

# Transit of Normal Rat Uterine Stromal Cells through G1 Phase of the Cell Cycle Requires Progesterone-Growth Factor Interactions

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## ABSTRACT

Understanding of cell cycle regulation in hormonally responsive cells lags behind studies in other systems because few models have been available to identify the role of steroid hormones and their receptors in this process. This study investigates progesterone-dependent effects on the progression of normal uterine stromal cells through early G1 phase of the cell cycle. Quiescent rat uterine stromal cells were stimulated to reenter the cell cycle by adding serum-free medium containing medroxyprogesterone acetate (MPA) and basic fibroblast growth factor (FGF). [<sup>3</sup>H]thymidine incorporation increased significantly ( $P = 0.025$ ) in cells stimulated with both FGF alone and MPA plus FGF compared with the control cells. Moreover, cells stimulated with MPA plus FGF incorporated significantly more ( $P = 0.01$ ) [<sup>3</sup>H]thymidine than cells treated with FGF alone, suggesting requisite interactions between progesterone and FGF for stromal cell entry into S phase. Flow cytometric analysis of stimulated stromal cells showed FGF alone and MPA plus FGF increased significantly ( $P = 0.002$ ) the percentage of cells in S phase at 12 h. Incorporation of bromodeoxyuridine into stromal cell nuclei indicated that FGF alone and MPA plus FGF increased the percentage of cells entering S phase at 18 and 24 h compared with the control cells. In

addition, MPA plus FGF increased significantly ( $P = 0.001$ ) the number of cells entering S phase at 24 h compared with FGF alone and sustained S phase entry compared with FGF alone, MPA alone, or the control cells. Stromal cells inhibited from G1 reentry by inhibition of mitosis showed accelerated entry into S phase in response to MPA plus FGF compared with FGF alone. Cyclin D1 messenger RNA increased in stromal cells treated with MPA plus FGF at 9, 12, and 15 h. Addition of RU 486 to cells stimulated with MPA plus FGF for 9 h reduced cyclin D1 messenger RNA accumulation by 40%. Western blot analysis of cyclin D1 immunoprecipitates indicated complex formation with both cyclin-dependent kinase 4 (Cdk4) and cyclin dependent kinase 6 (Cdk6). Cyclin D1-Cdk complexes and kinase activity correlated temporally with increased cyclin D1 expression in cells cultured with MPA plus FGF. Taken together, these results show that progesterone-FGF interactions increase cyclin D1 expression, correlating with accelerated stromal cell entry into S phase compared with cells treated with FGF alone. Moreover, progesterone plus FGF sustains the timing of stimulation for transit of uterine stromal cells through G1 into S phase compared with FGF alone. (*Endocrinology* 141: 637–648, 2000)

THE GROWTH and function of the uterus is dependent on regulated proliferation and differentiation of its cellular components. Within endometrial cells of the reproductive tract, hormones exert specific temporal, spatial, and interactive effects. Estrogens are associated generally with cell proliferation in uterine and breast tissues, while progesterone is considered more as the hormone promoting cellular differentiation in these organs. Mice lacking the progesterone receptor (PR) by targeted mutagenesis exhibit abnormalities in all aspects of reproduction including sexual behavior, mammary gland development, ovulation, and implantation (1). Implantation in PR null mice fails, in part, because the uterine stromal cells cannot undergo a decidual cell reaction (1). In spite of the preeminence of progesterone in female reproduction, the molecular mechanisms of action of this steroid on target cell proliferation and differentiation are not well understood (reviewed in Ref. 2). At day 3 of pregnancy

in the mouse (3), and day 4 of pregnancy in the rat (4, 5), there is a proliferative switch from uterine epithelial to stromal compartments. Stromal cells do not divide without progesterone, and proliferation is blocked by progesterone antibodies (6) and mifepristone (RU 486) (5, 7). The number of synchronously dividing stromal cells in the rat uterus increases in response to progesterone priming for 3 days followed by nidatory estrogen (5, 8). Progesterone alone increases DNA synthesis in the uterine stromal cells of hormonally sensitized rats (9), suggesting that this steroid can exert direct control on the genes involved in regulating cell cycle progression.

Alternatively, progesterone could mediate cell proliferation indirectly via control of growth factors or growth factor receptors (reviewed in Ref. 10). Progesterone regulates the expression of a variety of growth factors in uterine stromal and decidual cells including heparin binding-epidermal growth factor (11), insulin-like growth factor-1 (12), and tumor necrosis factor- $\alpha$  (13). In addition, growth factor receptors such as epidermal growth factor receptor (14) and tumor necrosis factor- $\alpha$ -RI (15) are induced by progesterone in the endometrium. Previous studies in our laboratory showed

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that the spatial and temporal distribution of fibroblast growth factor (FGF) in rat uterine stromal cells correlates with uterine stromal cell proliferation (5, 16). Furthermore, FGF expression is regulated by progesterone and estrogen in the rat uterus (17), and stromal cells express high-affinity FGF receptor 1 at the time of their proliferation (18).

While those earlier studies (16–18) were important to identify candidate molecules that regulate progesterone-dependent stromal cell proliferation *in vivo*, such an approach cannot be used to ascertain the molecular mechanisms involved. Progesterone, bound to PR, acts on target cells by stimulating or repressing the transcription of specific genes (19). In T47D breast cancer cells, the protooncogenes *c-fos* and *c-myc* are transiently induced by progesterone (20), and *c-fos* and *c-jun* are modulated by steroids in the rat endometrium (17, 21). Identifying protooncogenes as downstream effectors of steroid-mediated cell proliferation is complicated because these proteins function at multiple sites in signal transduction pathways including increasing synthesis of growth factors involved in cell cycle control. Moreover, recent experiments in rodents clearly show that, for some growth promoters (22) and steroid hormone receptors (23), there is a lack of correspondence between cell-specific expression in the endometrium and the proliferative response. The other candidate regulatory target genes for steroidal control of cellular proliferation include the G1 cyclins (cyclins C, D, and E) and G2 cyclins (cyclins A and B). Cyclins interact with their cyclin-dependent kinase (Cdk) partners and catalyze transitions both into S phase and into mitosis in the cell cycle (24, 25).

While considerable mechanistic information about the hormonal control of cell cycle transit has emerged from breast cancer cell studies (Refs. 26–28; reviewed in Ref. 29), the role of hormones, and in particular progesterone, is less well understood in normal uterine target cells. Isolated rodent stromal cells express normal permissive and instructive functions when reassociated with epithelium and grown *in vivo* (30). In addition, rat uterine stromal cells maintain progesterone-dependent proliferation in culture (31–34). Previous results from our laboratory showed that in a serum-free, phenol red-free chemically defined medium, quiescent rat uterine stromal cells required both progesterone and growth factors that bind to tyrosine kinase receptors to proliferate (33, 34). Of the growth factors we tested, FGF was chosen as the prototype for further studies since we had identified this growth factor as a potential coregulator of stromal cell proliferation in pregnant rats (5). Stromal cells stimulated to proliferate in culture with progesterone and FGF expressed both the A and B forms of PR and proliferation was inhibited by RU 486 (33). Addition of estrogen to cultures containing FGF alone and FGF plus progesterone did not significantly increase the proliferative response (33).

The objective of the following experiments was to determine the potential mechanisms involved in the progesterone-dependent control of stromal cell proliferation. While understanding the role of estrogen in stromal cell function is clearly important, the culture system we have developed and characterized (33, 34) provides a unique opportunity to discern the explicit role of progesterone as a mitogenic agent for uterine stromal cells. In this paper we provide greater insight

into the question of how progestins control cell proliferation in normal uterine cells.

## Materials and Methods

### *Stromal cell isolation and culture*

Stromal cells were isolated from the uterus of ovariectomized Sprague Dawley rats (Charles River Laboratories, Wilmington, MA) and characterized as described in detail elsewhere (33). Cells from the same passage (between passages 10–25) were used to determine treatment effects within an experiment by propagating a sufficient number of cells for each experiment in supplemented medium 199 containing 10% FBS. Quiescence was induced by culturing stromal cells for 72 h in serum-free, phenol red-free DMEM (Life Technologies, Inc., Gaithersburg, MD) and MCDB-105 (Sigma, St. Louis, MO) in a 3:1 mixture containing insulin (5  $\mu$ g/ml) and supplements as detailed elsewhere (33). Quiescent cells were stimulated to synchronously reenter the cell cycle by adding medroxyprogesterone acetate (MPA, 1  $\mu$ M) and FGF (50 ng/ml).

### *[<sup>3</sup>H]thymidine incorporation*

Uterine stromal cells ( $5 \times 10^4$ ) were seeded into 24-well plates and quiescence was induced by culture for 72 h in serum-free medium. Cells were stimulated to reenter the cell cycle by adding fresh serum-free medium containing MPA, FGF, the two agents together, or ethanol vehicle (negative control). Cells were incubated for 20 h and then pulsed for 2 h with 1  $\mu$ Ci [<sup>3</sup>H]thymidine/well (20–30 Ci/mmol, Amersham Pharmacia Biotech, Arlington Heights, IL) (32). The cells were washed twice in ice-cold PBS and placed at  $-20^\circ\text{C}$  for 60 min to detach the cells. The cells were washed in 10% trichloroacetic acid. Incorporated [<sup>3</sup>H]thymidine was separated from unincorporated by retention on nitrocellulose membrane filters (Whatman BA85, Fairfield, NJ). Filters were washed three times with ice-cold 5% trichloroacetic acid to remove unincorporated labeled thymidine. The filters were counted using liquid scintillation.

### *Flow cytometry*

Quiescent stromal cells were stimulated to reenter the cell cycle by adding fresh serum-free medium containing MPA, FGF, MPA plus FGF, and ethanol vehicle (control). Cells were collected at 12, 15, and 24 h after stimulation with additives. Stromal cells were loosened from dishes by incubation in single-strength trypsin-EDTA (Sigma, St. Louis, MO) for 30 sec at 37  $^\circ\text{C}$ . Cells were immediately scraped from the dishes and transferred to sterile 15-ml tubes containing 0.1 vol FBS. The cells were collected by centrifugation ( $500 \times g$ ), washed twice in PBS, and then stained for at least 4 h with a one-step propidium iodide solution (0.005% propidium iodide, 0.02% ribonuclease, 0.3% sodium phosphate, and 0.1% sodium citrate in distilled water) using standard methods (34, 35). Between  $1\text{--}2 \times 10^6$  cells per time and treatment within an experiment were analyzed on an EPICS 752 flow cytometer (Coulter Electronics, Hialeah, FL) with a laser output of 400 nW at 488 nm. Red fluorescent data were collected and DNA histograms were analyzed with the Modfit program (Verity Software, Topsham, ME). For the mitotic block experiments, quiescent stromal cells were stimulated with fresh serum-free medium containing MPA, FGF, MPA plus FGF, or ethanol vehicle. Nocodazole {methyl [5-(2-thienylcarbonyl)-1H-benzimidazol-2-yl]carbamate; Calbiochem, La Jolla, CA} was added (0.04  $\mu$ g/ml) from a stock solution made up in dimethyl sulfoxide. Stimulated cells were collected at times 0, 6, 9, 12, and 24 h and the percentage of cells in S phase and those blocked at the G2-M boundary were determined by flow cytometry.

### *Incorporation of bromodeoxyuridine (BrdU) into stimulated uterine stromal cells*

Uterine stromal cells ( $6\text{--}7 \times 10^3$ ) were plated into eight-well Lab-Tek chamber slides (Nunc Inc., Naperville, IL). The cells were cultured in serum-free medium for 72 h to induce quiescence. Cells were stimulated to reenter the cell cycle by adding fresh serum-free medium containing MPA, FGF, MPA plus FGF, and serum-free medium (control). At various timed intervals after stimulation, the cells were pulsed for 30 min with

BrdU-containing medium (10  $\mu\text{mol/liter}$ ). Samples were fixed with ethanol and stained with anti-BrdU antibody in the presence of nucleases according to the manufacturer's protocol (BrdU Labeling and Detection Kit, Roche Molecular Biochemicals, Indianapolis, IN). Bound anti-BrdU antibody was visualized by light microscopy. The number of labeled nuclei at 12, 18, 24, and 32 h after stimulation was counted from at least 500 cells for each time and treatment group. Quiescent stromal cells were also stimulated with serum-free medium containing BrdU and mitogens, and the number of labeled nuclei from at least 500 cells was counted after continuous BrdU (10  $\mu\text{mol/liter}$ ) labeling for 18 and 24 h.

### Northern blots

Total RNA was isolated from quiescent (time 0) and proliferating uterine (2–24 h) stromal cells using a single-step guanidine method (36). Samples (25  $\mu\text{g}$ ) of total RNA were dried under vacuum centrifugation, suspended in an RNA denaturing solution (0.4 M 3-*N*-morpholinopropanesulfonic acid, 0.1 M sodium acetate, 0.01 M EDTA, 2.2 M formaldehyde, 50% formamide), and loaded onto a 2.2 M formaldehyde-1% agarose gel. RNA was transferred by diffusion onto a nylon membrane (Micron Separation Inc., Westboro MA) for 18 h. The membrane was baked for 2 h at 60 C under vacuum. Hybridization probes were prepared by random prime labeling (RTS Rad Prime DNA Labeling System, Life Technologies, Inc., Gaithersburg, MD) the full-length murine cyclin D1 cDNA and rat  $\beta$ -actin. Northern blots were hybridized in 5 $\times$  SSPE, 5 $\times$  Denhardt's, 0.5% (wt/vol) SDS, and 100  $\mu\text{g/ml}$  sheared salmon testes DNA (Sigma) at 65 C for 18 h. Blots were washed using high stringency in 5 $\times$  SSPE [0.75 M NaCl, 0.05 M  $\text{NaH}_2\text{PO}_4$ , 5 mM EDTA, (adjusted to pH 7.4 with NaOH)]-0.5% SDS at 22 C, 1 $\times$  SSPE-0.5% SDS at 37 C, and 0.1 $\times$  SSPE-1% SDS at 65 C. Blots were exposed to Fuji x-ray film (Fisher Scientific, Pittsburgh, PA) using intensifying screens for 5–7 days at –80 C. The amount of cyclin D1 messenger RNA (mRNA) was determined by scanning densitometry using the NIH image software. Values were adjusted for assay variation by dividing the integrated optical density of cyclin D1 mRNA by the integrated optical density of  $\beta$ -actin mRNA in the same sample.

### Western blots

Uterine stromal cells were seeded at a density of  $2.5 \times 10^5$  cells/10  $\text{cm}^2$  in growth medium containing 10% FBS for 16 h. The cells were washed twice in PBS and cultured in serum-free medium for 72 h to induce quiescence. The cells were stimulated with fresh serum-free medium containing MPA, FGF, MPA plus FGF, or ethanol vehicle alone (Control). The cells were collected at timed intervals between 0–24 h. The cells were washed with PBS and lysed with lysis buffer [10 mM Tris-HCL, pH 7.5, 30 mM sodium pyrophosphate, 50 mM sodium chloride, 50 mM sodium fluoride, 5 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride, 10  $\mu\text{g/ml}$  leupeptin, and 10  $\mu\text{g/ml}$  aprotinin] for 30 min at 4 C. Protein extracts were centrifuged at  $13,000 \times g$  for 10 min at 4 C. The supernatants were transferred to a fresh tube and stored at –80 C. Protein extracts (150–300  $\mu\text{l}$ ) containing similar amounts of protein for each independent assay, as determined by absorbance at 280 nm, were incubated with cyclin D1 agarose-conjugated antibody (Mab D1-72-13G, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 6–14 h at 4 C. The agarose beads were collected by centrifugation and washed four times in PBS containing 0.1 M NaCl. The samples were boiled for 3 min in sample buffer (37) and cooled to 22 C, and the proteins were size fractionated by SDS-PAGE. Proteins were transferred to a nitrocellulose membrane using standard methods (18). The membrane was blocked in Tris-buffered saline [50 mM Tris, pH 7.4, 150 mM NaCl] containing gelatin (0.2%), powdered milk (5%), and Tween-20 (0.05%) at 4 C for 14 h. The proteins were reacted with primary antibodies [Cdk6 (MS-451, Neo Markers, Union City, CA) diluted 1:500; Cdk4 (sc-601-G, Santa Cruz Biotechnology, Inc.) diluted 1:500] for 90 min at 22 C. The blots were washed and reacted with species-specific alkaline phosphatase secondary antibodies (Sigma) diluted 1:1000. After washing, bound antibody was detected by incubating the blots with 0.3 mg/ml nitroblue tetrazolium and 0.15 mg/ml 5-bromo-4-chloro-3-indolyl phosphate in 0.1 M  $\text{NaHCO}_3$ , 1.0 mM  $\text{MgCl}_2$ , pH 9.8. The size of reactive bands was determined from prestained molecular size standards (Bio-Rad Laboratories, Inc., Hercules, CA) and the relative amount of reactive species was quantified using scanning den-

sitometry (NIH image software). Western blots processed without primary antibody or reacted with *c-fos* primary antibody did not show either of the reactive species (data not shown).

### Immune complex kinase assays

Quiescent stromal cells were stimulated with mitogens and approximately  $2-3 \times 10^6$  cells were suspended at the appropriate times in a lysis buffer (10 mM Tris-HCL, pH 7.5, 30 mM sodium pyrophosphate, 50 mM sodium chloride, 50 mM sodium fluoride, 5 mM EDTA, 1% Triton X-100, 1 mM sodium orthovanadate) containing 1 mM phenylmethylsulfonyl fluoride and protease inhibitor cocktails (38). Cells were incubated on ice for 30 min, and the samples were centrifuged ( $12,500 \times g$ ) for 10 min at 4 C. Cdk4 and Cdk6-cyclin complexes were immunoprecipitated from clarified cell lysates with agarose-conjugated antibodies to Cdk4 (sc-601, Santa Cruz Biotechnology, Inc.) and Cdk6 (sc-177, Santa Cruz Biotechnology, Inc.). The agarose-conjugated antibodies were suspended and incubated for 30 min at 30 C in a kinase buffer (50 mM HEPES, pH 7.5, 10 mM  $\text{MgCl}_2$ , 1 mM dithiothreitol, 0.1 mM sodium orthovanadate, 1 mM EGTA) containing 100  $\mu\text{M}$  ATP, 10  $\mu\text{Ci}$  [ $\gamma$ - $^{32}\text{P}$ ] ATP ( $>4000$  Ci/mmol, ICN Biochemicals, Inc., Costa Mesa, CA), and 0.5–2  $\mu\text{g}$  of purified glutathione-S-transferase-retinoblastoma protein (GST-pRb) substrate (Santa Cruz Biotechnology, Inc.) (39). Reactions were size fractionated by SDS PAGE (10%), and the gel was exposed to x-ray film for 30 min. The size of the radioactive bands was determined from prestained molecular size markers (Bio-Rad Laboratories, Inc.).

### Statistical analysis

Data were analyzed using the SPSS, Inc. (Chicago, IL) statistical software package. Differences among treatments were examined by ANOVA, and differences among means were determined through Scheffé's and Bonferroni's posthoc tests. The effects of treatment, time, and time-by-treatment interactions were assessed by least squares regression analysis. Differences between treatments in Fig. 6 were assessed by *t* test (two-tailed) for equality of means.

## Results

### MPA plus FGF action stimulates [ $^3\text{H}$ ]thymidine incorporation into uterine stromal cells

We showed previously that stromal cells proliferated significantly in response to progesterone plus FGF (33) and that those agents stimulated stromal cell transit through the cell cycle (34). Progesterone or FGF alone did not stimulate proliferation significantly; therefore, our first goal was to determine whether stromal cells treated with progesterone or FGF alone entered DNA replication. Since indistinguishable results were obtained in proliferation assays with progesterone and the synthetic progestin MPA, the latter was used routinely in these studies. We measured the incorporation of [ $^3\text{H}$ ]thymidine in cells cultured in MPA alone, FGF alone, the two agents together (MPA + FGF), or serum-free medium with ethanol vehicle. Proliferative agents were added for 20 h, and then the cells were pulsed for 2 h with [ $^3\text{H}$ ]thymidine (Fig. 1). Incorporation of [ $^3\text{H}$ ]thymidine increased significantly ( $P = 0.025$ ) in cells cultured with FGF alone and MPA plus FGF compared with cells in the control cultures. Cells stimulated with MPA plus FGF incorporated [ $^3\text{H}$ ]thymidine at a significantly higher ( $P = 0.01$ ) level than the cells treated with FGF alone. Incorporation of labeled thymidine into cells treated with MPA alone was significantly less ( $P = 0.01$ ) than the amount measured in cells treated with FGF alone or MPA plus FGF. Taken together, these results show that control of stromal cell entry into DNA replication, as measured by the incorporation of labeled thymidine, is enhanced by MPA plus FGF compared with FGF or MPA alone.

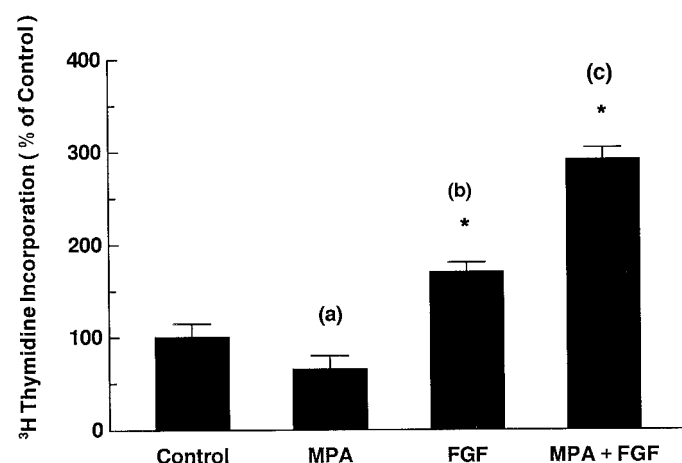


FIG. 1. Basic FGF and progesterone plus FGF stimulate stromal cell entry into DNA replication. Quiescent uterine stromal cells were stimulated to reenter the cell cycle by adding serum-free medium containing synthetic progestin (MPA, 1  $\mu$ M), FGF (50 ng/ml), and the two agents together (MPA + FGF). The control cultures received fresh serum-free medium with ethanol vehicle. Cells were cultured for 20 h and then pulsed for 2 h with [ $^3$ H]thymidine (1  $\mu$ Ci/well). [ $^3$ H]thymidine incorporation was determined by scintillation counting. Results are the mean  $\pm$  SEM for six observations for each treatment. \*,  $P = 0.025$  compared with the control cells. (a) vs. (b), (b) vs. (c), and (a) vs. (c),  $P < 0.01$  Scheffé's test.

*FGF alone and MPA plus FGF increase the percentage of cells in S phase*

To investigate further cell cycle-specific effects of progesterone, quiescent uterine stromal cells were stimulated to synchronously reenter the cell cycle in serum-free medium containing MPA, FGF, or the two agents together. Control cells received serum-free medium containing ethanol vehicle. The cells were collected between 0–24 h after mitogen addition, and the percentage of cells in S phase was determined using flow cytometry. Preliminary experiments showed that the percentage of cells in S phase in all treatment groups (MPA, FGF, MPA plus FGF) remained within 10% of the control cells (serum-free medium) until 12 h post stimulation (data not shown). Therefore, the percentage of cells in S phase was compared among the different treatments at times 0, 12, 15, and 24 h after addition of agents (Fig. 2). Stimulation of cells with FGF alone and MPA plus FGF increased significantly ( $P = 0.002$ ) the percentage of cells in S phase at 12 h after stimulation. Approximately 40% of the cells stimulated with FGF alone and MPA plus FGF were in S phase compared with 17% of the control cells at the same time. At 24 h poststimulation the percentage (28%) of cells in S phase was similar between cells treated with FGF alone and MPA plus FGF.

*MPA plus FGF increases the number of cells entering S phase and sustains the time of stimulation for cells to enter S phase*

To further clarify cell cycle-specific effects of progesterone on G1 transit and entry into S phase, BrdU incorporation into uterine stromal cell nuclei was investigated in cells stimulated with serum-free medium containing MPA, FGF, MPA plus FGF, and ethanol vehicle (control). Analysis of BrdU

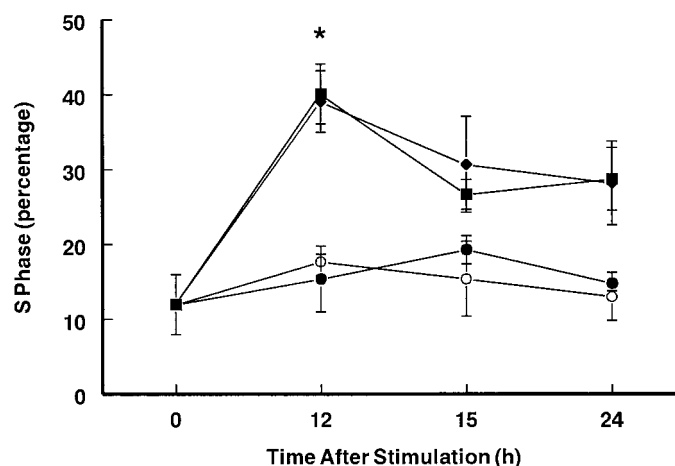


FIG. 2. Basic FGF alone and progesterone plus FGF increase the percentage of cells in S phase of the cell cycle. Quiescent uterine stromal cells were collected at times 0, 12, 15, and 24 h after the addition of synthetic progestin (MPA, 1  $\mu$ M), FGF (50 ng/ml), the two agents together (MPA plus FGF), or serum-free medium containing ethanol vehicle (control). The percentage of cells in S phase of the cell cycle was determined using flow cytometry as described in the text. Data are the mean percentages  $\pm$  SEM of cells in S phase from three independent experiments. \*,  $P = 0.002$  compared with the control cells. At all other times there were no significant differences. ◆, MPA plus FGF; ■, FGF alone; ●, MPA alone; ○, serum-free medium (control).

pulse labeling results by polynomial curve fitting showed that MPA not only stimulated the number of cells incorporating BrdU, but this steroid also sustained the time for stromal cell entry into S phase of the cell cycle (Fig. 3). At 18 h, BrdU incorporation into nuclei increased significantly ( $P = 0.001$ ) in cells treated with FGF alone and cells treated with MPA plus FGF. At 24 h after stimulation, BrdU incorporation continued to increase significantly in the cells treated with MPA plus FGF ( $P = 0.001$ ) and FGF alone ( $P = 0.023$ ) compared with the control cells. Importantly, however, cells stimulated with MPA plus FGF showed significantly more ( $P = 0.001$ ) BrdU-labeled nuclei than cells stimulated with FGF alone. The number of labeled nuclei in the cells treated with MPA alone was similar to that in the control cells. BrdU incorporation at 32 h poststimulation was similar among all of the treatment groups. We investigated the effects of treatment across time and found that the number of labeled nuclei in cells treated with MPA plus FGF increased significantly ( $P = 0.036$ ) at 24 h compared with 18 h. The number of BrdU-labeled nuclei in cells cultured with FGF declined at 24 h compared with the number at 12 h ( $P = 0.027$ ) and declined further at 32 h compared with the number at 18 h and 24 h ( $P = 0.034$  and  $P = 0.007$ , respectively). There were no differences across time for BrdU incorporation in cells treated with MPA alone.

To determine the proportion of cells that incorporated BrdU over this same time period, we stimulated quiescent stromal cells with serum-free medium containing MPA, FGF, MPA plus FGF, or vehicle control and added BrdU at the time of stimulation. The number of labeled nuclei was counted at 18 and 24 h after stimulation (Table 1). Consistent with the pulse-labeling experiments, FGF alone and MPA plus FGF significantly increased ( $P = 0.01$ ) the number of labeled nu-

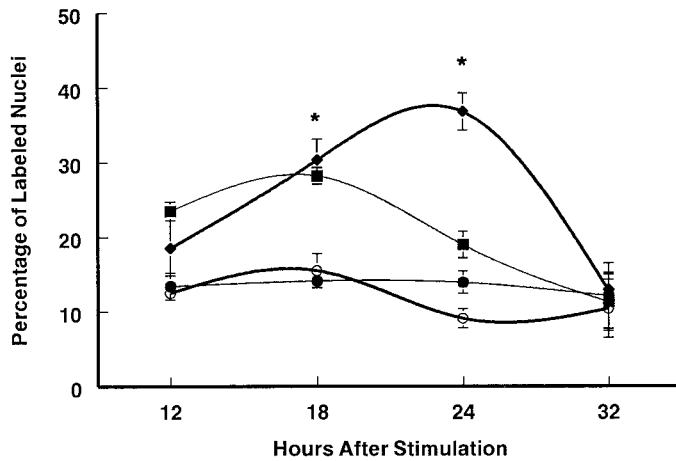


FIG. 3. Progesterone plus basic FGF increase the number of cells entering S phase and extend the time of stromal cell entry into DNA replication. Quiescent uterine stromal cells were stimulated to reenter the cell cycle with serum-free medium containing synthetic progestin (MPA, 1  $\mu$ M), FGF (50 ng/ml), the two agents together (FGF plus MPA), or ethanol vehicle (control). At the indicated times, cells were pulsed for 30 min with BrdU-containing medium (10  $\mu$ M/liter). Samples were fixed and stained with anti-BrdU antibody as detailed in the text. Data are the mean  $\pm$  SEM of three (18 h, 24 h) or two (12 h, 32 h) independent experiments. \*,  $P = 0.001$ , compared with the control cells. At 18 h, the percent of labeled nuclei increased in cells treated with FGF alone ( $P = 0.01$ ) and MPA plus FGF ( $P = 0.004$ ) compared with the control cells. At 24 h, the number of labeled nuclei in cells cultured with MPA plus FGF was significantly greater than for cells cultured in FGF alone ( $P = 0.001$ ), MPA alone ( $P = 0.001$ ), and the control medium ( $P = 0.001$ ). The number of labeled nuclei in cells treated with FGF alone remained increased ( $P = 0.023$ ) over that in the control cells at 24 h, Bonferroni posthoc tests. ◆, MPA plus FGF; ■, FGF; ●, MPA; ○, serum-free medium (control).

TABLE 1. Basic FGF alone and progesterone plus FGF increase the number of stromal cell nuclei incorporating BrdU

Treatment	Labeled nuclei (n)	Unlabeled nuclei (n)	Labeled nuclei (%)
Control	154	861	15.1
MPA	161	935	14.6
FGF	442	635	41.0 <sup>a</sup>
MPA + FGF	800	384	67.5 <sup>a</sup>

Uterine stromal cells were stimulated for 24 h in serum-free medium containing BrdU and ethanol vehicle (control), synthetic progestin (MPA, 1  $\mu$ M), FGF (50 ng/ml), and MPA + FGF. Data are the number of nuclei counted from three independent experiments.

<sup>a</sup>  $P = 0.001$  compared to the control cells. The number of labeled nuclei was significantly greater in cells cultured with MPA plus FGF compared to FGF alone ( $P = 0.002$ ), MPA alone ( $P = 0.001$ ), and the control medium ( $P = 0.001$ ).

clei compared with the control cells at 24 h. In addition, there were significantly more ( $P = 0.001$ ) labeled nuclei in the cells treated with MPA plus FGF compared with FGF alone. That increase in labeled nuclei occurred at some time after 18 h since the percentage of labeled nuclei was similar between cells treated with FGF alone (50%) and MPA plus FGF (47%) at 18 h. In cultures containing BrdU continuously, the number of labeled nuclei was similar between the cells treated with MPA compared with the control cells. Comparison of the incorporation into control cells pulsed with BrdU (Fig. 3) with the incorporation into cells continuously labeled with

BrdU (Table 1) indicated that cells in the control group did not progress through the cell cycle. Rather, there was a constant percentage of cells (12–15%) in the control medium that incorporated BrdU. Together, results from the BrdU labeling experiments showed that both FGF alone and MPA plus FGF increased the number of cells incorporating BrdU into nuclei compared with the control cells or cells cultured with MPA alone. Moreover, MPA plus FGF sustained the timing for stromal cell entry into S phase compared with FGF alone, MPA alone, or the control medium. This resulted in a significant ( $P = 0.001$ ) increase in the number of BrdU labeled nuclei in cells cultured with MPA plus FGF over the number in cells cultured with FGF alone.

#### MPA plus FGF accelerates stromal cell entry into S phase

To clarify MPA effects on the kinetics of stromal cell transit through G1 phase of the cell cycle, we inhibited the G1 reentry of stromal cells from M phase with nocodazole and monitored G1 exit into S phase by flow cytometry. Quiescent stromal cells stimulated with mitogenic agents and blocked in mitosis by nocodazole showed a significant increase in the percentage of cells in S phase at 9 and 12 h after treatment with FGF alone ( $P = 0.001$ ) and MPA plus FGF ( $P = 0.001$ ) compared with the control cells (Fig. 4). In addition, the percentage of cells in S phase at 9 h increased significantly ( $P = 0.002$ ) when cells were cultured with MPA plus FGF compared with FGF alone. The percentage of cells arrested at the G2-M boundary at 24 h after stimulation was 66% for cells treated with FGF alone compared with 75% for cells cultured with MPA plus FGF. In the control and MPA-

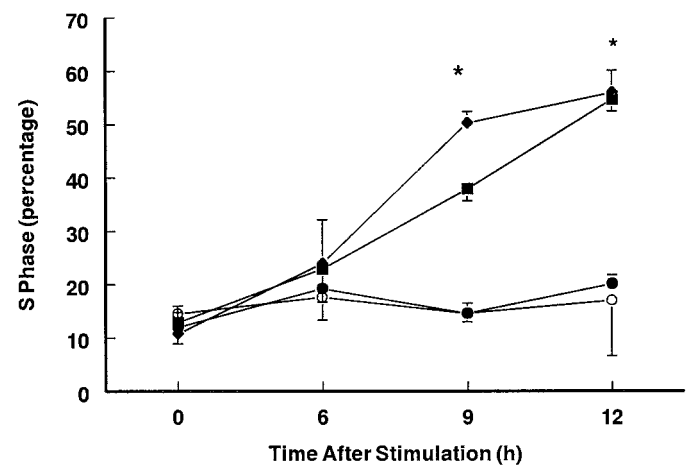


FIG. 4. Progesterone accelerates transit through G1 into S phase. Quiescent uterine stromal cells were stimulated to reenter the cell cycle with serum-free medium containing synthetic progestin (MPA, 1  $\mu$ M), FGF (50 ng/ml), the two agents together (MPA plus FGF), or ethanol vehicle (control). Culture medium also contained the mitotic inhibitor nocodazole (0.04  $\mu$ g/ml) to inhibit reentry from M phase. The percentage of cells in S phase of the cell cycle was determined using flow cytometry as described in the text. The percentage of cells in S phase increased significantly (\*,  $P = 0.001$ ) at 9 and 12 h when cells were stimulated with FGF alone and MPA plus FGF. At 9 h there was a significant increase ( $P = 0.002$ ) in the percent of cells in S phase after stimulation with MPA plus FGF compared with FGF alone. Data are the mean percentages  $\pm$  SEM of cells in S phase from three (0, 6, 9 h) or two (12 h) independent experiments. ◆, MPA plus FGF; ■, FGF alone; ●, MPA alone; ○, serum-free medium (control).

treated cells, the percentages at the G2-M border was similar (35% and 33%, respectively).

*The temporal appearance and amount of cyclin D1 mRNA is stimulated by MPA plus FGF*

The D cyclins have emerged as the major sensors for growth factor signal transduction (40). Activation of cyclin D1-Cdk is responsible for the phosphorylation of the pRb leading to the activation of the E2F transcription factor (41, 42). Progesterone accelerates the passage of breast cancer cells through G1 into S phase by stimulating transcription of the cyclin D1 gene (43). To determine whether cyclin D1 was a progesterone target in normal uterine cells the effects of MPA, FGF, and MPA plus FGF on cyclin D1 steady-state mRNA levels were investigated at various time points after addition of these agents to quiescent uterine stromal cells. Control cells received fresh serum-free medium with ethanol vehicle. Stromal cell total RNA (25  $\mu$ g) from the indicated time points was analyzed by Northern blotting using a murine cyclin D1 cDNA. The same blots were hybridized with  $\beta$ -actin to control for assay variation (Fig. 5, A and B). Cyclin D1 mRNA was readily detectable between 2 and 6 h after stimulating cells with MPA plus FGF. The steady-state mRNA levels in cells cultured with MPA plus FGF remained above those in the quiescent (time 0) and the control cells (Fig. 5A). Cyclin D1 mRNA in stromal cells cultured with FGF increased compared with quiescent cells between 6 and 24 h after addition of FGF (Fig. 5B). In the cells treated with MPA alone and in the control cells cyclin D1 mRNA was relatively low (compare Fig. 5A and 5B).

To gain further insight into the temporal and quantitative changes in cyclin D1 expression, steady-state mRNA levels in stromal cells from separate passages and experiments were measured from multiple Northern blots (Fig. 5C). The data shown in Fig. 5C are mean adjusted cyclin D1 values obtained by scanning densitometry of Northern blots from three independent experiments. Cyclin D1 steady-state mRNA increased in all treatment groups compared with the amount in the control cells after stimulation, but there were no significant ( $P > 0.05$ ) differences in treatments at 0, 2, 4, and 6 h after stimulation. The increase in cyclin D1 expression in cells cultured with MPA plus FGF approached significance ( $P = 0.053$ ) at 6 h compared with the other treatment groups. Between 9 and 15 h after stimulation, cells treated with MPA plus FGF expressed significantly more ( $P = 0.001$ , 9 h;  $P = 0.003$ , 12 h;  $P = 0.001$ , 15 h) cyclin D1 mRNA than cells treated with MPA alone, FGF alone, or the control cells. Peak levels of steady-state cyclin D1 mRNA (5-fold increase) were measured 9 h after addition of MPA plus FGF to quiescent cells compared with the amount in the control cells at the same time point. At 24 h after stimulation, the amount of cyclin D1 mRNA in cells treated with MPA plus FGF remained significantly higher ( $P = 0.015$ ) than the amount in the control and MPA-treated cells.

*PR antagonism reduces the progesterone-dependent increase in cyclin D1 mRNA*

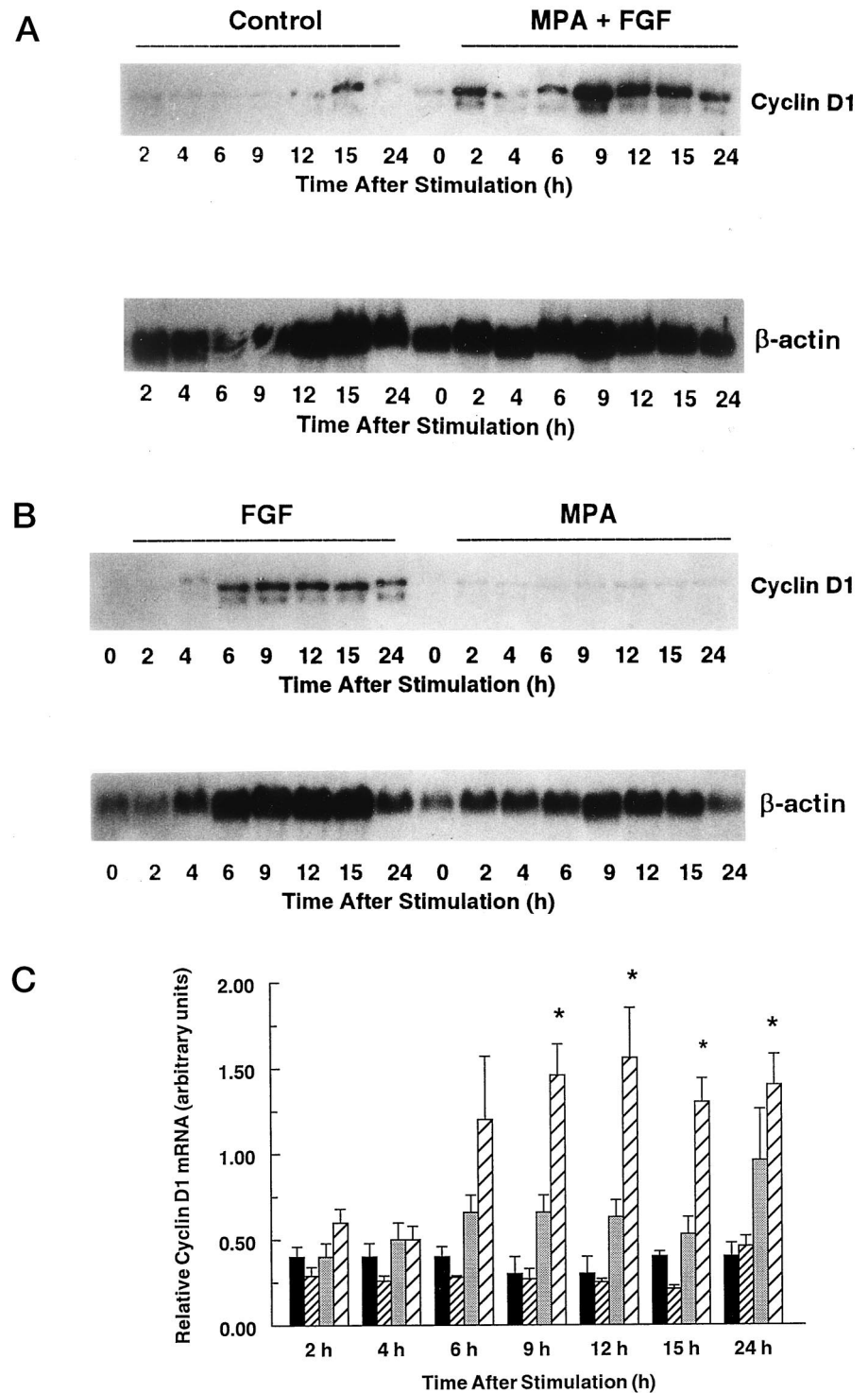
Previously we showed that progesterone-dependent proliferation was blocked in stromal cells cultured with 10-fold

molar excess of RU 486 (33). To investigate whether the antiproliferative effects of RU 486 were exerted on cyclin D1 expression, cells were stimulated with FGF alone and MPA plus FGF. Parallel cultures contained mitogenic agents and RU 486 (10  $\mu$ M), which we showed previously was cytostatic but not cytotoxic to uterine stromal cells (33). Total RNA was isolated at 6 and 9 h after addition of agents. Cyclin D1 mRNA was detected by Northern blotting of total RNA isolated from cells cultured without and with RU 486 (Fig. 6). Addition of RU 486 to cultures containing FGF did not change steady-state cyclin D1 mRNA (Fig. 6). Cyclin D1 mRNA decreased in cells cultured in medium containing MPA plus FGF and RU 486 at 6 h (data not shown) and 9 h (Fig. 6). Analysis of cyclin D1 mRNA levels adjusted to  $\beta$ -actin from three independent experiments showed that cyclin D1 steady-state mRNA decreased by 40% in cells cultured for 9 h in medium containing MPA plus FGF and RU 486 ( $0.2 \pm 0.05$  SEM) compared with the amount in cells cultured in MPA plus FGF ( $0.31 \pm 0.04$  SEM). These results suggest that the progesterone-dependent regulation of cyclin D1 mRNA is mediated, at least in part, via its receptor protein.

*Cyclin D1 forms complexes with Cdk4 and Cdk6 in stimulated stromal cells*

Mice lacking the retinoblastoma (rb) gene die *in utero*, demonstrating the importance of this protein in cell cycle regulation (reviewed in Ref. 41). The pRb sequesters positive and negative cell cycle regulators. When pRb is phosphorylated, the positive regulators such as E2F are released and activate target gene transcription. While the timing of synthesis and proteolysis of cyclin subunits regulates cell cycle progression, the modulation of the kinase activity of the Cdk partner through phosphorylation/dephosphorylation modification is also known to regulate cell cycle transit. Cdk4 and cdk6 code for the proteins that form the kinase components of the cyclin D1-Cdk complex (44). Western blot analysis from stromal cell lysates (6 mg of protein) immunoprecipitated with cyclin D1 and reacted with Cdk4 (Fig. 7A) and Cdk 6 (Fig. 7B) antibodies showed complexes of the expected size (34 kDa and 40 kDa, respectively) in quiescent and proliferating stromal cells. There were no obvious differences in the amount of complexes among the various treatments. To test whether cyclin D1 was depleted from the initial extracts, residual supernatants were reprecipitated with cyclin D1 antibody. When analyzed on Western blots, the reprecipitated samples contained no cyclin D1, indicating immunodepletion of cyclin D1 from the initial extracts (data not shown). Quantitative analysis of Cdk4 on two Western blots from independent assays did not measure any statistically significant differences among the treatments (data not shown). Cyclin D1-Cdk6 complexes analyzed from multiple Western blots showed a modest increase in complex formation in cells treated with MPA plus FGF compared with the other treatments. The amount of complex at 9 and 12 h increased significantly ( $P < 0.05$ ) in the stromal cells cultured with MPA plus FGF compared with cells cultured in the other treatment groups (Fig. 7C). There were no differences across time in cells cultured with MPA alone or cells cultured in the control medium. Across the times investigated, however, the amount of complexes was

**FIG. 5.** Interaction between progesterone plus basic FGF increase cyclin D1 steady-state mRNA levels. **A**, Quiescent uterine stromal cells were stimulated to reenter the cell cycle by the addition of synthetic progestin (MPA, 1  $\mu$ M) plus FGF (50 ng/ml), or ethanol vehicle (control). Total RNA (25  $\mu$ g) was isolated at the indicated times and size fractionated by gel electrophoresis. After transfer to a membrane, the RNA was hybridized with a radioactive full-length murine cyclin D1 cDNA. This same blot was hybridized with  $\beta$ -actin to control for assay variation. **B**, Quiescent uterine stromal cells were stimulated to reenter the cell cycle by addition of MPA (1  $\mu$ M) or FGF (50 ng/ml). Total RNA (25  $\mu$ g) was isolated at the indicated times and analyzed by Northern blotting. This same blot was hybridized with  $\beta$ -actin to control for assay variation. **C**, Quantitative analysis of cyclin D1 mRNA in stimulated uterine stromal cells. Steady-state cyclin D1 mRNA levels were measured from autoradiographs of Northern blots. Values were adjusted using  $\beta$ -actin mRNA to control for assay variation as described in the text. Data shown are the mean  $\pm$  SEM adjusted cyclin D1 mRNA levels from three independent assays. Between 0–6 h after addition of agents, no significant differences in cyclin D1 mRNA were measured. Cyclin D1 mRNA increased significantly (\*,  $P = 0.001$ , 9 h; \*,  $P = 0.003$ , 12 h; \*,  $P = 0.001$ , 24 h) in cells cultured with MPA plus FGF compared with MPA alone, FGF alone, or the control medium. At 24 h, cyclin D1 remained significantly ( $P = 0.015$ ) higher in cells treated with MPA plus FGF compared with cells treated with MPA alone or the control cells, but it was not significantly different from the amount in cells treated with FGF alone (Scheffé's posthoc tests). *Solid bars*, Control; *small striped bars*, MPA; *stippled bars*, FGF; *wide striped bar*, MPA plus FGF.



greater ( $P = 0.04$ ) in stromal cells cultured with FGF alone at 12 h compared with 4 h.

#### *Cyclin D-Cdk4 activity increases in cells treated with MPA plus FGF*

To determine whether kinase activity in the complexes differed among the treatment groups, we immunoprecipitated Cdk4 using clarified stromal cell lysates from cells that

had been stimulated with MPA, FGF, MPA plus FGF, and serum-free medium with ethanol vehicle (control). Kinase activity at the various time points was assayed using pRb as the substrate (Fig. 8). The relative amount of phosphorylated pRb substrate in lysates collected from the control cells was low (Fig. 8A, control). In stromal cells stimulated with MPA plus FGF, Cdk4 activity increased 15-fold at 8 h after stimulation compared with the control cells at 8 h in the same

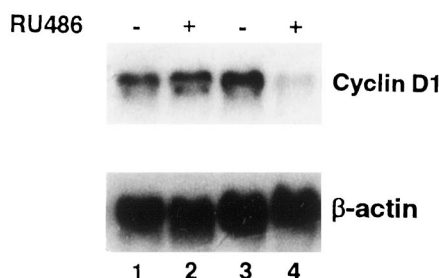


FIG. 6. PR antagonism reduces cyclin D1 steady-state levels in uterine stromal cells stimulated with progesterone plus basic FGF but not FGF alone. Quiescent uterine stromal cells were stimulated with FGF (50 ng/ml) alone and synthetic progestin (MPA, 1  $\mu$ M) plus FGF (50 ng/ml). Parallel cultures contained 10  $\mu$ M RU 486 in addition to additives. Total RNA was collected at 9 h and cyclin D1 steady-state mRNA levels were analyzed by Northern blotting. This same blot was hybridized with rat  $\beta$ -actin to assess assay variation. Lane 1, FGF; lane 2, FGF plus RU 486; lane 3, FGF plus MPA; lane 4, FGF plus MPA plus RU 486.

assay (Fig. 8A, MPA + FGF). Kinase activity in these extracts was reduced by 70% when immunodepleted with Cdk4 antibody (data not shown). The increased kinase activity at 8 h was 4-fold greater than the amount of substrate phosphorylated in cells treated with MPA plus FGF at 4 h and corresponds to increased cyclin D1 mRNA levels at these same time points (see Fig. 5C). It should be noted that in separate kinase assays, pRb phosphorylation consistently increased in cells stimulated with MPA plus FGF compared with quiescent or control cells (data not shown). Cdk4 activity in FGF-treated cells increased modestly (1.5-fold compared with quiescent cells in the same assay) 24 h after stimulation (Fig. 8B). This modest increase in Cdk4 activity in cells cultured with FGF correlated with maximal cyclin D1 mRNA levels measured in these cells at the same time (see Fig. 5C). No fold increase in Cdk4 activity was measured in extracts prepared from cells treated with MPA alone compared with the quiescent cells (time 0) in the same assay. Together, these results show that cyclin D-Cdk4 activity increases in cells treated with MPA plus FGF, and this activity corresponds temporally with the timing of maximal pRb phosphorylation and increased cyclin D1 mRNA.

#### Cyclin D-Cdk6 activity increases in cells treated with MPA plus FGF

Since Cdk6 also forms complexes with cyclin D1, we measured kinase activity in clarified stromal cell lysates after cells had been stimulated with MPA, FGF, MPA plus FGF, and serum-free medium with ethanol vehicle (control). Cdk6 was immunoprecipitated and kinase activity was detected at the various time points using pRb as the substrate (Fig. 9). The relative amount of phosphorylated pRb substrate in lysates collected from the control cells was low, and there were no notable increases in activity over time (Fig. 9A, control). Cdk6 activity increased at 9 h (~3-fold) in cells stimulated with MPA plus FGF and increased further (~9-fold) at 12 h compared with the quiescent cells (time 0) in the same assay (Fig. 9A, MPA + FGF). Cdk6 activity remained higher (~7-fold) at 24 h after stimulation with MPA plus FGF compared with the quiescent cells (time 0) in the same assay (Fig. 9A). There

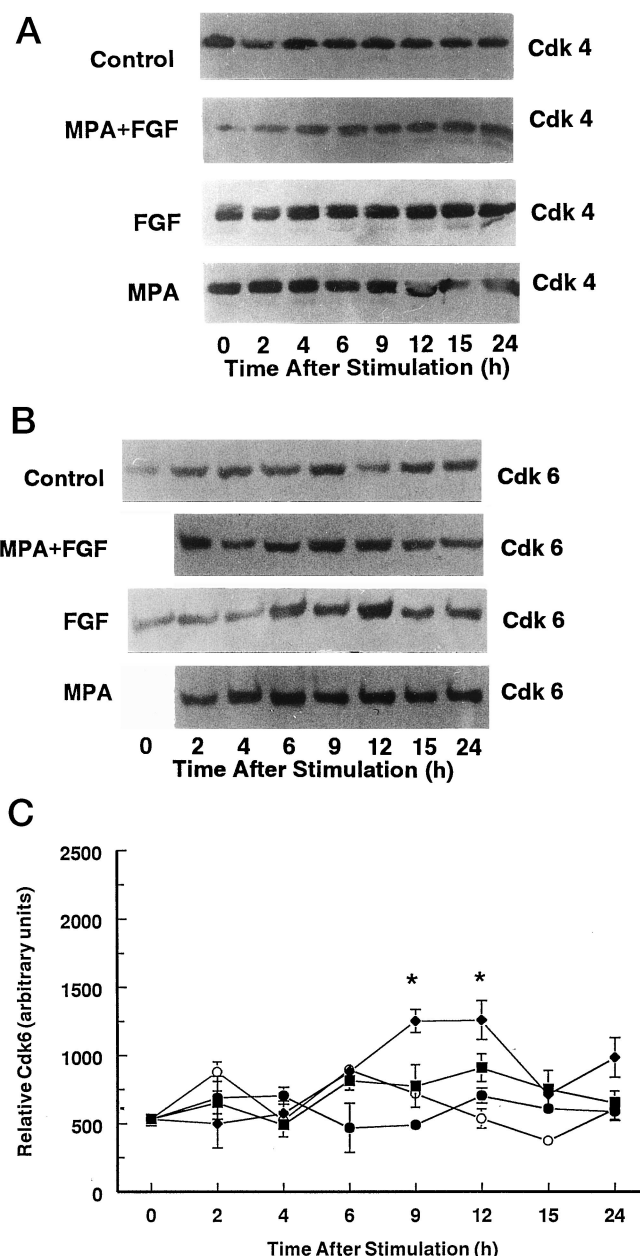


FIG. 7. Stromal cells express Cdk4 and Cdk6. Quiescent uterine stromal cells were stimulated to reenter the cell cycle with synthetic progestin (MPA, 1  $\mu$ M), FGF (50 ng/ml), the two agents together (MPA plus FGF), and ethanol vehicle (control). Cell extracts ( $1 \times 10^6$  cells, 6 mg protein) at each time point were collected and immunoprecipitated with a cyclin D1 antibody. The immunoprecipitates were analyzed for Cdk4 and Cdk6 on Western blots. A, Cyclin D1-Cdk4 complexes were detected in quiescent cells and at all times after addition of agents. Changes in the amount of the complexes were relatively modest. Similar results were obtained on two Western blots from independent assays. B, Cyclin D1-Cdk6 complexes were detected in quiescent cells and in all treatment groups. Similar results were obtained on three Western blots from independent assays. C, Quantitative analysis of cyclin D1-Cdk6 complexes in uterine stromal cells. Data shown are mean values  $\pm$  SEM of three independent assays. \*,  $P < 0.05$  compared with the control cells. At 9 h, the amount of cyclin D-Cdk6 complexes increased significantly ( $P = 0.003$ , MPA alone;  $P = 0.014$ , FGF alone) in the cells cultured with MPA plus FGF (Bonferroni posthoc tests).  $\diamond$ , MPA plus FGF;  $\blacksquare$ , FGF alone;  $\bullet$ , MPA alone;  $\circ$ , control.

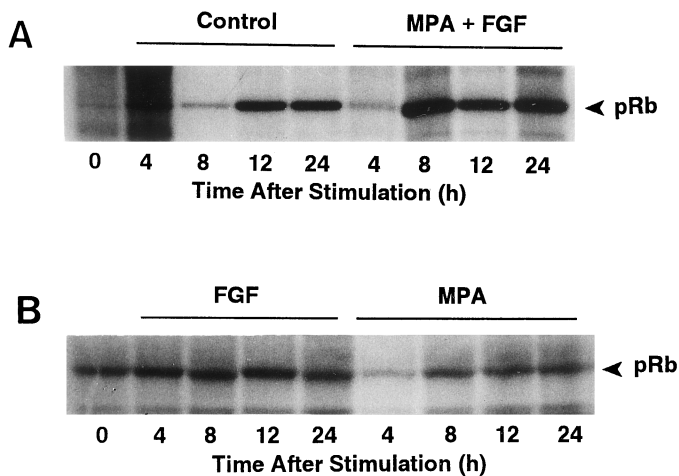


FIG. 8. Cyclin D-Cdk4 activity increases in stromal cells treated with MPA plus FGF. Quiescent uterine stromal cells were stimulated with the agents indicated for various time points. Cell extracts were prepared as described in the text. Cyclin D-Cdk4 complexes were immunoprecipitated using Cdk4 antibody. Kinase activity in the extracts was assessed by the phosphorylation of recombinant pRb. A, Stromal cells were stimulated with synthetic progestin (MPA, 1  $\mu$ M) plus FGF (50 ng/ml) or ethanol vehicle (control), and cell lysates ( $2 \times 10^6$  cells, 14 mg total protein) were collected. Reactions contained 0.5  $\mu$ g recombinant pRb as the phosphorylation substrate. Exposure time was 30 min. B, Quiescent stromal cells were stimulated with FGF (50 ng/ml) or MPA (1  $\mu$ M) alone, and cell extracts ( $2 \times 10^6$  cells, 14 mg protein) were collected at the indicated times. The extracts were immunoprecipitated with Cdk4 antibody, and the amount of cyclin D-Cdk4 activity was measured in the immunoprecipitates using 2  $\mu$ g of recombinant pRb as the phosphorylation substrate. Exposure time was 15 min.

was a modest increase in Cdk6 activity at 9 and 12 h after stimulation with FGF (1.5-fold) and at 9 h (2-fold) in cells treated with MPA alone compared with quiescent cells in the same assay (Fig. 9B).

### Discussion

Progress through the cell cycle requires the sequential activation of a number of genes, as well as transit through a series of crucial checkpoints (45). The lack of suitable model systems has prevented previous identification of the mechanisms by which progesterone regulates cellular proliferation in normal target cells. Earlier studies clearly demonstrate that progesterone is essential for stromal cell proliferation *in vivo* (5–7) and *in vitro* (31–34) but the mechanisms involved were not well understood. This investigation extends our earlier reports (33, 34) and shows that cyclin D1 is one of the early gene targets for progesterone-dependent stromal cell proliferation. Our data show a significant increase in the number of cells entering S phase after treatment with MPA plus FGF compared with FGF alone. Interestingly, in the absence of FGF, MPA is unable to augment cyclin D1 mRNA levels or stimulate cell cycle transit, providing direct evidence for requisite interactions between MPA and FGF for control of cyclin D1 expression. The reduction (40%) in cyclin D1 steady-state mRNA in stromal cells cultured with MPA plus FGF and RU 486 in this study, and our previous report (33) showing RU 486 blocked progesterone-dependent proliferation, indicates progesterone action on uterine stromal

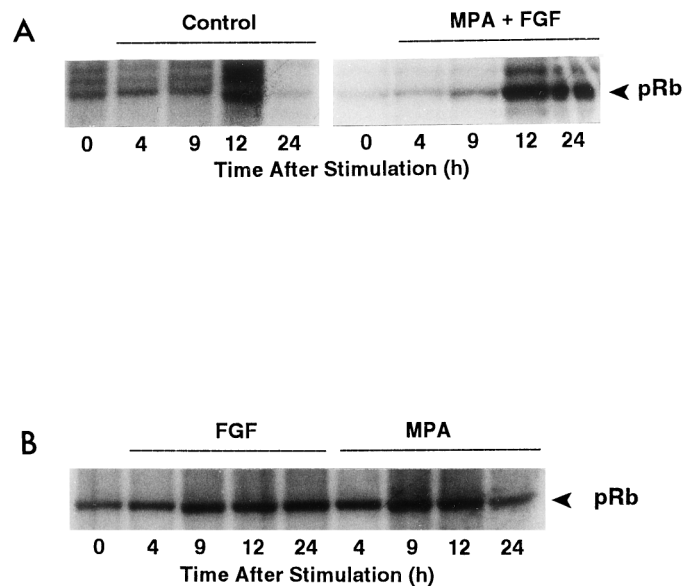


FIG. 9. Cyclin D-Cdk6 activity increases in uterine stromal cells treated with progesterone and FGF. Quiescent uterine stromal cells were stimulated with the agents indicated for various time points. Cell extracts were prepared as described in the text. Cyclin D-Cdk6 complexes were immunoprecipitated using Cdk6 antibody. Kinase activity in the extracts was assessed by the phosphorylation of recombinant pRb. A, Stromal cells were stimulated with synthetic progestin (MPA, 1  $\mu$ M) plus FGF (50 ng/ml) or ethanol vehicle (control), and cell lysates ( $2 \times 10^6$  cells, 14 mg total protein) were collected. Reactions contained 1.0  $\mu$ g recombinant pRb as the phosphorylation substrate. Exposure time was 30 min. B, Quiescent stromal cells were stimulated with FGF (50 ng/ml) or MPA (1  $\mu$ M) alone, and cell extracts ( $2 \times 10^6$  cells, 14 mg protein) were collected at the indicated times. The extracts were immunoprecipitated with Cdk6 antibody. The amount of cyclin D-Cdk6 activity was measured in the immunoprecipitates using 2  $\mu$ g of pRb as the phosphorylation substrate. Exposure time was 30 min.

cell proliferation is mediated via ligand-bound PR. Since we used a poorly metabolized progestin in the present experiments, incomplete antagonism between RU 486 and MPA could account for the failure of RU 486 to block cyclin D1 steady-state mRNA at a statistically significant level in cells cultured with MPA plus FGF and RU 486 compared with cells cultured with MPA plus FGF without PR antagonist.

In uterine stromal cells stimulated with MPA plus FGF, cyclin D1 mRNA increases significantly at 9, 12 and 15 h. The increase in cyclin D1 expression correlates with increased assembly of cyclin D1-Cdk complexes and kinase activity in cells stimulated with MPA plus FGF. Moreover, when stromal cells are prevented from reentering G1 phase by blocking mitosis with nocodazole, the percentage of cells in S phase increases significantly in response to MPA plus FGF compared with FGF alone, showing that progesterone exerts a direct effect on the transit of uterine stromal cells through G1 phase of the cell cycle. Stromal cells inhibited from reentering G1 phase by nocodazole progress temporally through G1 into S phase in conjunction with increased cyclin D1 mRNA (up to 5-fold), cyclin D1-Cdk complex formation, and kinase activity. These results indicate that changes in cyclin D1 steady-state mRNA levels in uterine stromal cells are indicative of biologically active protein under the conditions used

in our experiments. This temporal accumulation of cyclin D1 mRNA is dependent upon progesterone and FGF, and the resulting cyclin D1-Cdk complex formation and activity, although modest, promotes a significant increase in the number of stromal cells entering S phase.

Western blot analysis of cyclin D1 protein in stimulated uterine stromal cells over the same times and treatments we used to measure mRNA revealed no statistically significant differences in cyclin D1 protein (data not shown). Cyclin D1 does not oscillate with the magnitude observed for other cyclins because the D type cyclin proteins are normally present in cultured mammalian cells as long as growth factor stimulation persists (46). This dampening of cyclin D1 oscillation may be compounded further because eukaryotic cells cannot be perfectly synchronized in culture. When cells are synchronized in serum-free medium, typically 70% of the cells are arrested in G1 phase of the cell cycle and the other 30% are distributed in S and G2 phases of the cell cycle (34, 47, 48).

In the present experiments, a significant number of cells treated with FGF alone are in S phase of the cell cycle 12 h after stimulation, and in uterine stromal cells treated with FGF alone thymidine and BrdU incorporation is enhanced significantly compared with the control cells. Although the levels of cyclin D1 steady-state mRNA and cyclin D1-Cdk complexes appeared somewhat greater in FGF-treated cells than in the quiescent (time 0) or control (serum-free medium) cells, these differences were not statistically significant. Nevertheless, our results suggest that a subpopulation of uterine stromal cells are capable of entering S phase at a lower threshold of cyclin D1-Cdk complex formation and activation than the majority of cells in culture. Alternatively, we have shown previously that cyclin D3 mRNA levels are maximal at 9 and 12 h in cells cultured with FGF (49), and we cannot exclude the possibility that cyclin D3-Cdk complexes are involved in the FGF stimulation of stromal cell transit through G1 into S phase. However, only a cohort of the cells responding to FGF alone progress through M phase of the cell cycle because we have shown previously that although FGF alone augments cell proliferation, the differences are not statistically significant compared with the level of proliferation in the control cells (33). Cyclin D2 mRNA cannot be detected in quiescent or stimulated uterine stromal cells (49).

The lag time (~6–9 h) between the addition of MPA plus FGF to uterine stromal cell cultures and the subsequent accumulation of cyclin D1 mRNA suggests that progesterone-dependent regulation of cyclin D1 may occur through an intermediate factor. Transcription of cyclin D1 is controlled by progesterone in breast cancer cell lines but the mechanisms are not well understood (29). Progesterone response-like elements are not evident within 3 kb of the 5'-flanking DNA of the cyclin D1 gene (50). However, analysis of the human cyclin D1 promoter revealed a number of potential regulatory regions that included recognition sequences for Myc, E2F, OTF, Sp-1, FOS/JUN and CREB/ATF (50). Several of these potential cyclin D1 gene-regulatory proteins are controlled by steroids in the endometrium (17, 21), further suggesting the potential for indirect regulation.

Cyclin D1 mRNA is reported to have a short half-life (51). Examination of the rat cyclin D1 full-length cDNA revealed

five copies of the sequence motif AUUUA. Two of these motifs are positioned in an A/U-rich (71%) segment in the 3'-untranslated region of the cyclin D1 mRNA. These motifs, which are often positioned in A/U-rich regions, confer mRNA instability in certain lymphokine and immediate early gene transcripts (52). Interestingly, these motifs have been reported previously in the transcripts arising from the rat cyclin B gene (53, 54). It is notable that the regulation of cyclin B in the regenerating rat liver is primarily controlled by changes in mRNA stability rather than transcriptional activity although the mechanisms involved are not known (53). Steroid hormones promote the stability of transcripts from a number of hormone-responsive genes (55), but further studies on the control of progesterone-dependent cyclin D1 mRNA accumulation are necessary to clarify the mechanisms involved.

Since the timing of cyclin expression is a key step for activating its kinase partner (45, 46), our results show that progesterone exerts a direct effect on regulating cell cycle progression in normal uterine stromal cells. Cyclin D1-Cdk complex formation, and Cdk4/6 activities as measured by pRb phosphorylation, correlate temporally with increased cyclin D1 mRNA levels. Importantly however, progesterone also induces a more sustained stimulation of G1 to S phase progression than FGF alone. During this extended stimulation period, the number of cells entering S phase (as evidenced by [<sup>3</sup>H]thymidine and BrdU labeling) is significantly greater in MPA plus FGF-treated cells compared with cells treated with FGF alone. When cells are continuously labeled with BrdU in medium containing both MPA and FGF, the number of labeled nuclei increases significantly compared with other treatment groups. It seems likely that this sustained entry into S phase is stimulated by an increase in Cyclin D1-Cdk6 activity that begins at 9 h after cells are treated with MPA plus FGF, peaks at 12 h, and is maintained for at least 24 h. Taken together, these data now provide direct evidence for the recruitment of a significant number of uterine stromal cells treated with MPA plus FGF into S phase by accelerating entry into S phase and by sustained stimulation of uterine stromal cell transit through G1 owing to increased cyclin D1 expression and cyclin D1-Cdk6 assembly and activation. These results suggest that progesterone and FGF function at dual points early in the cell cycle and provide a molecular explanation for our earlier observations, which showed a significant increase in cell number after 48 h of culture in MPA plus FGF (33).

Since progesterone-dependent differentiation of uterine stromal cells involves endomitosis with resulting cellular DNA contents ranging from 4 n to 32 n (56, 57), separation of progesterone control of proliferation *vs.* differentiation is essential. It is tempting to speculate that the progesterone-dependent window that sustains stimulation of the G1 to S phase transit provides an opportunity for temporally controlled cues that direct cells from the proliferative cycle into a differentiation pathway. While our study shows that progesterone and FGF promote proliferation, the results suggest that additional molecules must be involved in uterine stromal cell differentiation. It is likely that these additional molecules will regulate exit from the cell cycle and entry into a differentiation program. Central to the differentiation cas-

cade in antimesometrial uterine stromal cells is the process of endoreplication, which involves the uncoupling of DNA replication from mitosis. This process of polyploidization also occurs in terminally differentiating bone marrow megakaryocytes, and it is coupled with cell cycle exit due to the inability of cyclin B to physically associate with Cdk1 (58). Mitosis in these cells does not occur in the absence of Cdk1 activation (59). The available evidence indicates that control of the uncoupling of DNA replication and mitosis may be cell type dependent and involves cyclin E-Cdk (59), cyclin A-Cdk (60), and cyclin B-Cdk (58). The data presented in this paper are the first to identify progesterone-dependent mechanisms for transit of normal uterine stromal cells through G1 phase of the cell cycle. Results from continuing studies are expected to identify further progesterone-dependent control of cell cycle progression. This knowledge will lay the foundation to systematically analyze cessation of cell proliferation for cellular differentiation.

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