Endocrine, *vol. 17, no. 3, 233–239, April 2002* 0969–711X/02/17:233–239/$11.75

Estrogen Stimulates Expression of p21$^{Waf1/Cip1}$ in Mouse Uterine Luminal Epithelium

Ming-Derg Lai,[1] Meei Jyh Jiang,[2] and Lih-Yuh C. Wing[1]

Departments of [1]Physiology and [2]Cell Biology and Anatomy, College of Medicine, National Cheng Kung University, Tainan, Taiwan

p21$^{Waf1/Cip1}$ was originally identified as an inhibitor of the cell cycle. Recent evidence suggests that it can act as a positive regulator of the cell cycle under the influence of some growth stimulators. We investigated the effects of ovarian steroids on the expression of p21, DNA synthesis, and mitosis in the uterus. Capsules containing 17β-estradiol (E_2) were subcutaneously implanted in ovariectomized mice that were sacrificed on different days. Their uteri were collected for p21 immunohistochemical staining. To study mitosis and DNA synthesis, colchicine and bromodeoxyuridine (BrdU) were injected into mice 3 or 5 h before sacrifice. The results showed that p21 expression, BrdU incorporation, and the mitotic index in uterine luminal epithelium increased 1 to 2 d after E_2 stimulation and then declined to basal levels between d 3 and 6. Furthermore, cotreatment with progesterone (P_4) and E_2 suppressed both p21 expression and the DNA synthesis stimulated by E_2 alone in uterine epithelial cells. Our results show that estrogen stimulates p21 expression and cell proliferation in uterine luminal epithelium and that cotreatment with P_4 prevents both effects, suggesting that p21 may act as a positive cell-cycle regulator.

Key Words: p21; proliferation; uterus; estrogen.

Introduction

The progression of the cell cycle is regulated by cyclins and their associated cyclin-dependent kinases (CDKs) *(1,2)*. Different cyclin/CDK complexes are activated at specific stages of the cell cycle. The activities of the cyclin-CDK complexes are in turn constrained by the CDK inhibitors, which include the INK4 and Cip/Kip families *(1,2)*. p21$^{Waf1/Cip1}$, the first CDK inhibitor identified *(3,4)*, belongs to the Cip/Kip family that includes p21^{Cip1}, p27^{Kip1}, and p57^{Kip2}. The amino-terminal domain of p21 is necessary for the inhibition of cyclin/CDK activity, and its carboxy-terminal domain is associated with proliferating cell nuclear antigen (PCNA), an accessory factor for DNA polymerase δ *(5)*. The binding of p21 with PCNA can inhibit DNA replication without affecting DNA repair *(6)*. In response to DNA damage, p21 expression is induced and the cell cycle is arrested *(7,8)*. Elevated expression of p21 is also found in cells undergoing differentiation *(9)*. However, increased p21 expression is not necessarily linked to growth arrest. Experimental evidence shows that p21 can be induced in response to growth signals, such as serum and growth factors *(10,11)*. The heperproliferation in the epidermis of E6/E7 transgenic mice is accompanied by increased levels of p21, cyclin D1, CDK2, CDK4, and CDK6 *(12)*. The assembly of CDK4 with the D types of cyclin and the activity of cyclin D–associated kinase can be promoted by p21 *(13)*. Therefore, p21 may act not only as a cell-cycle inhibitor, but also as a positive regulator of the cell cycle.

p21 has been detected in human and mouse uterus *(14–17)*. It was found that terminally differentiated endometrial epithelial cells exhibited variable expression of p21, and that endometrial hyperplasias and endometrial adenocarcinomas showed decreased expression of p21 *(14)*. It seems that p21 may play a negative role in regulating uterine cell proliferation. However, there was no clear pattern related to the hormonal regulation of cell proliferation and the expression of p21 in the uterus during the menstrual cycle *(14,15)*. Recently, it has been reported that androgen stimulates cell proliferation and upregulates p21 gene expression in prostatic epithelial cells *(18,19)*, suggesting a positive role for p21 in cell proliferation by sex steroid in its target organ. The administration of estrogen in adult ovariectomized (OVX) mice and rats stimulates cell proliferation specifically in uterine epithelial cells, but the coadministration of progesterone (P_4) suppresses its effect *(20–22)*. In the present study, we used immunostaining to investigate p21 expression and cell proliferation in the uterus under the influences of ovarian steroids. Our results show that estrogen stimulates and P_4 inhibits p21 expression specifically in uterine epithelium and that its expression is well correlated with DNA synthesis and mitosis.

Received January 18, 2002; Revised February 8, 2002; Accepted February 8, 2002.

Author to whom all correspondence and reprint requests should be addressed: Dr. Lih-Yuh C. Wing, Department of Physiology, College of Medicine, National Cheng Kung University, #1 University Road, Tainan, Taiwan 70101. E-mail: wing@mail.ncku.edu.tw

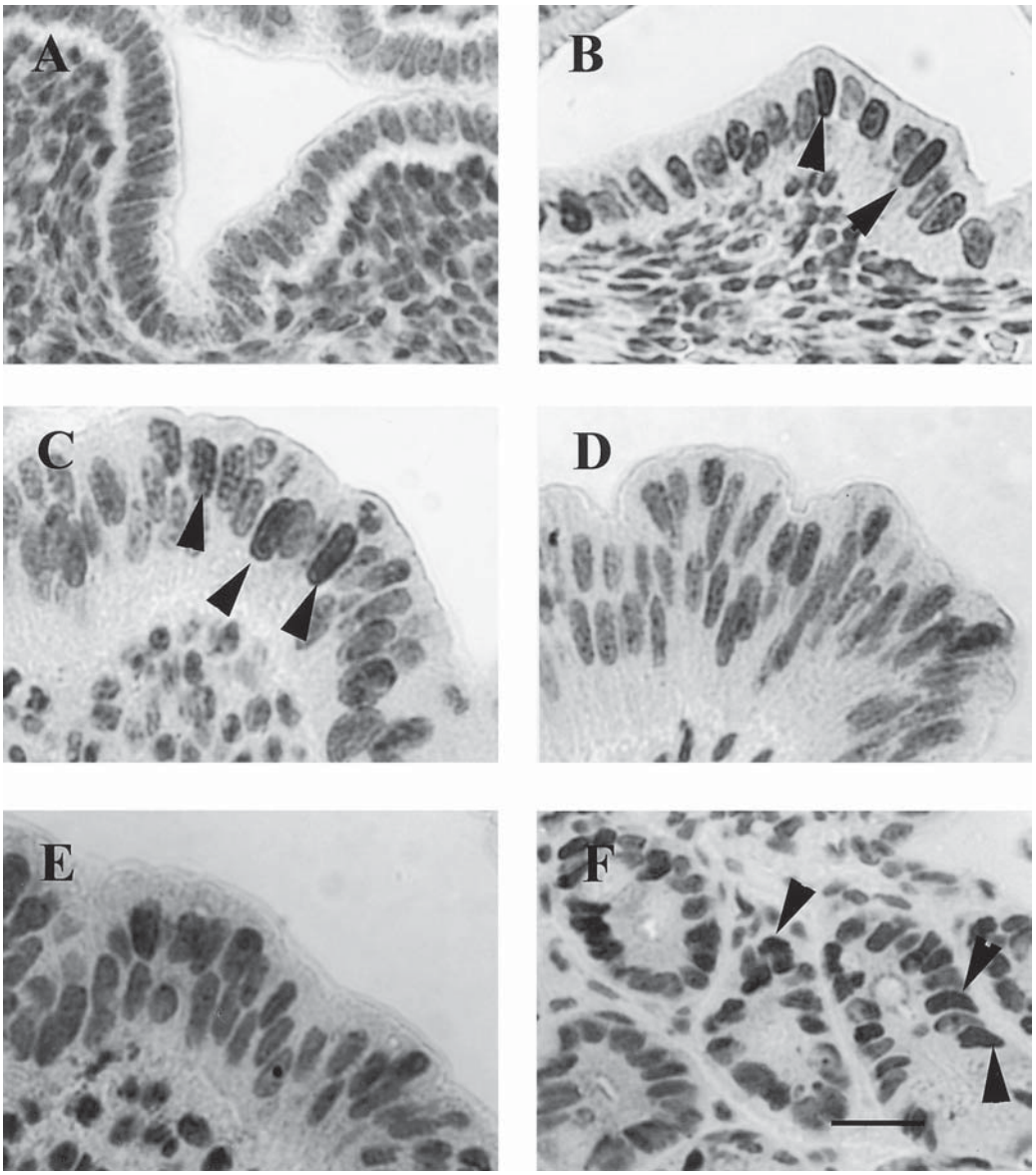

Fig. 1. Immunostaining of p21 in uterine luminal epithelium under estrogen treatment. OVX mice were implanted with E_2 capsules and sacrificed at (**A**) 0, (**B**) 1, (**C**) 2, or (**D**) 3 d after E_2 treatment. (**E**) Negative control: E_2-treated uterine section immunostained with normal rabbit IgG; (**F**) positive control: strong expression of p21 was detected in the epithelial nuclei of the small intestine of the irradiated mice. Bar = 100 μm.

Results

Figure 1 shows the immunostaining of p21 in mouse uterine epithelium after estrogen treatment. No p21 immunoreactivity was detected in OVX mice (Fig. 1A). The estrogen treatment induced a marked increase in the expression of p21 in uterine luminal epithelium 1 to 2 d after the hormonal stimulus (Fig. 1B,C), which decreased after 3–6 d of treatment with 17β-estradiol (E_2) (Fig. 1D). The effects of estrogen appeared to be epithelium specific since similar responses were also observed in the glandular epithelium (data not shown). Because it has been reported that p21 is induced in response to cell injury *(23)*, the small intestine from an X-ray-irradiated mouse was used to confirm the immunostaining of p21 (Fig. 1F).

To examine whether p21 is a positive regulator in estrogen-stimulated uterine epithelium, the changes in DNA synthesis and mitosis in the uterus after estrogen stimulation were investigated. Concomitant with the increased expression of p21, both the bromodeoxyuridine (BrdU) labeling index and the mitotic index in uterine luminal epithelium increased 1 to 2 d after hormonal stimulation. Also consistent with the changes in p21 expression, the BrdU labeling and mitotic indexes decreased with continuous treatment of E_2 for 3–6 d (Fig. 2). We also examined the changes of other cell-cycle regulatory proteins, including G1-phase

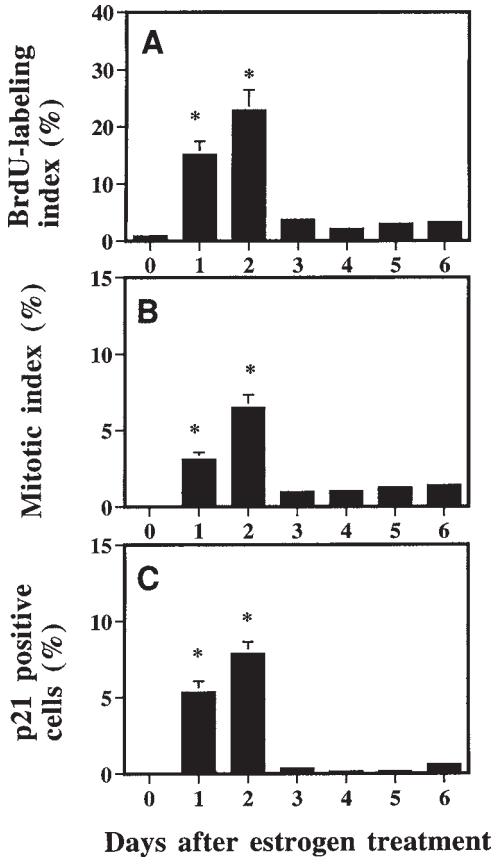

Fig. 2. (**A**) Effects of estrogen on percentage of BrdU-labeling index, (**B**) mitotic index, and (**C**) p21-positive cells in uterine luminal epithelium. OVX mice were implanted with E_2 capsules and sacrificed at 0, 1, 2, 3, 4, 5, and 6 d after hormonal treatment. The BrdU-labeling index, the mitotic index, and the percentage of p21-positive cells were determined as described in Materials and Methods. *$p < 0.05$ compared with d 0.

cyclin D1 and S-phase cyclin A, in the uterus after the stimulus of estrogen. In OVX mice, no cyclin D1 or A could be detected in the nuclei of the uterus (Fig. 3A,C); however, cyclin D1, cyclin A, and p21 were detected in the nuclei of uterine epithelial cells 2 d after estrogen treatment (Fig. 3B, D,F). These results show a perfect temporal correlation between p21 expression and different indices of cell proliferation.

It has been reported that estrogen stimulates and P_4 inhibits uterine epithelial cell proliferation *(20,22)*. We postulated that if p21 acts as a positive regulator of the cell cycle, then cotreatment with P_4 and E_2 should inhibit E_2-stimulated p21 expression. Thus, the expression of p21 was further analyzed in uteri treated with E_2, P_4, or both. The results showed that E_2 stimulated p21 expression and DNA synthesis while P_4 alone had no effect. As expected, the cotreatment suppressed the E_2-induced expression of p21 and DNA synthesis in luminal epithelial cells (Fig. 4).

Discussion

Estrogen has been shown to stimulate cell proliferation, to upregulate the expression of cell-cycle regulatory proteins (including cyclins D1, E, and A), and to increase the activities of related CDK in the uterus *(21,22,24)*. In the present study, we report that, specifically in mouse uterine epithelial cells, estrogen stimulates an expression of p21 that is accompanied by epithelial cell proliferation. Concomitant inhibition in E_2-stimulated p21 expression and cell proliferation was observed in the presence of P_4 or continuous E_2 treatment.

Although immunostaining has demonstrated the expression of p21 in the uterus of diethylstilbestrol (DES)-treated neonatal mice and in uterine stromal cells during decidualization *(16,17)*, Western blotting failed to detect p21 in the estrogen-treated adult uterus of OVX rats and mice *(22,25)*. In the present study, we used immunostaining to demonstrate that there was a cell-type-specific expression of p21 induced by estrogen in the mouse uterus. Estrogen stimulated p21 expression specifically in uterine epithelial cells, whereas P_4 suppressed estrogen-induced p21 expression. Interestingly, the expression of p21 in ovarian steroid-treated uteri correlated well with uterine epithelial cell proliferation. It has been reported *(25)* that estrogen stimulated the expression of the cell-cycle inhibitor $p27^{Kip1}$ in the rat uterus. Immunohistochemical evidence further showed that $p27^{Kip1}$ expression was restricted to the stroma where cell proliferation did not occur; however, the expression of cyclin D1 was predominant in the epithelium *(26)*. This suggests that cyclin D may mediate the mitogenic effect of estrogen in epithelial cells and that $p27^{Kip1}$ may prevent the same effect in the stroma. Our results that a perfect temporal and spatial correlation exists among p21, cyclin D1, and DNA synthesis in the epithelium under E_2 stimulation suggest that p21 may indeed act as a positive regulator of the cell cycle.

It is generally thought that p21 retards G1-phase progression and acts as a cell-cycle inhibitor; however, recent evidence suggests that p21 can also be a positive regulator of the cell cycle *(27)*. Both p21 and p27 have been shown to form complexes with cyclin D–CDK4 in proliferating cells and to increase the kinase activity of these complexes *(13)*. In p21/p27 double-null primary mouse embryonic fibroblasts, the assembly of cyclin D–CDK4 was impaired, but the progression of the cell cycle was not disturbed in these cells *(28)*. It seems, then, that the formation of cyclin D–CDK4-p21/p27 complexes may not be necessary for cell-cycle progression. Muraoka et al. *(29)*, however, reported that the association of cyclin D1 with CDK4 and the nuclear localization of cyclin D1 were impaired and cell proliferation decreased in $p27^{-/-}$ mammary epithelial cells. Weiss et al. *(30)* demonstrated that the transfection of vascular smooth muscle cells with antisense of p21 resulted in inhibition of platelet-derived growth factor–stimulated DNA synthesis and cell proliferation. These data support the role of p21

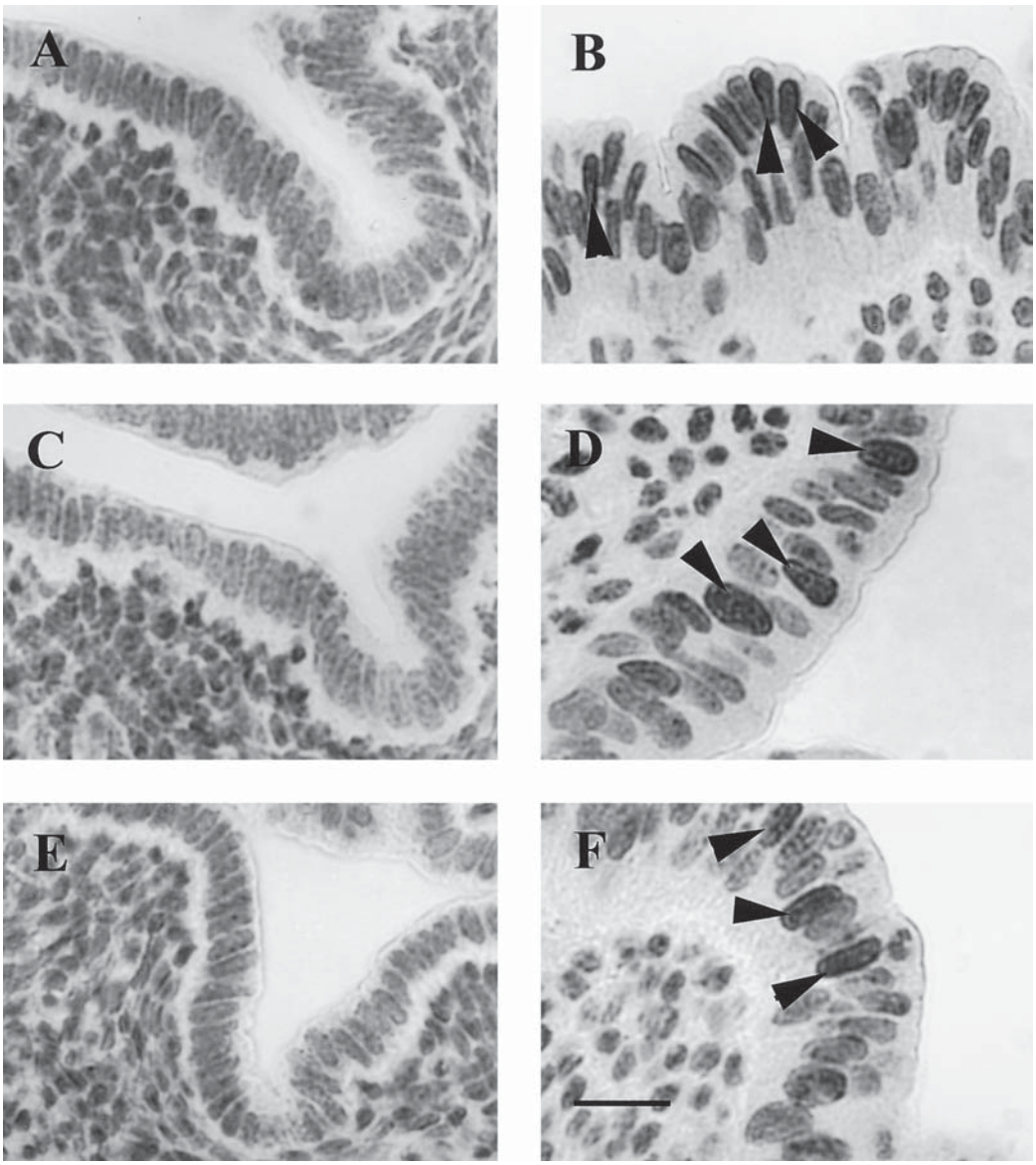

Fig. 3. Changes in expression of p21, cyclin D1, and cyclin A in uterine luminal epithelium after E_2 treatment. OVX mice received the E_2 capsule. After 2 d, animals were sacrificed and their uteri collected for immunostaining of (**A, B**) cyclin D1, (**C, D**) cyclin A, and (**E, F**) p21. (A, C, E) OVX uteri; (B, D, F) 2 d after E_2 treatment. Bar = 100 μm.

and p27 in the activation of cell-cycle progression. In a recent study, p21 and cyclin D3 were found to coexpress in decidualizing uterine stroma cells that exhibit a high percentage of polyploidy *(17)*. The correlation between p21/cyclin D3 expression and increased polyploidy in decidualizing stroma is consistent with the idea that p21 may act as a positive regulator for the entry of cells from G1 to S phase. Identification of the mechanisms that p21 may contribute to the estrogen-stimulated uterine epithelial cell proliferation awaits further investigation.

The p21 gene is regulated by diverse signals that may induce growth arrest, differentiation, and proliferation. p53-dependent regulation of p21 is critical for the response to DNA damage *(31)*. Androgen, a mitogen for prostatic epithelial cells, has been shown to stimulate the p21 gene through an androgen response element *(18)*. Moreover, transcription factor Sp1 has been reported to mediate androgen responsiveness *(32)*, and to be involved in the expression of several estrogen-regulated genes, including cyclin D1 *(33,34)*. The induction of cyclin D by E_2 in ZR75 breast cancer cells involved nuclear ERα/Sp1 interaction *(34)*. Because p21 promoter contains six Sp1-binding sites *(35)*, there is a strong possibility that the interaction of ER and Sp1 may be involved in estrogen-stimulated p21 expression.

In conclusion, our results show that estrogen stimulates the expression of p21, cyclin D1, and cyclin A specifically in mouse uterine epithelial cells. Estrogen-stimulated p21 expression was accompanied by mitosis and DNA synthe-

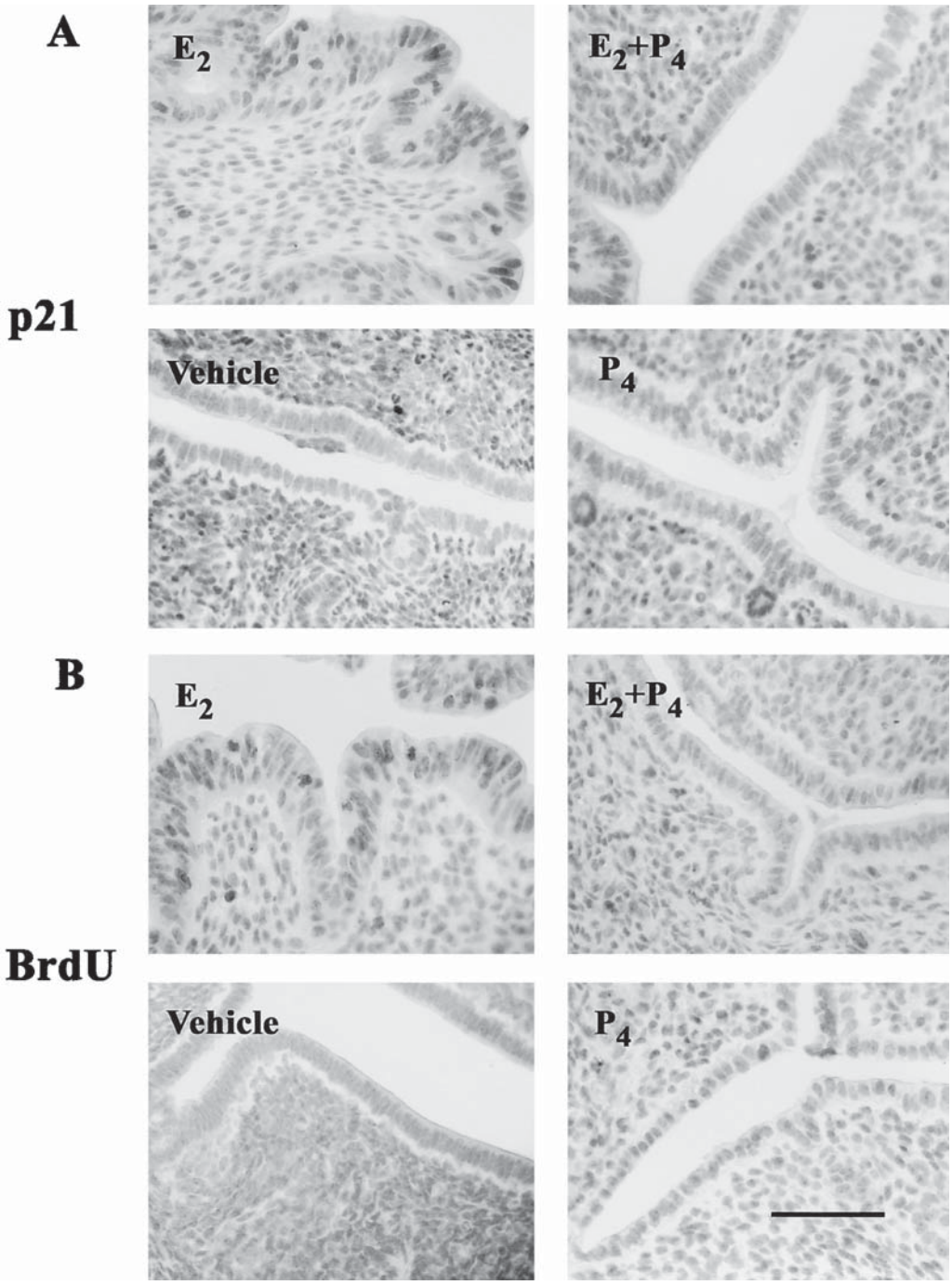

Fig. 4. Immunostaining of p21 and BrdU in response to E_2 and P_4 treatment in mouse uterus. OVX mice received different treatments for 2 d and were injected intraperitoneally with BrdU 5 h before sacrifice. Their uteri were removed for the immunostaining of (**A**) p21 and (**B**) BrdU. Vehicle = with capsules containing oil; E_2 = with capsules containing E_2; P_4 = with injection of P_4, 1 mg/(mouse·d); E_2 + P_4 = with E_2 capsules and injection of P_4. Bar = 100 μm.

sis, while the cotreatment of progesterone inhibited them all. The expression of p21 with cell proliferation in uterine epithelium indicates that p21 may act as a positive regulator for cell-cycle progression in uterine luminal epithelial cells. The mechanisms by which estrogen regulates p21 expression warrant further study.

Materials and Methods

Animals

Female C57BJ/6NCrj (B6) mice 8–10 wk old were obtained from our university's animal center and maintained on standard chow and water ad libitum in animal facilities illumi-

nated between 6:00 AM and 6:00 PM. All procedures were performed in accordance with the animal center's guidelines on the handling and training of laboratory animals. For the study of hormonal control of the uterus, mice were OVX at least 2 wk before being subcutaneously implanted with silastic capsules containing E_2, (25 μg/mL dissolved in sesame oil), prepared according to the procedures described by Cohen and Milligan *(36)*. After different time intervals, animals were sacrificed and their uteri removed. In another set of experiments, OVX mice were divided into four groups: control, E_2 treated, P_4 treated, and E_2/P_4 treated. The control group received capsules containing sesame oil, and the P_4 group was injected daily with progesterone (1 mg/[mouse·d]). The E_2-treated mice were implanted with E_2 capsules and the E_2/P_4-treated group received E_2 capsules and daily injections of progesterone. The treatments of ovarian steroids generated plasma E_2 and P_4 concentrations similar to those in early pregnancy *(37)*.

Measurement of Mitotic and BrdU Labeling Indexes

The mice were treated with BrdU and colchicine according to previously described procedures *(38)*. In brief, the mice were injected intraperitoneally with 5-bromo-2'-deoxyuridine (cell proliferation kit; Amersham, Piscataway, NJ) 5 h before sacrifice, and subcutaneously with colchicine (0.5 mg/100 g of body wt) (Sigma-Aldrich, St. Louis, MO) 3 h before sacrifice. The uterus was removed and fixed in 4% formalin solution and embedded in paraffin. To measure the mitotic index, tissue sections were stained with hematoxylin and eosin, and the cells undergoing mitosis were counted. A minimum of 800 cells per tissue was counted, and the results were expressed as a percentage (mitotic cells/total cells counted).

BrdU Staining

Tissue sections were deparaffinized, rehydrated, and then heated with 10 m*M* citrate buffer (pH 6.0) in a 640-W microwave oven for 10 min. After blocking with 3% H_2O_2 in phosphate-buffered saline (PBS) and washing with PBS, a monoclonal anti-BrdU antibody (Amersham) was added and incubated for 1 h at room temperature. After washing in PBS, sections were treated with peroxidase anti–mouse IgG (Amersham) for 30 min. Color development was achieved by incubation with an aminoethyl cabazole substrate kit (Zymed, South San Francisco, CA). For quantitative analysis, sections were randomly chosen and BrdU-immunoreactive cells were counted at a minimum of 800 cells for each tissue. The results were expressed as a percentage (BrdU-immunoreactive cells/total cells counted).

Immunohistostaining of p21, Cyclin D1, and Cyclin A

Tissue sections 5-μm thick on poly-L-lysine-coated slides were dewaxed through xylene, passed through 100% ethanol, and then incubated with 2% H_2O_2 in methanol. After being passed through a graded series of ethanol to distilled water, they were heated with 10 m*M* citrate buffer (pH 6.0) in a microwave oven for 30 min. After being blocked with 10% goat serum and washed with PBS, primary rabbit polyclonal antibody for p21 (1:100 dilution with 10% goat serum; Oncogene, Cambridge, MA), cyclin D1 (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), or cyclin A (1:200 dilution; Santa Cruz) was added to the slides and incubated at 4ºC overnight. After washing in PBS, tissue sections were incubated for 1 h with goat antirabbit antibody diluted in 5% mouse serum for p21, cyclin D1, or cyclin A immunostaining. After three washings with PBS, slides were incubated with an avidin-biotin complex reagent containing horseradish peroxidase (Vector, Burlingame, CA) in PBS, and color development was achieved by incubation with the aminoethyl cabazole substrate kit. The tissue sections were counterstained with hematoxylin. The quantitative analysis of p21-positve cells was performed as described for BrdU staining.

Statistical Analyses

Values are expressed as the mean ± SEM of at least four animals. The statistical significance of difference among respective groups was evaluated by analysis of variance followed by the Sheffe test.

Acknowledgments

We are grateful to Dr. James W. Wilson, Paterson Institute for Cancer Research, Manchester, England, UK, for help on the p21 immunostaining procedures. This work was supported by grant NSC86-2314-B006-082 from the National Science Council of Taiwan, Republic of China (to LCW).

References

1. Pestell, R. G., Albanese, C., Reutens, A. T., Segall, J. E., Lee, R. J., and Arnold, A. (1999). *Endocr. Rev.* **20,** 501–534.
2. Sherr, C. J. and Roberts, J. M. (1995). *Genes Dev.* **9,** 1149–1163.
3. El-Deiry, W. S., Tokino, T., Velculescu, V. E., Levy, D. B., Parsons, R., Trent, J. M., Lin, D., Mercer, W. E., Kinzler, K. W., and Vogelstein, B. (1993). *Cell* **75,** 817–825.
4. Harper, J. W., Adami, G. R., Wei, N., Keyomarsi, K., and Elledge, S. J. (1993). *Cell* **75,** 805–816.
5. Waga, S., Hannon, G. J., Beach, D., and Stillman, B. (1994). *Nature* **369,** 574–578.
6. Li, R., Waga, S., Hannon, G. J., Beach, D., and Stillman, B. (1994). *Nature* **371,** 534–537.
7. Leonardo, A. D., Linke, S. P., Clarkin, K., and Wahl, G. M. (1994). *Genes Dev.* **8,** 2540–2551.
8. Cayrol, C., Knibiehler, M., and Ducommun, B. (1998). *Oncogene* **16,** 311–320.
9. Steinman, R. A., Hoffman, B., Iro, A., Guillouf, C., Liebermann, D. A., and El-Houseini, M. E. (1994). *Oncogene* **9,** 3389–3396.
10. Michieli, P., Chedid, M., Lin, D., Pierce, J. H., Mercer, W. E., and Givol, D. (1994). *Cancer Res.* **54,** 3391–3395.
11. Mantel, C., Luo, Z., Canfield, J., Braun, S., Deng, C., and Broxmeyer, H. E. (1996). *Blood* **88,** 3710–3719.
12. Crish, J. F., Bone, F., Balasubramanian, S., Zaim, T. M., Wagner, T., Yun, J., Roke, E. A., and Eckert, R. L. (2000). *Carcinogenesis* **21,** 1031–1037.

13. LaBaer, J., Garrett, M. D., Stevenson, L. F., Slingerland, J. M., Sandhu, C., Chou, H. S., Fattaey, A., and Harlow, E. (1997). *Genes Dev.* **11,** 847–862.
14. Palazzo, J. P., Mercer, W. E., Kovatich, A. L., and McHugh, M. (1997). *Hum. Pathol.* **28,** 60–66.
15. Toki, T., Mori, A., Shimizu, M., Nikaido, T., and Fujii, S. (1998). *Mol. Hum. Reprod.* **4,** 1157–1164.
16. Yoshida, A., Newbold, R. R., and Dixon, D. (2000). *Toxicol. Pathol.* **28,** 237–245.
17. Tan, J., Raja, S., Davis, M. K., Tawfik, O., Dey, S. K., and Das, S. K. (2002). *Mech. Dev.* **111,** 99–113.
18. Lu, S., Liu, M., Epner, D. E., Tsai, S. Y., and Tsai, M. J. (1999). *Mol. Endocrinol.* **13,** 376–384.
19. Waltregny, D., Leav, I., Signoretti, S., Soung, P., Lin, D., Merk, F., Adams, J. Y., Bhattacharya, N., Cirenei, N., and Loda, M. (2001). *Mol. Endocrinol.* **15,** 765–782.
20. Martin, L., Pollard, J. W., and Fagg, B. (1976). *J. Endocr.* **69,** 103–115.
21. Quarmby, V. E. and Korach, K. S. (1984). *Endocrinology* **114,** 694–702.
22. Tong, W. and Pollard, J. W. (1999). *Mol. Cell. Biol.* **19,** 2251–2264.
23. Wilson, J. W., Pritchard, D. M., Hickman, J. A., and Potten, C. S. (1998). *Am. J. Pathol.* **153,** 899–909.
24. Geum, D., Sun, W., Paik, S. K., Lee, C. C., and Kim, K. (1997). *Mol. Reprod. Dev.* **46,** 450–458.
25. Altucci, L., Addeo, R., Cicatiello, L., Germano, P., Pacilio, C., Battista, T., Cancemi, M., Petrizzi, V. B., Bresciani, F., and Weisz, A. (1997). *Endocrinology* **138,** 978–984.
26. Zhuang, Y., Sarca, D., Weisz, A., Altucci, L., Cicatiello, L., Rollerova, E., Tuohimaa, P., and Ylikomi, T. (2001). *J. Steroid Biochem. Mol. Biol.* **78,** 193–199.
27. Sheer, C. J. and Roberts, J. M. (1999). *Genes Dev.* **13,** 1501–1512.
28. Cheng, M., Olivier, P., Diehl, J. A., Fero, M., Roussel, M. F., Roberts, J. M., and Sherr, C. J. (1999). *EMBO J.* **18,** 1571–1583.
29. Muraoka, R. S., Lenferink, A. E. G., Simpson, J., Brantley, D. M., Roebuck, L. R., Yakes, F. M., and Arteaga, C. L. (2001). *J. Cell Biol.* **153,** 917–931.
30. Weiss, R. H., Joo, A., and Randour, C. (2000). *J. Biol. Chem.* **275,** 10,285–10,290.
31. Macleod, K., Sherry, N., Hannon, G., Beach, D., Tokino, T., Kinzler, K., Vogelstein, B., and Jacks, T. (1995). *Genes Dev.* **9,** 935–944.
32. Lu, S., Jenster, G., and Epner, D. E. (2000). *Mol. Endocrinol.* **14,** 753–760.
33. Scholz, A., Truss, M., and Beato, M. (1998). *J. Biol. Chem.* **273,** 4360–4366.
34. Castro-Rivera, E., Samudio, I., and Safe, S. (2001). *J. Biol. Chem.* **276**, 30,853–30,861.
35. Gartel, A. L. and Tyner, A. L. (1999). *Exp. Cell Res.* **246**, 280–289.
36. Cohen, P. E. and Milligan, S. R. (1993). *J. Reprod. Fertil.* **99,** 219–223.
37. McCormack, J. T. and Greenwald, G. S. (1974). *J. Endocr.* **62,** 101–107.
38. Shinohara, Y., Matsumoto, H., and Mori, T. (1997). *In Vivo* **11,** 249–252.