Estradiol-17β regulates mouse uterine epithelial cell proliferation through insulin-like growth factor 1 signaling

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Edited by R. Michael Roberts, University of Missouri, Columbia, MO, and approved August 20, 2007 (received for review June 19, 2007)

Estradiol-17β (E_2) causes cell proliferation in the uterine epithelium of mice and humans by signaling through its transcription factor receptor α (ERα). In this work we show that this signaling is mediated by the insulin-like growth factor 1 receptor (IGF1R) expressed in the epithelium, whose activation leads to the stimulation of the phosphoinositide 3-kinase/protein kinase B pathway leading to cyclin D1 nuclear accumulation and engagement with the canonical cell cycle machinery. This cyclin D1 nuclear accumulation results from the inhibition of glycogen synthase kinase 3β (GSK3β) activity caused by an inhibitory phosphorylation by protein kinase B. Once the IGF1 pathway is activated, inhibition of ER signaling demonstrates that it is independent of ER. Inhibition of GSK3β in the absence of E_2 is sufficient to induce uterine epithelial cell proliferation, and GSK3β is epistatic to IGF1 signaling, indicating a linear pathway from E_2 to cyclin D1. Exposure to E_2 is the major risk factor for endometrial cancer, suggesting that downstream activation of this IGF1-mediated pathway by mutation could be causal in the progression to ER-independent tumors.

cell cycle | cyclin D | estrogen | glycogen synthase kinase 3β(GSK3β) | estrogen receptor

The adult mouse and human uterus undergoes waves of cell proliferation that are regulated by estradiol-17β (E_2) and progesterone (P_4). In both species, E_2 induces cell proliferation in the luminal and glandular epithelium, but in contrast to mice the human stroma also undergoes proliferation during this so-called proliferative phase (1). P_4 synthesized cyclically in the human or in response to copulation in the mouse inhibits this epithelial cell proliferation but primes the stromal cells to respond to E_2 by cell division (1). Because >50% of women seek medical advice due to uterine bleeding disorders, many of which are associated with proliferation at least once in their lives, and because E_2 is the major risk factor for endometrial and breast adenocarcinomas thought to be the result of the continuous stimulation of cell proliferation (1, 2), it is essential to understand the mechanism of action of these hormones in inducing cell division.

In most cell types the entry into DNA synthesis is regulated by the sequential activity of cyclins and their attendant cyclin-dependent kinases (CDK) in phosphorylating and inactivating the members of the Rb family of proteins (3). These cyclin/CDKs are, in turn, regulated by the expression of a variety of cyclin-dependent inhibitors that fall into two classes the CIP/KIP family and the pl6^{ink4} family, by phosphorylation of activating and inhibitory sites, as well as by their subcellular localization. In most cases, the initial sensing of external mitogenic stimuli is through altering the activity of D-type cyclins and their cognate CDK4 and CDK6 kinases (4, 5), which is also true in the uterine epithelium of mice in response to E_2. But in contrast to the mitogenic responses of cells to E_2 in culture, the level of cyclin D1 does not change acutely in these cells, but instead its localization is altered such that it becomes nuclear localized in response to E_2 (6).

The nuclear localization of cyclin D is regulated by phosphorylation of Thr^{286} by glycogen synthase kinase 3β (GSK3β). This phosphorylation results in egress of cyclin D1 from the nucleus through association with the nuclear export protein, CREM1 (7). In the uterine luminal epithelium, E_2 inhibits GSK3β action by the stimulation of a protein kinase B (AKT)-mediated inhibitory phosphorylation of Ser^9. AKT is in turn regulated through activation of phosphoinositide 3-kinase (PI3-kinase; ref. 8). Progesterone inhibits this pathway by blocking AKT phosphorylation and thus the inactivation of GSK3β with the resultant loss of nuclear cyclin D1 (8). Indeed, inhibition of GSK3β by LiCl (a selective GSK3β inhibitor) results in reversal of the P_4-induced block of cyclin D1 nuclear accumulation and subsequent phosphorylation of pRb and progress of the luminal epithelial cells toward but not into S phase (8).

Experiments using chimeric grafts of uterine epithelium and stroma whose estrogen receptor (ER) status differ because of an inactivating mutation suggests that E_2 signals by the stroma in a paracrine manner to regulate epithelial cell proliferation (9). Several paracrine mediators have been proposed, including insulin-like growth factor 1 (IGF1), keratinocyte growth factor, or members of the Wnt family (1). Here we show that IGF1 is required for E_2-induced uterine epithelial DNA synthesis.

Results

The central hypothesis of this work is that E_2 activates the PI3-kinase/AKT/GSK3β pathway in the uterine epithelial cell by IGF1 paracrine signaling and that this activation leads to proliferation. Our previous studies showed that the activity of GSK3β was inhibited by E_2 in these cells, an observation that predicts that inhibition GSK3β activity is central to the cell proliferative response to E_2 (8). If this prediction is the case, we hypothesized that a chemical inhibitor of GSK3β would induce uterine epithelial DNA synthesis in a manner analogous to E_2 in control ovariectomized mice that had not been exposed to E_2. To test this hypothesis we introduced the specific GSK3β inhibitor, SB415286, into the uterine lumen of ovariectomized mice. Using BrdU incorporation after an i.p. injection 2 h before death as a measure of DNA synthesis, we demonstrate that this inhibitor caused ≈40% of cells to enter into DNA synthesis 15 h after injection, a value significantly above the control level (Fig. 1 *A*

Author contributions: J.W.P. designed research; L.Z. performed research; L.Z. and J.W.P. analyzed data; and J.W.P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Abbreviations: AKT, protein kinase B; CDK, cyclin-dependent kinase; E_2, estradiol-17β; ER, estrogen receptor; GSK3β, glycogen synthase kinase 3β; IGF1, insulin-like growth factor 1; IGF1R, insulin-like growth factor 1 receptor; P_4, progesterone; PI3-kinase, phosphoinositide 3-kinase; PPP, picropodophyllin.

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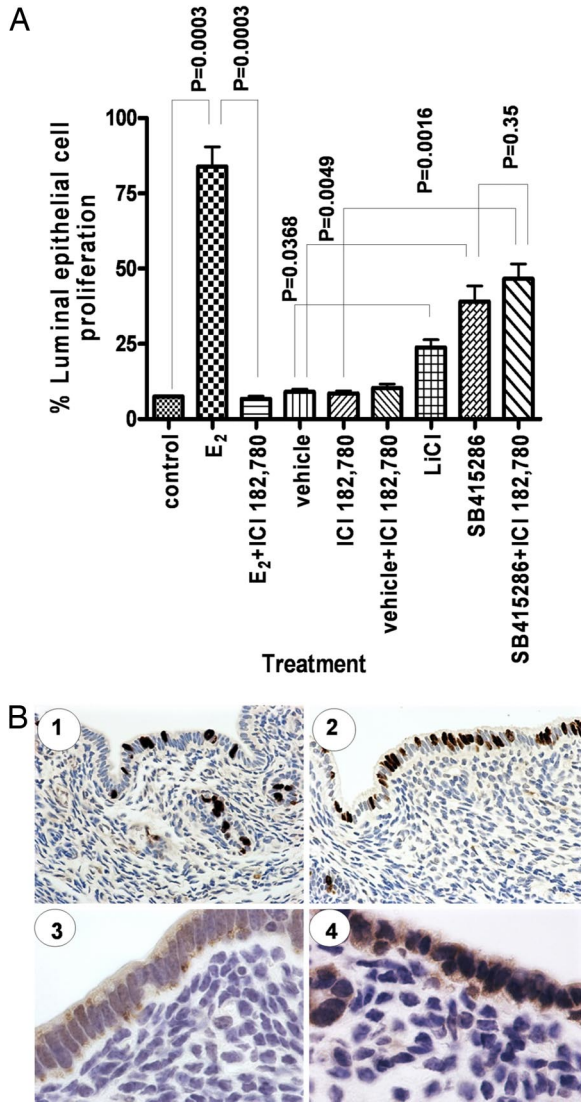

Fig. 1. Inhibition of GSK3β induces uterine luminal epithelial DNA synthesis. The GSK3β inhibitor, SB415286, was introduced into the uterine lumen of ovariectomized mice, and DNA synthesis was estimated by BrdU incorporation in transverse sections of uteri harvested 15 h after administration and 2 h after an i.p. injection of BrdU. As a control, 50 ng of E_2 was administered s.c. in oil, a regimen that results in maximal DNA synthesis 15 h after treatment. In some experiments, the pure estrogen antagonist ICI 182,780 was injected i.p. at time 0. (*A*) Quantitative estimates of BrdU incorporation expressed as percentage of luminal epithelial cells in S phase after the treatments shown on the *x* axis. Statistical comparisons were performed by Student's *t* test. (*B*) Representative transverse sections of mouse uteri immunostained for BrdU incorporation (*1* and *2*) or cyclin D1 nuclear localization (*3* and *4*) using appropriate antibodies in ovariectomized control uteri either untreated (*1* and *3*) or treated with SB415286 (*2*) or LiCl (*4*) administered intraluminally. These immunostaining experiments show the increased DNA synthesis after treatment that is restricted to the uterine epithelium and the nuclear localization of cyclin D1 after inhibition of GSK3β.

and *B*). Concordant to that observed with E_2, this DNA synthesis was restricted to the epithelial compartment (6). A similar induction of uterine epithelial DNA synthesis in ovariectomized mice was also obtained when we used LiCl, another, although slightly less specific and potent (10), inhibitor of GSK3β (Fig. 1*A*). Inhibition of GSK3β also, as predicted, resulted in cyclin D1 nuclear accumulation (Fig. 1*B*). Thus, these inhibitors act in a manner similar to estrogen in stimulating uterine epithelial cell proliferation in these cells.

We also hypothesized that once the PI3-kinase pathway is activated by E_2 it would become independent of ER activity. To test the dependence of this effect on ER signaling, we used the pure anti-estrogen ICI 182,780 that blocks ER signaling (11). In concord with previous experiments (12), this inhibitor completely blocked the E_2 stimulation of DNA synthesis after an i.p. injection bringing this level to that of the control untreated mouse (Fig. 1*A*). However, ICI 182,780 was completely ineffective at inhibiting the SB415286 stimulation of cell proliferation. Interestingly, the basal level of DNA synthesis observed in control ovariectomized mice was also independent of ER signaling (Fig. 1*A*). Thus, although E_2-induced uterine epithelial cell proliferation in the uterus depends on the ER, once the pathway is activated it becomes independent of ER signaling.

Next, to test the requirement of E_2-induced DNA synthesis on IGF1 signaling we used the intraluminal injection of the specific IGF1R inhibitor, picropodophyllin (PPP) (13) to determine whether it inhibited E_2-induced uterine epithelial cell proliferation. First, however, we determined the kinetics of effect of E_2 on IGF1R phosphorylation in the luminal epithelium by using an anti-IGF1R phosphotyrosine antibody. In lysates derived from the luminal epithelium of ovariectomized mice, the level of IGF1R phosphorylation was extremely low, although the unphosphorylated form of the IGF1Rβ can be easily detected (Fig. 2 *A* and *B*). E_2 treatment resulted in an increased level of phosphorylation that was detected within 2 h (the peak of ER occupancy under this treatment regimen (14) and reached a maximum 4 h after treatment (Fig. 2 *A* and *B*). E_2 also induced a small increase in IGF1R expression consistent with previously published data (15). Second, we wished to determine the potential source of the IGF1. Others have shown that the transcripts of this growth factor were up-regulated in the whole uterus by E_2 using Northern blotting (16) or in the uterine stroma and myometrium of rats (17). We therefore used *in situ* hybridization to identify its source in mouse uteri. In control, unstimulated uteri, the level of IGF1 mRNA was low (Fig. 2*C*) although easily detected in the luminal and glandular epithelia as well as the stromal compartments with a signal significantly above the sense control background level (Fig. 2*C*). E_2 treatment induced a dramatic up-regulation of IGF1 mRNA, particularly in the stroma with a more modest elevation in the epithelial cells 4 h after treatment (Fig. 2*C*). Thus, consistent with the previous data from Northern blotting and *in situ* experiments in rats (16), we show that E_2 dramatically elevates uterine IGF1 expression and signaling in mice.

E_2 treatment of ovariectomized mice causes an induction of DNA synthesis with a peak at 12–15 h after treatment that is confined to the luminal and glandular epithelium (14, 18) (Figs. 1 and 3.*2*). To test whether this E_2 effect depended on IGF1 signaling we blocked this signaling by using PPP introduced into the luminal space before treatment of ovariectomized mice with E_2. This treatment blocked the E_2 induction of IGF1R phosphorylation (Fig. 4 *A* and *B*) as assessed 4 h after treatment. Furthermore, it significantly reduced the DNA synthetic response induced by E_2 by ≈4-fold (Figs. 3.*3*, and 4*C*) measured by the percentage of cells incorporating BrdU. We had shown previously that E_2 also induces the phosphorylation of GSK3β at Ser^9, which results in the inhibition of this kinase (8), which, in turn, resulted in the nuclear accumulation of cyclin D1 because active GSK3β phosphorylates cyclin D1 at Thr^{286}, causing its nuclear egress (19). Thus, we queried whether inhibition of IGF1 signaling also blocked these downstream events. Indeed, treatment with PPP also inhibited the E_2-induced nuclear localization of cyclin D1 (Fig. 3.*5* and 3.*6*), consistent with its inhibition of DNA synthesis. We can conclude that E_2 signals through IGF1 to induce uterine epithelial DNA synthesis.

The next hypothesis that we tested was whether IGF1 signaling was upstream from GSK3β in a linear pathway. If this hypothesis

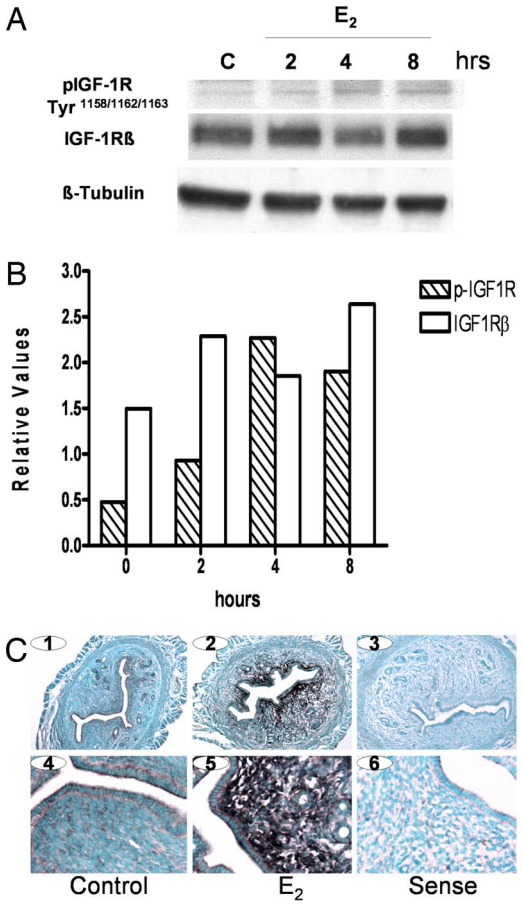

Fig. 2. E_2 treatment increases IGF1 expression in the uterine stroma and IGF1R signaling in the luminal epithelium. (*A*) Representative Western blot of uterine epithelial protein extracts isolated at the times shown after E_2 treatment of ovariectomized mice and probed with anti-phospho-IGF1Rβ, -IGF1Rβ, and -β-tubulin as a loading control as indicated. (*B*) Quantitative estimate by densitometry of the induction of IGF1 signaling in the epithelium after E_2 treatment relative to the β-tubulin control. Hatched bars, phospho-IGF1Rβ; open bars, IGF1Rβ subunit. (*C*) *In situ* hybridization of transverse sections of uteri of control (*1* and *4*) and E_2-treated (*2*, *3*, *5*, and *6*) mice probed by using antisense (*1*, *2*, *4*, and *5*) or sense (*3* and *6*) probes. Purple precipitate represents the hybridization signal and shows dramatic up-regulation after E_2 treatment principally in the stroma but also in the luminal and glandular epithelium. (Magnification: 1–3, ×10; 4–6, ×40.)

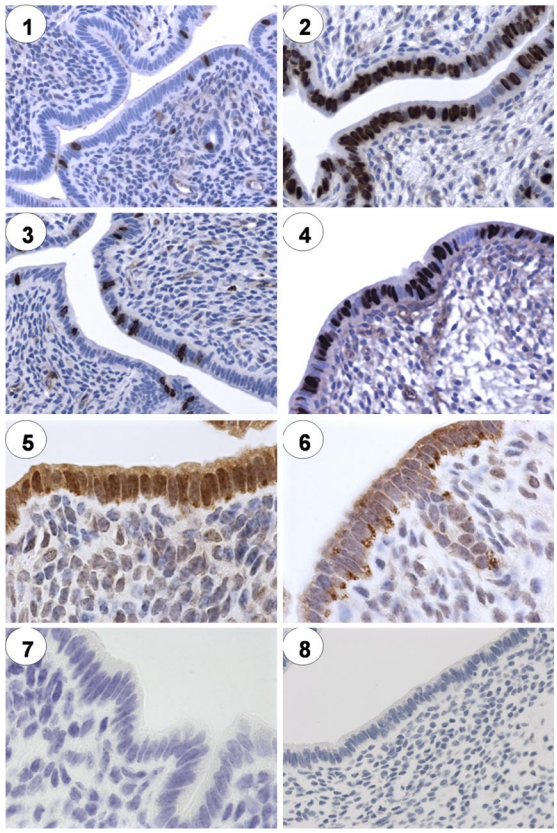

Fig. 3. E_2 signals through IGF1 and GSK3β to induce DNA synthesis in the uterine epithelium. (*1–4*) Transverse sections of uteri immunostained for BrdU incorporation from mice 15 h after the following treatments: control ovariectomized (*1*), s.c. E_2-treated (*2*), s.c. E_2 plus PPP given intraluminally (*3*), and s.c. E_2 plus PPP and SB415286 given intraluminally (*4*). (*5* and *6*) Transverse sections immunostained for cyclin D1 and harvested 4 h after treatment of E_2 alone (*5*) and E_2 with PPP administered intraluminally (*6*). The brown precipitate indicates the specific reaction, and the experiment shows the inhibition of nuclear cyclin D1 accumulation by PPP. (*7* and *8*) Similar sections stained in the absence of the primary antibody to cyclin D1 (*7*) or BrdU (*8*).

is true, we reasoned that inhibition of GSK3β would reverse the inhibitory effects of PPP on E_2 signaling to DNA synthesis. We thus introduced both inhibitors at the same time into the uterine lumen of mice followed by E_2 treatment. As mentioned above, PPP inhibited the E_2 induction of DNA synthesis by ≈4-fold (Figs. 3.*3* and 4*C*), which was completely reversed by the coinjection of the GSK3β inhibitor, SB415286, and the number of cells in DNA synthesis was not significantly different from that observed with E_2 treatment alone (Figs. 3.*4* and 4*C*). These data show that GSK3β is epistatic to IGF1R and E_2.

Discussion

Estrogen is the mitogen for the adult uterine epithelium causing the hormone-depleted cells to undergo a wave of DNA synthesis beginning 6 h after exposure with a peak at 12–15 h that is followed by progress through the G_2 and M phases of the cell cycle (1). This cell proliferation that occurs in the mouse every 4–5 days absolutely depends on the ER in the uterus and a direct action of estrogen (1). Tissue recombinants between epithelium and stroma transplanted into the kidney capsule of immunocompromised mice when one cell type lacked the ER because of an inactivating mutation suggested that the signal from E_2 derived from the stroma and acted on the epithelium in an ER-dependent manner (9). Our previous data showed that the regulation of epithelial cell proliferation centered on the nuclear translocation of cyclin D1 that was affected by an AKT-mediated inhibition of GSK3β whose phosphorylation of cyclin D1 at Thr^{286} results in nuclear egress (9). Consequent to this inhibition, the nuclear translocation of cyclin D1 resulted in Rb phosphorylation and the cascade of downstream events leading to S phase. To avoid problems of interpretation caused by application of inhibitors systemically, in this work we introduced PPP, a specific inhibitor of IGF1R into the uterine luminal space and showed definitively that IGF1 signaling in the epithelial cells triggers the signaling cascade in response to E_2, illustrated in Fig. 5.

Several predictions derived from consideration of this pathway were confirmed by the current experiments. The first prediction was that the inhibition of GSK3β by AKT-mediated phosphorylation was downstream from ER, which was demonstrated by using chemical inhibitors of GSK3β, treatments that resulted in an epithelial-restricted induction of DNA synthesis in the absence of E_2. In fact, by this classical estrogen assay, these GSK3β inhibitors would be considered as potent estrogen agonists. This, induction of DNA synthesis was not inhibited by the "pure" ER antagonist ICI 182,780 at doses that completely blocked the E_2

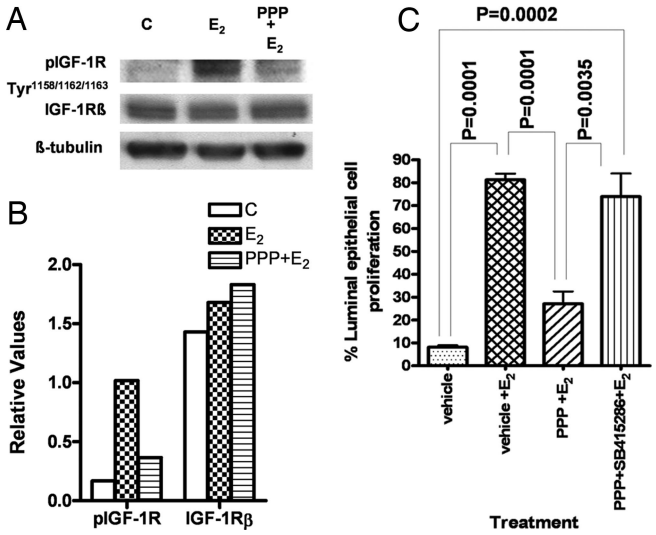

Fig. 4. Inhibition of IGF1R signaling blocks E_2-induced cell proliferation, which is reversed by inhibition of GSK3β. (*A* and *B*) Intraluminal injection of PPP blocks IGF1R phosphorylation. (*A*) Western blot showing that E_2-induced IGF1R phosphorylation is inhibited by PPP using the antibodies described in Fig. 1. (*B*) Quantitation of three independent Western blots showing the PPP inhibition. The inhibition is statistically different from the E_2-treated group. (*C*) Quantification of the uterine luminal epithelial DNA synthetic response to the treatments shown of the *x* axis. The PPP treatment significantly inhibits the E_2 response, which is significantly reversed by concurrent inhibition of GSK3β; *P* values are from Student's *t* test.

induction of DNA synthesis, confirming that GSK3β is downstream of ER. The second prediction was that the IGF1 signaling pathway to GSK3β was linear, which was shown to be the case because the inhibition of E_2-induced DNA synthesis by PPP was completely reversed by concurrent inhibition of GSK3β, showing they are epistatic. Furthermore, because IGF1R inhibition blocked and GSK3β inhibition caused nuclear cyclin D1 nuclear localization, we can conclude that this pathway is linear from IGF1 signaling to the phosphorylation of pRB.

The importance of IGF1 in uterine biology was shown by studies within IGF1-null mutant mice that were shown to have hypotrophic uteri with minimal development of the myometrium (20), which is consistent with studies showing that ^{125}I-IGF1

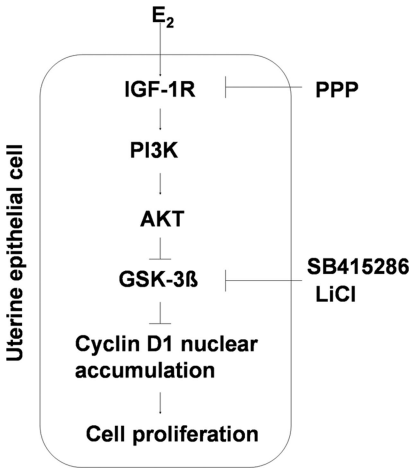

Fig. 5. E_2-induced pathway in the luminal epithelium that leads to the canonical cell cycle machinery. The points of inhibition of PPP, SB415285, and LiCl are indicated.

bound to cells in the myometrium (17). Similarly, using organ culture of immature uterus, IGF1 was shown to potentiate the proliferative response principally in the myometrium but also in the stroma (16). These data show the requirement of IGF1 in uterine, particularly myometrial development.

However, E_2 is not required for cell proliferation in the immature mouse uterus, with this proliferation being driven by factors derived from the hypothalamic–pituitary axis, whereas in adult mice it is absolutely required (1). In adult mice, E_2 treatment has been reported to induce IGF1 receptor activation in the uterine epithelium as determined by immunohistochemistry (21, 22), and this activation required ER (15). Furthermore, exogenously administered IGF1 stimulated the IGF1 receptor and induced the cell proliferation marker proliferating cell nuclear antigen, although only a small increase, if any, in DNA synthesis was reported (15). In organ cultures of rat uteri, both E_2 and IGF1 were required for cell division (17). IGF1 also activated epithelial ER, suggesting a direct requirement for this receptor in the epithelial cells, in contradistinction to the chimera experiments described above that showed that ER was not required in the epithelial cells (9, 15). Other studies in IGF1-null mutant mice showed that IGF1 is necessary for epithelial cells of prepubertal uteri in response to E_2 to progress through G_2 but not for their traverse through G_1 (23). In a recent transcriptome analysis, the transcripts of multiple genes associated with the IGF1 pathway were affected in response to E_2, further suggesting that this pathway is regulated by this hormone (24).

The studies described above have used whole uteri to determine biological effects of E_2. However, because the epithelium is the only cell type in adult murine uteri that responds acutely to E_2 by cell proliferation and this cell type represents only 5% of the total uterine cell number (18), the regulation of proliferation by E_2 in these studies is obfuscated by responses in other cell types that do not proliferate or that accumulate in the uterus in response to E_2. In contrast, the studies reported here used inhibitors applied locally to the luminal epithelium as well as biochemical analysis of responses in purified epithelial cell extracts. The data obtained clearly show that IGF1R signaling is required in the adult epithelial cells in the G_1 phase of the cell cycle and that the downstream activation of the canonical cell cycle pathway through this IGF1R pathway is independent of ER. This conclusion is consistent with the $\approx$40% reduction of response to E_2 in DNA synthesis caused by the transgenic overexpression of IGF1-binding protein 1 in the uterus (25). These data and the fact that prepubertal uteri exhibit normal epithelial proliferation in response to E_2 in *Igf1*$^{-/-}$ mice strongly suggest that the estrogen responses are different in prepubertal than pubertal uteri. Other candidate paracrine molecules in the immature uterus would be EGF, which has been shown to be mitogenic to these cells in this tissue (26).

The source of IGF1 has also been controversial. IGF1 mRNA and protein are expressed in the uterine epithelium at days 1 and 2 of pregnancy in mice, and IGF1 expression is potentiated by E_2 in ovariectomized mice (16, 17, 27–30). In ovariectomized rats treated with E_2, IGF1 mRNA was detected predominantly in the stroma and myometrium with lower expression in the epithelium (27). This pattern of expression was confirmed by this work in adult mice which showed by *in situ* hybridization a dramatic up-regulation of IGF1 mRNA in response to E_2 in the stroma with lesser although enhanced expression in the luminal and glandular epithelia. Despite these expression data, tissue-grafting experiments using uteri derived from IGF1-null mutant mice showed that systemic but not local IGF1 is required for E_2-induced uterine epithelial cell proliferation (31). Given the very dramatic up-regulation of IGF1 immediately after E_2 treatment coincident with IGF1R phosphorylation, our data would suggest a local source of this growth factor. However, the need for systemic IGF1 cannot be totally ruled out by the present

experiments, although it is unclear what action of ER in the stroma would make circulating IGF1 available within a short time span.

Exposure to unopposed estrogen is one of the major risk factors for endometrial and breast cancer (2). It has been hypothesized that this increase risk is because of mutations that accumulate in the epithelial cells during the repeated waves of cell proliferation caused by this hormone. The elucidation of this E_2 pathway acting within the epithelial cell through IGF1R, PI3-kinase, AKT, and GSK3β that in turn regulates the canonical cell cycle machinery is likely to give insights to the observed increased risks of cancer. Intriguingly, activated AKT is found in >40% of endometrial cancers, and phosphatase and tensin homolog deleted on chromosome 10 (PTEN) mutations (negative regulator of PI3-kinase) are also frequently associated with endometrial cancer (32, 33). Indeed, mice heterozygous for null mutations in PTEN succumb to endometrial hyperplasia and cancer (34). Thus, we can hypothesize that mutations that result in activation of the IGF1 to cyclin D1 pathway elucidated in this work would be causal in human endometrial and breast tumor progression to malignancy because they would render the cells ER-independent.

Materials and Methods

Mice and Treatment. Mice were obtained from Charles River Laboratories (Wilmington, MA), ovariectomized, rested for 2 weeks, and then primed with 100 ng of E_2 (Sigma, St. Louis, MO) given s.c. in oil as described. Six days later they were given 50 ng of E_2 s.c., a dose that mimics the proestrous estrogen surge and that stimulates a wave of DNA synthesis that peaks 12–15 h later in the luminal and glandular epithelium (14). Intraluminal injection of inhibitors or vehicle controls was performed under anesthesia 2 h before E_2 administration in a volume of 50 μl as described (8). The following compounds were injected either i.p., the ER antagonist ICI 182,780 (Tocris Bioscience, Ellisville, MO) or intraluminally, GSK3β inhibitor, SB415286 (Biomol International, Plymouth, PA) and LiCl (Sigma) and IGF1R antagonist PPP (Calbiochem, San Diego, CA). In some experiments in which DNA synthesis was measured, BrdU (Roche, Indianapolis, IN) was injected i.p. 2 h before killing (6).

Groups of three to five mice were killed at various times after treatment, and their uteri were removed and processed either for the preparation of an epithelial protein extract that is >95% pure as described or fixed for histology (14). Each experiment was repeated at least twice and usually three times, and consistent results were obtained.

Western Blotting. Epithelial protein extracts were separated by SDS/PAGE, blotted onto Immobilon-P membranes (Millipore, Billerica, MA), and probed with antibodies against IGF1Rβ: pTyr$^{1158/1162/1163}$-IGF1Rβ and β-tubulin (Santa Cruz Biotechnology, Santa Cruz, CA) as described (8). After development and film exposure, the relative signals were measured by densitometry and corrected for loading with the β-tubulin intensity.

Histology, Immunohistochemistry, and *in Situ* Hybridization. Five-micrometer transverse sections of uteri were immunostained with antibodies against BrdU (Roche) and cyclin D1 (Lab Vision, Fremont, CA) and counterstained with hematoxylin by using methods described before (8). The percentage of labeled cells was determined by counting luminal epithelial cells in these transverse sections.

In situ hybridization on frozen sections was performed by using digoxigenin-labeled sense or antisense probes to IGF1. Hybridization was detected by using the anti-digoxigenin-AP antibody and developed by nitroblue tetrazolium/ 5-bromo-4-chloro-3-indolyl phosphate (Roche) as described (35).

We thank Jim Lee for expert help with the maintenance of our mouse colony. This work was supported by National Institutes of Health Grants R01 CA 89617 (to J.W.P.) and P30 CA13330 (to the Albert Einstein Cancer Center). J.W.P. is the Sheldon and Betty E. Feinberg Senior Faculty Scholar in Cancer Research.

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