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Roles of estrogen receptor α and β in modulating urothelial cell proliferation

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Abstract

We reported previously that both subtypes of estrogen receptors, ER α and ER β , are expressed by human urothelial cells and mediate estrogen-induced cell proliferation in these cells. The aim of this study was to determine the extent to which each ER subtype contributes to urothelial cell proliferation and their possible involvement in the regulation of the cell cycle. We compared the expression of ER α and ER β mRNAs and protein quantitatively in primarily cultured human bladder urothelial cells obtained from six individuals with three immortalized urothelial (E6, E7, and UROtsa) and two bladder cancer cell lines (HTB-9 and T24). We found that all these cells express similar levels of ER β , but immortalized and cancer cells express much higher amounts of ER α than primary cells. Higher levels of ER α mRNA were also observed in the biopsies of bladder transitional cell carcinoma compared with sample from the same bladder unaffected by tumor. Using the ER α -selective agonist PPT, the ER β -selective agonist DPN, and specific small interfering RNA against ER α or ER β , we found that ER β predominantly mediates estrogen-induced G1/S transition and cell proliferation in the primary urothelial cells. By contrast, ER α predominantly mediates estrogen-induced G1/S transition and cell proliferation in bladder cancer cell lines. Furthermore, we found that 17 β -estradiol (E₂) rapidly induces phosphorylation of extracellular signal-regulated kinases, but U0126, a mitogen-activated protein kinase kinase (MEK) inhibitor, does not affect E₂-induced urothelial cell proliferation. E₂ up-regulated cyclin D1 and cyclin E expression in both the primary and bladder cancer cells, and the cancer cells have higher cyclin D1 and cyclin E expression during G0/G1 phases. Our data suggest that estrogen exerts its effects through different ER subtypes in urothelial cells. Increased expression of ER α may contribute to early induction of cyclin D1 and cyclin E during the cell cycle in bladder cancer cells.

Introduction

Estrogen hormones, particularly 17 β -estradiol (E₂), are potent regulators of cell growth in a wide array of tissues. These effects are mediated primarily through binding to the two major estrogen receptors (ER) subtypes, ER α and ER β , that are thought to be ligand-activated transcription factors. Ligand binding induces a conformational change within the ER that may stimulate high-affinity binding to specific estrogen response elements or regulate transcription at promoter elements by directly binding heterologous transcription factors through protein–protein interactions (Sabbah *et al.* 1999, Kushner *et al.* 2000). Recently,

extensive studies have shown that estrogen may induce rapid non-genomic effects mediated by ERs or other unrelated estrogen-binding sites residing in or near the cell plasma membrane through activation of signaling pathways such as mitogen-activated protein kinase (MAPK) or interaction with diverse growth factors (Manavathi & Kumar 2006, Song & Santen 2006).

ER α and ER β are encoded by unique genes located on different chromosomes and exhibit overlapping tissue distribution and function. Under normal conditions, ER α predominates in the anterior pituitary, mammary glands, uterus, vagina, cervix, and liver (Koike *et al.* 1987), whereas ER β is primarily detected in the ovary, thyroid, skin, spleen, lungs, hypothalamus, gastrointestinal tract, thymus, and urogenital tract (Mosselman *et al.* 1996). ER α and ER β display a high degree of conservation in the central DNA-binding domain and the carboxyl-terminal ligand-binding domain, with 97 and 60% amino acid homologies respectively, but have considerable divergence in the amino-terminal (18% homology; Enmark *et al.* 1997). Hence, ER α and ER β exhibit a similar, but not identical, binding affinity profile for different ligands. Recently, several compounds with relatively high selectivity for each ER subtypes have been produced (Stauffer *et al.* 2000, Meyers *et al.* 2001).

Epidemiological evidence and animal studies indicate that estrogen plays an important role in cell proliferation and survival in tumor-prone tissues responsive to hormones, such as the uterus and mammary glands. These tissues usually have overall higher levels of ER α than ER β . Studies have shown that ER α expression in many breast cancer cell lines is up-regulated, and estrogen-induced cell proliferation is mediated by ER α in these cells. Meanwhile, ER β has been shown to be down-regulated, and overexpression of ER β in these cells reduced cell proliferation with a concomitant decrease in many regulatory components of the cell cycle (Strom *et al.* 2004, Helguero *et al.* 2005). Transient reduction of ER α using small interfering RNA (siRNA) significantly suppressed cell cycle progression and cell proliferation in breast cancer cells (Liu *et al.* 2006). Transfection of HeLa cells with ER α increased cyclin D1 expression, while ER β overexpression decreased cyclin D1 (Liu *et al.* 2002). These observations have led to the controversial hypothesis that an important physiological role of ER β is to inhibit ER α actions and the loss of ER β contributes to the development of cancer (Helguero *et al.* 2005). Suggested mechanisms have focused on the less conserved amino-terminal A/B domains. Unlike ER α , ER β only has a weak activation function (AF)-1 but contains a repressor domain that may inhibit the overall transcriptional activity of the receptor (Paech *et al.* 1997, Hall & McDonnell 1999).

Urothelium refers to the specialized epithelial cells that line the renal pelvis, ureters, bladder, and urethra. It is one of the slowest growing epithelia in mammals under normal conditions. However, when stimulated appropriately, urothelium can undergo tremendous growth, particularly during wound healing and carcinogenesis (Walker 1960, Cohen & Ellwein 1993). More than 90% of bladder cancers arise in the urothelium, and bladder cancer is the fifth most frequently diagnosed cancer (Jemal *et al.* 2006). Several lines of evidence have linked estrogen to bladder carcinogenesis. Although men are thrice more likely to develop bladder cancer than women, when data are corrected for the influence of smoking, women appear to face an even greater risk than men (Castelao *et al.* 2001). It is interesting to note that epidemiological studies of risk factors for bladder cancer in pet dogs (a species that doesn't smoke) found one- to threefold higher risk in females (Kelsey *et al.* 1998). High levels of ER β mRNA and protein are expressed in the epithelium of the whole urogenital tract of both males and females, but expression of ER α is much lower (Hess *et al.* 1997, Makela *et al.* 2000). However, high levels of ER α have been found in a large number of human bladder transitional cell carcinomas (Kaufmann *et al.* 1998). Neonatal estrogenization of male rats increased expression of ER α and induced urogenital carcinogenesis (Prins *et al.* 1998). The purposes of the current study were to compare

expression of ER α and ER β mRNAs and protein in normal human bladder urothelial cells with that in different immortalized urothelial and bladder tumor cell lines and test the hypothesis that the ratio of ER α to ER β is lower in primary human urothelial than bladder cancer cells. Using ER α or ER β selective agonists and siRNA selectively against ER α or ER β , we also investigated their individual involvement in estrogen-induced cell cycle progression and cell proliferation.

Materials and methods

Cell culture and tissue specimens

All experimental protocols were reviewed and approved by the Health Sciences Human Subjects Committee of the University of Wisconsin. Normal human bladder urothelial cells (HBUC) were primarily cultured from discarded bladder mucosal layer obtained during surgeries performed at the University of Wisconsin Hospital. None of the bladders from which urothelial cells were obtained were affected by tumor or inflammation. Cells were plated on Petri dishes coated with type I rat tail collagen in Ham's F-12+ nutrient mixtures containing 2% fetal bovine serum (FBS) and used between passages 4 and 6. Ureteral urothelial cells immortalized with viral constructs containing human papillomavirus E6 or E7 were also maintained in F-12+ medium and cells passaged <35 times were used. HBUC immortalized with viral construct containing the SV40 large T antigen (UROtsa) were kindly provided by Dr Petia Simeonova (National Institute for Occupational Safety and Health, Morgantown, WV, USA) and maintained in Dulbecco's Modified Eagle's medium (DMEM) containing 5% FBS. Bladder tumor cell lines HTB-9 and T24 were purchased from The American Type Culture Collection (Manassas, VA, USA) and maintained in DMEM containing 5% FBS. All cultures were placed in a humidified incubator at 37 °C in 5% CO₂. All medium and supplements were purchased from Invitrogen. Phenol red-free medium and charcoal-treated FBS were used in all experiments. Paraffin-embedded samples of primary bladder carcinomas and matched samples of histologically normal tissues from the same bladder were obtained from six patients diagnosed with transitional cell carcinoma of the urinary bladder (grades 2–4) at the University of Wisconsin Hospital. Samples of total RNA were available from primary bladder carcinomas and matched samples of histologically normal tissues from the same bladders from ten patients diagnosed with transitional cell carcinoma of the urinary bladder (grades 2–4) at the University of Wisconsin Hospital. None of the patients had received systemic therapy. Bladder cancer tissue arrays with self-matching normal adjacent tissues were purchased from US Biomax (Rockville, MD, USA).

RNA interference

Sets of four siGENOME duplexes specifically designed against ER α or ER β were synthesized by Dharmacon (Lafayette, CO, USA). The duplex from each set that had the most significant inhibitory effects on urothelial cell proliferation was used in the remainder of this study. For siER α , the sense sequence used was 5'-UAUCUGAACCGUGUGGGAGUU-3'. For siER β , the sense sequence used was 5'-CAAGAAGAUU-CCCGGCUUUU-3'. Transfection of siRNA was performed at a concentration of 100 nM using Oligofectamine (Invitrogen) when cells achieved 30–50% confluence. Control studies to evaluate the specificity of siRNAs were performed using scrambled non-targeting siCONTROL siRNA (siCon, Dharmacon) under identical conditions. To quantify cell transfection efficiency, a cytotoxic siTOX (Dharmacon) was co-transfected into cells, and singly dispersed cell viability was determined by trypan blue exclusive assay 24 h after transfection. siGLO RISC-Free fluorescent-labeled siRNA (Dharmacon) was also co-transfected into cells to serve as both a negative control and an

efficiency marker. Nuclei were identified with Hoechst 33342 (Invitrogen) staining. More than 90% transfection efficiency was reproducibly achieved in all experiments.

Total RNA isolation and PCR analysis

Total RNA was isolated from cultured cells using QIAshredder and RNeasy Mini kit (Qiagen) or tissues using TRIzol reagent (Invitrogen) following the manufacturer's protocol. The cDNA pool was synthesized from 0.5 to 1 µg RNA using the MMLV Reverse Transcriptase kit (Promega). Conventional PCR for ERα or ERβ gene was performed using the HotStarTaq kit (Qiagen) in a Peltier Thermal Cycler (MJ Research, Waltham, MA, USA). Expression of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was determined as a reference gene. Primers for amplification were as follows. For ERα: forward –ACAGCATGAAGTGCAAGAACGTGG and reverse – TGGCTGTTCCCAACAGAAGACAGA; for ERβ: forward – TCGGCAGACCACAAGCCCAA and reverse – CCACACTTCACCATTCCCAC; and for GAPDH: forward – TCATCATCTCTGCCCCCTCTG and reverse – TCATCATCTCTGCCCCCTCTG. Quantitative real-time PCR was performed using SYBR Green Master Mix in a 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Experiments were performed in duplicate for each data point, and the abundance of human ribosomal protein S26 mRNA was determined as a reference gene. Primers for amplification were as follows. For ERα: forward – AGATCTTCGACATGCTGCTGGCTA and reverse – AGATCTTCGACATGCTGCTGGCTA; for ERβ: forward – ACACCTGGGCACCTTTCTCC TTTA and reverse – TCTTGCTTCACACCAGG GACTCTT; and for S26: forward – AACTAGGAA CGCATTTCACCCT and reverse – GGCTTC AAGAACGGCAACTCACTT.

Protein extraction and immunoblotting

Cells were lysed in M-PER Mammalian Protein Extraction Reagent (Pierce, Rockford, IL, USA) containing protease inhibitors (Roche). Supernatant was collected by centrifugation at 10 000 g for 10 min at 4 °C, and the protein content was measured using the BCA Protein Assay kit (Pierce). Thirty microgram extracts were electrophoresed on SDS-PAGE gels and transferred to nitrocellulose membranes (Bio-Rad). Antibodies used were mouse anti-human cyclin D1 (1:1000, HD11, sc-246), mouse anti-human cyclin E (1:1000, HE12, sc-247), mouse anti-human proliferating cell nuclear antigen (PCNA; 1:5000, PC-10, sc-56), rabbit anti-human ERα (1:5000, MC-20, sc-542), rabbit anti-human ERβ (1:5000, H-150, sc-8974), mouse anti-human p-ERK (1:5000, E-4, sc-7383), rabbit anti-human ERK1 (1:5000, K-23, sc-94), and mouse anti-human α-tubulin (1:5000, B-7, sc-5286), all from Santa Cruz (Santa Cruz, CA, USA) and diluted in TBST (50 mM Tris, 150 mM NaCl, 0.1% Tween 20 (pH 7.5)) containing 2% BSA. The membranes were blocked with SuperBlock (Pierce) for 2 h at room temperature and incubated with primary antibodies overnight at 4 °C. They were then incubated at room temperature for 2 h in 1:10 000 alkaline phosphatase-conjugated or horseradish peroxidase-conjugated anti-mouse or anti-rabbit antibodies (Santa Cruz) in TBST. Chemiluminescent signal was revealed with the ECL or CDPstar kit (Amersham) and detected by exposure to X-ray film. Band intensity was quantified with ImageJ (NIH) and normalized to that of α-tubulin in the same sample. Experiments were repeated at least four times.

Immunohistochemical staining

Slides of formalin-fixed and paraffin-embedded transitional cell carcinomas of the urinary bladder tissue were subjected to steam heat for 25 min in 10 mM citrate buffer (pH 6.0) for antigen retrieval after deparaffinization and dehydration. The antibodies used were mouse anti-ERα (aa 120–170, 1:200; Chemicon, Temecula, CA, USA), rabbit anti-human ERβ

(1:500, H-150; Santa Cruz), and normal rabbit serum (1:500, sc-2338; Santa Cruz) served as negative control and mouse breast tumor tissue served as positive control. Specific staining was developed using VECTASTAIN ABC and DAB kits (Vector, Burlingame, CA, USA). 1:200 Goat anti-rabbit antibody was used as secondary antibody and diaminobenzidine was used as a chromogen.

³H-Thymidine incorporation assay

Cells were aliquoted into 96-well tissue culture plates at a density of about 5000 cell/well and transiently transfected with 100 nM siRNAs. After serum starvation for 24 h, 1–100 nM E₂ (Sigma), 4,4',4''-(4-propyl-[1H]-pyrazole-1,3,5-triyl)trisphenol (PPT; Tocris, Ellisville, MO, USA), or 2,3-bis(4-hydroxyphenyl)-propionitrile (DPN; Tocris) was added to the wells in the absence or presence of 100 nM ICI 182 780 (Tocris, added 2 h prior to agonist treatments). Ten hours later, 0.5 µCi [methyl-³H] thymidine (Amersham) was added to each well. After 6 h, DNA was precipitated by 10% trichloroacetic acid (TCA) and washed with 70% ethanol. The acid-insoluble material was then dissolved with 0.5 M NaOH and collected on UniFilter-96 GF/C plates (Perkin-Elmer, Wellesley, MA, USA). Thirty microliters of Micro-Scint 20 were added into each well, and the radioactivity was counted with a TOPCount Microplate Scintillation Counter (Packard, Meriden, CT, USA). DPM (disintegrations per minute) was calculated by count per minute (CPM)/efficiency (~20–30%).

Flow cytometry assay and fluorescence-activated cell sorting (FACS)

Cells were harvested with trypsinization and stained in 20 µg/ml Hoechst 33342. Fluorescence intensity was measured with a Becton-Dickinson LSRII triple laser benchtop cytometer (BD PharMingen, San Diego, CA, USA) to estimate the proportion of cells in different phases of the cell cycle. Data were analyzed with BD Cell Quest software. Live cells were also sorted into G0/G1, S, and G2/M phase cells according to DNA content using a BD FACSCalibur cytometer, lysed in M-PER (Pierce), and expression of cyclins was assessed by immunoblotting.

Statistical analysis

All results are expressed as means±S.E.M. and accompanied by the number of observations. Data were obtained from groups containing four to six replicates. Paired Student's *t*-test was used to compare self-matched samples. Comparisons among treatments over time or within groups and between multiple groups were performed by one-way or two-way ANOVA using SAS/STAT (SAS Institute Inc., Cary, NC, USA). Fisher's LSD and Tukey's multiple tests were used to determine post hoc significance. *P*<0.05 was considered statistically significant.

Results

ERα and ERβ are differentially expressed in primary urothelial, immortalized urothelial, and bladder tumor cells

In general, we found that primary urothelial cells grow more slowly than immortalized urothelial or bladder tumor cells. The typical population-doubling time of early passages of HBUC is about 3–5 days, whereas the population-doubling time of immortalized or tumor cells is <1 day (Table 1). To investigate whether there are differences in ER expression among these cell types, we examined ERα and ERβ mRNA transcripts in total RNA preparations from three female donors (PF1–3), three male donors (PM1–3), as well as E6, E7, and UROtsa immortalized cells and bladder tumor cell lines originally from a male patient (HTB-9) or a female patient (T24) by quantitative real-time PCR. ERα mRNA

expression was significantly higher (by six- to seven-fold) in immortalized urothelial or bladder tumor cells than in primary cells (Fig. 1A). By contrast, all cells expressed similar levels of ER β mRNA (Fig. 1B). These observations were consistent with findings using conventional PCR (Fig. 1C). Immunoblotting for ER α and ER β proteins in whole-cell lysates of these cells demonstrated a similar pattern of ER expression as seen in mRNA levels (Fig. 2). These data showed that ER α and ER β are differentially expressed among primary, immortalized, and bladder cancer cells. Consistent with the observation that immortalized and cancer cells grow faster in culture, blots of PCNA showed that levels are significantly higher in immortalized urothelial and bladder tumor cells than in primary cells (Fig. 2). In formalin-fixed and paraffin-embedded bladder carcinomas and bladder cancer tissue arrays, both histologically normal and those tissues identified as transitional cell carcinomas stained positively for ER α and ER β (Fig. 3A). Real-time PCR analysis of RNA obtained from ten biopsies of bladder transitional cell carcinoma and matched sample from the same bladder unaffected by tumor showed that abundance of ER α mRNA was significantly greater (2.77-fold, $P=0.000017$ by paired t -test) in tumor than in normal tissues. By contrast, tumor and normal tissues express similar levels of ER β mRNA (paired t -test $P=0.49$; Fig. 3B).

ER α and ER β expression can be selectively reduced by siRNA

siRNAs designed to selectively suppress ER α or ER β were used to test the hypothesis that ER α and ER β have different effects in mediating estrogen-induced urothelial cell proliferation. Non-transfected cells, cells treated with only Oligofectamine, and cells transfected with non-targeting siRNA were used in all the experiments to control for non-specific effects of the transfection protocol. The specificity and efficiency of siRNAs were tested by transient co-transfection of cells with same amount (100 nM) of non-targeting siCon, a cytotoxic siTOX, or fluorescence-labeled siGLO RISC-Free with siER α or siER β . More than 90% transfection efficiency and nuclear and peri-nuclear localization of siRNAs was confirmed by staining cells with Hoechst 33342, allowing observation of nuclei (Fig. 4A). Quantitative real-time PCR confirmed that ER α mRNA was selectively inhibited by siER α by ~80% (Fig. 4B), and ER β mRNA was selectively inhibited by siER β by ~70% in both primary urothelial and T24 cells 24 h after transfection, and these observations were consistent with findings using conventional PCR (Fig. 4C). Immunoblotting for ER α and ER β protein levels in whole-cell lysates of these cells demonstrated that ER α protein was selectively reduced by siRNA against ER α by 50–60%, and ER β protein was selectively reduced by siER β by 60–80% (Fig. 5). Treatment with siER α did not affect the expression of ER β and vice versa. These results showed that ER α or ER β mRNA and protein can be selectively suppressed in urothelial cells by specific siRNAs with high efficiency.

Cell proliferation in response to selective ligands

Using urothelial cells transiently transfected with siER α or siER β , we evaluated the effects of E $_2$, the ER α -selective ligand PPT, and the ER β -selective ligand DPN on cell proliferation using the ^3H -thymidine incorporation assay following 16-h incubation with each compound in the presence or absence of the ER antagonist ICI 182 780. Concentration–response curves were generated for each reagent and demonstrated that treatment with 10 nM E $_2$, PPT, or DPN; 100 nM ICI 182 780; or 100 nM siRNAs were most effective (data not shown). In primary cells transfected with only siCon, 10 nM E $_2$, PPT, or DPN induced significant increases in DNA synthesis (11.4, 24.2, and 95.5% respectively, $n=6$; Fig. 6A). In primary cells transfected with only siER α , the increase induced by E $_2$ or PPT was reduced to 79.2 and 10.5% respectively. By contrast, DPN caused a higher increase in DNA synthesis (107.9%; Fig. 6B) relative to siCon-transfected cells. When cells were transfected with only siER β , the increase induced by E $_2$ or PPT was significantly reduced to 19.9 and 28.7% respectively. The effect of DPN on DNA synthesis (18.9%) in these cells was not significant

(Fig. 6C). In primary cells co-transfected with siER α and siER β , effects of E₂, PPT, and DPN were further reduced (Fig. 6D). The specific effects of DPN or siER β on DNA synthesis in primary cells indicate that ER β predominantly mediates estrogen-induced cell proliferation in these cells.

In T24 cells transfected with only siCon, E₂ or PPT induced significantly greater increases in DNA synthesis (183 and 129% respectively, $n=6$) than did DPN (38.8%; Fig. 6E). In T24 cells transfected with only siER α , the increase caused by E₂ or PPT was significantly reduced to 18 and 13.4% respectively. By contrast, DPN caused a higher increase in DNA synthesis (63.5%) than it did in siCon-transfected T24 cells (Fig. 6F). When T24 cells were transfected with only siER β , the increase induced by E₂ or PPT were 115.6 and 109.1% respectively. The effect of DPN on DNA synthesis (17.3%) was not significant in these cells (Fig. 6G). In T24 cells co-transfected with siER α and siER β , the effects of E₂, PPT, and DPN were abolished (Fig. 6H). The specific effects of PPT or siER α on DNA synthesis in T24 cells indicate that ER α predominantly mediates estrogen-induced cell proliferation in these cells. In all of these cells, ICI 182 780 abolished proliferative effects of estrogen to levels comparable with that in control cells (i.e., not exposed to E₂; data not shown). These results are consistent with patterns of ER expression in these cells and indicate that both ER subtypes contribute to cell proliferation in various urothelial cells.

Estrogen induction of G/S phase transition in primary urothelial and bladder cancer cells

The capacity of estrogen to stimulate cell cycle progression has been investigated extensively in breast cancer cells and has focused on inducing cell progress through G1 phase. To study the capacity of estrogen to induce entry into S phase in urothelial cells, we determined the percentages of cells in G0/G1, S, and G2/M phases in serum-starved primary urothelial or T24 cells treated with 10 nM E₂, PPT, or DPN alone or in combination with 100 nM ICI 182 780 for 6 or 16 h. Treatment for 6 h had minimal effects, and the results of 16-h treatments from three separate experiments are summarized in Table 2. E₂ caused a significant accumulation of cells in the S phase with a concomitant decrease in the proportion of those in the G0/G1 and G2/M phases in both primary (cells in S phase increased from 24.8 to 40.9%) and bladder tumor cells (cells in S phase increased from 32.3 to 45.1%). ICI 182 780 reduced E₂-induced G1/S transition to the levels in control cells. Only DPN, but not PPT, increased the percentage of primary urothelial cells entering into S phase to 40%. In T24 cells, PPT induced a greater proportion of cell entry into S phase than did DPN (44.6 and 38.9% respectively). We also found in primary cells, the effect of DPN was blocked by siER β , while in T24 cells the effect of PPT was blocked by siER α , and the effect of DPN was blocked by siER β similar to the effects of ICI 182 780. These data are consistent with cell proliferation results and suggest that both ER α and ER β mediate estrogen-induced G1/S transition in urothelial cells.

E₂-induced urothelial cell proliferation is independent of ERK signaling

After finding that estrogen affects DNA synthesis and cell cycle progression, we investigated whether the proliferative effects of estrogen are mediated via non-genomic signaling pathways entailing MAPK. Treatment with 10 nM E₂ rapidly induced a significantly increase in phosphorylation of ERK (5–30 min), and this was abolished by U0126, a specific MEK pathway inhibitor (Fig. 7A and B). However, in the presence of 3 μ M U0126, E₂ still induced a significant increase in DNA synthesis in both HBUC and T24 cells (Fig. 7C). These results suggest that E₂-induced urothelial cell proliferation is not altered by MAPK-mediated non-genomic effects of estrogen.

Cyclin D1 and cyclin E are induced by estrogen in urothelial cells

Expression of major subtypes of cyclins that govern G₁/S transition, cyclin A, cyclin B1, cyclin D1, cyclin E, and cyclin H was assessed by immunoblotting after treatment of serum-starved primary urothelial or T24 cells with E₂ for time intervals varying from 0 to 24 h. Only cyclin D1 and cyclin E protein levels were significantly increased. These reached maximal values after 4–6 h of E₂ stimulation and subsequently declined but remained relatively high for up to 12 h (Fig. 8). In the absence of E₂, cyclin D1 was found to be expressed at higher levels (approximately twofold) in T24 than in primary urothelial cells, but both cell types expressed similar level of cyclin E. Both cyclin D1 and cyclin E proteins were significantly increased (~60–90%) in primary and T24 cells after treatment with 10 nM E₂ for 6 h. This increased expression was completely abolished by ICI 182 780 (Fig. 9). These data suggest that estrogens exert their effects on cell cycle progression through up-regulation of both cyclin D1 and cyclin E in both primary urothelial and bladder cancer cells. Treatment of primary urothelial cells with 10 nM E₂ in the presence or absence of 3 μ M U0126 resulted in a similar increase in cyclin D1 and cyclin E, indicating that the effects of estrogen on expression of cyclin D1 and cyclin E were not dependent upon ERK phosphorylation (data not shown).

To investigate the question of how abundance of cyclin D1 and cyclin E correlate with different cell phases, live primary urothelial and T24 cells were sorted into G₀/G₁, S, and G₂/M phases, and the expression of cyclin D1 and cyclin E proteins in whole-cell extracts was determined using immunoblotting. In the presence or absence of treatment with 10 nM E₂, relatively higher levels of cyclin D1 were detected only at G₀/G₁ phase in primary cells, and tumor cells expressed significantly higher levels of cyclin D1 in both G₀/G₁ and S phase cells. Cyclin E was expressed at higher levels in primary urothelial cells in S and G₂/M phases. However, in T24 cells, cyclin E was expressed at higher levels during G₀/G₁ and S phases (Fig. 10). These results suggest that higher constitutive and induced expression of cyclin D1 and cyclin E during G₀/G₁ and S phases may contribute to accelerated growth of bladder tumor cells.

Discussion

Estrogen-induced cell proliferation is intimately linked with the development and progression of a number of human cancers, notably breast, uterine, and ovarian cancers. Previous studies in these tissues found that ER β expression declines when cells undergo malignant transformation, and this causes an increased ER α /ER β ratio (Pujol *et al.* 1998, Foley *et al.* 2000, Roger *et al.* 2001). In the present study, we demonstrate that the increase in the ER α /ER β mRNA or protein ratio in bladder cancer cells was rather due to a significant increase in ER α expression. Both ER α and ER β may mediate estrogen-induced cell proliferation through activation of cyclin D1 and cyclin E, and increased expression of ER α may contribute to dysregulated growth of bladder cancer cells.

The data presented here suggest that ER α expression in immortalized and tumor urothelial cells is significantly higher than in primary cells, but that all of these urothelial cells expressed similar levels of ER β . This result was consistent with the analysis of mRNA from bladder samples from patients with transitional cell carcinoma. Our finding that immortalized and tumor urothelial cells also express significantly higher levels of PCNA than primary cells supports the concept that immortalization, malignant transformation, recurrence, and the development of muscle invasion of human bladder tumor are accompanied by increased expression of PCNA (Syrigos *et al.* 2004). One study reported that bladder cancers express high levels of ER β but low levels of ER α ; however, this report considered tumors with 10% or fewer cells expressing ER α as negative for ER α (Shen *et al.* 2006). Thus, low expression of ER α was equaled with no expression. We observed that both

histologically normal bladder tissues and tissues from bladders affected with transitional cell carcinoma expressed ER α and ER β , but we did not attempt to quantify relative expression of ER α and ER β by immunohistochemistry. Analysis of total RNA extracted from histologically normal and neoplastic tissues from bladders affected by transitional cell carcinoma indicated that both tissues expressed similar abundance of ER β mRNA but that neoplastic tissues expressed approximately threefold more ER α mRNA than histologically normal tissue from the same bladder. This observation is consistent with our findings in bladder cancer cell lines and immortalized urothelial cells. These data do not prove a causal relationship between increased expression of ER α and malignant transformation of urothelial cells, but at the very least they do provide evidence of temporal association of this change with the presence of cancer of the bladder.

Several studies have shown that ER α is cancer promoting, while ER β is protective by repressing ER α activation (Lindberg *et al.* 2003, Helguero *et al.* 2005). However, these observations are not free of controversy. The HC11 cell line used by Helguero *et al.* (2005) is a particular mouse mammary cell line reflecting the situation in late pregnancy and lactation, during which mammary glands are resistant to the proliferative effects of estrogen (Shyamala & Ferenczy 1982). Although Lindberg *et al.* (2003) suggested that there is a 'ying-yang' relationship between ER α and ER β , it is interesting to note that in their experiments, ER β was able to mediate estrogen-induced gene transcription in the absence of ER α . ER β expression was induced in carcinogen-transformed human breast epithelial cells, and higher grade cancerous transformed cells expressed higher levels of ER β (Hu *et al.* 1998). In invasive breast tumors from postmenopausal women, there was a trend for ER β expression to increased cell proliferation and worse prognosis (O'Neill *et al.* 2004). ER β expression was also found to be associated with increased expression of cell proliferation markers Ki67 and cyclin A in human breast cancers (Jensen *et al.* 2001). High intake of dietary plant estrogen has been associated with a low incidence of breast cancer. In light of previous hypothesis, this might be attributed to the fact that most phytoestrogens have higher affinity for ER β than ER α . Curiously, at least two epidemiological studies showed that high dietary soy intake increased the risk of bladder cancer (Sun *et al.* 2002, 2004). These experimental and pathological studies indicate that ER β may also contribute to initiation and progression of tumorigenesis. Therefore, the hypothesis that ER α and ER β have opposite effects on cell proliferation and apoptosis and that repression of ER α is the major physiological function of ER β should not be extrapolated generally to all tissues, especially potential estrogen target tissues that predominantly express ER β such as normal urothelium.

Many investigations of estrogen-induced cell proliferation have focused on recruitment of noncycling cells into the cell cycle and induction of cycling cells to enter, progress, and exit the G1 phase. Progression through the G1 phase is under the control of cyclin-dependent kinases (Cdks) that are activated by two major types of cyclins: D and E. There is a strong correlation between cyclin D and cyclin E expression and increased ER α levels in breast cancer cells (Musgrove *et al.* 1993, Prall *et al.* 1998). Enhanced expression of cyclin D1 is also frequently (15% of cases) observed in bladder cancers (Hall & Peters 1996) and appears to be an early event in bladder transitional cell carcinogenesis (Shin *et al.* 1997). In MCF-7 breast cancer cells, overexpression of cyclin D1 is sufficient to mimic estrogen action (Prall *et al.* 1998) and cyclin D1 antisense or microinjection of cyclin D1 antibody blocked estrogen-induced entry into the cell cycle. The blockade imposed by antiestrogens in MCF-7 cells can be overcome by cyclin D1 overexpression (Lukas *et al.* 1996). These results indicate that cyclin D1 is necessary for estrogen action. Estrogen also activates formation of the cyclin E-cdk2 complex in MCF-7 cells preceding entry into S phase (Prall *et al.* 1998). We found that both cyclin D1 and cyclin E were induced by E₂ in primary and tumor urothelial cells. During G1 phase, the cancer cells constitutively expressed much higher

levels of cyclin D1 and cyclin E than primary cells. Our results suggest that both cyclin D1 and cyclin E play important roles in mediating E₂-induced cell cycle progression. The higher expression of cyclin D1 and cyclin E during G1/S phases may contribute to dysregulated growth in tumor cells. Our results are not in agreement with studies showing that ER α and ER β have opposing actions on expression of cyclins, in which ER α is reported to be an activator, while ER β is repressive (Liu *et al.* 2002, Helguero *et al.* 2005). Estrogen-activated non-genomic pathways, particularly MAPK, have been shown to play an important role in cell proliferation of non-reproductive tissues (Mendelsohn 2002, Song & Santen 2006). It has been observed in other cell types that estrogen may induce G1/S transition via non-genomic pathways entailing activation of PI3/akt and Ras/MAPK cascades that result in increased expression of cyclin D1 and decreased nuclear localization of p27 respectively (Castoria *et al.* 2004). Our data indicate that estrogen-dependent activation of ERK does not have this effect on urothelial cells. However, there is an ever-increasing number of proposed non-genomic effects of estrogen on cellular function, and while the time course of cellular response to estrogen seems to make this unlikely, our experiments certainly do not completely exclude the possibility of a role for non-genomic pathways in the response of urothelial cells to estrogen.

In summary, we observed that ER α expression is significantly greater in immortalized urothelial and bladder cancer cells than in primary urothelial cells, but all of these cells expressed similar levels of ER β . Abundance of ER α mRNA was greater in neoplastic than histologically normal tissues when matched tissue samples from patients with transitional cell carcinomas were compared. Our results suggest that both ER α and ER β contribute to estrogen-induced G1/S phase progression and cell proliferation in urothelial cells, increased ER α expression may induce early cyclin D1 and cyclin E expression, and result in dysregulated cell proliferation in bladder cancer cells.

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References

- Castelao JE, Yuan JM, Skipper PL, Tannenbaum SR, Gago-Dominguez M, Crowder JS, Ross RK, Yu MC. Gender- and smoking-related bladder cancer risk. *Journal of National Cancer Institute*. 2001; 93:538–545.
- Castoria G, Migliaccio A, Di Domenico M, Lombardi M, de Falco A, Varricchio L, Bilancio A, Barone MV, Auricchio F. Role of atypical protein kinase C in estradiol-triggered G1/S progression of MCF-7 cells. *Molecular and Cellular Biology*. 2004; 24:7643–7653. [PubMed: 15314172]
- Cohen SM, Ellwein LB. Use of cell proliferation data in modeling urinary bladder carcinogenesis. *Environmental Health Perspectives*. 1993; 101:111–113. [PubMed: 8013397]
- Enmark E, Peltö-Huikko M, Grandien K, Lagercrantz S, Lagercrantz J, Fried G, Nordenskjöld M, Gustafsson JA. Human estrogen receptor beta-gene structure, chromosomal localization, and expression pattern. *Journal of Clinical Endocrinology and Metabolism*. 1997; 82:4258–4265. [PubMed: 9398750]
- Foley EF, Jazaeri AA, Shupnik MA, Jazaeri O, Rice LW. Selective loss of estrogen receptor beta in malignant human colon. *Cancer Research*. 2000; 60:245–248. [PubMed: 10667568]
- Hall JM, McDonnell DP. The estrogen receptor beta-isoform (ER β) of the human estrogen receptor modulates ER α transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology*. 1999; 140:5566–5578. [PubMed: 10579320]
- Hall M, Peters G. Genetic alterations of cyclins, cyclin-dependent kinases, and Cdk inhibitors in human cancer. *Advances in Cancer Research*. 1996; 68:67–108. [PubMed: 8712071]

- Helguero LA, Faulds MH, Gustafsson JA, Haldosen LA. Estrogen receptors α (ER α) and β (ER β) differentially regulate proliferation and apoptosis of the normal murine mammary epithelial cell line HC11. *Oncogene*. 2005; 24:6605–6616. [PubMed: 16007178]
- Hess RA, Gist DH, Bunick D, Lubahn DB, Farrell A, Bahr J, Cooke PS, Greene GL. Estrogen receptor (α and β) expression in the excurrent ducts of the adult male rat reproductive tract. *Journal of Andrology*. 1997; 18:602–611. [PubMed: 9432133]
- Hu YF, Lau KM, Ho SM, Russo J. Increased expression of estrogen receptor β in chemically transformed human breast epithelial cells. *International Journal of Oncology*. 1998; 12:1225–1228. [PubMed: 9592178]
- Jemal A, Siegel R, Ward E, Murray T, Xu J, Smigal C, Thun MJ. Cancer statistics, 2006. *CA: A Cancer Journal for Clinicians*. 2006; 56:106–130. [PubMed: 16514137]
- Jensen EV, Cheng G, Palmieri C, Saji S, Makela S, Van Noorden S, Wahlstrom T, Warner M, Coombes RC, Gustafsson JA. Estrogen receptors and proliferation markers in primary and recurrent breast cancer. *PNAS*. 2001; 98:15197–15202. [PubMed: 11734621]
- Kaufmann O, Baume H, Dietel M. Detection of oestrogen receptors in non-invasive and invasive transitional cell carcinomas of the urinary bladder using both conventional immunohistochemistry and the tyramide staining amplification (TSA) technique. *Journal of Pathology*. 1998; 186:165–168. [PubMed: 9924432]
- Kelsey JL, Moore AS, Glickman LT. Epidemiologic studies of risk factors for cancer in pet dogs. *Epidemiologic Reviews*. 1998; 20:204–217. [PubMed: 9919439]
- Koike S, Sakai M, Muramatsu M. Molecular cloning and characterization of rat estrogen receptor cDNA. *Nucleic Acids Research*. 1987; 15:2499–2513. [PubMed: 3031601]
- Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiao AK, Uht RM, Webb P. Estrogen receptor pathways to AP-1. *Journal of Steroid Biochemistry and Molecular Biology*. 2000; 74:311–317. [PubMed: 11162939]
- Lindberg MK, Moverare S, Skrtic S, Gao H, Dahlman-Wright K, Gustafsson JA, Ohlsson C. Estrogen receptor (ER)- β reduces ER α -regulated gene transcription, supporting a 'ying yang' relationship between ER α and ER β in mice. *Molecular Endocrinology*. 2003; 17:203–208. [PubMed: 12554748]
- Liu MM, Albanese C, Anderson CM, Hilty K, Webb P, Uht RM, Price RH Jr, Pestell RG, Kushner PJ. Opposing action of estrogen receptors α and β on cyclin D1 gene expression. *Journal of Biological Chemistry*. 2002; 277:24353–24360. [PubMed: 11986316]
- Liu W, Konduri SD, Bansal S, Nayak BK, Rajasekaran SA, Karuppayil SM, Rajasekaran AK, Das GM. Estrogen receptor- α binds p53 tumor suppressor protein directly and represses its function. *Journal of Biological Chemistry*. 2006; 281:9837–9840. [PubMed: 16469747]
- Lukas J, Bartkova J, Bartek J. Convergence of mitogenic signalling cascades from diverse classes of receptors at the cyclin D-cyclin-dependent kinase-pRb-controlled G1 checkpoint. *Molecular and Cellular Biology*. 1996; 16:6917–6925. [PubMed: 8943347]
- Makela S, Strauss L, Kuiper G, Valve E, Salmi S, Santti R, Gustafsson JA. Differential expression of estrogen receptors α and β in adult rat accessory sex glands and lower urinary tract. *Molecular and Cellular Endocrinology*. 2000; 164:109–116. [PubMed: 11026563]
- Manavathi B, Kumar R. Steering estrogen signals from the plasma membrane to the nucleus: two sides of the coin. *Journal of Cellular Physiology*. 2006; 207:594–604. [PubMed: 16270355]
- Mendelsohn ME. Genomic and nongenomic effects of estrogen in the vasculature. *American Journal of Cardiology*. 2002; 90:3F–6F.
- Meyers MJ, Sun J, Carlson KE, Marriner GA, Katzenellenbogen BS, Katzenellenbogen JA. Estrogen receptor- β potency-selective ligands: structure–activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. *Journal of Medicinal Chemistry*. 2001; 44:4230–4251. [PubMed: 11708925]
- Mosselman S, Polman J, Dijkema R. ER β : identification and characterization of a novel human estrogen receptor. *FEBS Letters*. 1996; 392:49–53. [PubMed: 8769313]
- Musgrove EA, Hamilton JA, Lee CS, Sweeney KJ, Watts CK, Sutherland RL. Growth factor, steroid, and steroid antagonist regulation of cyclin gene expression associated with changes in T-47D

- human breast cancer cell cycle progression. *Molecular and Cellular Biology*. 1993; 13:3577–3587. [PubMed: 8497271]
- O'Neill PA, Davies MP, Shaaban AM, Innes H, Torevell A, Sibson DR, Foster CS. Wild-type oestrogen receptor beta (ER β 1) mRNA and protein expression in Tamoxifen-treated post-menopausal breast cancers. *British Journal of Cancer*. 2004; 91:1694–1702. [PubMed: 15477865]
- Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ, Scanlan TS. Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science*. 1997; 277:1508–1510. [PubMed: 9278514]
- Prall OW, Rogan EM, Musgrove EA, Watts CK, Sutherland RL. c-Myc or cyclin D1 mimics estrogen effects on cyclin E-Cdk2 activation and cell cycle reentry. *Molecular and Cellular Biology*. 1998; 18:4499–4508. [PubMed: 9671459]
- Prins GS, Marmar M, Woodham C, Chang W, Kuiper G, Gustafsson JA, Birch L. Estrogen receptor-beta messenger ribonucleic acid ontogeny in the prostate of normal and neonatally estrogenized rats. *Endocrinology*. 1998; 139:874–883. [PubMed: 9492016]
- Pujol P, Rey JM, Nirde P, Roger P, Gastaldi M, Laffargue F, Rochefort H, Maudelonde T. Differential expression of estrogen receptor-alpha and -beta messenger RNAs as a potential marker of ovarian carcinogenesis. *Cancer Research*. 1998; 58:5367–5373. [PubMed: 9850067]
- Roger P, Sahla ME, Makela S, Gustafsson JA, Baldet P, Rochefort H. Decreased expression of estrogen receptor beta protein in proliferative preinvasive mammary tumors. *Cancer Research*. 2001; 61:2537–2541. [PubMed: 11289127]
- Sabbah M, Courilleau D, Mester J, Redeuilh G. Estrogen induction of the cyclin D1 promoter: involvement of a cAMP response-like element. *PNAS*. 1999; 96:11217–11222. [PubMed: 10500157]
- Shen SS, Smith CL, Hsieh JT, Yu J, Kim IY, Jian W, Sonpavde G, Ayala GE, Younes M, Lerner SP. Expression of estrogen receptors-alpha and -beta in bladder cancer cell lines and human bladder tumor tissue. *Cancer*. 2006; 106:2610–2616. [PubMed: 16700038]
- Shin KY, Kong G, Kim WS, Lee TY, Woo YN, Lee JD. Overexpression of cyclin D1 correlates with early recurrence in superficial bladder cancers. *British Journal of Cancer*. 1997; 75:1788–1792. [PubMed: 9192983]
- Shyamala G, Ferenczy A. The nonresponsiveness of lactating mammary gland to estradiol. *Endocrinology*. 1982; 110:1249–1256. [PubMed: 7060524]
- Song RX, Santen RJ. Membrane initiated estrogen signaling in breast cancer. *Biology of Reproduction*. 2006; 75:9–16. [PubMed: 16571873]
- Stauffer SR, Coletta CJ, Tedesco R, Nishiguchi G, Carlson K, Sun J, Katzenellenbogen BS, Katzenellenbogen JA. Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists. *Journal of Medicinal Chemistry*. 2000; 43:4934–4947. [PubMed: 11150164]
- Strom A, Hartman J, Foster JS, Kietz S, Wimalasena J, Gustafsson JA. Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. *PNAS*. 2004; 101:566–1571.
- Sun CL, Yuan JM, Arakawa K, Low SH, Lee HP, Yu MC. Dietary soy and increased risk of bladder cancer: the Singapore Chinese Health Study. *Cancer Epidemiology, Biomarkers and Prevention*. 2002; 11:1674–1677.
- Sun CL, Yuan JM, Wang XL, Gao YT, Ross RK, Yu MC. Dietary soy and increased risk of bladder cancer: a prospective cohort study of men in Shanghai, China. *International Journal of Cancer*. 2004; 112:319–323.
- Syrgios KN, Karapanagiotou E, Harrington KJ. The clinical significance of molecular markers to bladder cancer. *Hybridoma and Hybridomics*. 2004; 23:335–342. [PubMed: 15684659]
- Walker BE. Renewal of cell populations in the female mouse. *American Journal of Anatomy*. 1960; 107:95–105. [PubMed: 13782694]

Figure 1.

ER α and ER β mRNAs are differentially expressed in primary, immortalized, and tumor urothelial cells. Total cellular RNA was extracted from primarily cultured human bladder urothelial cells PM1–PM3 (male donors), PF1–PF3 (female donors), human ureteral epithelial cells immortalized with human papillomavirus E6 or E7 (E6, E7), or human bladder urothelial cells immortalized with the SV40 large T antigen (UROtsa), and human bladder tumor cell lines HTB-9 and T24. Quantitative real-time PCR was performed using different specific primers and quantified from threshold cycle. Results are normalized to S26 ribosome RNA expression in the same sample. (A) The ratios of ER α /S26 mRNA in these cells are shown. Columns and vertical bars represent the mean $\pm$ S.E.M. of four separate experiments. (B) The ratios of ER β /S26 mRNA in the above cells. (C) Conventional RT-PCR for ER α , ER β , and GAPDH in these cells. Agarose gel representative of four separate experiments is shown.

Figure 2.

ERα, ERβ, and PCNA protein are differentially expressed in primary, immortalized, and tumor urothelial cells. Whole-cell lysates from primarily cultured human bladder urothelial cells, immortalized human ureteral epithelial cell lines E6 and E7, immortalized human bladder urothelial cells UROtsa, and human bladder tumor cell lines HTB-9 and T24 were blotted for human ERα, ERβ, PCNA, and α-tubulin. Blots representative of four separate experiments are shown.

Figure 3.

ERα and ERβ are differentially expressed in bladder carcinomas. (A) Immunohistochemical staining of transitional bladder carcinomas for ERα and ERβ (revealed by diaminobenzidine). Representative images of staining for ERα and ERβ from six randomly selected patients with self-matched normal tissues are shown. (a) ERα in normal urothelium, (b) ERβ in normal urothelium, (c) ERα in tumor tissue, (d) ERβ in tumor tissue. Scale bars, 50 μm. (B) The ratio of mRNA expression determined by real-time PCR in tumor tissue to self-matched normal tissue in ten randomly selected patients. Abundance of ERα mRNA was significantly greater (2.77-fold) in neoplastic relative to histologically normal tissues (paired *t*-test $P=0.000017$), but abundance of ERβ mRNA was similar in both tissues (paired *t*-test $P=0.49$). ** $P<0.01$.

Figure 4.

siRNA decreases ER mRNA expression in primary urothelial and bladder tumor cells. (A) T24 cells were co-transfected with 100 nM siER α , siER β , and siGLO. (a) Cell viability, (b) fluorescence of nuclei stained with Hoechst 33342, and (c) fluorescence of siRNAs in the same cells. (B) Cells were transiently transfected with 100 nM siCon, 100 nM siER α , 100 nM siER β , or 100 nM siER α +100 nM siER β . The ratio of ER α /S26 and ER β /S26 mRNAs are shown. Data represent the mean $\pm$ S.E.M. of four different primary cultures or four separate cDNA preparations from T24 cells. * P <0.05 compared with siCon treated same type of cells. (C) Conventional RT-PCR for ER α , ER β , and GAPDH in these cells. A gel representative of four separate experiments is shown.

Figure 5. siRNA decreases ER protein expression in primary urothelial and bladder tumor cells. Cells were transfected with 100 nM siCon, 100 nM siERα, 100 nM siERβ, or 100 nM siERα+100 nM siERβ. Whole-cell lysates were blotted for ERα, ERβ, and α-tubulin. Blots representative of four separate experiments are shown.

Figure 6. siER β and siER α inhibits estrogen-induced cell proliferation in primary urothelial and bladder cancer cells respectively. Cells were transfected with 100 nM siCon, 100 nM siER α , 100 nM siER β , or 100 nM siER α +100 nM siER β following serum starvation for 24 h. Cells were then treated with 10 nM 17 β -estradiol, 10 nM PPT, or 10 nM DPN for 16 h and labeled with 0.5 μ Ci [methyl-³H] thymidine. DNA was harvested with TCA and quantified using a scintillation counter to determine DPM. Relative increases in DNA were calculated by setting the average of vehicle-treated cells in each group to 100. Columns and vertical bars represent the mean $\pm$ S.E.M. of six replicates. * P <0.05 compared with vehicle-treated cells.

Figure 7.

E_2 induce ERK phosphorylation but this is independent of E_2 -induced cell proliferation. Cells were seeded at equal density, serum starved for 24 h, and then treated with 10 nM E_2 for up to 60 min in the presence or absence of 3 μ M U0126. (A) Whole-cell extracts were blotted for p-ERK and ERK-1. Blots representative of four separate experiments are shown. (B) Expression of p-ERK at different time points. The intensity of p-ERK bands was normalized to the intensity of ERK-1 in the same sample. * P <0.05. (C) [3 H] thymidine incorporation into E_2 -treated cells for 16 h in the presence or absence of 3 μ M U0126. * P <0.05 compared with vehicle-treated cells. # P <0.05 compared with E_2 -treated cells.

Figure 8.

Induction of cyclin D1 and cyclin E by E₂ in primary urothelial and bladder tumor cells in a time-dependent manner. Cells were seeded at equal density, serum starved for 24 h, and then treated with 10 nM E₂ for up to 24 h. Whole-cell lysates were blotted for cyclin D1, cyclin E, and α-tubulin. (A) Blots representative of four separate experiments are shown. (B) Expression of cyclin D1 and cyclin E at different time points in E₂-treated cells. The average of normalized intensity from control cells was arbitrarily set at 100%. Each point and vertical bars represent the mean±S.E.M. of four separate experiments. *P*<0.05 at 4, 6, and 12 h compared with vehicle-treated cells.

Figure 9.

E₂ stimulates cyclin D1 and cyclin E expression in primary urothelial and bladder tumor cells. Cells were seeded at equal density, serum starved for 24 h, and then treated with 10 nM E₂, 100 nM ICI 182 780, or a combination of the two compounds. Whole-cell lysates were blotted for cyclin D1, cyclin E, and α-tubulin. (A) Blots representative of four separate experiments are shown. (B) Relative intensity of cyclin D1 and cyclin E. The average density of normalized control samples was arbitrarily set at 100%. Columns and vertical bars represent the mean±S.E.M. of four separate experiments. **P*<0.05 compared with control, and #*P*<0.05 compared with cells in the absence of ICI 182 780.

Figure 10.

Cyclin D1 and cyclin E are differentially expressed in primary and bladder tumor cells at different phases of the cell cycle. Cells were stained with Hoechst 33342, and live diploids at G0/G1, S, and G2/M phases of the cell cycle were collected into separate tubes with a BD FACSCalibur. Whole-cell lysates from 2000 sorted cells were sequentially blotted for cyclin D1, cyclin E, and α -tubulin. Blots representative of three separate experiments are shown in (A). The relative abundance of cyclin D1 or cyclin E at different cell phases is shown in (B) by arbitrarily setting the intensity of primary cells at G0/G1 phases at 100%. Data represent the mean $\pm$ S.E.M. of three separate experiments. * $P < 0.05$.

Table 1

Population-doubling time for human urothelial cells under normal culture conditions

Cell type	Passage	Doubling time under normal culture conditions (h)
Human urothelial cells primarily cultured from ureter	1–3	48–60
Human urothelial cells primarily cultured from ureter	4–7	24–36
Human urothelial cells primarily cultured from bladder	1–3	60–90
Human urothelial cells primarily cultured from bladder	4–7	36–48
E6, E7, and urotsa immortalized cells, HTB-9	All	24
T24 cells	All	16

Table 2

Changes in cell cycle phase distribution following estrogen treatments

Primary HBUC	% DNA			% DNA		
	G0/G1	S	G2/M	T24 cells	G0/G1	S
Control	68.9	24.8	6.4	Control	65.7	32.3
E ₂ 10 nM	59.1	40.9	0.1	E ₂ 10 nM	54.8	45.1
ICI 100 nM	71.2	22.4	6.4	ICI 100 nM	64.0	33.5
E ₂ +ICI	67.5	21.6	10.9	E ₂ +ICI	59.6	32.7
PPT 10 nM	62.5	27.4	10.2	PPT 10 nM	55.4	44.6
DPN 10 nM	60.0	40.0	0.0	DPN 10 nM	57.5	38.9
siControl	63.7	24.7	11.6	siControl	65.2	34.8
siER α 25 nM	66.5	17.7	15.7	siER α 25 nM	66.7	27.4
siER β 25 nM	64.8	20.3	14.9	siER β 25 nM	65.2	30.8
siER α +PPT	65.4	20.2	14.4	siER α +PPT	65.2	30.7
siER β +DPN	62.3	25.5	12.2	siER β +DPN	64.3	31.2

Representative results from three separate experiments. Cells were treated with estrogen or combinations of agonists and antagonists in the absence or presence of siRNAs for 16 h and stained with Hoechst 33342. The proportion of live diploids in G0/G1, S, and G2/M phases of the cell cycle was determined with a BD LSR II cytometer. Data were analyzed by BD Cell Quest.