REPRODUCTIVE ENDOCRINOLOGY AND INFERTILITY

Stromal cell–specific apoptotic and antiestrogenic mechanisms may explain uterine defects in humans after clomiphene citrate therapy

Magdalena Nutu, PhD; Yi Feng, MD, PhD; Emil Egecioglu, PhD; Birgitta Weijdegård, MSc; Elisabet Stener-Victorin, RPT, PhD; Ruijin Shao, MD, PhD

OBJECTIVE: The purpose of this study was to investigate clomiphene citrate (CC)–induced modulation of uterine cell function in vivo.

STUDY DESIGN: Prepubertal female Sprague-Dawley rats were treated intraperitoneally with CC for 6 or 24 hours or with a combination of CC and/or 17-β-estradiol (E2) for 4 days.

RESULTS: Chronic CC treatment induced apoptosis in a fraction of uterine stromal cells by activating the caspase-3-mediated apoptotic pathway. The damage was prevented by successive E2 treatment; however, pretreatment or concomitant treatment with E2 did not protect against CC-induced uterine apoptosis. CC decreased the protein expression of estrogen receptor α and increased its phosphorylation but did not affect estrogen receptor β expression or phosphorylation. Furthermore, changes in Hoxa11, p27, and progesterone receptor protein levels and localization were associated with CC treatment.

CONCLUSION: We provide novel mechanistic insights into cellular and molecular events by which CC regulates uterine stromal cell function and hence the implantation process and pregnancy outcome.

Key words: apoptosis, clomiphene citrate, implantation, uterus

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Clomiphene citrate (CC), a nonsteroid tissue-selective estrogen receptor (ER) modulator,[1] is used commonly to treat various infertilities in women, most notably polycystic ovary syndrome (PCOS) and unexplained infertility.[2,3] Several clinical and epidemiologic studies have indicated that successful ovulation occurs in 70–99% of women who are treated with CC,[2,4] whereas ultimate pregnancy rates are only 27–40%.[2,5] The total CC-treated pregnancy rates are approximately 10 times lower than "natural" rates.[6] Furthermore, spontaneous abortion and miscarriage occur frequently in women who receive CC therapy.[2,4,7] Although it has been proposed that the antiestrogenic effects of CC may lead to early pregnancy failure,[4] the precise molecular events underlying CC-induced uterine defects remain incompletely understood.

★ EDITORS' CHOICE ★

The mammalian uterus is a dynamic reproductive organ that undergoes cyclic changes in response to ovarian steroid hormones.[8] The biologic effects of 17-β-estradiol (E2) and CC are mediated primarily by 2 nuclear receptors, ER-α and/or ER-β, which are encoded by 2 different genes and function as transcription factors.[9] Although the distribution and relative levels of ER-α and ER-β expression are tissue specific and diverse,[10] both ER-α and ER-β are expressed in the rodent uterus and human endometrium.[11] It has been reported that inappropriate activation or inhibition of ER subtypes may cause or contribute to a variety of uterine diseases, such as endometriosis and endometrial cancer.[11] The protective effects of E2 in uterine homeostasis are evident both from in vivo and in vitro studies: the presence of E2 inhibits uterine apoptosis in vivo;[12] however, human endometrial cells undergo apoptosis when E2 is withdrawn.[13] Previous studies from our laboratory and others have shown that CC enhances apoptotic processes in the ovaries, fallopian tubes, villi, and decidual tissues.[14-16] Less is known, however, about

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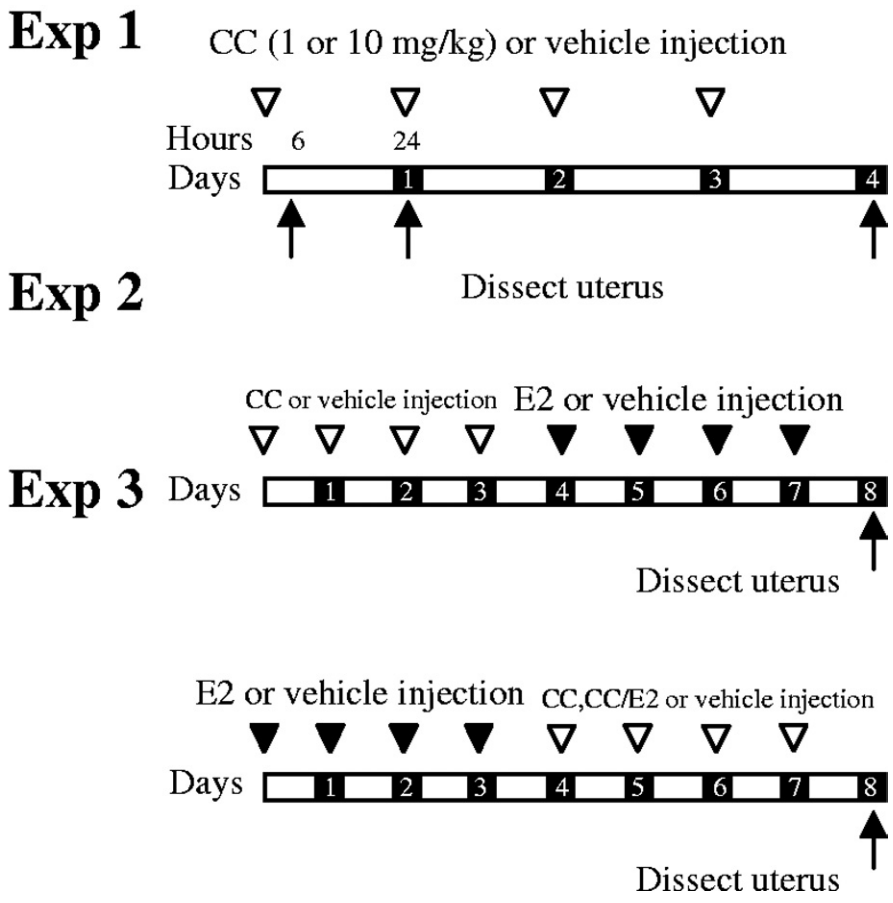
FIGURE 1
Schematic of the experimental design



CC, clomiphene citrate; *E2*, 17-β-estradiol; *Exp*, experiment.

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the effects of CC on the apoptotic machinery in the uterus.

The maternal endometrium shows prominent steroid-dependent cyclic changes in structure and function in preparation for the process of implantation.[8] Successful implantation requires precise coordination between the embryo and uterus under the influence of ovarian steroids.[17] After fertilization, specific uterine cell types undergo differentiation and proliferation to provide a suitable environment for embryo implantation and development.[17] Genomic endometrial responses to estrogen are essential for the regulation of the "implantation window."[18] The transformation of endometrial stromal cells into decidual cells has been recognized as a fundamental step during the process of implantation.[18,19] Previously, it has been shown that in vivo treatment with CC delays and/or inhibits implantation in rodents,[18,20] probably because of abnormalities that are seen in the reproductive tract (including the uterus) after CC treatment in both rats[16,21] and humans.[22] Furthermore, a significant decrease in the implantation rate has also been observed in rabbits that were treated with CC before and after ovulation.[23] These observations, combined with clinical studies, have given rise to the hypothesis that CC may contribute to implantation-related complications through an unidentified regulatory process in the uterus. The goals of this study were (1) to determine whether CC treatment induces uterine cell apoptosis and (2) to investigate the molecular regulation of ERs and other potential implantation and cell cycle regulators[17,24] in the uteri of rats that were treated with CC.

Materials and Methods

Animals

All experimental procedures and protocols were approved by the ethics committee at Gothenburg University. Prepubertal female Sprague-Dawley rats (20 days old) were obtained from Taconic M&B (Copenhagen, Denmark) and maintained in cages that contained wood chips under defined conditions (temperature, 21 ± 2°C; relative humidity, 45–55%; and 12-hour light/dark illumination schedule). Animals were acclimated to the animal facilities for 5 days before the initiation of the experiments. All animals had free access to tap water and were fed ad libitum with standard laboratory diet.

Experimental design

Three experiments were carried out (Figure 1) in 25-day-old rats. The rats were randomized to receive intraperitoneal injections of CC (Sigma-Aldrich, St. Louis, MO), E2 (Sigma-Aldrich), or both. Controls were treated with vehicle only. Bodyweight was recorded throughout the experiment. Rats were killed under light anesthesia with sodium pentobarbital (0.5 mL/kg bodyweight). Aliquots of serum were prepared from trunk blood after heart puncture and stored at −80°C until analysis. The uteri were dissected grossly with the removal of contaminating tissues (eg, adipose tissues), weighed, and immediately frozen in liquid nitrogen or fixed in formalin.

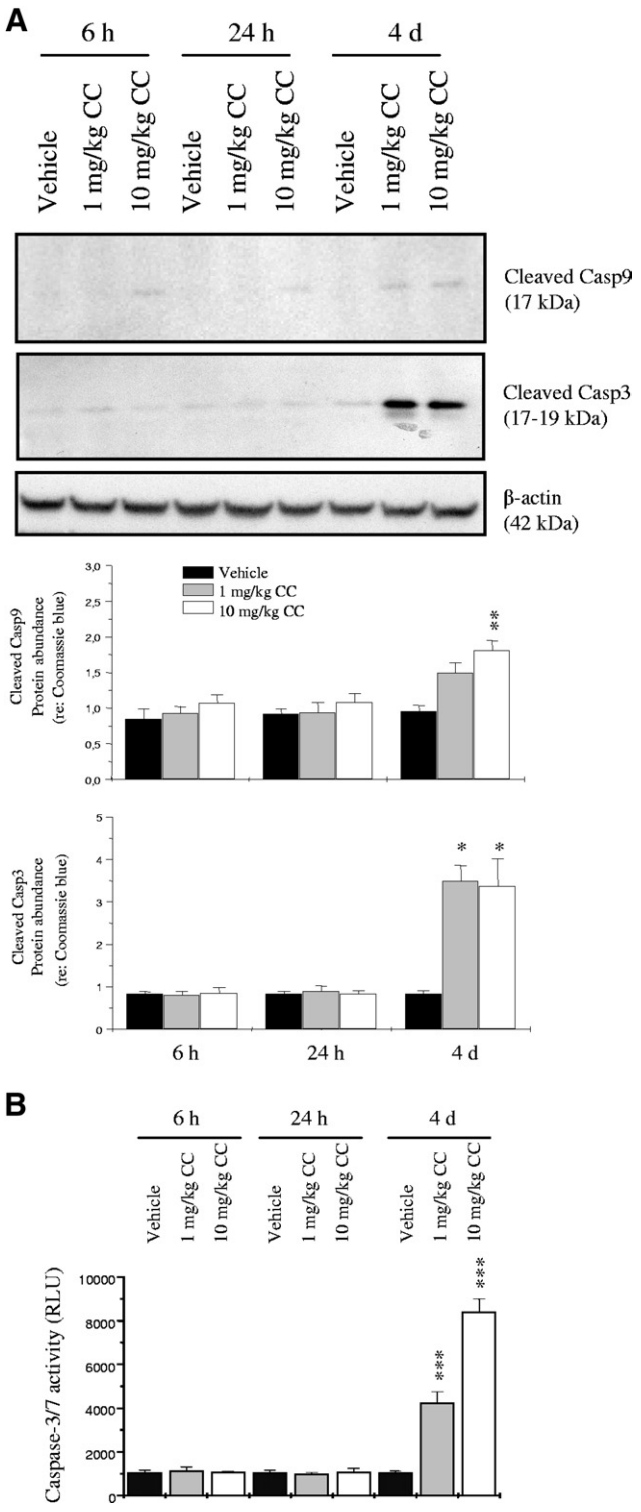
Experiment 1

Rats received CC (1 or 10 mg/kg intraperitoneally) or an equivalent volume of vehicle (0.9% NaCl). Uteri were collected 6 and 24 hours (acute effect) after injection or on day 4 (chronic effect) after daily intraperitoneal injections (n = 8/group). For the selected doses and treatment schedule, CC is effective in rat fallopian tube and uterus in vivo.[16,25]

Experiment 2

Rats received injections of CC (10 mg/kg intraperitoneally) or vehicle once daily for 4 consecutive days. Twenty-four hours after the last injection, E2 (0.3 mg/kg in 100 μL sesame oil) or vehicle was injected for 4 consecutive days.

FIGURE 2
Chronic treatment with clomiphene citrate (*CC*) induces uterine apoptosis



Uteri were dissected from rats that were treated with clomiphene citrate for 6 or 24 hours or 4 consecutive days. **A,** Western blot analysis of cleaved caspase-9 and -3 relative to whole protein. Gels were stained with Coomassie blue (n = 3 rats/group). In addition, β-actin served as a loading control. **B,** Caspase-3/7 activity was determined in whole uterine tissues from rats that were treated with clomiphene citrate for 6 or 24 hours or 4 consecutive days (n = 5 rats/group). Values are means ± SEM. The *single asterisk* denotes $P < .05$; the *double asterisks* denote $P < .01$; the *triple asterisks* denote $P < .001$ vs vehicle-treated controls.

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Experiment 3

Rats were injected with E2 or vehicle once daily for 4 consecutive days, as in experiment 2. Twenty-four hours after the last injection, CC (10 mg/kg), CC (10 mg/kg) and E2 (0.3 mg/kg), or vehicle was injected intraperitoneally for 4 consecutive days. In experiments 2 and 3, the rats were killed, and uteri were collected 24 hours after the final injection (n = 5/group).

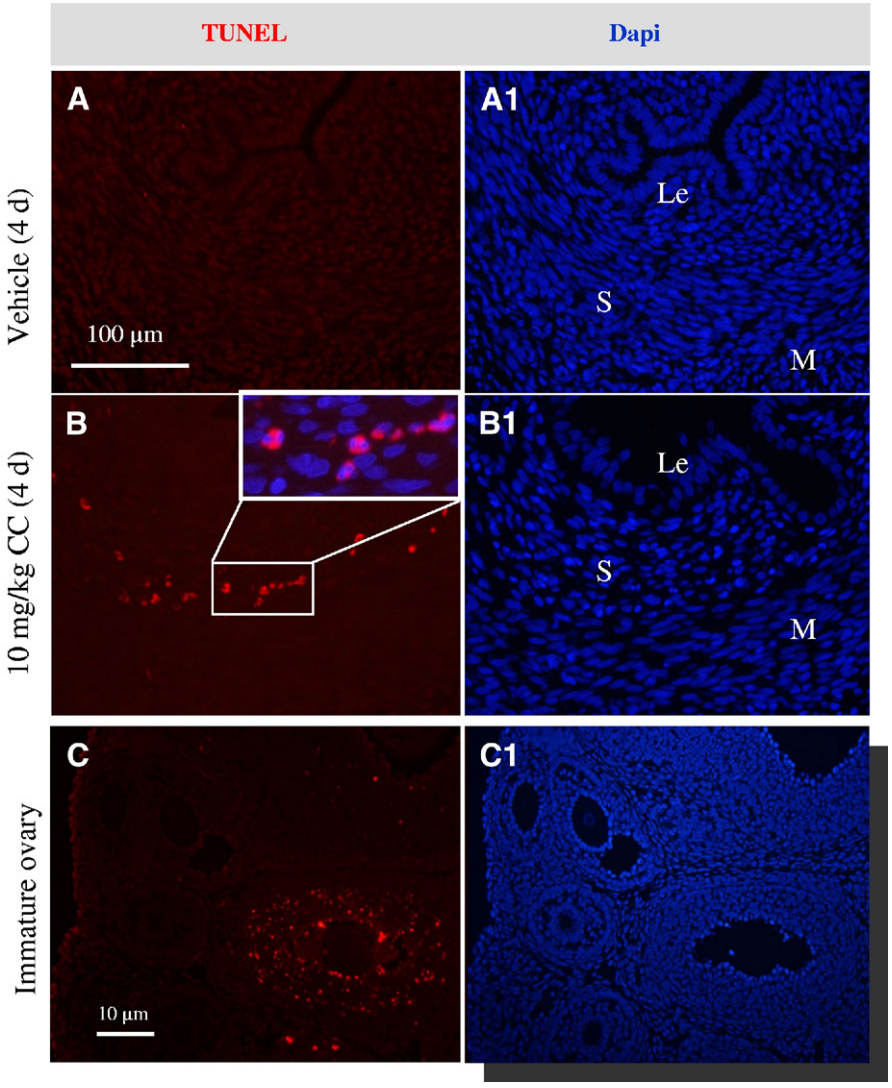
Antibodies

The primary antibodies that were used for Western blot (WB) analysis and immunofluorescence were obtained from commercial sources (anticleaved caspase 3 and anticleaved caspase 9 [Cell Signaling Technology, Beverly, MA]; anti-ERá, antiphospho-ERâ [Ser87], anti-Hoxa10, anti-Hoxa11, anti-p27, and anti-p53 [Santa Cruz Biotechnology, Santa Cruz, CA]; antiphospho-ERá [Ser118] and anti-ERâ [Upstate Biotechnology, Lake Placid, NY]; anti-pan-cytokeratin, anti-á-smooth muscle actin and anti-â-actin [Sigma-Aldrich]; and antiprogesterone receptor [PR; Novocastra Laboratories, Newcastle Upon Tyne, UK] antibodies). The secondary antibodies for WB were goat-antimouse immunoglobulin G (Sigma-Aldrich), goat-antirabbit immunoglobulin G (AC31RL; Tropix, Bedford, MA), and donkey anti-goat immunoglobulin G (Santa Cruz Biotechnology), which were all conjugated with alkaline phosphatase. The Cy3-conjugated antimouse antibody was from Jackson ImmunoResearch Laboratories (West Grove, PA).

Histologic evaluation and immunofluorescence staining

Uteri were fixed in neutral buffered 10% formalin, decalcified, dehydrated, and embedded in paraffin. Series sections (5 μm) were prepared and stained with hematoxylin/eosin to visualize cell nuclei (Histocenter, Västra Frolunda, Sweden). Uterine sections were subjected to immunofluorescence studies to test the lo-

FIGURE 3
Clomiphene citrate (CC)–induced uterine apoptosis is cell type specific



A, Vehicle treatment in the uterus compared with **B,** chronic treatment with clomiphene citrate (10 mg/kg)-induced DNA fragmentation (*red*) of the stromal cell layer. Apoptotic cells (**B**) in the stromal cell layer are shown in the *inset* at higher magnification. Sections were counterstained with 4′,6-diamidino-2-phenylindole (DAPI, **A1,** and **B1**; *blue*) to visualize cell nuclei. Ovarian sections (**C** and **C1**) were used as experimental controls. The pictures in **A-C** are representative of stainings in tissues from different rats (n = 5/group). All photographs were taken with a ×10 or a ×40 magnification; the exact scale is given in the Figure.

Le, luminal epithelial cells; *M*, muscle cells; *S*, stromal cells.

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calization of progesterone receptor A isoform, as described previously.[26]

In situ detection of DNA fragmentation

Tissue sections were dewaxed, protease digested, and incubated with terminal transferase mixture according to the instructions of the manufacturer, with the use of an in situ apoptosis detection kit (Roche Diagnostics GmbH, Mannheim, Germany). Briefly, sections were incubated in a permeabilization solution, which contained 0.1% Triton X-100 and 0.1% sodium citrate, and then were incubated with the terminal deoxynucleotidyl transferase mediated 2′-deoxyuridine, 5′-triphosphate nick-end-labeling (TUNEL) reaction mixture, which included the enzyme solution (terminal deoxynucleotidyl transferase) and label solution (tetramethylrhodamine isothiocyanate-labeled nucleotides), in a humidified chamber for 1 hour at 37°C. After being washed with phosphate-buffered saline solution, the sections were evaluated with a confocal laser microscopy. The enzyme solution was omitted in the negative control. Sections that were treated this way remained unstained.

Protein extraction and WB analysis

Whole-cell extracts from uterine tissues were analyzed by WB, as described previously.[26]

Caspase activity assay

For the measurement of CASP-3/7 activity in whole uterine tissues, frozen tissue was homogenized in lysis buffer (100 mmol/L HEPES, pH 7.4, 140 mmol/L NaCl, and protease inhibitors), and the crude homogenate was centrifuged at 12,000*g* for 30 minutes at 4°C. Cellular caspase activity was determined with the use of the Caspase-Glo-3/7 assay kit (Cell Signaling Technology), as previously described.[16]

Statistical analysis

Data were analyzed with SPSS software (version 13.0; SPSS, Inc, Chicago, IL). Two-way analysis of variance was used to assess the main effects of treatment and time and to identify interactions between them. If significant interactions between the fixed factors were observed, within-group analyses were performed with a 1-way analysis of variance followed by Bonferroni's multiple comparison test. Values are shown as means ± SEM. Significance was accepted at a probability value of $< .05$.

RESULTS

Chronic, but not acute, CC treatment induces cell type-specific apoptosis in the uterus

Rats were given 1 acute injection of CC (1 or 10 mg/kg) or vehicle, and the ex-

pression of cleaved caspase 9 and caspase 3 (Casp3) was measured in the uterus at 6 and 24 hours. Alternatively, CC was administered daily for 4 days (chronic treatment), and the expression of the same apoptotic molecules was measured in the uterus. WB analysis revealed elevated levels of cleaved caspase 9 and Casp3 in the uteri from rats that had been treated with CC for 4 days but not at 6 and 24 hours (Figure 2, A). Using the caspase-Glo 3/7 assay, we confirmed that elevated Casp3 activity was found only in uteri from rats that had been treated with CC for 4 days (Figure 2, B), which is consistent with the levels of cleaved Casp3 (Figure 2, A). Next, DNA fragmentation was detected in situ with TUNEL staining. TUNEL-positive cells were present specifically in the stromal cell layer in rats that had been treated with CC for 4 days (Figure 3, B) but not in control rats (Figure 3, A). The luminal (Figure 3, A and B) and glandular (data not shown) epithelial cells and the smooth muscle cell layers (Figure 3, A and B) in both control and CC-treated rats showed no staining. Ovarian sections (Figure 3, C and C1) were used as experimental controls.

E2 aids recovery from CC-induced uterine apoptosis but is not protective

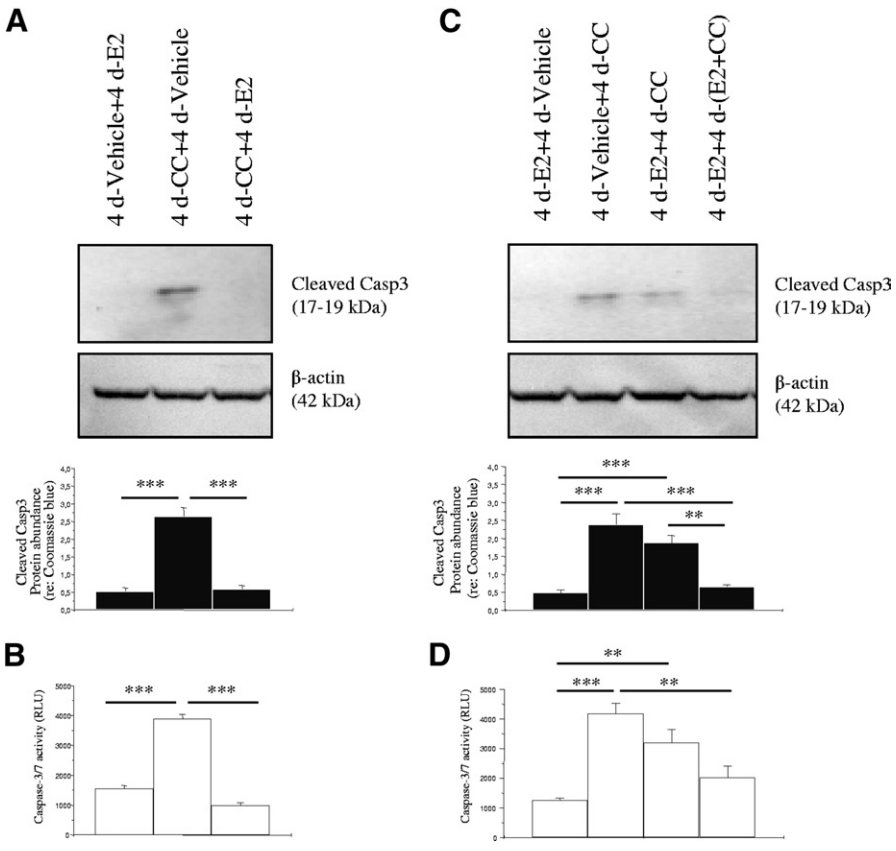
To test whether E2 might contribute to recovery from CC-induced uterine apoptosis, rats were treated sequentially with E2 or vehicle for 4 days after chronic treatment with CC. Cleaved Casp3 levels (Figure 4, A) and activity (Figure 4, B) were lower in uteri from the E2-treated rats. Neither pretreatment nor concomitant treatment with E2 significantly affected the ability of chronic CC treatment to decrease cleaved Casp3 expression (Figure 4, C) and activity (Figure 4, D).

Chronic treatment with CC results in changes in uterine morphologic condition

As shown in the Table, although bodyweight was unaffected by acute or chronic treatment with low-dose or high-dose CC, uterine weight increased after 24-hour and 4-day treatments at both doses. In control rats, individual epithelial cells contained large round or

FIGURE 4

E2 treatment enables recovery from clomiphene citrate (*CC*)–induced uterine apoptosis but is not protective



Rats were treated with 17-β-estradiol (*E2*) **A, B,** after or **C, D,** before 4 days of clomiphene citrate treatment. Western blot analysis (**A** and **C**) was used to measure cleaved caspase-3 protein level relative to whole protein. Gels were stained with Coomassie blue (n = 5 rats/group). β-actin served as a loading control. Caspase-3/7 activity (**B** and **D**) was determined in whole uterine tissues of rats (n = 5 rats/group). Values are means ± SEM (n = 5 rats/group). The *double asterisks* denote $P < .01$; the *triple asterisks* denote $P < .001$ vs 17-β-estradiol–treated rats.

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oval basal nuclei with very little surrounding cytoplasm (Figure 5, A1). Rats that were treated with CC for 4 days had increases in epithelial height, muscular thickness, and luminal space (Figure 5, C and C1) compared with control rats (Figure 5, A and A1). Essentially, the epithelium changed in character from cuboidal to columnar cells. In CC-treated rat uteri, the nuclei were less dark, and the long axis of the nuclei of the epithelium was parallel to the basement membrane (Figure 5, C1). Although the uterine morphologic condition of E2-treated rats (Figure 5, B and B1) was quite similar to that of CC-treated rats, treatment with E2 had less of an effect on the epithelium. Moreover, clear cells in the epithelium were occasionally present in CC-treated rats. This "bubbly" appearance may indicate the lipid and/or glycogen accumulation. There was less connective tissue between stromal cells in CC-treated rats (Figure 5, C1) than in E2-treated rats (Figure 5, B1). Histologic findings also showed a lack of integrity in the epithelium of CC-treated rats after an additional 4-day vehicle treatment (Figure 5, D and D1).

Chronic treatment with CC activates ER-α but not ER-β in the uterus

WB analysis revealed that chronic treatment with both low-dose and high-dose

TABLE
Effects of clomiphene citrate (*CC*) on bodyweight and uterine tissue weights in rats

Treatment	Weight		
	Body, g	Uterus, mg	Uterus/body, mg
Acute: 6 h			
Vehicle	58.74 ± 0.50	10.13 ± 0.42	0.17 ± 0.007
1 mg/kg CC	58.24 ± 0.81	10.49 ± 0.39	0.18 ± 0.006
10 mg/kg CC	61.06 ± 1.98	10.08 ± 0.32	0.17 ± 0.005
Acute: 24 h			
Vehicle	60.98 ± 0.68	10.17 ± 0.16	0.17 ± 0.003
1 mg/kg CC	62.98 ± 0.49	17.93 ± 0.98[a]	0.28 ± 0.014[a]
10 mg/kg CC	60.58 ± 1.38	18.32 ± 0.36[a]	0.30 ± 0.005[a]
Chronic: 4 days			
Vehicle	79.92 ± 2.16	10.68 ± 0.24	0.13 ± 0.005
1 mg/kg CC	73.68 ± 2.07	30.90 ± 0.68[b]	0.42 ± 0.016[b]
10 mg/kg CC	72.98 ± 2.15	31.50 ± 0.60[b]	0.43 ± 0.013[b]

Values are means ± SEM ($n = 5$ in all groups).

[a] $P < .05$ vs vehicle (24 hours; Bonferroni's post hoc test after significant 2-way analysis of variance time and treatment interaction); [b] $P < .05$ vs vehicle (4 days; Bonferroni's post hoc test after significant 2-way analysis of variance time and treatment interaction).

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CC resulted in a significant decrease in ER-α expression (Figure 6). Because the stability and activity of ER subtypes are affected by ligand-dependent ER phosphorylation,[9] it is important to note that chronic treatment with CC significantly enhanced the phosphorylation of ER-α, but not ER-β (Figure 6).

Chronic treatment with CC regulates Hoxa11, p27, and the progesterone receptor A isoform in the uterus

WB analysis showed that, although the levels of Hoxa11 and p27 were decreased, neither Hoxa10 nor p53 levels were altered by chronic treatment with CC (Figure 6). Chronic treatment with low-dose or high-dose CC caused a mobility shift of pan-cytokeratin compared with controls (Figure 6, A), although the cause of this shift in the apparent molecular weight of cytokeratin remains unknown. There were no changes in the levels of α-smooth muscle actin in uteri after CC treatment (Figure 6, A). Because the PR antibody (NCL-L-PGR-312) recognizes only the progesterone receptor A (PRA) isoform in tissue sections with the immunohistochemical analysis,[27] only PRA expression was analyzed in successive sections of the uterus. Chronic treatment with CC (Figure 7, B) or E2 (Figure 7, C) increased the number of PRA-positive cells in the stromal cell layer compared with controls (Figure 7, A). Moreover, concomitant CC and E2 treatment for 4 days decreased the number of PRA-positive cells in the stromal cell layer (Figure 7, D) compared with E2 treatment alone (Figure 7, C).

Comment

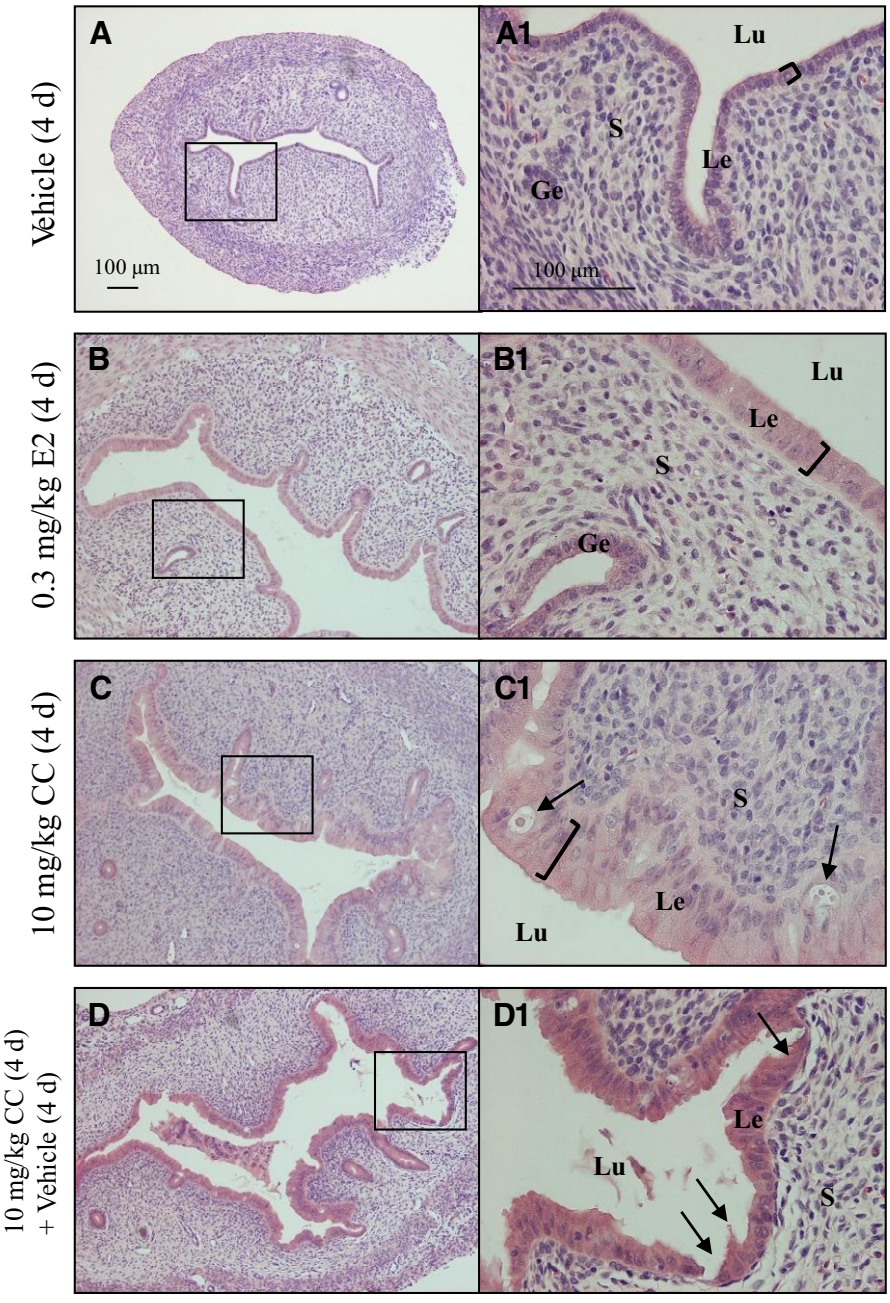
To our knowledge, this is the first study to demonstrate that chronic treatment with CC can induce not only stromal cell apoptosis but also morphologic abnormalities in the uteri of rats in a dose-independent manner. The present study highlights the potential role of the ER-α signaling pathway in the uterus after chronic CC treatment. Furthermore, the high ovulation rates combined with low pregnancy rates in women who were treated with CC may be explained by the aberrant expression of Hoxa11, p27, and PRA because of repression of ER-α expression and consequent disruption of the implantation process.

CC is a racemic mixture of zuclomiphene (38%) and enclomiphene (62%) that provides both estrogenic and antiestrogenic activities in a variety of E2-target tissues.[1,2] The effects of CC in the rodent uterus and human endometrium are inconsistent, however. For example, CC has both antagonistic and agonistic activities in the rat uterus,[25] whereas it displays a pure ER agonistic effect in the mouse uterus[28] in contrast to the human endometrium.[29-32] Our previous work has demonstrated that chronic treatment with CC induces epithelial cell apoptosis in rat fallopian tubes in a region-specific manner.[16] Here we show that, within the uterus, the stromal cells are the main cell type to be targeted by the apoptotic effects of chronic treatment with CC. In addition, in the rat uterus, CC displays agonistic activity at low concentration, although it functions as an ER antagonist at high concentration in vivo.[25] Furthermore, only chronic treatment with CC induces uterine cell apoptosis, which indicates that the agonistic and/or antagonistic effects of CC are also dependent on the duration of treatment.[21,33] These observations suggest that CC acts as an ER agonist and/or antagonist depending on the species, target tissue/cell specificity, dose range, and regimen.

We show that the effects of CC in the uterus are time dependent rather than dose dependent and that the damage can be reversed with E2, which suggests that CC, as an apoptotic inducer, blocks the antiapoptotic effect of E2 in the uterus. The clear lack of protective effects of pretreatment or concomitant with E2 could be because CC exhibits much stronger antiestrogenic activity in the presence of an E2-stimulated rat uterus.[16] It is well accepted that the endometrium undergoes morphologic and biochemical changes that are required for successful implantation and pregnancy.[8,17] Previous studies have shown that E2 is necessary for the induction of decidualization[34] and is inhibited by CC treatment in rats.[18] Several clinical studies have demonstrated that luteal E2 action in the endometrium is blocked in women who

have been treated with CC.[35,36] Although increased stromal cell proliferation, but not the apoptosis, is considered an initiator of decidualization and inappropriate uterine cell apoptosis results in the failure of implantation,[37] our results suggest that aberrant apoptotic activities of uterine stromal cells after chronic CC treatment may be a mechanism whereby the implantation process is disrupted. In agreement with a previous study, Grunert et al[38] reported that chronic treatment with CC blocked the action of E2 only in stromal cells and not in epithelial cells. Although in vitro tissue recombinant studies have shown that stromal cells are able to regulate epithelial cell proliferation in response to E2 stimulation,[19] our finding of massive damage to the epithelial cell layer in rats that are treated with CC when E2 is absent (Figure 5, D) indicates that stromal cells may fail to support and maintain their epithelial structure in response to CC when antagonizing E2 in vivo. Indeed, the addition of E2 after CC treatment in women has been shown to improve cervical mucus that results in increased endometrial thickness and conception rates.[39] Collectively, our results suggest that negative effects of CC on the transformation of stromal cells (direct) and the disintegration of stromal-epithelial interactions that lead to epithelial cell destruction (indirect) may inhibit implantation in the uterus. Although chronic CC treatment increases endogenous E2 levels in rats,[16] it has been suggested that endometrial changes in women who are treated with CC are due to an antiestrogenic effect of CC itself rather than changes in CC-induced hormonal levels.[40] The molecular mechanism of E2 supplementation in the uterus of CC-treated rats suggests that estrogen status of patients who are appropriate candidates for CC therapy should be taken into consideration to increase efficacy. A better understanding of the CC-induced apoptotic mechanism in the uterus could reveal new therapeutic strategies for the improvement of implantation rates and reduction of the risk of miscarriage in humans who are treated with CC. For example, additional treatment with the antioxidant N-acetylated cys-

FIGURE 5
Histologic condition of the rat uterus



Hematoxylin/eosin staining of uterine tissues from rats treated with **A, A1,** vehicle, **B, B1,** 17-β-estradiol (*E2*; 0.3 mg/kg) or **C, C1,** clomiphene citrate (*CC*; 10 mg/kg) for 4 consecutive days plus 4 additional days of vehicle injections (**D, D1**). Note that, although treatment with both 17-β-estradiol (**B1**) and clomiphene citrate (**C1**) increases epithelial cell height, clear cells in the epithelial cell layer (**C1,** *arrows*) are present only in clomiphene citrate-treated rats. Incomplete epithelial cell layers were found in the 4-day clomiphene citrate plus 4-day vehicle-treated group (**D1,** *arrow*). Uterine tissues from different rats (n = 5/group) were evaluated. All photographs were taken with a ×10 or a ×40 magnification; the exact scale is given in the Figure.

Ge, glandular epithelial cells; *Le*, luminal epithelial cells; *Lu*, lumen; *S*, stromal cells.

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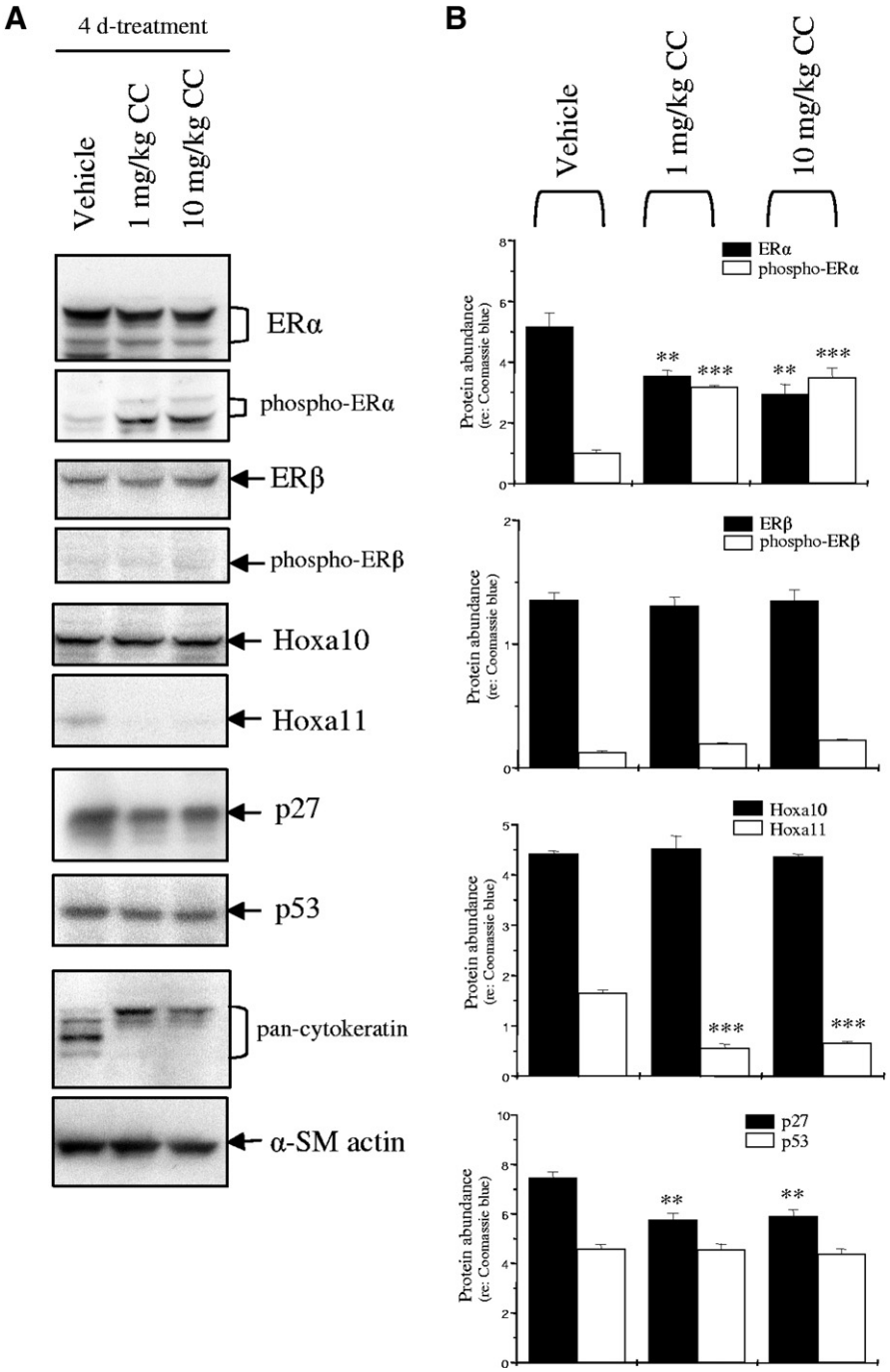
FIGURE 6
Estrogen receptor (*ER*) subtype activation and cell markers for implantation during clomiphene citrate (*CC*)–induced uterine apoptosis



Western blot analysis was used to measure estrogen receptor α, phospho-estrogen receptor α, estrogen receptor β, phospho-estrogen receptor β, Hoxa10, Hoxa11, p27, and p53 relative to whole protein; gels were stained with Coomassie blue (n = 5 rats/group). Expression of pan-cytokeratin and α-smooth muscle (*SM*) actin was evaluated in the same samples. Values are means ± SEM. The *double asterisks* denote $P < .01$; the *triple asterisks* denote $P < .001$ vs vehicle-treated controls.

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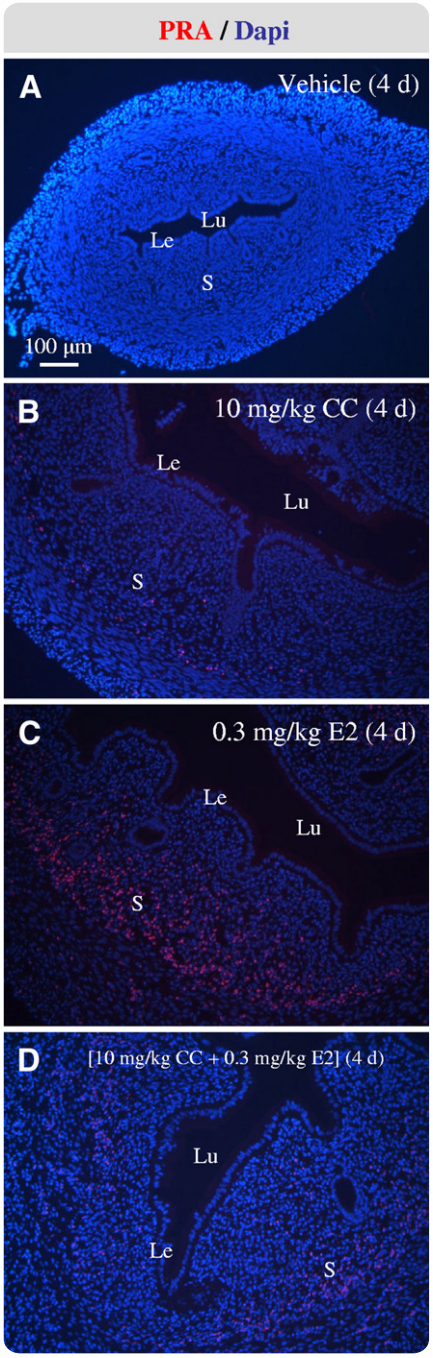
teine (an antiapoptotic agent) significantly improves endometrial function and leads to increases in ovulation and pregnancy rates, compared with CC treatment alone, in women with polycystic ovary syndrome.[41]

One of the most important findings of this study is that, although CC binds to both ER-α and ER-β with approximately the same affinity in vitro,[42] the cellular response to CC is dependent on ER-α expression and phosphorylation in the rat uterus in vivo, which indicates that ER-α and ER-β are not functionally equivalent in the response to CC treatment. Both ER subtypes are expressed in rodent uteri and human endometrium,[11] and either subtype can display diverse transactivational properties in a ligand-dependent manner when ER-α and ER-β are coexpressed.[43,44] Furthermore, ER-β has the capacity to regulate ER-α function in vitro[43,44] through the formation of functional heterodimers (instead of homodimers) for ER-mediated transcription.[45] Our studies cannot eliminate the possibility that the agonist/antagonist activities of CC may also be influenced by the relative expression of the 2 receptors in the uterus in vivo.

Successful implantation involves a complex sequence of signaling events.[8,17] Several genetically modified mouse models with gene disruptions in ER, PR, Hoxa10, Hoxa11, p27, and p53 show implantation defects,[9,17,24] which suggests that the expression and activation of these genes are essential to the implantation process in vivo. In the mouse uterus, treatment with E2 increases ER-α expression in stromal cells but not in epithelial cells.[11] It has been shown that the disruption of ER-α, but not ER-β, leads to failure of implantation in female mice,[9,11] which provides evidence for a vital and distinctive role of the 2 ER subtypes in the uterus. Moreover, studies that have used ER-α knockout mice in vivo and uterine tissue recombinants in vitro have revealed that the estrogenic regulation of PR expression requires stromal ER-α but is independent of epithelial ER-α.[46] In addition, the maintenance of stromal PR expression is required for the establishment of the "implantation window."[17,24] Previous

FIGURE 7
Expression of progesterone receptor A (*PRA*) isoform in the stromal cell layers of the rat uterus



Uterine progesterone receptor A immunoreactivity is absent in the **A,** vehicle group but is seen in the **B,** clomiphene citrate (*CC*)–treated group. Note that the immunoreactivity of progesterone receptor A is less intense in the **B,** clomiphene citrate-treated and the **D,** clomiphene citrate + 17-β-estradiol (E2)-treated groups compared with the **C,** 17-β-estradiol–treated group. These stainings were repeated in 5 rats/group with similar results. All photographs were taken with a ×10 magnification; the exact scale is given in the Figure.

Le, luminal epithelial cells; *Lu,* lumen; *S,* stromal cells.

Nutu. Clomiphene citrate treatment and rat uterus. Am J Obstet Gynecol 2010.

studies, however, could not distinguish fully which stromal PR isoform was responsive to E2 stimulation. Although PRA is the functional PR isoform that is responsible for the regulation of uterine stromal differentiation during implantation,[9,11] it is interesting to note that CC is able not only to increase PRA expression when E2 is absent but is also able to block E2-induced PRA expression in rat uterine stromal cells, which suggests that CC converts from an estrogenic agonist to an estrogenic antagonist in the presence of E2. Previous studies from our laboratory and others have shown that p27 is a target gene of PR signaling in the uterus.[26,47] Because CC does not bind directly to the uterine PR[18] and chronic treatment with CC fails to change endogenous progesterone levels,[16] we propose that the antiestrogenic effects of CC on the regulation of p27 expression seem to be mediated by ER-α and do not require the presence of progesterone directly. There is no change in the expression of p53 after CC treatment in the uterus, which suggests that divergent regulatory pathways between p27 and p53 are triggered by CC treatment. Although both Hoxa10 and Hoxa11 are downstream targets of the action of estrogens in the mouse uterus,[37] we observed only the down-regulation of Hoxa11 in the CC-treated rat uterus. Because Hoxa11 is expressed only in the stromal cells, our results support the idea that the CC-induced expression of stromal signals may control the epithelial fate. Together, these data highlight the major role of the ER-α signaling pathway in the antiestrogenic effect of CC on the uterine stromal cells. Whether these molecules are independent of 1 another in CC-induced implantation defects will require further investigation.

In terms of the cell-type selection mechanism in the rat uterus in vivo, we have reported direct molecular mechanisms by which the antiestrogenic effects of CC induce stromal cell-specific apoptosis and regulate the expression of molecules that are important for the implantation process through the ER-α signaling pathway in the rat uterus. At least in part, the present study has resulted in a cellular and molecular mechanism-based explanation for the discrepancy between high ovulation rates and low successful pregnancy rates in women who are treated with CC.[2,4,5] Moving forward, it is noteworthy that there is also a need to evaluate such changes in women who undergo CC treatment. The use of endometrial biopsies in CC-treated women who do not become pregnant might help to explore our hypotheses that have been generated from this study. ■

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