Apoptotic Action of 17β-Estradiol in Raloxifene-Resistant MCF-7 Cells *In Vitro* and *In Vivo*

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Background: Resistance to tamoxifen, a selective estrogen receptor modulator (SERM), involves changes that prevent apoptosis and enhance cell proliferation and survival. Paradoxically, estrogen treatment inhibits the growth of long-term tamoxifen-treated breast tumors. Because of the increasing use of raloxifene, another SERM, to prevent osteoporosis and potentially reduce breast cancer risk, some women will develop raloxifene-resistant breast cancer. We developed a raloxifene-resistant MCF-7 cell model (MCF-7/Ral) and investigated the nature of raloxifene-resistant breast cancer and its response to estradiol. *Methods:* Raloxifene resistance and hormone responsiveness were assessed by proliferation assays and cell cycle analysis in parental MCF-7 and MCF-7/Ral cells. Nuclear factor κB (NF-κB) activity was investigated with a transient transfection assay. Apoptosis was investigated by annexin V staining, mRNA was measured by real-time polymerase chain reaction, and protein was measured by western blotting. Tumorigenesis was studied by injecting MCF-7 or MCF-7/Ral cells into ovariectomized athymic mice (10 per group) and monitoring tumor size weekly. All statistical tests were two-sided. *Results:* Basal NF-κB activity was higher in MCF-7/Ral cells (1.6 U, 95% confidence interval [CI] = 1.2 to 2.0 U) than in MCF-7 cells (0.8 U, 95% CI = 0.4 to 1.1 U; P = .004). When cultured with 1 μM raloxifene, MCF-7/Ral cells grew statistically significantly (P<.001) faster than MCF-7 cells. Estradiol treatment of MCF-7/Ral cells arrested cells in G_2/M phase of the cell cycle, decreased NF-κB activity (0.2 U, 95% CI = 0.2 to 0.3 U; P<.001), increased expression of Fas protein and mRNA (4.5-fold, 95% CI = 2.8- to 6.3-fold versus 0.5-fold, 95% CI = 0.3- to 0.8-fold for control treatment; P<.001), and induced apoptosis. Treatment with either raloxifene or tamoxifen stimulated MCF-7/Ral tumor growth, suggesting that such tumors were resistant to both drugs. When a 9-week raloxifene or tamoxifen treatment was followed by a 5-week estradiol treatment, estradiol statistically significantly reduced the size of tumors stimulated by raloxifene or tamoxifen (at week 14, P = .004 for raloxifene and P<.001 for tamoxifen). *Conclusions:* Growth of raloxifene-resistant MCF-7/Ral cells *in vitro* and *in vivo* is repressed by estradiol treatment by a mechanism involving G_2/M-phase arrest, decreased NF-κB activity, and increased Fas expression to induce apoptosis. [J Natl Cancer Inst 2003;95:1586–97]

Selective estrogen receptor modulators (SERMs) are a novel class of compounds that have multiple applications in women's health. Tamoxifen, the first clinically useful SERM, has antiestrogenic effects in breast tissue and can be used to treat *(1)* or

prevent *(2)* breast cancer. SERMs also have estrogen-like activity in bone and can be used to treat and prevent osteoporosis *(3)*. Raloxifene is the first SERM approved for the treatment and prevention of osteoporosis in postmenopausal women *(4)*. In a randomized clinical trial of patients with osteoporosis, raloxifene treatment was found to be associated with a statistically significant decrease in the incidence of breast cancer *(5,6)*. Consequently, raloxifene is currently being evaluated to determine whether it is an effective chemopreventive agent in women who were determined by the Gail model to be at high risk for breast cancer *(7)*. The Study of Tamoxifen and Raloxifene (STAR) trial is recruiting 19 000 high-risk postmenopausal women to establish whether raloxifene is more or less effective than tamoxifen, the current standard of care, as a chemoprevention agent for breast cancer but with fewer side effects.

Resistance to tamoxifen, in the form of tamoxifen-stimulated growth, is well documented in the laboratory and the clinic *(8–10)*. In contrast, there is only limited information about the development of resistance to raloxifene. The widespread use of raloxifene to prevent osteoporosis, or perhaps in the future to prevent breast cancer, means that increasing numbers of women will be developing raloxifene-exposed breast cancer. Thus, it is important to elucidate the mechanism of raloxifene resistance in breast cancer and to develop an effective therapy for raloxifene-resistant tumors.

Resistance to SERMs results from a complex series of changes that prevent apoptosis and thus enhance cell proliferation and survival. Alterations in several signal transduction pathways have been described for tamoxifen resistance, including enhanced activity of the activating protein 1 (AP1) *(11–13)* and phosphatidylinositol 3-kinase/protein kinase B (PKB or AKT) pathways *(14,15)* and altered expression of protein kinase Cα *(16)*, erbB-2 *(17–19)*, and insulin-like growth factor I *(20)*; all of these events have been linked to the activation of nuclear factor κB (NF-κB) *(21–24)*. Increased NF-κB activity is also important for hormone-independent growth and for ICI 182,780 (fulvestrant) resistance *(25,26)*. Thus, NF-κB pathways may also play a central role in raloxifene resistance. After resistance to one SERM appears, the breast tumors often exhibit cross-resistance to other antiestrogens *(27–31)*, which limits the effectiveness of secondary endocrine therapy. However, the process that causes resistance to SERMs appears to begin with a stage in which both estrogen and the SERM stimulate growth through the estrogen receptor (ER) *(8)* and then progress to a stage in which cell proliferation is dependent on the SERM or on prolonged estrogen deprivation but is inhibited by estrogen. Physiologic levels of estrogen inhibit the growth of long-term tamoxifen-stimulated breast tumors *(32,33)*, T47D cells stably transfected with protein kinase Cα (T47D:protein kinase Cα cells) inoculated into athymic mice *(16)*, long-term estrogen-deprived T47D and MCF-7 cells in culture *(34–36)*, and raloxifene-resistant ECC1 endometrial cancer cells *(37)*. Therefore, we propose that a patient's own estrogens might ultimately destroy SERM-resistant tumor cells after the SERM therapy is stopped.

In this article, we report the development of a reproducible raloxifene-resistant cell model, termed MCF-7/Ral. We used this model to investigate raloxifene resistance in breast cancer and to provide a preclinical basis for possible therapy for raloxifene-resistant tumors. We also used this model to explore the mechanism for raloxifene resistance and responsiveness to 17β-estradiol (hereafter estradiol), with particular attention to the regulation of NF-κB activity and apoptosis initiated through Fas, a member of the tumor necrosis factor receptor family *(38,39)*.

MATERIALS AND METHODS

Breast Cancer Cells

MCF-7 cells used in this study *(40)* were cloned from an ERα-positive human MCF-7 breast cancer cells originally obtained from the American Type Culture Collection (Manassas, VA). They were maintained in full serum medium composed of RPMI-1640 medium, 10% fetal bovine serum, 2 m*M* glutamine, penicillin at 100 U/mL, streptomycin at 100 μg/mL, 1× nonessential amino acids (all from Invitrogen, Carlsbad, CA), and bovine insulin at 6 ng/mL (Sigma-Aldrich, St. Louis, MO). Raloxifene-resistant MCF-7 (termed MCF-7/Ral) cells were derived by culturing MCF-7 cells for more than 12 months in 1 μ*M* raloxifene in estrogen-free medium composed of phenol red–free minimal essential medium, 5% calf serum (treated three times with dextran-coated charcoal), 2 m*M* glutamine, bovine insulin at 6 ng/mL, penicillin at 100 U/mL, streptomycin at 100 μg/mL, and 1× nonessential amino acids. 4-Hydroxytamoxifen and estradiol were purchased from Sigma-Aldrich. Raloxifene used in cell cultures was a generous gift from Lilly Research Laboratories (Indianapolis, IN). Raloxifene (Evista) tablets used in animal studies were commercially available (Lilly Research Laboratories). ICI 182,780 (fulvestrant) and tamoxifen were provided by AstraZeneca Pharmaceuticals (Macclesfield, U.K.).

Proliferation Assays

MCF-7 or MCF-7/Ral cells were cultured in estrogen-free medium for 4 days before beginning the proliferation assay (day 0) by plating 1.5×10^4 cells in 1 mL of estrogen-free medium per well in 24-well plates. Medium containing the appropriate test compound was added on day 1. All compounds were dissolved in 100% ethanol (i.e., vehicle) and added to the medium at a 1 : 1000 dilution. Compound-containing medium was changed on days 3 and 5, and the experiment was stopped on day 7. The DNA content of cells was measured as described previously *(41)* with a VersaFluor fluorometer (Bio-Rad Laboratories, Hercules, CA).

To measure cell proliferation rates, 7.5×10^4 MCF-7 or MCF-7/Ral cells were plated into T-25 flasks in 5 mL of medium. Medium containing the appropriate test compound was added on day 1, and the cell number was counted each day with a hemacytometer.

To determine the effect of extended estradiol treatment on the proliferation of MCF-7/Ral cells, the cells were grown in estrogen-free medium without raloxifene for 4 days before starting experiments. For passage 0, cultures containing 2.5×10^5 cells were treated with vehicle control, 1 μ*M* raloxifene, 1 n*M* estradiol, or a combination of 1 μ*M* raloxifene and 1 n*M* estradiol for 6 days. After trypsinization, the cells in a single-cell suspension were counted with a hemacytometer. For passage 1, 2.5×10^5 cells from each treatment group were plated and continuously treated with the respective treatment for another 6 days. The cells were trypsinized and counted. This procedure was repeated for passages 2 and 3.

Cell Cycle Analysis

Cell cycle analysis was performed in the flow cytometry core facility (Robert H. Lurie Comprehensive Cancer Center, Northwestern University, Chicago, IL), as described *(42,43)*. Briefly, single-cell suspensions of MCF-7 or MCF-7/Ral cells were washed with phosphate-buffered saline and resuspended at 1×10^6 cells per milliliter, and 1 mL of cell suspension was mixed with 1 mL of a solution containing propidium iodide at 50 µg/mL, RNase at 180 U/mL, 0.1% Triton X-100, and 3% polyethylene glycol in 3.36 m*M* sodium citrate (pH 7.8). After a 20-minute incubation at room temperature in the dark, 1 mL of a solution containing propidium iodide at 50 µg/mL, 0.1% Triton X-100, 3.56 *M* NaCl, and 0.3% polyethylene glycol was added. The tubes were incubated at 4 °C in the dark for a minimum of 6 hours before flow cytometry analysis (Beckman Coulter Epics XL-MCL; Beckman Coulter, Miami, FL). Data were analyzed with Modfit (version 5.2; Verity Software House, Topsham, ME).

Apoptosis

Approximately 2×10^5 cells from a treatment group were stained with an annexin V fluorescein isothiocyanate kit (Beckman Coulter), and the number of apoptotic cells was determined by flow cytometry. To visualize apoptotic cells, the cells were grown on cover slides and stained with annexin V. Micrographs were taken with a Zeiss LSM510 confocal microscope (Carl Zeiss, Thornwood, NY). Phase-contrast live cell images were also taken with a Zeiss Invertoskop inverted microscope (Carl Zeiss).

Western Blot Analysis

Whole-cell lysates were extracted in protein extraction buffer, as described previously *(44)*. Protein concentration was measured with the Bio-Rad Protein Assay kit (Bio-Rad Laboratories). Antibodies against ERα (product G-20) and Fas (products B-10 and C-20) were from Santa Cruz Biotechnology (Santa Cruz, CA). Antibodies against Fas ligand (FasL; product F37720) and retinoblastoma protein (product 554136) were from BD Biosciences (San Jose, CA). Antibody against NF-κB p65 (product 06-418) was from Upstate Biotechnology (Waltham, MA). Antibody against β-actin (product AC-15; Sigma-Aldrich) was used to standardize loading. The appropriate secondary antibody conjugated to horseradish peroxidase (Santa Cruz Biotechnology) was used to visualize bands with an enhanced chemiluminescence (ECL) visualization kit (Amersham, Arlington Heights, IL).

Transient Transfection and Luciferase Assays

MCF-7 or MCF-7/Ral cells were grown in the estrogen-free medium 4 days before the transient transfection assay. Approximately 5×10^6 cells were mixed with 1 µg of a reporter plasmid, either VitA$_2$-ERE$_3$-luciferase (where VitA$_2$ is vitellogenin A$_2$ and ERE$_3$ is three estrogen response elements) *(45)* or pNF-κB-Luc (where Luc is luciferase; product 6053-1; BD Biosciences Clontech, Palo Alto, CA), and 0.2 µg of pCMV-β-galactosidase (where CMV is cytomegalovirus). Cells were electroporated (950 µF, 320 V) with a Bio-Rad Gene Pulser II (Bio-Rad Laboratories). Luciferase and β-galactosidase activities were measured as previously described *(45)*.

Athymic Mouse Model

Approximately 1×10^7 MCF-7 or MCF-7/Ral cells suspended in saline solution (product 20012027; Invitrogen) were bilaterally inoculated into mammary fat pads of ovariectomized BALB/c nu/nu mice (Harlan Sprague Dawley, Madison, WI) as described previously *(46)*. Inoculated mice were randomly divided into groups of 10 and were treated with estradiol, tamoxifen, or raloxifene or were not treated. For the estradiol treatment, silastic estradiol capsules [1 cm long *(47)*] were implanted subcutaneously in the mouse's back on the day of cell inoculation and replaced after 8–10 weeks of treatment. These capsules produced a mean serum estradiol level of 380 pg/mL *(48)*. Preparation of tamoxifen and raloxifene were described in detail previously *(31)*. Tamoxifen and raloxifene were administered orally by gavage at 1.5 mg/day per mouse for 5 days a week for 9 or 14 weeks, as indicated. Tumors were measured weekly with vernier calipers. The cross-sectional tumor area was calculated by multiplying the length (*l*) by the width (*w*) and π and dividing by 4 (i.e., $lw\pi/4$).

The Animal Care and Use Committee of Northwestern University approved all of the procedures involving animals.

Real-Time Polymerase Chain Reaction

Total RNA (100 ng) was reverse transcribed by use of random hexamers (TaqMan Reverse Transcription Reagents; Applied Biosystems, Foster City, CA). Primers and probes for Fas and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed with Primer Express 1.5 software (Applied Biosystems). The sequences for all primers and probes are as follows: GAPDH forward primer, 5′-GAAGGTGAAGGTCGGAGTCA-3′; GAPDH reverse primer, 5′-GAAGATGGTGATGGGATTTC-3′; GAPDH probe, 5′-FAM-CAAGCTTCCCGTTCTCAGCC-QSY7–3′; Fas forward primer, 5′-TGGAAGGCCTGCAT-CATGA-3′; Fas reverse primer, 5′-CAGTCCCTAGCTTTCC-TTTCACC-3′; and Fas probe, 5′-FAM-CCAATTCTGCC-ATAAGCCCTGTCCTCC-3′. All probes were labeled with 6-carboxyfluorescein (FAM) as reporter and with QSY7 (a nonfluorescent diarylrhodamine derivative) as quencher. The primers and probes were synthesized by MegaBases (Evanston, IL). The TaqMan polymerase chain reaction (PCR) Core Reagent Kit (Applied Biosystems) was used for PCR. A 25-µL reaction mixture contained 2 µL of the cDNA, probe at 100 n*M* , and each primer at 200 n*M*. PCRs were performed with the ABI Prism 7700 Sequence Detection System (Applied Biosystems). The PCR conditions were 50 °C for 2 minutes and 95 °C for 10 minutes, followed by 40 cycles of 95 °C for 15 seconds and 60 °C for 1 minute. Human GAPDH was used as an internal control, and each total RNA sample was normalized to the content of GAPDH mRNA.

Statistical Analysis

All data are expressed as the mean (with 95% confidence interval [CI]) of at least three determinations, unless stated otherwise. Paired *t* test was used when only two groups were compared. The interaction between estradiol and raloxifene was determined with a two-way analysis of variance (SPSS software; SPSS, Chicago, IL). When the interaction was statistically significant, each pairwise comparison was made with a one-way analysis of variance followed by Tukey's honestly significant difference. All statistical tests were two-sided.

RESULTS

Raloxifene Resistance of MCF-7 Cells *In Vitro*

Growth characteristics of raloxifene-resistant MCF-7/Ral cells and parental MCF-7 cells were investigated. When cultured with 1 μM raloxifene, MCF-7/Ral cells (at day 6, 152×10^4 cells, 95% CI = 130×10^4 to 174×10^4 cells; $P = .006$) grew statistically significantly faster than MCF-7 cells (at day 6, 55×10^4 cells, 95% CI = 44×10^4 to 64×10^4 cells) (Fig. 1, A). In fact, raloxifene at 1 μM statistically significantly (on day 4, $P = .001$; on day 5 and day 6, $P<.001$) inhibited the growth of cultured MCF-7 cells (Fig. 1, A), as reported previously *(49)*, and cell cycle analyses demonstrated that such MCF-7 cells were arrested in G_0/G_1 phase (Fig. 1, B).


Because the ERα plays an important role in the development and growth of breast cancer *(3)*, raloxifene resistance could be a consequence of changes in the expression and/or transactivation activity of ERα. We measured the level of ERα protein by western blot analysis in MCF-7 and MCF-7/Ral cells and found that ERα protein levels were slightly lower in MCF-7/Ral cells than in MCF-7 cells (Fig. 2, A). In both cell lines, addition of 1 μM raloxifene had essentially no effect on the level of ERα protein, whereas addition of 1 nM estradiol decreased the level of ERα protein dramatically. Both cell lines had similar basal ERE$_3$–luciferase activities. Addition of 1 nM estradiol statistically significantly increased ER transcriptional activity ($P<.001$), and addition of 1 μM raloxifene inhibited the estradiol-induced ER transactivation activity in both cell lines (Fig. 2, B). Addition of estradiol also induced expression of endogenous ER-regulated genes, such as cyclin D1, p53, and BCL2 in both MCF-7 and MCF-7/Ral cells (data not shown). Because MCF-7/Ral cells appeared to express a functional ERα, as do MCF-7 cells, and raloxifene did not increase ER transactivation activity, enhanced estrogenic activity of the raloxifene–ERα complex is probably not the primary mechanism of raloxifene resistance in MCF-7 cells.


Fig. 1. Proliferation of MCF-7 and MCF-7/Ral cells *in vitro*. **A**) Proliferation rate. Approximately 7.5×10^4 MCF-7 or MCF-7/Ral cells were plated in T-25 flasks. MCF-7 cells were treated with ethanol vehicle (MCF-7 Control) or 1 μM raloxifene (MCF-7 + 1 μM Ral) in full serum medium. MCF-7/Ral cells were grown in estrogen-free medium alone [MCF-7/Ral (-Ral)] or containing 1 μM raloxifene (MCF-7/Ral). Three samples were counted every day. Data are the means; **error bars** are 95% confidence intervals. Statistically significant differences compared with MCF-7 treated with 1 μM raloxifene cells are as follows: *a*, $P<.001$; *b*, $P = .041$; *c*, $P = .006$. The experiment was repeated twice. **B**) Cell cycle analysis. MCF-7 cells were grown in full serum medium containing ethanol vehicle (Control) or 1 μM raloxifene (Ral) for 4 days, and MCF-7/Ral cells were grown in medium containing vehicle (Control) or 1 μM raloxifene for 4 days. * $P<.001$ compared with the corresponding control. All statistical tests were two-sided.

Fig. 2. Expression of functional estrogen receptor α (ERα) in MCF-7 and MCF-7/Ral cells. **A**) Western blot analysis for ERα in MCF-7 and MCF-7/Ral cells grown in estrogen-free medium. The cells were treated with ethanol vehicle (**lanes C**), 1 μM raloxifene (**lanes Ral**), 1 nM estradiol (**lanes E$_2$**), or a combination of 1 μM raloxifene and 1 nM estradiol (lanes Ral + E$_2$) for 4 days. β-Actin was used as the loading control. The blot is representative of three experiments, all with similar results. **B**) Transcriptional activity of the estrogen receptor in MCF-7 and MCF-7/Ral cells, as measured by VitA$_2$-ERE$_3$-luciferase transient transfection assays (where VitA$_2$ is vitellogenin A$_2$ and ERE$_3$ is three copies of estrogen response elements). RLU = relative light units. *$P<.001$ compared with the corresponding control groups. All statistical tests were two-sided.

Enhanced NF-κB Activity in MCF-7/Ral Cells

We next investigated whether NF-κB activity plays a role in raloxifene resistance. Short-term treatment (up to 48 hours) with estradiol or raloxifene had essentially no effect on NF-κB activity in either cell line, as measured with NF-κB reporter activity (data not shown). However, as shown in Fig. 3, statistically significantly higher basal NF-κB activity was detected in MCF-7/Ral cells (1.6 U, 95% CI = 1.2 to 2.0 U) than in MCF-7 cells (0.8 U, 95% CI = 0.4 to 1.1 U; P = .004). In addition, a higher level of NF-κB p65 protein, as measured by western blot analysis, was found in MCF-7/Ral cells than in MCF-7 cells (Fig. 3, inset). Thus, enhanced NF-κB activity may play a role in raloxifene resistance in MCF-7 cells.

Inhibitory Effect of Estradiol in MCF-7/Ral Cells

To investigate potential cross-resistance to other SERMs, we used DNA assays to measure the proliferation in MCF-7 and MCF-7/Ral cells treated with vehicle control, 1 nM estradiol, 1 μM raloxifene, or 1 μM 4-hydroxytamoxifen for 6 days. As shown in Fig. 4, A, proliferation of MCF-7 cells was statistically significantly higher in estradiol-treated cultures than in untreated control cell cultures (P<.001; Fig. 4, A). Both raloxifene and 4-hydroxytamoxifen inhibited estradiol-induced increased proliferation of MCF-7 cells (data not shown). MCF-7/Ral cells proliferated more rapidly than MCF-7 cells (P<.001) in estrogen-free medium. Although a 6-day estradiol treatment did not appreciably affect the proliferation of MCF-7/Ral cells (Fig. 4, A), cells were larger in estradiol-treated cultures than in untreated cultures, and apoptotic-like and multinucleated cells were observed in estradiol-treated cultures (Fig. 4, B). In contrast, MCF-7/Ral cultures treated for 6 days with a combination of raloxifene and estradiol contained normal-sized cells (Fig. 4, B). Thus, estradiol may be involved in the regulation of cell proliferation and apoptosis in raloxifene-resistant cells.

To study long-term effects of estradiol, MCF-7/Ral cells were treated with vehicle control, 1 μM raloxifene, 1 nM estradiol, or a combination of raloxifene and estradiol for four passages (passages 0–3). As shown in Fig. 4, C, treatment with estradiol statistically significantly inhibited the proliferation of MCF-7/

Ral cells after 12–24 days compared with cells in raloxifene-treated cultures (for example, for passage 3, P = .001). Interestingly, extended estradiol treatment of MCF-7/Ral cells also statistically significantly inhibited NF-κB activity (0.2 U, 95% CI = 0.2 to 0.3 U, compared with 1.6 U, 95% CI = 1.2 to 2.0 U for baseline control; P<.001) (Fig. 4, D) without affecting the level of NF-κB p65 protein (data not shown). This result suggests that elevated NF-κB activity plays a role in raloxifene resistance in MCF-7 cells. In contrast, estradiol treatment did not statistically significantly affect NF-κB activity in MCF-7 parental cells (estradiol = 0.6 U, 95% CI = 0.4 to 0.8 U; control = 0.8 U, 95% CI = 0.5 to 1.1 U; P = .225).

Effect of Estradiol on the Cell Cycle and Apoptosis in MCF-7/Ral Cells

Because a decrease in cell number can result from decreased cell proliferation, increased cell death, or both, we first evaluated the effect of estradiol on the cell cycle in MCF-7 and MCF-7/Ral cells. In parental MCF-7 cells, estrogen deprivation blocked the transition from G_1 phase to S phase, whereas treatment with 1 nM estradiol relieved the block as shown by the decreased number of cells in G_0/G_1 phase and the increased number in S phase (for both, P<.001), apparently by inducing phosphorylation of the retinoblastoma protein (Rb) (Fig. 5, A). The estradiol-mediated Rb phosphorylation was blocked by raloxifene. In contrast, a 6-day estradiol treatment did not affect the cell cycle of MCF-7/Ral cells (data not shown), but a treatment of 2 weeks or longer with 1 nM estradiol statistically significantly increased the percentage of cells in G_2/M phase to 12.5% (95% CI = 10.6% to 14.3%) compared with 7.6% for vehicle control treatment (95% CI = 5.1% to 10.0%; P = .003), 7.7% for raloxifene (95% CI = 5.6% to 9.8%; P = .004), and 7.6% for estradiol plus raloxifene (95% CI = 4.9% to 10.2%; P = .003). A treatment of 2 weeks or longer with 1 nM estradiol also statistically significantly decreased the percentage of cells in G_0/G_1 phase to 69.7% (95% CI = 65.0% to 74.4%) compared with 80.1% for vehicle control treatment (95% CI = 74.2% to 85.9%; P = .006), 77.5% for raloxifene (95% CI = 72.1% to 82.8%; P = .041), and 77.3% for estradiol plus raloxifene (95% CI = 72.7% to 82.0%; P = .045) (Fig. 5, B). Rb was phosphorylated in raloxifene-treated MCF-7/Ral cells, and addition of estradiol did not induce additional phosphorylation of Rb (Fig. 5, B, lower panel).

Estradiol induces apoptosis in long-term estrogen-deprived cells *(36)* and, in fact, we observed apoptotic cells in MCF-7/Ral cultures treated with estradiol for 6 days (Fig. 4, B). To confirm that apoptotic cells were present in estradiol-treated MCF-7/Ral cultures, we used annexin V binding. Untreated control MCF-7/Ral cultures or MCF-7/Ral cultures treated with 1 μM raloxifene for up to 24 days had a minimal number of apoptotic cells (Fig. 6, B), an observation that reflects enhanced survival of MCF-7/Ral cells (Fig. 3) that may be mediated by their high activity of NF-κB, a known survival factor *(50–54)*. After a 6-day estradiol treatment (passage 0), the percentage of apoptotic MCF-7/Ral cells was not statistically significantly different than that in untreated control cultures or cultures treated with 1 μM raloxifene or a combination of estradiol and raloxifene (P = .063). Extended estradiol treatment (passages 1–3; 12–24 days), however, increased the percentage of apoptotic cells, and the addition of raloxifene essentially blocked this effect
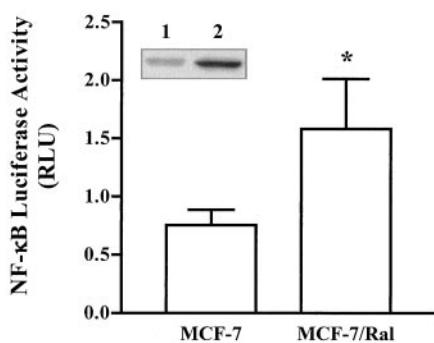

Fig. 3. Basal activity of nuclear factor-κB (NF-κB) in MCF-7 and MCF-7/Ral cells. Activity was measured by NF-κB-luciferase transient transfection assays. MCF-7 and MCF-7/Ral cells were grown in estrogen-free medium for 4 days before the assays. **Inset)** Protein levels of NF-κB p65 measured by western blot analysis in MCF-7 (**lane 1**) and MCF-7/Ral (**lane 2**) cells. RLU = relative light units. *P = .004 compared with MCF-7 cells. All statistical tests were two-sided.
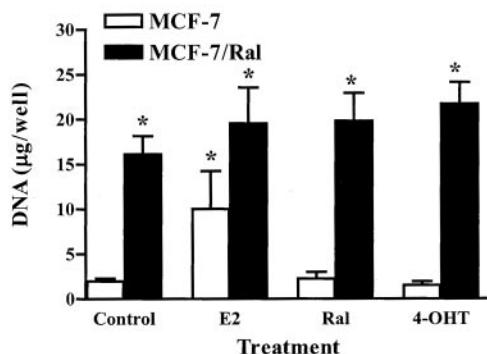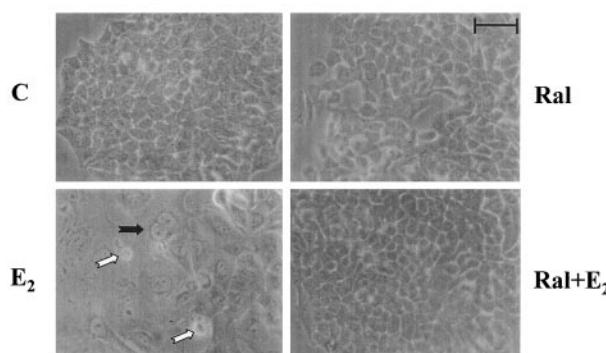
Fig. 4. Hormone-dependent growth of MCF-7 and MCF-7/Ral cells. All cells were grown in estrogen-free medium for 4 days before the assays. Cells were treated with ethanol vehicle (C or control), 1 nM 17β-estradiol (E$_2$), 1 μM raloxifene (Ral), or 1 μM 4-hydroxytamoxifen (4-OHT) for 6 days. **A**) DNA assay. For MCF-7 cells, values were as follows: control = 2.0 μg of DNA per well (95% CI = 1.6 to 2.3 μg); E$_2$ = 10.0 μg (95% CI = 5.8 to 14.3 μg); Ral = 2.2 μg (95% CI = 1.5 to 3.0 μg); and 4-OHT = 1.5 μg (95% CI = 1.1 to 1.9 μg). For MCF-7/Ral cells, values were as follows: control = 16.1 μg (95% CI = 14.1 to 18.2 μg); E$_2$ = 19.6 μg (95% CI = 15.6 to 23.6 μg); Ral = 19.8 μg (95% CI = 16.7 to 22.9 μg); and 4-OHT = 21.7 μg (95% CI = 19.3 to 24.1 μg). * $P<.001$ compared with the MCF-7 control cells. **B**) Cell morphology. Micrographs are phase-contrast images. **Open arrow** = apoptotic-like cells; **solid arrow** = double nuclei. **Scale bar** = 50 μm. **C**) Estradiol inhibition of MCF-7/Ral cell growth after an extended treatment *in vitro*. Approximately 2.5 × 10^4 cells were grown in estrogen-free medium for each passage (0–3) in the absence (C) or presence of 1 μM raloxifene (Ral), 1 nM estradiol (E$_2$), or the combination of raloxifene and estradiol (E$_2$ + Ral). Experiments were repeated five times. The statistical interaction of estradiol and raloxifene was determined with a two-way analysis of variance. The interaction was statistically significant in passages 1 ($P = .022$), 2 ($P = .023$), and 3 ($P = .029$). All six pairwise comparisons among the four groups were calculated with Tukey's honestly significant difference. *Statistically significant compared with the values in raloxifene treatment group as follows: for passage 1, $P<.001$; for passage 2, $P = .006$; and for passage 3, $P = .001$. **D**) Estradiol treatment and nuclear factor κB (NF-κB) activity in MCF-7 and MCF-7/Ral cells. MCF-7 or MCF-7/Ral cells were grown in estrogen-free medium for 4 days before the NF-κB-luciferase transient transfection assay. MCF-7 + E$_2$ or MCF-7/Ral + E$_2$ cells were treated with 1 nM estradiol for 6 days and then grown in estrogen-free medium for 4 days before the transient transfection assay. * $P<.001$ compared with MCF-7/Ral cells. All statistical tests were two-sided.
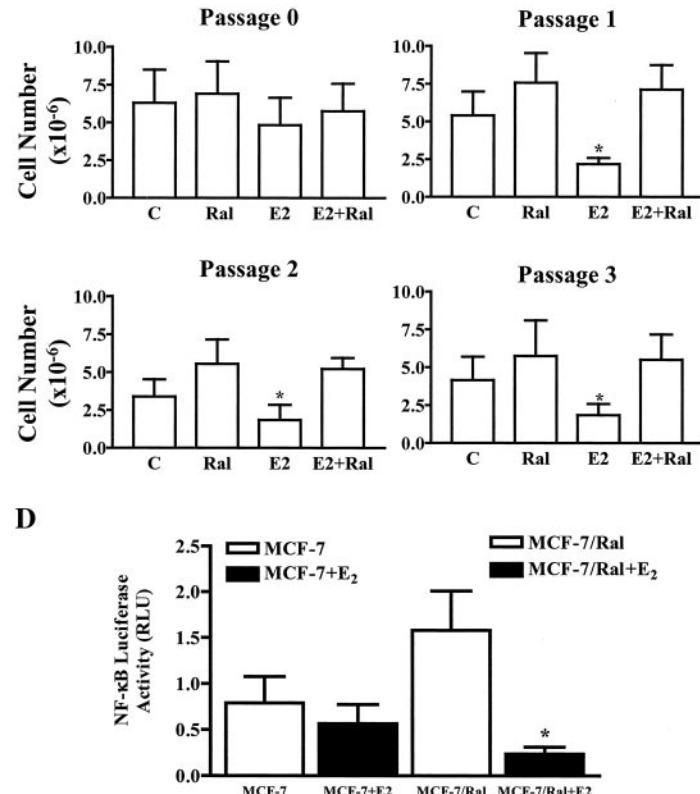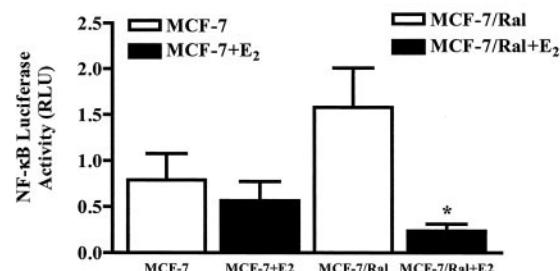
(Fig. 6, B). Apoptosis was observed in untreated control and raloxifene-treated MCF-7 cell cultures, and estradiol treatment decreased the percentage of apoptotic MCF-7 cells (Fig. 6, B). Thus, estradiol treatment inhibited apoptosis in parental MCF-7 cells, and estradiol treatment promoted apoptosis in raloxifene-resistant MCF-7/Ral cells.

Estradiol and the Induction of Fas Expression

Estradiol regulates FasL expression in a variety of cells and tissues *(55–59),* including long-term estrogen-deprived MCF-7 cells *(36).* Untreated MCF-7 cells express a low or nondetectable level of Fas receptor *(36,60),* but long-term estrogen deprivation of MCF-7 cells increases Fas expression *(36).*

We investigated whether estradiol plays a role in the regulation of the Fas/FasL system in MCF-7/Ral and MCF-7 cells by determining their levels of Fas and FasL expression. Both MCF-7 and MCF-7/Ral cells expressed FasL but in an estradiol-

independent manner. Although more apoptotic cells were observed in untreated control and raloxifene-treated MCF-7 cultures (Fig. 6, B), Fas protein was barely detected in MCF-7 cells. Estradiol treatment of parental MCF-7 cells did not appreciably affect Fas expression (Fig. 6, C) but did prevent apoptosis (Fig. 6, B), indicating that apoptosis in MCF-7 cells does not appear to be mediated by the Fas/FasL pathway. Fas protein was not detected in untreated control or raloxifene-treated MCF-7/Ral cells (Fig. 6, C). Estradiol treatment dramatically increased the level of Fas protein and mRNA in MCF-7/Ral cells (Fig. 6, C and D). For example, Fas mRNA levels increased 4.5-fold (95% CI = 2.8- to 6.3-fold) after estradiol treatment compared with 0.5-fold (95% CI = 0.3- to 0.8-fold) after control treatment, 0.8-fold (95% CI = 0.7- to 1.0-fold) after 1 μM raloxifene, and 0.7-fold (95% CI = 0.4- to 1.1-fold) after raloxifene plus estradiol (all $P<.001$). Addition of raloxifene blocked estradiol-induced increases in Fas protein and mRNA levels (Fig. 6, C and
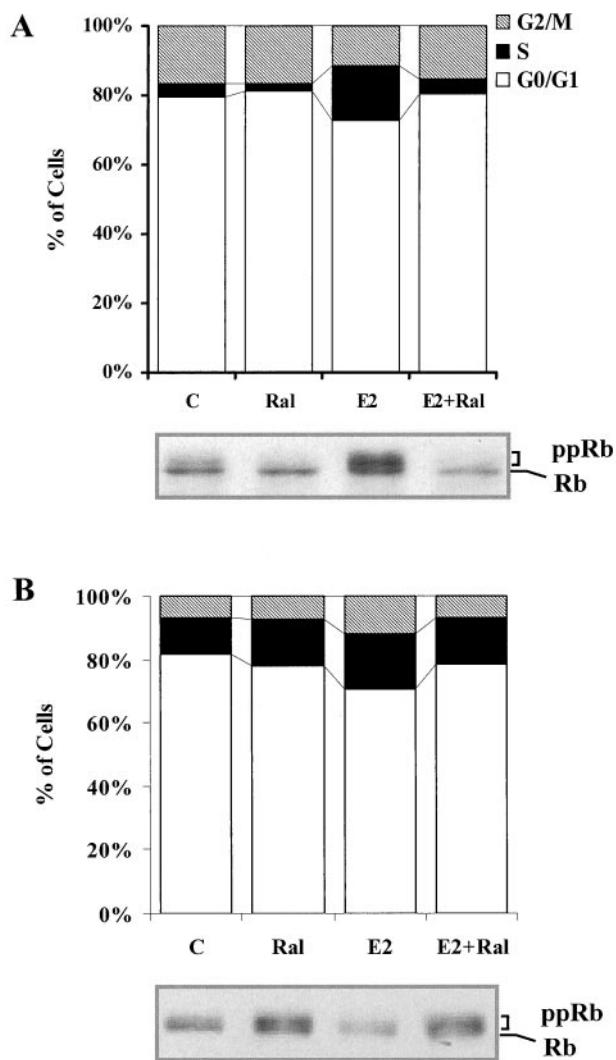
Fig. 5. Hormone-dependent effects on the cell cycle in MCF-7 (**A**) and MCF-7/Ral (**B**) cells. Cell cycle analysis is shown in the **upper panels**, and western blot analysis for the retinoblastoma protein (Rb) and phosphorylated Rb (ppRb) is shown in the **lower panels**. MCF-7 cells (**A**) and MCF-7/Ral cells (**B**) were treated with vehicle control (C), 1 μM raloxifene (Ral), 1 nM 17β-estradiol (E$_2$), or 1 μM raloxifene plus 1 nM 17β-estradiol (E$_2$ + Ral) for 6 days. Because there was no statistically significant difference in results from MCF-7/Ral cells in passages 1–3, the results from these passages were pooled for analysis.

D). In MCF-7/Ral cells, the effects of estradiol on Fas expression correspond to those on apoptosis and indicate that estradiol-induced apoptosis might be mediated by the Fas/FasL pathway in these cells.

Hormone-Dependent Growth of MCF-7/Ral Cells *In Vivo*

To further study raloxifene resistance and examine the unique inhibitory effect of estradiol on the growth of SERM-sensitized breast cancer cells, we examined the ability of MCF-7/Ral cells and parental MCF-7 cells to form tumors in athymic mice. As previously reported *(8,61),* treatment with estradiol, but not with raloxifene, stimulated the growth of MCF-7 cell tumors in ovariectomized athymic mice (Fig. 7, A). MCF-7/Ral tumors, in contrast, required treatment with a SERM to grow; both raloxifene and tamoxifen, but not estradiol, statistically significantly stimulated the growth of MCF-7/Ral tumors in athymic mice (Fig. 7, B). Further-

more, if SERM treatment of the tumor-bearing athymic mice was stopped after 9 weeks and replaced by estradiol treatment for 5 weeks, tumors decreased in size in both groups of mice (*P* = .004 for raloxifene and *P*<.001 for tamoxifen), and some tumors disappeared altogether. These data are consistent with the effect of estradiol on the MCF-7/Ral cells *in vitro*.

DISCUSSION

The increased use of SERMs to prevent breast cancer (with tamoxifen) or osteoporosis (with raloxifene) has added a new therapeutic dimension to clinical practice. The results of clinical trials now underway may establish SERMs as multifunctional medicines for the prevention of osteoporosis, coronary heart disease, and breast cancer *(3)*. However, the clinical use of these agents must be accompanied by rigorous laboratory studies to elucidate the mechanisms involved in the development of SERM-resistant breast cancer. The process of tamoxifen-resistant breast cancer development is well documented, but it is important to examine the potential for drug resistance to SERMs in general. We now report data from a new study of the evolution of raloxifene-resistant breast cancer in the laboratory that builds on our previous studies with tamoxifen *(8,29,30,33,62)*. Understanding the mechanism of raloxifene resistance should contribute important information for effective breast cancer therapy. We have confirmed and extended our original observation that low concentrations of estrogen shift the survival of SERM-resistant breast cancer cells by initiating apoptosis. Unlike our previous work that was entirely *in vivo (32,33,63),* we now report results from both *in vitro* and *in vivo* studies using a new model of raloxifene-resistant breast cancer cells, MCF-7/Ral cells. We have defined the shift from cell survival induced by raloxifene in breast cancer to cell death induced by a modest concentration (1 nM) of estradiol in pre-sensitized cells.

Most research on SERM-resistant breast cancer has previously focused on tamoxifen. Apparent increased expression of ERα *(64,65)* and decreased expression of the nuclear receptor corepressor (i.e., N-CoR) *(66)* were detected in tamoxifen-resistant cells and tumors. An altered balance between ER coactivators and corepressors has been suggested as the mechanism for tamoxifen resistance *(67,68)*. Consequently, we initially examined the expression of ERα, ER coactivators (such as steroid receptor coactivators SRC-1, SRC-2, and SRC-3, and p300/CBP [cAMP response element–binding protein binding protein]), and ER corepressors (N-CoR and silencing mediator for retinoid and thyroid receptors) in MCF-7 and MCF-7/Ral cells with real-time PCR. We found no statistically significant differences in the expression levels of ERα and the ERα co-regulators in MCF-7 cells and MCF-7/Ral cells grown in estrogen-free medium or treated with 1 μM raloxifene for 24 or 48 hours (data not shown). Therefore, we conclude that changes in the expression of ERα or ER coregulators are not the primary mechanism for raloxifene resistance.

NF-κB is a key transcriptional factor for cell growth *(69)* and survival *(50–54)*. Increased NF-κB activity is involved in drug resistance in breast cancer *(70–72)*, and ERα represses NF-κB activity in the presence of estradiol *(52–54,73,74)*. Because raloxifene is an antiestrogen, we anticipated that raloxifene would block estradiol-mediated repression of NF-κB activity and thus increase NF-κB activity. In fact, we found higher levels of NF-κB p65 protein and activity in MCF-7/Ral cells than in
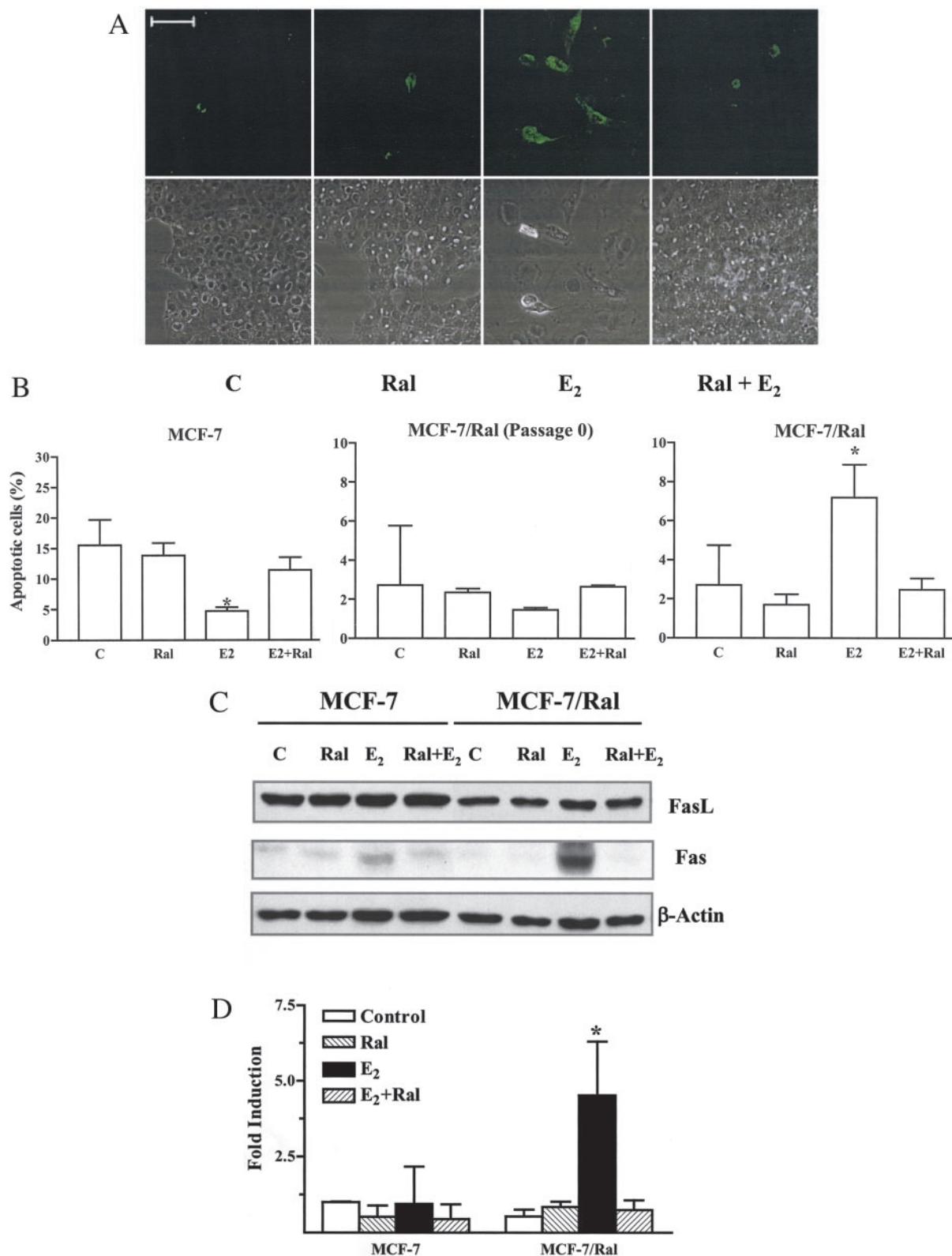
Fig. 6. Hormones and apoptosis in MCF-7 and MCF-7/Ral cells. C = vehicle control; Ral = raloxifene; E₂ = estradiol. **A)** Confocal images of annexin V staining in MCF-7/Ral cells. **Upper panel** = annexin V staining; **lower panel** = phase-contrast image. **Scale bar** = 50 μm. **B)** Apoptosis as measured by annexin V staining and flow cytometry. For panels MCF-7 and MCF-7/Ral (passage 0), cells were treated for 6 days with vehicle control, 1 μM raloxifene, 1 nM estradiol, or 1 μM raloxifene plus 1 nM estradiol. For panel MCF-7/Ral, cells were treated as indicated for passages 1–3 (12–24 days) and data from passages 1–3 were pooled. **C)** Western blot analysis for Fas and FasL in MCF-7 and MCF-7/Ral cells treated as indicated for two passages. β-Actin was used as the loading control. **D)** Real-time polymerase chain reaction analysis of Fas mRNA expression. MCF-7 cells were treated as indicated for 6 days, and MCF-7/Ral cells were treated as indicated for passages 0–3 (12–24 days). * $P < .001$ compared with any other group of MCF-7/Ral cells. All statistical tests were two-sided.
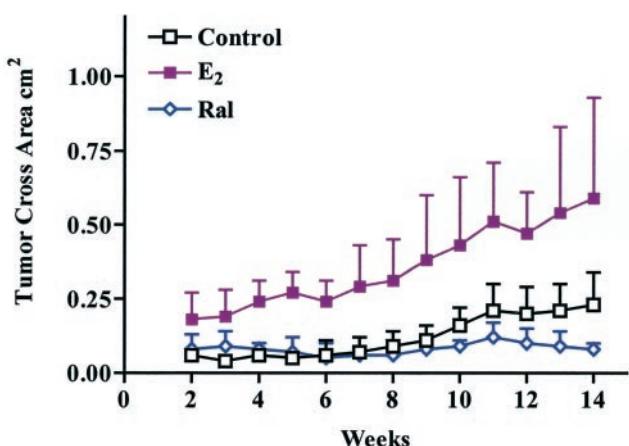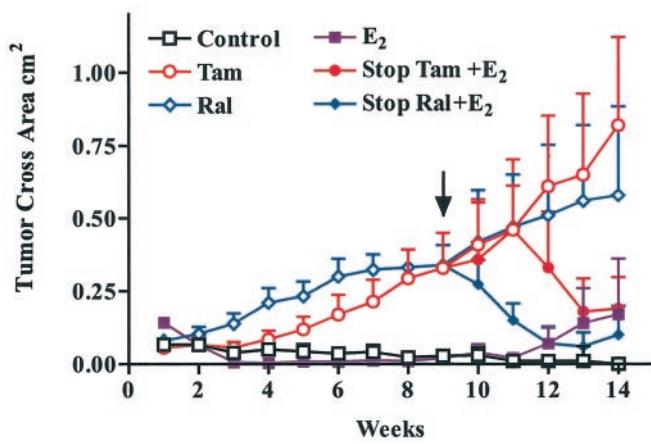
Fig. 7. Growth of MCF-7 (**A**) and MCF-7/Ral (**B**) tumors in ovariectomized athymic mice. Each group had 10 mice. The mice were treated with a 1-cm estradiol (E_2) capsule, 1.5 mg of tamoxifen (Tam), 1.5 mg of raloxifene (Ral), or vehicle control. At week 9 (**arrow**), raloxifene- and tamoxifen-stimulated MCF-7/Ral tumors were split into two arms: 1) continue raloxifene or tamoxifen treatment or 2) stop raloxifene or tamoxifen treatment and start estradiol treatment for 5 weeks (i.e., Stop Ral + E_2 or Stop Tam + E_2). For both treatments in the second arm, tumors at 14 weeks were statistically significantly decreased in size in both groups of mice ($P = .004$ for raloxifene and $P<.001$ for tamoxifen). Results are shown as mean tumor size with 95% confidence interval (only the upper confidence limit for each point is shown).

MCF-7 cells (Fig. 3). Thus, the NF-κB signal transduction pathway could be involved in the survival of raloxifene-resistant MCF-7/Ral cells.

The finding that raloxifene-resistant MCF-7/Ral cells are also resistant to tamoxifen has important therapeutic implications. Tamoxifen is used to treat all stages of ER-positive breast cancer and is used as a chemopreventive agent in women at high risk for breast cancer (2). In vitro (Fig. 4, A) and in vivo (Fig. 7) data show that cells and/or tumors resistant to raloxifene are also resistant to tamoxifen and indicate that tamoxifen should not be given to patients with raloxifene-exposed breast cancer. The converse is also true (31). It is perhaps prudent to consider treatment with an aromatase inhibitor or fulvestrant (i.e., Faslodex), a pure antiestrogen, in patients who have been exposed to raloxifene.

However, the most important observation for the outcome of long-term SERM-resistant breast cancer is the effectiveness of estrogen to induce apoptosis. Antitumor activity of estradiol has been reported for estrogen-deprived breast cancer cells *in vitro* (34–36), tamoxifen-stimulated breast tumors *in vivo* (16,32,33), and raloxifene-resistant ECC1 endometrial cancers *in vivo* (37). More important, these findings have been extended to clinic settings, where estrogen is an effective treatment for breast cancers that have developed resistance to successive antiestrogenic therapies (75). In this study, we found that a 6-day estradiol treatment (passage 0) did not inhibit MCF-7/Ral cell growth (Figs. 4, A, and 6, B), whereas an extended estradiol treatment (passages 1–3) of 12–24 days statistically significantly inhibited MCF-7/Ral cell growth (Fig. 4, C). Although the causes of the delayed inhibitory effect of estradiol are still not known, estradiol treatment also caused a delayed decrease in NF-κB activity (Fig. 4, D) without changing the level of NF-κB p65 protein (data not shown). Estradiol binds to ERα, and the estradiol–ERα complex suppresses NF-κB activity by inhibiting the binding of NF-κB to DNA or by competing with p300/CBP (52,53,76,77), independent of new protein synthesis. However, because a 24- or 48-hour estradiol treatment did not inhibit NF-κB activity in MCF-7 and MCF-7/Ral cells (data not shown), the delayed inhibitory effect of estradiol on NF-κB activity may require the synthesis of new protein(s). This hypothesis is consistent with the report that estradiol inhibits NF-κB activity by increasing the expression of p105, the precursor of NF-κB (78). However, further studies are necessary to understand the mechanism through which estradiol induces the delayed inhibition of NF-κB activity and growth in MCF-7/Ral cells.

If NF-κB plays an important role in raloxifene resistance, the decreased NF-κB activity induced by estradiol should increase the number of cells undergoing apoptosis. In fact, we observed that an extended estradiol treatment statistically significantly increased the number of apoptotic MCF-7/Ral cells (Fig. 6, A and B), as reported in long-term estrogen-deprived MCF-7 cells (36) and in T47D:protein kinase Cα cells (16). Song et al. (36) suggested that estradiol induces apoptosis in long-term estrogen-deprived MCF-7 cells via the Fas/FasL pathway, and they noted that the level of Fas is elevated in the long-term estrogen-deprived MCF-7 cells and that treatment with estradiol increases the expression level of FasL and induces apoptosis through the elevated level of Fas. Consequently, we examined the expression of Fas and FasL in MCF-7 and MCF-7/Ral cells in response to estradiol treatment. We found that estradiol did not induce expression of FasL in MCF-7 or MCF-7/Ral cells or the expression of Fas in MCF-7 cells but did induce the expression of Fas protein and mRNA in MCF-7/Ral cells (Fig. 6, D). This result is perplexing because, to our knowledge, there are no reports that estrogen stimulates the expression of Fas; however, increased Fas expression is observed in tamoxifen-resistant MCF-7 cells treated with estradiol, which presages apoptosis *in vivo* (63).

In MCF-7/Ral cells, estradiol treatment induced cell cycle arrest at G_2/M phase (Fig. 5, B) and generated multinucleated cells (Fig. 4, B), although the mechanism is currently unclear. Estradiol stimulates the expression of genes that are involved in both inhibiting and promoting G_2/M-phase arrest. In MCF-7 cells, estrogen induces the estrogen-responsive finger protein (79,80), which targets the 14–3–3σ protein for proteolysis. [14–3–3σ is a p53-regulated gene that, along with p21$^{Cip-1/Waf-1}$, plays a role in p53-mediated cell cycle arrest at G_2/M phase (81,82).] Because estradiol also induces p53 (83), the estrogen-responsive finger protein and p53 might be involved in the

estradiol-mediated G_2/M-phase arrest observed in MCF-7/Ral cells. We are currently investigating this hypothesis.

In summary, the MCF-7/Ral model is the first reproducible raloxifene-resistant breast cancer grown in athymic mice. Using this model, we demonstrated that breast tumors and/or cells resistant to tamoxifen are also resistant to raloxifene, and so tamoxifen would not be an appropriate therapy for breast cancer after raloxifene resistance is diagnosed. More importantly, we have established that estradiol treatment causes tumor regression in SERM-sensitized cells by inducing G_2/M-phase arrest and apoptosis. Although the mechanism is not completely elucidated, the balance between survival and apoptosis may be mediated through the Fas/FasL pathway by the increasing Fas expression and the decreasing NF-κB activity. Overall, these data, and those in the companion study *(63)* using a tamoxifen-resistant model *in vivo,* suggest that it is possible for a patient's own estrogen to act as an anticancer agent in SERM-resistant breast cancers. Clearly, a clinical strategy to use an aromatase inhibitor after SERM resistance may have some short-term benefit for patients, but it is possible that a novel strategy of briefly treating patients with estrogen before re-instituting estrogen-deprivation therapy may benefit certain women. Indeed, preliminary studies *(75)* have demonstrated the beneficial effects of estrogen in patients for whom repeated estrogen-deprivation treatments have failed.

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