Improved endometrial assessment using cyclin E and p27

Rebecca L. Dubowy, M.D.,[a] Ronald F. Feinberg, M.D., Ph.D.,[a,b] David L. Keefe, M.D.,[c] Gustavo F. Doncel, M.D.,[d] Shaun C. Williams, M.D.,[d,e] Juliette C. McSweet, B.S.,[a] and Harvey J. Kliman, M.D., Ph.D.[a]

Yale University School of Medicine, New Haven, Connecticut

Objective: To evaluate endometrial expression of cyclin E and p27 in fertile and infertile women.

Design: Retrospective clinical study.

Setting: University medical center and private practice.

Patient(s): Thirty-three fertile volunteers, 83 women seeking infertility treatment, and 23 women undergoing mock cycles.

Intervention(s): Endometrial biopsy.

Main Outcome Measure(s): Cyclin E and p27 immunohistochemistry.

Result(s): Glandular cyclin E and p27 expression dramatically changed in intensity and subcellular localization throughout the menstrual cycle. In normal control biopsies, glandular cyclin E progressed from the basal to the lateral cytoplasm (midproliferative phase) to the nucleus (days 18 to 19) and was absent in biopsies after day 20. First appearing on days 17 to 19, p27 was found only in the nuclei. Cyclin E was more frequently seen after day 20 in infertility patients. In the hyperstimulated cycles, staining for cycle E in proliferative samples was more intense than in the natural cycles, but p27 staining was unchanged.

Conclusion(s): Cyclin E and p27 may be clinically useful markers of development in the endometrium. As cell cycle regulators, cyclins reveal underlying biochemical processes driving endometrial progression and may partly represent the means by which estrogen and progesterone regulate this dynamic tissue. (Fertil Steril® 2003;80:146–56. ©2003 by American Society for Reproductive Medicine.)

Key Words: Endometrium, cyclin E, p27, endometrial receptivity, endometrial dating, infertility

Implantation is a critical step in successfully achieving pregnancy. Because some cases of unexplained infertility may result from implantation failure, there is a need to assess the endometrium accurately for defects that could preclude implantation (1). Current tools for endometrial evaluation, however, are limited (2). Clinically, assessments are generally based exclusively on the histologic appearance of hematoxylin and eosin (H&E) stained tissue (3) using the eight morphologic markers proposed by Noyes, Hertig, and Rock (4). Their system, developed in 1950, was the first qualitative attempt to assess the endometrium; it has subsequently been shown to have limitations. Most notable are the high levels of interobserver and intraobserver variation (5–7). This lack of reproducibility limits the ability to definitively diagnose endometrial abnormalities, such as the often cited "luteal phase defect" (8) whose definition itself remains unsettled (9).

Incremental improvements in endometrial evaluation and modifications to the Noyes, Hertig, and Rock system of dating the biopsy sample based on the most advanced component have been proposed. For example, Hendrickson and Kempson (10) developed a system using independent morphometric measurements of the glands and stroma. The usefulness of evaluating individual endometrial features has been supported by studies indicating that only a subset of features is closely associated with chronologic dating (11), and another set of exclusively glandular features is associated with a population of women with unexplained infertility (12). In addition to modifying the histologic evaluation, the use of markers has been investigated as a way to better assess endometrial development, and in-

directly, endometrial receptivity (1, 13, 14). These markers, predominantly cell products, include the $\alpha v \beta 3$ integrin (15–17), MUC1 (18), leukemia inhibitory factor (19), interleukin-1 receptor type I (20), colony-stimulating factor-1 (1), MAG (mouse ascites Golgi) mucin (21), HOXA10 (14, 22), glycodelin-A (1, 23), insulin-like growth factor binding protein-1 (14), and estrogen and progesterone (P) receptors (19, 24, 25). Pinopods, dome-like structures that form on the apical aspects of endometrial surface epithelial cells, have also been proposed as a marker of endometrial receptivity (13, 26). Modifications to the histologic system have not generally been adopted clinically because $\alpha v \beta 3$ integrin is the only one of these markers that is commercially available.

To improve endometrial assessment our first and foremost goal was to develop a useful, easily applied, informative system that would work in formalin fixed, paraffin embedded biopsy samples, and that could be easily and accurately read by pathologists. Ideally the markers would undergo a standard progression of expression during the menstrual cycle such that the levels of cellular differentiation on individual cycle days could be distinguished. In addition, these markers would help us to understand the factors controlling this dynamic tissue. Our search included antibodies to the cyclins E, A, D1, and D3; the cyclin-dependent kinases 2 and 4; the cyclin-dependent kinase inhibitors p21, p27, and p57; topoisomerase II; bcl II; and Ki67, and yielded two markers that fit our criteria: cyclin E and p27.

A cell's progression through the mitotic cycle is controlled by cyclins, cyclin-dependent kinases (cdk), and cyclin-dependent kinase inhibitors. In particular, cyclin E with its partner cdk2 is thought to be the rate-limiting activator of the mitotic G1 to S phase transition, whereas the cyclin-dependent kinase inhibitor p27 prevents this cell cycle progression (27, 28). The activity of these factors depends on their interactions. For example, cyclin E requires cdk2 to be active and the pair is inactivated by an interaction with p27 (29). Activity also depends on subcellular localization. For example, p27 is only active when present in the nucleus (30–32). Further, there is evidence that estrogen positively regulates cyclin E while P induces the transition to a p27-dominated state (33–35).

Previous investigations of cyclin E and p27 in the endometrium have focused on their roles in neoplasia. For example, elevated cyclin E expression has been demonstrated in endometrial cancers (36), and p27, a putative tumor suppressor gene, was found at reduced levels in endometrial carcinomas (37, 38). Our current study attempts to use the expression of cyclin E and p27 in a novel way to improve our understanding of normal endometrial development and receptivity.

MATERIALS AND METHODS

Endometrial Biopsies

The use of archival pathology materials in this study was approved by the Yale University Human Investigations Committee. Each specimen had been obtained by Pipelle sampling from the uterine cavity, immediately fixed in 10% neutral phosphate-buffered formalin for at least 24 hours, and then embedded in paraffin. Three sets of endometrial biopsy samples were collected. [1] Eighty-eight endometrial biopsies were collected from 33 volunteers with no history of infertility, as previously described elsewhere (13, 23). Thirty-eight of these samples, from 10 patients, were not analyzed because inadequate tissue remained in the paraffin blocks, which left 48 biopsies from 23 patients for our analysis. [2] One-hundred and thirty biopsies were analyzed from 83 naturally cycling women who had sought treatment for infertility. These specimens represented a random sampling of infertility patients whose biopsy samples were previously obtained between January 1, 2000, and October 3, 2001, as part of their infertility workup. [3] Forty biopsies were taken on cycle days 15 and/or 24 of a mock cycle from 23 infertile women who had undergone one or more mock cycles in preparation for oocyte donation.

Endometrial Dating

Standard hematoxylin and eosin stained sections were examined for dating. Histologic diagnosis and endometrial dating of the stroma and glands were performed according to the general criteria of Noyes et al. (4) as more fully detailed in Hendrickson and Kempson's (10) decision tree for endometrial dating (Table 1). As described by these investigators, only the portions of each biopsy from the functionalis layer were used for dating. Both the stroma and the glands were dated independently. When dyssynchrony was noted between the stromal and glandular dating, the two dates and the percentage of glands with the dyssynchronous date were recorded. Cycle days were defined in relation to an idealized 28-day cycle with the LH surge occurring on day 13, ovulation occurring on day 14, and the first full day of P treatment in a mock cycle set as cycle day 14.

Immunohistochemistry

Formalin-fixed, paraffin-embedded biopsy samples were immunohistochemically stained using diaminobenzidine (DAB) (Sigma-Aldrich, St. Louis, MO) as the chromagen, as previously described (21). The only modification to the protocol was that after deparaffinization in xylene and rehydration with graded concentrations of alcohol, antigen retrieval was achieved by heating each section in a 750-watt microwave at 60% power or in a hot water bath maintained at 95° to 99°C in 0.01 M citrate buffer (pH 6.0). The slides were allowed to heat for 5 minutes with occasional fluid replacement for evaporation losses, followed by cooling at room temperature for 1 hour. Anti-cyclin E (clone HE12) and anti-p27 (clone DCS-72.F6), both purified mouse mono-

Endometrial dating by histologic criteria.

Cycle day	Major histologic features
1–4	Crumbling stroma, hemorrhage; intravascular fibrin thrombi; neutrophils present; regenerative changes prominent in late menstrual
5–8	Straight gland structure
9–11	Coiled gland structure, stromal edema
12–14	Coiled gland structure, little stromal edema
15	Scattered subnuclear vacuoles present, but with less than 50% of the glands exhibiting uniform subnuclear vacuolization
16	>50% of the glands exhibit uniform subnuclear vacuolization, leading to exaggerated nuclear pseudostratification; mitotic figures frequent
17	Subnuclear vacuoles and nuclei uniformly aligned; scattered mitotic figures
18	Vacuoles assume luminal position; mitotic figures rare
19	Vacuoles infrequent; secretions in lumens of glands
20	Secretions prominent
21	Beginning stromal edema
22	Maximal stromal edema
23	Spiral arteries first prominent
24	Thick periarterial cuffs of predecidua
25	Islands of predecidua in superficial compactum
26	Beginning coalescence of predecidual islands; large granulated lymphocytes appear in stroma
27	Confluence of predecidual islands; large granulated lymphocytes prominent
28	Extravasation of red cells in stroma; prominence of stromal neutrophils

Source: Adapted from Hendrickson and Kempson's decision tree of endometrial dating (10).

Dubowy. Cyclin C and p27 endometrial expression. Fertil Steril 2003.

clonal antibodies, type IgG1, purchased from Neo-Markers (distributed by Lab Vision Corporation, Fremont, CA), were used at dilutions of 1:100 and 1:800, respectively. Selective samples were stained in parallel with nonimmune mouse ascites (NMA; Sigma-Aldrich) as primary antibody for a negative control. The sections were counterstained with hematoxylin.

Interpretation of Immunohistochemical Staining

The specific staining of cyclin E and p27 was assessed by the presence of DAB precipitates in the nucleus and/or cytoplasm of the glandular epithelial cells. Biopsy tissues that were shown to be strongly positive were used as positive controls for subsequent studies. Endothelial cells within each section were found to act as an internal positive control. The percentage of the glandular epithelial cells that stained, the nuclear and cytoplasmic staining intensity (ranging from 0 for no staining to 3+ for the strongest staining), and the distribution within the glandular epithelium and stroma were evaluated for each specimen. Due to the variability in the amount of surface

present in the biopsy samples, surface expression was not included in our analysis.

Statistics

Characteristics of normal fertile patients, infertility patients, and patients undergoing mock cycles in preparation for oocyte donor embryo transfer were compared using one-way ANOVA, the Aspin-Welch t test and the Mann-Whitney test for continuous data, and the Fisher exact test for categorical data; $P<.05$ was considered statistically significant.

RESULTS

Dating, Cyclin E, and p27 Expression in the Endometrial Biopsy Samples From Normal Fertile Women

Forty-seven of the 48 biopsies examined from the normal fertile controls had known LH surges. Of these, 36 (77%) exhibited histologic cycle dates within 2 days of the cycle day determined for the LH surge. Twenty of the normal fertile control biopsy samples were dated as cycle day >20 based on stromal characteristics. Four (20%) of these biopsies showed glandular stromal dyssynchrony (GSD, defined as ≥30% of the glands appearing to be at cycle day <20 with stroma appearing to be at cycle day >20).
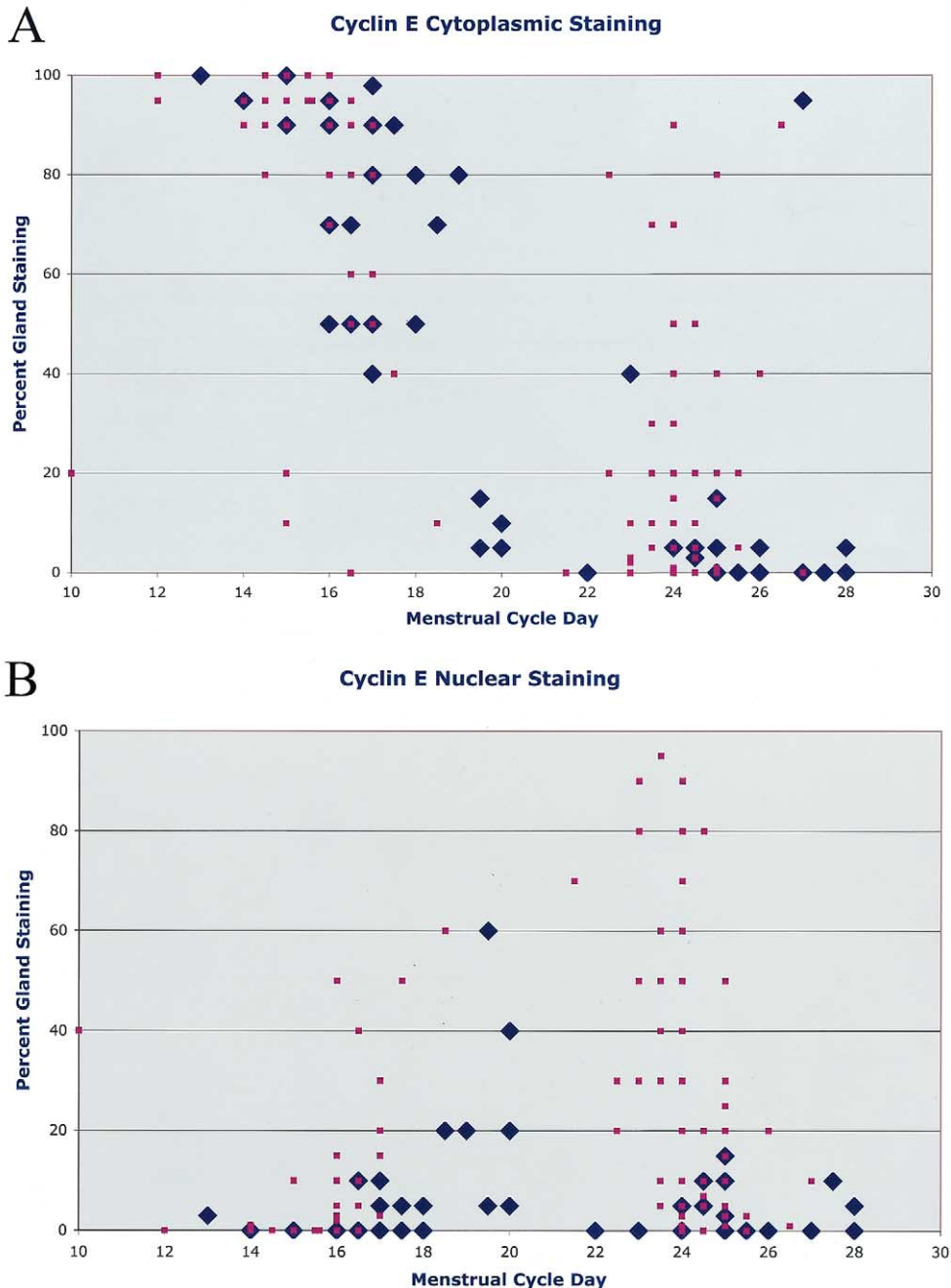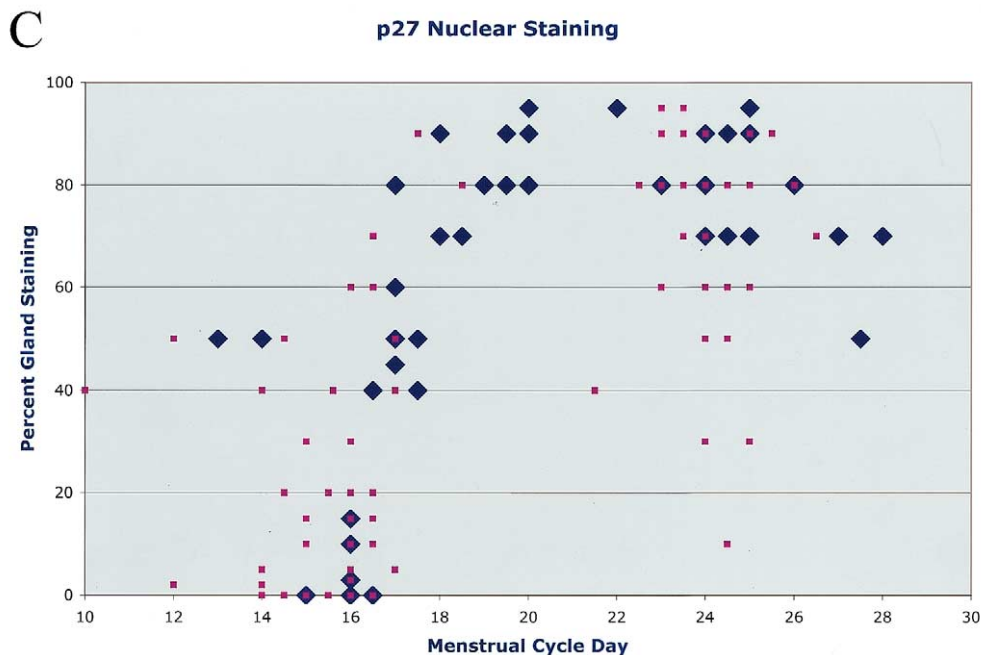
Immunohistochemistry

Immunohistochemical staining of midproliferative through late secretory endometrial biopsies from normal, fertile women revealed a progressive series of changes in the expression of both cyclin E and p27 in the endometrial glands (Fig. 1). Stromal expression of neither cyclin E nor p27 changed appreciably during the menstrual cycle, except in the late luteal biopsy samples, which demonstrated increased stromal cyclin E and p27 reactivity. The cytoplasm of endothelial cells stained consistently and intensely (3+) for both cyclin E and p27 throughout the cycle.

In addition to changes in the total percentage of glandular cells staining, the subcellular localization of cyclin E in the endometrial gland cells throughout the menstrual cycle was found to change dramatically. Midproliferative gland cells exhibited strong (approximately 3+ intensity) lateral, membrane-associated, cytoplasmic staining (Fig. 2A). From days 16 to 17 the endometrial gland cells exhibited decreased lateral cytoplasmic and increased basal staining relative to the midproliferative cells (see Fig. 2B, C). From days 18 to 19 there was a reciprocal pattern of decreasing cytoplasmic and increasing nuclear staining. Day 19 biopsy samples exhibited maximal nuclear staining (see Fig. 2D, E). Biopsy samples from cycle days 23 to 27 revealed no or only trace cytoplasmic or nuclear staining (see Fig. 2F), although one of these 20 biopsies (5%) had persistent gland nuclear expression of cyclin E.

FIGURE 1

Cyclin E and p27 expression through the menstrual cycle in fertile and infertile patients. (**A**), Cytoplasmic cyclin E. Cyclin E cytoplasmic staining in fertile patients (♦) was high up to cycle day 17, after which it declined rapidly so that the majority of the biopsy samples expressed less than 10% reactivity after cycle day 20. Cyclin E cytoplasmic reactivity was similar in the fertile and infertile groups before cycle day 17, but was more often seen in >20% of the glands in the infertile group (■) after cycle day 20. (**B**), Nuclear cyclin E. Cyclin E nuclear staining was low until cycle day 17 in the fertile patients (♦). Between days 18 and 20 cyclin E nuclear staining reached a maximum and then quickly dropped to below 10% for the majority of specimens. Cyclin E nuclear staining in the infertile group (■), however, was significantly different from that of the fertile group. Compared to the fertile group, a greater number of biopsies in the infertile group showed nuclear cyclin E reactivity before cycle day 18. In addition, more biopsies in the infertile group showed >10% cyclin E nuclear reactivity after cycle day 20. (**C—next page**), p27 expression. In the fertile patients, p27 reactivity was most commonly noted to begin between cycle days 16 and 17 (♦). Peak p27 staining began on cycle day 19 and was maintained at this level to the late luteal phase. The pattern of p27 expression for infertile patients (■) was not significantly different from that seen in the fertile patients, although more infertile patients exhibited decreased p27 staining after cycle day 20 than was seen in the fertile patients.

Dubowy. Cyclin C and p27 endometrial expression. Fertil Steril 2003.

FIGURE 1. CONTINUED.

C

p27 Nuclear Staining

Unlike cyclin E, the changes in subcellular localization of p27 in the endometrial gland cells throughout the menstrual cycle were simple. None to trace nuclear staining and no cytoplasmic staining were noted in midproliferative to day 16 biopsies (Fig. 3A). Nuclear p27 staining progressively increased from day 17 to day 19 (see Fig. 3B). Day 23 to 27 biopsy samples exhibited strong p27 nuclear staining, with none of the specimens exhibiting less than 50% glandular nuclear staining.

Dating, Cyclin E, and p27 Expression in the Endometrial Biopsy Samples of Women Seeking Infertility Treatment

Of the 130 biopsy samples examined from the women seeking infertility treatment, 106 had known LH surges. Of these, 92 (87%) exhibited histologic cycle dates within 2 days of the cycle day determined as the LH surge. Seventy-five of the biopsies were dated as cycle day >20 by stromal characteristics, with 23 (31%) of these exhibiting glands characteristic of cycle day <20 consistent with glandular stromal dyssynchrony (GSD).

Immunohistochemistry

Immunohistochemistry for cyclin E and p27 in the biopsy samples from infertile women revealed a pattern of expression different from that of the fertile controls, especially for cyclin E. The subcellular localization of cyclin E in these biopsy samples appeared to parallel the patterns seen in the fertile controls up to about cycle day 19. Whereas the majority of fertile control biopsies exhibited no to minimal (≤10% glands staining) cyclin E

staining after cycle day 20, 45 (60%) of the biopsy samples from the infertile group revealed persistent cyclin E staining (>10% glands staining), especially nuclear staining (Fig. 4).

The differences between the fertile controls and infertile patients for p27 were less dramatic (see Fig. 1B vs. 1C). Forty-four of the biopsy samples dated as cycle day >20 by stromal characteristics from the infertile group were stained with p27. Of these, 4 (9%) had less than 50% of the gland nuclei reactive for p27 (as compared to 0 out of 20 from the fertile group).

Dating, Cyclin E, and p27 Expression in the Endometrial Biopsy Samples of Women Undergoing Mock Cycles

Of the 40 biopsies examined from women undergoing a mock cycle, 40 (100%) exhibited histologic cycle dates within 2 days of the cycle day determined as the start of P. Twenty-one of the biopsy samples were dated as cycle day >20 by stromal characteristics and of these, 10 (48%) showed GSD. Because we only had biopsies available from cycle days 15 and/or 24 of these mock cycles, we were not able to generate a full cycle pattern for cyclin E and p27. We did note, however, that the staining intensity for cyclin E in the day 15 mock cycle biopsy samples were significantly stronger than the typical natural cycle biopsy taken on cycle day 15. Eight (38%) of the day 24 biopsies revealed persistent cyclin E staining (>10% glands staining), especially nuclear staining.

FIGURE 2

Cyclin E immunohistochemistry in representative fertile controls throughout the menstrual cycle. (**A**), Cyclin E expression in a midproliferative gland demonstrates strong (3+) lateral cytoplasmic staining (membrane associated pattern). At this time in the cycle, cyclin E is limited to the cytoplasm without evidence of nuclear or microvillus staining (*arrow heads*). Note the positively staining endothelial cells (*arrow*). (**B**), Beginning on cycle day 16 the cyclin E becomes more prominent in the basal aspect of the glandular cells (*arrow heads*), with only minimal apical or lateral staining. (**C**), By cycle day 16 to 17 cyclin E is found exclusively in the basal portion of the glandular cells (*arrow heads*) with no apical or lateral reactivity. The nuclei remain negative. (**D**), Between cycle days 17 and 18 there is a progressive loss of cytoplasmic cyclin E staining with a concomitant increase in nuclear staining. In this gland from cycle day 18, only minimal basal staining remains (*arrow heads*) and approximately half of the nuclei are now reactive, some strongly (*arrows*). (**E**), Maximal glandular nuclear reactivity is reached on cycle day 19. (**F**) After cycle day 19 there is a rapid loss of cyclin E reactivity. In this gland from cycle day 25, no cytoplasmic cyclin E and only trace nuclear cyclin E are present. Note the cytoplasm which is positive for cyclin E in the decidualized stromal cells (*arrow heads*). (A–F: magnification ×400.)
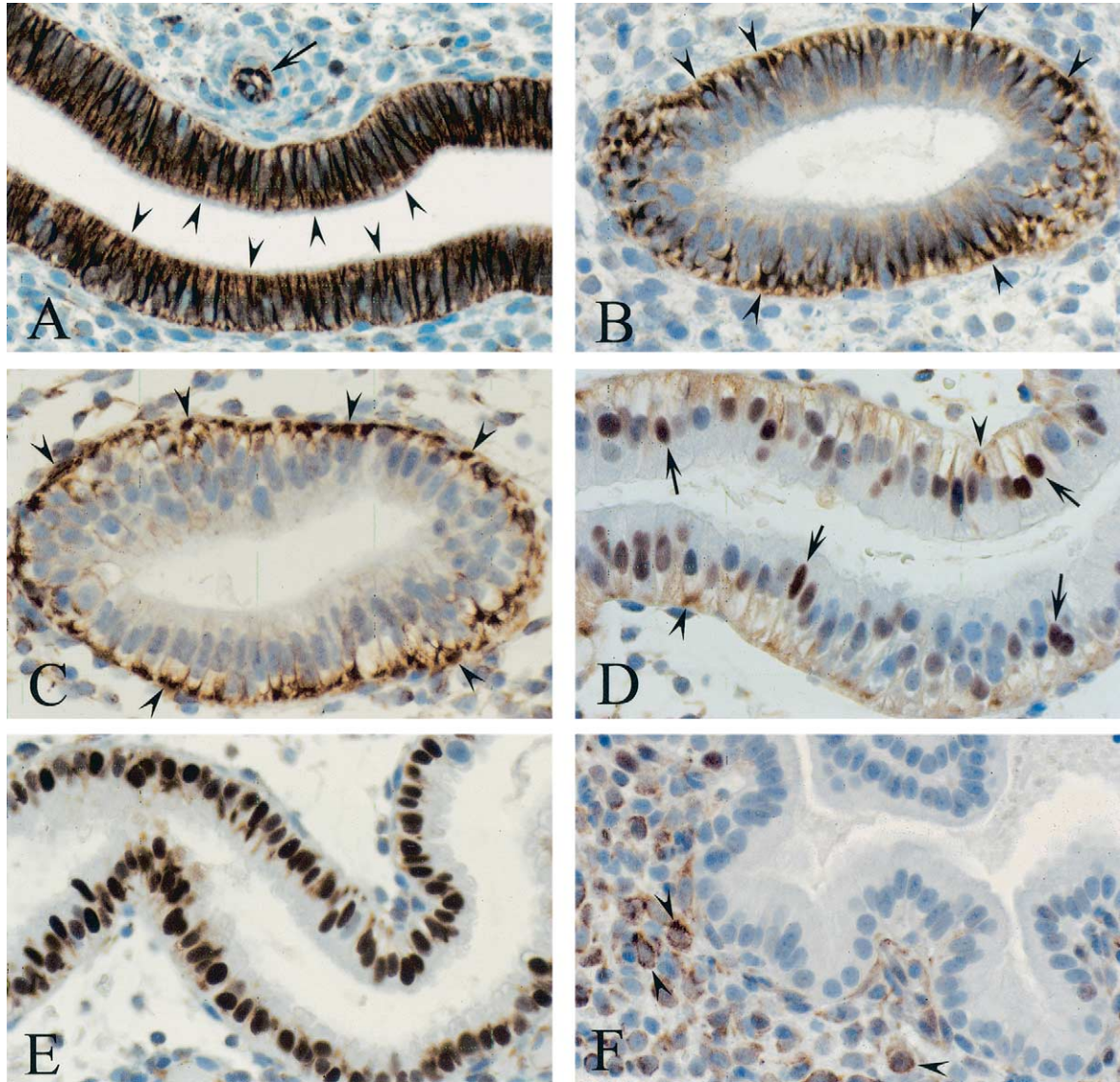
Dubowy. Cyclin C and p27 endometrial expression. Fertil Steril 2003.

FIGURE 3

p27 immunohistochemistry in representative fertile controls throughout the menstrual cycle. (**A**), Up to cycle day 16 there was no nuclear or cytoplasmic expression of p27 in the endometrial glands (*G*). However, note the strong endothelial cell cytoplasmic staining (*arrow heads*). (**B**), Beginning on cycle day 17, nuclear reactivity progressively increased until cycle day 19 when strong nuclear staining in the gland cells was noted. As with the cyclin E studies, there was strong p27 staining in the endothelial cells throughout the menstrual cycle. (A, B: magnification ×600.)
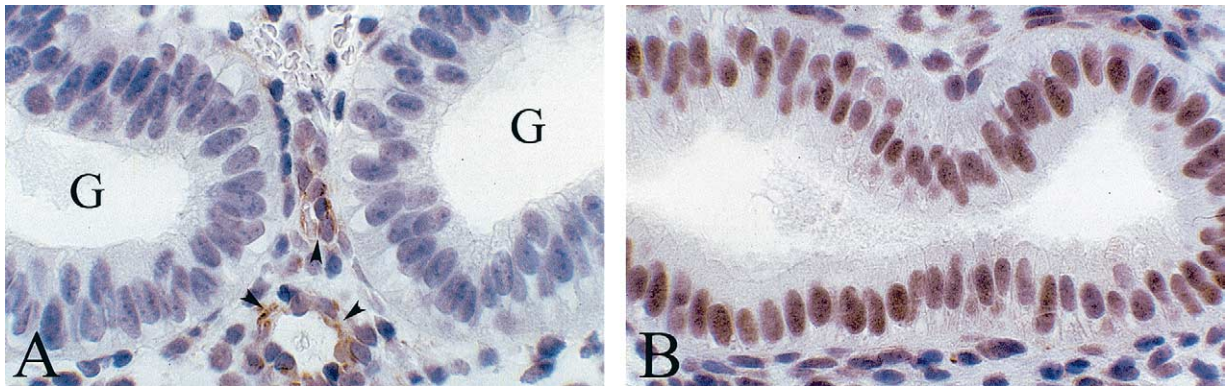
Dubowy. Cyclin C and p27 endometrial expression. Fertil Steril 2003.

Statistical Comparisons of Biopsy Samples From Normal Fertile, Infertile, and Mock Cycle Patients

Histologic Dating

The mean (±SD) of the differences between LH cycle day and histologic dating was 0.42 ± 2.5 for the fertile group, 0.49 ± 2.1 for the infertile group, and -0.11 ± 0.7 for the mock cycle group. These differences were not statistically different by one-way ANOVA. Individual comparisons among groups confirmed no statistically significant differences using the Aspin-Welch t test for assumed unequal variances or the nonparametric Mann-Whitney test.

FIGURE 4

Abnormal cyclin E staining patterns in biopsy samples from infertile women. (**A**), An endometrial gland from a woman seeking infertility treatment revealed a stroma with a cycle day of 24, but >90% of the glands were consistent with cycle days 17 to 18, consistent with glandular stromal dyssynchrony (GSD). Cyclin E staining revealed that 50% of the glandular cells had basal cytoplasmic staining (*arrow heads*) and 60% of the nuclei were positive, consistent with glandular developmental arrest (GDA) at cycle day 18. (**B**), An endometrial gland from a different woman seeking infertility care revealed a stroma with a cycle day of 24 and 60% of the glands consistent with cycle days 18 to 19 (GSD). Cyclin E staining revealed only trace cytoplasmic staining, but 90% of the nuclei were strongly positive, consistent with GDA at cycle day 19. (A, B: magnification ×400.)
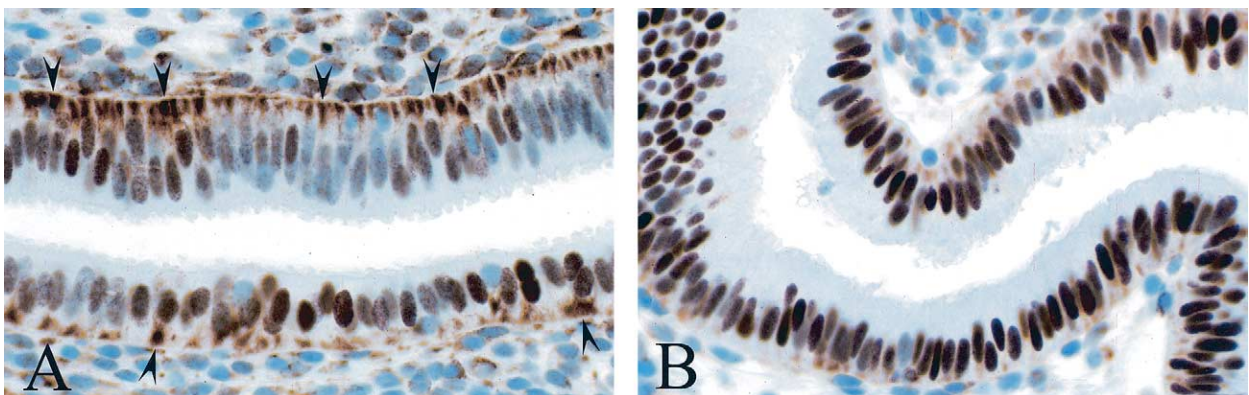
Dubowy. Cyclin C and p27 endometrial expression. Fertil Steril 2003.

Glandular Stromal Dyssynchrony (GSD)

Using Fisher's exact test (two-tailed $P=.41$ for fertile compared to infertile and two-tailed $P=.10$ for fertile compared to mock cycle) and defining 30% or greater GSD as abnormal resulted in no statistically significant differences among the three groups.

Glandular Developmental Arrest (GDA)

Defining greater than 10% cyclin E nuclear staining in a biopsy sample dated as cycle day 21 or greater resulted in statistically significant differences between the fertile and infertile ($P=.00000065$, Fisher's exact test) and fertile and mock cycle groups ($P=.02$, Fisher's exact test). An abnormal cyclin E result predicted an infertile patient with a sensitivity of 60%, specificity of 95%, PPV of 98%, a conventional OR of 12 (1.8–81.8 95% CI), and a weighted for prevalence OR of 45 (33.6–60.3 95% CI). An abnormal cyclin E result predicted a mock cycle patient with a sensitivity of 38%, specificity of 95%, PPV of 89%, a conventional OR of 7.6 (1.05–55 95% CI), and a weighted for prevalence OR of 8 (4.0–16.0 95% CI).

p27

Defining less than 50% p27 nuclear reactivity in a cycle day 21 or later biopsy sample as abnormal resulted in no statistically significant difference between the fertile and infertile groups ($P=.32$, Fisher's exact test).

DISCUSSION

In gynecologic practice, though it is often important to evaluate the endometrium, the available tools have been limited. High levels of interobserver and intraobserver variability mark the histologic evaluation using the criteria set forth by Noyes, Hertig, and Rock (4). Using their criteria, most pathologists date the endometrium based on the most advanced portion of the biopsy, and internal dyssynchrony between glandular and stromal development is not always appreciated. Of the currently available markers, which are predominantly products of the differentiated cell, only $\alpha v \beta 3$ integrin is commercially available (15). In addition, the clinical utility of $\alpha v \beta 3$ integrin has recently been questioned (15, 16).

Given the importance of assessing the endometrium and the limitations of the currently available methods, we developed a novel system that is easy to use and provides insight into the underlying biology of this dynamic tissue. Our system involves immunohistochemically staining the endometrium with markers for the mitotic regulators cyclin E (the rate-limiting activator of the mitotic G1 to S phase transition) and p27 (an inhibitor of cyclin E). On a practical level, the system may be used with the commonly prepared formalin-fixed, paraffin-embedded specimens. In addition, it produces easy to visualize patterns of intracellular brown staining in the glands, which can be used to differentiate

individual cycle days between the midproliferative and late secretory phases of the menstrual cycle (Fig. 5). Stromal staining was not found to change significantly throughout the menstrual cycle. Conveniently, endothelial cells are consistently strongly stained with both markers throughout the menstrual cycle and are therefore useful as an internal positive control.

In addition to simply delineating the endometrium's developmental stage, these cell cycle regulators shed light on the underlying regulation of the endometrium, which drives it to cycle between phases of proliferation and differentiation. Cyclin E plays the role of the gas pedal, driving proliferation, while p27 is the brake, allowing differentiation to occur. Our observations of these cyclins in the endometrial glands are consistent with this model, with cyclin E normally being found only in the estrogen-controlled proliferative phase and the early secretory phase and p27 found only in the nonproliferating, P controlled secretory phase. The observation that the artificially stimulated mock cycle biopsy samples exhibited significantly greater amounts of follicular phase cyclin E supports the conclusion that E2 drives cyclin E production. Given their fundamental roles in controlling endometrial development, these markers provide insight into the actual biochemical and developmental state of each endometrial gland.

Using our system, the stroma is dated based on its histologic appearance according to the criteria established by Noyes et al. (4) as further defined by Hendrickson and Kempson (10) (see Table 1). The glands are then reviewed and dated and their expression patterns of cyclin E and p27 are recorded (see Fig. 5). After dating the stroma and glands, the percentage of glands at the various stages of development is noted. The importance of evaluating the stroma and glands independently in the normally developing endometrium is supported by previous morphometric analyses indicating that only some of the individual endometrial features have a statistically significant correlation with the endometrial date (11). Our results confirm that when dating abnormalities are noted they are most often associated with glands that are developmentally arrested.

A central reason for developing this system was to go beyond simple endometrial dating and to create an endometrial function test (EFT) to easily identify an abnormally developing endometrium. Our results confirmed the limitations of the simple endometrial dating that others had questioned (5–9, 39). Even our more sophisticated analysis that determined the degree of GSD was not able by itself to distinguish fertile and infertile patients, in spite of GSD having been previously reported both in untreated infertility patients (12, 40) and in infertility patients undergoing hyperstimulated cycles (41).

Identification of luteal cyclin E expression, especially nuclear cyclin E, was able to distinguish the biopsy samples from fertile and infertile patients. Beyond simply being a

FIGURE 5

Diagram of cyclin E and p27 glandular expression through the menstrual cycle. Specific antibody staining is represented by brown and hematoxylin staining by purple. (**Top**) p27 immunostaining through the menstrual cycle revealed [1] no nuclear staining or only trace nuclear staining in midproliferative to day-16 biopsy samples, [2] nuclear staining that progressively increased from day 17 to 19, and [3] day 20 to 28 endometria with strong nuclear staining. (**Bottom**) Cyclin E immunostaining through the menstrual cycle revealed [1] midproliferative glands with strong basal and lateral cytoplasmic staining, [2] cytoplasmic lateral staining that from day 16 to 17 decreased, while the basal staining increased, [3] a reciprocal pattern of decreasing cytoplasmic and increasing nuclear staining from day 17 to 19, and [4] endometria with either no nuclear staining or only trace nuclear staining from day 20 to 28. Note the reciprocal expression pattern for cyclin E and p27 between days 17 and 18 and the concomitant strong nuclear staining for both markers on day 19.
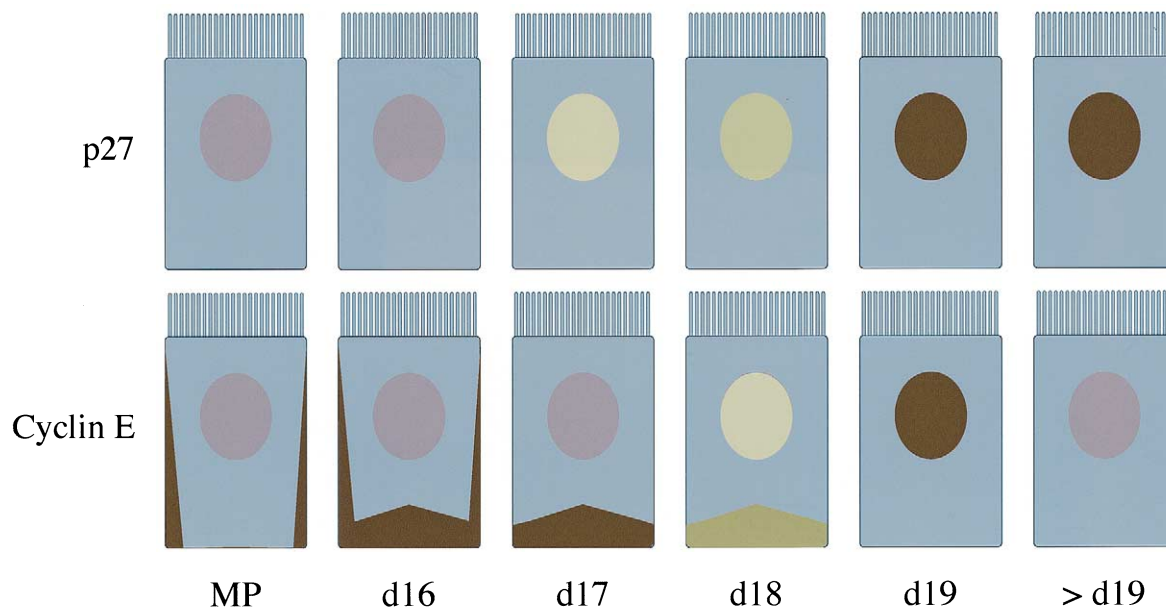
Dubowy. Cyclin C and p27 endometrial expression. Fertil Steril 2003.

histologic artifact, the continued expression of cyclin E indicates that the cells are actually developmentally delayed and arrested at an earlier stage of the menstrual cycle. Given that this delay, which we have termed glandular developmental arrest (GDA) (42), is found in increased rates in women with infertility, it may be a manifestation of defective uterine receptivity. The assertion that the glands are truly arrested in an early phase of development is consistent with other marker studies. For example, work with the $\alpha v \beta 3$ has shown that this integrin is closely correlated with cellular histologic maturation in both synchronous and dyssynchronous biopsy samples, thus indicating that the dyssynchronous glands are truly functionally delayed (43). This lag in integrin expression is also found in increased rates in women with primary, unexplained infertility (44), further supporting the contention that GDA may represent an underlying cause of some cases of infertility. Work with MAG mucin (21) showed the same association between GDA and infertility patients (42).

In addition to revealing features of normal and abnormal endometrium development, this work has helped us to better understand cyclin E and p27. The prime contribution of our work to the current understanding of these important cell cycle regulators lies in the fact that we have examined them in situ and have studied their intracellular movement as the cells progress from proliferation to differentiation. Previous research has relied primarily on protein electrophoresis, immunoprecipitation, and Western blotting to examine such features as expression levels (45), molecular pathways of phosphorylation and proteolysis by which the factors are regulated (46), and interactions between the individual cyclins and their regulators (47). By examining expression levels during the hormonally controlled menstrual cycle, we were able to evaluate the roles of estrogen and P in regulating cyclin E and p27. In particular, our work validates the contention that cyclin E is positively regulated by estrogen while P stimulates p27 activity and levels (33–35, 48).

Our study of the cycling endometrium has revealed a unique progression of glandular cyclin E from the lateral cytoplasm to the basal surface and finally to the nucleus, where it was found in association with p27 in the early days of luteal differentiation. The dynamic presence of cyclin E in the cytoplasm during proliferation indicates that it may be functional when concentrated within this compartment. It is

possible that cyclin E, with its partner cdk2, may act upon its substrates within the cytoplasm. Alternatively, the high levels within the cytoplasm may act as a reservoir for nuclear cyclin E, where it might be active both in stimulating proliferation and in repressing p27 expression; that is, cyclin E-cdk2 is known to negatively regulate p27 activity by phosphorylation, leading to the elimination of p27 from the cell (47). This conclusion is consistent with previous work by Orend et al. (32) who found cytoplasmic displacement of cyclin E in proliferating and transformed anchorage-independent fibroblasts and tumor cells. Cytoplasmic localization, it was concluded, helped to keep the cyclin E-cdk2 complexes active in the nucleus (32). Because of the insensitivity of immunohistochemical staining to low substrate levels, we were not able to detect nuclear cyclin E in follicular phase glandular nuclei, though it must be present in these mitotically active cells.

The movement of cyclin E from the cytoplasm of the proliferating cells to the nucleus of the differentiating cells, where it is temporally associated with p27, indicates that cyclin E with its cdk2 partner may be physically bound by the p27 (49). This would allow the nuclear cyclin E concentration to increase to visible levels as the cytoplasmic depots are depleted in the absence of renewed cyclin E synthesis. The movement of cyclin E from the cytoplasmic stores into the nucleus would help to explain the reciprocal decrease in cytoplasmic and increase in nuclear staining during this early part of the secretory phase. Once bound by p27, the cyclin E is inactivated and soon broken down. This could explain why the cyclin E is not normally observed in the later part of the secretory phase.

As opposed to cyclin E, which moves through the cytoplasm and into the nucleus, glandular p27 was found exclusively in the nuclei of the nonproliferating, differentiated cells. This association is consistent with the fact that p27 inhibits cyclin E-cdk2 and plays a critical role in preventing proliferation and allowing differentiation. This function is emphasized by findings in tumor cell lines. For example, abnormally low levels of p27 are frequently found in human carcinomas, and these low levels often correlate directly with both histologic aggressiveness and patient mortality. Also, Fero et al. (50) found that p27 heterozygous mice have a lower than normal ability to suppress cellular proliferation and are significantly more likely to develop a variety of tumors when challenged with gamma-irradiation or a chemical carcinogen.

In our current immunohistochemical study of the endometrial expression of cyclin E and p27, we have developed an endometrial function test (EFT), which allows us to date normal endometrium, and to differentiate between normally and abnormally developing endometrium. Ultimately, we hope this test will prove useful to reproductive endocrinologists in evaluating implantation potential. In addition, given that cyclin E and p27 are regulated by estrogen and p, this test may have a role in evaluating the effects of these hormones' exogenous administration in infertility treatments as well as in other circumstances such as hormone replacement therapy of perimenopausal and postmenopausal women. The full potential of these markers will need to be established with prospective, case-controlled multicenter trials that examine different groups of infertility patients to investigate the accuracy of the EFT in predicting pregnancy outcomes in natural cycles, IVF, and donor oocyte patients.

References

1. Lindhard A, Bentin-Ley U, Ravn V, Islin H, Hviid T, Rex S, et al. Biochemical evaluation of endometrial function at the time of implantation. Fertil Steril 2002;78:221–33.
2. Martin J, Dominguez F, Avila S, Castrillo JL, Remohi J, Pellicer A, et al. Human endometrial receptivity: gene regulation. J Reprod Immunol 2002;55:131–9.
3. Key JD, Kempers RD. Citation classics: most cited articles from *Fertility and Sterility*. Fertil Steril 1987;47:910–5.
4. Noyes FW, Hertig AT, Rock J. Dating the endometrial biopsy. Fertil Steril 1950;1:3–25.
5. Li TC, Dockery P, Rogers AW, Cooke ID. How precise is histologic dating of endometrium using the standard dating criteria? Fertil Steril 1989;51:576–82.
6. Gibson M, Badger GJ, Byrn F, Lee KR, Korson R. Error in histologic dating of secretory endometrium: variance component analysis. Fertil Steril 1991;56:242–7.
7. Smith S, Hosid S, Scott L. Endometrial biopsy dating: interobserver variation and its impact on clinical practice. J Reprod Med 1995;40:1–3.
8. Scott RT, Snyder RR, Strickland DM, Tyburski CC, Bagnall JA. The effect of interobserver variation in dating endometrial histology on the diagnosis of luteal phase defects. Fertil Steril 1988;50:888–92.
9. Castelbaum AJ, Lessey BA. Insights into the evaluation of the luteal phase. Infert Reprod Med Clin N Am 1995;6:199–213.
10. Hendrickson MR, Kempson RL. Surgical pathology of the uterine corpus. Major Probl Pathol 1980;12:36–98.
11. Li TC, Rogers AW, Dockery P, Lenton EA, Cooke ID. A new method of histologic dating of human endometrium in the luteal phase. Fertil Steril 1988;50:52–60.
12. Li TC, Dockery P, Rogers AW, Cooke ID. A quantitative study of endometrial development in the luteal phase: comparison between women with unexplained infertility and normal fertility. Br J Obstet Gynaecol 1990;97:576–82.
13. Acosta AA, Elberger L, Borghi M, Calamera JC, Chemes H, Doncel GF, et al. Endometrial dating and determination of the window of implantation in healthy fertile women. Fertil Steril 2000;73:788–98.
14. Daftary GS, Taylor HS. Molecular markers of implantation: clinical implications. Curr Opin Obstet Gynecol 2001;13:269–74.

15. Lessey BA, Castelbaum AJ, Wolf L, Greene W, Paulson M, Meyer WR, et al. Use of integrins to date the endometrium. Fertil Steril 2000;73:779–87.
16. Ordi J, Creus M, Ferrer B, Fabregeus F, Carmona F, Casamitjana R, et al. Midluteal endometrial biopsy and αVβ3 integrin expression in the evaluation of the endometrium in infertility: implications for fecundity. Int J Gynecol Pathol 2002;21:231–8.
17. Lessey BA. Implantation defects in infertile women with endometriosis. Ann N Y Acad Sci 2002;955:265–80.
18. Hey NA, Li TC, Devine PL, Graham RA, Saravelos H, Aplin JD. MUC-1 in secretory phase endometrium: expression in precisely dated biopsies and flushings from normal and recurrent miscarriage patients. Hum Reprod 1995;10:2655–62.
19. Cullinan EB, Abbondanzo SJ, Anderson PS, Pollard JW, Lessey BA, Stewart CL. Leukemia inhibitory factor (LIF) and LIF receptor expression in human endometrium suggests a potential autocrine/paracrine function in regulating embryo implantation. Proc Natl Acad Sci USA 1996;93:3115–20.
20. Simon C, Pellicer A, Polan ML. Interleukin-1 system crosstalk between embryo and endometrium in implantation. Hum Reprod 1995;10(Suppl 2):43–54.
21. Kliman HJ, Feinberg RF, Schwartz LB, Feinman MA, Lavi E. A mucin-link glycoprotein identified by MAG (mouse ascites golgi) antibodies. Am J Pathol 1995;146:166–81.
22. Daftary GS, Troy PJ, Bagot C, Young SL, Taylor HS. Direct regulation of β3-integrin subunit gene expression by HOXA10 in endometrial cells. Mol Endocrinol 2002;16:571–9.
23. Brown SE, Mandeline E, Oehninger S, Toner J, Seppala M, Jones H. Endometrial glycodelin-A expression in the luteal phase of stimulated ovarian cycles. Fertil Steril 2000;74:130–3.
24. Moyer DL, Felix JC. The effects of progesterone and progestins on endometrial proliferation. Contraception 1998;57:399–403.
25. Bourgain C, Ubaldi F, Tavananiotou A, Smitz J, Steirteghem A, Devroey P. Endometrial hormone receptors and proliferation index in the periovulatory phase of stimulated embryo transfer cycles in comparison with natural cycles and relation to clinical pregnancy outcome. Fertil Steril 2002;78:237–44.
26. Nardo LG, Sabatini L, Rai R, Nardo F. Pinopode expression during human implantation. Eur J Obstet Gynecol Reprod Biol 2002;101:104–8.
27. Hamel PA, Hanley-Hyde J. G1 cyclins and control of the cell division cycle in normal and transformed cells. Cancer Invest 1997;15:143–52.
28. Gillett CE, Barnes DM. Demystified: cell cycle. Br Med J 1998;51:310–6.
29. Clurman BE, Porter P. New insights into the tumor suppression function of P27(kip1). Proc Natl Acad Sci USA 1998;95:15158–60.
30. Reynisdottir I, Massague J. The subcellular locations of p15(Ink4b) and p27(Kip1) coordinate their inhibitory interactions with cdk4 and cdk2. Genes Dev 1997;11:492–503.
31. Singh SP, Lipman J, Goldman H, Ellis FH Jr, Aizenman L, Cangi MG, et al. Loss or altered subcellular localization of p27 in Barrett's associated adenocarcinoma. Cancer Res 1998;58:1730–5.
32. Orend G, Hunter T, Ruoslahti E. Cytoplasmic displacement of cyclin E-cdk2 inhibitors p21Cip1 and p27Kip1 in anchorage-independent cells. Oncogene 1998;16:2575–83.
33. Musgrove EA, Sutherland RL. Cell cycle control by steroid hormones. Semin Cancer Biol 1994;5:381–9.
34. Shiozawa T, Li SF, Nakayama K, Nikaido T, Fujii S. 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. Mol Hum Reprod 1996;2:745–52.
35. Musgrove EA, Swarbrick A, Lee CSL, Cornish AL, Sutherland RL. Mechanisms of cyclin-dependent kinase inactivation by progestins. Mol Cell Biol 1998;18:1812–25.
36. Milde-Langosch K, Bamberger A, Goemann C, Rossing E, Rieck G, Kelp B, et al. Expression of cell-cycle regulatory proteins in endometrial carcinomas correlations with hormone receptor status and clinicopathologic parameters. J Cancer Res Clin Oncol 2001;127:537–44.
37. Bamberger AM, Riethdorf L, Milde-Langosch K, Bamberger CM, Thuneke I, Erdmann I, et al. Strongly reduced expression of the cell cycle inhibitor p27 in endometrial neoplasia. Virchows Arch 1999;434:423–8.
38. Shiozawa T, Nikaido T, Nakayama K, Lu X, Fujii S. Involvement of cyclin-dependent kinase inhibitors p27Kip1 in growth inhibition of endometrium in the secretory phase and of hyperplastic endometrium treated with progesterone. Mol Hum Reprod 1998;4:899–905.
39. The Reproductive Medicine Network. The endometrial biopsy as a diagnostic tool in the cytoplasm of the infertile patient [abstract no. O-4]. In: Abstracts of the Scientific Oral and Poster Sessions of the 58th Annual Meeting. Seattle, Washington: American Society for Reproductive Medicine, 2002:S2.
40. Dockery P, Pritchard K, Warren MA, Li TC, Cooke ID. Changes in nuclear morphology in the human endometrial glandular epithelium in women with unexplained infertility. Hum Reprod 1996;11:2251–6.
41. Benadiva CA, Metzger DA. Superovulation with human menopausal gonadotropins is associated with endometrial gland-stroma dyssynchrony. Fertil Steril 1994;61:700–4.
42. Kliman HJ, FeinbergRF, Van Deerlin P, Barmat LI, Moomjy M, Grifo JA, et al. Glandular developmental arrest (GDA): a unifying model of reproductive endometrial pathology [abstract no. P-009]. In: Abstracts of the Scientific Oral and Poster Sessions of the 53rd Annual Meeting. Cincinnati, OH: American Society for Reproductive Medicine, 1997:S96.
43. Creus M, Balasch J, Ordi J, Fabregues F, Casamitjana R, Quinto L, et al. Integrin expression in normal and out-of-phase endometria. Hum Reprod 1998;13:3460–8.
44. Lessey BA, Castelbaum AJ, Sawin SW, Sun J. Integrins as markers of uterine receptivity in women with primary unexplained infertility. Fertil Steril 1995;63:535–42.
45. Kwon TK, Nordin AA. Overexpression of cyclin E and cyclin-dependent kinase inhibitor (p27Kip1): effect on cell cycle regulation in HeLa cells. Biochem Biophys Res Commun 1997;238:534–8.
46. Tsvetkov LM, Yeh KH, Lee SJ, Sun H, Zhang H. p27(Kip1) ubiquitination and degradation is regulated by the SCF(Skp2) complex through phosphorylated Thr187 in p27. Curr Biol 1999;9:661–4.
47. Sheaff RJ, Groudine M, Gordon M, Roberts JM, Clurman BE. Cyclin E-CDK2 is a regulator of p27Kip1. Genes Dev 1997;11:1464–78.
48. Amanatullah DF, Zafonte BT, Pestell RG. The cell cycle in steroid hormone regulated proliferation and differentiation. Minerva Endocrinol 2002;27:7–20.
49. Sherr CJ, Roberts JM. CDK inhibitors: positive and negative regulators of G1-phase progression. Genes Dev 1999;13:1501–12.
50. Fero ML, Randel E, Gurley KE, Roberts JM, Kemp CJ. The murine gene p27Kip1 is haplo-insufficient for tumour suppression. Nature 1998;396:177–80.