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Molecular mechanism of cell proliferation in rodent uterus during the estrous cycle

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ABSTRACT

The rodent uterus is a widely studied target tissue for sexual steroid hormone action. The aim of the present study was to assess the molecular mechanism that participates in the initiation of cell proliferation of the rat uterine epithelial cells during the estrus (E)–metestrus (M) transition. Cell proliferation, ERα, c-fos, cyclin D1 and D3, cdk4, and cdk6 proteins were assessed in these animals by immunohistochemistry. Estradiol (E_2) and progesterone (P_4) plasma levels were assessed by RIA. The results indicate that the glandular epithelium starts to proliferate at 21:00 h on estrus day, and initiates at least 3 h before the luminal epithelium does. Fos expression was markedly increased during the afternoon of estrus day, and its increase was in parallel to ERα expression. Interestingly, both, cyclin D1 and D3 were abundantly expressed in the luminal and glandular epithelia, and nuclear immunolabelling of cyclin D1 and D3 precedes BrdU incorporation in the cell. cdk4 and cdk6 were localized in the nuclei in both epithelia throughout the studied time course. In addition, cdk4 was more abundant throughout estrus and metestrus days than cdk6. The overall results indicate that ERα, Fos and cyclins D1 and D3, cdk4 and cdk6 are expressed in both glandular and luminal epithelia of the rat uterus during the E–M transition. In conclusion, there is a good correlation between sequential expression of these proteins and cell cycle progression in the rat uterine epithelial cells during the estrous cycle. However, the differences observed in the cellular localization, time course of expression and the cellular types that express both cyclins between physiological and pharmacological conditions, demonstrated different mechanisms of regulation and should be due to the complex hormonal milieu during the estrous cycle.

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1. Introduction

Sex steroids are widely recognized as primary regulators of proliferation, differentiation and function of many different target tissues at the molecular level. The rodent uterus has served as a valuable model for monitoring tissue responsiveness to ovarian steroid hormone stimulation. In adult ovariectomized (ovx) rat and mouse, the major effects of acute stimulation with estrogen are rapid onset of DNA synthesis and cell proliferation in the endometrium, preceded by specific changes in cell cycle related gene expression [1–4].

The mechanism of estrogen action in target tissues is mediated by its interaction with the two estrogen receptor (ER) subtypes, denoted ERα and ERβ. Both ERs are widely distributed throughout the body, displaying distinct but overlapping expression patterns in a variety of tissues. However, in the rat uterus, ERα has been reported to be the dominant subtype in the luminal (LE) and glandular epithelia (GE) [5,6]. In several mammals, including rodents, cyclical hormone levels regulate ERα activity in uterus and oviduct [5,7]. These intracellular proteins are members of a large superfamily of nuclear receptors that function as ligand-activated transcription factors. ER ligand complex interact with estrogen response elements (ERE) located in the promoter region of target genes [8]. This complex recruits coactivators (or corepressors) and other regulatory proteins that form the active transcription complex. The ER ligand activating complex induces the expression of a variety of genes, including early induced genes such as the proto-oncogenes, c-fos, c-jun, jun-B, and jun-D, among others [9–12]. Fos and Jun proteins form the AP-1 complex, which participates in cell proliferation and differentiation. It is known that AP-1 complexes regulate different growth factors such as IL-2, TGFα and TGFß [13]. Although these genes modify cellular proliferation, it

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remains unclear how the AP-1 complex regulates DNA replication after mitogenic stimulus in the rat uterus during the estrous cycle.

Two AP-1 sites (5′-TGACTCA-3′ and 5′-TAACGTCA-3′) have been identified in the promoter region of human *cycD1* gene [14–16]. These regulatory sequences were proposed to be the mechanism by which estrogens modulate DNA replication and cellular proliferation processes in the reproductive tract of ovx rats because they can induce cyclins which participate in the G1-S transition of the cell cycle. However, the promoter regions of cyclins D1 and D3 in rodents do not seem to have AP-1 regulatory sequences [17–19].

The best known regulators of G1 progression in the mammalian cell cycle are represented by the three D-type cyclins (D1–D3), whose concentrations in the cell fluctuate characteristically during progression through G1 in response to mitogenic stimuli [20–23]. They act predominantly by associating with the catalytic subunits of their specific cyclin-dependent kinase (CDKs) partners, cdk4 and cdk6. Their primary action is to phosphorylate members of the retinoblastoma (pRB, p107 and p130) family of proteins, which results in release of E2F transcription factors (E2F 1–7) from an inhibitory pRB complex and activation of genes required for cell cycle progression, such as cyclin E, cyclin A, cdc2, and cdk2 [24–31]. The actions of cyclins and their associated kinases are regulated by two families of cyclin-dependent kinase inhibitors (CKI), CIP/KIP and INK4 [32–35].

Several studies have demonstrated the effects of E_2 on the expression of cyclin and CDK activity in ovx adult rat uterus. The results indicate that cdk4, cdk5, and cdk6 but not cdk2 are significantly activated in the uterus within the first few hours of hormonal stimulation, accompanied by expression of D-type (D1–D3), A and E cyclin messenger RNAs. Analysis of RNA extracted from E_2-stimulated rat endometria shows early accumulation of D1 and D3, but not D2 cyclin mRNA, preceded by transient accumulation of c-fos mRNA [22]. It has also been reported that cyclin D1 and D3 mRNAs were induced 2.5-fold after c-fos and before thymidine kinase mRNA peaks in an immature mouse pharmacological model. The E_2-induced cyclin D1 and D3 gene expression was blocked by the antiestrogens tamoxifen and ICI 182,780. The administration of cycloheximide (CHX) to E_2 primed animals produce a superinducibility of cyclin D3, but not cyclin D1 mRNA. These results suggest that both cyclin D1 and D3 mRNAs are constitutively expressed in uterine tissues and induced by E_2 during the G1 phase of the mouse uterine cell cycle. However, the superinducibility and temporal shift of cyclin D3 by CHX suggest that there is a different regulatory mechanism underlying cyclin D1 and D3 gene expression in mouse uterine cell cycle progression [36]. Interestingly a previous study indicate that treating to ovx rats with estradiol induces nuclear expression of cyclin D1 and D3 proteins in the luminal and glandular epithelium respectively, indicating a differential regulation of proliferation in these cellular types [37].

Some of the mechanisms for estrogen-dependent uterine proliferation have been previously investigated in E_2 treated ovx animals. However, there are no studies showing the expression pattern of proteins that initiate cell proliferation in uterine epithelial cells on the rat uterus during the estrus (E)–metestrus (M) transition. In the present study, cell proliferation, ERα, c-fos, cyclin D1 and D3, cdk4 and cdk6 protein expression was assessed in intact animals during different hours through the transition from estrus–metestrus. In addition, correlation with estradiol (E_2) and progesterone (P_4) plasma levels was analysed.

2. Materials and methods

2.1. Animals

All animal maintenance and handling was carried out in accordance with the Guidelines of the Mexican Federal Law of Animal Protection. Intact adult female Wistar rats (200–250 g) were used, which presented at least four regular 4-day estrous cycles, as determined by daily vaginal smears. Animals were maintained under a 12:12 h light:dark cycle with food and water available ad libitum. Groups of five rats were killed at 13:00, 17:00 and 21:00 h on the day of estrus (E), and at 00:00, 03:00, 06:00, 09:00 and 13:00 h on the day of metestrus (M). Uteri were dissected, trimmed of connective tissue, and immediately fixed with ice-cold 4% paraformaldehyde in phosphate buffer saline (PBS) for 2 h. Tissues were dehydrated through a series of increasing ethanol concentrations and finally cleared with xylene. Tissues were then embedded in paraplast (OxfordLabware, St. Louis, MO).

2.2. BrdU immunostaining

Two hours prior to killing, each animal received an i.p. injection of 5-bromo-2'-deoxyuridine (BrdU) in PBS at a dose of 50 mg/kg. All animals were sacrificed at the hours previously described during the transition from estrus (E) to metestrus (M). The uteri were dissected, trimmed of connective tissue, and embedded in paraplast. Tissue sections (5 μM thick) were cut and mounted on poly-L-lysine coated slides. The BrdU Labelling and Detection Kit (Roche, Indianapolis, IN) was used to detect cells that had incorporated BrdU into their DNA, following the manufacturer's protocol. Briefly, rehydrated sections were microwaved (two cycles of 10 min each) in 0.01 M sodium citrate, pH 6, and cooled to room temperature. After washing with PBS, the slides were incubated in 0.5% Triton X-100 for 10 min, and non specific immunoglobulin binding was blocked by incubating sections in 5% bovine serum albumin (BSA) for 30 min. Sections were incubated with an anti-BrdU mouse monoclonal antibody at a 1:10 dilution in incubation buffer (66 mM Tris buffer, 0.66 mM $MgCl_2$, 1 mM 2-mercaptoethanol) for 60 min at 37 °C. As negative control, primary antibody was omitted. After washing with PBS, sections were incubated for 2 h at room temperature with fluorescein-conjugated rabbit anti-mouse IgG diluted 1:10 in PBS. Slides were washed with PBS and covered with DAKO Fluorescent Mounting Medium (DAKO Corporation, Carpinteria, CA). The sections were observed in an Eclipse E600 fluorescent microscope (Nikon Co., Ltd., Tokyo, Japan) using a B-2A filter from Nikon. The proliferation rates of the luminal and glandular epithelia were assessed by counting the number of BrdU positive nuclei in a section and expressed as percent of the total number of epithelial cells. Five sections were analyzed from each animal. $N = 5$ animals per day.

2.3. Immunohistochemistry

Tissue sections (5 μm thick) were cut and mounted on poly-L-lysine coated slides. Sections were cleared of paraplast with xylene, rehydrated and processed for immunohistochemistry, as previously described [2]. Briefly, rehydrated sections were microwaved (two cycles of 10 min each) in 0.01 M sodium citrate, pH 6, and cooled to room temperature. Endogenous peroxidase was blocked by incubation in 3% hydrogen peroxide in PBS for 30 min. After rinsing in PBS, the slides were incubated in 0.5% Triton X-100, and non specific immunoglobulin binding was blocked by incubating sections in 5% bovine serum albumin for 30 min. The sections were incubated overnight with primary antibody at the specified dilutions: ERα: 1:200; c-fos: 1:200; cyclin D1: 1:100; cyclin D3: 1:100; cdk4: 1:100; cdk6: 1:100 (sc-542, sc-52, sc-717, sc-182, sc-260, sc-7181, respectively, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in a humidified chamber at 4 °C. As negative control, two different procedures were used. In the first one, primary antibody was omitted, and in the second one, slides were incubated with normal rabbit IgG (sc-2027, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) in place of the first antibody. After washing with PBS, the sections were incubated for 2 h at room temperature with biotin-conjugated

Table 1
Nuclear immunostaining of the studied proteins in the uterine luminal epithelium during the estrus–metestrus transition.

	E 13:00	E 17:00	E 21:00	M 00:00	M 03:00	M 06:00	M 09:00	M 13:00
BrdU	–	–	–	–	+	++	+++	+++
ERα	++	++	++	++	++	+++	+++	+++
Fos	+	++	++	+++	+++	+++	+++	+++
cyc D1	–	–	+++	+++	++	++	+	–
cyc D3	–	+	+++	++	–	–	–	+
cdk4	+++	+++	+++	+++	+++	+++	+++	+++
cdk6	+++	++	++	++	++	+++	+++	+++

–, no immunopositive nuclei present; +, low number of immunopositive nuclei; ++, moderate number of immunopositive nuclei; +++, high number of immunopositive nuclei. BrdU: bromodeoxyuridine incorporation (proliferation); cyc D1: cyclin D1; cyc D3: cyclin D3.

goat anti-rabbit IgG (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) at a 1:100 dilution and then washed with PBS to remove unbound secondary antibody, and incubated for 1 h at room temperature in peroxidase-conjugated avidin–biotin reagent (Santa Cruz Biotechnology, Inc., Santa Cruz CA). The slides were washed with PBS, developed with DAB substrate, and counterstained with Mayer's hematoxylin. The slides were analyzed under a Nikon E600 microscope. Five sections were analyzed for each animal, $N = 5$ animals per studied hour. Nuclear staining was assigned the following scores: –, no immunopositive nuclei present; +, low number of immunopositive nuclei; ++, moderate number of immunopositive nuclei; and +++, high number of immunopositive nuclei. The histologic score was assessed by three independent observers.

2.4. Radioimmunoassay

Specific radioimmunoassays were performed in serum samples from all studied animals during the E–M transition. All samples were stored at −70 °C until E_2 and P_4 were determined, as previously described [38]. The sensitivity of the assays was 5.43 pg/mL for E_2, 8.3 ng/mL for P_4. The intra and interassay variation coefficients were 5.3% and 8% for E_2 respectively and 6.5% and 10% for P_4 respectively.

2.5. Statistical analysis

BrdU immunostaining and radioimmunoassay data were analyzed using one-way analysis of variance (ANOVA) followed by a Tukey's multiple comparison test. The Prism 2.01 program (Graph Pad, San Diego, CA) was used for calculating probability values.

3. Results

3.1. Proliferation pattern in luminal and glandular epithelia of the rat uterus during the estrus–metestrus transition of the estrous cycle

In order to determine the proliferation pattern of uterine epithelial cells during the transition from estrus (E) to metestrus (M), we evaluated the incorporation of BrdU in luminal and glandular epithelial cells of the rat uterus, as an index of DNA synthesis.

During the E–M transition, we observed that the glandular epithelium was the first to present labelled cells, starting at 21:00 h of E day (Figs. 1 and 2). A significant increase of labelled cells was observed at 00:00 h of M day (30.9 ± 4.9%) and the highest labelling index was detected at 03:00 h on M day (56.9 ± 8.3%), maintaining these high levels of proliferation until 13:00 h on M day (Figs. 1 and 2).

In contrast, luminal epithelium proliferation begins at 03:00 h on M day. At that hour, the BrdU index of the LE was of 10.1 ± 1.7% and continued to increase during the next hours, presenting its highest levels (38.7 ± 5.5%) at 13:00 h on that same day (Figs. 1 and 2). Interestingly, we observed that in the luminal epithelium, as shown in Fig. 1C and D, the cells proliferate forming "proliferation islets" composed of several proliferating cells surrounded by non proliferating areas.

3.2. Appearance of ERα protein in uterine epithelia during the E–M transition

ERα protein in uterus of intact animals during the E–M transition was assessed by immunohistochemistry. ERα immunoreactivity was detected in the cytoplasm and nuclei of both LE and GE (Fig. 3). The lowest number of ER immunopositive nuclei in luminal epithelia was observed at 13:00 h on E day (Fig. 3A, Table 1). A significant increase in the number of immunopositive nuclei in the luminal epithelia was observed on E 21:00 h (Fig. 3C). The number of immunopositive nuclei continue to increase until M 09:00 h (Fig. 3G), and these high levels are maintained until M 13:00 h (Fig. 3I and Table 1). In the glandular epithelium, intense nuclear staining was observed throughout the time course (Fig. 3 B, D, F, H, J and Table 2).

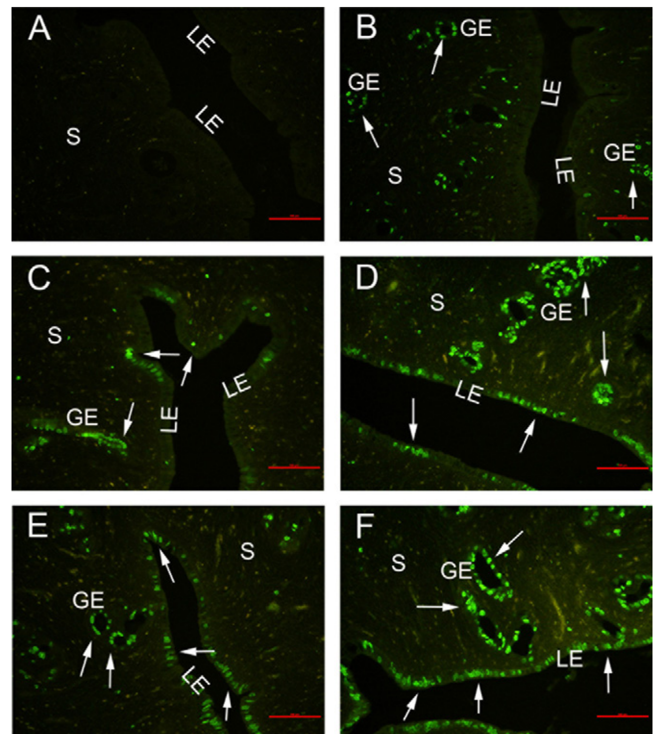


Fig. 1. Rat uterine glandular epithelium proliferates before than the luminal epithelium during the estrous cycle. Uterine epithelial proliferation during the E–M transition of the estrous cycle was assessed by BrdU incorporation in uterine tissues of the rat at each of the studied times. (A) E 21:00 h, (B) M 00:00 h, (C) M 03:00 h, (D) M 06:00 h, (E) M 09:00 h, (F) M 13:00 h. Arrows indicate marked cells. LE, luminal epithelium; GE, glandular epithelium; S, stroma. Bar = 100 μm.

Table 2
Nuclear immunostaining of the studied proteins in the uterine glandular epithelium during the estrus–metestrus transition.

	E 13:00	E 17:00	E 21:00	M 00:00	M 03:00	M 06:00	M 09:00	M 13:00
BrdU	–	–	–	++	+++	+++	+++	+++
ERα	+++	+++	+++	+++	+++	+++	+++	+++
Fos	+++	+++	+++	+++	+++	+++	+++	+++
cyc D1	+++	+++	+++	+++	+++	++	++	–
cyc D3	–	+	+++	++	+	–	–	+
cdk4	+++	+++	+++	+++	+++	+++	+++	+++
cdk6	+++	+++	++	+++	+++	+++	+++	+++

–, no immunopositive nuclei present; +, low number of immunopositive nuclei; ++, moderate number of immunopositive nuclei; +++, high number of immunopositive nuclei. BrdU: bromodeoxyuridine incorporation (proliferation); cyc D1: cyclin D1; cyc D3: cyclin D3.

Stromal cells also presented immunopositive nuclei during the studied time course. However, a significant increase in the number of immunopositive nuclei was observed at M 09:00 h (Fig. 3G, H)

3.3. Appearance of c-fos protein in uterine epithelia during the E–M transition

To determine whether there is a correlation between the initiation of endometrial epithelial cell proliferation and c-fos expression in the uterus of intact animals, c-fos protein was detected by immunohistochemistry. The lowest number of c-fos immunopositive nuclei in the luminal epithelia was detected at 13:00 and 17:00 h of E day (Fig. 4A, Table 1). A marked increase in c-fos nuclear immunostaining was observed from 21:00 h of E day until 13:00 h of M day (Fig. 4 C, E, G and I). In the glandular epithelia, intense nuclear immunostaining was detected during the entire time course (Fig. 4 B, D, F, H, J and, Table 2).

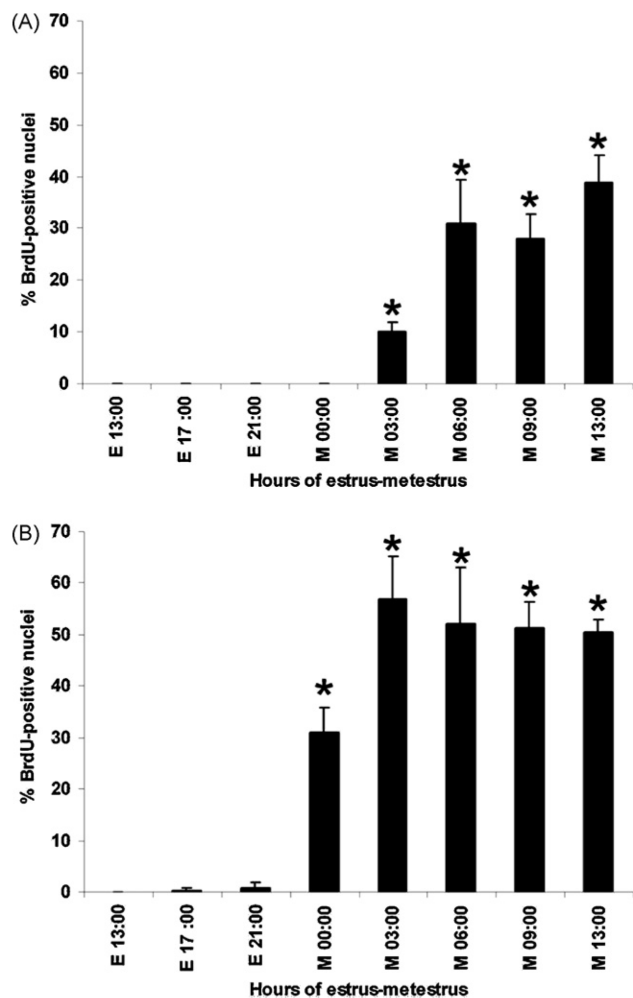


Fig. 2. Quantitative representation of BrdU incorporation in the uterine epithelia during the E–M transition of the estrous cycle. Immunofluorescent nuclei were determined in (A) luminal epithelium and (B) glandular epithelium. E, estrus; M, metestrus. Results are expressed as the mean ± SD. In (A) $^*p < 0.05$ vs. E 13:00, 17:00, 21:00 h and M 00:00. In (B) $^*p < 0.05$ vs. E 13:00, 17:00 and 21:00 h.

3.4. Time course appearance of cyclins D1 and D3 during uterine epithelial cell proliferation

In order to determine whether there is a difference in the cell type expression pattern of cyclins D1 and D3 during the estrus–metestrus transition of the estrous cycle, we assessed cyclin D1 and D3 protein localization by immunohistochemistry. Cyclin D1 and D3 were detected in both epithelia, and showed nuclear and cytoplasmic localization (Figs. 5 and 6).

In the luminal epithelium, cyclin D1 is predominantly localized in the cytoplasm during E 13:00 and E 17:00 h, while cyclin D3 is the first one to be detected in the nuclei at E 17:00 h. There is an increase in nuclear immunostaining of cyclin D1 and D3 proteins from 13:00 to 21:00 h on E day, which precedes BrdU cell incorporation. At 21:00 h of E day we detected the highest number of cyclin D1 and D3 immunolabelled nuclei (Figs. 5C and 6C, Table 1). A reduction in cyclin D1 and D3 nuclear immunostaining was detected in luminal epithelium from 00:00 to 13:00 h during metestrus (Figs. 5 and 6, Table 1). However, cyclin D3 nuclear localization decreased more rapidly than cyclin D1. At M 13:00 h there was strong cytoplasmic immunostaining of both cyclins but no nuclear localization of cyclin D1 was observed, while some nuclei still presented cyclin D3 immunostaining (Figs. 5I and 6I). In the glandular epithelium, cyclin D1 is found in the nuclei beginning at E 13:00 h (Fig. 5B, Table 2), presents the highest nuclear immunostaining at E 21:00 h (Fig. 5D), and slowly decreased during the next hours (Fig. 5F and H), presenting predominantly cytoplasmic localization at M 13:00 (Fig. 5J). In contrast, cyclin D3 was found predominantly in the cytoplasm at E 13:00 h (Fig. 6B), it translocates to the nucleus at E 17:00 h (Table 2), and is found predominantly in the cytoplasm from M 00:06 to M 09:00 h (Fig. 6H). At M 13:00 h some nuclei present cyclin D3 immunostaining (Fig. 3J, Table 2). The time period of nuclear localization and initial site of induction of cyclins D1 and D3 in both, luminal and glandular epithelial cells, is summarized in Fig. 9.

3.5. cdk4 and cdk6 present constant nuclear localization throughout the estrus–metestrus transition

The best characterized partners of D-type cyclins during G1-S are CDK's 4 and 6. We studied the expression of these proteins during the E–M transition of the estrous cycle. cdk4 immunostaining was detected in nuclei and cytoplasm in both epithelia and remained almost constant during the studied period (Fig. 7, Tables 1 and 2). In contrast, in the luminal epithelium, the number of cdk6 immunopositive nuclei presented a slight decrease from E 13:00 to M 00:00 h. In the following hours (M 03:00 to M 13:00 h) the number of immunopositive nuclei increased to similar levels as observed at E 13:00 h (Fig. 8).

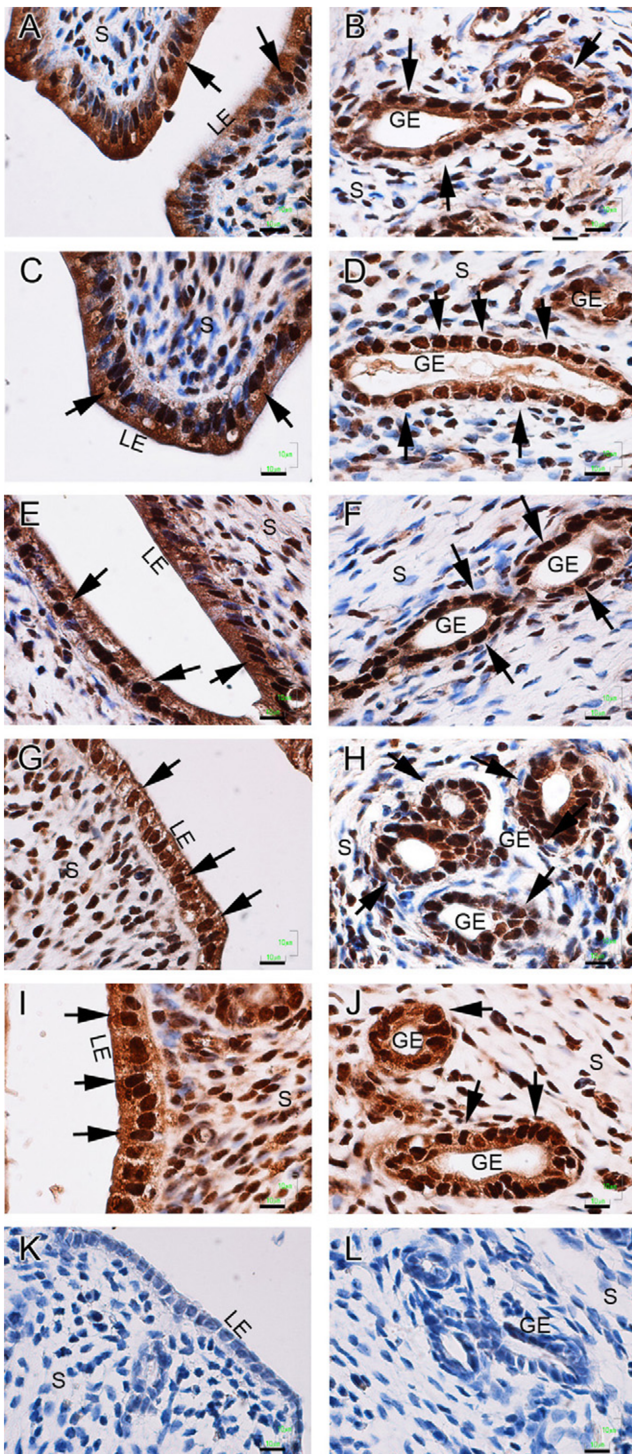


Fig. 3. ERα nuclear localization precedes proliferation in the rat uterus during the E–M transition of the estrous cycle. Immunohistochemical localization of ERα protein in: (A and B) estrus 13:00 h; (C and D) estrus 21:00 h; (E and F) metestrus 03:00 h; (G and H) metestrus 09:00 h; (I and J) metestrus 13:00 h; (K and L) Negative control. Arrows indicate marked cells. LE, luminal epithelium; GE, glandular epithelium; S, stroma. Bar = 10 μm.

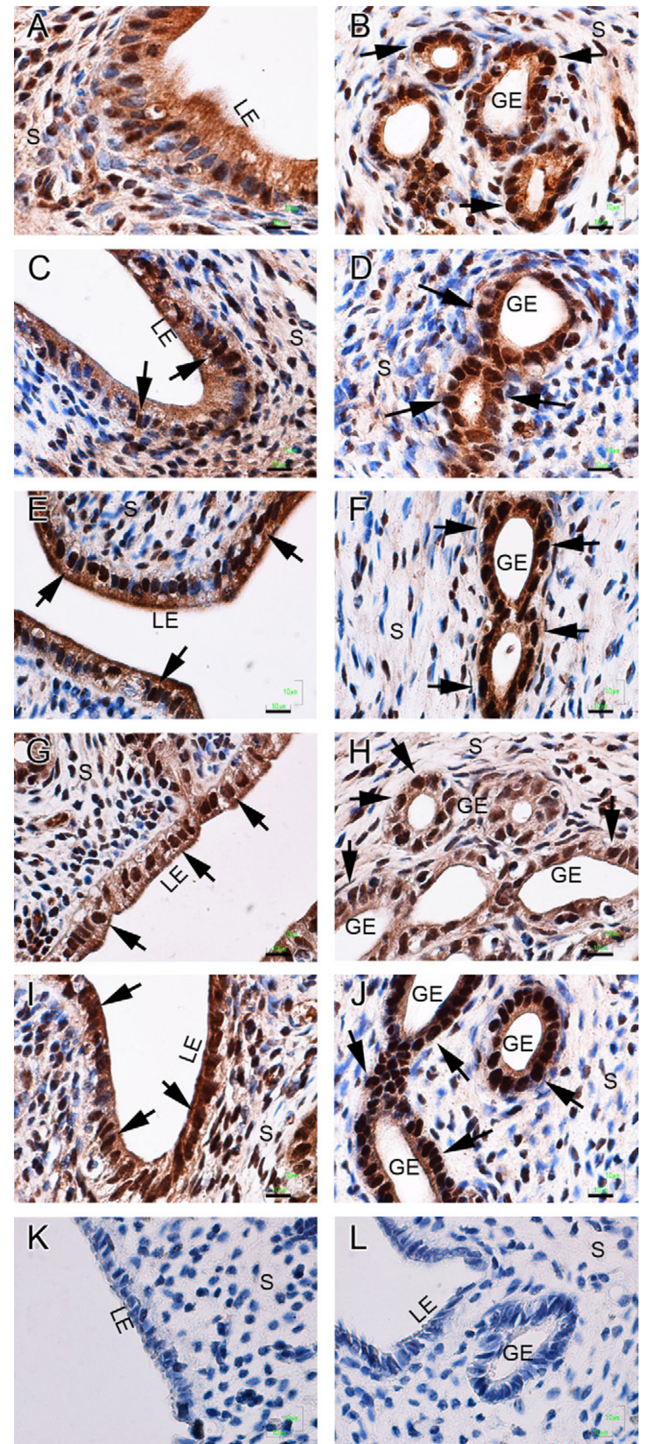


Fig. 4. Immunohistochemical localization of Fos protein in the rat uterus during the E–M transition of the estrous cycle. (A and B) estrus 13:00 h; (C and D) estrus 21:00 h; (E and F) metestrus 03:00 h; (G and H) metestrus 09:00 h; (I and J) metestrus 13:00 h; (K and L) negative control. LE, luminal epithelium; GE, glandular epithelium; S, stroma. Arrows indicate marked cells. Bar = 10 μm.

3.6. Radioimmunoassay

E_2 and P_4 levels were determined in all studied animals. Interestingly, the lowest E_2 levels were found during the E–M transition as compared to the proestrus and diestrus days. Conversely, the highest P_4 levels were found at 13:00 h on metestrus day (Table 3).

4. Discussion

One of the main effects of estrogens on the uterus and the mammary gland is the induction of cellular proliferation. The molecular mechanism by which estradiol induces uterine cellular proliferation has been extensively studied in ovx rats [1,39–42]. However, few studies have been performed under physiological conditions [5].

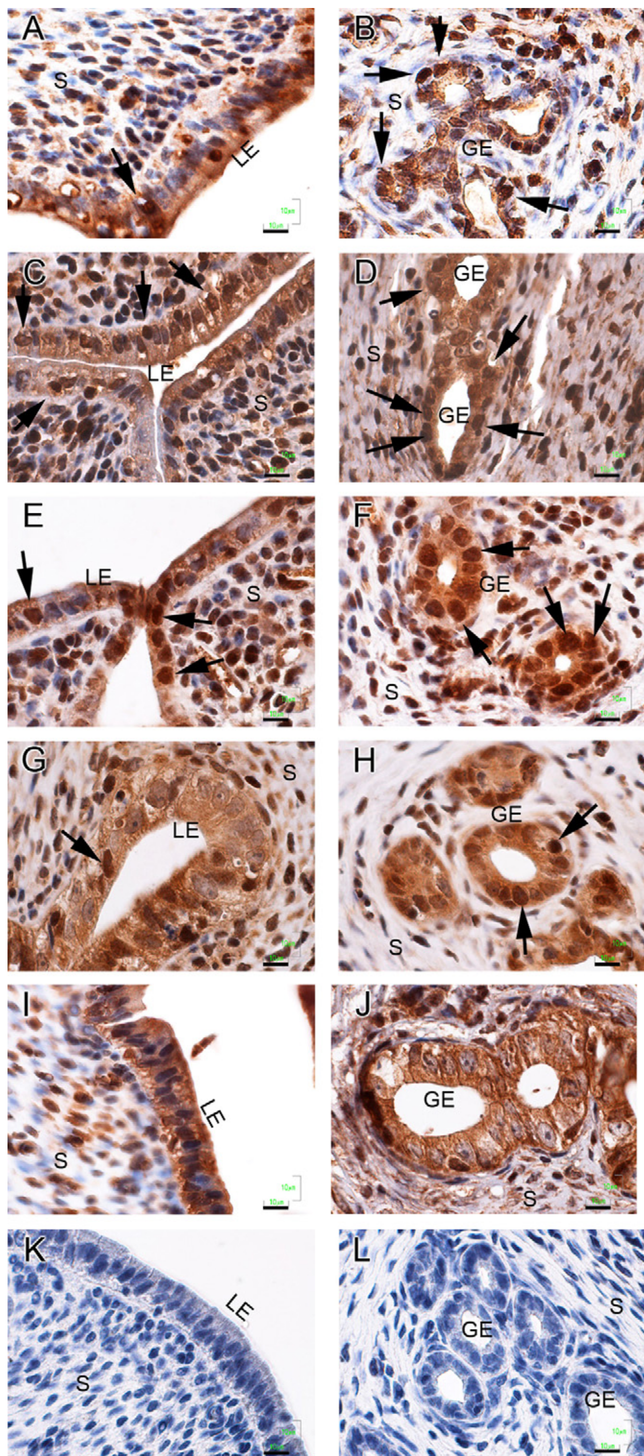


Fig. 5. Changes in cyclin D1 localization from the cytoplasm to the nucleus of the epithelial cells of the rat uterus during the E–M transition of the estrous cycle. Immunohistochemical localization of cyclin D1 protein in (A and B) estrus 13:00 h; (C and D) estrus 21:00 h; (E and F) metestrus 03:00 h; (G and H) metestrus 09:00 h; (I and J) metestrus 13:00 h; (K and L) negative control. Arrows indicate marked cells. LE, luminal epithelium; GE, glandular epithelium; S, stroma. Bar = 10 μm.

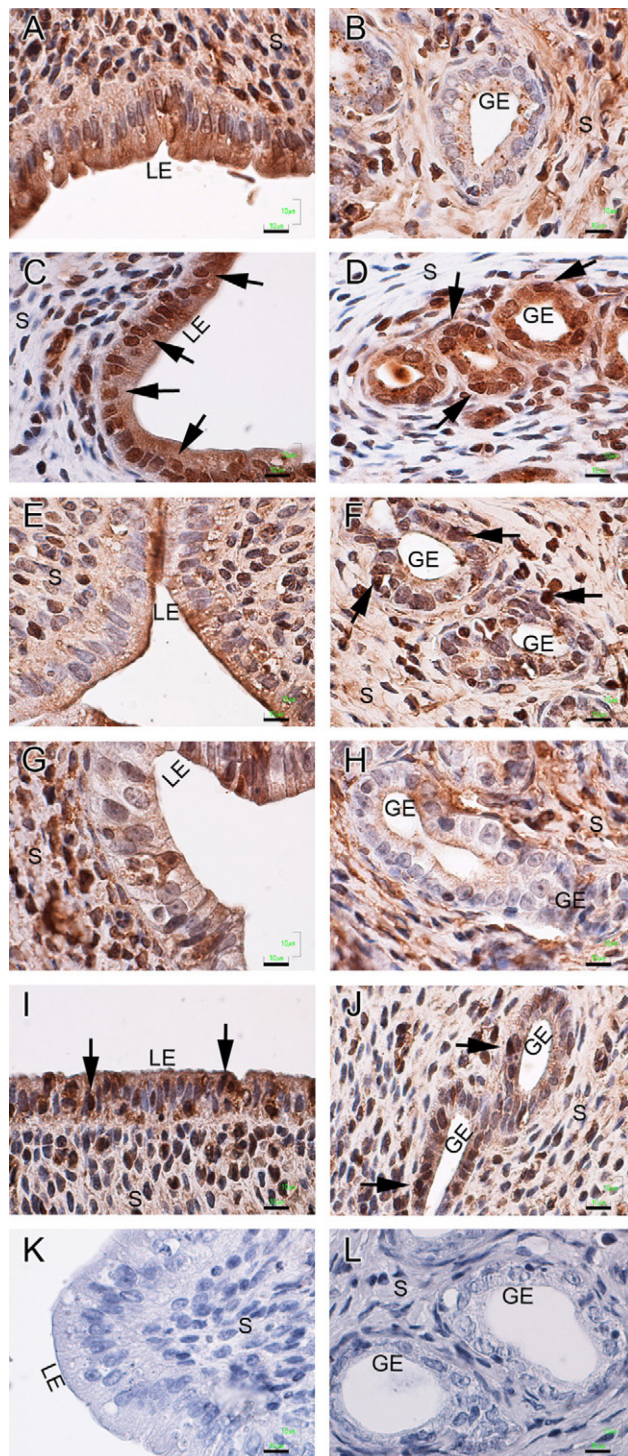


Fig. 6. Cyclin D3 presents a brief nuclear expression in the rat uterine epithelia during the E–M transition of the estrous cycle. Immunohistochemical localization of cyclin D3 protein in: (A and B) estrus 13:00 h; (C and D) estrus 21:00 h; (E and F) metestrus 03:00 h; (G and H) metestrus 09:00 h; (I and J) metestrus 13:00 h; (K and L) negative control. Arrows indicate marked cells. LE, luminal epithelium; GE, glandular epithelium; S, stroma. Bar = 10 μm.

In order to establish the time of initiation of proliferation (S phase entry) in uterine epithelia during the estrus–metestrus transition, we performed time course experiments using BrdU incorporation [43]. We observed that 30% of the glandular epithelial cells are in S phase at 00:00 h of metestrus day, while in the luminal epithelium 10% of the cells are in S phase at 03:00 h of metestrus (Figs. 1 and 2). Interestingly, during the estrous cycle, estradiol levels reach their peak during the morning of proestrus day (Table 3) [44] and epithelial cells start to proliferate during the first hours of metestrus day (00:00 to 03:00 h), approximately 40 h after the highest levels of E_2 were detected. This is in contrast to E_2 treated ovx animals, which present the highest epithelial cellular

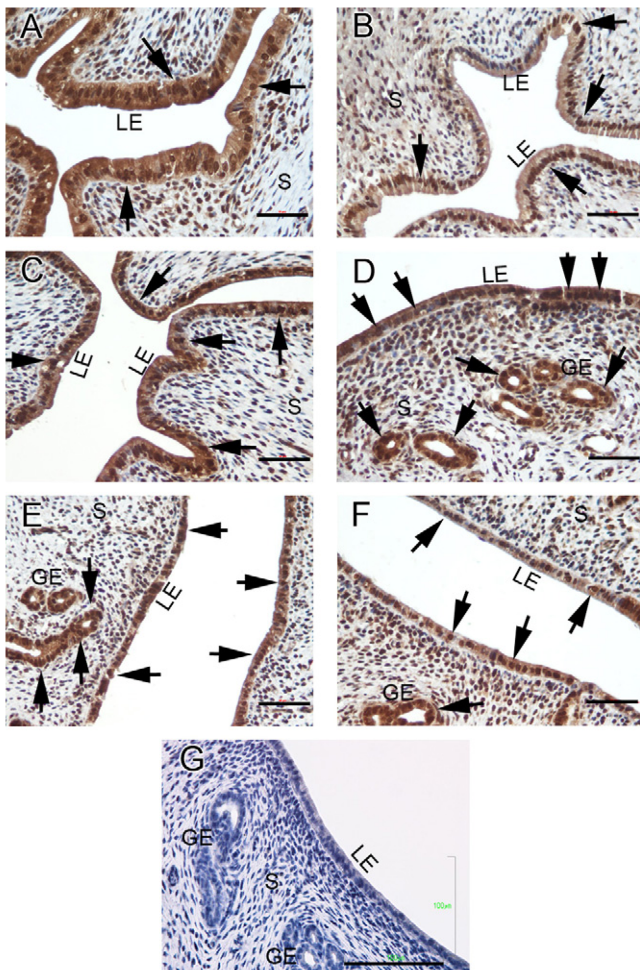


Fig. 7. Permanent nuclear localization of cdk4 in the rat uterine epithelia during the E–M transition of the estrous cycle. Immunohistochemical localization of cdk4 protein in: (A) 13:00 h; (B) estrus 21:00 h; (C) metestrus 00:00 h; (D) metestrus 03:00 h; (E) metestrus 09:00 h; (F) metestrus 13:00 h; (G) negative control. Arrows indicate marked cells. LE, luminal epithelium; GE, glandular epithelium; S, stroma. (A–F) Bar = 50 μm. (G) Bar = 100 μm.

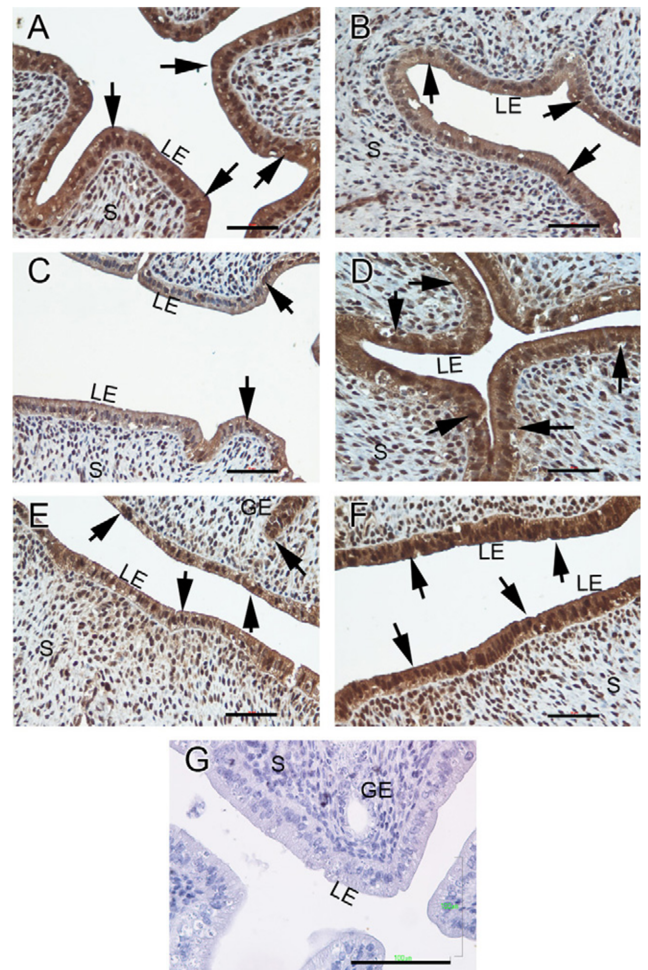


Fig. 8. Immunohistochemical localization of cdk6 protein in the rat uterine epithelia during the E–M transition of the estrous cycle. (A) estrus 13:00 h; (B) estrus 21:00 h; (C) metestrus 00:00 h; (D) metestrus 03:00 h; (E) metestrus 09:00 h; (F) metestrus 13:00 h; (G) negative control. Arrows indicate marked cells. LE, luminal epithelium; GE, glandular epithelium; S, stroma. (A–F) Bar = 50 μm. (G) Bar = 100 μm.

proliferation 12–24 h after E_2 treatment [1–4], exhibiting the highest E_2 serum levels between 1 to 6 h after treatment, depending on the administration procedure [45]. The differences in the timing of the response suggest that under physiological conditions, the mitogenic action of estradiol may involve different signalling cascades of gene activation.

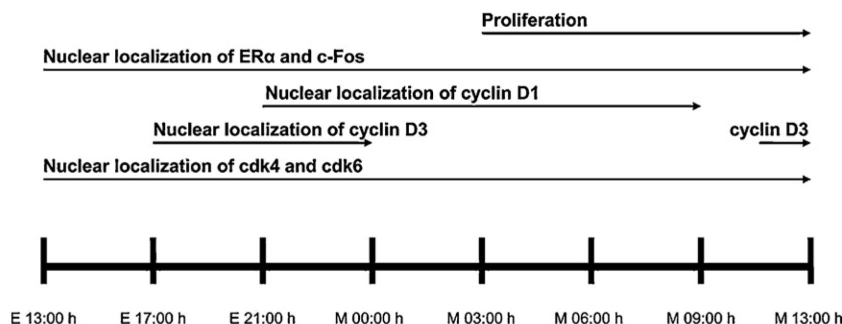
Interestingly, we identify groups of BrdU labelled cell in both epithelia, suggesting an epithelial stem cell-like/progenitor niche which may be responsible for endometrial renewal, in a similar manner to what has been previously observed in mouse endometrial cells [46,47].

In order to establish the participation of key molecules in the induction of cell cycle progression, we evaluated ERα, Fos, cyclin D1, cyclin D3, cdk4 and cdk6 protein expression patterns during the E–M transition of the estrous cycle. Our results show that uterine epithelial proliferation is preceded by the sequential nuclear expression of these proteins, as is observed in Tables 1 and 2 and Fig. 9. In both glandular and luminal epithelia, ERα and Fos were detected in the nuclei beginning at E 13:00 h (Figs. 3, 4, 9, and

Table 3
Variations of estradiol (E_2) and progesterone (P_4) serum levels during the estrous cycle.

Day of the cycle	E_2 pg/mL (pmol/L)	P_4 ng/mL (nmol/L)
Proestrus 13:00 h	65.3 ± 7.89** (239.72 ± 28.96)	16.9 ± 4.11 (53.74 ± 13.07)
Estrus 13:00 h	16.6 ± 2.17 (60.94 ± 7.97)	10.3 ± 3.69 (32.75 ± 11.73)
Estrus 17:00 h	18.7 ± 5.21 (68.65 ± 19.13)	23.7 ± 6.19 (75.37 ± 19.68)
Estrus 21:00 h	14.3 ± 3.73 (52.49 ± 13.69)	18.3 ± 5.02 (58.19 ± 15.96)
Metestrus 00:00 h	18.3 ± 4.27 (67.18 ± 15.68)	19.0 ± 8.49 (60.42 ± 27.00)
Metestrus 03:00 h	25.5 ± 2.07 (93.61 ± 7.60)	29.9 ± 7.89** (95.08 ± 25.09)
Metestrus 06:00 h	15.7 ± 4.68 (57.63 ± 17.18)	22.9 ± 7.12 (72.82 ± 22.64)
Metestrus 09:00 h	28.5 ± 4.68 (104.62 ± 17.18)	29.5 ± 6.07* (93.81 ± 19.30)
Metestrus 13:00 h	24.5 ± 2.60 (89.94 ± 9.54)	33.8 ± 6.54** (107.48 ± 20.80)
Diestrus 13:00 h	33.0 ± 5.11* (121.14 ± 18.76)	8.4 ± 0.69 (26.71 ± 2.19)

Steroid levels were determined by radioimmunoassay. Data are expressed as the mean ± SEM of the five animals/studied hour of the cycle. In E_2 $^{**}p < 0.001$ vs. all the other studied hours; $^{*}p < 0.05$ vs. E 13:00, E 17:00, E 21:00, M 00:00 and M 06:00. In P_4 $^{**}p < 0.01$ vs. P 13:00, E 13:00, E 21:00, D 13:00; $^{*}p < 0.05$ vs. P 13:00, E 13:00, D 13:00.



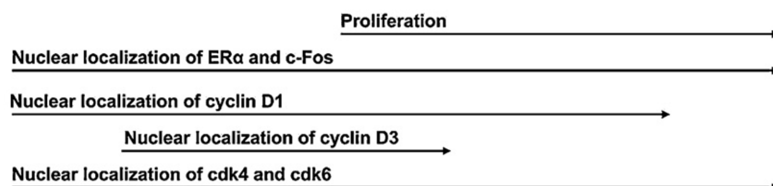


Fig. 9. Schematic representation of the sequence of events that are involved in the induction of uterine epithelia proliferation. (A) Luminal epithelium. (B) Glandular epithelium. The arrows represent the time course during which the studied proteins and BrdU incorporation are detected in the nuclei.

Tables 1 and 2). However, in the glandular epithelium there were a higher number of immunopositive nuclei than in the luminal epithelium during the initial studied hours. In addition, in the glandular epithelium, cyclin D1 was also detected in the nuclei early in estrus (E 13:00 h) (Figs. 5, 9 and Table 2) and cyclin D3 was observed in the nuclei beginning at E 17:00 h (Figs. 6, 9 and Table 2). In contrast, in the luminal epithelium, cyclins were observed in the nuclei several hours later E 21:00 h (Figs. 5, 6, 9 and Table 1). These differences in the time of induction of nuclear cyclins could account for the differences in the proliferation observed in both epithelia, being the glandular epithelia the one that proliferates earlier. These results agree with data reported by Zhuang et al. [37] showing that in ovx rats stimulated with E_2 there was a slight difference in S phase entry kinetics between the luminal and glandular epithelium: glandular epithelial cells expressed S phase markers (cyclin A and PCNA) earlier and to a somewhat greater extent than luminal epithelial cells.

The D-type cyclins, particularly cyclin D1, have been the focus of significant attention because of their key role in normal and malignant cell proliferation. It is well known that D-type cyclins are components of the cell cycle machinery and govern progression through G1 phase in response to extracellular signals. In the case of the uterus, it has been recognized that the major regulators of proliferation are estrogens. Participation of cyclin D1 and D3 in the regulation of proliferation of the uterine epithelia has been studied in E_2 treated ovx rodents [22,37,42]. In that model, cyclin D1 protein concentration does not change, but shows relocalization from the cytoplasm to the nucleus. Cyclin D1 was detected in the nuclei of both epithelia, but was more evident in the glandular epithelia and nuclear localization was observed before presenting S phase markers (4–12 h after E_2 treatment), while cyclin D3 was observed almost exclusively in the nuclei of luminal epithelial cells and after expression of S phase markers (32 h after treatment). This indicates that they might act in a cell-specific manner [37]. In agreement with these results, we observed in the luminal epithelium that cyclin D3 was the first to be detected in the nuclei at E 17:00 h, while in the glandular epithelium cyclin D1 was the first one to be detected in the nuclei at E 13:00 h (Fig. 9). Nevertheless, we observed that both cyclins, D1 and D3, are present in the nuclei of both glandular and luminal epithelial cells, before proliferation was detected (Figs. 5, 6, 9, Tables 1 and 2). These results suggest that, although cyclin D3 might initiate proliferation in the luminal epithelium while cyclin D1 may initiate it in the glandular epithelium, both cyclins participate in the regulation of proliferation of both epithelia during the estrous cycle. Interestingly, we observed that cyclin D3 was maintained in the nuclei of both epithelia for a shorter period of time than cyclin D1. These results suggest that there is a different regulatory mechanism underlying cyclin D1 and D3 gene expression in the rat uterus during the estrous cycle. These results are on line with the data reported by Geum et al. in immature mice primed with E_2 [36].

Cyclin D1 has been recognized to be the most powerful inducer of proliferation and has been observed in different normal and cancer cell types [23]. D cyclins are differentially expressed in different tissues and display several molecular effects. Indeed, recently they have been reported to have an important participation in different cellular metabolic pathways including those of nuclear receptors, particularly PPARγ, estrogen, androgen and thyroid hormone receptors, as well as in mitochondrial functions and lipolysis [48–50]. The expression of both cyclins in the glandular and luminal epithelia introduce a new intriguing question about the role of these proteins in other cell functions, apart from cell cycle regulation in the uterus, and deserves to be investigated.

Our observation of the different time course of nuclear localization of cyclins D1 and D3 in both epithelia may be in relation to their participation on cell cycle progression. Cyclin D1 is induced earlier in glandular epithelial cells and may initiate the transition of G1 to S in the cell cycle. In contrast, cyclin D3 is induced first in the luminal epithelium (Fig. 9, Tables 1 and 2). More experiments are required to determine the exact role for each cyclin in the transition and metabolic processes of the cell cycle in uterine tissue.

In contrast to cyclins, CDK's 4 and 6 were detected in the nuclei and cytoplasm of both epithelia during the entire time course studied. These results agree in part with previous studies in ovx animals [42] in which both cdk4 and cdk6 were localized in the nucleus and cytoplasm of uterine epithelial cells of both ovx and E_2 treated ovx animals. In contrast with our results, that study showed that cdk6 levels remained high under E_2 treatment over a 15-h time course,

while we detected a slight decrease of this protein from E 17:00 to M 03:00 h (Table 1). Additionally, in Tong's study [42] cdk4 nuclear association was increased following E_2 treatment, showing maximal levels 4 h after treatment. In our study, we did not observe changes in nuclear staining of both epithelia (Tables 1 and 2). It has been reported that cdk4 and cdk6 proteins are stably expressed in most cell types. Their regulation is believed to be imposed through the regulation of their partners, the D-type cyclins [51]. Our results suggest that this may also be the case in uterine epithelial cells during the E–M transition.

In conclusion, the overall results indicate that there is a good correlation between sequential expression of ERα, Fos, nuclear localization of cyclins and CDK's 4 and 6 and cell cycle progression in the rat uterine epithelial cells during the estrous cycle. However, the differences observed in the cellular localization, time course of expression and the cellular types that express both cyclins between physiological and pharmacological conditions, demonstrated different mechanisms of regulation and should be due to the complex hormonal milieu during the estrous cycle.

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