COUP-TFII Mediates Progesterone Regulation of Uterine Implantation by Controlling ER Activity

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Progesterone and estrogen are critical regulators of uterine receptivity. To facilitate uterine remodeling for embryo attachment, estrogen activity in the uterine epithelium is attenuated by progesterone; however, the molecular mechanism by which this occurs is poorly defined. COUP-TFII (chicken ovalbumin upstream promoter transcription factor II; also known as NR2F2), a member of the nuclear receptor superfamily, is highly expressed in the uterine stroma and its expression is regulated by the progesterone–Indian hedgehog–Patched signaling axis that emanates from the epithelium. To further assess COUP-TFII uterine function, a conditional COUP-TFII knockout mouse was generated. This mutant mouse is infertile due to implantation failure, in which both embryo attachment and uterine decidualization are impaired. Using this animal model, we have identified a novel genetic pathway in which BMP2 lies downstream of COUP-TFII. Epithelial progesterone-induced Indian hedgehog regulates stromal COUP-TFII, which in turn controls BMP2 to allow decidualization to manifest in vivo. Interestingly, enhanced epithelial estrogen activity, which impedes maturation of the receptive uterus, was clearly observed in the absence of stromal-derived COUP-TFII. This finding is consistent with the notion that progesterone exerts its control of implantation through uterine epithelial-stromal cross-talk and reveals that stromal-derived COUP-TFII is an essential mediator of this complex cross-communication pathway. This finding also provides a new signaling paradigm for steroid hormone regulation in female reproductive biology, with attendant implications for furthering our understanding of the molecular mechanisms that underlie dysregulation of hormonal signaling in such human reproductive disorders as endometriosis and endometrial cancer.

Introduction

Establishment of uterine receptivity is mandatory for successful embryo apposition, attachment, and implantation; failure to manifest this uterine state is an underlying cause of most pregnancy failures in women. A multitude of signaling molecules have been shown to play key roles in the elaboration of this uterine response through mesenchymal–epithelial interaction. Among numerous factors involved in these primary events of pregnancy, two steroid hormone receptors, progesterone receptor (PR) and estrogen receptor (ER), and their cognate ligands, undoubtedly play central roles in this biological process [1–3]. Although estrogen activity is essential for an integrated uterine response, it has been shown that excessive estrogen activity can prematurely close the implantation window [4], suggesting that estrogen activity is tightly controlled during the peri-implantation period to allow normal development of the receptive uterus. Importantly, progesterone is known to attenuate estrogen-induced gene expression in uterine epithelial cells [5]. Intriguingly, this suppression is mediated by stromal progesterone receptors [6,7], suggesting that the coordinated action of estrogen and progesterone depends on crosstalk between the epithelial and stromal compartments of the uterus. Although the inhibitory effect of progesterone on epithelial estrogen activity has been described [6,7], the mechanism by which progesterone suppresses estrogen action remains poorly defined.

Lydon et al have shown that female PR-null mice are infertile [8]. The expression of Indian hedgehog (Ihh), a gene highly expressed in the uterine epithelium, is greatly reduced in these null mutants, indicating that Ihh is a downstream target of the progesterone receptor [9]. To understand the role of Ihh in reproduction, conditional null mutant mice of Ihh were generated [10]. These mutants exhibit defects in...
The Roles of COUP-TFI in the Uterus

Author Summary

Pregnancy is established and maintained through a series of precisely choreographed cellular and molecular events that are controlled by two sex hormones, estrogen and progesterone. Both hormones exert their actions through their distinct nuclear receptors. During the peri-implantation period, estrogen activity is attenuated by progesterone to facilitate epithelial remodeling and embryo attachment, but the detailed molecular mechanism of how this process is achieved remains largely undefined. COUP-TFI (chicken ovalbumin upstream promoter transcription factor II; also known as NR2F2), a member of the nuclear receptor superfamily, is highly expressed in the uterine stroma, and its expression is controlled by progesterone–Indian hedgehog–Patched signaling from the epithelium to the stroma. To assess the uterine function of COUP-TFI, uterine-specific COUP-TFI knockout mice were generated. These mutant mice are infertile due to failure of implantation. We identified a novel genetic pathway in which the epithelial Ihh regulates the stroma COUP-TFI to control BMP2 and regulates decidualization. Interestingly, enhanced epithelial estrogen activity, which impedes the maturation of receptive uterus, was clearly noted in the absence of COUP-TFI. This finding reveals that COUP-TFI plays a critical role in maintaining the balance between estrogen and progesterone activities to establish proper implantation. This finding also provides new insights into women’s health care associated with uncontrolled estrogen activity, such as breast cancer and endometriosis.

Results

COUP-TFI Expression Is Efficiently Ablated in the Uterus of COUP-TFI Mutant Mice

Conditional knockout mice of COUP-TFI, PRCre/+ COUP-TFIcre/+ were generated by crossing PR-Cre knockin mice with COUP-TFIcre/+ mice. PR is highly expressed in the uterine stroma and epithelium, while COUP-TFI is highly expressed in the stroma, but rarely expressed, if ever, in the uterine epithelium [18,22]. Immunohistochemistry of the reproductive tract indicated that COUP-TFI is efficiently ablated in the stroma of the mutant uterus by the PR-Cre (Figure 1A and 1B). It is also evident from this figure that COUP-TFI is highly expressed in the stroma compartment but is hardly detectable in the luminal and the glandular epithelia of the uterus. In contrast, PR is expressed in the granulosa cells, while COUP-TFI is expressed in the theca cells of the ovary [18,22]. Since PR and COUP-TFI are not expressed in the same cell, COUP-TFI is not ablated in the theca cells. As expected, the expression of COUP-TFI in the theca cells of the ovary is not altered in the conditional COUP-TFI mutants comparison with controls as shown by immunostaining (Figure 1C–1E). To ensure there is no disruption of ovarian function in COUP-TFI mutants, we transferred ovaries from control and mutant mice to wild-type recipients and observed reproduction for a 6-mo period. Healthy newborns were yielded from PRCre/+ COUP-TFIcre/+ ovaries in a similar manner as the controlled PRCre/+ and PRCre/+ COUP-TFIcre/+ ovaries (Table 1). In addition, the litter size from the mutant ovaries was not significantly reduced...
Figure 1. COUP-TFII Is Efficiently Deleted by PR-Cre, and Resulting Mutants Are Infertile

However, COUP-TFII is not deleted in the ovary and, thus, mutants exhibit no ovarian defects. (A–B) Immunohistological detection of COUP-TFII in the uterus. (A) Control, 4.5 dpc. COUP-TFII is highly expressed in the endometrial stroma, but is undetectable in the epithelial compartment. (B) Mutant, 4.5 dpc. COUP-TFII expression is efficiently ablated in the mutant uterus. (C–D) Immunohistological detection of COUP-TFII in the ovary. (C) Control, 4.5 dpc. COUP-TFII is highly expressed in the theca cell layer. (D) Mutant, 4.5 dpc. COUP-TFII expression is maintained in the theca cell layer. (E) The gene expression of COUP-TFII, assayed by quantitative real-time RT-PCR. COUP-TFII expression is efficiently ablated in the uterus but not affected in the ovary. White bar: control; black bar: mutant. *p < 0.001 (t-test, n = 9). (F) Summary of breeding studies. PR^{Cre+} COUP-TFII^{flox/flox} mutants are infertile, while both controls are normal in fertility. doi:10.1371/journal.pgen.0030102.g001
Compared with that of controls, indicating that the $PR^{Cre/+}$ COUP-TFII females have no ovarian defects (Table 1). Thus, the implantation failure observed in our conditional mutants is likely due to impaired uterine, but not ovarian, function.

**COUP-TFII Mutants Are Infertile Due to Failure of Embryo Attachment**

$PR^{Cre/+}$ COUP-TFII$^{floxed}$ mutant mice and COUP-TFII$^{floxed}$ control mice were mated with wild-type males (B6SJLF1; Taconic) and observed for 6 mo to compare breeding capacity. $PR$-Cre mice were also used as a control to distinguish the contribution of the $PR$-Cre allele. Pups were not born from mutant females, while both types of controls gave birth regularly (Figure 1F), indicating that ablation of COUP-TFII in the uterus leads to infertility. The hormone profile during pregnancy showed no significant difference in estradiol (control, 45.3 ± 3.8 pg/ml; mutant, 47.9 ± 3.2 pg/ml; n = 14, 3.5 d postcoitus [3.5 dpc]) and progesterone levels (control, 15.8 ± 4.4 ng/ml; mutant, 18.6 ± 2.9 pg/ml; n = 12, 3.5 dpc) between mutants and controls, further supporting the fact that $PR^{Cre/+}$ COUP-TFII$^{floxed}$ mice have no obvious ovarian defect as stated above (Figure 1C–1E; Table 1).

To dissect the cause of infertility, we examined whether embryos properly attach to the uterine lumen, an early event of pregnancy that is initiated at midnight of pregnancy day 4 (4 dpc). We dissected mice on the morning of pregnancy day 5 (4.5 dpc) and counted the number of implantation sites by injecting Chicago Blue dye. Implantation sites were not detected in the mutant uterine horns, while normal implantation sites were scored in the controls (Figure 2A–2C). Histological examination also showed embryos failed to attach to the uterine lumen of mutant mice, while normal attachment and induction of the decidual response was observed in all controls (Figure 2D–2E). Embryo-attachment failure is most likely caused by an altered uterine receptivity response in the mutant model, since the blastocyst still contains an unaltered COUP-TFII allele, and even mutant embryos are able to implant in wild-type mothers as previously described [17].

**COUP-TFII Mutants Lose the Potential for Decidualization**

Decidualization is the subsequent step in the implantation process [23]. Although it is not possible to compare the decidual response in natural pregnancies of these mice, decidualization was assayed after hormonal induction [24]. Induction of decidualization was normal in COUP-TFII$^{floxed}$ control mice using two types of stimuli (oil injection into the uterine lumen or needle scratching on the antimesometrial side of luminal epithelia). Uterine horns of $PR^{Cre/+}$ COUP-TFII$^{floxed}$ mutant mice failed to decidualize under treatment of either stimuli (Figure 3A, 3B, and 3E), and alkaline phosphatase activity, an indicator of stromal cell differentiation in response to decidualization, was absent (Figure 3C and 3E). In addition to the failure of decidualization, stromal cell proliferation is also affected since the size of mutant uterine horns appear small at 4.5 dpc (Figure 2A and 2B). Immunostaining of phosphorylated histone H3 (phospho-H3) demonstrated that stromal cell proliferation was significantly decreased as indicated by the phospho-H3–positive cells (Figure 3F and 3G). The numbers of phospho-H3–positive cells in stroma are quantified and shown in Figure 3H. In contrast to the stroma, the numbers of phospho-H3–positive cells in the epithelia are increased in the mutant (Figure 3F and 3G). The increase in the numbers of proliferating cells in the mutant epithelium are quantified and shown in Figure 3I. In addition to the decreased proliferation in the stroma, vessel density visualized by lectin staining was also lower in the mutant uterus (Figure 3J and 3K). Reduced angiogenesis could partly contribute to the decrease in size of the uterine horn.

### Table 1. Ovaries of COUP-TFII Mutant Mice Were Functionally Normal Compared with Control Mice

| Genotype          | Sample Size | Litter Size | Interval of Litters, Days |
|-------------------|-------------|-------------|--------------------------|
| PR-Cre            | n = 3       | 5.8 ± 0.8   | 21.6 ± 1.3               |
| $PR^{Cre/+}$ COUP-TFII$^{floxed}$/ $^{floxed}$ | n = 5       | 6.4 ± 0.6   | 22.5 ± 1.3               |
| $PR^{Cre/+}$ COUP-TFII$^{floxed}$/ $^{floxed}$ | n = 7       | 4.8 ± 0.7   | 21.2 ± 0.6               |

Ovaries from 6-wk-old controls $PR^{Cre/+}$ or $PR^{Cre/+}$ COUP-TFII$^{floxed}$/ $^{floxed}$ or mutant $PR^{Cre/+}$ COUP-TFII$^{floxed}$/ $^{floxed}$ mice were transferred to a B6SJF1 female mouse. At 2 wk after transfer, the mice were mated with B6SJL-F1 male mice for a period of 2 to 6 mo. Each litter was genotyped.

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![Image](https://example.com/image1.png)
BMP2 Is the Major Downstream Effector of COUP-TFII for Decidualization

BMP2 is a known specific marker for decidualization in the uterus, and its expression is greatly induced upon decidualization [25,26]. To explore the molecular mechanism of decidualization failure in the COUP-TFII mutant mice, we asked whether expression of BMP2 is altered. Basal Bmp2 expression levels were unaffected in the mutants in comparison with the controls. However, the induced expression of Bmp2 upon decidualization was greatly diminished in the mutant uterus (Figure 4A). Immunohistochemistry confirmed no stromal expression of BMP2 in the mutant uterus (Figure 4B and 4C).

The above results suggest that BMP2 is a downstream target of COUP-TFII that regulates the decidual response. To address this, we asked whether BMP2 could rescue the decidualization defect exhibited by the COUP-TFII conditional mutant. Along with artificially stimulating the uterus, recombinant human BMP2 was administered into the uterine lumen. Mice were dissected 48 h later, and the decidual response was measured.

Figure 3. COUP-TFII Mutants Are Defective in Decidualization
(A–E) PRCre/+; COUP-TFIIfloxflox mutants have decidualization failure. (A) Control, 48 h after stimuli. The right horn (R) was stimulated, and the left horn (L) was unstimulated. Only right horn is decidualized. (B) Mutant, 48 h after stimuli. Neither right (stimulated) nor left (unstimulated) horn is decidualized. (C) Control, right horn. Alkaline phosphatase activity is detected in the mutant uterus. (D) Mutant, right horn. No alkaline phosphatase activity is detected in the mutant uterus. (E) Ratio of right to left horn in weight. Mutant uterine horns fail to decidualize. White bar: control; black bar: mutant. *p < 0.001 (t-test, n = 6).

(F–G) Cell proliferation is altered in PRCre/+; COUP-TFIIfloxflox mutants. (F) Control, phospho-H3, 3.5 dpc. (G) Mutant, phospho-H3, 3.5 dpc. Some positive cells in the epithelium of the mutant are marked by an arrowhead.

(H–I) Percentage of phospho-H3–positive cells in the stroma (H) and in the epithelium (I). The number of cells were counted on multiple sections and averaged in each mouse. Stromal cell proliferation is decreased, while epithelial proliferation is enhanced in the mutant uterus. White bar: control; black bar: mutant. *p = 0.007; **p = 0.001 (t-test, n = 7).

(J–K) PRCre/+; COUP-TFIIfloxflox mutants have an angiogenesis defect. (J) Control, 3.5 dpc. Lectin was intravenously administered and adhered to the inner lumen of vessels. (K) Mutant, 3.5 dpc. Vascular density is apparently reduced in the mutant uterus.

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Figure 4. BMP2 Mediates COUP-TFII in Decidualization
(A–C) BMP2 induction is diminished in the mutant uterus. (A) The gene expression of Bmp2 in the stimulated horn (D) and the unstimulated horn (ND), assayed by quantitative real-time RT-PCR at 48 h after stimuli. Bmp2 expression is induced by stimuli for decidualization in the control, but is not sufficiently induced in the mutant uterus. White bar: control; black bar: mutant. *p < 0.001, (t-test, n = 6). (B) Control, right horn, 72 h after stimuli. BMP2 is immunohistologically detected in the secondary decidual zone. (C) Mutant, right horn, 72 h after stimuli. BMP2 is not detected in the mutant stroma.

(D–I) Decidualization is rescued by the administration of recombinant human BMP2. (D) Control, 48 h after stimuli. The right horn was treated with BMP2, and the left horn was treated with BSA (vehicle). Both horns are decidualized. (E) Control, right horn. (F) Control, left horn. Alkaline phosphatase activity is detected in both uterine horns. (G) Mutant, 48 h after stimuli. Only the right horn is decidualized. (H) Mutant, right horn. (I) Mutant, left horn. Alkaline phosphatase activity is detectable in the BMP2-treated horn, but not in the BSA-treated horn of mutant uterus.

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BMP2 is a known specific marker for decidualization in the uterus, and its expression is greatly induced upon decidualization [25,26]. To explore the molecular mechanism of decidualization failure in the COUP-TFII mutant mice, we asked whether expression of BMP2 is altered. Basal Bmp2 expression levels were unaffected in the mutants in comparison with the controls. However, the induced expression of Bmp2 upon decidualization was greatly diminished in the mutant uterus (Figure 4A). Immunohistochemistry confirmed no stromal expression of BMP2 in the mutant uterus (Figure 4B and 4C). The above results suggest that BMP2 is a downstream target of COUP-TFII that regulates the decidual response. To address this, we asked whether BMP2 could rescue the decidualization defect exhibited by the COUP-TFII conditional mutant. Along with artificially stimulating the uterus, recombinant human BMP2 was administered into the uterine lumen. Mice were dissected 48 h later, and the decidual response was measured.
BMP2 treatment restored the decidual response in the mutant uterine horns (Figure 4D and 4G) as measured by the enhancement of alkaline phosphatase activity in the stimulated horns, while no activity was detected in the vehicle (BSA)–treated mutant horns (Figure 4E, 4F, 4H, and 4I). These results strongly support that BMP2 is a major COUP-TFII effector that lies downstream of COUP-TFII to mediate uterine decidualization. BMP2 has also been shown as a downstream target of hedgehog signaling in other tissues [27,28], and conditional ablation of Bmp2 results in decidualization defects, but embryo attachment is unaffected (Lee et al., unpublished data). Therein, our finding provides new evidence in support of the existence of a uterine Ihh–COUP-TFII–BMP2 axis that is required for decidualization.

**Estrogen Activity Is Enhanced in the Uterine Epithelia of COUP-TFII Mutants**

The lack of embryo attachment indicates that ablation of COUP-TFII not only affects the physiology of uterine stromal cells but also affects the endometrial epithelial compartment. One of the major roles of progesterone is to down-regulate ER activity in the uterine luminal epithelium, which consequently opens the uterine receptivity window. Since COUP-TFII mutants have a receptivity defect (Figure 2A–2E), we wondered whether COUP-TFII is a mediator of progesterone’s suppression of ER activity in the epithelia. If so, ER activity in this compartment should increase in COUP-TFII mutants. To address this, the expression level of estrogen-responsive genes was examined by quantitative real-time RT-PCR analysis (qRT-PCR). The expression of lactoferrin (Ltf), a known estrogen-responsive target in the uterine epithelia [29], is significantly elevated in the mutant uterus at 3.5d pc (Figure 5A). To exclude the possible involvement of other factors, we also examined the expression of Ltf in mice exogenously treated with hormones, mimicking 3.5 dpc of pregnancy (30 h after progesterone and estrogen [Pe] treatment; see Materials and Methods). Although the fold changes vary, Ltf expression level is consistently significantly higher in mutant mice (Figure 5B). Immunohistological staining detected high lactoferrin expression in mutant epithelia (Figure 5C–5F), demonstrating that estrogen activity is indeed enhanced in the uterine epithelial compartment. Other well-documented estrogen-responsive genes in the

**Figure 5. COUP-TFII Regulates ER Activity in the Epithelia**

Estrogen-responsive genes are upregulated in the mutant uterine epithelia.

(A) The gene expression of Ltf, assayed by qRT-PCR at 3.5 dpc. Ltf expression is high in the mutant uterus. White bar: control; black bar: mutant. *p = 0.012 (t-test, n = 6).

(B) Ltf, qRT-PCR, 30 hPe. Ltf expression is consistently high in the mutant uterus. White bar: control; black bar: mutant. *p < 0.001 (t-test, n = 9).

(C) Control, immunohistological detection of lactoferrin, 30 hPe.

(D) Mutant, lactoferrin.

(E) Control, DAPI.

(F) Mutant, DAPI. Upregulated expression of lactoferrin is observed in the epithelial compartment.

(G) The gene expression of C3, assayed by qRT-PCR at 30 hPe. C3 expression is high in the mutant uterus. White bar: control; black bar: mutant. *p = 0.015 (t-test, n = 9).

(H) Clca3, qRT-PCR, 30 hPe. Clca3 expression is high in the mutant uterus. White bar: control; black bar: mutant. *p = 0.002 (t-test, n = 9).

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High Estrogen Activity Alters Uterine Receptivity in COUP-TFII Mutants

Mucin 1 (MUC1) is known to be one of the important markers determining uterine receptivity [32]. MUC1 is an estrogen-responsive target, and its expression is attenuated at the time of implantation to facilitate epithelial remodeling [33,34]. Persistent expression of MUC1 in the mutant uterus (Figure 6A). In addition, immunohistochemistry detected high expression levels of MUC1 in the apical surface of mutant luminal epithelia (Figure 6B and 6C). These results suggest that high estrogen activity might be the underlying cause of the uterine receptivity defect displayed by the mutant model. Consistent with this notion, Clea3, a gene important for the overproduction of mucus protein [35], is also shown to be highly upregulated in the mutant uterus (Figure 5H). Therefore, upregulation of many ER target genes suggests that stromal COUP-TFII is essential for the PRL-mediated downregulation of ER activity in the epithelium to open up the receptivity window.

The membrane transformation of uterine epithelia is well documented as a marker of uterine receptivity [36]. Long microvilli of the epithelial surface are characteristically present under estrogen influence, while progesterone shortens these structures. Microvilli flattening occurs before implantation and is an important process to facilitate embryo attachment [36]. Electron microscope (EM) studies revealed that mutant epithelia fail to undergo appropriate remodeling to flatten the microvilli (Figure 6D and 6E). In addition, mutant microvilli exhibit increased glyocalyx expression (Figure 6F and 6G), which is consistent with high expression of MUC1 [36]. Both MUC1 expression and glyocalyx formation prevent embryo attachment [34,37,38]. It has been reported that a series of glycosylation enzymes are involved in the glycosylation of mucins, and among them, UDP-N-acetyl-alpha-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase 1 (GALNT1) is a key enzyme [39]. We examined Galnt1 expression in qRT-PCR and observed that its expression level is increased by 20% (Figure 6H), and, most remarkably, another glycosylation enzyme Galnt7 expression is upregulated by almost 70% in the mutant uterus (Figure 6I). Although it has not been well established in the mouse uterus, activation of GALNT7 catalytic activity requires prior glycosylation by other enzymes [40], and GALNT7 cooperatively functions with GALNT1 [41]. The high expression of these enzymes might account for hyperglycosylation of the apical surface of the mutant uterine luminal epithelium.

Another important parameter for uterine epithelial maturation is the presence of desmosomes [36,42,43], adherent junctions of the lateral plasma membrane. Desmosomes are normally lost before implantation to facilitate embryo invasion into the uterine stroma. However, desmosomes are persistently present in the mutant epithelia (Figure 6I and 6J). As expected, the expression level of desmocollin-2 (Dsc2), one of the ubiquitous desmosomal components [44], is high in the mutant uterus (Figure 6K). This inappropriate regulation of Dsc2 might contribute to desmosome dysregulation. Taken together, the high estrogen activity observed in the mutant epithelium alters the uterine receptivity in the mutant mice, which is reflected by striking structural abnormalities in the apical–lateral regions of mutant luminal epithelial cell.

Downregulation of PR Expression in the Uterine Stroma of COUP-TFII Mutants

PR in the stroma has been implicated to play a critical role in modulating ER activity in the epithelium [6,7]. Since the
activity of ER is enhanced in COUP-TFII mutants, an important question is whether ablation of COUP-TFII in the uterine stroma alters the expression level of stromal PR. To address this possibility, we used PR-specific immunostaining to assess the expression of PR in the uterus of controls and mutants. The result clearly shows that downregulation of PR in the absence of COUP-TFII could disrupt stromal–epithelial interactions and contribute to the enhanced ER activity.

The Expression of Epithelial ER and Its Coactivator SRC-1 Is Upregulated in COUP-TFII Mutants

In an attempt to further dissect the molecular mechanism of enhanced estrogen activity in the mutant uterine epithelium, we first asked whether uterine ERα levels are altered in mutants. qRT-PCR showed a 40% increase in levels of ERα (Esr1) mRNA in the whole-uterine tissues of COUP-TFII mutants (Figure 7C). Immunohistological staining using
expression levels, we isolated uterine epithelia from whole mutants (Figure 7D and 7E). To quantify the difference in and gain receptivity for embryo attachment.

Figure 8. Working Model of COUP-TFII in Mediating Progesterone Function in the Uterus

Progesterone activates Ihh–Ptc signaling to induce COUP-TFII expression in the stroma compartment. COUP-TFII, in turn, regulates stromal cell differentiation (decidualization) through the induction of BMP2. COUP-TFII also mediates the suppression of epithelial estrogen activity through inhibiting the expression of SRC-1 and ERα2 as well as ERα activation, which allows the uterine epithelium to undergo transformation and gain receptivity for embryo attachment.

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ERα-specific antibody further confirmed an increased expression of ERα in the epithelial compartment of COUP-TFII mutants (Figure 7D and 7E). To quantify the difference in expression levels, we isolated uterine epithelia from whole uterus and examined ERα expression by western blot analysis. The result showed that ERα expression is increased 2- to 3-fold in the mutant uterine epithelium (Figure 7F). To further ask whether these receptors are activated or not, we examined the phosphorylation status of ERα using antiphosphorylated ERα antibody and observed increased phosphorylation of ER in the uterine epithelium of COUP-TFII mutants (Figure 7F–7H). Increased phosphorylation levels of ER seem proportional to increased expression levels of ER, but this modification has been shown to couple with growth factor signaling, which might be controlled under paracrine mechanism [45] and is less likely to be an autophosphorylation; therefore, this finding really supports the notion that stromal–epithelial communication is dysregulated in the COUP-TFII mutant uterus. In addition to ER, members of the steroid receptor coactivator (SRC)/p160 family, SRC-1 and SRC-2, have been shown to play a major role in regulating ER activity and uterine function [46–48]. Thus, we examined the expression of coactivators by both immunohistochemistry and western blot analysis. We showed that SRC-1 is upregulated in mutant uterine epithelium (Figure 7F, 7I, and 7J), while SRC-2 and SRC-3 are unchanged (unpublished data). Taken together, the increase in ER, phosphorylated ER, and SRC-1 levels in the mutant uterine epithelium can together contribute to enhanced uterine ER activity in the COUP-TFII mutant.

Discussion

Uterine receptivity has been intensely studied in recent years because of its clinical importance [49,50]. Mouse models generated by gene-knockout technology revealed that multi-
associated with a receptive uterus are lost [52]. Since observed phenotypes in LIF-null mice are similar to COUP-TFIIflox/flox mutant mice, LIF also could be placed in our scheme. LIF is known to be estrogen responsive; when examined, we did not find significant changes in LIF by qRT-PCR and by immunocytochemistry in mutants in comparison to the controls. It is possible that more complex mechanism underlies our model, but it is still unequivocal that COUP-TFIIflox/flox has access to the principal part of steroid receptor regulation in the uterine biology.

The finding that COUP-TFIIflox/flox antagonizes ER action is intriguing. ER has been shown to regulate the expression of many glycoproteins during the peri-implantation period [34,53]. Downregulation of the expression of such glycoproteins (including MUC1) is known to pave the way for remodeling of the epithelial surface to facilitate embryo attachment. Although COUP-TFIIflox/flox has been shown to compete with ER binding in vitro in the regulation of LIF [54,55], COUP-TFIIflox/flox is not expressed in the same compartment as lactoferrin and MUC1, and thus it is unlikely that it regulates their expression directly in vivo. Using tissue-recombinant studies, Buchanan et al. showed that epithelial lactoferrin expression is not only regulated by epithelial ER but also regulated by stromal ER [56]. This raises the possibility that COUP-TFIIflox/flox might compete with stromal ER and alter the epithelial ER function. Another possible mechanism is that COUP-TFIIflox/flox regulates local estrogen levels, since COUP-TFIIflox/flox has been shown to compete with SF-1 to regulate aromatase expression [57]. However, aromatase expression was not altered in the COUP-TFIIflox/flox conditional mutant mice (unpublished data). We also showed that the expression of ER, phosphorylated ER, and SRC-1 are all increased in the COUP-TFIIflox/flox mutants. Enhanced expression of these molecules will no doubt contribute to the observed increased ER activity and the subsequent activation of the downstream ER targets. Since COUP-TFIIflox/flox is highly expressed in the stroma but is barely detectable in the epithelia, the up-regulation of ER activity in the epithelium is unlikely a consequence of direct regulation of the above molecules by COUP-TFIIflox/flox. It is more likely that the stromal COUP-TFIIflox/flox regulates PR to control a paracrine signal, which acts through its epithelial receptor to suppress epithelial ER activity as well as ER and its coregulator expression. Unlike as it might be, we can not exclude the possibility that the low levels of epithelial COUP-TFIIflox/flox expression is sufficient to synergize with other epithelial factors to suppress epithelial ER activity directly.

In conclusion, COUP-TFIIflox/flox controls early molecular and cellular changes in the uterus that are required for embryo implantation and subsequent decidualization. Based on our previous observation that COUP-TFIIflox/flox is a mediator of the Shh pathway in motor neurons and the stomach [11,14], it is not surprising that COUP-TFIIflox/flox mediates progesterone–Ihh signaling to regulate decidualization. We also show that BMP2 can rescue the decidual defect elicited by the loss of COUP-TFIIflox/flox, which places BMP2 downstream of the COUP-TFIIflox/flox pathway. Unexpectedly, stromal COUP-TFIIflox/flox also promotes PR expression to mediate progesterone-induced suppression of estrogen activity in the uterine epithelium; local suppression of estrogen activity is required to establish a receptive uterus. Therefore, progesterone control of epithelial estrogen activity is projected from the stromal compartment via COUP-TFIIflox/flox through a complex epithelial–stromal cross-

communication pathway. The abnormal increase in estrogen activity following the removal of COUP-TFIIflox/flox may help our understanding of the molecular events that control uterine receptivity as well as female reproductive health.

Materials and Methods

Animals and chemicals. Generation of COUP-TFIIflox/flox mice and PR-Cre knockout mice has been previously described [14,22]. To obtain uterine tissues of pregnant mice, we started mating with wild-type males (B6SJLF1; Taconic, http://www.taconic.com) at 7 wk of age and designated the day of vaginal plug as pregnant day 1. Ovariectomy was performed at 6 wk of age and followed by the hormone regimen as described below. For priming with 1 μg of 17β-estradiol (E2; Sigma-Aldrich, http://www.sigmaaldrich.com) was dissolved in 1 ml sesame oil (Sigma-Aldrich), and 0.1 ml was subcutaneously administered in a single dose for each mouse. For daily treatment of Pe, 10 mg progesterone (Sigma-Aldrich) and 67 ng 17β-estradiol (nadir estrogenic)) were dissolved in 1 ml sesame oil, and 0.1 ml was subcutaneously administered in a single dose for each mouse. In the implantation study, 1% Chicago Sky Blue 6B (Sigma-Aldrich) was prepared in 0.9% saline, and 0.1 ml was intravenously injected for each mouse before dissection for the rescue of decidualization, 25 μg recombinant human BMP2 (Fitzgerald Industries International, http://fitzgerald-fii.com) was reconstituted by 10% BSA, and 0.1 ml was administered for each uterine horn. All procedures for animal study were approved by the institutional animal care guidelines at Baylor College of Medicine. All assays were repeated at least three times.

Ovary transfer. We followed the ovary transfer procedure described previously [38]. Ovaries from 6-wk-old controls, PRCre/− or PRCre/−; COUP-TFIIflox/flox mice, or mutant PRCre/−; COUP-TFIIflox/flox plus mice were isolated and then transferred to a B6129-F1 female mouse. At 2 wk after transfer, the mice were mated with B6SJL-F1 male mice for a period of 2 to 6 mo. Each litter was genotyped in order to characterize the origin of the pups. When two litters came from the transferred ovary, the mating was stopped and the experiment was considered a success.

Induction of decidualization. The details of this method have been previously described [24]. Briefly, after 2 wk of ovariectomy, we first primed mice with 100 ng of estradiol (E2) for 3 d and then started the daily treatment of 1 mg progesterone and 6.7 ng E2 (Pe) 2 d later. Mechanical stimulation was added 54 h after the first Pe treatment (54 hPe), and mice were dissected 48 h later for decidual response measurement. The same hormone regimen was used for exogenous hormone treatment mimicking 3.5 dpf. Tissues were isolated at 30 hPe.

Immunohistochemical staining. Isolated uterine tissues were fixed in 4% paraformaldehyde (PFA)/PBS, dehydrated through graded ethanol, and processed for paraffin embedding. Primary antibodies used in this study are as follows: mouse monoclonal anti-COUP-TFIIflox/flox (1:1,000; Perseus Proteomics, http://ppmx.com), rabbit polyclonal anti-phospho-H3 (1:200; Upstate Biotechnologies, http://www. upstate.com), goat polyclonal anti-BMP2 (1:100; Santa Cruz Biotechnology, http://www.scbt.com), rabbit polyclonal anti-lactoferrin (1:5,000; Abcam, http://www.abcam.com), rabbit polyclonal anti-MUC1 (1:400; Abcam), rabbit polyclonal anti-PR (1:200; Dako, http:// www.dako.com), rabbit polyclonal anti-ERα (1:500; Santa Cruz Biotechnology), rabbit polyclonal anti-phosphorylated ERα (S118, 1:100; Abcam), and rabbit polyclonal anti-SRC-1 (1:500; Santa Cruz Biotechnology). Biotinylated antibodies (1:400; Jackson Immunoresearch, http://www.jacksommuno.com) were used as secondary antibodies, followed by horseradish peroxidase–conjugated streptavidin (1:200; Molecular Probes, http://probes.invitrogen.com), and signals were developed with 3,3′-diaminobenzidine (DAB) substrate kit (Vector Laboratories, http://www.vectorlabs.com) or Alexa fluor 488–conjugated tyramide signal amplification (TSA) kit (Molecular Probes). Hematoxylin or methyl green (Vector Laboratories) was used for counterstaining in immunohistochemistry.

Alkaline phosphatase staining. Isolated tissues were fixed in 2% PFA/PBS, cryoprotected at 30% sucrose/PBS, and embedded in OCT compounds (Sakura, http://www.sakura.com). The sections were stained with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro blue tetrazolium chloride (NBT) solution (pH 9.5; Roche, http://www.roche.com). Nuclear Fast Red (Vector Laboratories) was used for counterstaining.

Lectin staining. A total of 25 μg biotinylated–lycopersicon esculentum lectin (Vector Laboratories) was intravenously injected, and then uterine tissues were isolated, fixed in 4% PFA/PBS,
cyp19a1a and cyp19a1b, the expression of which is strongly increased during the secretory endometrial stage. We also performed in situ hybridization study using antisense riboprobes for these two genes. The expression of these two genes was increased in the luminal epithelial cells in gravid uterus, indicating hormonal stimulation of aromatase gene expression.

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References

1. Wang H, Dey SK (2006) Embryo implantation: clues from mouse models. Nat Rev Genet 7: 185–199.
2. Dey SK, Lim H, Das SK, Reese J, Paria BC, et al. (2004) Molecular cues to interactions in hormonal responses. Arch Histol Cytol 67: 417–434.
3. You LR, Lin FJ, Lee CT, DeMayo FJ, Tsai MJ, et al. (2005) Suppression of Notch signaling by the COUP-TFII transcription factor regulates vein identity. Nature 435: 98–104.
4. De-Fo V (1967) Decidualization. In: Wynn RM, editor. Cellular biology of the uterus. New York: Appleton-Century-Crofts. pp. 191–203.
5. Finn CA, Martin L (1972) Endocrine control of the timing of endometrial sensitivity to a decidua stimulus. Biol Reprod 7: 82–86.
6. Ying Y, Zhao Q (2000) Detection of multiple bone morphogenetic protein messenger ribonucleic acids and their signal transducer, Smad1, during muscle decidualization. Biol Reprod 63: 1781–1786.
7. Robey PG (1999) Decidualization of mouse endometrium. In: Liao SS, editor. Biology of the endometrium. New York: Plenum. pp. 75–97.
8. Haig D, Brown A, Black JD (2000) The contribution of the trophoblast to the maternal genome. Science 287: 1947–1950.
9. Millar TB, Carrell DT, Crozier RJ, Bongso A, Weyers G, et al. (2000) Human sperm swim up through conditioned media from decidualized endometrial stromal cultures. Biol Reprod 63: 482–490.
10. Zhang Q, Tsai SY, Hsiao H,3,4 Chiang H, Greider CW, et al. (2000) Regulation of human implantation by decidualization. Science 288: 347–350.
11. O'Malley BW (2000) Tissue development and disease: the role of nuclear hormone receptors and their ligands. Am J Pathol 157: 1663–1668.
12. Greenblatt MB, Caniggia I, Boulet R, Neill JD, Lebovic J, et al. (2000) The role of COUP-TFII in the control of estrogen receptor beta expression in the mouse uterus. Biol Reprod 63: 295–300.
13. O'Hara RC, Reijo-Poutiainen LS, Rosenthal S, Doody S, Lebeau MM, et al. (2000) Inactivation of the estrogen receptor beta gene in the uterus results in delayed implantation and embryonic lethality. Nat Genet 24: 294–298.
14. Wang H, Dey SK, Cook RG, Beattie WG, Tsai MJ, et al. (1998) COUP-TFII is essential for radial and anteroposterior patterning of the stomach. Development 125: 2179–2189.
15. Wang LH, Tsai SY, Cook RG, Beattie WG, Tsai MJ, et al. (1999) COUP-TFII is a member of the steroid receptor superfamily. Nature 400: 163–166.
16. Tsai SY, Tsai MJ (1997) Chick ovalbumin upstream promoter-transcription factor (COUP-TF)-C in 0.5% casein containing primary RIPA buffer (150 mM NaCl, 10 mM Tris-Cl [pH 7.5], 0.1% SDS, 1% Triton X-100, 1% deoxycholate, and 5 mM EDTA) containing protease inhibitors and phosphatase inhibitors. The whole lysate was harvested and then postfixed by 1% OsO4 in 0.1 M cacodylate buffer. The uterine luminal epithelial cell lysates were obtained from 24 h to 2 days postimplantation and used to visualize by transmission electron microscopy (Applied Biosystems, http://www.appliedbiosystems.com). Gene expression assay was performed by running the ABI PRISM 7700 Sequence Detector System (Applied Biosystems, Foster City, CA). Universal Master Mix and inventoried primer/probe mixture (Applied Biosystems) were used for the reaction. The primers/probes used in this study are the following: Bmp2 (Mm01962382_m1), Lif (Mm00434787_m1), C3 (Mm00437858_m1), Clec3 (Mm00489959_m1), Mac1 (Mm01036481_m1), Gata1 (Mm00489148_m1), Gata2 (Mm00319998_m1), Dsc2 (Mm00516355_m1), Esr1 (Mm00433141_m1), COUP-TFII (Mm00772898_m1). Standard curves were generated by serial dilution of a preparation of total RNA, and mRNA quantities were normalized against 18S RNA determined by using eukaryotic 18S RNA endogenous control reagents (Applied Biosystems).

Steroid hormone assay. The serum progesterone and estradiol levels were measured with radioimmunoassay by the core laboratory of University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core.

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expression of osteoblasts in response to hedgehog signaling. Mol Cell Biol 26: 6197–6208.
29. McMaster MT, Teng CT, Dey SK, Andrews GK (1992) Lactoferrin in the mouse uterus: Analyses of the preimplantation period and regulation by ovarian steroids. Mol Endocrinol 6: 101–111.
30. Sundstrom SA, Komm BS, Ponce-de-Leon H, Yi Z, Teuscher C, et al. (1989) Estrogen regulation of tissue-specific expression of complement C3. J Biol Chem 264: 16941–16947.
31. Jeong JW, Lee KY, Lydon JP, DeMayo FJ (2006) Steroid hormone regulation of Clic3 expression in the murine uterus. J Endocrinol 189: 473–484.
32. Lagow E, DeSouza MM, Carson DD (1999) Mammalian reproductive tract mucins. Hum Reprod Update 5: 280–292.
33. Braga VM, Gendler SJ (1993) Modulation of Muc-1 mucin expression in the mouse uterus during the estrus cycle, early pregnancy and placentaion. J Cell Sci 105 (Part 2): 397–405.
34. Surveyor GA, Gendler SJ, Pemberton L, Das SK, Chakraborty I, et al. (1995) Expression and steroid hormonal control of Muc-1 in the mouse uterus. Endocrinology 136: 3639–3647.
35. Nakanishi A, Morita S, Iwashita H, Sagita Y, Ashida Y, et al. (2001) Role of gob-5 in mucus overproduction and airway hyperresponsiveness in asthma. Proc Natl Acad Sci U S A 98: 5175–5180.
36. Murphy CR (2004) Uterine receptivity and the plasma membrane translocation. Cell Res 14: 259–267.
37. Achache H, Revel A (2006) Endometrial receptivity markers, the journey to successful embryo implantation. Hum Reprod Update 12: 731–746.
38. Chavez DJ, Anderson TL (1985) The glycocalyx of the mouse uterine epithelium during estrus, early pregnancy and placentaion. J Cell Sci 105 (Part 2): 397–405.
39. Ten Hagen KG, Fritz TA, Tabak LA (2003) All in the family: The UDP-N-acetylgalactosaminyltransferases. Glycobiology 13: 156–162.
40. Bennett EP, Hassan H, Hollingsworth MA, Claussen H (1999) A novel human UDP-N-acetyl-D-galactosamine:polypeptide N-acetylgalactosaminyltransferase, GalNac-T17, with specificity for partial GalNac-glycosylated acceptor substrates. FEBS Lett 466: 226–230.
41. Tetact D, Ten Hagen KG, Richet C, Boersma A, Gagnon J, et al. (2001) Glycopeptide N-acetylgalactosaminyltransferase specificities for O-glycosylated sites on MUC5AC mucin motif peptides. Biochem J 357: 313–320.
42. Illingworth IM, Kiszka I, Bagley S, Ireland GW, Garrod DR, et al. (2000) Desmosomes are reduced in the mouse uterine luminal epithelium during the preimplantation period of pregnancy: A mechanism for facilitation of implantation. Biol Reprod 63: 1764–1773.
43. Preston AM, Lindsay LA, Murphy CR (2006) Desmosomes in uterine epithelial cells decrease at the time of implantation: An ultrastructural and morphometric study. J Morphol 267: 103–108.
44. Huber O (2003) Structure and function of desmosomal proteins and their role in development and disease. Cell Mol Life Sci 60: 1872–1890.
45. Driggers PH, Segars JH (2002) Estrogen action and cytoplastic signaling pathways. Part II: The role of growth factors and phosphorylation in estrogen signaling. Trends Endocrinol Metab 13: 422–427.
46. DeMayo FJ, Zhao B, Takamoto N, Tsai SY (2002) Mechanisms of action of estrogen and progestrone. Annu N Y Acad Sci 955: 48–59.
47. Xu J, Qu Y, DeMayo FJ, Tsai SY, Tsai MJ, et al. (1998) Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. Science 279: 1922–1925.
48. Mukherjee A, Soyal SM, Fernandez-Valdivia R, Gehin M, Chambon P, et al. (2006) Steroid receptor coactivator 2 is critical for progesterone-dependent uterine function and mammary morphogenesis in the mouse. Mol Cell Biol 26: 6571–6583.
49. Ota B, Li TC (2006) Implantation failure following in-vitro fertilization. Curr Opin Obstet Gynecol 18: 440–445.
50. Daftary GS, Taylor HS (2001) Molecular markers of implantation: Clinical implications. Curr Opin Obstet Gynecol 13: 269–274.
51. Simon C, Garcia Velasco JJ, Valbuena D, Peinado JA, Moreno C, et al. (1998) Increasing uterine receptivity by decreasing estradiol levels during the preimplantation period in high responders with the use of a follicle-stimulating hormone step-down regimen. Fertil Steril 70: 234–239.
52. Fouldi-Nadra AA, Jones CJ, Nijjar N, Mohamet L, Smith A, et al. (2005) Characterization of the uterine phenotype during the peri-implantation period for LIF-null, MFI strain mice. Dev Biol 281: 1–21.
53. Kimber SJ, Stones RE, Sidhu SS (2001) Glycosylation changes during differentiation of the murine uterine epithelium. Biochimie Soc Trans 29: 156–162.
54. Liu Y, Teng CT (1992) Estrogen response module of the mouse lactoferrin gene contains overlapping chicken ovalbumin upstream promoter transcription factor and estrogen receptor-binding elements. Mol Endocrinol 6: 355–364.
55. Kline MJ, Silver BF, Driscoll MD, Sathya G, Bambara RA, et al. (1997) Chicken ovalbumin upstream promoter-transcription factor interacts with estrogen receptor, binds to estrogen response elements and half-sites, and inhibits estrogen-induced gene expression. J Biol Chem 272: 31465–31474.
56. Buchanan DL, Setiawan T, Lubahn DB, Taylor JA, Kurita T, et al. (1999) Tissue compartment-specific estrogen receptor-alpha participation in the mouse uterine epithelial secretory response. Endocrinology 140: 484–491.
57. Zeroun K, Takayama K, Michael MD, Bulun SE (1999) Stimulation of aromatase P450 promoter (II) activity in endometriosis and its inhibition in endometrium are regulated by competitive binding of steroidogenic factor-1 and chicken ovalbumin upstream promoter transcription factor to the same cis-acting element. Mol Endocrinol 15: 233–253.
58. Nagy A, Gertsenstein M, Vintersten K, Behringer R (2003) Manipulating the mouse embryo: A laboratory manual. New York: Cold Spring Harbor Laboratory Press. 764 p.
59. Bigg Jr RM, Cooke PS, Cunha GR (1986) A simple efficient method for separating murine uterine epithelial and mesenchymal cells. Am J Physiol 251: E630–E636.