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Progesterone Inhibits the Estrogen-Induced Phosphoinositide 3-Kinase→AKT→GSK-3β→Cyclin D1→pRB Pathway to Block Uterine Epithelial Cell Proliferation

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The mammalian cell cycle is regulated by the cyclin/cyclin-dependent kinase (CDK) phosphorylation of the retinoblastoma (pRB) family of proteins. Cyclin D1 with its CDK4/6 partners initiates the cell cycle and acts as the link between extracellular signals and the cell cycle machinery. Estradiol-17β (E_2) stimulates uterine epithelial cell proliferation, a process that is completely inhibited by pretreatment with progesterone (P_4). Previously, we identified cyclin D1 localization as a key point of regulation in these cells with E_2 causing its nuclear accumulation and P_4 retaining it in the cytoplasm with the resultant inhibition of pRB phosphorylation. Here we show that E_2 stimulates phosphoinositide 3-kinase to activate phosphokinase B/AKT to effect an inhibitory phosphorylation of glycogen synthase kinase (GSK-3β). This pathway is suppressed by P_4. Inhibition of the GSK-3β activity in P_4-treated uteri by the specific inhibitor, LiCl, reversed the nuclear accumulation of cyclin D1 and in doing so, caused pRB phosphorylation and the induction of downstream genes, proliferating cell nuclear antigen and Ki67. Conversely, inhibition of phosphoinositide 3 kinase by LY294002 or Wortmanin reversed the E_2-induced GSK-3β Ser^9 inhibitory phosphorylation and blocked nuclear accumulation of cyclin D1. These data show the reciprocal actions of E_2 and P_4 on the phosphoinositide 3-kinase through to the GSK-3β pathway that in turn regulates cyclin D1 localization and cell cycle progression. These data reveal a novel signaling pathway that links E_2 and P_4 action to growth factor-mediated signaling in the uterus. (*Molecular Endocrinology* 19: 1978–1990, 2005)

EXPOSURE TO ESTROGENS is the main risk factor for breast and endometrial cancers (1). Estrogens induce uterine epithelial cell proliferation, and this effect is thought to be the cause of this increased cancer risk upon prolonged exposure to this hormone. Thus, exposure of postmenopausal women to the selective estrogen response modulator, tamoxifen, that is an estrogen agonist in the uterus (2), increases the risk of endometrial cancer (3). In normal physiology, estradiol-17β (E_2) synthesized at every estrus or menstrual cycle causes uterine epithelial cells to undergo cell proliferation (4). Progesterone (P_4) completely inhibits this E_2-induced cell proliferation. Both of these hormones act through their nuclear transcription factor receptors because inhibition of their action either by inhibitors or by gene deletion results in the failure of the hormone to regulate cell proliferation. Thus, the estrogen receptor (ER)-negative uteri are hypoplastic, and the progesterone receptor-negative ones are hyperplastic in response to E_2 (5–7). Therefore, therapeutically, P_4 is used to treat estrogen-dependent endometrial cancers and as a prophylactic to antagonize estrogen action in hormone replacement therapy (8, 9).

The cellular dynamic in the uterus during the estrus cycle and early pregnancy can be precisely mimicked in ovariectomized mice by exogenous sex steroid hormone treatment (10–13). In this system, E_2 treatment of ovariectomized mice results in cell proliferation that is restricted to the luminal and glandular epithelium. DNA synthesis peaks with approximately 80% of the cells in S-phase at 12–15 h after treatment. P_4 pretreatment completely suppresses this induction of cell proliferation and also reduces the basal rate of proliferation to zero but sensitizes uterine stromal cells to respond to E_2 with a wave of cell proliferation following a time course similar to those observed in the epithelium (4, 11, 14).

The mammalian cell cycle is regulated by the sequential action of cyclins acting with their cyclin-de-

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Abbreviations: BrdU, Bromodeoxyuridine; CDK, cyclin-dependent kinase; DTT, dithiothreitol; E_2, estradiol-17β; ER, estrogen receptor; GSK, glycogen synthase kinase; Mcm, minichromosome maintenance; P_4, progesterone; PCNA, proliferating cell nuclear antigen; PH, pleckstrin homology; PI3 kinase, phosphoinositide 3 kinase; PIP3, phosphatidyl inositol-3,4,5-triphosphate; pRB, phosphorylation of the retinoblastoma.

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pendent kinase partners (CDKs) (15). Their primary action is to phosphorylate members of the retinoblastoma (pRB) family of proteins. Phosphorylation of pRB results in release of E2F transcription factors from an inhibitory pRB complex and activation of genes required for cell cycle progression (16). The first acting cyclins belong to the D family, and these together with their catalytic partners CDK4/6 act as intracellular sensors of extracellular signals (17). In the canonical pathway, their phosphorylation of pRB is required for further phosphorylation by CDK2 acting together with cyclin E initially and then cyclin A, as cells progress through the cell cycle. The action of cyclins and their associated kinases are inhibited by cyclin-dependent kinase inhibitors (CKI) that fall into two families, the Ink4 and Cip/Kip families (18). Cyclin D-CDK4/6 complexes are subjected to negative regulation by both the Ink4 and the Cip/Kip families of inhibitors, whereas cyclin E/Cdk2 complexes are negatively controlled only by the Cip/Kip family such as $p21^{Cip/Waf1}$, $p27^{Kip1}$, and $p57^{Kip2}$ (19).

It is generally accepted that, in cultured cells, cyclin D1 concentration is elevated in response to growth factors, and this initiates the cell cycle both by activating CDK4/6 and by titering away the cell cycle inhibitor $p27^{kip1}$ from cyclin E/CDK2 complexes. However, in contrast to this and also that observed in hormone-dependent breast cancer cells in culture (20), in the uterine epithelial cells *in vivo* E_2 does not dramatically change the cellular concentration of cyclin D1 but instead causes the relocation of cyclin D1-CDK4 from the cytoplasm to the nucleus (21). This gives the active complex access to its pRB substrate with its resultant phosphorylation. Thereafter, cyclin E is elevated, and this together with the activation of CDK2 results in further pRB phosphorylation and a dramatic up-regulation of cyclin A, coincident with progression of the epithelial cells into the S phase of the cell cycle. The E_2-induced nuclear accumulation of cyclin D1 is completely suppressed by P_4 treatment (21). This prevents E_2-induced pRB phosphorylation and consequently blocks the downstream induction of CDK2 and completely inhibits cell cycle progression. These data suggest that the cellular localization of cyclin D1 is a central point of regulation for sex steroid hormones in uterine epithelial cells.

The molecular basis for this nuclear accumulation of cyclin D1 could be either increased transport to or enhanced egress from the nucleus. Diehl and coworkers (22, 23) have shown that glycogen synthase kinase-3β (GSK-3β), a key regulatory kinase in numerous signaling pathways, can phosphorylate cyclin D1 specifically on Thr^{286}, which increases its rate of nuclear export relative to nuclear import by facilitating the association of cyclin D1 with the nuclear exportin chromosome region maintenance (CRM) 1. In resting cells, GSK-3β is constitutively active and its activity is negatively regulated primarily through Ser^9 phosphorylation (24–26) and further enhanced by Tyr^{216} phosphorylation. In this study, we analyzed the mechanism of the sex steroid hormone regulation of cyclin D1 nuclear localization and showed that E_2 and P_4 exert opposite actions of GSK-3β activity by regulating the activity of phosphokinase B/AKT through a phosphoinositide 3 kinase (PI3 kinase) mediated pathway. This provides a novel insight into E_2 action that might be exploited therapeutically in E_2-dependent cancers.

RESULTS

Progesterone Antagonizes Estrogen's Inhibition of GSK-3β Activity to Exclude Cyclin D1 from the Nucleus

Our studies have suggest that the cellular localization of cyclin D1 is a critical regulation point in the uterine epithelial cell cycle progression because E_2 induces nuclear accumulation of cyclin D1 in early G1 phase, which is completely blocked by P_4 treatment (21). GSK-3β has been shown to be involved in the regulation of the subcellular localization of cyclin D1 in cultured cells (22, 23, 27). To explore whether female sex steroid hormones might regulate cyclin D1 localization through modifying GSK-3β activity, we examined the phosphorylation status of GSK-3β using an antiphospho-Ser^9-specific antibody in uterine epithelial cells following the different hormone treatments (Fig. 1). The total level of GSK-3β in uterine epithelial cells remained constant under all the hormone treatments compared with that in the untreated control mice. In control and P_4-treated mice, the extent of GSK-3β-Ser^9 phosphorylation was at or below the level of detection. In contrast, E_2 treatment induced a sharp increase of GSK-3β phosphorylation at Ser^9 that was first detected 2 h after treatment and peaked at approximately 8 h with a progressive loss thereafter until 16 h. Pretreatment with P_4 largely suppressed the E_2-induced GSK-3β phosphorylation at Ser^9 but not completely because there was still some weak signal at 4 h and 8 h after this treatment compared with the P_4 alone group. In addition to this inhibitory Ser^9 phosphorylation, GSK-3β is also subjected to the phosphorylation at Tyr^{216} that stimulates its activity (28). Using an anti-GSK-3β-Tyr^{216}-specific antibody, we determined whether E_2 and P_4 regulated GSK-3β activity through this Tyr^{216} phosphorylation (Fig. 1). Similar to previous studies (28, 29), GSK-3β was constitutively phosphorylated even in epithelial extracts of untreated control mice and the different hormone treatments did not change the phosphorylation level of GSK-3β at Tyr^{216}.

This result suggests that sex steroid hormones regulate GSK-3β solely through Ser^9 phosphorylation and that E_2 and P_4 act antagonistically through the regulation of GSK-3β activity that in turn determines cyclin D1 nuclear localization, and subsequently pRB phosphorylation and epithelial cell progression. To test this hypothesis, we used LiCl to determine the role of this kinase in the regulation of cyclin D1 cellular distribu-

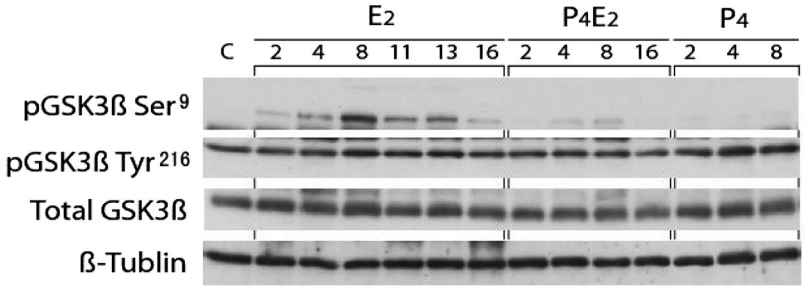

Fig. 1. P_4 Inhibits the E_2-Induced Phosphorylation of GSK-3β

Purified uterine epithelial cell extracts from ovariectomized mice killed at the times indicated in hours after treatment with either vehicle alone (C) or treated with E_2, P_4, or P_4E_2 as described in *Materials and Methods*, were separated by SDS-gel electrophoresis and blotted onto nylon membranes. These were probed as indicated with an antibody against total GSK-3β or against the Tyr^{216} or Ser^9-phosphorylated forms of GSK-3β. Detection of β-tubulin with an anti-β-tubulin antibody was used as a loading control.

tion. LiCl is a chemical inhibitor of GSK-3β that has been demonstrated to specifically inhibit GSK-3β function with a IC_{50} of 2 mM by competition for Mg^{2+} ion but not for ATP or substrate binding (30, 31). LiCl is relatively impermeable to cells and therefore to ensure effective inhibition of GSK-3β concentrations of 30–100 mM are commonly used in tissue culture (32, 33). To expose uterine epithelial cells to this inhibitor, 50 μl of LiCl was injected directly in a pluronic gel over this range of concentrations (20–100 mM) into the uterine lumen of the mouse under anesthesia 2 h before E_2 or P_4E_2 treatment. Titration of the responses described below showed that 50 mM resulted in the maximal effect, a result that was also found in cultured cells by others (32). Seven hours after the E_2 treatment and 9 h after LiCl, the localization of cyclin D1 was determined with an anticyclin D1 antibody either by immunohistochemistry of transverse uterine sections or by Western blotting of nuclear extracts with the detection of lamin A/C as a loading control. Our studies (21) have shown that cyclin D1 stays predominantly in the cytoplasm of the uterine epithelial cells of control mice but enters into the nuclei after E_2 treatment. This was confirmed by the present studies using immunohistochemistry (Fig. 2, A1 and A2) and Western blotting (Fig. 2B) that showed a significant accumulation in the nucleus after E_2 treatment. In contrast, P_4 pretreatment completely inhibited this E_2-induced localization of cyclin D1 in the nucleus (Fig. 2, A5 and B) as we also described previously (21). However, intraluminal injection of LiCl reversed the P_4-induced block of nuclear localization of cyclin D1 and instead, after this treatment it now became localized in the nucleus (Fig. 2, A6 and B). The extent of the effects was somewhat variable from mouse to mouse but consistently involved 30–90% of luminal epithelial cells. Consistent with this, in control hormone untreated mice where GSK-3β would be constitutively active in the epithelial cells (no Ser^9 phosphorylation), LiCl treatment caused an enhancement of nuclear cyclin D1 accumulation with approximately 30% of cells being positive compared with approximately 3% in untreated mice (Fig. 2, A1 and A3). In contrast, the E_2-induced nuclear accumulation was unaffected by intrauterine injection of LiCl (Fig. 2, A2, A4, and B), a result that would have been expected because of GSK-3β's inhibited state after this hormone treatment. Control injections of an equal volume of PBS in any hormone treatment did not alter cyclin D1 nuclear accumulation (Fig. 2B), thus ruling out the possibility that the nuclear accumulation of cyclin D1 by LiCl in P_4E_2-treated uteri was due to the side effect of the increased intraluminal hydraulic pressure after injection. Furthermore, we have shown that LiCl does not change expression or cellular localization of the progesterone receptor (data not shown) indicating that the P_4 function is unaffected at this level.

In cultured fibroblasts, the GSK-3β phosphorylation of cyclin D1 results not only in nuclear egress, but also in degradation of cyclin D1 protein (22). In contrast, in these epithelial cells the concentration of total cellular cyclin D1 was unaffected by either of the hormone treatments as has been described before (21) or by LiCl injection (Fig. 2B). This may allow the rapid recovery of proliferative state that follows removal of P_4, which *in vivo* would permit those mice that did not become pregnant to rapidly move into estrogen-driven estrus that is accompanied by uterine epithelial cell proliferation.

Inhibition of GSK-3β Results in a Reversal of Progesterone's Inhibition of pRB Phosphorylation

Phosphorylation of pRB proteins is the central event during the G1-S phase transition (16). In our earlier research, we showed that E_2-induced pRB phosphorylation was inhibited by pretreatment with P_4 (21). Because LiCl can trigger cyclin D1 nuclear accumulation in the P_4E_2-treated mouse uterine epithelial cells, we tested whether pRB phosphorylation was changed in response to this alteration in localization of cyclin D1 in the luminal epithelial extracts 7 h after different hormone treatments in the presence or absence of 50 mM LiCl (Fig. 3). E_2 induced an enhanced phosphory-

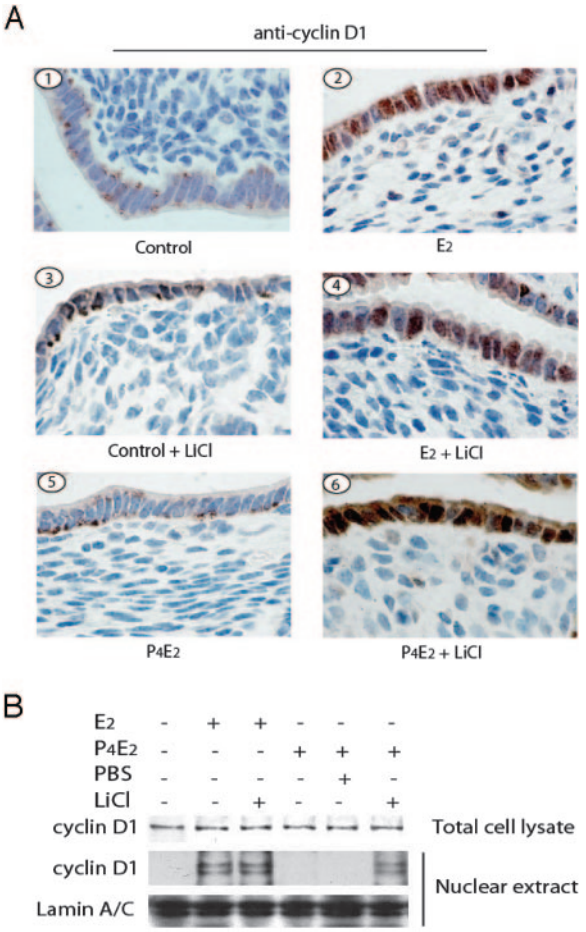

Fig. 2. The P_4-Induced Nuclear Exclusion of Cyclin D1 Is Reversed by Inhibition of GSK-3β

A, 1–6, Immunohistochemistry with anticyclin D1 antibody of transverse sections of uteri isolated from CD1 mice 7 h after the various hormonal and LiCl treatments *in vivo* as indicated. The *brown color* indicates positive staining and the columnar cells are the luminal epithelium (×100). B, Western blots of total cellular and nuclear extracts of uterine epithelial cells from CD1 mice treated as indicated across the *top* and probed with antibodies listed on the *side*. Three panels show sequentially total cellular cyclin D1 concentration, cyclin D1 content in the nuclear extract and lamin A/C as its control for protein loading of this extract.

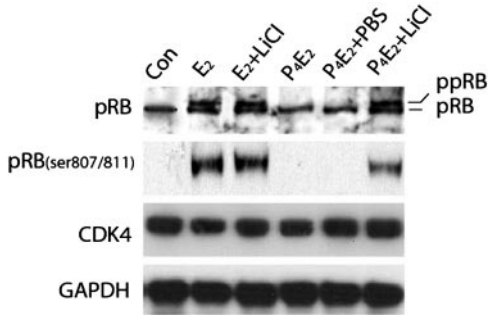

Fig. 3. Inhibition of GSK-3β Reverses the P_4 Inhibition of pRB Phosphorylation

Western blots of total uterine epithelial cellular extracts from mice treated as indicated on *top*. Blots were probed with antibodies from *top* to *bottom*, to pRB, to the phos-Ser $^{807/811}$ epitope of pRB and to CDK4 as indicated and to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) as a loading control. ppRB, Hyperphosphorylated form of pRB.

lation of pRB, shown by the slower migrating band that was unaffected by the intraluminal addition of LiCl. In extracts from P_4E_2-treated mice, only the hypo-phosphorylated form of pRB could be detected. However, treatment with LiCl significantly elevated the phosphorylation of pRB to a level comparable with that observed after E_2 treatment in these P_4E_2-treated mice (Fig. 3).

Phosphorylation of pRB can be effected by several CDKs acting with their coordinate cyclin partners (34, 35). Thus, to confirm that cyclin D1 contributed to this pRB phosphorylation, we used a phosphospecific antibody that specifically recognized pRB proteins when phosphorylated at Ser$^{807/811}$, the site whose phosphorylation is catalyzed by cyclinD1-CDK4/6 kinases (36). E_2 enhanced phosphorylation at these sites on pRB compared with control mice, whereas essentially no phosphorylation of pRB could be detected in mice treated with P_4E_2 (Fig. 3). However, intraluminal administration of LiCl substantially increased the phosphorylation of pRB at Ser$^{807/811}$ in the extracts from P_4E_2-treated mice but did not inhibit the E_2-induced level (Fig. 3, *middle panel*). We have previously shown that the concentration of the cyclin D1 partner CDK4 was unaltered in response to P_4E_2 treatment when compared with E_2-treated epithelial cells (21). To determine whether LiCl inhibition of GSK-3β changed the levels of CDK4, we performed Western blots of uterine epithelial cells extracts isolated 7 h after hormone treatment using appropriate antibodies. These data not only confirmed our previous observations (21) but also showed that LiCl did not significantly affect the concentrations of this protein (Fig. 3, *bottom panel*). These data suggest that the LiCl inhibition of GSK-3β in P_4-treated mice caused cyclin D1 to be relocalized to the nucleus by E_2, thereby gaining access to its major substrate pRB with the resultant phosphorylation of this molecule at CDK4-specific sites.

Inhibition of GSK-3β Reverses the Progesterone-Mediated Block of the pRB Pathway and Propels Cells toward S-Phase

The induction of pRB phosphorylation by inhibition of GSK-3β in P_4E_2-treated mice suggests that this would activate a set of genes in this pathway that are necessary for S phase entry, such as cyclin A expression and proliferating cell nuclear antigen (PCNA) nuclear localization, and drive the cells through G1 into S phase. Therefore, we analyzed the expression of these genes by either Western blotting or immunohistochemistry. Consistent with the effects of the steroid hormones on cell proliferation and as demonstrated

before, cyclin A was strongly induced in the epithelial cells by E_2, and this induction was inhibited by P_4 treatment (21) (Fig. 4A). LiCl treatment of P_4E_2-treated mice, however, resulted in an induction of cyclin A at 3 h after treatment with a peak at 15 h in a manner similar to that observed in the uterine epithelial cells of mice given E_2 alone (Fig. 4A). This suggests that the simple relocalization of cyclin D1 is sufficient to reverse the cell cycle inhibition by P_4. Thus, we analyzed DNA synthesis in the uterus by three indices, nuclear PCNA and Ki67 staining (37) as well as a true measure of DNA synthesis, incorporation of bromodeoxyuridine (BrdU) into DNA. In the untreated ovariectomized mice, there was a low level of nuclear PCNA and Ki67 immunostaining in the luminal and glandular epithelium (Fig. 4, B1 and C1). Fifteen hours after E_2 treatment, the majority of the luminal epithelial cells became PCNA and Ki67 positive consistent with the induction of cell division by this hormone (14, 21) (Fig. 4, B2 and C2). This E_2-induced induction in the epithelium was completely abolished by P_4 pretreatment as well as the basal level of cell proliferation, but it stimulated expression in stromal cells as described before (21) (Fig. 4, B3 and C3). However, treatment of mice exposed to the P_4E_2 hormonal regimen with an intraluminal injection of LiCl induced PCNA accumulation and the number of Ki67-positive cells in the luminal and glandular epithelium (Fig. 4, B4 and C4). Therefore, the LiCl inhibition of GSK-3β activity is sufficient to overcome the P_4-induced block in the expression of the surrogate markers of DNA synthesis, Ki67, and PCNA in response to E_2.

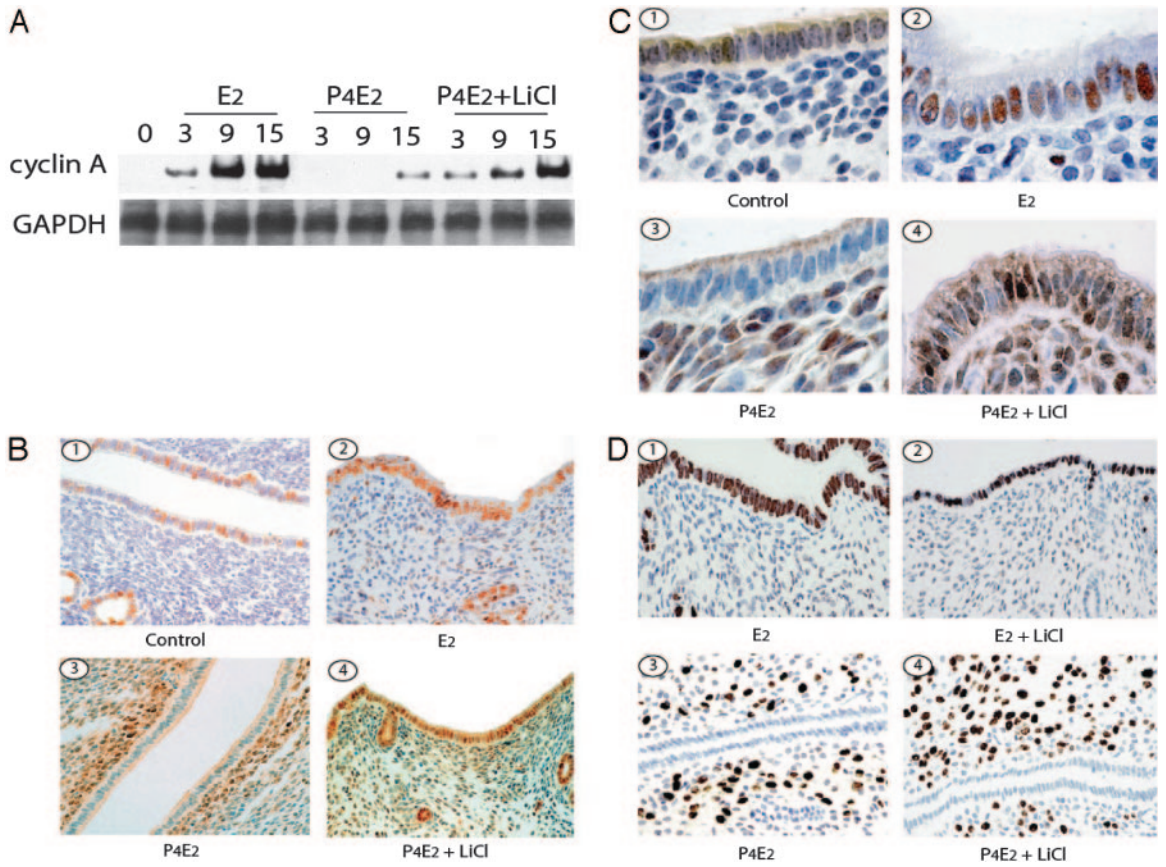

Fig. 4. Inhibition of GSK-3β Reverses the P_4 Block of Cyclin A, PCNA, and Ki67 Expression But Not of Uterine Epithelial Cell DNA Synthesis

A, Western blot of uterine epithelial cell extracts isolated at 0, 3, 9, and 15 h after the treatments indicated and probed with an anticyclin A antibody. Detection of GAPDH (glyceraldehyde-3-phosphate dehydrogenase) was used as a protein loading control. B, Immunostaining for PCNA on uterine transverse sections isolated 15 h after the treatments shown. P_4E_2 (B3) blocks the E_2-induced nuclear PCNA staining (B2) in the uterine epithelium and this is reversed in the P_4E_2-treated uterine epithelium by inhibition of GSK-3β with LiCl (B4) ($\times$40). C, Similar sections to B but immunostained for Ki67. As in panel B, these results show that inhibition of GSK-3β activity reverses the P_4-block of E_2-induced Ki67 expression ($\times$100). D, Immunohistochemistry of transverse uterine sections isolated 15 h after mice were given the treatments shown and 2 h after an ip injection of BrdU. Sections were stained with anti-BrdU antibody whose *brown* immunostaining indicates cells undergoing DNA synthesis. E_2 stimulates epithelial cell proliferation (D1) and this is not blocked by LiCl (D2). P_4 permits E_2 induction of stromal cell proliferation and blocks epithelial cell proliferation (D3) but neither the luminal epithelial proliferation block nor the stromal proliferation is affected by treatment intraluminally with LiCl (D4) ($\times$40).

To confirm whether DNA synthesis was indeed activated by LiCl in the P_4E_2-treated uterine epithelium, we injected BrdU ip into the variously hormonal-treated mice, and incorporation of DNA was determined by immunohistochemistry using an anti-BrdU antibody. As described before (21) E_2 stimulated essentially all uterine epithelial cells to incorporate BrdU (Fig. 4D1), and this induction of DNA synthesis was completely inhibited by P_4 even though P_4 permitted E_2-induced DNA synthesis in the uterine stroma (Fig. 4D3). The E_2 induction was unaffected by LiCl treatment (Fig. 4D2). However, unlike the surrogate markers for DNA synthesis, PCNA and Ki67 described above, intraluminal injection of LiCl was unable to reverse the P_4 inhibition of epithelial cell DNA synthesis (Fig. 4D3). Stromal BrdU incorporation was also unaffected in these P_4E_2 and LiCl-treated mice (Fig. 4D2). This acted as another positive control showing that LiCl does not indiscriminately inhibit the incorporation of BrdU into DNA. Thus, although LiCl treatment was able to drive the cell cycle engine and activate elements of the pRB pathway in P_4E_2-treated uteri leading to expression of proliferation-associated markers, PCNA and Ki67, it was unable to fully commit cells to enter into DNA synthesis.

P_4 Attenuates the E_2-Induced PI3 Kinase/AKT Pathway, Thereby Affecting Downstream GSK-3β Activity and Cyclin D1 Distribution

GSK-3β is a well-documented downstream target of the PI3 kinase/AKT pathway that regulates various cellular processes, such as proliferation, cell growth, apoptosis, and cytoskeletal rearrangement (26). The generation of phosphatidyl inositol-3,4,5-triphosphate (PIP3) by PI3 kinase on the plasma membrane, after PI3 kinase activation by the receptor tyrosine kinases, recruits AKT, a serine/threonine kinase, by direct interaction with its pleckstrin homology (PH) domain. AKT is phosphorylated on Thr^{308} by another PH domain-containing serine/threonine kinase PDK1 at the membrane (26). However for maximal activation, AKT needs additional phosphorylation at Ser^{473} by PDK2 (38). Using antiphospho-specific antibodies directed against these sites on AKT, we explored the regulation of AKT by E_2 and P_4. Under all hormone treatment regimens, AKT protein appeared to be constitutively expressed (Fig. 5A). Furthermore, in either the control untreated or P_4 alone-treated mice, the phosphorylation of AKT was at, or below, the level of detection. However, E_2 caused a marked elevation of phosphorylation at both Ser^{473} and Thr^{308} that was detected within 2 h of treatment and reaching a maximum at 8 h before declining toward control levels by 16 h (Fig. 5A). P_4 pretreatment significantly attenuated this E_2-induced phosphorylation at both Ser^{473} and Thr^{308}, although there was still a modest elevation that also reached a maximum at 4–8 h after E_2 treatment (Fig. 5A). This declined to undetectable levels by 16 h (Fig. 5A). Thus, E_2 and P_4 regulate AKT activation in a

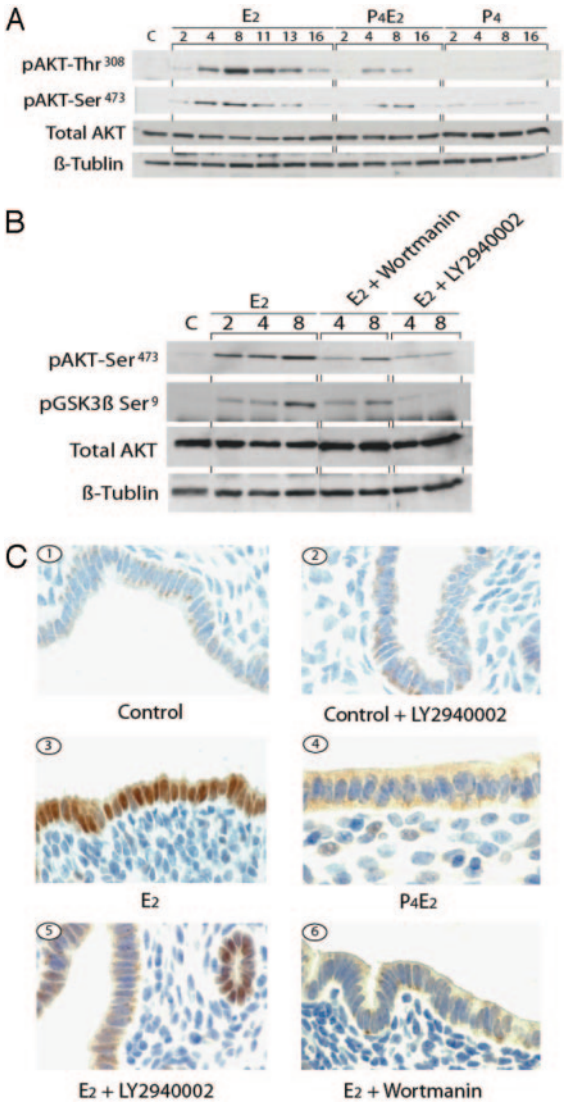

Fig. 5. P_4 Inhibits the E_2 Activation of AKT that Is Required for the Nuclear Accumulation of Cyclin D1

A, Western blots of uterine epithelial cell lysates from mice treated with hormonal regimens as described in Fig. 1 probed with antibodies against the specific phosphorylated epitopes on Thr^{308} and Ser^{473} of AKT as indicated. Total cellular AKT was also determined with an anti-AKT antibody and β-tubulin was used as a protein loading control as described in Fig. 1. B, Western blots of epithelial cell lysates from mice killed at the times indicated after treatment with E_2 or E_2 and the PI3 kinase inhibitors, wortmanin and LY294002, given intraluminally immediately before the E_2. Blots were probed with antibodies to the phospho-Ser^{473} and Phospho-Ser^{9}-specific epitopes on AKT and GSK-3β, respectively. Total AKT and β-tubulin were determined as described in A. These blots show that the PI3 Kinase inhibitors reduce the activity of PI3 kinase in the luminal epithelial cells as assessed by the extent of phosphorylation of the downstream targets AKT and its target GSK-3β. C, Immunohistochemistry using anticyclin D1 antibodies on transverse uterine sections from mice killed 4 h after the treatments shown. LY294002 had no effect on the cyclin D1 localization in control uteri (C1 and C2). However, both wortmanin and LY294002 (C5 and C6) inhibit the E_2-induced (C3) nuclear localization of cyclin D1 in the luminal epithelial cells to produce a state reminiscent of the P_4E_2-treated uteri (C4) ($\times$100)

similar pattern to the GSK-3β Ser^9 phosphorylation in the uterine epithelium.

Given the data presented above that showed that the activity of GSK-3β regulates cyclin D1 localization, we further tested whether this was through a PI3 kinase-mediated mechanism by blocking the activity of this enzyme in the luminal epithelial cells by uterine intraluminal injection of the PI3 kinase-specific inhibitors, Wortmanin and LY294002, followed by determination of the nuclear localization of cyclin D1. Before examining the effects on cyclin D1 distribution, we first analyzed their efficacy by the extent of phosphorylation of the downstream substrate AKT at Ser^{473} using phosphospecific antibodies and Western blotting. Both Wortmanin and LY294002, although not altering the cellular concentration of AKT, significantly inhibited the E_2-induced phosphorylation of AKT at Ser^{473} with LY294002 being the more effective drug (Fig. 5B). This relative efficacy is consistent with the increased stability of LY294002 *in vivo*. This inhibition of PI3 kinase also resulted in a significant inhibition of GSK-3β phosphorylation at Ser^9, showing that this is downstream of the PI3 kinase (Fig. 5B). These data indicate that the intraluminal injection of these drugs reaches a sufficient concentration to inhibit the E_2 induction of PI3 kinase activity that leads the phosphorylation of AKT and GSK-3β and that these effects persist for at least 4–8 h.

We next determined the cellular location of cyclin D1 by immunohistochemistry. As previously reported, in control mice cyclin D1 is predominantly in the cytoplasm and that E_2 treatment caused it to be detected predominantly in the nuclear compartment of the luminal epithelial cells (Fig. 5, C1 and C3). In contrast, P_4 pretreatment resulted in its exclusion from the nucleus (Fig. 5C4). Inhibition in E_2-treated mice of uterine PI3 kinase with either drug resulted in a dramatic relocalization of cyclin D1 from the nucleus to the cytoplasm in the luminal epithelial cells (compare Fig. 5, C3, C5, and C6). Interestingly, this effect is not observed in the glandular epithelium (Fig. 5C3), suggesting that the effects of the drugs are very local, only influencing the luminal epithelium. As expected, these drugs did not alter the cellular localization of cyclin D1 in the control untreated uteri where PI3 kinase is inactive (Fig. 5C2). Together, these data confirm that the regulation by E_2 and P_4 of epithelial cyclin D1 localization, and therefore the pRB pathway, is through the PI3 kinase→AKT→GSK-3β pathway with these hormones exerting opposite effects on key points of regulation by protein phosphorylation.

DISCUSSION

The female steroid hormones E_2 and P_4 coordinately regulate the cell proliferation, differentiation, and remodeling of the uterus in a temporal and cell-specific manner to provide an environment suitable for blastocyst implantation and pregnancy. These hormones act through transcription factor receptors located in the target tissues that serve to give tissue specificity to the hormone response (39, 40). In adult mice, the estrogen surge at proestrus causes ovulation and mating behavior at estrus and induces cell proliferation that is restricted solely to the luminal and glandular epithelium (12, 14, 41). This E_2 exposure is essential for efficient implantation approximately 4.5 d later. If copulation occurs, then the P_4 synthesized by the newly formed corpus lutea suppresses this epithelial cell proliferation and induces these epithelial cells to differentiate to receive the blastocyst (13). P_4 also prepares the uterine stroma to respond to the nidatory E_2 to undergo a single round of cell division that is required for these cells to undergo a decidual response (13, 42).

The regulation of cell proliferation by female sex steroid hormones has mostly been studied in breast cancer cells in culture (43, 44). In these cells, E_2 first exerts its influence by the dramatic induction of cyclin D1 mRNA and protein resulting in CDK4 activation and the consequent elevation of CDK2 activity. This together with the down-regulation of the cell cycle inhibitors, $p27^{kip1}$ and $p21^{waf1}$ results in the sequential action of these CDKs on pRB phosphorylation and progression into S-phase (45–47). P_4, in contrast, down-regulates CDK4 as well as CDK2 activity through an inhibition of cyclin D1 expression and induction of the cell cycle inhibitors $p27^{kip1}$, $p21^{waf1}$ and Ink4c (p18) activity that act in concert to inhibit these CDKs (43, 44, 48). These data are very similar to that which is observed after growth factor stimulation of resting cells in culture. However, it is likely that these breast cancer cells have become adapted to culture conditions and/or altered by their neoplastic transformation and their responses to sex steroid hormones do not necessarily reflect the action of these hormones upon normal mammary epithelium *in vivo*. In addition, these studies may not reveal the mechanisms that control P_4 and E_2 regulation of uterine epithelial cell proliferation *in vivo*. In respect to this, it is noticeable in the mammary gland that gene ablation of cyclin D1 affects only lobuloalveolar proliferation in response to P_4 and E_2 (49) and the loss of $p27^{kip1}$, although somewhat controversial (50), does not affect mammary development *in vivo* (51). Furthermore, in our previous studies, we have shown that null mutations in both *Ink4c* and *Ink4d*, either as single mutations or together, do not affect female fertility, indicating that, in the absence of these proteins, the uterus responds normally to steroid hormones (52). In addition, P_4 did not reverse the E_2-induced down-regulation of $p27^{kip1}$ in the uterine epithelial cells, and mice carrying null mutations in this gene show normal uterine cell cycle responses to E_2 and P_4 (14, 21, 53, 54). Furthermore, we have shown in the uterine epithelium that E_2 does not result in the elevation of cyclin D1 protein from the very low levels that are observed in cell culture but instead regulated its cellular localization (21). These

data emphasize the need to study the relevant responses in particular tissues *in vivo*.

Our experiments with adult ovariectomized mice given exogenous E_2 and P_4 have indicated the cellular localization of cyclin D1 is a essential point in the regulation of the uterine epithelial cell cycle (21). E_2 causes the nuclear localization of cyclin D1 that is maximal within 4 h of E_2 treatment and persists through S-phase. This nuclear accumulation permits phosphorylation of the nuclear localized pRB and the subsequent progression of cells into S-phase. Indeed, if cyclin D1 is missing through gene ablation in mice, cyclin D2 completely compensates for its loss, and this protein now shows nuclear accumulation and cyclin D2-dependent CDK4 activity (55). The cyclin D1 relocalization is completely blocked by pretreatment of mice with P_4, and this prevents pRB phosphorylation and cell cycle progression (21). These data suggest that this early localization of cyclin D1 is an essential regulatory point for sex steroid hormone action in the uterus. Consistent with this is the failure of P_4 to block the E_2 induction of cell proliferation if administered 3 h after E_2, although it is fully potent before that time (56).

There have been several mechanisms that have been shown in tissue culture cells to be involved in the cellular localization of cyclin D1. For example, binding of cyclin D1 with heat shock protein 70 and the CKI p21 can stabilize cyclin D1 and induce its transport into the nucleus (57). However, we have not been able to demonstrate significant levels of p21 in the uterine epithelial cells after E_2 treatment (Tong, W., and J. W. Pollard, our unpublished observations) suggesting that this mechanism is an unlikely cause for the cyclin D1 nuclear accumulation after this treatment. Another mechanism is through the phosphorylation on Thr^{286} of cyclin D1 by GSK-3β. This results in the binding of cyclin D1 to the nuclear exportin, chromosome region maintenance (CRM) 1, and its subsequent nuclear egress (22). In this paper, we show that E_2 causes GSK-3β to be subjected to an inhibitory phosphorylation on Ser^9 with a time course that parallels the accumulation of cyclin D1 in the nucleus of these epithelial cells. P_4 blocks the E_2-induced inhibitory phosphorylation of GSK-3β that we speculated would result in the observed cytoplasmic localization of cyclin D1. To determine whether GSK-3β is the central kinase regulating the localization of cyclin D1, we treated mice with an intraluminal injection of LiCl, a specific inhibitor of GSK-3β (58). As anticipated, this had no effect upon cyclin D1 localization in E_2-treated uterine epithelial cells where GSK-3β is inactivated by phosphorylation but caused a marked accumulation of nuclear cyclin D1 in the mice exposed to P_4E_2 treatment. Consistent with this cyclin D1 nuclear accumulation, LiCl treatment resulted in pRB phosphorylation in a situation, whereby it is normally completely inhibited by P_4. The activation of this pRB pathway is sufficient to induce some S-phase genes such as cyclin A, PCNA, and Ki-67 in P_4E_2-treated uterine epithelial cells. The inhibition of GSK-3β by LiCl in P_4-treated uteri resulting in cyclin D1 accumulation and pRB phosphorylation, together with our earlier studies, provides strong evidence that this is a central regulatory point for the action of these sex steroid hormones in the uterine epithelium *in vivo*.

It was noticeable, however, that the induction of cyclin D1 nuclear accumulation by LiCl was not sufficient to cause these cells to enter into DNA synthesis, as assessed by BrdU incorporation. These data suggest that another pathway is required for DNA replication to start that runs parallel to the pRB pathway and which converges at the onset of DNA synthesis. Indeed, the pRB pathway must also be integrated with other pathways that are essential for DNA synthesis to commence such as rRNA and protein synthesis (59). For example, before the onset of DNA synthesis the pre-RC assembly begins with the binding of a six-subunit origin recognition complex to specific origin sites on the chromatin and serves as a landing platform for additional initiation factors. The origin recognition complex recruits two loading factors, CDC6 and Cdt1, which in turn facilitate the loading of the heterohexameric minichromosome maintenance (Mcm2–7) proteins onto chromatin at the origin of replication (60). This complex is known as the licensing complex because it is required for assembly of the polymerase complex that performs the DNA synthesis (61). Exactly how these pathways are integrated is still uncertain, although it appears that some members of the complex such as CDC6 are targets of the E2F transcription factors (61, 62). In other studies, we have shown that P_4 inhibits the expression of MCM proteins involved in the prereplication complex and disrupts the E_2-induced prereplication complex assembly on the chromatin within 1–2 h after hormone treatment (Pan, H., and J. W. Pollard, unpublished data). Because this licensing complex is an obligate part of S-phase initiation, LiCl would need to reverse both pathways to induce DNA synthesis, but its action is later than the observed effects of P_4 on the MCM proteins. Thus, we suggest that LiCl is only able to activate the pRB pathway, and thus the cells stall before the onset of DNA replication because of the requirement of these other pathways to generate the full cellular response.

GSK-3β is a major node in the complex web of intracellular signaling pathways receiving input from many sources. These include the canonical Wnt pathway that inhibits GSK-3β activity, such that β-catenin becomes dephosphorylated and stabilized. β-Catenin is then translocated to the nucleus, where it becomes associated with the lymphoid enhancer factor, Tcf/Lef, transcription factor to initiate transcription of target genes (26). While this paper was under review, a study reported that Wnt signaling also plays a role in the regulation of uterine epithelial cell proliferation (63). These studies showed that E_2 rapidly activates the canonical Wnt signaling through up-regulation of Wnt4, wnt5a and the Wnt receptor Frizzed-2 in an ER-independent manner, and this promotes the nu-

clear accumulation of its downstream transcription cofactor β-catenin. Similarly, Wnt5a pathway is required for epithelial-mesenchymal signaling in the uterus (64). The early E_2 response of the Wnt signaling pathway appears to be critical for the DNA synthetic response because abrogation of this pathway by *in vivo* delivery of SFRP-2, a Wnt antagonist, attenuated DNA synthesis (63). We have also shown a down-regulation of Wnt4 and Frizzed-2 mRNA expression within 3 h after P_4E_2 administration (Pan, H., and J. W. Pollard, unpublished data), suggesting that P_4 may also regulate this pathway. Thus, it seems likely that this represents another pathway modulated by the female sex steroid hormones, all of which converge to promote DNA synthesis after E_2 treatment and which are inhibited by P_4.

Growth factors, such as epidermal growth factor, can also signal through the GSK-3β-inactivating kinase $p90^{RSK}$ (25). Whereas insulin causes inactivation of GSK-3β through Ser^9 phosphorylation by a PI3 kinase-dependent mechanism (25), active PI3 kinase in turn generates PIP3 at the plasma membrane and thereby recruits AKT, a serine/threonine kinase, by direct interaction with its PH domain. Once recruited, AKT is phosphorylated on Thr^{308} by another PH domain-containing serine/threonine kinase PDK1 at the membrane (65). However, for maximal activation, AKT needs additional phosphorylation at Ser^{473} by PDK2 (38). AKT then phosphorylates GSK-3β at Ser^9. Interestingly, this insulin-regulated pathway and the Wnt pathway appear to be insulated from one another because its activation does not cause β-catenin accumulation and in turn, Wnt signaling does not affect insulin signaling (66, 67). In this study, we show that E_2 induced phosphorylation of AKT at the activating Ser^{473} and Thr^{308} sites in the luminal epithelial cells with a peak level of phosphorylation detected at 8 h after treatment. This parallels the inhibitory phosphorylation of GSK-3β at Ser^9 that also shows a maximum at 8 h. This direct effect of PI3 kinase through AKT was confirmed by inhibitor studies that showed that the inhibition of PI3 kinase reduced GSK-ser^9 phosphorylation after E_2 stimulation.

This activation of AKT by E_2 is dramatically attenuated by pretreatment of the mice with P_4, although there is still a small elevation over that detected in mice treated with P_4 alone. PI3 kinase has multiple roles in the cell and it is notable that E_2 stimulates many processes independent of whether the mice had been pretreated or not with P_4 including protein synthesis, rRNA synthesis, and the induction of many immediate early genes (68–71). It is likely that PI3 kinase is involved in some of these steps, perhaps through spatially restricted activation, and this is the reason why there is a small degree of PI3 kinase activation after P_4E_2 treatment. Despite this, it is clear that the PI3 kinase to AKT to GSK-3β pathway is the signal transduction mechanism that E_2 and P_4 use to regulate the pRB pathway because inhibition of PI3 kinase blocked the E_2-induced nuclear accumulation of cyclin D1 and as discussed above, the inactivation of GSK-3β in P_4-treated uteri resulted in nuclear accumulation of cyclin D1 and pRB phosphorylation.

Previous studies have indicated that E_2 induces uterine IGF-I expression (72, 73) as well as receptor activation as detected by phospho-tyrosinated IRS1 and IGF-1R (74, 75). This resulted in activation of AKT as assessed by phosphorylation at Ser^{473} (75). Interestingly treatment of ovariectomized mice with IGF-I alone was also able in an ER-dependent manner, to induce PCNA nuclear accumulation and to a much lesser extent epithelial cell proliferation in the uterine epithelium (75). Although the biochemical studies were performed upon whole uteri making discrimination of the cell types in which the signaling occurred impossible to determine, it seems likely that the epithelial cells are the primary targets. Interestingly, in MCF-7 breast cancer cells in culture, IGF-I causes nuclear accumulation of cyclin D1 through a GSK-3β mediated mechanism (76). In this case, IGF-I acts synergistically with E_2 to promote cell proliferation (76, 77). Together, these data are consistent with ours and suggest that sex steroid hormones in the uterine epithelium regulate the pRB pathway by activating IGF-I signaling. Studies by Cunha and associates (78, 79) using tissue recombinants have suggested that this mechanism is through the paracrine action of E_2 on the uterine stroma. Thus, it seems likely as summarized in Fig. 6 that E_2 triggers stromal synthesis or release of IGF-I that stimulates the PI3 kinase to AKT to GSK-3β pathway that leads to nuclear accumulation of cyclin D1 and activation of the pRB pathway. This is blocked by P_4 either at the PI3 kinase step or through blockage of synthesis of IGF-I or its binding to the IGF receptor. These data are therefore the first to link female sex steroid hormone action by growth factor signaling to the canonical cell cycle machinery *in vivo*.

In summary, as described in Fig. 6, we have identified that E_2 couples directly to the cell cycle regulatory machinery in the uterine epithelium *in vivo* via PI3 kinase→AKT→GSK-3β. In this scenario, PI3 kinase triggers PIP3 synthesis that binds AKT that is activated by phosphorylation and then inhibits GSK-3β action through Ser^9 phosphorylation. This results in the inhibition of cyclin D1 phosphorylation, causing it to accumulate in the nucleus. This is sufficient to activate the pRB pathway and that this pathway acts as a primary sensor for E_2 action on uterine epithelial cell proliferation. The data also show that P_4 blocks this PI3 kinase pathway to cause cell cycle arrest through inhibiting the nuclear localization of cyclin D1 and thereby blocking the pRB pathway. The PI3 kinase pathway is the major one that couples the female sex steroid hormones to the cyclin-regulated cell cycle motor and suggests that members of this pathway may be targets for

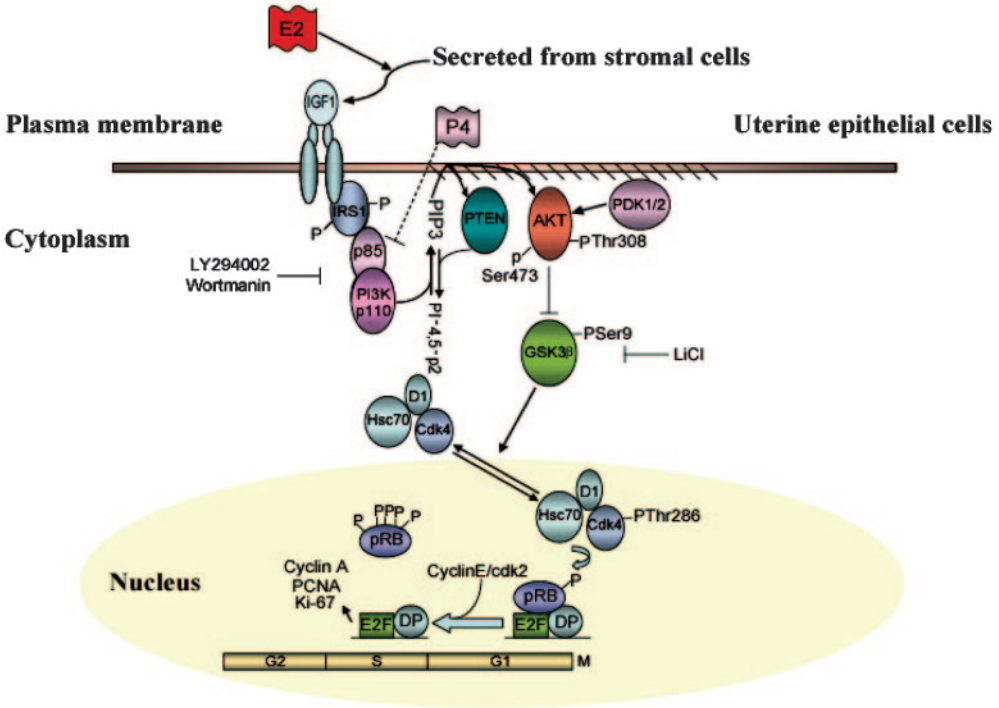

Fig. 6. Model for the Regulation of the Uterine Epithelial Cell Cycle by E_2 and P_4

The figure summarizes the data that E_2 is coupled directly to the cell cycle machinery by activation of the PI3 kinase pathway and suggests that this is through a paracrine mechanism involving IGF-I. This activation of PI3 kinase results in PIP3 synthesis (shown as \\\\) that recruits AKT to the cell membrane where it is activated by phosphorylation on Ser^{473}. Active AKT then causes an inhibitory Ser^{9} phosphorylation of GSK-3β with the resultant redistribution of cyclin D1 from the cytoplasm to the nucleus and subsequent pRB phosphorylation. P_4 blocks this PI3 kinase pathway and consequently inhibits cyclin D1 nuclear localization, pRB phosphorylation, and therefore the E_2-induced cell proliferation. PTN, Phosphatase and tensin homolog; DP, $DRFT_1$-polypeptide. Also shown are the points of action of the inhibitors LY294002, wortmannin, and LiCl, that were used in this study to verify the involvement of specific components of this hormone regulated pathway.

therapeutic intervention in cancers of the breast and uterus.

MATERIALS AND METHODS

Animal Treatment

Adult female CD1 mice were purchased from Charles River and kept in a barrier facility at the Albert Einstein College of Medicine. The Albert Einstein Animal Use committee approved all animal procedures. The mice were treated with physiological hormonal regimens as described (21). Briefly they were ovariectomized via a dorsal incision under tribromoethanol anesthesia. Two weeks after the surgery, mice were primed with 100 ng of E_2 (Sigma, St. Louis, MO) in peanut oil by sc injection for 2 d before the experiment. Six days later, groups of two to five mice were killed by cervical dislocation at different time points after one of the following treatments: 1) no treatment (control), 2) one sc injection of 50 ng of E_2 in 0.05 ml of peanut oil (E_2 treatment), 3) the same treatment as 2) but an intraluminal injection of PBS, LiCl (Sigma), 100 or 200 nM wortmannin, or 30–50 μM LY294002 (Cell Signaling Technology, Beverly, MA) given before the E_2 injection as indicated in the results, 4) 4 d of sc injections of 1 mg of P_4 (Sigma) with one sc injection of 50 ng of E_2 at the same time as the last P_4 injection (P_4E_2 treatment) 5) 4 d of sc injections of 1 mg of P_4 with one sc injection of 50 ng of E_2 at the same time as the last P_4 injection (P_4E_2 treatment) 2 h after an intraluminal injection of PBS (P_4E_2 + PBS treatment) or with one of the inhibitors as in 3). Intraluminal injections were 50 μl in volume in a pleuronic gel. The intraluminal injections of solutes in pleuronic gels is relatively inefficient with only approximately 50% of mice injected showing gel in the uterus 4 h after injection. The remainder is presumably lost due to muscular contractions as the result of the surgical manipulation before the gel can set. Thus, in the LiCl-treated groups mice were screened for efficacy using immunohistochemistry of one horn before biochemistry was performed upon the other. This represented approximately 40% of the mice treated. All experiments reported were performed in duplicate and repeated at least once and usually three to four times.

Preparation of Uterine Epithelial Extracts

After hormone treatment, uteri were removed and split longitudinally and an epithelial extract greater than 95% pure was prepared as described (80). Lysates were sonicated and clarified by centrifugation. For each experiment, equal amounts of protein, measured by Bradford assay (Bio-Rad Laboratories, Inc., Hercules, CA), were used. The extraction buffer contained 10 mM HEPES-KOH (pH 7.5), 0.1 M NaCl, 1 mM EDTA, 2.5 mM EGTA, 10 mM β-glycerophosphate, 10% glycerol, 1 mM dithiothreitol (DTT), 1 mM NaF, 0.1 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg aprotinin/ml, 10 μg leupeptin/ml, 10 μg pepstatin A/ml. The washing buffer contained 90 mM HEPES-KOH (pH 7.5), 0.2 M NaCl, 1 mM

EDTA, 2.5 mM EGTA, 0.2% Tween 20, 10% glycerol, 10 mM β-glycerophosphate, 1 mM DTT, 1 mM NaF, 0.1 mM Na_3VO_4, 0.2 mM phenylmethylsulfonyl fluoride, 10 μg aprotinin/ml, 10 μg leupeptin/ml, 10 μg pepstatin A/ml. The extraction buffer was combined with the washing buffer to constitute the immunoprecipitation (IP) buffer.

Preparation of a Nuclear Fraction from the Uterine Epithelium

Uterine epithelial cell lysates were prepared as described before (21) with some modification. In brief, a sucrose-based extraction buffer containing 10 mM HEPES (pH 7.5), 50 mM NaCl, 0.5 M sucrose, 1 mM EDTA, 0.25 mM EGTA, 1 mM DTT, 0.6 mM spermidine, and protease inhibitors cocktail (Roche, Indianapolis, IN) was used. After filtration, Nonidet P-40 was added to a final concentration of 0.7%. The lysates were vortexed for 20 sec followed by repeated passing through a 22-gauge needle. Nuclei were collected by centrifugation at 800 × *g* for 10 min at 4 C and washed once with extraction buffer. The nuclei were then subjected to Western blotting analysis.

Western Blotting, Immunohistochemistry, and Antibodies

Polyclonal antibodies for cdk4 (sc-260), cyclin A (sc-596), and Lamin A/C (sc-20681) were obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal antibodies for RB (G3–245) were obtained from Pharmingen (San Diego, CA); those for cyclin D1 (DCS-6, DCS-11), and Ki-67 (Ab-3) were obtained from Neomarkers, Lab Vision Corp. (Fremont, CA); and that for PCNA (PC10) was obtained from Roche Applied Sciences (Indianapolis, IN); for Akt, antiphospho-Akt (T308), antiphospho-Akt (S473), antiphospho-Gsk (S9) and antiphospho-RB (S807) antibodies, and LY294002 and wortmanin were from Cell Signaling Technology. Gsk and antiphospho-Gsk (Y216) were purchased from BD Transduction Laboratory (San Jose, CA).

For Western blotting, equal amounts of protein or cell number equivalents were separated by SDS-PAGE and transferred to Immobilon P membranes (Millipore, Billerica, MA). The membranes were blocked in Tris-buffered saline with 0.1% (vol/vol) Tween 20 and 5% (wt/vol) nonfat dry milk for 3 h. They were then incubated for 1–3 h with a dilution of the specific antibody in blocking solution and subsequently incubated for 0.5–1 h with a horseradish peroxidase-linked secondary antibody [Amersham (Piscataway, NJ) or Santa Cruz Biotechnology] as described (21, 55). Immunodetection was achieved with an enhanced chemiluminescence system (ECL; Amersham).

Immunohistochemical procedures were performed as described before (21, 55). Briefly, transverse 5-μm paraffin sections of the uterus were deparaffinized and subjected to antigen retrieval by boiling the samples in 0.01 M sodium citrate buffer (pH 6.0) for 10 min. Nonspecific binding was blocked by incubating sections with 10% normal goat or rabbit serum for 30 min. After the sections were incubated with the appropriate antibody as detailed above, they were washed and exposed to biotin-conjugated secondary antibodies (Vector Laboratories, Inc., Burlingame, CA) for 30 min, followed by incubation with avidin DH-biotinylated horseradish peroxidase H complex (Vector Laboratories, Inc.) for 30 min. This complex was detected with the metal-enhanced diaminobenzidene substrate kit (Pierce, Rockford, IL) Sections were counterstained with hematoxylin (Sigma).

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