C/EBPβ is a critical mediator of steroid hormone-regulated cell proliferation and differentiation in the uterine epithelium and stroma

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During early pregnancy, steroid hormones estrogen (E) and progesterone (P) regulate a complex series of interactions between the implanting embryo and the uterus by controlling the proliferation and differentiation of uterine epithelium and stroma in a timely manner. To identify the steroid-regulated genes that control these functions, we performed messenger RNA profiling of mouse uterine tissues at the time of implantation. Our analysis revealed that the expression of the transcription factor CCAAT/enhancer-binding protein β (C/EBPβ) is rapidly induced in the pregnant uterus at the time of blastocyst attachment. The expression of C/EBPβ increased further during the decidualization phase of pregnancy and was localized in the proliferating as well as the decidualized stromal cells surrounding the implanted embryo. Administration of E or P to ovariectomized females induced C/EBPβ expression in both uterine epithelium and stroma, showing a dual regulation of this gene by these hormones. The female C/EBPβ-null mice are infertile. We, therefore, assessed steroid-hormone-dependent responses in the uteri of these mice. We observed that E-induced proliferation of uterine epithelial cells is markedly compromised in the absence of C/EBPβ. Most strikingly, there was a complete lack of response of the C/EBPβ-deficient uteri to an artificial decidualogenic stimulus, indicating a critical role of this transcription factor in regulating the decidualization program. Further analysis revealed defects in steroid-induced stromal cell proliferation and differentiation in C/EBPβ-null uteri. Collectively, our studies established that C/EBPβ is a key mediator of steroid responsiveness of the epithelium and stroma in the mouse uterus.

The physiological functions of the mammalian uterus are profoundly influenced by the concerted actions of steroid hormones estrogen (E) and progesterone (P). Waves of steroid-hormone-induced cell proliferation and differentiation dictate the cyclical changes that occur in the uterine epithelium during the reproductive cycle (1–4). During pregnancy, these hormones orchestrate the changes in the uterine epithelium that make it competent to attach to the blastocyst to initiate the process of implantation (1–4). Subsequently, E and P regulate a series of complex interactions between the developing embryo and the cells in the stromal compartment leading to the formation of the decidua, which nourishes the embryo and maintains early pregnancy. Although the details of these events vary in different species, the central roles played by E and P in controlling various phases of early pregnancy are common to many mammals (5).

The mouse has served as an important animal model to study the regulation of uterine functions by E and P (6–8). The development of mutant mouse models lacking the estrogen receptor (ER) α and progesterone receptor (PR) has firmly established the requirement of these hormones and their downstream signaling pathways for successful establishment and maintenance of pregnancy (7, 9). The ERα-null mice are impaired in the growth and function of the female reproductive tract and its preparedness for blastocyst attachment (9). The PR-null mice display a refractory uterus that fails to respond to an artificial decidualogenic stimulus (7). A number of recent studies employing gene expression profiling have explored steroid-hormone-regulated molecular pathways that control epithelial and stromal functions during implantation (10–13). Although these studies have identified several potential target genes for both ER and PR, the identities of molecules that mediate critical steroid-regulated functions in the uterus remain largely unknown.

In this study, we have used a delayed-implantation mouse model to identify steroid-regulated gene networks that have functional relevance in implantation. In mice, implantation is initiated 4 days after fertilization, when the blastocyst reaches the uterus (14). Removal of the ovaries several hours before implantation blocks this process because of a lack of ovarian steroids. Continued administration of P alone to these ovariectomized pregnant animals allows the blastocysts to remain viable, but the attachment of the embryo to the uterine epithelium does not occur in the absence of E. Administration of E to the P-primed mice promotes attachment of the blastocyst trophectoderm to the luminal epithelium within 12–24 h (1, 2). The E-induced attachment of the embryo to the uterine epithelium triggers the process of decidualization, which then progresses primarily under the influence of P. During the decidual phase, uterine stromal cells surrounding the implanting embryo initially proliferate and then differentiate to form the implantation chamber (3). The experimentally induced delayed-implantation model, therefore, presents a unique opportunity to identify E- and P-regulated factors that control the critical phases of implantation.

We used DNA microarrays to analyze changes in uterine mRNA profiles in response to E in pregnant mice undergoing delayed implantation. Our studies revealed that CCAAT/enhancer-binding protein β (C/EBPβ), present at a low level in the P-primed delayed mice, is markedly and rapidly induced upon E administration. We also observed that P acting through PR further increases C/EBPβ expression in the pregnant uterus during decidualization. Using a C/EBPβ-null mouse model, we showed that this transcription factor is a critical mediator of E-induced epithelial cell proliferation in the uteri of nonpregnant mice. We also provided strong evidence that C/EBPβ controls stromal cell proliferation and differentiation, which are critical for decidualization.

Results

E Induces C/EBPβ Expression in the Uterus During Delayed Implantation in Mice. To understand the molecular basis of steroid hormonal regulation of implantation, it is critical to identify the steroid-
regulated gene networks that underlie this process. In this study, we used a well-established delayed-implantation mouse model in which embryo attachment to the uterus depends on the administration of E to P-primed ovariectomized pregnant uteri (14). Using mouse oligonucleotide microarrays (Affymetrix, Santa Clara, CA) representing ~11,000 genes, we compared mRNA profiles of uteri of pregnant mice treated with either P alone or P plus an implantation-initiating dose of E. Our studies identified several mRNAs that were up- or down-regulated in the uteri of mice undergoing delayed implantation within 1 h of E administration (Y.-P.C. and I.C.B., unpublished results). One of the mRNAs whose level was robustly enhanced after E treatment encoded C/EBPβ, a well-studied transcription factor known to have an essential role in female reproduction (15).

The C/EBPβ protein expression during delayed implantation was investigated by immunohistochemistry (IHC) (Fig. 1). A low level of C/EBPβ expression was observed in the epithelial cells of uterine sections of P-treated delayed animals before E administration (Fig. 1A, 0h). The level of C/EBPβ protein increased dramatically in both epithelial and stromal cells within 1 h of E treatment (Fig. 1A, 1h). Whereas the level of C/EBPβ protein in the epithelium declined sharply at 3 h and 12 h after E administration, a relatively lesser reduction in the expression of this protein was seen in the stromal compartment at these times (Fig. 1A, 3h and 12h).

Both E and P Receptors Regulate Uterine C/EBPβ Expression. Because E enhanced C/EBPβ expression in the P-primed uterus during delayed implantation, we further examined the regulation of this gene by each of these hormones and their cognate receptors in nonpregnant ovariectomized mice. Treatment with E alone markedly and rapidly enhanced C/EBPβ protein expression in uterine epithelial and stromal cells within 1 h of hormone administration (Fig. 2A). The elevated C/EBPβ expression in both compartments declined at 24 h. Coadministration of ICI 182,780, an antagonist of ER, strongly inhibited E-induced expression of C/EBPβ in the uterus, indicating that this process is mediated by nuclear ERs (Fig. 2B).

Our studies also showed that treatment of ovariectomized mice with P for 1 h (Fig. 2Ca and data not shown). However, when these mice were treated with P for 1, 2, or 3 consecutive days, the expression of C/EBPβ protein gradually increased in both epithelial and stromal cells (Fig. 2C b–d). When ovariectomized PR knockout (PRKO) females were similarly treated with P, no induction of C/EBPβ protein could be detected (Fig. 2C, compare e and f). These results clearly indicated that P-induced C/EBPβ expression in the uterine compartments is mediated by PR.

E-Induced Uterine Epithelial Cell Proliferation Is Impaired in C/EBPβ-Null Mice. Uterine epithelium is a well characterized site of estrogenic action. Previous studies established that treatment of nonpregnant ovariectomized mice with E leads to multiple rounds of epithelial cell proliferation and moderate wet-weight gain in the uterus (16, 17). Our observation that E rapidly induced C/EBPβ in these cells raised the possibility that this transcription factor or its...
downstream target genes may play a role in this cell proliferation. To test this possibility, we compared the E-induced proliferation of uterine epithelia of C/EBPβ KO mice and their WT littermates by examining the immunostaining for Ki67 antigen, a widely used marker of cell proliferation (18). Upon ovariectomy, the uteri of both WT and mutant mice became atrophic. Administration of E clearly induced marked epithelial cell proliferation, as indicated by strong Ki67 expression in these cells (Fig. 3A a–c). In contrast, E treatment elicited only a weak immunostaining for Ki67 in the uterine epithelium of the C/EBPβ KO mice, indicating a poor proliferative response in the absence of this transcription factor (Fig. 3A d–f). Interestingly, we noted that the expression of ERα, PR, and a previously well characterized E-regulated gene, lactotransferrin (19), remained unaltered in the C/EBPβ-null epithelium (Fig. 3B, ER and PR data not shown). These results suggested that the lack of E-induced epithelial cell proliferation in C/EBPβ-null mouse is not due to a general loss of responsiveness to this hormone.

PR Regulates C/EBPβ Expression in Uterine Stromal Cells During Decidualization. We next examined the profile of expression of C/EBPβ protein in the normal pregnant mouse uterus during the periimplantation period by employing IHC (Fig. 4A). Uterine sections obtained from day-4 pregnant mice (12 noon) exhibited a low level of C/EBPβ expression in the undifferentiated stromal cells (Fig. 4A, D4). A marked increase in the level of C/EBPβ protein was observed in the uterine sections of mice on day 5 (afternoon) of gestation (Fig. 4A, D5). The enhanced C/EBPβ expression was observed in both primary and secondary decidual zones on days 5 and 6 of pregnancy (Fig. 4A, D5 and D6). The staining was initially seen predominantly in the antimesometrial region. Interestingly, the C/EBPβ expression on day 7 was restricted to the decidualized stromal cells immediately surrounding the implanted embryo (Fig. 4A, D7). C/EBPβ expression spread into the mesometrial region on day 8 and then declined sharply by day 9 of pregnancy (data not shown). These results showed that C/EBPβ expression in the stromal compartment of the pregnant uterus precisely overlaps with the decidual phase.

The program of decidualization is tightly controlled by P acting through PR in the differentiating stromal cells (7). Our observation that administration of P induces C/EBPβ expression in the non-pregnant, ovariectomized uterus prompted us to examine whether its expression during decidualization is under PR regulation. To test this possibility, we treated pregnant mice on day 6 (afternoon) of pregnancy with RU486, a well known PR antagonist, and assessed the impact of this treatment on stromal C/EBPβ expression. We observed a sharp reduction in the level of C/EBPβ protein on day 7 (morning) in response to RU486 treatment, indicating the involvement of PR in the regulation of C/EBPβ in the pregnant uterus during decidualization (Fig. 4B).

C/EBPβ Is a Critical Regulator of Deciduization. The steroid-regulated induction of C/EBPβ in the stroma during decidualization prompted us to analyze the functional role of this transcription factor during this process. The C/EBPβ-null mice are infertile, presumably because of defects in ovulation and luteinization (15). To initially examine whether these mutant mice can support pregnancy at all, we performed embryo-transfer experiments (Fig. 5A). Blastocysts were collected from uteri of WT females on day 4 of pregnancy. These blastocysts were then transferred directly into the uteri of pseudopregnant WT or C/EBPβ KO females on day 4 of pseudopregnancy. Five days after the embryo transfer, the numbers of implanted embryos and the size of the decidua in the recipient uteri were examined. We found that the majority (>85%) of the transferred embryos did not implant in C/EBPβ KO recipients, and a few that were present in the uterus did not grow or form any significant decidua (Fig. 5A). These results indicated...
that uterine functions are severely compromised in the C/EBPβ-null mice.

Considering the possibility that the impairment in implantation and decidualization in the null mice could arise from a potential inadequacy of ovarian hormone synthesis or release, we administered steroid hormones to these mice and assessed the response of the hormone-primed uterus to an artificial decidual stimulation (13). In this experiment, uteri of ovariectomized WT and C/EBPβ KO mice were treated with a well-established regimen of E and P, and then a decidualization reaction was initiated in the left uterine horn by mechanical stimulation with a needle, and the right horn was left unstimulated. We then examined the gross anatomy of the stimulated and unstimulated uterine horns of WT and C/EBPβ KO mice. As expected, the uterine horn of WT mice exhibited a robust decidual response within 72 h after receiving the artificial stimulation (Fig. 5B Left). In contrast, the C/EBPβ KO uteri under identical conditions failed to show any significant decidualization (Fig. 5B Right). When an assessment of the decidual response was done by measurement of uterine wet weight, the C/EBPβ KO uteri exhibited a poor response compared with the WT uteri, in which a marked weight gain was recorded (Fig. 5C). Morphological examination of WT and KO uteri further revealed that the stimulated WT horn was packed with decidual cells (Fig. 5D Left). In contrast, similarly treated uterine horns of C/EBPβ KO animals were largely devoid of decidual cells (Fig. 5D Right). Our studies, therefore, clearly indicated a severe impairment of the decidualization process in the C/EBPβ-null mice and suggested a critical role of this transcription factor in supporting the stromal cell differentiation program in the steroid-hormone-primed uterus.

C/EBPβ Regulates Stromal Cell Proliferation and Differentiation During Decidualization. During decidualization, the uterine stromal cells undergo mitotic expansion, followed by differentiation (20, 21). To further investigate the decidualization defect in C/EBPβ-null mice, we monitored the proliferation and differentiation of steroid-hormone-primed stromal cells in response to a mechanical decidual stimulus, by using known markers of these events. As shown in Fig. 6A, uteri of WT mice exhibited an intense staining for Ki67, predominantly in the stromal cells, within 24 h of receiving the decidual stimulus, clearly indicative of extensive stromal cell proliferation, which is also seen during normal pregnancy in response to embryo attachment. In contrast, the uterine sections from C/EBPβ KO mice showed greatly reduced Ki67 staining under identical conditions, implying a lack of stromal cell proliferation. It is important to mention that the stromal expression pattern of PR in the C/EBPβ KO mice is comparable with that of the WT mice (data not shown). The loss of stromal cell proliferation in the null mice is, therefore, not due to an aberrant expression of PR in this compartment but, rather, a direct functional consequence of C/EBPβ deficiency.

An analysis of uterine sections of WT and C/EBPβ KO mice also revealed a defect in stromal cell differentiation in the absence of C/EBPβ. The expression of alkaline phosphatase, a classical marker of decidualization of stromal cells, was prominent in the antimesometrial decidual cells of the WT uteri at 72 h after decidual stimulation. In contrast, no expression of this decidual marker was seen in the uteri of C/EBPβ KO mice at a similar time point, indicating a complete blockade of stromal cell differentiation in these mice (Fig. 6B). Taken together, our results indicated that
C/EBPβ is a critical regulator of stromal cell proliferation and differentiation during decidualization.

**Discussion**

In this study, we have identified C/EBPβ as a critical mediator of steroid hormone responsiveness in the uterus. This transcription factor belongs to a C/EBP family of basic leucine-zipper proteins that includes, among other members, C/EBPα and C/EBPδ (22). Previous studies indicated that C/EBPβ is a critical regulator of proliferation and/or differentiation in multiple tissues, including the liver, adipose tissue, immune system, and mammary gland (22–26). The role of C/EBPβ in reproduction came as a surprise when it was found that homozygous mutant female mice are infertile (15). The infertility of C/EBPβ-null mice was initially attributed to an impaired corpus luteal function. Consistent with this finding, transplantation of WT ovaries into C/EBPβ-null mice fully restored the formation and function of corpora lutea. This intervention, however, resulted in only a poor rescue of the pregnancy outcome (15). These results hinted at additional reproductive abnormalities, presumably at the level of the uterus, in the mutant female. We now report that a lack of C/EBPβ in the uterus leads to multiple functional abnormalities that include an impaired epithelial cell proliferation in response to E and a loss of decidual reaction to an artificial stimulus. These defects were observed in the presence of exogenously administered steroid hormones, indicating that they were independent of ovarian malfunction and intrinsic to the uterine tissue.

Our study has uncovered an important role of C/EBPβ in the pathway of E-induced epithelial cell proliferation in uterus of nonpregnant mice. Numerous earlier studies addressed the role of pubertal E in the growth and function of the female reproductive tract (16, 17, 27). These studies documented that the uterine epithelial cells undergo mitotic divisions in response to E (28). Studies employing ER-null mice confirmed that the proliferative effects of E in the uterus were, indeed, mediated by the ERα isoform (6). Our results showed that in the uteri of C/EBPβ null mice, epithelial proliferation in response to E is drastically reduced (Fig. 3A). It is important to point out that the E-responsiveness of the adult uterus is not fully compromised in the absence of C/EBPβ during development. The expression profiles of E and P receptors and hormonal inducibility of well known E-regulated genes, such as lactotransferrin, remain unaffected in the uteri of C/EBPβ-null mice. The defective E-induced proliferation of uterine epithelium in these mice is, therefore, likely due to the absence of expression and function of C/EBPβ and its downstream target genes rather than a global developmental deficiency or lack of hormone responsiveness.

It is postulated that ERα, a ligand-inducible transcription factor, generates downstream signaling molecules that impact on the cell cycle to trigger mitosis (17). Several growth-regulatory molecules, such as epidermal and insulin-like growth factors, their receptors, and protooncogenes, such as c-fos and c-myc, were identified as downstream targets of ER in the uterus (19, 29–33). None of these factors, however, could be functionally linked to the E-induced proliferative effects in the uterine epithelium. It is in this context that our discovery of C/EBPβ as a factor that mediates the proliferative action of E in the uterine epithelium assumes significance. The precise mechanism by which C/EBPβ regulates uterine epithelial cell proliferation remains to be defined. Previous studies have documented a role for C/EBPβ in regulation of cell cycle in other tissues (23, 24). In the reproductive liver, C/EBPβ is required for a normal proliferation of hepatocytes. Interestingly, cyclin B and E gene expression was dramatically reduced in C/EBPβ-null hepatocytes, providing a direct link between this transcription factor and cell-cycle control (23).

C/EBPβ expression is rapidly induced in response to E in epithelial and stromal compartments of the P-primed delayed pregnant uterus (Fig. 1). This induction is clearly mediated by ER, because treatment with ICI 182,780, an ER antagonist that blocks the transcriptional activity of the receptor, suppressed this expression. Interestingly, we observed that the E-induced C/EBPβ expression in the uterine epithelium is transient in nature and subsided within 3 h of hormone administration. It is unclear how this rapid burst of C/EBPβ expression in the epithelium by E functionally impacts the implantation process. The uterine epithelial cells cease to proliferate as P surges at the onset of pregnancy and are committed to the differentiation pathway. It is conceivable that signaling downstream of C/EBPβ may contribute to epithelial cell differentiation that allows them to interact with the blastocyst or signal to the stroma in a productive manner.

In contrast to the transient nature of E-induced C/EBPβ expression in pregnant uterine epithelium, its expression in the stromal cells continued to increase during the decidualization phase of pregnancy. After blastocyst attachment to the luminal epithelium, the underlying stromal cells undergo extensive proliferation and differentiation that result in their transformation into the decidual cells. In mice and a few other species, decidualization can also be triggered by a variety of artificial stimuli in steroid-hormone-
primed uteri. We found that the expression of C/EBPβ is robustly induced in the decidual tissue during normal and artificial decidual reaction (Fig. 4A, data not shown). Analysis of the C/EBPβ KO mice revealed that the uterus of the mutant mouse is refractory to a decidualizing stimulus even after treatment with exogenous steroid hormones. The complete lack of decidual response in the C/EBPβ-deficient mice pointed to a potential role of this transcription factor in the stromal compartment.

The expression pattern of C/EBPβ in the decidual uterus likely arises from a complex interplay of E and P within the uterine compartments. Although E administration initiates induction of C/EBPβ in the stromal compartment of the pregnant uterus, our results indicated that P is also a critical regulator of this gene in the differentiating stromal cells. This view is strongly supported by our observations that (i) administration of P alone to ovariectomized mice induced C/EBPβ expression in the uterus, and (ii) RU486, an antagonist of PR, efficiently suppressed C/EBPβ expression in the decidual uterus. However, it remains to be established whether C/EBPβ is a primary target of regulation by ER or PR in the uterus.

We have recently scanned the 5′-regulatory region of the mouse C/EBPβ gene for the presence of putative ER elements (EREs) and PR elements (PREs). This in silico analysis has revealed the existence of multiple potential EREs and PREs within sequences 5 kb upstream from the transcription start site of this gene. Further studies are necessary to determine whether ER or PR functions by directly interacting with any of these sites.

The process of decidualization involves stromal cell proliferation, followed by their differentiation. A defect in decidualization could arise from either compromised cell proliferation or an arrest in the differentiation program of proliferated stromal cells. Morphological analysis of uterine sections of C/EBPβ KO uterus in response to a decidual stimulus showed a severely reduced stromal/decidual cell mass, indicating a defect in cell proliferation in these mice (Fig. 5D). This observation is also corroborated by our immunohistochemical studies using cell proliferation marker Ki67. The failure of induction of this marker in C/EBPβ KO uteri after artificial decidual stimulation indicated that the reduced number of uterine stromal/decidual cells results from an overall reduced cell proliferation (Fig. 6A). These results suggested that, in C/EBPβ KO mice, stromal cells fail to proliferate in response to a decidual stimulation. There was also a striking lack of expression of alkaline phosphatase, a classical marker of stromal cell differentiation, indicating that decidualization is not accomplished.

In summary, the identification of C/EBPβ as a mediator of proliferating effects of E in the uterine epithelium of pubertal, nonpregnant mice is a unique finding. Our studies have also revealed that C/EBPβ is a critical downstream target of E and P in the stromal compartment during decidualization. The same transcription factor, therefore, mediates separate functions of E and P in different uterine cell types, presumably by activating distinct molecular pathways. C/EBPβ is also a known regulator of several genes that are involved in the inflammatory response, including those coding for cytokines and their receptors and acute phase proteins (34). Because implantation is considered an inflammatory reaction, it is possible that the expression of this transcription factor in the decidual tissue regulates immune-cell function during pregnancy. Future studies will explore the pathways that work downstream of C/EBPβ in mediating the steroid hormone regulation of cell proliferation, differentiation, and immune response in the uterine tissue at critical phases of female reproduction.

Materials and Methods

Animals and Tissue Collection. Female CD1 mice in proestrus, were mated with adult males. The presence of a vaginal plug after mating was designated as day 1 of pregnancy. The animals were killed at various stages of gestation, and the uteri were collected. In some experiments, mice were subjected to ovariectomy, and treated with E, or P, or vehicle as described in ref. 11. Delayed implantation and artificial decidualization were described as performed in refs. 12 and 13.

IHCl. Polyclonal antibodies against C/EBPβ, Ki67, and lactotransferrin were obtained from Santa Cruz Biotechnology. Paraffin-embedded uterine tissues were subjected to IHC as described in ref. 11.

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