

Estrogen Receptor β Inhibits Human Breast Cancer Cell Proliferation and Tumor Formation by Causing a G₂ Cell Cycle Arrest

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ABSTRACT

Studies indicate that estrogen receptor (ER) α mediates breast cancer-promoting effects of estrogens. The role of ER β in breast cancer is unknown. Elucidating the role of ER β in the pathogenesis of breast cancer is important because many human breast tumors express both ER α and ER β . We show that adenovirus-mediated expression of ER β changes the phenotype of ER α -positive MCF-7 cells. Estradiol increases cell proliferation and causes tumor formation of MCF-7 cells expressing only ER α . In contrast, introducing ER β into MCF-7 cells causes an inhibition of proliferation *in vitro* and prevents tumor formation in a mouse xenograft model in response to estradiol. ER β inhibits proliferation by repressing c-myc, cyclin D1, and cyclin A gene transcription, and increasing the expression of p21^{Cip1} and p27^{Kip1}, which leads to a G₂ cell cycle arrest. These results demonstrate that ER α and ER β produce opposite effects in MCF-7 cells on cell proliferation and tumor formation. Natural or synthetic ER β -selective estrogens may lack breast cancer promoting properties exhibited by estrogens in hormone replacement regimens and may be useful for chemoprevention of breast cancer.

INTRODUCTION

Clinical, epidemiological, and biological evidence indicate that estrogens participate in the initiation and progression of breast cancer (1–3). The Women's Health Initiative Trial provided the most definitive evidence that estrogens in hormone replacement therapy increase the incidence of breast cancer (4). Estrogen effects are mediated through two estrogen receptors (ERs), ER α and ER β (5–8). Understanding the role of each ER in the pathogenesis of breast cancer is vital, because an urgent need exists to develop estrogens for long-term hormone replacement therapy that do not promote breast cancer.

ER knockout mice clearly indicate that ER α or ER β have distinct roles in breast development. ER α knockout mice have primitive mammary development (9), whereas ER β knockout mice develop normal mammary glands (10). These observations demonstrate that only ER α is required for growth and differentiation of the mouse mammary gland. The precise roles of ER α or ER β in breast cancer are unknown. Some studies indicate that ER α mediates the tumor promoting effects of estrogens. Estradiol stimulates proliferation of MCF-7 breast cancer cells that express only ER α (11). A MCF-7 cell line that lost ER α does not proliferate with E₂, but recovers its capacity to proliferate when ER α is reintroduced (12). A mutation in ER α has been identified in human premalignant breast hyperplasias that leads to enhanced proliferation of MCF-7 cells in response to E₂ (13).

The role of ER β in breast cancer remains elusive (14, 15). Most studies correlating the presence of ERs in human breast tumors with clinical outcomes use antibodies that only detect ER α . However, more recent studies demonstrate that ER β is also expressed in many human breast tumors (14). Approximately 70% of breast tumors express ER β , and most tumors coexpress both ER α and ER β (16, 17). Several studies indicate that ER β expression in human breast tumors is associated with a poorer prognosis, compared with tumors that only express ER α (18). ER β expression is associated with elevated cell proliferation markers, Ki67 and cyclin A, in human breast tumors (19). ER β mRNA is also elevated significantly in the tamoxifen-resistant tumors compared with tamoxifen-sensitive tumors (20). These studies suggest that ER β may promote cell proliferation and breast tumor formation.

In contrast, other studies indicate that the presence of ER β in breast tumors confers a more favorable prognosis compared with tumors that contain only ER α (21). The levels of ER β are highest in normal mammary tissue and it decreases as tumors progress from preinvasive to invasive tumors (22, 23). ER β expression is associated with negative axillary node status, low-grade tumors, and low S phase fraction (24), and a greater disease-free survival rate (21). ER β expression also showed a strong association with the presence of progesterone receptors and well-differentiated breast tumors (25). The presence of ER β in >10% of cancer cells confers a better survival in women treated with tamoxifen (26). These studies indicate that ER β may function as a tumor suppressor and that the loss of ER β promotes breast carcinogenesis. Clearly, additional studies are needed to clarify the role of ER β in breast cancer. Because many breast tumors express both ER α and ER β , we investigated the effects of ER β on proliferation and tumor formation of MCF-7 breast cancer cells that contain only ER α .

MATERIALS AND METHODS

Cell Proliferation Assays. Adenoviruses (Ads) expressing human ER α or ER β (530 amino acids) were prepared according to the manufacturer's protocol (BD Biosciences Clontech, Palo Alto, CA). The control virus, Ad-lacZ, was purchased from BD Biosciences Clontech. MCF-7 breast cancer cells were cultured in phenol red-free DMEM/F-12 medium containing 5% fetal bovine serum, 2 mM glutamine, 50 units/ml penicillin, and 50 μ g/ml streptomycin. Before the addition of 17 β -estradiol (E₂), cells were grown in DMEM/F-12 medium containing 4% stripped fetal bovine serum for 1 week. Cells (5000) were plated in 24-well plates and treated with vehicle or 1 nM E₂ for 10 days. [³H]thymidine incorporation was used to quantify DNA synthesis. All of the experiments were done at least three times, and the data were similar between experiments. The data points were done in triplicate, and SE was <10%.

Xenograft Studies in Nude Mice. MCF-7 cells grown in DMEM/F-12 medium containing 4% stripped fetal bovine serum were infected with Ads for 24 h. The cells were collected, and 250,000 cells were aggregated in suspension and then resuspended in 200 μ l of neutralized collagen (27). After an overnight incubation, the cells were then grafted under the kidney capsule of nude mouse as described and illustrated in detail elsewhere.⁵ One month after grafting, tumors were harvested, fixed in 10% phosphate-buffered formalin (Fisher Scientific, Fairlawn, NJ), embedded in paraffin, sectioned, and stained

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⁵ Internet address: <http://mammary.nih.gov/tools/mousework/Cunha001/index/html>.

with H&E. Immunohistochemistry of paraffin sections of the tumors was done with Ki67 antibodies (Novocastra Laboratories Ltd., Newcastle, United Kingdom), and the proliferation index was determined as described (27). The animal studies were carried out with approval from the University of California, San Francisco committee on animal care.

Immunoblotting. Cells infected with Ad-LacZ or Ad-ER β were grown in six-well tissue culture plates and treated with 10 nM E₂ for times indicated in the figures. At the end of treatment, proteins were extracted in radioimmuno-precipitation assay buffer containing protease and phosphatase inhibitors. Immunoblotting of proteins was performed using standard procedures using antibodies to ER α (DAKO Corporation, Carpinteria, CA), ER β (Genetex, San Antonio, TX), and c-myc, cyclin D1, cyclin A, and actin (Oncogene Research Products, Boston, MA). Proteins were visualized using ECL kits (Amersham Life Science, Arlington Heights, IL).

Flow Cytometry. Cells infected with Ad-LacZ or Ad-ER β were treated with 1 nM E₂ for 24, 48, or 96 h. The cells were lysed in 1 ml hypotonic DNA staining solution (0.5 mg/ml propidium iodide, 0.1% sodium citrate, and 0.05% Triton X-100). Nuclear emitted fluorescence with wavelength >585 nm was measured with a Coulter Elite instrument with laser output adjusted to deliver 15 mW at 488 nm. Nuclei (10,000) were analyzed from each sample at a rate of 300–500 cells/s. The percentages of cells in the G₁, S, and G₂-M phases of the cell cycle were determined with the multicycle computer program (Phoenix Flow Systems, San Diego, CA) as described (28).

Real-Time Quantitative Reverse Transcription-PCR Analysis. Cells infected with Ad-LacZ or Ad-ER β were washed with PBS, and then 1 ml Trizol (Life Technologies, Inc., Grand Island, NY) was added to the cells. Total RNA was prepared according to the manufacturer's protocol. Real-time quantitative PCR was performed using SYBR Green Supermix (BIO-RAD, Hercules, CA) with an iCycler thermal cycler (BIO-RAD). We used the following primers: p21 Forward 5'-GGCGGCAGACCAGCATGACAGATT-3' and Reverse 5'-GCAGGGGGCGGCCAGGGTAT-3'; p27 Forward 5'-GGGGCTCGTCTTTTCGGGGTGT-3' and Reverse 5'-GAGCGGGAGGGCGGAGAGGAG-3'; c-myc Forward 5'-GCCCCCTAACGTTAGCTTCA-3' and Reverse 5'-TTCAGATATCCTCGCTGGG-3'; Cyclin D1 Forward 5'-AACTACCTGGACCGCTTCT-3' and Reverse 5'-CCACTTGAGCTTGTTACCA-3'; and GUS Forward 5'-CTCATTTGGAATTTTGCCGATT-3' and Reverse 5'-

CCGAGTGAAGATCCCCCTTTT-3'. The data were collected and analyzed using the comparative Ct (threshold cycle) method using GUS expression as the reference gene.

RESULTS

ER β Inhibits MCF-7 Cell Proliferation. We selected MCF-7 cells to introduce ER β because E₂ stimulates the proliferation of MCF-7 cells that express exclusively ER α (11), whereas ER-negative cells stably transfected with ER α display anomalous behavior, because estrogens inhibit proliferation (29). Furthermore, MCF-7 cells are the best-characterized ER-positive cell line in terms of known genes regulated by estrogens that promote proliferation. The MCF-7 cells used for these studies expressed ER α (Fig. 1A, top panel, Lane 0) by immunoblotting, but not ER β (Fig. 1A, bottom panel, Lane 0). The cells were infected for 24 h with 50 or 100 multiplicity of infection (MOI) Ad-ER α , Ad-ER β , or Ad-LacZ to control for potential nonspecific effects of the virus. The infected cells were then grown for 10 days in the absence or presence of E₂, after which DNA synthesis was measured by [³H]thymidine incorporation *in vitro*. The expression of ER β resulted in a 48% reduction in cell proliferation of MCF-7 cells in the absence of E₂ compared with cells infected with 50 MOI of Ad-LacZ (Fig. 1B). E₂ augmented the inhibition of cell proliferation to 71% in the Ad-ER β -infected cells. Similar results were observed using 100 MOI of Ad-ER β (Fig. 1B).

ER β Induces a G₂ Arrest of MCF-7 Cells. To explore the mechanism whereby ER β inhibits proliferation, we studied the effect of expressing ER β on the cell cycle. MCF-7 cells were infected with 50 MOI Ad-LacZ or Ad-ER β and then treated with E₂. The DNA content in the infected cells was measured by flow cytometry. The data obtained from these studies demonstrate that ER β induces a G₂ cell cycle arrest. At 96 h, flow cytometric analysis of cells infected with Ad-ER β showed about a 4-fold increase in the percentage of cells in

Fig. 1. Estrogen receptor (ER) β inhibits proliferation of MCF-7 cells by inducing a G₂ cell cycle arrest. A, MCF-7 cells express ER α , but not ER β . MCF-7 cells were infected with 0, 50, or 100 multiplicity of infection (MOI) adenovirus (Ad)-LacZ or Ad-ER β for 24 h, and cellular lysates were immunoblotted for the presence of ER α (top panel) or ER β (bottom panel). The positive ER β control (+) is from cellular lysates of U2OS cells stably transfected with ER β . B, effect of ER β on proliferation of MCF-7 cells. Cells were infected with Ad-LacZ, Ad-ER α , or Ad-ER β and then grown for 10 days in the absence (□) or presence of 1 nM E₂ (■). DNA synthesis was determined by [³H]thymidine incorporation in triplicate samples. The data are expressed as % growth inhibition of cells infected with ER α or Ad-ER β relative to cells infected with Ad-LacZ; bars, $\pm$ SE. C, effect of ER β on cell cycle in MCF-7 cells. Cells were infected with 50 MOI Ad-LacZ or Ad-ER β and then treated with vehicle or 1 nM E₂ for 96 h, and the cell cycle profile was determined using flow cytometry.

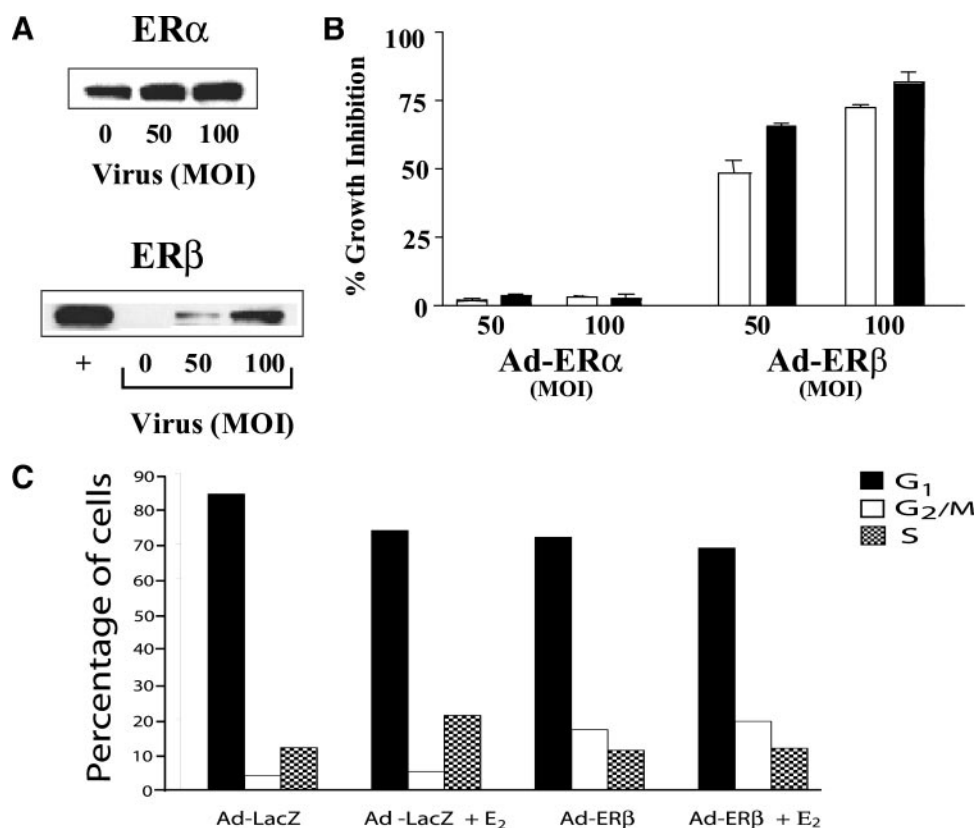
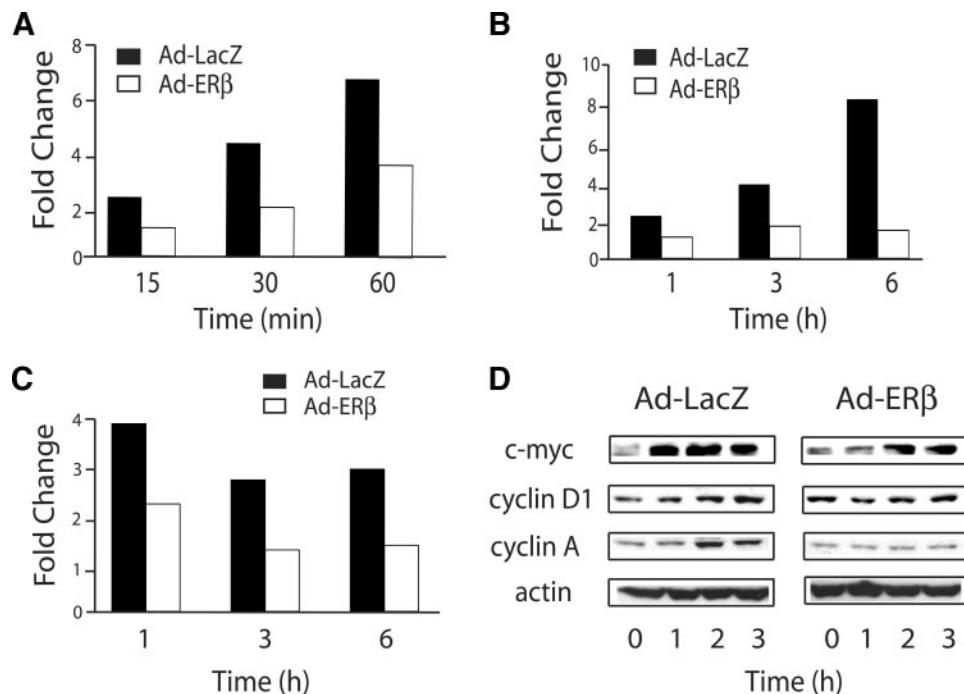


Fig. 2. Estrogen receptor (ER) β down-regulates c-myc, cyclin D1, and cyclin A in MCF-7 cells. MCF-7 cells infected with adenovirus (Ad)-LacZ (■) or Ad-ER β (□) were treated with vehicle or 10 nM E₂ for the times indicated in the figures, and total RNA was isolated. A, real-time quantitative PCR for c-myc expression. B, real-time quantitative PCR for cyclin D1 expression. C, real-time quantitative PCR for cyclin A expression. D, immunoblot of c-myc, cyclin D1, cyclin A, and actin. MCF-7 cells infected with Ad-LacZ or Ad-ER β were treated with vehicle or 10 nM E₂ for the indicated times, and cellular lysates were immunoblotted with antibodies against c-myc, cyclin D1, cyclin A, or β -actin. Lanes were loaded with 20 μ g of cellular proteins.



G₂-M phase (17%) compared with cells infected with Ad-LacZ (5%; Fig. 1C). The addition of E₂ produced an additional increase in G₂-M cells to 19.5%. Similar results were observed after 24 and 48 h of treatment with E₂.⁶ These results indicate that ER β inhibits proliferation of MCF-7 cells by causing a G₂ cell cycle arrest independently of E₂ and that E₂ produces a modest G₂ arrest enhancement.

ER β Down-Regulates c-Myc, Cyclin D1, and Cyclin A. Estrogens regulate the production of multiple proteins involved in cell proliferation and cell cycle regulation (30). Cyclin D1 (31), cyclin A (32), and c-myc (33) are known to be estrogen-inducible genes in MCF-7 cells. These genes are likely targets for ER β to cause inhibition of cell proliferation and cell cycle arrest, because they cause quiescent cells to progress through the cell cycle. Cyclin D1 interacts with cyclin-dependent kinase 4 and 6, which causes progression through G₁, whereas cyclin A interacts with cyclin-dependent kinase 2 to promote the transition from the S phase to G₂ (34). To investigate whether ER β regulates cyclin D1, cyclin A, or c-myc gene expression, we infected MCF-7 cells with Ad-LacZ or Ad-ER β and then measured mRNA levels by real-time quantitative PCR and protein levels by immunoblotting. E₂ produced a time-dependent increase in c-myc, cyclin D1, and cyclin A mRNA (Fig. 2, A, B, and C, respectively) and protein levels (Fig. 2D) in cells infected with Ad-LacZ. The increase in gene expression and protein production is mediated by ER α because only ER α is expressed in these cells (11). ER β inhibited the induction of c-myc, cyclin D1, and cyclin A mRNA (Fig. 2, A, B, and C, respectively) and protein by immunoblotting (Fig. 2D).

ER β Increases Transcription of p21 and p27. The down-regulation of c-myc is likely to be a key mechanism whereby ER β inhibits proliferation and tumor formation. One mechanism by which c-myc induces proliferation involves the repression of the cyclin-dependent kinase cyclin inhibitors genes, p21 and p27 (35). Carroll *et al.* (36) found that antisense oligonucleotides to c-myc prevented E₂-induced proliferation of MCF-7 cells and caused a cell cycle arrest by enhancing p21 synthesis. We hypothesized that the repression of c-myc by ER β induces a G₂ arrest by increasing the production of p21 and p27.

To test this hypothesis, the effects of ER β on p21 and p27 gene expression were examined in MCF-7 cells infected with Ad-LacZ or Ad-ER β . ER β produced a ligand-independent increase of p21 and p27 mRNA (Fig. 3A) and protein (Fig. 3B) levels in MCF-7 cells. These results indicate that ER β reduces MCF-7 cell proliferation by inhibiting the induction of cell proliferation genes and activating the antiproliferation genes, p21 and p27.

ER β Prevents Tumor Formation in Mouse Xenografts. We next explored the effects of expressing ER β on tumor formation in a mouse xenograft model. MCF-7 cells infected with Ads that express LacZ, ER α , or ER β were initially aggregated, then resuspended in polymerized collagen gel and grafted under the kidney capsule of female nude mice. The mice were also implanted with an estrogen pellet to stimulate tumor cell growth. One-month after the cells were grafted, tumors of comparable size developed (Fig. 4A) from uninfected MCF-7 cells, and cells infected with Ad-LacZ. The size of the tumor derived from cells infected with Ad-ER α were not larger than uninfected MCF-7 cells, suggesting the level of endogenous ER α is sufficient to produce a maximal stimulation of growth. ER β produced a dose-dependent inhibition of tumor formation. A small tumor developed with 50 MOI Ad-ER β , whereas no significant tumor developed from MCF-7 cells infected with 100 MOI Ad-ER β (Fig. 4A).

H&E staining and immunohistochemistry for the proliferation marker Ki67 was done to assess the histology of the tumor and magnitude of tumor cell proliferation, respectively. A large tumor of MCF-7 cells formed from noninfected cells (first row) and cells infected with Ad-LacZ or Ad-ER α (Fig. 4B) as measured by H&E staining. In contrast, no significant tumor was observed in MCF-7 cells infected with ER β by H&E staining. The Ki67 proliferation index found that ~70% of noninfected MCF-7 cells and cells infected with Ad-LacZ or Ad-ER α stained for Ki67 compared with 5% of cells infected with Ad-ER β (Fig. 4C). Ki67-positive cells also stained with antibodies to keratin 8, a specific marker for MCF-7 cells, confirming that the proliferating cells were derived from the tumor.⁷ Our studies demonstrate that introducing Ad-ER β into MCF-7 cells but not

⁶ V. Kerekatte, unpublished observations.

⁷ H. Parmar, unpublished observations.

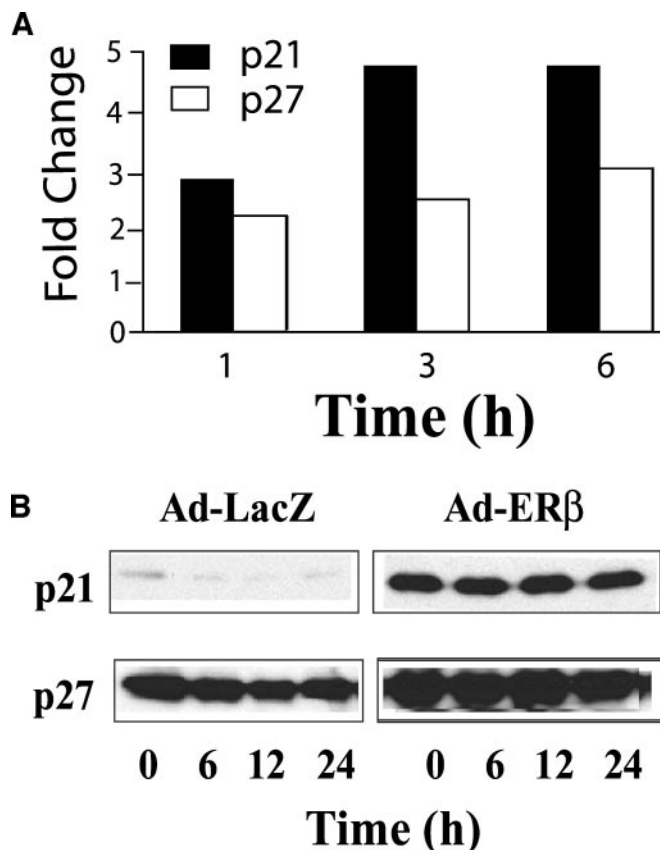


Fig. 3. Estrogen receptor (ER) β activates p21 and p27 gene expression in MCF-7 cells. A, real-time quantitative PCR for p21 (■) or 27 (□) expression. MCF-7 cells infected with adenovirus (Ad) -LacZ or Ad-ER β were treated with vehicle or 10 nM E₂ for the indicated times and total RNA was isolated. B, immunoblot of p21 and p27. MCF-7 cells infected with Ad-LacZ or Ad-ER β were treated with vehicle or 10 nM E₂ for the indicated times, and cellular lysates were immunoblotted with antibodies against p21 and p27. Lanes were loaded with 20 μ g of cellular proteins.

Ad-ER α prevents tumor formation in mouse xenografts. Similar levels of expression of ER α and ER β from the Ads were detected in the infected cells by immunoblots (Fig. 1A) making it unlikely that our results are due to overexpression and nonspecific squelching of co-factors or transcription factors by ER β . Furthermore, if squelching was the mechanism whereby ER β prevents tumor formation then similar results should have been observed with cells infected with Ad-ER α .

DISCUSSION

ER knockout mice clearly indicate that ER α and ER β have distinct roles in mammary gland development (9). However, the exact role of ER α and ER β in the pathogenesis of breast cancer is unclear. Whereas many breast tumors express ER β , it is unclear if ER β participates in breast carcinogenesis, tumor progression, or resistance to antiestrogens. Furthermore, studies correlating the presence of ER β in human breast tumors with prognosis have been inconsistent. In this study, we used another approach to explore the role of ER β in breast cancer by introducing ER β into MCF-7 cells that express exclusively ER α to mimic the majority of ER-positive breast tumors. Our studies provide additional evidence that ER α and ER β have distinct roles in breast cancer cells. We found that ER α promotes proliferation in MCF-7 cells, whereas ER β inhibits cell proliferation and tumor formation. The opposite effects exhibited ER α and ER β on breast cancer cells are consistent with the proposal by Weihua *et al.* (37) that ER α and ER β have yin/yang relationship in some tissues. The growth inhibi-

tory effects of ER β suggest that ER β functions as a tumor suppressor in breast cells. A tumor suppressor function for ER β is consistent with the observations that ER β knockout mice develop prostate hyperplasia (10) and leukemia (38).

ER β can form a heterodimer with ER α (39, 40) to inhibit ER α -mediated transcriptional activation of a classical estrogen response element (41, 42) and the cyclin D1 promoter (43). These results indicate that ER β might inhibit cell proliferation and tumor formation of MCF-7 cells by functioning as a dominant negative of ER α -mediated induction of growth promoting genes, such as cyclin D1 (43), cyclin A, and c-myc. However, we found that ER β did not inhibit some other genes induced by ER α ,⁸ demonstrating that ER β does not function as a dominant negative of all ER α -inducible genes. ER β also inhibits proliferation of an ER-negative breast cancer cell (44). Furthermore, we found that most genes regulated by ER α in response to E₂ are distinct from those regulated by ER β in U2OS osteosarcoma cells.⁹ These observations suggest that in addition to antagonizing the effects of ER α , ER β may inhibit cell proliferation by directly regulating distinct genes or by exerting other mechanisms independent of ER α .

The inhibition of cell proliferation by ER β and activation of p21 and p27 was predominantly or totally ligand independent, respectively. It is possible that this observation results from residual E₂ remaining in stripped serum or retained in cells infected with ER β . Alternatively, several studies indicate that steroid receptors can elicit ligand-independent effects. Ciana *et al.* (45) found that ERs can activate gene transcription in the absence of ligand in a transgenic mouse model engineered with an estrogen response element-luciferase construct. These studies found that the luciferase reporter was active in some nonreproductive tissues in ovariectomized adult mice and immature mice that do not produce estrogens. Progesterone receptor A receptor also regulates several genes in the absence of ligand (46).

Our studies demonstrate that ER β changes the phenotype of MCF-7 cells in response to E₂. In ER α -expressing MCF-7 cells, E₂ causes proliferation and tumor formation. In contrast, when ER β is expressed along with ER α , MCF-7 cells are directed to antiproliferation and antitumor pathways even in the presence of estrogens. These results suggest that ER β can alter the response to estrogens and provide a possible explanation for the findings that ER β expression in breast tumors is associated with a more favorable prognosis (21–24). A potentially important clinical application of our studies is that ER β -selective estrogens may be more potent at eliciting antiproliferative pathways compared with ER nonselective estrogens, such as E₂, that also activate ER α . High intake of dietary plant estrogens (phytoestrogens) is associated with a low incidence of breast cancer (47, 48). Whereas E₂ binds equally to ER α and ER β , phytoestrogens selectively bind to ER β (49, 50) and recruit coregulators to ER β to trigger transcriptional activation and repression (11). Our results indicate that phytoestrogens and estrogens designed to selectively trigger ER β transcriptional pathways might not promote breast cancer, making them a safer alternative to estrogens used in current hormone replacement therapy formulations.

Selective ER modulators, such as tamoxifen and raloxifene, reduce the incidence of ER-positive breast cancer tumors (51, 52). These drugs block transcriptional activation of growth promoting genes by recruiting corepressor proteins to ERs bound to the promoter region (53, 54). However, selective ER modulators are not ideal drugs for breast cancer chemoprevention, because they can cause serious ad-

⁸ S. Paruthiyil, unpublished observations.

⁹ M. K. Tee *et al.* Estradiol and selective estrogen receptor modulators differentially regulate target genes with estrogen receptors α and β . *Mol. Biol. Cell.*, in press, 2004.

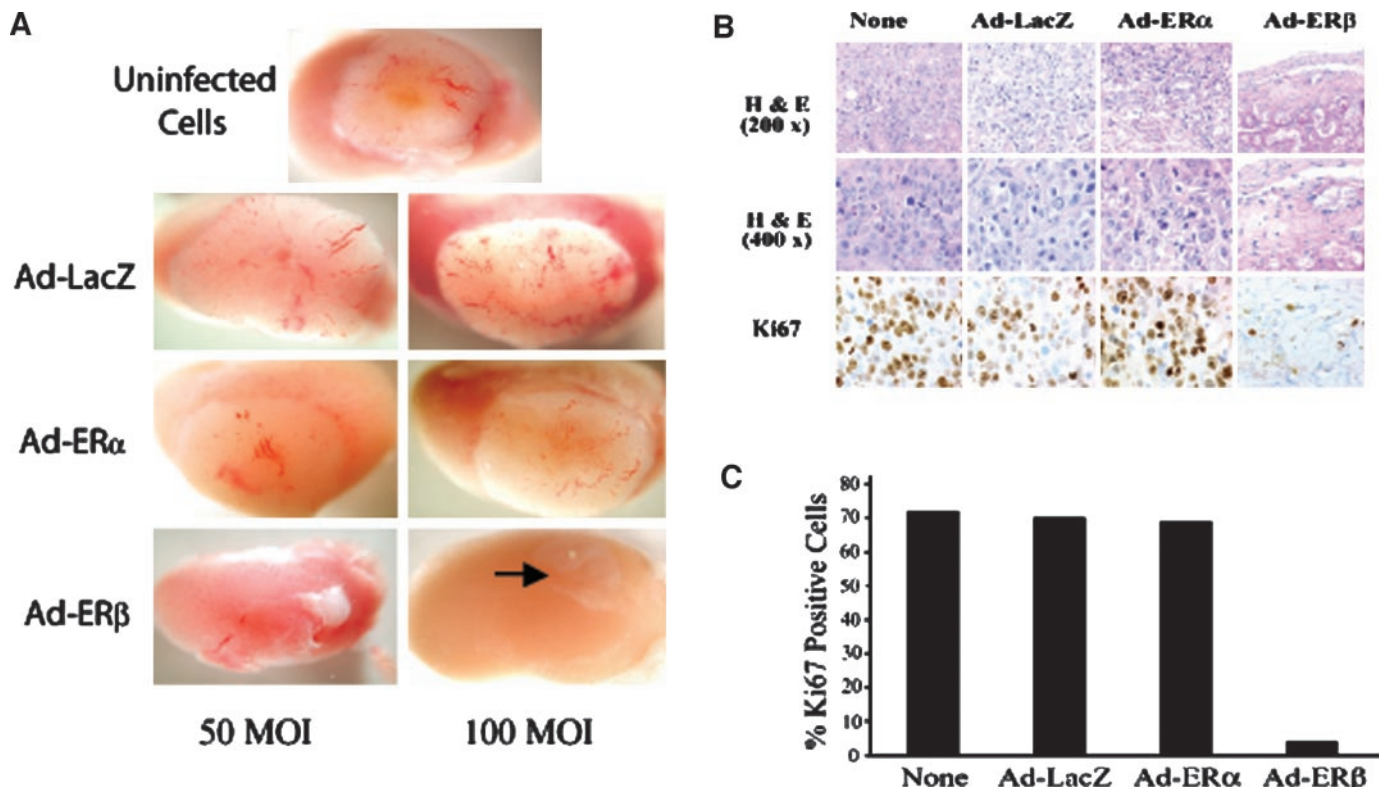


Fig. 4. Estrogen receptor (ER) β inhibits tumor formation of MCF-7 cells in mice xenografts. Uninfected MCF-7 cells, or cells infected with 50 or 100 multiplicity of infection adenovirus (Ad) -LacZ, Ad-ER α , or Ad-ER β were grafted under the kidney capsule of female nude mice. After 1 month, the tumors were removed and analyzed for size, histology, and proliferation. A, gross morphology of the xenografts. The arrow points to the site of grafting of MCF-7 cells infected with Ad-ER β . B, histology of tumors stained with H&E at $\times 200$ (top panels) or $\times 400$ (middle panels), and immunohistochemistry of tumor sections stained with an antibody to Ki67 (bottom panels). C, Ki67 proliferation index of tumor cells infected with Ad-LacZ, Ad-ER α , or Ad-ER β .

verse effects, such as thromboembolisms (51, 52). Our results showing that ER β inhibits proliferation and tumor formation of breast cancer cells suggests that dietary or synthetic ER β -selective estrogens may be an alternative to selective ER modulators for chemoprevention of breast cancer.

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