

# Dominant Negative ER Induces Apoptosis in GH<sub>4</sub> Pituitary Lactotrope Cells and Inhibits Tumor Growth in Nude Mice

EUN JIG LEE, W. RACHEL DUAN, MONIKA JAKACKA, BARRY D. GEHM, AND J. LARRY JAMESON

Division of Endocrinology, Metabolism, and Molecular Medicine and Northwestern University Medical School, Chicago, Illinois 60611

The ER plays an important role in the proliferation and differentiation of lactotrope tumor cells. GH<sub>4</sub> cells were infected with adenoviral vectors (AdL540Q and Ad1-536) to investigate the ability of dominant negative ER mutants to affect the regulation of gene expression and cell growth by endogenous ER. The dominant negative mutants suppressed estradiol stimulation of an estrogen-responsive reporter gene and the PRL promoter in these cells. AdL540Q or Ad1-536 infection also inhibited GH<sub>4</sub> cell growth and induced apoptosis, increasing the expression of the proapoptotic Bax protein and decreasing the expression of antiapoptotic Bcl-2. AdwtER-infected cells also showed decreased Bcl-2 protein. E2-induced

activation of p38 MAPK, an enzyme that may participate in apoptosis, was observed in cells infected with AdwtER, AdL540Q, and Ad1-536. Consistent with the apoptotic effects *in vitro*, infection of GH<sub>4</sub> cells with AdL540Q or Ad1-536 inhibited the ability of the cells to form tumors in nude mice. These results indicate that dominant negative ER mutants induce apoptosis of GH<sub>4</sub> cells and suppress tumor formation and development. The delivery of dominant negative ERs by adenoviral vectors may provide an alternative modality for the targeted therapy of pituitary lactotrope adenomas and other estrogen-responsive tumors. (*Endocrinology* 142: 3756–3763, 2001)

THE PITUITARY LACTOTROPE is a well established target of estrogens. Estrogens stimulate PRL synthesis, storage, and release as well as lactotrope proliferation (1, 2). There are several indications that estrogens are associated with the pathogenesis of pituitary lactotrope adenomas. The prevalence of prolactinoma is higher among women (3), and long-term stimulation with estrogen induces pituitary lactotrope tumors in animal models (4, 5). ER has been detected in prolactinoma cells (6–8). Some prolactinomas respond to treatment with antiestrogens such as tamoxifen (9). These findings suggest that the ER plays an important role in the proliferation and differentiation of lactotrope tumor cells. The down-regulation of normal ER function could, therefore, provide a potential therapy for this type of tumor.

The pure antiestrogens ICI 164384 and ICI 182780 have been shown to down-regulate ER and to block the transcription of ER-regulated genes. These antiestrogens effectively inhibit cell growth and induce apoptosis in ER-positive breast cancer MCF-7 cells (10) and GH<sub>3</sub> pituitary mammosomatotrope tumor cells (11). Dominant negative forms of the ER have been suggested as an alternative method to inactivate the ER. Several dominant negative ER mutants have been generated (12–14): truncated receptors (ER1-530 and ER1-536, missing the last 65 or 59 amino acid residues), a point mutant (L540Q), and a frameshift mutant (S554fs).

Abbreviations: CMV, Cytomegalovirus; ERE, estrogen response element; hER $\alpha$ , human ER $\alpha$ ; HSV-TK/GCV, herpes simplex virus thymidine kinase/ganciclovir; MOI, multiplicity of infection; p(A), polyadenylation; PFU, plaque-forming units; SV40, simian virus 40; TUNEL, terminal deoxynucleotidyltransferase-mediated UTP end labeling; wtER $\alpha$ , wild-type ER $\alpha$ .

Lazennec *et al.* (15) demonstrated that adenovirus-directed expression of the frame-shifted ER (S554fs) suppressed the proliferation of ER-positive breast cancer cells.

GH<sub>3</sub> cells are derived from rat pituitary tumors that occur after long-term treatment with estrogen (16, 17) and are widely used as an *in vitro* model of lactotropes or somatotropes. These cells express ER $\alpha$ , ER $\beta$ , and the truncated ER product lacking exons 1–4 of ER $\alpha$  (18–20). GH<sub>4</sub>C<sub>1</sub> (hereafter referred to as GH<sub>4</sub>) cells are derived from GH<sub>3</sub> cells and secrete less GH (21). These cells exhibit many features of lactotropes (22, 23). In the present study we used adenoviral vectors carrying the dominant negative ER mutants L540Q and ER1-536 and examined their effects on gene transcription, cell proliferation, and apoptosis in GH<sub>4</sub> tumor cells *in vitro* and in a tumor-bearing animal model.

## Materials and Methods

### Generation of recombinant adenoviral vectors

A cassette containing the human ER $\alpha$  (hER $\alpha$ ) cDNA (provided by Dr. Pierre Chambon, Universite Louis Pasteur, Strasbourg, France) driven by the cytomegalovirus (CMV) promoter/enhancer with a simian virus 40 (SV40) polyadenylation [p(A)] sequence was subcloned into an adenoviral transfer plasmid (24) based on pCDNA3 (Invitrogen, Carlsbad, CA). The dominant negative ERs, L540QhER $\alpha$  and 1-536hER $\alpha$ , were created using site-directed mutagenesis and were exchanged for the wild-type ER $\alpha$  (wtER) in the adenoviral transfer plasmid. The resulting plasmids, pCwtER, pCL540Q, and pC1-536, were used to generate recombinant adenoviruses. Linearized transfer plasmids containing 5' 393 bp of adenoviral sequence and expression cassette were ligated with *Cla*I-digested Ad5 309/356 DNA representing map units 3.0–100. (Ad5 309/356 is a recombinant adenovirus in which the E3 region is deleted. *Cla*I digestion removes the E1a region, resulting in a replication-deficient virus.) The ligation products were transfected into 293 cells, in which cellular expression of the E1a protein allows replication of the E1-deleted

recombinant viruses. The cloned and purified adenoviral vectors were titrated by plaque assay. Recombinant adenoviruses carrying wild-type hER $\alpha$ , L540QhER $\alpha$ , and 1-536hER $\alpha$  were designated AdwtER, AdL540Q, and Ad1-536, respectively. AdGal, which contains  $\beta$ -galactosidase driven by CMV promoter, was used as a control.

An adenoviral reporter vector, AdERE-Luc, was created to investigate transcriptional activity of the wild-type or dominant negative ER by adenoviral vectors. The ERE2-TK109 promoter sequence was excised from ERE2-tk109-luc (25) and ligated into the pGL3-promoter plasmid (Promega Corp., Madison, WI) from which the SV40 promoter had been deleted (*NheI* to *HindIII*). A portion of the resulting plasmid containing the upstream synthetic p(A) signal, two consensus estrogen response elements (EREs), a 109-bp fragment of the thymidine kinase promoter, the firefly luciferase gene, and the downstream SV40 p(A) signal, was subcloned into the adenoviral transfer plasmid. The resulting plasmid, pC-ERE-Luc, was used to generate AdERE-Luc. The sequences of the expression cassettes in the adenoviral vectors were confirmed by automated DNA sequencing. Structures of the adenoviral vectors are shown in Fig. 1.

#### Cell culture and infection with recombinant adenoviruses

GH<sub>4</sub> cells and HEK293 embryonic kidney cells were obtained from the American Type Culture Collection (Manassas, VA). HEK293 cells were maintained in DMEM and 10% FBS, and GH<sub>4</sub> cells were maintained in DMEM/Ham's F-12 and 10% FBS. All media were supplemented with 100 U/ml penicillin and 100  $\mu$ g/ml streptomycin, and all cells were incubated at 37 C in 5% CO<sub>2</sub>.

For infection with adenoviral vectors, cells were first depleted of estrogen for 3 d using phenol red-free DMEM/Ham's F-12 containing 5% dextran/charcoal-stripped FBS. The transduction efficiency of the adenoviral vectors in cell lines was tested using AdGal.  $\beta$ -Galactosidase expression was detected in 95–100% of GH<sub>4</sub> cells at 48 h after infection with AdGal at a multiplicity of infection (MOI) of 5 plaque-forming units (PFU)/cell (data not shown). Therefore, subsequent experiments were performed using similar amounts (5 or 10 PFU/cell) of recombinant adenoviral vectors.

The transcriptional activities of wtER and the dominant negative mutants were assayed using an artificial estrogen-responsive reporter in a viral vector (AdERE-luc) and the naturally estrogen-responsive PRL promoter in a reporter plasmid (2.5 PRL-luciferase) (26). Briefly, 12-well plates of GH<sub>4</sub> cells were infected overnight with 5 PFU/cell AdERE-Luc

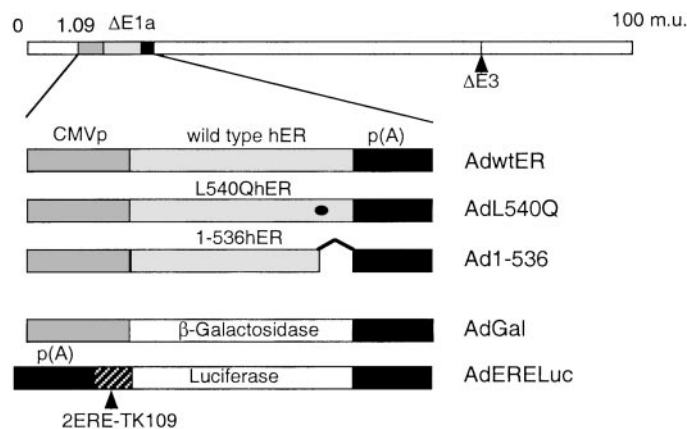


FIG. 1. Structures of recombinant adenoviruses. The adenoviral vector contains a backbone derived from adenovirus type 5 (Ad5 309/356), in which the E3 regions have been deleted. The genes of interest (shaded area) were inserted in place of the E1a region. Five different recombinant adenoviral vectors were generated: AdwtER, AdL540Q, Ad1-536, AdGal, and AdERE-Luc. AdGal, which contains the  $\beta$ -galactosidase gene driven by the CMV promoter, was used to determine the efficiency of gene transduction. AdERE-Luc, which carries two EREs, the minimal thymidine kinase promoter (109 bp; 2ERE-TK109), and the luciferase gene, was used to investigate the transcriptional activity of wild-type and dominant-negative ER expressed by adenoviral vectors.

and increasing amounts (1, 5, and 10 PFU/cell) of AdwtER, AdL540Q, or Ad1-536. Fresh medium with or without estradiol (E2) was added, incubation was continued for 24 h, and luciferase activity was assayed. GH<sub>4</sub> cells were also transfected with 500 ng/well PRL-luciferase plasmid (provided by Dr. Richard A. Maurer, Oregon Health Sciences University, Portland, OR) using Lipofectamine Plus (Life Technologies, Inc., Gaithersburg, MD), followed by infection with adenoviral vectors as described above.

#### Immunofluorescent detection of ER expression

GH<sub>4</sub> cells were collected, washed twice with PBS, and mounted on glass slides 48 h after infection with adenoviral vectors. After 20 min of air-drying, slides were fixed in ice-cold methanol and acetone for 10 min each. After preincubation with serum-blocking solution (ABC kit, Vector Laboratories, Inc., Burlingame, CA) for 10 min, specimens were incubated with mouse monoclonal antihuman ER (1:50; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) for 1 h at room temperature. After washing with Tris-buffered saline and 0.025% Tween, staining was performed using biotinylated secondary antibodies (ABC kit, Vector Laboratories, Inc.) and streptavidin-FITC (1:100; Vector Laboratories, Inc.). Cell images were analyzed using a Carl Zeiss microscope (Axioskop, Carl Zeiss, Oberkochen, Germany) and Fuji Photo Film Co., Ltd. color film (1600 Super HG, Fuji Photo Film Co., Ltd., Tokyo, Japan).

#### Terminal deoxynucleotidyltransferase-mediated UTP end labeling (TUNEL) assay

GH<sub>4</sub> cells were infected with adenoviral vectors (5 PFU/cell), treated with 1 nM E2 for 6 d, then washed twice with PBS and mounted on glass slides. Cells were fixed for 30 min in 4% paraformaldehyde and permeabilized with buffer containing 0.1% sodium acetate and 0.4% Triton X-100 for 10 min on ice. After washing with PBS, a modified TUNEL was performed using the *In Situ* Cell Death Detection Kit, Fluorescein (Roche Molecular Biochemicals, Indianapolis, IN). Cells were visualized and photographed as described above.

#### Western blot analysis of Bcl-2, BAX, and p38MAPK expression

Cells were plated in 10-cm culture dishes at a density of  $5 \times 10^6$  cells/dish. The following day, they were infected with adenoviral vectors at an MOI of 5 PFU/cell for 5 h. After the addition of fresh medium, the cells were incubated for 48 or 72 h with or without 1 nM E2. Cells were washed twice with PBS, and whole cell lysates were prepared with lysis buffer [25% glycerol, 0.5 M NaCl, 1.5 mM MgCl<sub>2</sub>, 20 mM HEPES (pH 7.9), 1 mM phenylmethylsulfonyl fluoride, 0.2 mM EDTA, 25 mM NaF, and protease inhibitor cocktail tablets (Roche Molecular Biochemicals)]. Equal amounts of protein (20  $\mu$ g) were resolved by SDS-PAGE on 10% gel and transferred to nitrocellulose paper. The membranes were blocked with 3% nonfat milk in PBS for 1.5 h and then incubated overnight at 4 C with primary antibodies. Mouse monoclonal anti-Bcl-2 (1:1000; Santa Cruz Biotechnology, Inc.) and mouse monoclonal anti-Bax (1:1000; Santa Cruz Biotechnology, Inc.), were used for the detection of apoptosis-associated proteins. The activated p38MAPK (the Thr<sup>180</sup>/Tyr<sup>182</sup>-phosphorylated p38MAPK) and p38MAPK were detected by phospho-p38MAPK (1:1000) and p38MAPK (1:2000) polyclonal antibodies (New England Biolabs, Inc., Beverly, MA), respectively.

After three washes in 0.1% Tween-20 in PBS, immunoreactive proteins were detected using an antimoser or rabbit horseradish peroxidase-conjugated antibody (1:5000; Promega Corp.) and the enhanced chemiluminescence system (Amersham Pharmacia Biotech, Arlington Heights, IL). Bands were detected with X-Omat film (Eastman Kodak Co., Rochester, NY).

#### Effect of dominant negative ERs on GH<sub>4</sub> cell growth *in vitro*

The effect of dominant negative ERs on GH<sub>4</sub> cell growth was measured with a nonradioactive cell proliferation assay according to the manufacturer's protocol (Cell Titer 96 Aqueous NonRadioactive Cell Proliferation Assay, Promega Corp.). Cells were seeded in 96-well plates at a density of  $5 \times 10^3$  cells/well and infected on the following day with

adenoviral vectors at different MOIs (0, 5, and 10 PFU/cell). Medium was replaced at 5 h after infection and every 2 d thereafter. To measure the effects of different doses of E2 (1, 10, and 100 nM), quadruplicate wells were assayed for viable cell density on d 6. Relative density was calculated as the absorbance at 490 nm divided by that of the uninfected, 1 nM E2-treated cells and expressed as a percentage (mean  $\pm$  SD). In a separate experiment cell density was assayed at 2-d intervals over an 8-d period with a fixed (1-nM) E2 concentration.

#### Effect of dominant negative ER on growth of GH<sub>4</sub> cells in nude mice

GH<sub>4</sub> cells were infected with 5 PFU/cell of adenoviruses and incubated at 37 C for 24 h. Cells were collected, washed twice with PBS, resuspended in medium, and injected ( $2 \times 10^6$  cells) into the flanks of adult (8-wk-old) athymic female nude mice (Harlan-Sprague Dawley, Indianapolis, IN) that had been sc implanted with 60-d estrogen pellets (Innovative Research of America, Sarasota, FL) 7 d earlier. The mice were divided into five groups: group A, no virus (n = 8); group B, AdGal (n = 6); group C, AdwtER (n = 9); group D, AdL540Q (n = 8); and group E, Ad1-536 (n = 8). Animals were examined for tumor formation every 2 d, and the size of the tumor was measured with calipers in three dimensions. Tumor size (cubic millimeters) was calculated using the formula:  $(3.14 \times \text{length} \times \text{width} \times \text{depth})/6$ . The experiment was terminated 2 wk after cell injection because control (no virus and AdGal) mice began to show morbidity. All studies involving the use of nude mice were approved by the Northwestern University Medical School animal care and use committee.

## Results

#### Expression of hER delivered by adenoviral vector

Because GH<sub>4</sub> cells express endogenous rat ER, a specific antihuman ER antibody was used to detect human ER delivered by adenoviral vectors. AdGal-infected cells served as a negative control. The human ER was detected by immunofluorescence in the nuclei of GH<sub>4</sub> cells infected with AdwtER, AdL540Q, and Ad1-536, but not in cells infected with AdGal. ER expression was detected in 95–100% of GH<sub>4</sub> cells infected with 5 PFU/cell of adenoviral vectors (Fig. 2).

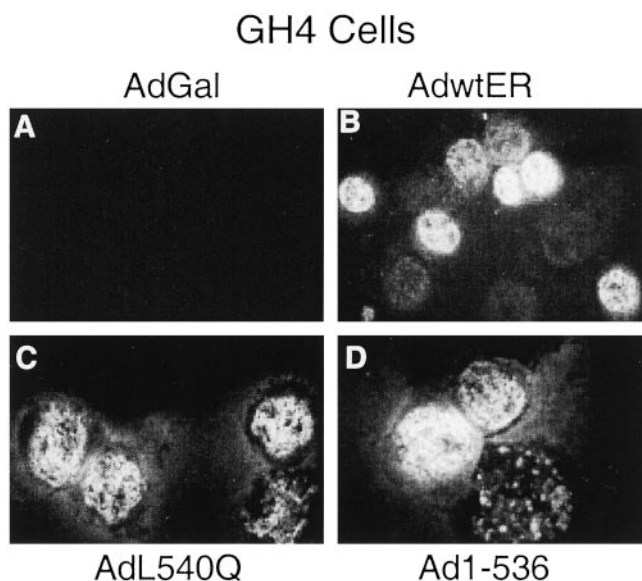


FIG. 2. Expression of the human ER $\alpha$  in GH<sub>4</sub> cells infected with adenoviral vectors. GH<sub>4</sub> cells infected with AdGal (A), AdwtER (B), AdL540Q (C), and Ad1-536 (D) were subjected to immunofluorescence staining with mouse monoclonal antihuman ER $\alpha$ , as described in *Materials and Methods*.

The expected reduction in molecular weight for the 1-536 truncation mutant and comparable levels of protein expression for the wtER and mutants were confirmed by Western blot analysis of infected MDA-MB-231 cells, an ER-negative breast cancer cell line (data not shown).

#### Effect of dominant negative ER on transcriptional activity of the endogenous ER in GH<sub>4</sub> cells

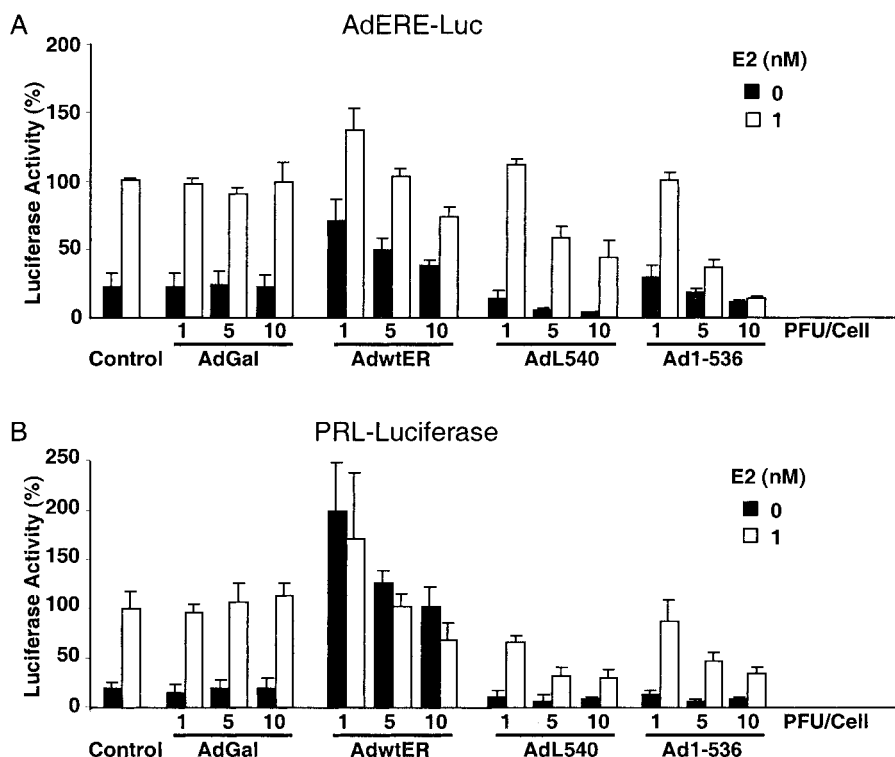
To investigate whether expression of a dominant negative ER affects the ability of the endogenous ER to activate an estrogen-responsive reporter gene, AdERE-Luc was coinfecting into GH<sub>4</sub> cells with adenoviruses carrying dominant negative ERs. As shown in Fig. 3A, E2 (1 nM) treatment stimulated ERE reporter gene activity 6- to 8-fold over background in control (uninfected and AdGal-infected) cells. AdwtER infection increased reporter activity 3-fold without ligand, presumably due to residual estrogen in the medium. AdwtER infection at an MOI of 1 PFU/cell also increased reporter response somewhat in the presence E2. However, both unstimulated and E2-stimulated activities declined at higher doses of the vector. Infection with adenoviral vectors expressing dominant negative ERs also suppressed reporter activity in both the absence and presence of E2. The suppression was greater in cells infected with Ad1-536 (64% and 86% at 5 and 10 PFU/cell, respectively) than with AdL540Q (42% and 57%).

The effect of dominant negative ERs was also tested using the PRL promoter (Fig. 3B). E2 treatment stimulated PRL promoter activity 6- to 8-fold in control (uninfected and AdGal-infected) cells. AdwtER infection increased PRL reporter response by 20-fold over background without ligand. However, higher doses of AdwtER diminished reporter activity. In cells infected with AdwtER, reporter activity was independent of or slightly inhibited by treatment with E2. This may be due to cooperative activation by endogenous Pit-1 and unliganded wild-type ER (27) or enhanced sensitivity to residual traces of E2 in the stripped serum when wild-type ER is overexpressed. In contrast to AdwtER, infection with adenoviral vectors expressing dominant negative ERs suppressed PRL promoter activity in a dose-dependent manner, with or without E2. This suppression was greater in cells infected with AdL540Q (69% and 70% at 5 and 10 PFU/cell, respectively) than in those infected with Ad1-536 (54% and 65%).

#### Effect of dominant negative ER on GH<sub>4</sub> cell growth in vitro

To investigate whether the disruption of ER signaling by dominant negative ER expression could influence cell growth, we analyzed the proliferation of GH<sub>4</sub> cells infected with two different doses (5 and 10 PFU/cell) of adenoviral vectors. As shown in Fig. 4, A and B, GH<sub>4</sub> cell growth was stimulated by 6-d E2 treatment (1–100 nM) in uninfected cells. AdL540Q or Ad1-536 infection suppressed the growth of E2-treated GH<sub>4</sub> cells in a pattern dependent on viral dose (65–75% with 5 PFU/cell, 75–85% with 10 PFU/cell). AdGal had little effect on growth of GH<sub>4</sub> cells. Infection with 5 PFU/cell of AdwtER caused minimal growth inhibition, but 10 PFU/cell of AdwtER also induced 60–70% growth inhibition in the presence of E2. Although their effects were most

FIG. 3. Effect of dominant negative ERs on transcriptional activity by the endogenous ER of GH<sub>4</sub> cells. ER transcriptional activity was assayed using AdERE-luc (A) and PRL-luciferase (B), as described in *Materials and Methods*. Three independent experiments were normalized to the activity of control GH<sub>4</sub> cells treated with E2, and results are plotted as the mean  $\pm$  SD in A and B.



pronounced in the presence of E2, AdL540Q, and Ad1-536, but not wtER, produced growth inhibition in the absence of added hormone (48% and 70%, respectively, at 10 PFU/cell).

To assess the time course of dominant negative ER effects on cell growth, cells were treated with E2 (1 nM) over an 8-d period (Fig. 4, C and D). Growth inhibition by Ad1-536 or AdL540Q was apparent by d 4 or 6. AdwtER had little effect on cell growth at 5 PFU/cell, but 10 PFU/cell inhibited growth significantly. The growth rate of GH<sub>4</sub> cells was not affected by the AdGal virus, indicating that growth inhibition was not due to nonspecific effects of viral infection.

#### Effect of dominant negative ER on induction of apoptosis, BAX and Bcl-2 expression, and p38 MAPK activation in GH<sub>4</sub> cells

The TUNEL reaction was used to investigate whether dominant negative ERs induce apoptosis. GH<sub>4</sub> cells were infected with adenoviral vectors, treated with E2, and assayed as described in *Materials and Methods*. A positive TUNEL reaction was obtained in about 40–50% of cells infected with AdL540Q and Ad1-536, but in only 5–10% of cells infected with AdwtER (Fig. 5, B and C, D). The TUNEL reaction was negative in AdGal-infected cells (Fig. 5A) and uninfected cells regardless of estrogen treatment (data not shown).

Western blotting was used to examine expression of specific proteins associated with apoptosis. GH<sub>4</sub> cells were infected with adenoviral vectors and treated with E2 for 48 or 72 h. Bax expression was increased, and Bcl-2 expression was decreased in cells infected with either AdL540Q or Ad1-536 (Fig. 6, A and B). The levels of Bax expression were markedly increased in Ad1-536-infected cells. AdwtER-infected cells also showed decreased Bcl-2 expression.

To analyze the effect of dominant negative ERs on p38 MAPK activity, Western blot analyses were performed with an antibody that specifically recognizes phosphorylated p38 MAPK. Lysates were isolated from GH<sub>4</sub> cells treated with E2 over a range of times (0 and 15 min and 6, 12, 24, 48, and 72 h). An increase in phosphorylated p38 MAPK was detected at 48 and 72 h in E2-treated cells infected with AdwtER, AdL540Q, and Ad1-536, but not with AdGal control virus (Fig. 6C).

#### Effect of dominant negative ER on growth of GH<sub>4</sub> cells in nude mice

Based on the findings that dominant negative ERs inhibit cell growth and induce apoptosis *in vitro*, we hypothesized that expression of a dominant negative ER might inhibit tumor formation by pituitary prolactinoma cells in nude mice (see Fig. 7). GH<sub>4</sub> cells were injected sc into estrogen-treated female athymic mice as described in *Materials and Methods*. Tumors developed within 6 d in mice injected with uninfected or AdGal-infected cells. These tumors grew very rapidly and reached half the size of the mouse at the end of 2 wk. In mice injected with cells infected with AdwtER, tumor formation was delayed until 10 d after injection. The rate of growth and the size of the tumors were greatly reduced compared with those in control groups (no virus and AdGal). Even more striking, there was almost complete suppression of tumor formation in the animals injected with cells infected with AdL540Q or Ad1-536.

## Discussion

We used GH<sub>4</sub> cells as an *in vitro* model to study the effects of dominant negative ER mutants. Estrogen treatment activated an estrogen-responsive reporter gene and the PRL

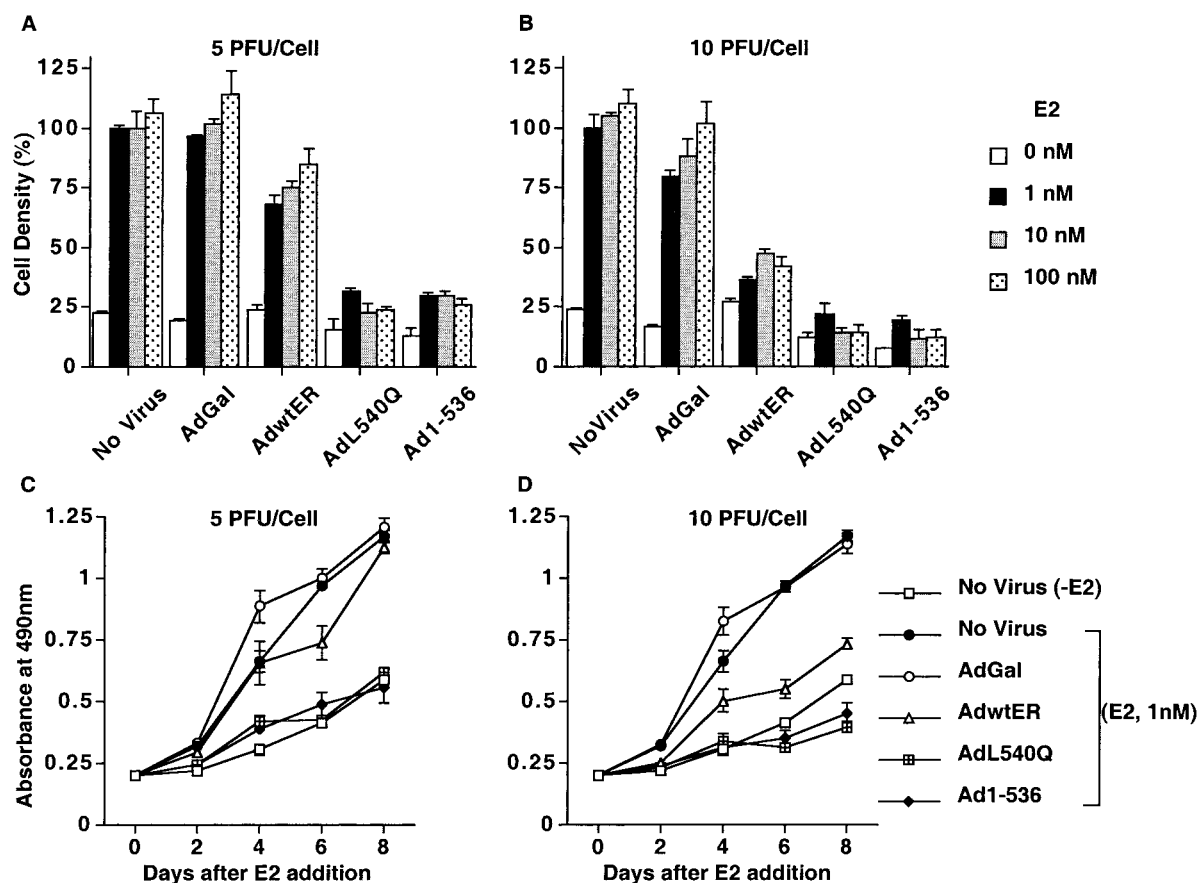


FIG. 4. Effect of dominant negative ERs on the growth of GH<sub>4</sub> cells. Cells were plated, infected, and treated as described in *Materials and Methods*. After treatment for 6 d with various doses of E2 (A and B) or at intervals over an 8-d treatment with 1 nM E2 (C and D), cell growth was determined by a colorimetric assay. For A and B, cell density is expressed as a percentage, normalized to E2-treated (1 nM) uninfected cells. All results are plotted as the mean  $\pm$  SD for three or more independent experiments.

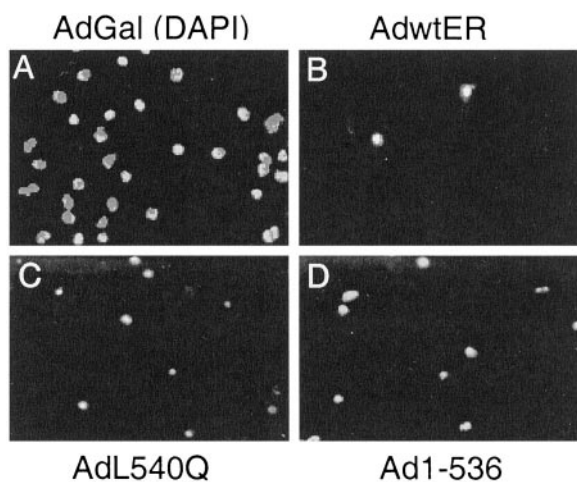


FIG. 5. Induction of positive TUNEL reaction. GH<sub>4</sub> cells were infected with AdGal (A), AdwtER (B), AdL540Q (C), and Ad1-536 (D) at an MOI of 5 PFU/cell. After 6 d of treatment with E2 (1 nM), TUNEL assays were performed. Fluorescent staining indicates TUNEL labeling. In A, AdGal-infected cells were also stained with 4',6-diamidino-2-phenylindole (*violet*) to show cell density.

promoter, and also stimulated the proliferation of GH<sub>4</sub> cells, suggesting that the ER is one of the major regulators of gene transcription and cellular proliferation in these cells.

As dominant negative ER mutants are known to suppress the transcription of genes regulated by wtER, it has been suggested that such mutants could influence the proliferation of ER-positive tumor cells. An efficient gene delivery system is required to achieve high levels of expression of ER mutants in target cells. A recent report (15) demonstrated that adenovirus-directed expression of the frame-shifted ER mutant S554fs, a dominant negative ER, induced apoptosis of ER-positive breast cancer cells. A similar strategy might be applied to pituitary lactotrope adenoma cells. In this study, using adenoviral vectors carrying different dominant negative ER mutants (L540Q and 1-536), we demonstrated the induction of apoptosis in ER-positive lactotrope GH<sub>4</sub> cells and suppression of GH<sub>4</sub> tumor growth in nude mice.

Pituitary lactotrope cells express ER $\alpha$  and ER $\beta$  (18–20), both of which regulate PRL gene transcription (2, 27). Due to heterodimerization, dominant negative mutants of each are able to inhibit the activity of both isoforms (28). We confirmed that L540QhER $\alpha$  and 1-536hER $\alpha$  suppress ER $\beta$  transcriptional activity effectively in transiently transfected TSA cells (data not shown). When expressed by adenoviral vectors, both mutants suppressed the transcriptional activity of endogenous ERs in GH<sub>4</sub> cells, as assessed using ERE reporter genes or the rat PRL promoter (Fig. 3B). Interestingly, Ad1-536 was a more effective inhibitor of the artificial estrogen-

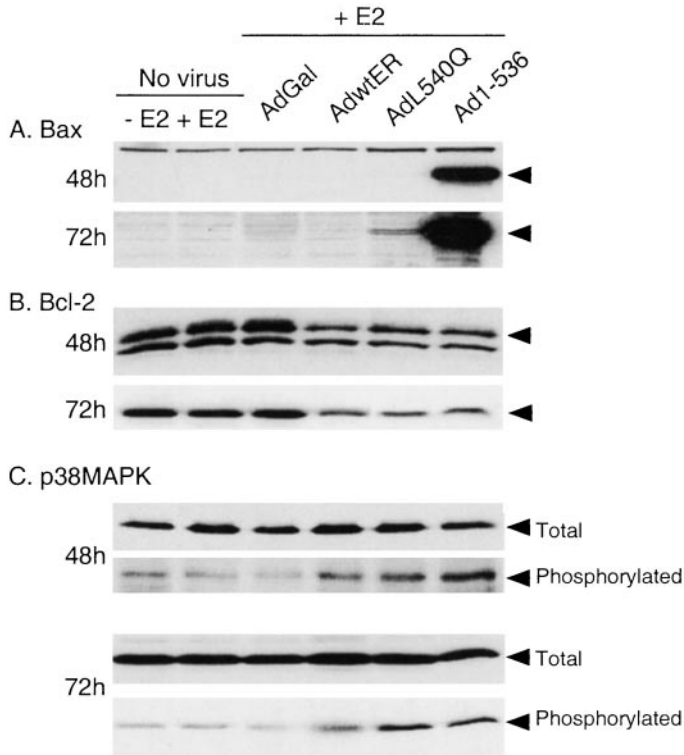


FIG. 6. Expression of Bax and Bcl-2, and activation of p38 MAPK in GH<sub>4</sub> cells. Cells were infected with 5 PFU/cell of adenoviral vectors and treated with E2 for 48 h or 72 h. Equal amounts of whole cell extracts were resolved by SDS-PAGE and immunoblotted with Bax or Bcl-2 mouse monoclonal antibody, phospho-p38MAPK, and p38MAPK polyclonal antibodies. A, Bax; B, Bcl-2; C, total and phospho-p38 MAPK.

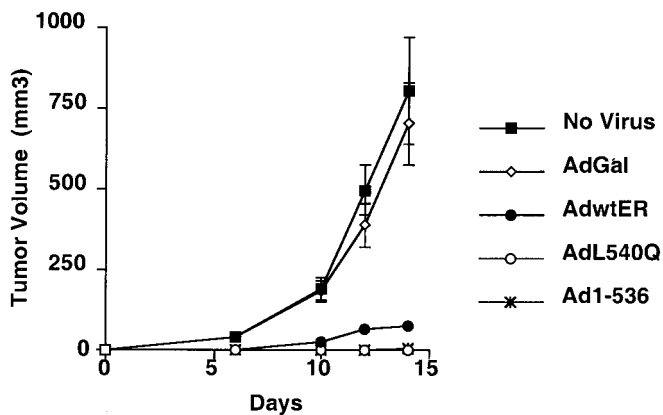


FIG. 7. Effect of dominant negative ERs on the growth of GH<sub>4</sub> cells in nude mice. GH<sub>4</sub> cells were uninfected or infected with 5 PFU/cell of AdGal, AdwtER, AdL540Q, and Ad1-536. After incubation for 24 h at 37°C, cells were collected, washed twice with PBS, and injected into the flank of nude mice ( $1 \times 10^6$  cells). The size of the tumor was measured with calipers in three dimensions every 2 d for 2 wk. Each point represents the mean  $\pm$  SD of tumor volumes in six to nine mice, as described in *Materials and Methods*.

responsive reporter gene, whereas AdL540Q was more effective against the natural PRL promoter. Thus, the relative efficacy of the dominant negative mutants appears to vary with respect to different reporter genes.

Apoptosis, or programmed cell death, plays an important role in maintaining cellular homeostasis to ensure the bal-

ance between the rates of cellular proliferation and cell loss. Apoptosis is inhibited by the Bcl-2/Ced-9 family of proteins (29). The *bcl-2* gene is overexpressed in many tumors, including breast cancers (30, 31). This gene is also expressed in about 60% of prolactinomas, a higher expression rate than in any other subset of pituitary tumors (32). A positive correlation of Bcl-2 expression with markers of angiogenesis was also demonstrated in prolactinomas (33). The induction of apoptosis in GH<sub>3</sub> cells by bromocriptine is accompanied by decreased Bcl-2 expression (34). These results suggest that *bcl-2* gene expression is an important factor in the survival of pituitary lactotrope tumor cells. Estrogen is known to up-regulate *bcl-2* transcription in ER-positive MCF-7 and T47D human breast cancer cells (31, 35, 36). A recent report revealed that estrogen induction is mediated by two EREs present in the *bcl-2*-coding region (37). Consistent with these findings, we show that infection of AdL540Q and Ad1-536 decreased estrogen-induced Bcl-2 expression. However, AdwtER had a similar effect, suggesting that the ER regulation of this antiapoptotic gene may involve a nonclassical transcriptional mechanism.

The dominant negative ER mutants also increased expression of the proapoptotic Bax protein. These results indicate that in GH<sub>4</sub> cells, apoptosis induced by dominant negative ERs is associated with down-regulation of Bcl-2 and up-regulation of Bax. In many cancer cells, Bax overexpression produces increased sensitivity to stressful stimuli, resulting in decreased cell survival and increased apoptosis. The ratio of Bcl-2 to Bax, rather than the absolute level of either protein, may therefore determine the sensitivity to apoptosis (38). However, in the present experiments this ratio did not correlate to apoptosis by AdL540Q or Ad1-536, suggesting that another pathway might be involved in apoptosis induced by dominant negative ERs in GH<sub>4</sub> cells.

AdwtER-infected cells showed growth inhibition and induction of apoptosis when treated with estrogen. These results were not entirely unexpected, because growth inhibition has been reported previously in cells transiently or stably transfected with the ER (39–42). In addition, we observed similar results in ER-positive T47D breast cancer cells infected with AdwtER or adenovirus encoding mouse ER $\alpha$  (unpublished results). Although the mechanism of cell death remains unknown, it is possible that high levels of ER expression titrate transcription factors that are necessary for cell proliferation or induce the expression of estrogen-regulated growth inhibitory/cytotoxic genes. Of note, the AdwtER was less effective than the dominant negative mutants for inhibiting *in vitro* cell proliferation, inducing DNA fragmentation, and suppressing tumor growth, suggesting that different mechanisms may be involved in the induction of apoptosis by the AdwtER and mutants (43).

The p38 MAPK pathway is also known to be strongly correlated to apoptosis, although the underlying mechanisms are not well understood. p38 MAPK is activated by several environmental stresses, such as UV light, heat shock, and osmotic shock. p38 MAPK is also activated by the proinflammatory cytokines IL-1 and TNF- $\alpha$ . These stressful stimuli induce growth inhibition or apoptosis in cells, and SB203580, a selective p38 MAPK inhibitor, abolishes these effects (44, 45), suggesting that the activation of p38 MAPK

plays an important role in apoptosis. A similar result was observed in bromocriptine-induced apoptosis of pituitary GH<sub>3</sub> cells (46). A recent report showed that activation of the p38 MAPK pathway is involved in E2 induction of apoptosis in HeLa cells stably expressing significant levels of ER (47). In our study overexpression of wtER increased p38 MAPK activation in estrogen-treated GH<sub>4</sub> cells. Expression of dominant negative ERs had a similar effect. The activation of p38 MAPK by UV light or genotoxic stress is known to phosphorylate the p53 tumor suppressor (45, 48), which results in increased transcription of genes involved in apoptosis. The present study also demonstrates that induction of apoptosis by dominant negative ERs in GH<sub>4</sub> cells is associated with up-regulation of the proapoptotic protein Bax, which is regulated positively by wild-type p53 (49). However, at present it is unknown whether Bax induction is related to p53 phosphorylation through the activation of p38 MAPK.

The GH<sub>4</sub> cell tumors in control groups of nude mice (uninfected or AdGal-infected) grew very rapidly and reached half the size of the mice by the end of 2 wk, suggesting that GH<sub>4</sub> cells are highly malignant. These rapidly growing tumors may not be an appropriate model for human pituitary prolactinomas, which are usually benign and slow-growing. The rate of tumor growth may affect the choice of the appropriate gene for gene therapy; for example, the herpes simplex virus thymidine kinase/ganciclovir (HSV-TK/GCV) suicide system is mitosis dependent. In a previous study using the HSV-TK/GCV system we observed a highly cytotoxic effect on rapidly growing tumors in nude mice (24). In contrast, the HSV-TK/GCV system under control of the PRL promoter was not effective for lowering PRL levels in a rat model of pituitary lactotrope hyperplasia, which may more closely resemble human prolactinomas (50). Presumably this is due to the decreased effectiveness of GCV in slowly dividing cells. Delivery of apoptosis-inducing or directly toxic genes may therefore be more effective for slowly growing tumors, and it will be interesting to examine the AdL540Q and Ad1–536 dominant negative mutants in the rat lactotrope-hyperplasia model. The clinical application of this strategy must await further analyses of efficacy and safety of the recombinant adenoviruses.

In conclusion, we have demonstrated that adenovirus-directed expression of dominant negative ERs induces growth suppression and apoptosis in pituitary lactotrope adenoma cell lines *in vitro* and inhibits tumor growth *in vivo* in nude mice. These results suggest that dominant negative ER mutants have the potential to suppress growth or induce apoptosis of ER-positive tumor cells, and that the delivery of dominant negative ERs by adenoviral vectors may be an alternative modality for the targeted therapy of pituitary lactotrope adenomas.

### Acknowledgments

We are grateful to Fred Martinson for his help with animal experiments. We also thank Dr. Pierre Chambon for providing cDNA of hER $\alpha$ , Dr. Richard A. Maurer for providing 2.5 PRL-luciferase reporter gene plasmid, and Tom Kotlar for critical reading and discussion.

Received December 21, 2000. Accepted May 11, 2001.

Address all correspondence and requests for reprints to: J. Larry Jameson, M.D., Ph.D., Division of Endocrinology, Metabolism, and

Molecular Medicine, Northwestern University Medical School, 303 East Chicago Avenue, Chicago, Illinois 60611. E-mail: ljameson@northwestern.edu.

This work was supported by a grant from the Northwestern Memorial Foundation, by a Center of Excellence grant from Knoll Pharmaceutical Co., and National Institute Specialized Program of Research Excellence (SPORE) Grant IP50 CA-89018-01. Additional support was provided by U.S. Army Medical Research and Materiel Command Breast Cancer Research Program Grants DAMD17-94-J-4082 (to J.L.J.) and DAMD17-99-1-9334 (to B.D.G.).

### References

- Lieberman ME, Maurer RA, Gorski J 1978 Estrogen control of prolactin synthesis *in vitro*. Proc Natl Acad Sci USA 75:5946–5949
- Maurer RA 1982 Estradiol regulates the transcription of the prolactin gene. J Biol Chem 257:2133–2136
- Drange MR, Fram NR, Herman-Bonert V, Melmed S 2000 Pituitary tumor registry: a novel clinical resource. J Clin Endocrinol Metab 85:168–174
- Burgett RA, Garris PA, Ben-Jonathan N 1990 Estradiol-induced prolactinomas: differential effects on dopamine in posterior pituitary and median eminence. Brain Res 531:143–147
- Walker BE, Kurth LA 1993 Pituitary tumors in mice exposed prenatally to diethylstilbestrol. Cancer Res 53:1546–1549
- Jaffrain-Rea ML, Petrangeli E, Ortolani F, et al. 1996 Cellular receptors for sex steroids in human pituitary adenomas. J Endocrinol 151:175–184
- Shupnik MA, Pitt LK, Soh AY, Anderson A, Lopes MB, Laws Jr ER 1998 Selective expression of estrogen receptor alpha and beta isoforms in human pituitary tumors. J Clin Endocrinol Metab 83:3965–3972
- Chaidarun SS, Swearingen B, Alexander JM 1998 Differential expression of estrogen receptor- $\beta$  (ER $\beta$ ) in human pituitary tumors: functional interactions with ER alpha and a tumor-specific splice variant. J Clin Endocrinol Metab 83:3308–3315
- Lamberts SW, de Quijada M, Klijn JG 1980 The effect of tamoxifen on GH and PRL secretion by human pituitary tumors. J Endocrinol Invest 3:343–347
- Coopman P, Garcia M, Brunner N, Derocq D, Clarke R, Rochefort H 1994 Anti-proliferative and anti-estrogenic effects of ICI 164,384 and ICI 182,780 in 4-OH-tamoxifen-resistant human breast-cancer cells. Int J Cancer 56:295–300
- Newton CJ 1995 Estrogen receptor blockade by the pure antiestrogen, ZM 182780, induces death of pituitary tumour cells. J Steroid Biochem Mol Biol 55:327–336
- Ince BA, Zhuang Y, Wrenn CK, Shapiro DJ, Katzenellenbogen BS 1993 Powerful dominant negative mutants of the human estrogen receptor. J Biol Chem 268:14026–14032
- Ince BA, Schodin DJ, Shapiro DJ, Katzenellenbogen BS 1995 Repression of endogenous estrogen receptor activity in MCF-7 human breast cancer cells by dominant negative estrogen receptors. Endocrinology 136:3194–3199
- Chien PY, Ito M, Park Y, Tagami T, Gehm BD, Jameson JL 1999 A fusion protein of the estrogen receptor (ER) and nuclear receptor corepressor (NCoR) strongly inhibits estrogen-dependent responses in breast cancer cells. Mol Endocrinol 13:2122–2136
- Lazennec G, Alcorn JL, Katzenellenbogen BS 1999 Adenovirus-mediated delivery of a dominant negative estrogen receptor gene abrogates estrogen-stimulated gene expression and breast cancer cell proliferation. Mol Endocrinol 13:969–980
- Yasumura Y, Buonassisi V, Sato G 1966 Clonal analysis of differentiated function in animal cell cultures. I. Possible correlated maintenance of differentiated function and the diploid karyotype. Cancer Res 26:529–535
- Tashjian Jr AH, Yasumura Y, Levine L, Sato GH, Parker ML 1968 Establishment of clonal strains of rat pituitary tumor cells that secrete growth hormone. Endocrinology 82:342–352
- Shupnik MA, Gordon MS, Chin WW 1989 Tissue-specific regulation of rat estrogen receptor mRNAs. Mol Endocrinol 3:660–665
- Friend KE, Ang LW, Shupnik MA 1995 Estrogen regulates the expression of several different estrogen receptor mRNA isoforms in rat pituitary. Proc Natl Acad Sci USA 92:4367–4371
- Mitchner NA, Garlick C, Steinmetz RW, Ben-Jonathan N 1999 Differential regulation and action of estrogen receptors  $\alpha$  and  $\beta$  in GH3 cells. Endocrinology 140:2651–2658
- Tashjian AH, Jr 1979 Clonal strains of hormone-producing pituitary cells. Methods Enzymol 58:527–535
- Kiley SC, Parker PJ, Fabbro D, Jaken S 1991 Differential regulation of protein kinase C isozymes by thyrotropin-releasing hormone in GH4C1 cells. J Biol Chem 266:23761–23768
- Shimon I, Huttner A, Said J, Spirina OM, Melmed S 1996 Heparin-binding secretory transforming gene (hst) facilitates rat lactotrope cell tumorigenesis and induces prolactin gene transcription. J Clin Invest 97:187–195
- Lee EJ, Anderson LM, Thimmapaya B, Jameson JL 1999 Targeted expression of toxic genes directed by pituitary hormone promoters: a potential strategy for adenovirus-mediated gene therapy of pituitary tumors. J Clin Endocrinol Metab 84:786–794

25. **Gehm BD, McAndrews JM, Jordan VC, Jameson JL** 2000 EGF activates highly selective estrogen-responsive reporter plasmids by an ER-independent pathway. *Mol Cell Endocrinol* 159:53–62
26. **Maurer RA** 1989 Both isoforms of the cAMP-dependent protein kinase catalytic subunit can activate transcription of the prolactin gene. *J Biol Chem* 264:6870–6873
27. **Schaufele F** 1999 Regulation of estrogen receptor activation of the prolactin enhancer/promoter by antagonistic activation function-2-interacting proteins. *Mol Endocrinol* 13:935–945
28. **Ogawa S, Inoue S, Orimo A, Hosoi T, Ouchi Y, Muramatsu M** 1998 Cross-inhibition of both estrogen receptor  $\alpha$  and  $\beta$  pathways by each dominant negative mutant. *FEBS Lett* 423:129–132
29. **Raff MC** 1992 Social controls on cell survival and cell death. *Nature* 356:397–400
30. **Kyprianou N, English HF, Davidson NE, Isaacs JT** 1991 Programmed cell death during regression of the MCF-7 human breast cancer following estrogen ablation. *Cancer Res* 51:162–166
31. **Teixeira C, Reed JC, Pratt MA** 1995 Estrogen promotes chemotherapeutic drug resistance by a mechanism involving Bcl-2 proto-oncogene expression in human breast cancer cells. *Cancer Res* 55:3902–3907
32. **Wang DG, Johnston CF, Atkinson AB, Heaney AP, Mirakhor M, Buchanan KD** 1996 Expression of bcl-2 oncoprotein in pituitary tumours: comparison with c-myc. *J Clin Pathol* 49:795–797
33. **Turner HE, Nagy Z, Gatter KC, Esiri MM, Wass JA, Harris AL** 2000 Proliferation, bcl-2 expression and angiogenesis in pituitary adenomas: relationship to tumour behaviour. *Br J Cancer* 82:1441–1445
34. **Yin D, Tamaki N, Kokunai T, Yasuo K, Yonezawa K** 1999 Bromocriptine-induced apoptosis in pituitary adenoma cells: relationship to p53 and bcl-2 expression. *J Clin Neurosci* 6:326–331
35. **Wang TT, Phang JM** 1995 Effects of estrogen on apoptotic pathways in human breast cancer cell line MCF-7. *Cancer Res* 55:2487–2489
36. **Dong L, Wang W, Wang F, et al.** 1999 Mechanisms of transcriptional activation of bcl-2 gene expression by 17 $\beta$ -estradiol in breast cancer cells. *J Biol Chem* 274:32099–32107
37. **Perillo B, Sasso A, Abbondanza C, Palumbo G** 2000 17 $\beta$ -Estradiol inhibits apoptosis in MCF-7 cells, inducing bcl-2 expression via two estrogen-responsive elements present in the coding sequence. *Mol Cell Biol* 20:2890–901
38. **Butt AJ, Firth SM, King MA, Baxter RC** 2000 Insulin-like growth factor-binding protein-3 modulates expression of Bax and Bcl-2 and potentiates p53-independent radiation-induced apoptosis in human breast cancer cells. *J Biol Chem* 275:39174–39181
39. **Kushner PJ, Hort E, Shine J, Baxter JD, Greene GL** 1990 Construction of cell lines that express high levels of the human estrogen receptor and are killed by estrogens. *Mol Endocrinol* 4:1465–1473
40. **Maminta ML, Molteni A, Rosen ST** 1991 Stable expression of the human estrogen receptor in HeLa cells by infection: effect of estrogen on cell proliferation and c-myc expression. *Mol Cell Endocrinol* 78:61–69
41. **Jiang SY, Jordan VC** 1992 Growth regulation of estrogen receptor-negative breast cancer cells transfected with complementary DNAs for estrogen receptor. *J Natl Cancer Inst* 84:580–591
42. **Lee Y, Renaud RA, Friedrich TC, Gorski J** 1998 Estrogen causes cell death of estrogen receptor stably transfected cells via apoptosis. *J Steroid Biochem Mol Biol* 67:327–332
43. **Kousteni S, Bellido T, Plotkin LI, et al.** 2001 Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors. dissociation from transcriptional activity. *Cell* 104:719–730
44. **Itoh S, Hattori T, Hayashi H, et al.** 1999 Antiproliferative effect of IL-1 is mediated by p38 mitogen-activated protein kinase in human melanoma cell A375. *J Immunol* 162:7434–7440
45. **Huang C, Ma WY, Maxiner A, Sun Y, Dong Z** 1999 p38 kinase mediates UV-induced phosphorylation of p53 protein at serine 389. *J Biol Chem* 274:12229–12235
46. **Kanasaki H, Fukunaga K, Takahashi K, Miyazaki K, Miyamoto E** 2000 Involvement of p38 mitogen-activated protein kinase activation in bromocriptine-induced apoptosis in rat pituitary GH<sub>3</sub> cells. *Biol Reprod* 62:1486–1494
47. **Zhang CC, Shapiro DJ** 2000 Activation of the p38 mitogen-activated protein kinase pathway by estrogen or by 4-hydroxytamoxifen is coupled to estrogen receptor-induced apoptosis. *J Biol Chem* 275:479–486
48. **Sanchez-Prieto R, Rojas JM, Taya Y, Gutkind JS** 2000 A role for the p38 mitogen-activated protein kinase pathway in the transcriptional activation of p53 on genotoxic stress by chemotherapeutic agents. *Cancer Res* 60:2464–2472
49. **Han J, Sabbatini P, Perez D, Rao L, Modha D, White E** 1996 The E1B 19K protein blocks apoptosis by interacting with and inhibiting the p53-inducible and death-promoting Bax protein. *Genes Dev* 10:461–477
50. **Southgate TD, Windeatt S, Smith-Arica J, et al.** 2000 Transcriptional targeting to anterior pituitary lactotrophic cells using recombinant adenovirus vectors *in vitro* and *in vivo* in normal and estrogen/sulpiride-induced hyperplastic anterior pituitaries. *Endocrinology* 141:3493–3505