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Estrogen Receptor β Inhibits Human Breast Cancer Cell Proliferation and Tumor Formation by Causing a G_2 Cell Cycle Arrest

Sreenivasan Paruthiyil,[1,3] Hema Parmar,[2,3] Vaishali Kerekatte,[4] Gerald R. Cunha,[2,3] Gary L. Firestone,[4] and Dale C. Leitman[1,3]

[1]Departments of Obstetrics, Gynecology, and Reproductive Sciences, Cellular and Molecular Pharmacology and [2]Anatomy, and [3]Center for Reproductive Sciences, University of California, San Francisco, and [4]Department of Molecular and Cellular Biology, University of California, Berkeley, California

ABSTRACT

Studies indicate that estrogen receptor (ER) α mediates breast cancer-promoting effects of estrogens. The role of ERβ in breast cancer is unknown. Elucidating the role of ERβ in the pathogenesis of breast cancer is important because many human breast tumors express both ERα and ERβ. We show that adenovirus-mediated expression of ERβ changes the phenotype of ERα-positive MCF-7 cells. Estradiol increases cell proliferation and causes tumor formation of MCF-7 cells expressing only ERα. In contrast, introducing ERβ into MCF-7 cells causes an inhibition of proliferation *in vitro* and prevents tumor formation in a mouse xenograft model in response to estradiol. ERβ 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 G_2 cell cycle arrest. These results demonstrate that ERα and ERβ produce opposite effects in MCF-7 cells on cell proliferation and tumor formation. Natural or synthetic ERβ-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), ERα and ERβ (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 ERα or ERβ have distinct roles in breast development. ERα knockout mice have primitive mammary development (9), whereas ERβ knockout mice develop normal mammary glands (10). These observations demonstrate that only ERα is required for growth and differentiation of the mouse mammary gland. The precise roles of ERα or ERβ in breast cancer are unknown. Some studies indicate that ERα mediates the tumor promoting effects of estrogens. Estradiol stimulates proliferation of MCF-7 breast cancer cells that express only ERα (11). A MCF-7 cell line that lost ERα does not proliferate with E_2, but recovers its capacity to proliferate when ERα is reintroduced (12). A mutation in ERα has been identified in human premalignant breast hyperplasias that leads to enhanced proliferation of MCF-7 cells in response to E_2 (13).

The role of ERβ 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 ERα. However, more recent studies demonstrate that ERβ is also expressed in many human breast tumors (14). Approximately 70% of breast tumors express ERβ, and most tumors coexpress both ERα and ERβ (16, 17). Several studies indicate that ERβ expression in human breast tumors is associated with a poorer prognosis, compared with tumors that only express ERα (18). ERβ expression is associated with elevated cell proliferation markers, Ki67 and cyclin A, in human breast tumors (19). ERβ mRNA is also elevated significantly in the tamoxifen-resistant tumors compared with tamoxifen-sensitive tumors (20). These studies suggest that ERβ may promote cell proliferation and breast tumor formation.

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

MATERIALS AND METHODS

Cell Proliferation Assays. Adenoviruses (Ads) expressing human ERα or ERβ (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 μg/ml streptomycin. Before the addition of 17β-estradiol (E_2), 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 E_2 for 10 days. [^{3}H]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 μl 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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Requests for reprints: Dale Leitman, HSE 1619, Center for Reproductive Sciences, 513 Parnassus Avenue, University of California San Francisco, San Francisco, CA 94143-0556. Phone: (415) 502-5261; Fax: (415) 753-3271; E-mail: leitmand@obgyn.ucsf.edu.

[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-ERβ were grown in six-well tissue culture plates and treated with 10 nM E_2 for times indicated in the figures. At the end of treatment, proteins were extracted in radioimmunoprecipitation assay buffer containing protease and phosphatase inhibitors. Immunoblotting of proteins was performed using standard procedures using antibodies to ERα (DAKO Corporation, Carpinteria, CA), ERβ (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-ERβ were treated with 1 nM E_2 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 G_1, S, and G_2-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-ERβ 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′-GGGGCTCGTCTTTTCGGGGTGTTT-3′ and Reverse 5′GAGCGGGAGGGCGGAGAGGAG-3′; c-myc Forward 5′-GCCCCTCAACGTTAGCTTCA-3′ and Reverse 5′ TTCCAGATATCCTCGCTGGG-3′; Cyclin D1 Forward 5′-AACTACCTGGACCGCTTCCT-3′ and Reverse 5′-CCACTTGAG CTTGTTCACCA-3′; and GUS Forward 5′-CTCATTTGGAATTTTGCCGATT-3′ and Reverse 5′-CCGAGTGAAGATCCCCTTTTTA-3′. The data were collected and analyzed using the comparative Ct (threshold cycle) method using GUS expression as the reference gene.

RESULTS

ERβ Inhibits MCF-7 Cell Proliferation. We selected MCF-7 cells to introduce ERβ because E_2 stimulates the proliferation of MCF-7 cells that express exclusively ERα (11), whereas ER-negative cells stably transfected with ERα 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 ERα (Fig. 1*A*, *top panel, Lane 0*) by immunoblotting, but not ERβ (Fig. 1*A*, *bottom panel, Lane 0*). The cells were infected for 24 h with 50 or 100 multiplicity of infection (MOI) Ad-ERα, Ad-ERβ, 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 E_2, after which DNA synthesis was measured by [^{3}H]thymidine incorporation *in vitro*. The expression of ERβ resulted in a 48% reduction in cell proliferation of MCF-7 cells in the absence of E_2 compared with cells infected with 50 MOI of Ad-LacZ (Fig. 1*B*). E_2 augmented the inhibition of cell proliferation to 71% in the Ad-ERβ-infected cells. Similar results were observed using 100 MOI of Ad-ERβ (Fig. 1*B*).

ERβ Induces a G_2 Arrest of MCF-7 Cells. To explore the mechanism whereby ERβ inhibits proliferation, we studied the effect of expressing ERβ on the cell cycle. MCF-7 cells were infected with 50 MOI Ad-LacZ or Ad-ERβ and then treated with E_2. The DNA content in the infected cells was measured by flow cytometry. The data obtained from these studies demonstrate that ERβ induces a G_2 cell cycle arrest. At 96 h, flow cytometric analysis of cells infected with Ad-ERβ showed about a 4-fold increase in the percentage of cells in

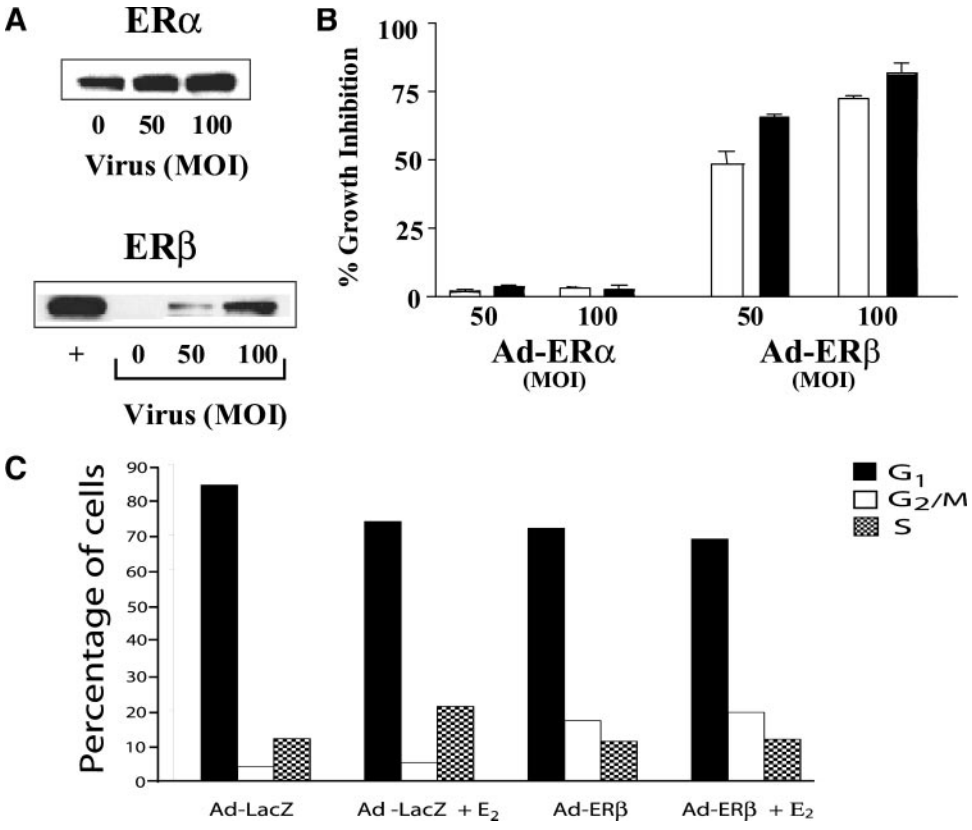

Fig. 1. Estrogen receptor (ER) β inhibits proliferation of MCF-7 cells by inducing a G_2 cell cycle arrest. *A*, MCF-7 cells express ERα, but not ERβ. MCF-7 cells were infected with 0, 50, or 100 multiplicity of infection (MOI) adenovirus (Ad) -LacZ or Ad-ERβ for 24 h, and cellular lysates were immunoblotted for the presence of ERα (*top panel*) or ERβ (*bottom panel*). The positive ERβ control (+) is from cellular lysates of U2OS cells stably transfected with ERβ. *B*, effect of ERβ on proliferation of MCF-7 cells. Cells were infected with Ad-LacZ, Ad-ERα, or Ad-ERβ and then grown for 10 days in the absence (□) or presence of 1 nM E_2 (■). DNA synthesis was determined by [^{3}H]thymidine incorporation in triplicate samples. The data are expressed as % growth inhibition of cells infected with ERα or Ad-ERβ relative to cells infected with Ad-LacZ; bars, ± SE. *C*, effect of ERβ on cell cycle in MCF-7 cells. Cells were infected with 50 MOI Ad-LacZ or Ad-ERβ and then treated with vehicle or 1 nM E_2 for 96 h, and the cell cycle profile was determined using flow cytometry.

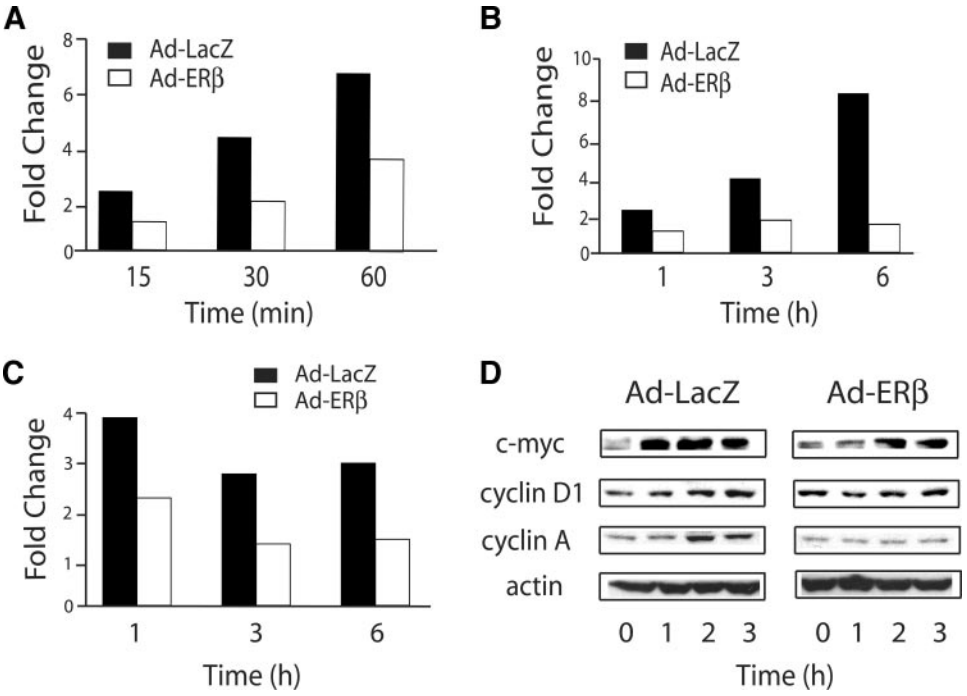

Fig. 2. Estrogen receptor (ER) β down-regulates c-myc, cyclin D1, and cyclin A in MCF-7 cells. MCF-7 cells infected with adenovirus (Ad)-LacZ (■) or Ad-ERβ (□) were treated with vehicle or 10 nM E_2 for the times indicated in the figures, and total RNA was isolated. *A*, real-time quantitative PCR for 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-ERβ were treated with vehicle or 10 nM E_2 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 μg of cellular proteins.

G_2-M phase (17%) compared with cells infected with Ad-LacZ (5%; Fig. 1*C*). The addition of E_2 produced an additional increase in G_2-M cells to 19.5%. Similar results were observed after 24 and 48 h of treatment with E_2.[6] These results indicate that ERβ inhibits proliferation of MCF-7 cells by causing a G_2 cell cycle arrest independently of E_2 and that E_2 produces a modest G_2 arrest enhancement.

ERβ 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 ERβ 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 G_1, whereas cyclin A interacts with cyclin-dependent kinase 2 to promote the transition from the S phase to G_2 (34). To investigate whether ERβ regulates cyclin D1, cyclin A, or c-myc gene expression, we infected MCF-7 cells with Ad-LacZ or Ad-ERβ and then measured mRNA levels by real-time quantitative PCR and protein levels by immunoblotting. E_2 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. 2*D*) in cells infected with Ad-LacZ. The increase in gene expression and protein production is mediated by ERα because only ERα is expressed in these cells (11). ERβ inhibited the induction of c-myc, cyclin D1, and cyclin A mRNA (Fig. 2, *A*, *B*, and *C*, respectively) and protein by immunoblotting (Fig. 2*D*).

ERβ Increases Transcription of p21 and p27. The down-regulation of c-myc is likely to be a key mechanism whereby ERβ 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 E_2-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 ERβ induces a G_2 arrest by increasing the production of p21 and p27. To test this hypothesis, the effects of ERβ on p21 and p27 gene expression were examined in MCF-7 cells infected with Ad-LacZ or Ad-ERβ. ERβ produced a ligand-independent increase of p21 and p27 mRNA (Fig. 3*A*) and protein (Fig. 3*B*) levels in MCF-7 cells. These results indicate that ERβ reduces MCF-7 cell proliferation by inhibiting the induction of cell proliferation genes and activating the antiproliferation genes, *p21* and *p27*.

ERβ Prevents Tumor Formation in Mouse Xenografts. We next explored the effects of expressing ERβ on tumor formation in a mouse xenograft model. MCF-7 cells infected with Ads that express LacZ, ERα, or ERβ 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. 4*A*) from uninfected MCF-7 cells, and cells infected with Ad-LacZ. The size of the tumor derived from cells infected with Ad-ERα were not larger than uninfected MCF-7 cells, suggesting the level of endogenous ERα is sufficient to produce a maximal stimulation of growth. ERβ produced a dose-dependent inhibition of tumor formation. A small tumor developed with 50 MOI Ad-ERβ, whereas no significant tumor developed from MCF-7 cells infected with 100 MOI Ad-ERβ (Fig. 4*A*).

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-ERα (Fig. 4*B*) as measured by H&E staining. In contrast, no significant tumor was observed in MCF-7 cells infected with ERβ by H&E staining. The Ki67 proliferation index found that ~70% of noninfected MCF-7 cells and cells infected with Ad-LacZ or Ad-ERα stained for Ki67 compared with 5% of cells infected with Ad-ERβ (Fig. 4*C*). 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-ERβ into MCF-7 cells but not

[6] V. Kerekatte, unpublished observations.

[7] H. Parmar, unpublished observations.

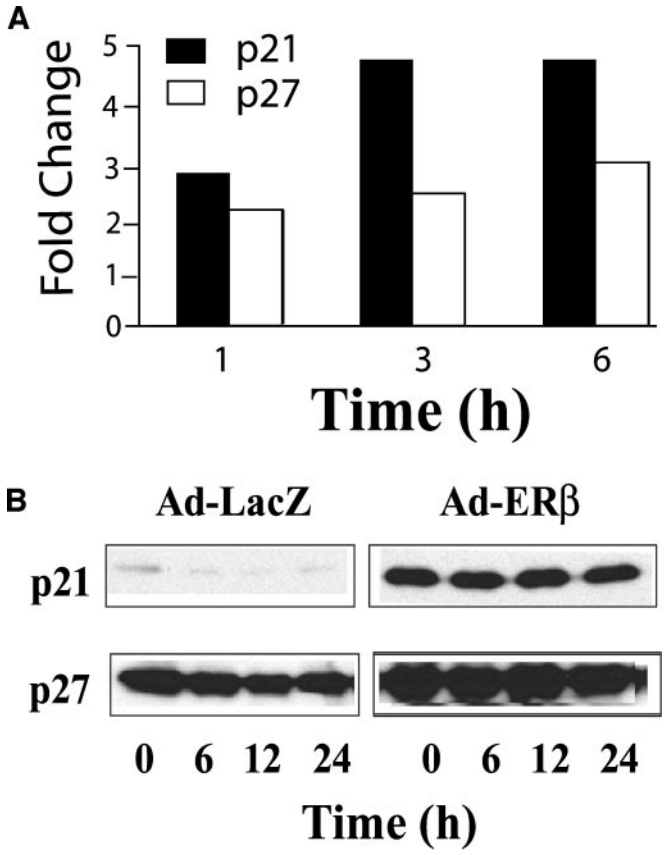

Fig. 3. Estrogen receptor (ER) β activates p21 and p27 gene expression in MCF-7 cells. *A*, real-time quantitative PCR for p21 (■) or 27 (□) expression. MCF-7 cells infected with adenovirus (Ad) -LacZ or Ad-ERβ were treated with vehicle or 10 nM E_2 for the indicated times and total RNA was isolated. *B*, immunoblot of p21 and p27. MCF-7 cells infected with Ad-LacZ or Ad-ERβ were treated with vehicle or 10 nM E_2 for the indicated times, and cellular lysates were immunoblotted with antibodies against p21 and p27. Lanes were loaded with 20 μg of cellular proteins.

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

DISCUSSION

ER knockout mice clearly indicate that ERα and ERβ have distinct roles in mammary gland development (9). However, the exact role of ERα and ERβ in the pathogenesis of breast cancer is unclear. Whereas many breast tumors express ERβ, it is unclear if ERβ participates in breast carcinogenesis, tumor progression, or resistance to antiestrogens. Furthermore, studies correlating the presence of ERβ in human breast tumors with prognosis have been inconsistent. In this study, we used another approach to explore the role of ERβ in breast cancer by introducing ERβ into MCF-7 cells that express exclusively ERα to mimic the majority of ER-positive breast tumors. Our studies provide additional evidence that ERα and ERβ have distinct roles in breast cancer cells. We found that ERα promotes proliferation in MCF-7 cells, whereas ERβ inhibits cell proliferation and tumor formation. The opposite effects exhibited ERα and ERβ on breast cancer cells are consistent with the proposal by Weihua *et al.* (37) that ERα and ERβ have yin/yang relationship in some tissues. The growth inhibitory effects of ERβ suggest that ERβ functions as a tumor suppressor in breast cells. A tumor suppressor function for ERβ is consistent with the observations that ERβ knockout mice develop prostate hyperplasia (10) and leukemia (38).

ERβ can form a heterodimer with ERα (39, 40) to inhibit ERα-mediated transcriptional activation of a classical estrogen response element (41, 42) and the cyclin D1 promoter (43). These results indicate that ERβ might inhibit cell proliferation and tumor formation of MCF-7 cells by functioning as a dominant negative of ERα-mediated induction of growth promoting genes, such as cyclin D1 (43), cyclin A, and c-myc. However, we found that ERβ did not inhibit some other genes induced by ERα,[8] demonstrating that ERβ does not function as a dominant negative of all ERα-inducible genes. ERβ also inhibits proliferation of an ER-negative breast cancer cell (44). Furthermore, we found that most genes regulated by ERα in response to E_2 are distinct from those regulated by ERβ in U2OS osteosarcoma cells.[9] These observations suggest that in addition to antagonizing the effects of ERα, ERβ may inhibit cell proliferation by directly regulating distinct genes or by exerting other mechanisms independent of ERα.

The inhibition of cell proliferation by ERβ and activation of p21 and p27 was predominantly or totally ligand independent, respectively. It is possible that this observation results from residual E_2 remaining in stripped serum or retained in cells infected with ERβ. 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 ERβ changes the phenotype of MCF-7 cells in response to E_2. In ERα-expressing MCF-7 cells, E_2 causes proliferation and tumor formation. In contrast, when ERβ is expressed along with ERα, MCF-7 cells are directed to antiproliferation and antitumor pathways even in the presence of estrogens. These results suggest that ERβ can alter the response to estrogens and provide a possible explanation for the findings that ERβ expression in breast tumors is associated with a more favorable prognosis (21–24). A potentially important clinical application of our studies is that ERβ-selective estrogens may be more potent at eliciting antiproliferative pathways compared with ER nonselective estrogens, such as E_2, that also activate ERα. High intake of dietary plant estrogens (phytoestrogens) is associated with a low incidence of breast cancer (47, 48). Whereas E_2 binds equally to ERα and ERβ, phytoestrogens selectively bind to ERβ (49, 50) and recruit coregulators to ERβ to trigger transcriptional activation and repression (11). Our results indicate that phytoestrogens and estrogens designed to selectively trigger ERβ 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.

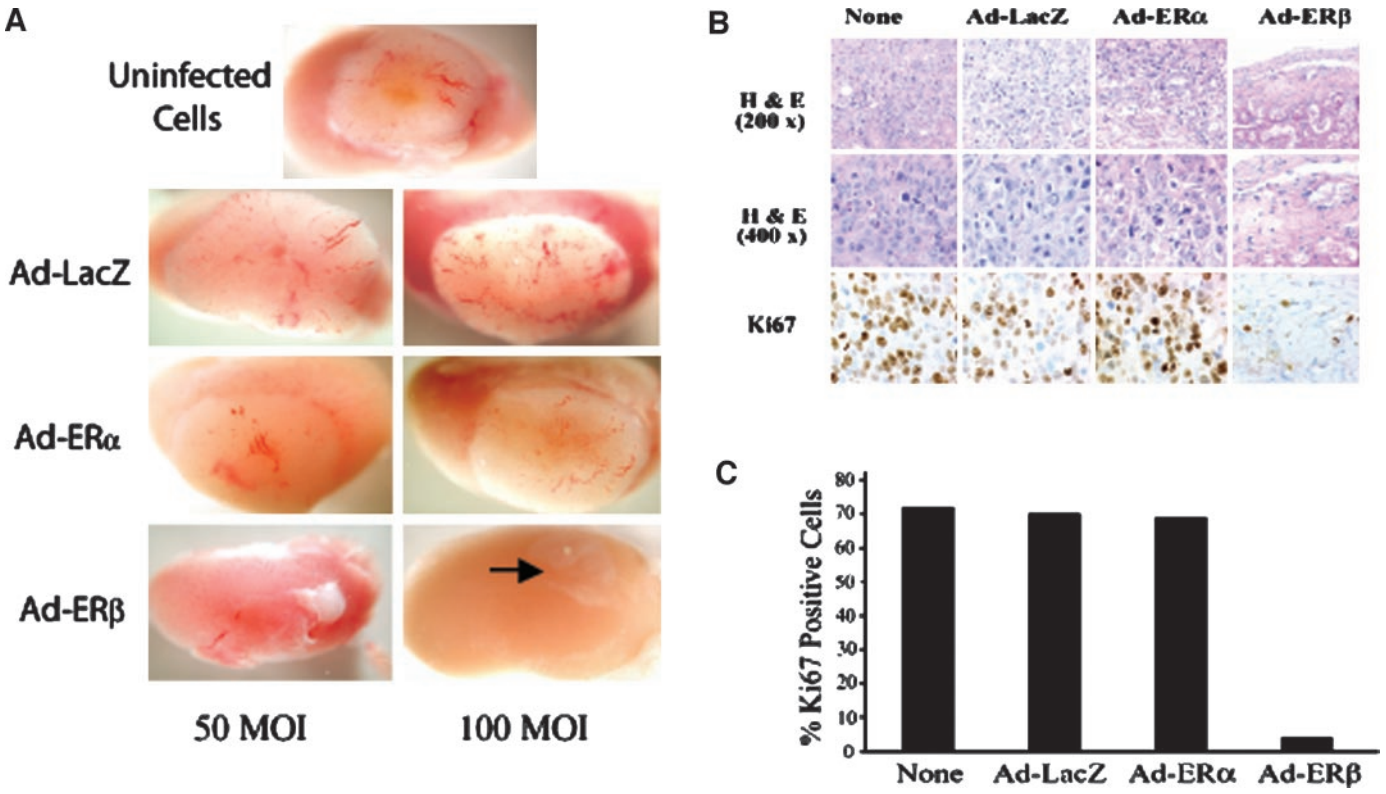

Fig. 4. Estrogen receptor (ER) β inhibits tumor formation of MCF-7 cells in mice xenografts. Uninfected MCF-7 cells, or cells infected with 50 or 100 multiplicity of infection adenovirus (Ad) -LacZ, Ad-ERα, or Ad-ERβ were grafted under the kidney capsule of female nude mice. After 1 month, the tumors 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-ERβ. *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- ERα, or Ad-ERβ.

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

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