

# Involvement of Estrogen Receptor $\beta$ in Ovarian Carcinogenesis

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## ABSTRACT

Knockout and expression studies suggest that estrogen receptor  $\beta$  (ER $\beta$ ) plays a prominent role in ovarian function and pathology. Moreover, ovarian cancers are characterized by high morbidity and low responsiveness to anti-estrogens. Here we demonstrate, using quantitative PCR to measure ER $\alpha$  and ER $\beta$  levels in 58 ovarian cancer patients, that ER $\beta$  expression decreased in cysts and ovarian carcinomas as compared with normal ovaries and that this decrease is attributable only to a selective loss in ER $\beta$  expression during cancer progression. To address the question of a possible involvement of ER $\beta$  in ovarian cancers, we restored ER $\alpha$  and ER $\beta$  expression in two human ovarian cancer cell lines PEO14 (ER $\alpha$ -negative) and BG1 (ER $\alpha$ -positive) using adenoviral delivery. ER $\alpha$ , but not ER $\beta$ , could induce progesterone receptor and fibulin-1C. Moreover, ER $\alpha$  and ER $\beta$  had opposite actions on cyclin D1 gene regulation, because ER $\beta$  down-regulated cyclin D1 gene expression, whereas ER $\alpha$  increased cyclin D1 levels. Interestingly, ER $\beta$  expression strongly inhibited PEO14 and BG1 cell proliferation and cell motility in a ligand-independent manner, whereas ER $\alpha$  had no marked effect. Induction of apoptosis by ER $\beta$  also contributed to the decreased proliferation of ovarian cancer cells, as shown by Annexin V staining. This study shows that ER $\beta$  is an important regulator of proliferation and motility of ovarian cancer and provides the first evidence for a proapoptotic role of ER $\beta$ . The loss of ER $\beta$  expression may thus be an important event leading to the development of ovarian cancer.

## INTRODUCTION

Ovarian cancer is, after breast cancer, the second most common gynecological cancer in terms of incidence but the first one in terms of morbidity in Western countries (1). Ovarian carcinogenesis mechanisms have not yet been elucidated but appear to be different from those of breast tumor progression. Indeed, about two-thirds of breast cancer patients with estrogen receptor (ER)-positive tumors respond to tamoxifen therapy. However, although 40–60% of ovarian cancers express ER $\alpha$  (2, 3), only a minor proportion of patients (ranging from 7–18%) respond clinically to anti-estrogen treatment (4, 5). The role of estrogens has been recently highlighted by the results of three large prospective studies showing that estradiol uptake in postmenopausal stage increased the risk of ovarian cancer incidence and mortality in women who used long-term estrogen replacement therapy (4, 6–8). Estrogen effects are mediated by two estrogen receptors, ER $\alpha$  (NR3A1) and ER $\beta$  (NR3A2), which belong to a large family of nuclear receptors (9, 10). The generation of the different estrogen receptor knockout (ERKO) mice provides ideal models to analyze the distinct roles of both estrogen receptors in various tissues (11, 12). The ovarian phenotype of ER $\alpha$ KO female mice is distinct from that of ER $\beta$ KO mice. ER $\alpha$ KO females are infertile whereas ER $\beta$ KO females

exhibit inefficient ovarian function and subfertility. Interestingly, double knockout mice (ER $\alpha$  $\beta$ KO) exhibit phenotypes that most heavily resemble those of the ER $\alpha$ KO, with the exception of the ovarian phenotype, characterized by progressive germ cell loss accompanied by re-differentiation of the surrounding somatic cells, suggesting an important role for both ER forms in this tissue (13).

In the uterus, ER $\beta$ KO mice show increased cell proliferation and an exaggerated response to 17 $\beta$  estradiol (E2; ref. 14). Moreover, at least in the prostate, ER $\beta$  is involved in the regulation of epithelial growth, and its absence results in hyperplasia of the prostatic epithelium (15). In the rodent mammary gland, 90% of ER $\beta$ -bearing cells do not proliferate (16), confirming the involvement of ER $\beta$  in the control of the proliferation *in vivo*. Several *in vitro* studies also show some evidence that ER $\beta$  may negatively regulate cellular proliferation and have a protective role in normal breast and prostate (17–19). A loss of ER $\beta$  expression or a decreased ER $\beta$ /ER $\alpha$  ratio in epithelial ovarian cancer as compared with normal tissues has been reported consistently by several groups (20–22). This loss of ER $\beta$  could thus constitute a crucial step in ovarian carcinogenesis and hormone unresponsiveness.

In this study we compared ER $\alpha$  and ER $\beta$  expression in different ovarian samples and showed that the increase in the ER $\alpha$ /ER $\beta$  mRNA ratio observed earlier in ovarian carcinomas (20–22) was attributable to a selective decrease in ER $\beta$  mRNA expression without significant variations in ER $\alpha$  levels. To analyze ER $\beta$  role, we engineered ER $\alpha$ -positive and ER $\alpha$ -negative ovarian cancer cell lines to express functional ER $\beta$ . Interestingly, ER $\beta$  expression had major effects on proliferation, motility, and apoptosis of ovarian cancer cells. All these results suggest that ER $\beta$  could exert a protective role against ovarian cancer development.

## MATERIALS AND METHODS

**Tissue Collection.** Twenty-two normal ovaries, 7 ovarian cysts and 29 cancer specimens were obtained from patients after surgical therapy between 1994 and 1999 in Department of Gynecology, University Hospital of Turin. All tissues were collected for therapeutic or diagnostic purpose according to the ethical rules of Helsinki (1984), modified in Tokyo, with the approval of the local ethics committee, and tissue experiments were undertaken under informed consent of each patient. Tumors were staged according to the International Federation of Gynecologists and Obstetricians criteria. Grading information was established according to the criteria of Day *et al.* (23). The histological subtypes of the 29 ovarian tumors and informations concerning the patients are indicated in Table 1.

**Quantitative RT-PCR.** Real-Time quantitative PCR method using Taqman technology was allowed to measure ER $\alpha$ , ER $\beta$ , and ribosomal 18S RNA levels in ovarian tissues, using ABI Prism 7700 Sequence Detector System (PE Applied Biosystems, Foster City, CA). The primers used for ERs and 18S are described in Table 2. The thermal PCR conditions comprised an initial step at 95°C for 15 minutes and a two-step PCR for 45 cycles (20 seconds at 94°C followed by 20 seconds at 68°C and 15 seconds at 94°C followed by 20 seconds at 65°C for ER $\alpha$  and ER $\beta$ , respectively) or 35 cycles (15 seconds at 94°C followed by 15 seconds at 67°C for 18S). To create a standard curve for each gene, RNAs were produced by *in vitro* transcription from linearized templates corresponding to ER $\alpha$ , ER $\beta$ , and 18S cDNA constructs using T7 or T3 polymerases and reverse transcribed to cDNA.

**Cell Lines.** The human ovarian cancer cell lines PEO14 (ER-negative cells) and BG1 (ER $\alpha$ -positive cells) were used in this study. PEO14 (obtained

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Table 1 Pathoclinical details of ovarian tumors used in the study

Clinical parameters	Number of patients
Patients	
Total in study	58
Age ≥50	37
Age <50	21
Group	
Normal	22
Cysts	7
Tumors	29
Tumor stage	
I	5
II	4
III	17
IV	3
Tumor histological grade	
0	3
I	1
II	9
III	16
Tumor histotype	
Serous papillary	12
Mucinous	3
Endometrioid	7
Clear cell	2
Undifferentiated	5

from Dr. Langdon, Hospital of Edinburg, United Kingdom) cells were cultured in RPMI 1640 (Life Technologies, Inc., Eraguy, France) supplemented with 10% FCS and gentamicin in the presence of 5% CO<sub>2</sub>. BG1 cells (ref. 24; obtained from Dr. C. E. Welander, Emory University, Atlanta, GA) were cultured in Mc Coy's medium (Life Technologies, Inc., Eraguy, France) containing 10% FCS. The human breast cancer cell line MCF-7 was cultured in DMEM-F12 medium supplemented with 10% FCS. To wean the cells off steroids, they were cultured in phenol red-free RPMI (PEO14 cells for 2 days) or in phenol red-free DMEM-F12 (BG1 and MCF-7 cells for 5 days) supplemented with 10% charcoal dextran-treated FCS (CDFCS).

**Recombinant Adenovirus Construction, Propagation, and Infection.** The adenoviruses Ad5, Ad-ERα and Ad-ERβ used in this study have been described previously (17). PEO14 and BG1 cells were infected overnight with the different viruses. Ethanol control vehicle or E2 treatment (10<sup>-8</sup> mol/L) began 18 hours after infection. The optimal infection conditions were determined for both cell lines using a β-galactosidase encoding virus to determine the optimal multiplicity of infection (MOI). We observed a very efficient infection of cells when increasing the MOI from 0 to 100, leading to an infection of about 90% of the cells at MOI 100 for PEO14 cells and MOI 25 for BG1 cells (data not shown).

**Plasmids.** The luciferase reporter plasmid ERE<sub>2</sub>-TK-LUC contains two copies of the consensus estrogen-responsive element (ERE) cloned upstream of the minimal herpes simplex virus thymidine kinase promoter. Cytomegalovirus (CMV)-ERα and CMV-ERβ correspond to the wild type human ERα and ERβ cDNAs cloned into CMV5 plasmid under the control of the CMV promoter and were a kind gift of Dr. B. S. Katzenellenbogen. A CMV-Gal reporter was used as an internal control and corresponds to the β-galactosidase gene cloned in CMV5.

**Transient Transfection and Reporter Assays.** Approximately 3 × 10<sup>5</sup> PEO14 cells were seeded in 6-well plates 48 hours before transfection in phenol red-free RPMI 1640 supplemented with 10% CDFCS. Transfections were performed overnight with Lipofectamin 2000 (Invitrogen) according to the manufacturer's protocols, using 1 μg of luciferase-reporter construct, 0.5 μg of the internal reference CMV-GAL and 0.15 μg of CMV-ER expression vectors or recombinant viruses per well at MOI 100. Cells were then treated with 10<sup>-8</sup> mol/L E<sub>2</sub> or with ethanol vehicle. Cells were harvested 48 hours

after E<sub>2</sub>-treatment and cell extracts prepared. The cells were lysed directly in the plates with 200 μl of cell culture lysis reagent (Promega). β-Galactosidase and luciferase were determined as described previously (17).

**Detection of ERα and ERβ Protein by Immunohistochemistry.** PEO14 cells were seeded in 10% CDFCS DMEM-F12 on sterile coverslips in 6-well plates and infected with Ad5, Ad-ERα, or Ad-ERβ at MOI 50. Two days after infection, the cells were fixed (4% formaldehyde 12 minutes/methanol 5 minutes/acetone 2 minutes) and washed with PBS. The coverslips were incubated for 30 minutes with PBS containing non-immune rabbit serum (1:40). Then the cells were incubated with the primary antibody (ERα SRA-1000 1:2000, Stressgen; ERβ 503 immunoglobulin Y 1:2500; ref. 16) in PBS for 60 minutes at room temperature. The cells were then incubated with the secondary antibody (antimouse or antichickn peroxidase conjugate, 1:3000, Sigma, St. Louis, MO) in PBS-bovine γ globulin for 30 minutes at room temperature. Finally, the cells were incubated with a diaminobenzidine chromogen solution [0.66 mg/ml in PBS +0.08% H<sub>2</sub>O<sub>2</sub> (30 vol)] for 10 minutes at room temperature. The cells were counterstained with hematoxylin.

**RNA Isolation and Reverse Transcription PCR.** Total RNA was isolated using RNeasy mini kit (Qiagen) according to protocols provided by the manufacturer. Five micrograms of total RNA were subjected to a reverse transcription step using the M-MuLV Reverse Transcriptase RNase H- and random-hexamer primers (Amersham Biosciences) in a 20-μl reaction volume. PCR co-amplification was performed using 1:20 reverse transcription reaction using DyNAzyme EXT DNA polymerase and with different primers in the same reaction. Cycles of 30 seconds at 94°C, 1 minute at 60°C, and 1 minute at 72°C were done 29 times. One-tenth of each PCR was run on 1.5 to 2% agarose gel with ethidium bromide. Primers used were described previously for ERα (25), ERβ (26), and hypoxanthine phosphoribosyl transferase (27).

**Northern Blot Analysis.** Twenty micrograms of total RNA were loaded on a 1% agarose-formaldehyde gel. RNA was then transferred to nylon membranes. Membranes were then hybridized with the different probes at 42°C in hybridization solution (ULTRAhyb, Ambion). The probes were <sup>32</sup>P-labeled by multistep DNA synthesis. The washes were performed as described previously (28). When required, the membranes were stripped of probe by boiling in 0.5% SDS solution and rehybridized. A probe for human 18S was used to confirm equal loading of RNA in all of the wells. Signals were quantified using PC BAS 1000 reader (Fujix).

**Cell Proliferation Studies.** PEO14 and BG1 cells were maintained respectively for 48 hours and for 5 days in 10% Charcoal Dextran (CD)-FCS phenol red-free medium and then seeded at 20,000 cells/well in 24-well dishes. Cells were infected overnight with the different viruses. The next morning, the medium was removed and replaced with fresh 10% CDFCS medium. Treatment with E2 or ICI 182,780 began at the same time. After 2, 4, and 6 days of treatment, the total cell DNA was quantified by 3,5-diaminobenzoic acid assay as described previously (17).

**Wound-Healing Assay.** Cells were plated in 6-well dishes in RPMI 1640 containing 10% CDFCS. Cells were infected with the different viruses overnight. The next morning, ethanol or E2 treatment began. After 24 hours of treatment, wound-induced migration was triggered by scraping the cells at day 1 with a blue tip, and the wound was pictured immediately (*t* = 0 hours). Twenty-four hours later the cells were pictured again. The percentage of wound filling was calculated by measuring the remaining gap space on the pictures.

**Apoptosis Detection by Staining with Annexin V.** Apoptosis was assessed by staining DNA with Hoechst 33258 and by cell surface binding of Annexin V. The Annexin-V-Alexa 568 (Roche Diagnostic, Meylan, France) was used to detect apoptosis by fluorescence microscopy. PEO14 and BG1 cells were weaned off steroids for 4 days and then seeded in 12-well dishes. Cells were infected overnight with the different viruses. The next day, cells

Table 2 Primers and TaqMan probes used for detection by real-time quantitative PCR

Gene	Oligonucleotide	Sequences 5'-3'	PCR product size (pb)	TaqMan probes 5'-3'
ERα	Forward	GCCAGCAGGTGCCCTACTAC	132	CGCCGGCATTTCTACAGGCCAAA
	Reverse	TGGTACTGGCCAATCTTTCTCTG		
ERβ	Forward	AAGAGCTGCCAGGCTGCC	268	CTCACCCTCTGGAGGCTGAGCCGC
	Reverse	GCGCACTGGGGCGGCTGATCA		
18S	Forward	CTACCACATCCAAGGAAGGCA	71	CGCGCAAATTACCCACTCCGCAC
	Reverse	TTTTTCGTCACTACTCTCCCG		

were treated with control vehicle ethanol or E2. After 48 hours of treatment, the cells were harvested, washed twice in PBS, and then resuspended in a binding buffer [HEPES, 10 mM (pH 7.4), 140 mM NaCl, 5 mM NaCl<sub>2</sub>], before staining with Annexin-V-Alexa 568 incubation reagent and Hoechst. Cells were then incubated in the dark for 15 minutes at room temperature. Cells were washed twice in PBS and immediately laid down on microscope slides with subsequent mounting in the anti-fading agent Mowiol. The scoring of apoptotic cells (%) was calculated by dividing the number of apoptotic cells (Annexin V) by the total of cells counted per cross-section (Hoechst). Counting by slide was performed, and this was repeated in three different experiments.

**Statistics.** Data indicated in the text represent mean  $\pm$  SD. Statistical differences within the populations were determined by Kruskal-Wallis non-parametric tests for quantitative parameters.  $P < 0.05$  was considered as significant.

## RESULTS

Ovarian cancer is characterized by a rapid evolution, a high morbidity, and a relatively low responsiveness to adjuvant treatments (1, 4). One interesting hypothesis is the possible involvement of ER $\beta$  in the etiology of this cancer because normal ovary is one of the major sites of expression of this receptor. Our first goal was to assess whether such event occurred during ovarian carcinogenesis.

**ER $\alpha$  and ER $\beta$  mRNA Expression in Ovarian Tissues.** We first analyzed by quantitative reverse transcription (RT)-PCR ER $\alpha$  and ER $\beta$  RNA expression in normal ovaries, ovarian cysts, and ovarian carcinomas (Fig. 1). All ovarian tissues analyzed expressed detectable levels of ER $\alpha$  mRNA, and there was no significant difference in ER $\alpha$  levels mRNA among the group of normal ovaries and the groups of ovarian cysts and ovarian carcinomas ( $P = 0.45$ ; Fig. 1). In contrast, ER $\beta$  mRNA expression varied among the three groups of ovarian tissues. ER $\beta$  was detectable in most normal ovaries (ER $\beta$ <sup>+</sup>: 16 of 22, 72.7%), whereas ER $\beta$  levels decreased in ovarian cysts (ER $\beta$ <sup>+</sup>: 4 of 7, 57.1%) and in ovarian carcinomas (ER $\beta$ <sup>+</sup>: 9 of 29, 31%). Statistical analysis showed a significant difference between the three groups (Kruskal-Wallis test,  $P = 0.001$ ), with a marked decrease in ER $\beta$  mRNA levels in ovarian cancers as compared with normal ovaries

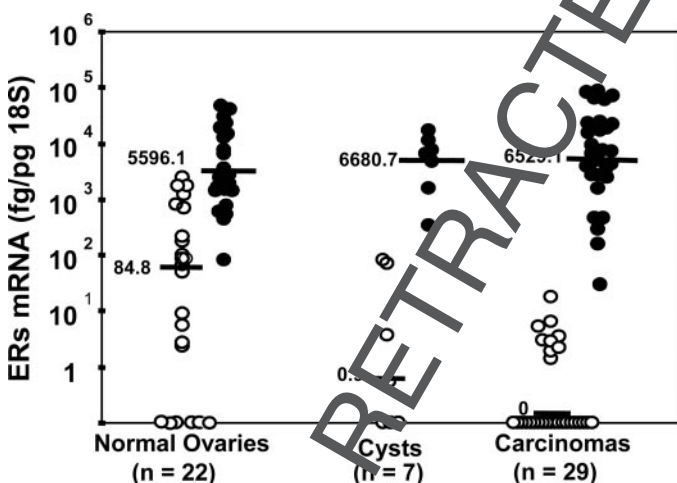


Fig. 1. ER $\alpha$  and ER $\beta$  mRNA levels in ovarian tissues. ER $\alpha$  and ER $\beta$  mRNA levels were measured in normal ovaries ( $n = 22$ ), ovarian cysts ( $n = 7$ ), and ovarian carcinoma ( $n = 29$ ) by real-time quantitative RT-PCR. For data quantification, ER $\alpha$  and ER $\beta$  mRNA levels were normalized to 18S rRNA levels and expressed as femtogram of ER mRNA/picogram 18 seconds rRNA. Individual values of ER $\alpha$  and ER $\beta$  mRNA levels are represented by dots and the median ER values for the various groups of ovarian tissues are indicated by a horizontal dash. \*, statistical differences within populations were determined by Kruskal-Wallis nonparametric tests for quantitative parameters.  $P < 0.05$  was considered as significant. There was no significant difference in ER $\alpha$  mRNA levels among the three groups ( $P = 0.45$ ). ER $\beta$  mRNA levels showed a significant difference among the three groups ( $P = 0.001$ ), with a marked decrease in ER $\beta$  mRNA levels in ovarian cancers as compared with normal ovaries ( $P = 0.0003$ ).

( $P = 0.0003$ ), suggesting that the loss of ER $\beta$  expression may be an important early step of ovarian tumor progression.

**Adenoviral-Mediated Expression of ER $\alpha$  and ER $\beta$ .** To test this hypothesis, we restored ER $\alpha$  and ER $\beta$  expression in ovarian cancer cells lines using adenoviruses encoding hER $\alpha$  or hER $\beta$  (Ad-ER $\alpha$  and Ad-ER $\beta$ ). The expression of ER $\alpha$  and ER $\beta$  in Ad-ER $\alpha$ - and Ad-ER $\beta$ -infected PEO14 cells was checked by RT-PCR (Fig. 2A). We were not able not detect the expression of ER $\alpha$  and ER $\beta$  RNAs in PEO14 cells non-infected or infected with the nonrecombinant virus Ad5. After infection with Ad-ER $\alpha$  or Ad-ER $\beta$ , a high expression of corresponding ER $\alpha$  and ER $\beta$  mRNA could be observed. Immunohistochemistry experiments using ER $\alpha$ - and ER $\beta$ -specific antibodies show that ER $\alpha$  and ER $\beta$  proteins were not detected in Ad5-infected cells (Fig. 2B, c and d), whereas ER $\alpha$ - and ER $\beta$ -infected cells displayed a nuclear staining (respectively, Fig. 2B, a and b).

To demonstrate the functionality of the receptors introduced, we analyzed their capacity to activate an estrogen-responsive reporter gene. We observed a strong activation of the reporter by ER $\alpha$  encoding virus and plasmid in the presence of E2 (Fig. 2C). ER $\beta$  encoding virus and plasmid were able to activate the transcription in the presence of E2, but the stimulation was half of that obtained with ER $\alpha$ . The pure anti-estrogen ICI 182,780 did not stimulate ER $\alpha$  or ER $\beta$  activity but completely abolished E2-induced activities of ER $\alpha$  and ER $\beta$  (Fig. 2C).

**ER $\alpha$  and ER $\beta$  Effects on Endogenous Target Genes.** As another control of the functionality of ER $\beta$  in this system, we next determined whether the expression of endogenous genes known to be regulated by E2 in ovarian or breast ER-positive cells could also be modulated by ER $\alpha$  or ER $\beta$  in infected PEO14 cells (Fig. 3, A and B). ER $\alpha$  was able to activate the expression of fibulin 1C and progesterone receptor (PR) in the presence of E2, whereas ER $\beta$  had no effect. pS2 mRNA could not be detected in ovarian cancer cells infected with ER $\alpha$  or ER $\beta$  viruses. pS2 gene is likely to be altered in ovarian cancers. Indeed, although some ovarian tumors are producing pS2, pS2 is not regulated by estrogens (29, 30). The mechanisms underlying this unresponsiveness remain to be determined. The pure anti-estrogen ICI 182,780 was not able to modify the levels of fibulin-1C and PR expression. Interestingly, ER $\alpha$  and ER $\beta$  also had an opposite effect on expression of cyclin D1 mRNA levels. Indeed, ER $\alpha$  induced cyclin D1 in the presence of E2, whereas ER $\beta$  inhibited cyclin D1 expression in a ligand-independent manner. Moreover, treatment of the cells with ICI 182,780 was not able to alleviate this inhibition. We have also analyzed the expression fibulin-1C and cdk1 p21<sup>WAF-1</sup> genes in ER $\alpha$ -positive BG-1 cells infected with Ad-ER $\beta$  virus (Fig. 3, C and D). Northern blot experiments show that fibulin-1C RNA levels were increased after E2-treatment in non-infected or Ad5-infected BG-1 cells. Introduction of ER $\beta$  in these cells did not alter this regulation. p21<sup>WAF-1</sup> RNA was down-regulated by E2 in control cells, suggesting that the cells were proliferating more rapidly in the presence of E2. On the other hand, ER $\beta$  was able to increase by about 2-fold p21<sup>WAF-1</sup> levels in a ligand-independent manner. Interestingly, neither E2 nor ICI was able to modulate these effects.

**ER $\beta$  Is a Potent Inhibitor of the Proliferation of Ovarian Cancer Cells.** The down-regulation of cyclin D1 and up-regulation of p21<sup>WAF-1</sup> expression by ER $\beta$  led us to investigate whether ER $\beta$  could modulate the proliferation of ovarian cancer cells. PEO14 cells not infected or infected with the nonrecombinant virus Ad5 were used as control (Fig. 4, A and B). Control cells presented a similar growth pattern in the absence or in the presence of E2 (Fig. 4A, left panel). When PEO14 cells were infected with Ad-ER $\alpha$  virus, a moderate ligand-independent inhibition of proliferation occurred (Fig. 4A, middle panel). Conversely, ER $\beta$  strongly inhibited the proliferation in a ligand-independent manner (Fig. 4A, right panel). The pure anti-

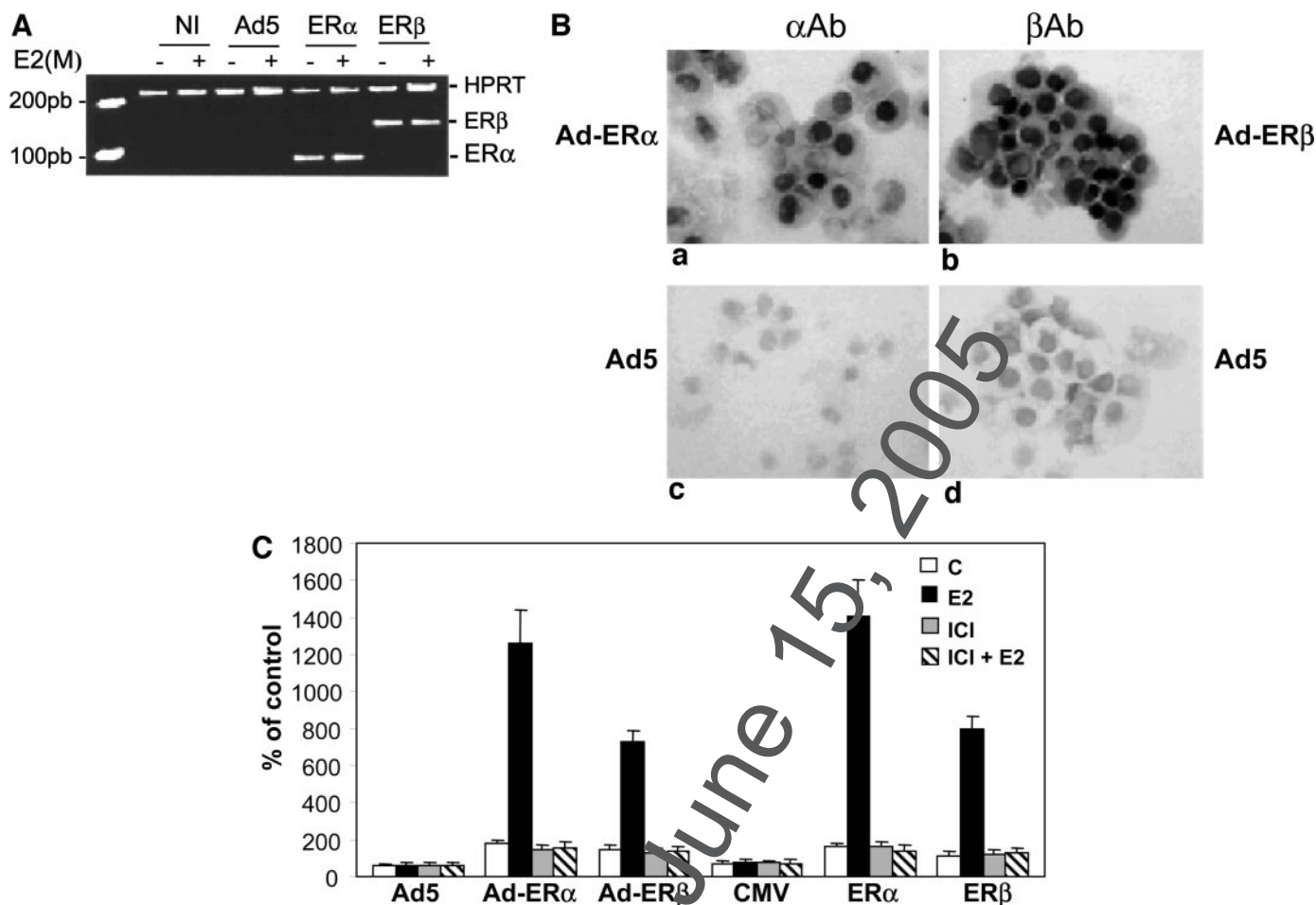


Fig. 2. Adenoviral expression of ER $\alpha$  and ER $\beta$  in PEO14 cells. A, PEO14 cells were infected with Ad5, Ad-ER $\alpha$ , or Ad-ER $\beta$  viruses. After 48 hours of treatment with  $10^{-8}$  mol/L E2, hER $\alpha$ , and hER $\beta$ , expressions were checked by RT-PCR using primers located in the ligand-binding domain. The PCR products have a size of 108 and 143 bp for ER $\alpha$  and ER $\beta$ , respectively. *HPRT*, hypoxanthine phosphoribosyl transferase. B, PEO14 cells were infected with Ad5 (c and d), Ad-ER $\alpha$  (a), and Ad-ER $\beta$  (b) viruses at MOI 50, and ER $\alpha$  and ER $\beta$  expressions were visualized by immunohistochemistry using ER $\alpha$  ( $\alpha$ Ab)- and ER $\beta$  ( $\beta$ Ab)-specific antibodies. C, PEO14 cells were either infected with Ad5, Ad-ER $\alpha$ , Ad-ER $\beta$  viruses or transfected with empty CMV5 vector (CMV), CMV-ER $\alpha$  (ER $\alpha$ ), or CMV-ER $\beta$  (ER $\beta$ ) along with ERE $_2$ -TK-LUC- and CMV-GAL-reporter constructs. Cells were grown for 48 hours in the presence of control vehicle ethanol (C),  $10^{-8}$  mol/L E2,  $10^{-6}$  mol/L ICI 182,780, or the combination of E2 and ICI 182,780 ( $10^{-8}$  mol/L and  $10^{-6}$  mol/L, respectively). Results are expressed as the percentage of LUC activity in noninfected cells and represent the mean of luciferase activity after normalization for  $\beta$ -galactosidase activity.

estrogen ICI 182,780 had no effect by itself on the proliferation of naive cells and could not modulate the proliferation rate of ER $\alpha$ - or ER $\beta$ -expressing cells (Fig. 4B). These results confirm that inhibition of the proliferation by ER $\beta$  (or ER $\alpha$ ) are ligand-independent, when their expression is restored in ER-negative ovarian cancer cells. We next determined whether this inhibitory effect of ER $\beta$  could be obtained in ER $\alpha$ -positive BG1 cell line (Fig. 4C and D). The proliferation of these cells is increased by estrogens (30, 31) as shown in control cells (non-infected or infected with the nonrecombinant virus Ad5; Fig. 4, C and D). When BG1 cells were infected with Ad-ER $\beta$  virus, the proliferation was inhibited by 60%. This inhibition appears to be E2-independent, as suggested by the lack of effect of ICI 182,780 on the proliferation rate, and to involve an increase in p21<sup>WAF-1</sup> expression as shown above.

**ER $\beta$  Inhibits the Motility of PEO14 Cells.** Because tumor development involves not only proliferation but also invasion, it was important to determine whether ER $\beta$  expression could affect the motility of ovarian cancer cells. We performed wound healing-induced migration experiments on PEO14 cells (Fig. 5A). After 24 hours of migration, Ad5-infected PEO14 cells had filled 75% of the wound (Fig. 5B). ER $\alpha$ -infected cells had filled about 65% of the wound in the absence or the presence of E2. However, ER $\beta$  expressing cells had a very slow migration ability as they filled only 20 to 30% of the gap.

**ER $\beta$  Induces Apoptosis of Ovarian Cancer Cells.** The decreased proliferation observed in PEO14 cells infected with Ad-ER $\beta$  virus could be the result not only of cell cycle blockage, as suggested by cyclin D1 down-regulation, but it could also involve an increased apoptosis. Simultaneous staining with Annexin V and Hoechst non-vital dye made it possible to distinguish between intact cells (stained positive for Hoechst only) and early apoptotic cells death (stained positive for Hoechst and Annexin V). When PEO14 cells were infected with the nonrecombinant Ad5 virus or Ad-ER $\alpha$  virus, about 8% of cells were in early apoptosis (Fig. 6, A and C), whereas 20% of ER $\beta$ -infected cells were undergoing apoptosis. E2 treatment of PEO14 infected with Ad-ER $\alpha$  increased by 2-fold the percentage of apoptotic cells, whereas E2 had no effect on ER $\beta$ -induced apoptosis. Introduction of ER $\beta$  in BG1 cells led to a 2-fold increase of apoptosis in the absence of E2 (Fig. 6, B and D). In BG1 cells, E2 had antiapoptotic properties as shown in non-infected, Ad5-infected, but also ER $\beta$ -expressing cells, suggesting that the reduction of apoptosis may be attributable to the presence of endogenous ER $\alpha$ .

## DISCUSSION

The aim of this study was to determine the extent to which ER $\beta$  contributes to ovarian carcinogenesis and its possible involvement in

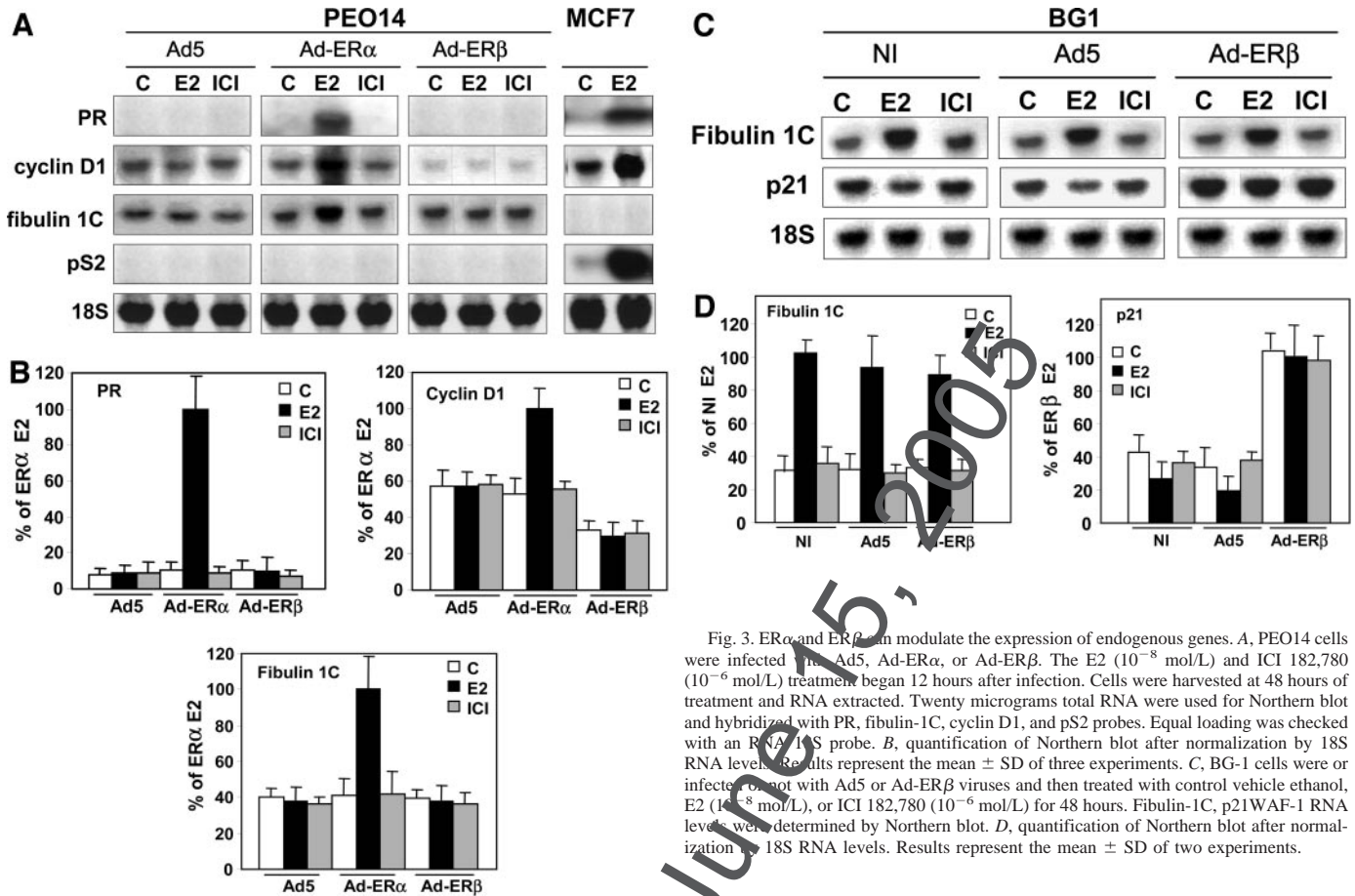


Fig. 3. ER $\alpha$  and ER $\beta$  can modulate the expression of endogenous genes. *A*, PEO14 cells were infected with Ad5, Ad-ER $\alpha$ , or Ad-ER $\beta$ . The E2 ( $10^{-8}$  mol/L) and ICI 182,780 ( $10^{-6}$  mol/L) treatment began 12 hours after infection. Cells were harvested at 48 hours of treatment and RNA extracted. Twenty micrograms total RNA were used for Northern blot and hybridized with PR, fibulin-1C, cyclin D1, and pS2 probes. Equal loading was checked with an RPLA 18S probe. *B*, quantification of Northern blot after normalization by 18S RNA levels. Results represent the mean  $\pm$  SD of three experiments. *C*, BG-1 cells were infected with Ad5 or Ad-ER $\beta$  viruses and then treated with control vehicle ethanol, E2 ( $10^{-8}$  mol/L), or ICI 182,780 ( $10^{-6}$  mol/L) for 48 hours. Fibulin-1C, p21WAF-1 RNA levels were determined by Northern blot. *D*, quantification of Northern blot after normalization by 18S RNA levels. Results represent the mean  $\pm$  SD of two experiments.

mechanisms such as proliferation, motility, or apoptosis, which are the basis of cancer development.

At the clinical level, we found by quantitative PCR, using samples from 58 patients, that ER $\beta$  RNA levels are reduced during ovarian tumor progression. By contrast, similar expression of ER $\alpha$  mRNA was observed in normal ovaries and ovarian carcinomas. Numerous breast cancer studies suggest that ER $\beta$  expression declines when cells turn cancerous (32–35), whereas ER $\beta$  expression in ovarian cancers is less documented, because studies have only used a limited sample number (21, 22). The fact that such decrease is observed not only in ovarian but also in breast, colon, and prostate cancers (32–37) suggests that the loss of ER $\beta$  expression could be an important event in the pathogenesis of cancers and may reflect a general cellular mechanism contributing to the carcinogenesis of estrogen-dependent tissues.

On the basis of the decreased expression of ER $\beta$  in ovarian cancer, our goal was to determine the possible protective effect of ER $\beta$  against abnormal proliferation. To investigate this hypothesis, we introduced ER $\beta$  in ovarian cancer cells and studied its effects on cell growth. Our work shows that ER $\beta$  is involved in the regulation of proliferation of ovarian cancer cells, because ER $\beta$  inhibits their proliferation. In breast and prostate cancers, exogenous ER $\beta$  expression also reduces cell proliferation (17, 19), suggesting that this is a general mechanism in ER $\alpha$ -positive breast cells. In ER $\alpha$ -positive BG-1 cells, we also observed a decreased proliferation when ER $\beta$  was expressed in these cells. These findings are in agreement with the results of Omoto *et al.* (18) who showed that MCF-7 cells expressing stably ER $\beta$ 1 (ER $\beta$ wt) or ER $\beta$ 2 (ER $\beta$ cx) display a reduced cell growth and colony formation in an anchorage-independent situation. Recently, two studies showed that the expression of ER $\beta$  in ER $\alpha$ -positive breast

cancer cells inhibits their growth (38, 39). Interestingly, ER $\beta$  knock-out analysis show that in prostate ER $\beta$  controls, the regulation of epithelial growth and its absence results in hyperplasia of the prostatic epithelium (15). All together, these data clearly demonstrate that ER $\beta$  is an inhibitor of proliferation, not only in ER-negative but also in ER $\alpha$ -positive breast and ovarian cancer cells, supporting a general antiproliferative role for this receptor. The decreased proliferation that we observed in ovarian cancer cells could be the result at least in part of the inhibition of cyclin D1 expression by ER $\beta$ . This down-regulation of cyclin D1 expression confirms findings that showed how ER $\beta$  completely inhibits cyclin D1 gene expression in HeLa cells, whereas ER $\alpha$  activates cyclin D1 mRNA levels (40). Two other studies also suggest that ER $\beta$  might reduce cell proliferation by inhibiting cyclin D1 gene expression in breast (38, 39). Several results raise the possibility that overexpression of cyclin D1 may contribute to the pathogenesis of epithelial ovarian cancers (41, 42). On the other hand, p21<sup>WAF-1</sup> up-regulation by ER $\beta$  could also account for the decreased proliferation triggered by ER $\beta$ . Several groups have also reported such regulation when ER $\beta$  is expressed in breast cancer cells (17, 38, 39).

In addition to cyclin D1 regulation, we show that in the presence of E2, ER $\alpha$  induced the expression of PR, fibulin-1C in PEO14-infected cells, whereas ER $\beta$  has no effect on these genes. Very little is known about potential estrogen-regulated genes in ovarian tissue. This holds true for ER $\beta$  target genes. To our knowledge, our work is the first evidence that expression of ER $\beta$  in ovarian cancer cells can regulate endogenous gene levels. To date, most of the studies performed in different cell types have shown that ER $\beta$  was generally less active than ER $\alpha$  (43). Moreover, except for a few cases (44), all genes that are regulated by ER $\beta$  are also regulated by ER $\alpha$  in breast cancer cells

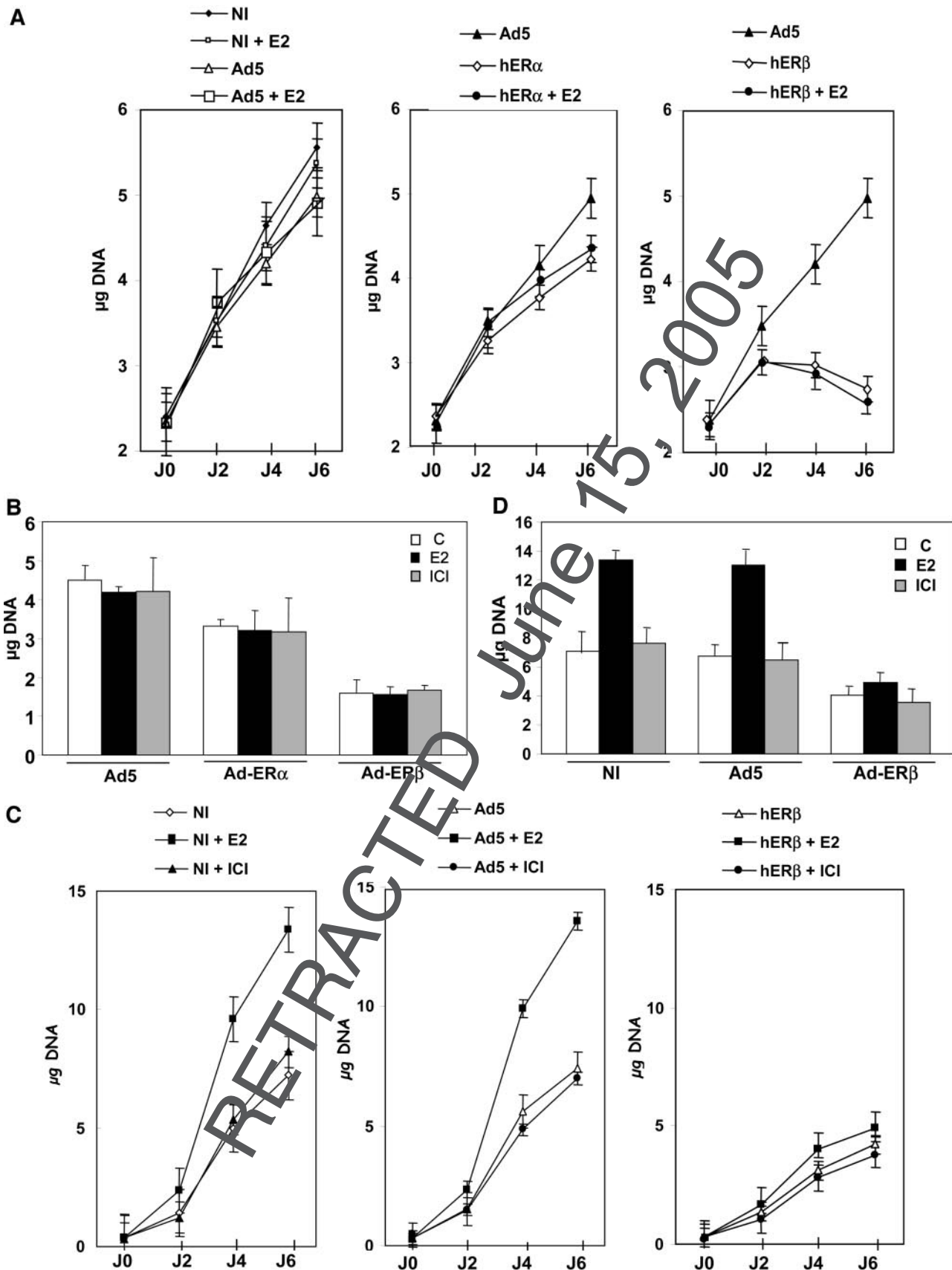


Fig. 4. Effect of ER $\alpha$  and ER $\beta$  on PEO14 and BG1 cell proliferation. A, PEO14 cells, not infected (NI) or infected with different viruses, were treated with vehicle ethanol (C) or E2 ( $10^{-8}$  mol/L). Proliferation rate was determined by 3,5-diaminobenzoic acid assay at days 0, 2, 4, and 6. Results represent the mean  $\pm$  SD of three experiments. B, PEO14 cells were treated or not with E2 ( $10^{-8}$  mol/L) or ICI 182,780 ( $10^{-6}$  mol/L). On day 6, the number of cells was evaluated by the 3,5-diaminobenzoic acid method. Results represent the mean  $\pm$  SD of three experiments. C, The BG1 cells were not infected or infected with Ad5 or Ad-ER $\beta$  and then treated like PEO14 cells. Proliferation rate was determined by 3,5-diaminobenzoic acid assay at days 0, 2, 4, and 6. D, quantification of effect of ER $\beta$  on BG1 cells proliferation at day 6.

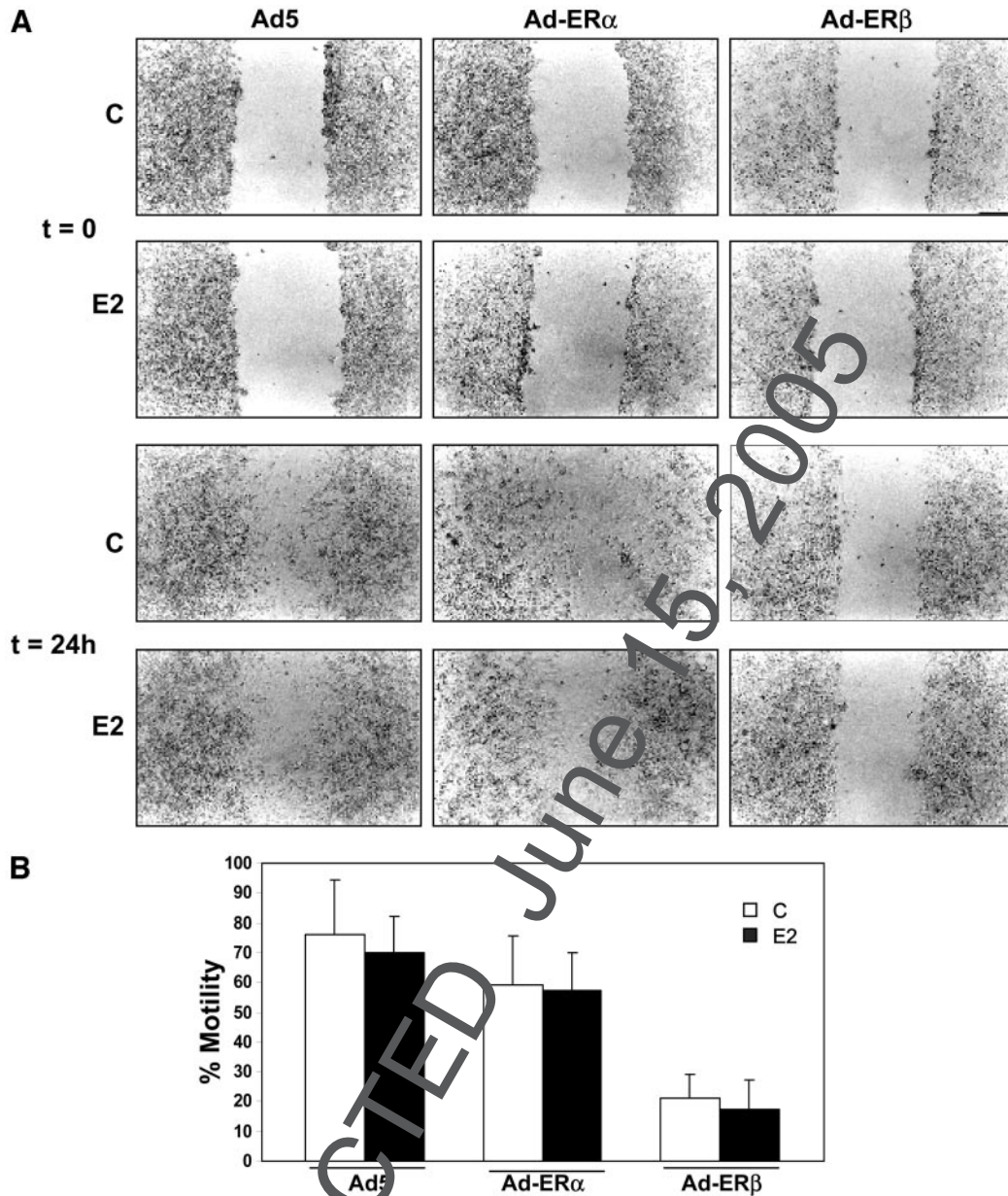


Fig. 5. ER $\beta$  is able to inhibit motility of PEO14 cells. PEO14 cells were not infected (NI) or infected with different viruses. Twelve hours after infection, cells were treated with control vehicle ethanol (C) or E2 ( $10^{-8}$  mol/L). After 48 hours of E2 exposure, cells were scratched with a blue tip and pictured ( $t = 0$ ). The wound was pictured 8 hours, 12 hours, and 24 hours after the scratch. A, pictures of a representative assay at  $t = 0$  and  $t = 24$  hours are shown here. B, results are shown as the percentage of wound filling after 24 hours of migration and represent the mean  $\pm$  SD of three experiments.

(17, 18). On the other hand, in bone, most of the genes that are regulated by estrogens in wild-type mice are also regulated in the same manner in ER $\beta$ KO mice, suggesting that ER $\alpha$  is the main ER transactivator in bone (45). More recently, an extensive search of ER $\beta$  target genes in osteosarcoma cells has revealed that most of ER $\beta$  target genes were also ER $\alpha$ -regulated genes (46). All together, these data suggest that despite regulating a small set of specific genes, ER $\beta$  may have major effects on tumor proliferation, survival, and invasion.

Ovarian cancer cells are characterized by their ability to invade freely the peritoneal cavity, which also accounts for their high aggressiveness and high morbidity (31). We investigated the potential modulation of motility by ER $\beta$ , as another key event occurring during tumor development. We indeed observed that ER $\beta$  drastically inhibits motility of ER-negative ovarian cancer cell line as observed previously in breast and prostate cancer models exogenously expressing ER $\beta$  (17, 19).

It was also of great interest to analyze the effects of ER $\beta$  on apoptosis, because cell growth results from the balance of both cell cycle events and apoptosis regulation. Indeed, ovarian physiology involves apoptosis, because during the repair process of the ovarian surface epithelial cells after incessant ovulation, ovarian surface epithelium that are sequestered in inclusion cysts are normally eliminated by apoptosis, thereby removing potential sites of ovarian tumorigenesis (47). Ghahremani *et al.* (47) proposed that the etiology of ovarian cancer stems from the loss of the apoptotic pathways that normally eliminate ovarian surface epithelium in inclusion cysts. Therefore, malignant transformation occurs when there is a disruption of the normal balance between cellular proliferation and apoptosis. We thus hypothesized that the loss of ER $\beta$  and its potential proapoptotic function could represent an early process in ovarian transformation. And in fact, the introduction of ER $\beta$  in ovarian cancer cells led to increased apoptosis as shown by Annexin V staining. This is the first

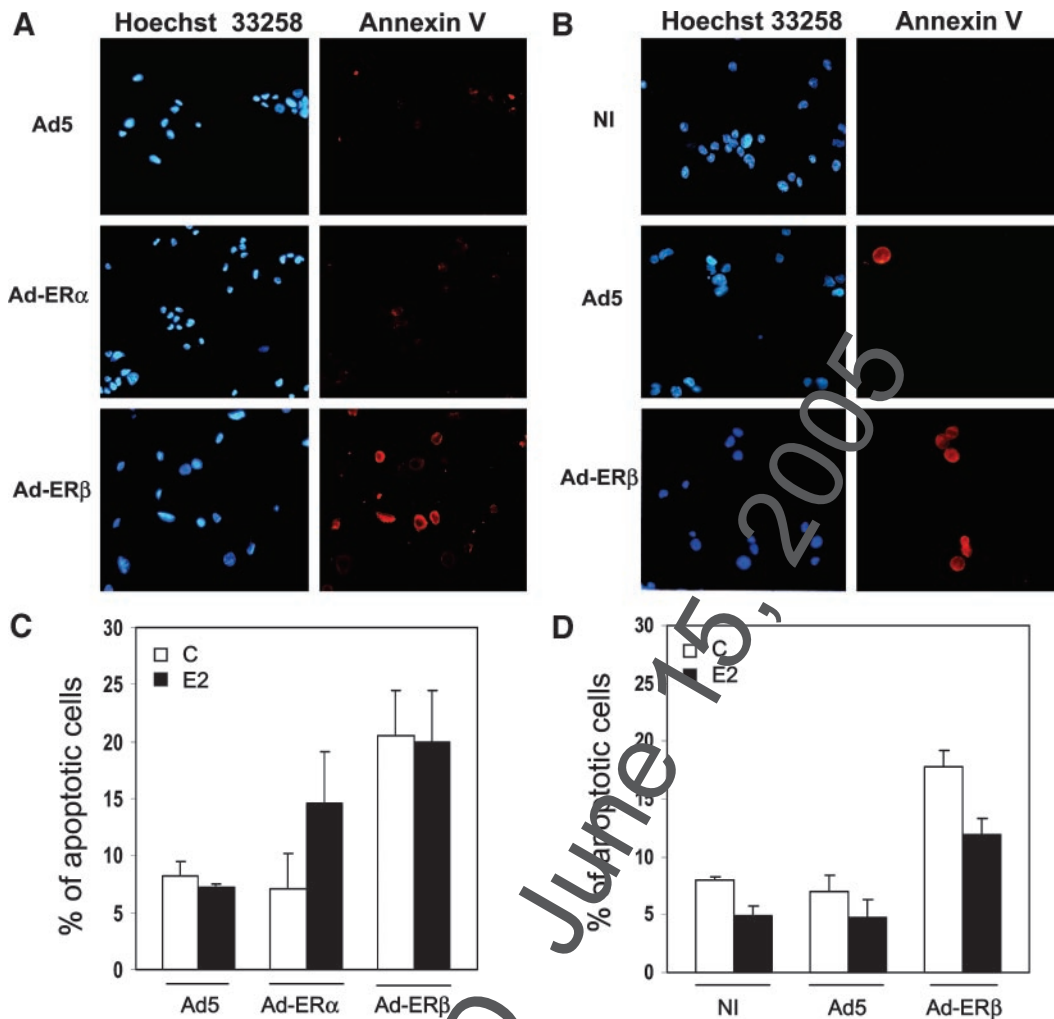


Fig. 6. ER $\beta$  induces apoptosis of ovarian cancer cells. A, PEO14 cells were infected with different viruses and then treated during 48 hours with vehicle ethanol (C) or E2 ( $10^{-8}$  mol/L). Cells were stained with annexin V and Hoechst and pictured with microscope. B, The BG1 cells were not infected or infected with Ad5 or Ad-ER $\beta$  and then treated like PEO14 cells. C, results for PEO14 cells are shown as the percentage of apoptotic cells after 48 hours of treatment and represent the mean  $\pm$  SD of three experiments. D, results for BG1 cells are shown as the percentage of apoptotic cells after 48 hours of treatment and represent the mean  $\pm$  SD of three experiments.

observation that ER $\beta$  controls apoptosis of ovarian cancer cells. Recent work has also suggested that in normal ovary, ER $\beta$  could up-regulate FasL, a major apoptotic protein ligand (48). In addition, we have shown recently that exogenous expression of ER $\beta$  in prostate cancer cells leads also to apoptosis (19). The precise mechanisms underlying apoptosis control by ER $\beta$  will need to be investigated in the future.

In summary, our data show that ER $\beta$  appears to be a key regulator of ovarian carcinogenesis. The loss of ER $\beta$  expression during ovarian carcinogenesis may be a crucial and early step leading to dysregulated growth. Moreover, introduction of ER $\beta$  in ovarian cancer cells has allowed us to show for the first time that this receptor exhibits protective properties against cancer development by increasing apoptosis and decreasing proliferation and motility. This suggests that ER $\beta$  could act as a tumor suppressor in the ovary. The identification of ER $\beta$ -regulated specific genes involved in epithelial proliferation and apoptosis may thus be a clue for understanding the progression of ovarian cancer and for the design of new target therapies.

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