

# Relationship between the expression of cyclins/cyclin-dependent kinases and sex-steroid receptors/Ki67 in normal human endometrial glands and stroma during the menstrual cycle

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Cell cycle regulatory molecules were analysed in normal human endometrial tissue using antibodies against cyclins D1, E, A, and B1 and cyclin-dependent kinases (CDKs) cdk4, cdk2, and cdc2. The expression of these regulatory molecules in gland cells and stromal cells was compared with the expression of oestrogen receptors (ER), progesterone receptors (PR), and Ki67 (a growth-related molecule). In general, a substantially higher percentage of the gland cells stained positive for cyclins and CDKs during the proliferative phase of the menstrual cycle. Cyclin E, cdk2 and/or cdk4 were especially apparent in the cytoplasm of most of the gland cells as well as in the stromal cells. In contrast, most of the regulatory molecules were undetectable in the gland cells by the end of the secretory phase of the cycle, but they did not decline in the stromal cells. The data also revealed that ER, PR, and Ki67 in both gland cells and stromal cells follow the same basic pattern of expression as the cyclins and CDKs. These results suggest that cyclins and CDKs are functionally involved in the rhythmic proliferation of normal human endometrial tissue, and the action of these agents may be related to the endometrial levels of sex steroids and Ki67.

**Key words:** cyclin/cyclin-dependent kinase/endometrium/Ki67/steroid receptors

## Introduction

Proliferation and differentiation of the human endometrium are regulated by ovarian steroid action on oestrogen receptors (ER) and progesterone receptors (PR). Oestradiol stimulates proliferation of both gland cells and stromal cells, whereas progesterone prevents this effect and induces secretory changes in gland cells and decidual changes in stromal cells (Strauss and Gurpide, 1991). This cyclic growth and differentiation of the endometrium is indispensable for human fertility.

Cyclins are a group of proteins that are expressed periodically during progression of the cell cycle (Sherr, 1993). Cyclins achieve their functions by forming complexes with cyclin-dependent kinases (CDKs). Cyclin E binds to cdk2 and cyclin D to cdk4, and these complexes are known to phosphorylate retinoblastoma gene products (RB) and propel the cell cycle from G<sub>1</sub> to S phase (Arion *et al.*, 1988; Kato *et al.*, 1993; Sherr, 1994). Also, complex formations of cyclins A and B with cdc2 are indispensable in G<sub>2</sub>/M phase transition (Nurse, 1994). The actions of these cell cycle regulators in yeast (Lee and Nurse, 1987) and sea urchin eggs (Evans *et al.*, 1983) are conserved in human cells (Sherr, 1993).

In studies on the proliferation of endometrial tissue, growth-related molecules such as Ki67 (Gerdes *et al.*, 1985) and proliferating cell nuclear antigen (PCNA) (Bravo *et al.*, 1987) have been analysed (Pickarts *et al.*, 1990; Li *et al.*, 1993); however, interactions of such growth factors with cell cycle regulatory molecules have not been reported. Therefore, to obtain further information on the growth mechanism of the normal human endometrium during the menstrual cycle, this study examines the expression of a number of different cell

cycle regulatory molecules using specific antibodies against cyclins D1, E, A, and B1, and against three CDKs (cdk4, cdk2, and cdc2). The expression of these regulatory molecules in endometrial glandular and stromal cells is compared to the expression of ER, PR, and Ki67 in the same endometrial cells.

## Materials and methods

### Histological materials

Normal endometrial tissue was obtained from 49 women (aged 35–45 years) all of whom had a previous history of pregnancy. The experimental tissue was extirpated at the time of hysterectomy, following diagnosis of leiomyoma or carcinoma *in situ* of the uterine cervix. The tissue was used with the approval of the Ethical Committee of Shinshu University, Japan, after obtaining written consent from the patients. Each specimen was obtained from the posterior wall of the uterus, and was immediately fixed in 10% phosphate-buffered formalin for 24 h and embedded in paraffin. Serial sections of 3 µm thickness were stained with haematoxylin and eosin (H&E) prior to immunostaining. Histological diagnosis and endometrial dating (Noyes *et al.*, 1950) were performed on each of the stained slides. Of the 49 endometrial sources, 20 were in the proliferative phase (days 6–14), 12 in the early secretory phase (days 15–21), nine in the mid-secretory phase (days 22–24), and eight in the late secretory phase (days 25–28).

### Immunohistochemistry

Indirect immunostaining was performed using selected anti-cyclin and anti-CDK antibodies. Antibodies for cyclin D1 (HD11), cyclin E (HE1), cyclin A (BF683), cyclin B1 (GNS-1), cdc2 (17), cdk2 (M2), and cdk4 (C-22) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies for ER (ER1D5), PR (PR10A),

**Table I.** Percentage of cells that stained positive for oestrogen receptors (ER), progesterone receptors (PR), Ki67, cyclins and cyclin-dependent kinases (CDKs) in gland cells

	Sites stained		Proliferative phase (n = 20)	Secretory phase		
				early (n = 12)	mid (n = 9)	late (n = 8)
ER	functionalis	nucleus	94.8 ± 6.4	33.6 ± 14.6*	0*	0*
	basalis	nucleus	95.9 ± 4.8	37.6 ± 7.2*	0*	16.7 ± 11.0*
PR	functionalis	nucleus	98.7 ± 1.7	86.7 ± 19.1	10.6 ± 7.5*	0.1 ± 0.4*
	basalis	nucleus	98.7 ± 2.3	85.4 ± 20.8	17.3 ± 7.1*	0*
Ki67	functionalis	nucleus	24.5 ± 6.9	2.2 ± 1.8*	0.1 ± 0.3*	0*
	basalis	nucleus	12.9 ± 8.2	3.7 ± 1.9*	0*	0*
Cyclin D1	functionalis	cytoplasm	0	0	0	0
		nucleus	tr	0	0	0
	basalis	cytoplasm	0	0	0	0
		nucleus	tr	tr	0	0
Cyclin E	functionalis	cytoplasm	39.3 ± 33.1	5.6 ± 4.3*	3.3 ± 2.5*	0*
		nucleus	0.5 ± 0.4	0.3 ± 0.3	1.2 ± 0.3*	0.1 ± 0.1
	basalis	cytoplasm	26.8 ± 22.7	4.3 ± 3.4*	2.8 ± 2.6*	0*
		nucleus	tr	0	tr	0
Cyclin A	functionalis	cytoplasm	0.3 ± 0.1	tr*	0*	0*
		nucleus	tr	0	0	0
	basalis	cytoplasm	0.2 ± 0.1	tr*	0*	0*
		nucleus	0	0	0	0
Cyclin B1	functionalis	cytoplasm	3.9 ± 1.9	tr*	0*	0*
		nucleus	tr	0	0	0
	basalis	cytoplasm	0.3 ± 0.2	tr*	0*	0*
		nucleus	0.2 ± 0.1	0.1 ± 0.1	tr*	0*
cdk4	functionalis	cytoplasm	74.8 ± 12.8	23.2 ± 19.5*	5.8 ± 3.7*	tr*
		nucleus	tr	0	0	0
	basalis	cytoplasm	58.6 ± 18.3	17.7 ± 14.5*	3.3 ± 2.5*	0*
		nucleus	0	0	0	0
cdk2	functionalis	cytoplasm	75.0 ± 19.7	37.3 ± 24.5*	29.2 ± 16.9*	0*
		nucleus	1.1 ± 0.5	0.4 ± 0.3*	0.1 ± 0.1*	0*
	basalis	cytoplasm	53.7 ± 27.6	32.7 ± 21.1*	21.7 ± 20.4*	0*
		nucleus	0.7 ± 0.5	0.3 ± 0.3*	0*	0*
cdc2	functionalis	cytoplasm	0.8 ± 0.4	tr*	0*	0*
		nucleus	0.1 ± 0.1	0	0	0
	basalis	cytoplasm	0.5 ± 0.3	tr*	0*	0*
		nucleus	tr	0	0	0

\*Significantly different compared with the proliferative phase.

tr = trace, positive cells are observed but are &lt;0.1%.

and Ki67 (MIB-1) were purchased from Immunotech (Marseille, France). Each immunohistochemical staining procedure was carried out by the avidin–biotin–peroxidase complex method using a Histofine SAB-PO detector kit (Nichirei Co., Tokyo, Japan). Briefly, after deparaffinization in xylene and rehydration through graded concentrations of alcohol, each section was treated by microwave in 0.01 M citrate buffer (pH 6.0) for 15 min. Endogenous peroxidase activity was blocked by 0.03% hydrogen peroxide in methyl alcohol for 30 min. Then, 10% normal rabbit serum (for mouse primary antibodies), or goat serum (for rabbit primary antibodies), was applied to minimize non-specific reactivities. The sections were then incubated with specific primary antibodies [diluted 1:500 with phosphate-buffered saline (PBS)/bovine serum albumin (BSA) for anti-cdk2 and anti-cdk4 antibodies, and 1:100 for the other antibodies], or with non-immunized mouse or rabbit serum (as controls) at 4°C overnight. After rinsing with PBS, biotinylated anti-mouse or anti-rabbit immunoglobulin (Ig)G was applied for 30 min at room temperature. After rinsing again with PBS, peroxidase-conjugated streptavidin solution was applied for 30 min and the antigen-antibody reaction was visualized by 0.05% 3,3'-diaminobenzidine (DAB). The tissue was lightly counterstained with haematoxylin.

#### Interpretation of immunohistochemical staining

The specific staining of cyclins, CDKs, steroid receptors, and Ki67 was identified by the presence of brown-coloured products in the

nucleus and/or cytoplasm. All of the control slides yielded negative staining. Positive staining was tabulated as the percentage of positive cells per population of 1000 cells in each section, and the results were recorded as the mean percentage of positive cells ± SD in each histological group. When the mean number of positive cells detected by a given immunohistochemical stain was <0.1%, the result was reported simply as a trace (tr). The intensity of the staining varied from weak to strong, and any detectable staining was regarded as positive.

#### Statistical analysis

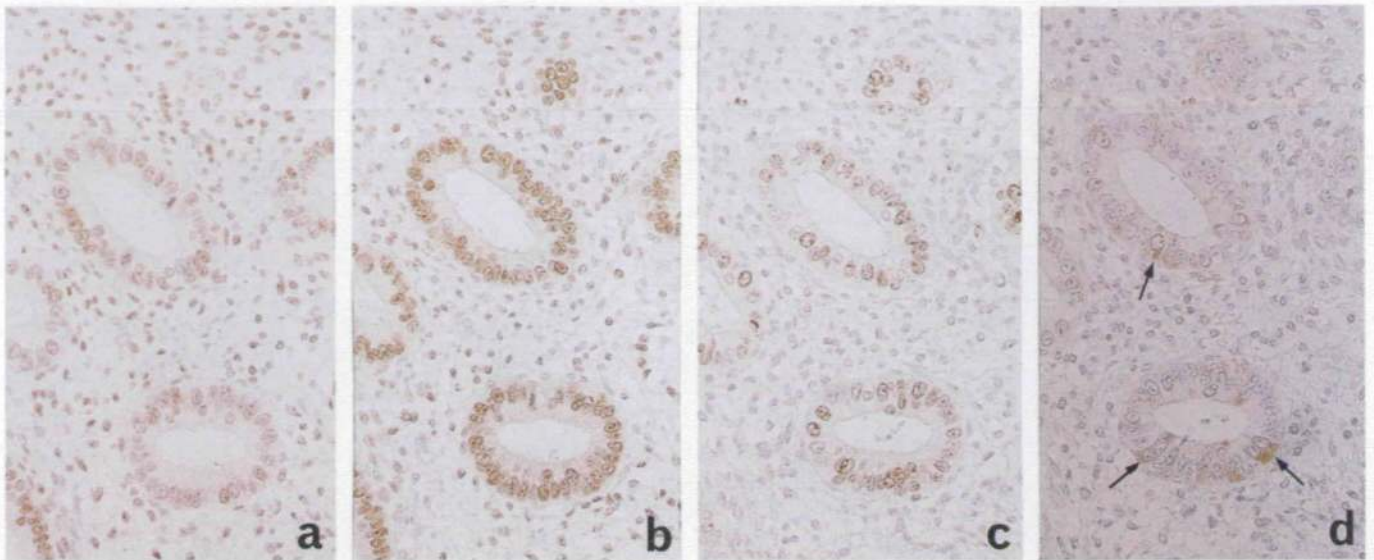
Statistical analysis was performed by the Mann–Whitney test (Statview System, Macintosh). Differences were considered significant when  $P < 0.05$ .

## Results

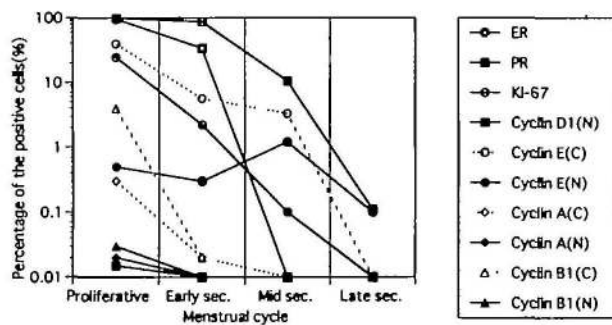
### Immunohistochemical staining of gland cells

#### Expression of ER, PR, and Ki67

In the proliferative phase of the menstrual cycle, almost all of the gland cells in both the functional and the basal layers of the endometrium stained positive for ER and PR (Table I, Figures 1a,b and Figure 2). However, by the early secretory phase there was a marked decrease in ER-positive cells and a



**Figure 1.** Serial sections with staining of (a) oestrogen receptors (ER); (b) progesterone receptors (PR); (c) Ki67 and (d) cyclin A during the proliferative phase of the menstrual cycle. ER and PR are distributed diffusely among both gland cells and stromal cells. The receptor-positive cells contain Ki67, and the cyclin A positive cells (arrows) are also positive for Ki67 (original magnification  $\times 175$ ).



**Figure 2.** Percentage of gland cells (functionalis) staining positive for oestrogen receptors (ER), progesterone receptors (PR), Ki67 and cyclins at different stages of the menstrual cycle. All of the factors (except for nuclear staining of cyclin E) are expressed mainly in the proliferative phase and decrease during the secretory phase of the menstrual cycle. sec = secretory; C = cytoplasm; N = nucleus.

moderate decline in PR-positive cells. The down-regulation of these steroid receptors was much more significant by the middle of the secretory phase and remained low until the beginning of the next proliferative phase of the menstrual cycle. Although the percentage of cells that stained positive for Ki67 (Figure 1c) was substantially less than those that stained for ER and PR, the expression of this growth-related molecule during the menstrual cycle followed a pattern that was proportional to steroid receptor expression, especially to ER expression (Table I, Figure 2).

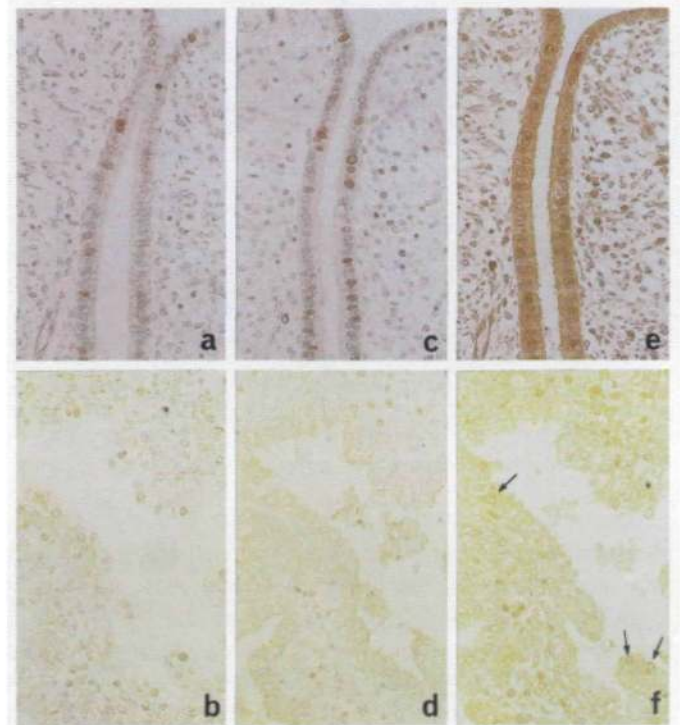
### Expression of cyclins and CDKs

#### Cyclin D1

A few nuclei were positive for cyclin D1 in the proliferative phase (Table I, Figure 2).

#### Cyclin E

Cyclin E was the principal cell cycle regulatory molecule, and was positive in both the cytoplasm and nucleus (Table I, Figures 2, 4a,b). Cyclin E staining in the cytoplasm was



**Figure 3.** Immunostaining of cyclin E (a, b), Ki67 (c, d) and cdk2 (e, f). In the proliferative phase, serial sections reveal that the gland cells with nuclear staining for cyclin E (a) also stain positive for Ki67 (c) and cdk2 (e). Also, there is cytoplasmic staining for cyclin E and cdk2 in gland cells, as well as in stromal cells (a, e). In the mid secretory phase, serial sections show that gland cells express cyclin E (b) and cdk2 (f, arrows) in the nucleus, but these cells are negative for Ki67 (d). Yet, during this phase, stromal cells still contain cyclin E in cytoplasm (b) and cdk2 in the cytoplasm as well as in the nucleus (f) (original magnification  $\times 175$ ).

diffusely observed in the proliferative phase (Figure 3a) and it markedly decreased in the secretory phase (Figure 3b). Cyclin E staining in the nucleus was observed throughout the

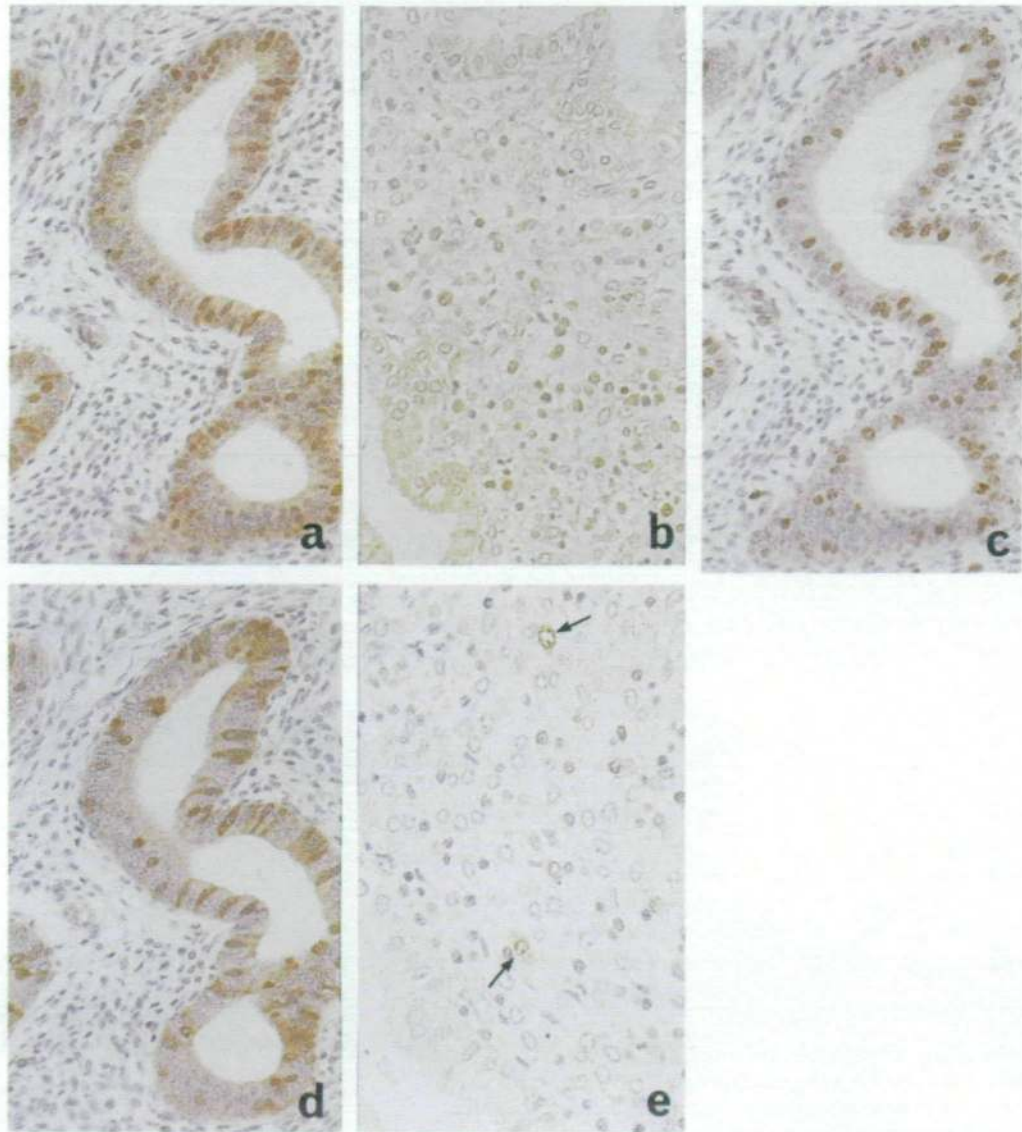


Figure 4. Legend opposite.

menstrual cycle with the highest positive rate in the mid-secretory phase. The nuclear-positive rate was significantly higher in the functionalis compared with the basalis during the menstrual cycle. The cells with nuclear staining of cyclin E in the proliferative phase (Figure 3a) were usually positive for both Ki67 (Figure 3c) and steroid receptors, but those in the secretory phase (Figure 3b) were negative for them (Figure 3d).

Cyclin A

Cyclin A was mainly positive in the cytoplasm (Table I, Figure 1d, Figure 2). Positive cells were observed in the proliferative phase (Figure 1d). The positive cells markedly decreased in the secretory phase, and disappeared in the mid-late secretory phase. Cyclin A positive cells were included among those cells with mitotic figures, and with positivity for Ki67 (Figure 1c).

Cyclin B1

Cyclin B1 showed positive staining in the cytoplasm and nucleus (Table I, Figures 2, 4a,b). Cytoplasmic positive cells

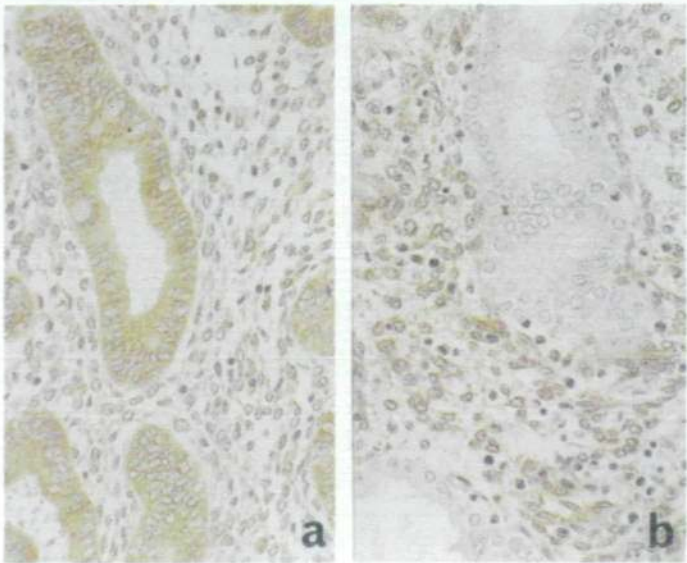


Figure 5. Legend opposite.

were observed mainly in the proliferative phase (Figure 4a) and they markedly decreased in the secretory phase (Figure 4b). The cytoplasmic positive rate was significantly higher in the functionalis compared to the basalis. Nuclear positive cells were also observed in both the basalis and functionalis, and the positive rate was significantly higher in the former compared with the latter. These cytoplasmic as well as nuclear positive cells were included among those cells with mitotic figures, and positivity for Ki67 (Figure 4c).

#### *cdk4*

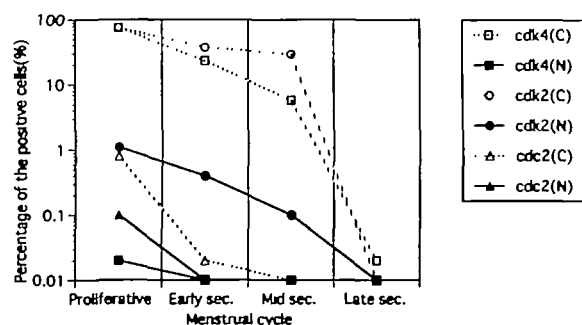
Cdk4 was usually positive in the cytoplasm (Table I, Figures 5a,b, Figure 6). Positive cells were observed in both the functionalis and the basalis, mainly in the proliferative phase (Figure 5a) with decrease in the secretory phase (Figure 5b).

#### *cdk2*

Cdk2 showed positive staining in both the cytoplasm and nucleus (Table I, Figures 2, 3e,f, Figure 6). Cytoplasmic positive cells were observed in the proliferative phase (Figure 3e), and they gradually decreased in the secretory phase (Figure 3f). Nuclear positive cells were observed throughout the menstrual cycle, but the positive rate was significantly higher in the proliferative phase compared to the secretory phase. Nuclear positive cells were topographically correlated with the cells positive for cyclin E (Figure 3a,b,e,f).

#### *cdc2*

Cdc2 showed positive staining in both the cytoplasm and nucleus (Table I, Figures 2, 4d,e, Figure 6). The rate of cytoplasmic positive cells was the highest in the proliferative phase (Figure 4d), it decreased in the secretory phase, and no cells were positive in the mid-late secretory phase (Figure 4e). Nuclear positive cells were also observed in the proliferative phase, and they disappeared in the secretory phase. Cdc2



**Figure 6.** Percentage of gland cells (functionalis) staining for cyclin-dependent kinases (CDKs). CDKs are mainly expressed in the proliferative phase, and they diminish during the secretory phase.

positive cells were topographically correlated with the cells positive for cyclin B1 (Figure 4a,d)

### **Immunohistochemical staining of stromal cells**

#### **Expression of ER, PR, and Ki67**

During the proliferative phase of the menstrual cycle most of the stromal cells in both the functional and the basal layers stained positive for the two steroid receptors (Table II, Figure 1a,b and Figure 7). With progression of the secretory phase, the steroid receptors were moderately, but significantly, down-regulated, especially in the functional layer. On the other hand, during the proliferative phase Ki67 was present in a lesser percentage of functional stromal cells (Figure 1c) compared with ER and PR expression, and this growth-related molecule was rarely observed in only a few of the basal stromal cells (Table II). In the earliest stage of the secretory phase, Ki67 expression decreased significantly in the functional stromal cells, but the percentage of cells that stained positive for this factor increased back to the level found in the proliferative phase by the end of the secretory phase (Table II, Figure 7).

### **Expression of cyclins and CDKs**

#### **Cyclin D1**

Cyclin D1 was positive in the nucleus (Table II, Figure 7). A small number of positive cells were observed in the proliferative phase and in the mid-secretory phase.

#### **Cyclin E**

As in the gland cells, cyclin E was the principal cell cycle regulatory molecule expressed in endometrial stromal cells, and was mainly positive in the cytoplasm (Table II, Figures 3a,b and Figure 7). Positive cells were frequently observed in the proliferative phase and mid-late secretory phases.

#### **Cyclin A**

Cyclin A showed positive staining in the cytoplasm (Table II, Figure 7). A small number of positive cells was observed in the functionalis of the proliferative phase and no positive cells were observed in the secretory phase.

#### **Cyclin B1**

Cyclin B1 was positive in the nucleus (Table II, Figures 4a,b and Figure 7). Positive cells were observed throughout the menstrual cycle with the highest rate in the late secretory phase.

#### **cdk4**

Cdk4 showed positive staining in both the cytoplasm and nucleus (Table II, Figures 5a,b and Figure 8). Cytoplasmic positive cells were observed throughout the menstrual cycle, frequently in the functionalis. Nuclear positive cells were also

**Figure 4.** Immunostaining of cyclin B1 (a, b), Ki67 (c) and cdc2 (d, e). In the proliferative phase, serial sections show that cyclin B1 (a) and cdc2 (d) is occasionally present in the nucleus as well as in the cytoplasm of the gland cells, and that the distribution of cyclin B1 and in gland cells occurs in conjunction with Ki67 (c) and cdc2 (d). In the late secretory phase, gland cells are negative for cyclin B1 (b) and cdc2 (e) but stromal cells are positive for cyclin B1 (b) and cdc2 (e, arrows) (original magnification  $\times 175$ ).

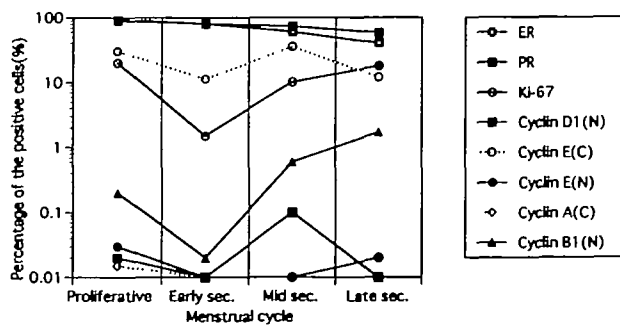
**Figure 5.** Immunostaining of cdk4 in the proliferative phase (a) and in the secretory phase (b). In the proliferative phase there was diffuse cytoplasmic staining for cdk4 in the cytoplasm of gland cells, while most of the stromal cells also had this kinase not only in the cytoplasm, but also in their nuclei (a). By the secretory phase, gland cells did not contain cdk4, but most of the stromal cells continued to express this kinase in both the nucleus and the cytoplasm (b) (original magnification  $\times 175$ ).

**Table II.** Percentage of cells that stained positive for oestrogen receptors (ER), progesterone receptors (PR), Ki67, cyclins and cyclin-dependent kinases (CDKs) in stromal cells

	Sites stained		Proliferative phase (n = 20)	Secretory phase		
				early (n = 12)	mid (n = 9)	late (n = 8)
ER	functionalis	nucleus	88.8 ± 6.1	80.7 ± 5.0	60.7 ± 8.5*	40.3 ± 17.6*
	basalis	nucleus	78.8 ± 11.3	66.7 ± 5.8	62.0 ± 14.2*	63.3 ± 5.3*
PR	functionalis	nucleus	91.6 ± 4.4	80.4 ± 5.5*	73.5 ± 13.1*	58.5 ± 12.3*
	basalis	nucleus	89.3 ± 5.8	72.0 ± 16.2*	74.4 ± 8.7*	71.5 ± 3.4*
Ki67	functionalis	nucleus	20.2 ± 7.5	1.5 ± 1.2*	10.3 ± 3.8*	18.0 ± 6.7
	basalis	nucleus	0.8 ± 0.6	0.9 ± 0.9	1.2 ± 1.1	1.9 ± 0.9
Cyclin D1	functionalis	cytoplasm	0	0	0	0
		nucleus	tr	0	0.1 ± 0.1	0
	basalis	cytoplasm	0	0	0	0
		nucleus	0	0	tr	0
Cyclin E	functionalis	cytoplasm	30.1 ± 23.0	11.4 ± 8.5*	35.6 ± 26.4	12.1 ± 7.6*
		nucleus	tr	0	0	tr
	basalis	cytoplasm	2.3 ± 2.4	0.4 ± 1.4*	2.7 ± 3.6	0*
		nucleus	0	0	0	0
Cyclin A	functionalis	cytoplasm	tr	0	0	0
		nucleus	0	0	0	0
	basalis	cytoplasm	0	0	0	0
		nucleus	0	0	0	0
Cyclin B1	functionalis	cytoplasm	0	0	0	0
		nucleus	0.2 ± 0.2	tr	0.6 ± 0.6	1.7 ± 0.5*
	basalis	cytoplasm	0	0	0	0
		nucleus	tr	tr	0.1 ± 0.1	0.2 ± 0.1*
cdk4	functionalis	cytoplasm	60.8 ± 20.6	38.6 ± 22.8*	70.0 ± 12.0	68.3 ± 11.7
		nucleus	75.6 ± 26.4	23.0 ± 20.0*	80.8 ± 16.3	50.8 ± 9.7*
	basalis	cytoplasm	42.7 ± 26.0	13.2 ± 13.0*	11.0 ± 4.2*	30.0 ± 20.1
		nucleus	65.8 ± 28.9	0.4 ± 0.3*	1.8 ± 1.2*	12.2 ± 6.3*
cdk2	functionalis	cytoplasm	44.2 ± 28.9	22.7 ± 16.3	55.0 ± 23.4	48.0 ± 17.9
		nucleus	19.1 ± 8.5	5.8 ± 4.7*	11.2 ± 6.3	25.6 ± 10.2
	basalis	cytoplasm	6.3 ± 6.1	1.3 ± 2.3*	8.3 ± 4.1	8.0 ± 2.7
		nucleus	1.0 ± 0.6	0.4 ± 0.3	1.3 ± 2.3	1.3 ± 1.8
cdc2	functionalis	cytoplasm	0.4 ± 0.3	tr	0.3 ± 0.2	0.2 ± 0.2
		nucleus	0.1 ± 0.1	0*	tr*	0*
	basalis	cytoplasm	tr	0	0	0
		nucleus	tr	0	0	0

\*Significantly different compared with the proliferative phase.

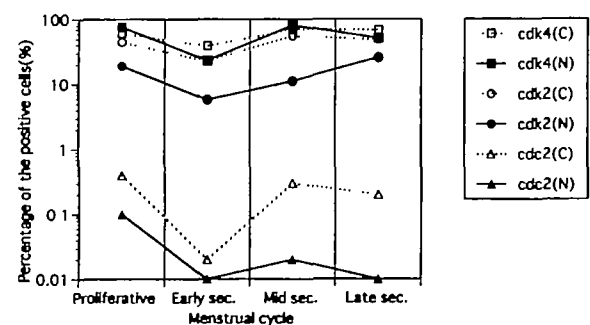
tr: trace, positive cells are observed but are &lt;0.1%.

**Figure 7.** Percentage of stromal cells (functionalis) staining for oestrogen receptors (ER), progesterone receptors (PR), Ki67 and cyclins. ER and PR are moderately down-regulated in the secretory phase. Ki67 expression was elevated in the proliferative phase and during the mid- to late secretory phase. Cytoplasmic cyclin E is expressed in a large percentage of the stromal cells throughout the menstrual cycle. Cyclin B1 increased in the nucleus during the mid- to late secretory phase.

observed throughout the menstrual cycle with the highest rate in the functionalis of the mid-secretory phase.

#### *cdk2*

Cdk2 showed positive staining in both the cytoplasm and nucleus (Table II, Figures 3e,f and Figure 8). Cytoplasmic

**Figure 8.** Percentage of stromal cells (functionalis) staining positive for cyclin-dependent kinases (CDKs). The CDKs were expressed throughout the menstrual cycle, with cdk4 and cdk2 being the more common of the three kinases.

positive cells were observed throughout the menstrual cycle. Nuclear positive cells were also observed throughout the menstrual cycle with the highest positive rate in the late secretory phase.

#### *cdc2*

Cdc2 showed positive staining in both the cytoplasm and nucleus (Table II, Figures 4d,e, Figure 8). Cytoplasmic positive cells were observed throughout the menstrual cycle with the

highest positive rate in the proliferative phase. Nuclear positive cells were also observed throughout the menstrual cycle with the highest positive rate in the proliferative phase.

## Discussion

In this study, both ER and PR were expressed in the majority of the gland cells in the proliferative phase, and ~30% of these cells stained positive for Ki67. In the secretory phase, the expression of these receptors was down-regulated, and Ki67 positive cells were markedly diminished and even disappeared in the mid- and late secretory phase. These patterns of expression of ER, PR, and Ki67 in gland cells of the endometrium during the menstrual cycle are consistent with the previous report of Pickarts *et al.* (1990). Like in the gland cells, a high percentage of stromal cells expressed ER, PR, and Ki67 in the proliferative phase. However, in the secretory phase, there was a modest down-regulation of the two steroid receptors, while positive staining for Ki67 remained relatively high. Therefore, growth activity in the stromal cells appears to be elevated in both the proliferative phase and the secretory phase of the menstrual cycle.

The results also demonstrate a rhythmic expression of cyclins and CDKs in the endometrium that was correlated with expression of the two sex steroid receptors and with Ki67 antigen during the menstrual cycle. In the literature on cell cycle phenomena, cells reportedly express Ki67 from the G<sub>1</sub> to the M phase (Gerdes *et al.*, 1985). However, cyclins such as cyclins D1, E, A, and B1 are more cell cycle specific (Sherr, 1993) than Ki67. In mammalian cells, cyclins D1 and E are expressed in the G<sub>1</sub> phase (Sherr, 1994), cyclin A in late G<sub>1</sub> to M phase, and cyclin B1 in G<sub>2</sub> to M phase (Nurse, 1994). Therefore, the cells expressing these cyclins are theoretically included in the cells that were positive for Ki67.

Cyclin D1 is an important G<sub>1</sub>-phase cyclin that forms a complex with cdk4 (Sherr, 1994). Unlike other cyclins, the expression of cyclin D1 is induced by growth stimuli such as growth factors and hormones (Sherr, 1994). In addition, the expression of cyclin D1 is involved in oestrogen-induced growth of breast cells (Sicinski *et al.*, 1995). The present data confirm that cyclin D1 is expressed in only a few gland cells of the endometrium, as reported previously (Barkova *et al.*, 1994). The short half-life of the cyclin D1 molecule (Withers *et al.*, 1991) may be partly responsible for its low detection rate by immunohistochemistry. In contrast, the pattern of cdk4 expression correlated with that of Ki67 in the gland cells.

The present study demonstrated that cyclin E, a molecule of the late G<sub>1</sub> to early S phase, which forms a complex with cdk2 (Sherr, 1993), was expressed in gland cells of the proliferative phase with two staining patterns. One pattern, involving both cytoplasmic and nuclear staining for cyclin E and cdk2, was correlated with the expression of Ki67. The other pattern, showing diffuse cytoplasmic staining for cyclin E and cdk2 without nuclear staining, was unrelated to the expression of Ki67. The former staining pattern is interpreted as a 'functioning' status of these molecules as they contribute to the growth of gland cells, while the latter pattern is considered to be a 'prefunctioning' status in cell proliferation.

However, in the secretory phase, nuclear staining for cyclin E, with or without nuclear staining for cdk2, was often observed with negative expression of Ki67. The significance of this nuclear staining for cyclin E with or without cdk2 is unclear. However, over-expression of the cyclin E/cdk2 complex has been reported to occur in senescent or G<sub>1</sub>-arrested cells (Dulic *et al.*, 1993). Therefore, the nuclear expression of cyclin E with or without cdk2 in the gland cells of the secretory phase might indicate that these cells are in either a senescent or a G<sub>1</sub>-arrested status.

Cyclin A, which forms a complex with cdk2 in late G<sub>1</sub>-S phase and with cdc2 in the G<sub>2</sub>/M phase of the cell cycle (Minshull *et al.*, 1990; Sherr, 1993), was mainly observed in the cytoplasm of gland cells of the proliferative phase in correlation with the expression of Ki67. Previous reports of the transportation of cyclin A from the cytoplasm to the nucleus (Maridor *et al.*, 1993) and of the existence of a cyclin A/cdc2 complex in the nucleus (Pines and Hunter, 1991; Pagano *et al.*, 1993) suggest that cytoplasmic expression of cyclin A is correlated with the proliferative-type activity of gland cells.

Cyclin B1, which forms a complex with cdc2 in the G<sub>2</sub>/M phase (Nurse, 1994), and cdc2 itself, were observed in the cytoplasm of gland cells of the proliferative phase in a pattern correlating with the expression of Ki67. Cyclin B1 and cdc2 then diminished in the secretory phase. In studies with animal oocytes, the cyclin B1/cdc2 complex is localized in the cytoplasm during the G<sub>2</sub> phase (Ookata *et al.*, 1992) and is activated by cdc25 phosphatase in the cytoplasm, but this complex shifts to the nucleus during the M phase (Strausfeld *et al.*, 1991). This suggests that the cytoplasmic complex of cyclin B1/cdc2 may have a biological role in the growth of endometrial glands. Also, a small number of nuclear positive cells for cyclin B1 and cdc2 was observed in the proliferative phase of the menstrual cycle. However, those cells that were positive for cyclin B1/cdc2 in the nucleus did not always display mitotic symbols. This suggests that cells exhibiting nuclear expression of the cyclin B1/cdc2 complex might be in a pre-mitotic phase, or in the early stages of mitosis.

At present, it is technically difficult to identify the actual phase of the cell cycle for each cell *in situ*. Also, chronological analysis of the cell cycle progression of each cell *in situ* is not possible. However, the expression of cyclins are generally believed to be highly cell cycle-specific. Therefore, the present findings allow the speculation that endometrial glandular tissue accomplishes cell growth by the stepwise expression of cyclins. Furthermore, the expression of cyclins (and especially cyclins A and B1) in Ki67-positive gland cells suggests that these regulatory molecules might serve as potential markers to predict the cell cycle in normal gland cells. Collectively, these findings suggest that the different classes of cyclins/CDKs are functionally involved in the growth of gland cells in the normal endometrium.

In the staining of stromal cells, even in serial sections, it was difficult to detect the expression of more than one regulatory molecule in an individual cell. Therefore, the relationships among the different cyclins, CDKs, and Ki67 were not as clear. However, at least in the hormonal environment of

the proliferative phase, it was possible to distinguish differences in the expression and/or distribution of cyclins/CDKs between the stromal cells and the gland cells. The observable differences may be the result of variations in the turnover of cell cycle-related molecules between the two different types of endometrial cells. In the secretory phase, the expression of Ki67 in the stromal cells indicates that such cells are involved in growth activity even though local progesterone levels are relatively high. This finding is congruent with other reports (Pickarts *et al.*, 1990; Li *et al.*, 1993; Amso *et al.*, 1994). The growth activity of stromal cells in the proliferative phase at the time when gland cells are also undergoing replication is to be expected; however, it is somewhat uncertain why the stromal cells might continue to proliferate when the gland cells have ceased dividing. Under the same hormonal milieu of the secretory phase, the stromal cells seem to respond quite differently, which is a further indication that stromal cells might have growth-regulating mechanisms different from those of the gland cells. Such an interpretation is feasible, since continuous growth potential in the stromal cells might set the stage for decidualization of the endometrium and help maintain the necessary environment for pregnancy. Moreover, the stromal cells exhibited different patterns of expression of cyclins and CDKs when compared to the gland cells. For example, stromal cells did not express appreciable amounts of cyclins E, or A in the nucleus, or of cyclins A, or B1 in the cytoplasm during either phase of the menstrual cycle. Therefore, the difference in the growth pattern between glandular cells and stromal cells during the secretory phase might be due in part to differences in the responsiveness of stromal cells to progesterone-induced expression of individual cyclins and CDKs. In addition, the fact that the cell cycle-related molecules were mainly detected in the stromal cells of the functionalis suggests a topographical difference in proliferative-type activity by the stromal cells during the secretory phase. Such conditions would be advantageous to oocyte implantation. Thus, taken together, the present data suggest that the growth control mechanism for endometrial gland cells is different from that of stromal cells.

In summary, the cyclical expression of cyclins and CDKs in the endometrium during the menstrual cycle can be correlated with the expression of sex steroid receptors and Ki67 antigen. These observations suggest that cell cycle-related molecules are functionally involved in the growth of normal human endometrial tissue. Further characterization of the involvement of such molecules in endometrial growth might be obtained by a more detailed analysis of the action of sex steroids on their target cells in the endometrium during the menstrual cycle.

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