the level of transcription, Sox17+/− mice were ovarioctomized, rested for 2 weeks, and then administered P4. After 6 hours, uterine horns were flash frozen and the tissue was subject to the ChIP-seq assay. The

Fig. 2 Dysregulated estrogen and progesterone signaling in Sox17-deficient uterus during the window of receptivity. a Immunohistochemical staining of ESR1, phosphorylated ESR1 and PGR in GD 3.5 uteri from Sox17+/− and Sox17+/−/− mice (n = 3). IgG staining as the negative control. Scale bars: 100 μm. b Quantification of ESR target genes (Lif, Greb1, Lif, Lifr), as well as PGR target genes (Areg, Ihh) in GD 3.5 uteri from Sox17+/− (black dots) and Sox17+/−/− (red dots) mice (n = 7). *P < 0.05 (Student’s t test). Data are presented as means ± S.E.M.
SOX17 ChIP-seq demonstrated enriched binding at promoter and 5’ untranslated regions (Supplementary Fig. 3a, b). Homer de novo motif analysis described SOX17 primarily bound at locations with nuclear receptor response elements, in which SOX, GATA families as well as PGR were among top-enriched motifs (Supplementary Fig. 3c). As a result, 11,277 genes were identified as SOX17-bound within 25 kb upstream and/or downstream of the gene boundary (Supplementary Data 5 and 6).

To identify genes potentially regulated by SOX17, differentially expressed genes between Sox17+/− and control uteri were overlapped with the SOX17-bound gene list. Of the 1595 differentially regulated genes, 807 genes were identified as containing SOX17-binding sites within 25 kb upstream and/or downstream of the gene boundary (Fig. 6a and Supplementary Data 7). Of particular interest, 78 genes have been shown to be associated with uterine receptivity and implantation, 34 of which are uterine epithelial-specific genes (Fig. 6a). Based on the findings that Sox17+/− and Foxa2+/− transcriptomes largely overlap and Sox17 is a target of PGR, which is regulated by GATA2 during the early pregnancy, we evaluated common binding peaks by comparing SOX17 ChIP-seq dataset with three other independent mouse ChIP-seq data sets (FOXA2, PGR, and GATA2). This analysis identified 624 common peaks (assigned to 737 genes) (Fig. 6b). When further overlapping with 1595 differentially regulated genes in Sox17+/−-ablated uteri, 92 genes were identified as SOX17 target genes with shared binding sites by FOXA2, PGR, and GATA2. Many of them (Areg, Btg2, Cnn3, Cobl, Glul, Ihh, Irg1, Lrp2, Pfkfb3, Sox17, and Sprr2f) are well-known uterine epithelial-specific genes, indicating SOX17 may interact with other transcription factors to regulate uterine gene expression.

Interestingly, in vivo SOX17-binding events occurred at putative enhancer 19 kb upstream of Ihh gene locus (Fig. 6c). ChIP-seq analysis has demonstrated that FOXA2, PGR, and GATA2 are also bound in this region (Fig. 6c). This region 19 kb upstream of the Ihh gene is located at the intragenic region of Nhej1 gene. Nhej1−/− mice have been generated and the disruption did not alter the Ihh region. The Nhej1−/− mice were viable, and fertile. Functional analysis of the role of the Ihh region can be conducted without any concerns that altering the expression of Nhej would confound the analysis of the uterine phenotype. Therefore, to evaluate the significance of these binding sites on uterine receptivity, particularly Ihh signaling, we used CRISPR/Cas9 technology to ablate this putative enhancer in vivo (Fig. 6c). Thus, the mouse line (Ihh19d/d) was generated with the deletion of 577 bp containing the SOX17 putative enhancer (Fig. 6c and Supplementary Fig. 3d).
Fig. 4 SOX17 regulation of uterine transcriptome. a Heatmap of uterine function-associated genes differentially regulated between Sox17fl/fl and Sox17ed/ed mouse uteri at GD 3.5 generated from microarray analysis. b Enrichment of functional annotation in differentially expressed genes between Sox17fl/fl and Sox17ed/ed mouse uteri at GD 3.5 by IPA analysis. c Overlaps and correlations of Sox17 KO with Foxa2 KO or Arid1a KO transcriptomes generated by NextBio gene expression profile comparison. d Immunohistochemical staining of ARID1A in GD 3.5 uteri from Sox17fl/fl and Sox17ed/ed mice (n = 3; row 1), as well as SOX17 in GD 3.5 uteri from Arid1af/f and Arid1ad/d (PgrCreArid1af/f) mice (n = 3; row 2) Scale bars: 100 µm.
An endometrial enhancer for Ihh controls uterine receptivity. Mice bearing the deletion 19 kb upstream of the Ihh gene were born at the normal Mendelian frequencies with no obvious skeletal defects as reported in mice with germ line deletion of Ihh. This deletion did not impact the expression for Ihh that is required for normal development. We then assayed the impact of Ihh19 deletion on the expression and tissue specificity of Ihh in the intestine (Fig. 7a). Immunohistochemical staining further confirmed the downregulation of IHH in the Ihh19d/d uteri at GD 2.5 (Supplementary Fig. 4a). However, the loss of IHH expression was not complete as cells positive for IHH could be identified in the Ihh19d/d uteri at GD 2.5 (Supplementary Fig. 4a). As the mice did not show the other non-uterine phenotypes associated with Ihh ablation, the deletion of the enhancer of the Ihh flanking region impacted was specific to the uterus.

The phenotypic consequences of the deletion of the Ihh19-binding region were then assayed based on the ability of the uterus to support pregnancy (including implantation sites), undergo an artificial induced decidual reaction, IHH signaling in the uterus and uterine morphology. Ihh19d/d females showed subfertility, as evidenced by the lower number (P < 0.05) of pups born per female and pups per litter in 6-month breeding trials compared with the Ihh19+/+ mice (Fig. 7b). Further, the lower number (P < 0.05) of implantation sites at GD 5.5 (Fig. 7c, d) and compromised decidualization (Fig. 7e, f) may account for such subfertility of Ihh19d/d females. The decrease in implantation sites was not due to an impact on ovulation or embryo development as there was no difference in the number of embryos flushed from the uterus and oviduct at GD 2.5 between Ihh19d/d and control mice at GD 2.5 (Supplementary Fig. 4b). There was no difference in the serum P4 level between these two groups, indicating a normal endocrine regulation of hormone secretion (Supplementary Fig. 4c).

Immunohistochemical analyses showed the expression levels and distribution of uterine epithelial SOX17 and FOXA2 in the uterine GE were not different between Ihh19+/+ and Ihh19d/d mice at GD 3.5 (Fig. 7h). Also, there was no TRP63-positive staining for basal cells in the uterine epithelia between them (Fig. 7h). However, analysis of the IHH signaling axis showed that Ihh19d/d mice exhibited significant reduction of PTCH1, COUP-TFI, HAND2 in the stromal cells, and increases in pFRS2 mostly in the epithelial cell of uteri at GD 3.5 with human endometrial receptivity biomarkers. Green font denotes gene downregulation; red font denotes gene upregulation.
3.5 (Fig. 7j). Total ESR1 was upregulated mainly in the stromal cells and pESR1 increased in both epithelial and stromal compartments of Ihh19d/d uteri as compared with that of Ihh+/+ mice at GD 3.5 (Fig. 7i). In addition, PGR decreased in epithelial and stromal cells of Ihh19d/d uteri as compared with that of Ihh+/+ mice (Fig. 7i). Although the uterine ablation of Ihh rendered mice completely infertile, Ihh19d/d mouse exhibited a hypomorphic phenotype of the uterine Ihh deletion most likely due to an incomplete ablation of Ihh as evidenced by detection (P < 0.05) of Ihh mRNA in decidua rather than the contralateral horn of Ihh19d/d uterus at DD2 (equivalent to GD6.5) (Fig. 7g).
Fig. 7 The SOX17-binding region 19 kb upstream of Ihh gene governs the window of uterine receptivity. **a** Quantification of Sox17, Areg, Ihh gene expression in uteri and/or intestine from Ihh+/+ and Ihh19d/d female mice at GD2.5 (n = 5). **b** Pups born from Ihh+/+ and Ihh19d/d female mice during 6-month breeding trial (n = 4). **c, d** Embryo implantation sites (c) and quantification (d) in uteri from Ihh+/+ and Ihh19d/d female mice at GD5.5 (n = 5). Scale bars: 10 mm. **e, f** Artificial deciduoma formation (e) and ratio of decidual to control horn weight (f) in Ihh+/+ and Ihh19d/d female mice at DD2 (n = 6) and DD5 (n = 5). Scale bars: 10 mm. **g** Quantification of Ihh gene in decidual and contralateral horn from Ihh+/+ and Ihh19d/d female mice at DD2 (n = 6). **h-j** Immunohistochemical staining of SOX17, TRP63, FOXA2, ESR1, pESR1, PGR, PTCH1, COUP-TFI, HAND2, and pFRS2 in GD 3.5 uteri from Ihh+/+ and Ihh19d/d mice (n = 3). Scale bars: 100 μm. Black dots denote Ihh+/+ and yellow dots denote Ihh19d/d. *P < 0.05 (Student’s t test for a, b, d, and ANOVA with Tukey’s post hoc test for f, g). Data are presented as means ± S.E.M.
Pregnancy, in which SOX17 and PGR co-regulated the PGR binding region distal to the Ihh regulated by SOX17 is development and differentiation, embryo implantation and the regulating uterine epithelial cell proliferation, uterine gland here we demonstrate that uterine epithelial SOX17 is critical for Discussion

Here we demonstrate that uterine epithelial SOX17 is critical for regulating uterine epithelial cell proliferation, uterine gland development and differentiation, embryo implantation and the ability of the uterus to support pregnancy (Fig. 8). Transcriptomic and cistromic analyses have demonstrated that SOX17 regulates the transcription of uterine epithelial genes. Among the genes regulated by SOX17 is Ihh. Ablation of Sox17 or the SOX17-binding region distal to the Ihh gene alters proper epithelial–stromal cross-talk in the uterus required for pregnancy, in which SOX17 and PGR co-regulated the PGR signaling and IHH signaling. The impaired IHH signal transmission from epithelial to stroma, subsequently affected the stromal PGR via its regulator, the stromal COUP-TFII (Fig. 8). SOX17 expression is conserved in the human endometrium and many of the genes regulated by SOX17 in the mouse are shown to be markers of uterine receptivity in humans.

Sox17 has been identified as altered in endometriol cancer. Mice generated in this report (i.e., Sox17^+/− and Sox17^+/−) do not develop endometrial cancer. This indicates that Sox17 is not a primary driver of endometrial cancer. However, this analysis shows that SOX17 inhibits uterine epithelial cell proliferation. Also, the transcriptomic alterations in response to epithelial Sox17 ablation overlap with Arid1a-ablated transcriptome. ARID1A is highly mutated in endometriosis associated cancers such as ovarian cancer and endometrial cancer. This indicates that the role of Sox17 in the development of cancer of the uterus may be in the modification of a critical regulator of endometrial cell homeostasis.

Previously, it has been reported that female mice with Sox17 haploinsufficiency are subfertile owing to impaired implantation. This was not observed in the Sox17^+/− or Sox17^+/− in this study, in which Sox17^+/− (PgrCre/−;Sox17^+/+) produced an average litter size of 5.3 ± 0.79 pups/litter (n = 5 dam), whereas Sox17^+/− females gave litters of 5.9 ± 0.89 pups/litter (n = 4 dam), suggesting that the decrease in fertility in the Sox17^+/− mice is likely owing to the non-uterine actions of SOX17. It has also been reported that mice with epithelial deletion of Sox17 using the Spry2^cre mice are infertile with normal adenogenesis. Our findings further show that ablating Sox17 in the uterus epithelium by the Lif^Cre results in complete abolition of embryo implantation, which only agrees with the previous report but also provides a more detailed functional timeline of epithelial SOX17 on fertility. The Spry2^Cre-mediated Sox17 knockout model also show the presence of glands in the uterus, which differs from that of the Pgr^Cre-mediated Sox17 ablation model. It was speculated that the persistence of glands was due to an involvement of SOX17–expressing stromal cells. Our results identified the SOX17–expressing cells in the stromal compartment as endometrial cells. Moreover, deleting Foxa2 in uterine epithelium after puberty also allows establishment of glands despite of infertility. Therefore, the presence of glands in both Spry2^Cre and Lif^Cre models is likely owing to the timing of Sox17 ablation. In both models, gene recombination is delayed until after puberty.

The role of Sox17 in the mouse uterus is twofold. First, it regulates uterine gland development and differentiation. Second, it regulates the expression of key genes regulating uterine fertility. The loss of epithelial Sox17 postnatally reduced the number of glands and caused the epithelial stratification in the 2-month old mouse uterus. Postpubertal ablation of Sox17 resulted in a delay in epithelial stratification and no glandular reduction in the 8-month old uterus. However, uterine gland function was altered as evidenced by a decrease in Lif expression. This suggests that, like Foxa2, Sox17 regulates postnatal adenogenesis and once the uterine glands are developed they regulate gland function. Furthermore, the significant reduction of FOXA2 in the GE of Sox17-ablated uteri at GD 3.5, and no binding of SOX17 to the Foxa2 gene locus suggest an indirect regulation of SOX17 on Foxa2 gene expression. Notably, the loss of Lif in this model was not the only cause of the infertility as administration of recombinant LIF did not rescue the implantation phenotype. This observation suggests that additional pathways parallel to the FOXA2-LIF axis are also required for postpubertal gland functions.

The epithelial stratification with the expression of basal cell marker TRP63 in Sox17^+/− KO is similar to the uterine phenotypes seen after ablation of β-catenin, Wnt5a, or Gata2 and conditional uterine activation of smoothened. This places SOX17 as a regulator of uterine epithelial integrity along with P4, WNT, and hedgehog signaling. SOX17 is reported to inhibit WNT signaling but work in concert with these signaling pathways to regulate uterine epithelial differentiation. Furthermore, the significant reduction of FOXA2 in the GE of Sox17-ablated uteri at GD 3.5, and no binding of SOX17 to the Foxa2 gene locus suggest an indirect regulation of SOX17 on Foxa2 gene expression. Notably, the loss of Lif in this model was not the only cause of the infertility as administration of recombinant LIF did not rescue the implantation phenotype. This observation suggests that additional pathways parallel to the FOXA2-LIF axis are also required for postpubertal gland functions.
and glandular differentiation. Similar transcriptomic alteration between Sox17+/−ed and Foxa2+/−ed further suggest the essential role of SOX17 in adenogenesis and maintenance of functional glands in the uterus. Transcriptomic, cistronic, and genome editing analyses indicate that the role SOX17 plays in regulating uterine function may not be secondary to alterations in uterine epithelial differentiation, but may be due to direct regulation of genes critical for uterine fertility. Analysis of the binding sites for SOX17 demonstrated 737 genes that share the common binding sites (624 peaks) for PGR, GATA2, and FOXA2. Among those genes, 92 were identified in response to SOX17 regulation via the shared uterine-specific enhancers, i.e., non-coding regions harboring cis-regulatory elements that are bound by transcription factors and may define cellular identity.33,34. The putative enhancer 19 kb upstream to the Ihh gene was identified based on SOX17, FOXA2, and PGR, and GATA2 ChIP-seq data from uterine tissue. Thus, Ihh enhancer was further deleted in vivo using CRISPR/Cas9 technology and validated by sequencing. The mice were subfertile with impaired IHH signaling only in uterine tissue, confirming the unique distal enhancer bound by SOX17 that regulates Ihh gene. The analysis of this enhancer region has identified a class of transcription factor binding sites that may be critical for uterine epithelial gene expression. It is easy to speculate that some combinations of these transcription factors would allow the differentiation of progenitor cell to achieve a uterine epithelial cell phenotype. Uterine epithelial cells are difficult to culture in vitro, therefore understanding regulation of and by these transcription factors may lead to the development of better in vitro models for uterine epithelial function.

Methods

Human endometrial samples. This study was carried out in accordance with federal regulations governing human subjects research. All procedures were approved by the following ethics committees: Institutional Review Board/Committee-A (IRB) of Greenville Health System under IRB file # Pro00000993 and Pro00013885 and the University of Cape Hill at North Carolina IRB under file #: 05-1757. Informed consent was obtained from all patients before their participation in this study. To examine SOX17 expression patterns of eutopic endometrium between women with and without endometriosis (control vs. endometriosis group), 28 samples in total were used from proliferative and secretory phases (n = 7 per phase per group).

Animals. All animal studies were conducted in accordance with the National Institute of Environmental Health Sciences (NIEHS) Animal Care and Use Committee guidelines and in compliance with NIEHS-approved animal protocol. Mice carrying the Sox17+/− allele (stock no: 007686, the Jackson Laboratory, Bar Harbor, ME) were bred to Ltficre/+ or Pgrcre/+ mice to generate Sox17+/− or Pgr+/− mice to generate Ltficre/+ or Pgrcre/+ Sox17+/− or Pgr+/− Sox17+/− mice, respectively.

CRISPR/Cas9-mediated deletion of the enhancer region in vivo. CRISPR/Cas9 system targeting Ihh enhancer region were identified using MIT CRISPR Design tool (http://crispr.mit.edu55–57, i.e., GTGACA-GCTGGAGCGGCTTTA, and GTTACATGAGGACCGACTAT). Each sgRNAs were cloned into the pDR274 plasmid vector, and in vitro transcribed using the MEGAscriptT T7 kit (Life Technologies). Cas9 mRNA was in vitro synthesized. Cas9 mRNA (30 ng µl−1) and sgRNAs (25 ng µl−1 for each) were microinjected into the cytoplasm of fertilized eggs of superovulated C57BL/6 female mice (JAX) and implanted into oviducts of pseudopregnant fosterers. Founder mice were bred with wildtype C57BL/6 mice to obtain F1 heterozygous (Ihh19+/−) mice. F1 mice with an identical genotype were interbred to generate F2 homozygous (Ihh19−/−) mice. Immunohistochemistry and dual immunofluorescence. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Embedded tissues were sectioned at 5 µm and baked 1 hour at 60°C. Upon cooling, slides were dewaxed using Citrotsolv clearing agent (catalog no. 22-143-975, Thermo Fisher, Waltham, MA, USA) in a decreasing gradient of pure ethanol. For hematoxylin and eosin (H&E) staining, tissues were adequately stained with H&E and were then dehydrated before coverslips were applied. For immunohistochemistry, antigen retrieval was performed according to manufacturer’s instructions (Vector Labs Antigen Unmasking Solution H-3300). Endogenous peroxide was blocked using 3% hydrogen peroxide diluted in methanol. The tissue was blocked with 5–10% normal donkey serum before application of primary antibody overnight at 4°C. Secondary antibody was diluted in 1% bovine serum albumin at a concentration of 1:1000. The ABO secondary antibody was applied to tissues before applying a manual stimulus to a single uterine horn (protocol outlined previously by Finn and Martin, 1972)38. After ovariectomy and 2 weeks of rest to eliminate endogenous ovarian steroids, mice were administered daily E2 (100 ng per mouse) injections for 3 days. After 2 days of rest, mice were treated with E2 (6.7 ng per mouse) and P4 (1 mg per mouse) for 3 days. On the third day of treatment, mice were administered daily injection of 0.05 mg estradiol benzoate to the right uterine horn. Mice were administered E2 and P4 for 5 more days and killed on the fifth day (DD5). Uterine wet weights for the stimulated and control horns were recorded. Weight ratios were calculated by dividing stimulated horn weight by unstimulated horn weight.

Superovulation assay. Superovulation was induced in female mice by i.p. injection of five international units (IU) of pregnant mare’s serum gonadotropin (EMD Millipore, Billerica, MA), followed by 5 IU of human chorionic gonadotropin (Pregnyl, Merck & Co., Inc., Whitehouse Station, NJ) 48 h later and placed with wildtype C57BL/6 male mice. The mice were killed 24 h later by cervical dislocation while under the anesthetic, Avertin (2,2-tribromoethyl alcohol, Sigma-Aldrich, St. Louis, MO) and oocytes were flushed from the oviducts and counted.

LIF rescue assay. The attempt to rescue the implantation defect in the Sox17+/−ed mice was performed using the protocol described by Kelleher et al.33. In brief, female mice received two intraperitoneal (i.p.) injections (one at 1000 h and one at 1800 h) of vehicle or 10 μg recombinant mouse LIF on GD 3.5, and embryo implantation sites were evaluated on GD 5.5.

Immunohistochemistry and dual immunofluorescence. Tissues were fixed in 4% paraformaldehyde and embedded in paraffin. Embedded tissues were sectioned at 5 µm and baked 1 hour at 60°C. Upon cooling, slides were dewaxed using Citrotsolv clearing agent (catalog no. 22-143-975, Thermo Fisher, Waltham, MA, USA) in a decreasing gradient of pure ethanol. For hematoxylin and eosin (H&E) staining, tissues were adequately stained with H&E and were then dehydrated before coverslips were applied. For immunohistochemistry, antigen retrieval was performed according to manufacturer’s instructions (Vector Labs Antigen Unmasking Solution H-3300). Endogenous peroxide was blocked using 3% hydrogen peroxide diluted in methanol. The tissue was blocked with 5–10% normal donkey serum before application of primary antibody overnight at 4°C. Secondary antibody was diluted in 1% bovine serum albumin at a concentration of 1:1000. The ABO secondary antibody was applied to tissues before applying a manual stimulus to a single uterine horn (protocol outlined previously by Finn and Martin, 1972)38. After ovariectomy and 2 weeks of rest to eliminate endogenous ovarian steroids, mice were administered daily E2 (100 ng per mouse) injections for 3 days. After 2 days of rest, mice were treated with E2 (6.7 ng per mouse) and P4 (1 mg per mouse) for 3 days. On the third day of treatment, mice were administered daily injection of 0.05 mg estradiol benzoate to the right uterine horn. Mice were administered E2 and P4 for 5 more days and killed on the fifth day (DD5). Uterine wet weights for the stimulated and control horns were recorded. Weight ratios were calculated by dividing stimulated horn weight by unstimulated horn weight.

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delta Ct values were calculated using 18 S control amplification results to acquire relative mRNA levels per sample. Information for all primers is provided in Supplementary Table 2.

**Microarray analysis.** RNA quality was assessed using the Agilent 2100 Bioanalyzer (Agilent Technologies). Microarray analysis was performed by the Genomic and RNA Profiling Core at Baylor College of Medicine. After library amplification and labeling, individual cDNA samples were hybridized to the Agilent G3 Mouse GE 8 × 60 k array according to manufacturer’s instructions (Agilent Technologies). Array data were analyzed using Bioconductor with quantile normalization. Genes with an unadjusted p value < 0.01, and an absolute fold change ≥ 1.4 were identified as differentially regulated.

**Chromatin immunoprecipitation and sequencing.** Whole uterine tissue was flash frozen and sent to Active Motif company for Factor-Path chromatin immunoprecipitation and sequencing (ChiP-seq) analysis. The tissue was fixed and then promptly sheared into small fragments before immunoprecipitation with the SOX17 antibody (AF1924; R&D Systems). SOX17-bound DNA was purified and amplified to generate a library and sequenced using Illumina’s Genome Analyzer 2. The raw ChiP-Seq reads (36 nt, single-end) were processed and aligned to the mouse reference genome (mm9; Genome Reference Consortium Mouse Build 37 from July 2007) by Active Motif. SOX17-bound intervals were identified using Model-based analysis of ChiP-seq (MACS 1.3.7.1) and mapped to nearby genes within 25 Kb by developing a Perl script.

**Data analysis.** The differentially expressed genes generated from microarray data were analyzed using Ingenuity Pathway Analysis software (IPA, http://www.ingenuity.com) and Database for Annotation, Visualization, and Integrated Discovery (DAVID, http://david.ncifcrf.gov/). The similar transcriptional to the Sox17 gene expression profile were identified by searching available data sets in the NextBio database (https://www.nextbio.com/). The ChiP-Seq data were analyzed using the Cistrome software (http://cistrome.org/ap/). The GraphPad Prism software was implemented for one-way ANOVA, multiple comparison test, and Student’s t test analyses for qRT-PCR and decidual wet weights. Hormone response elements were identified using HOMER de novo motif analysis (http://homer.salk.edu/homer/). Hierarchical clustering heatmaps were generated using Partek Genomics Suite 6.6 software.

**Data availability.** The data that support the findings of this study are available from the article and Supplementary Information files, or from the corresponding author upon request. Raw FASQ data files (GSE18264) and Genome-wide sequencing data (GSE118327) are deposited in the NCBI Gene Expression Omnibus database under SuperSeries accession number GSE118328.

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Author contributions

X.W., X.L., F.J.D and F.J.D designed the experiment. X.W. and X.L conducted experiments including: animal fertility assay, embryo implantation assessment, artificial decidualization assay, immunohistochemistry, immunofluorescence, qRT-PCR, Microarray, and ChIP-seq analyses. X.L. and F.J.D established and performed initial characterization of the Ihh19 deletion mouse line. J.J. and T.H.K. conducted experiments related to the Arid1a deletion mouse model and human endometrial tissues. S.L.Y. and B.A.L. collected human endometrial specimens. X.W., T.W., S.W. and R.B.L. each performed a subset of data analysis. X.W., J.J. and F.J.D. wrote the manuscript.

Additional information

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