

Tumor Repressive Functions of Estrogen Receptor β in SW480 Colon Cancer Cells

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Abstract

Estrogen receptor β (ER β) is the predominant ER in the colorectal epithelium. Compared with normal colon tissue, ER β expression is reduced in colorectal cancer. Our hypothesis is that ER β inhibits proliferation of colon cancer cells. Hence, the aim of this study has been to investigate the molecular function of ER β in colon cancer cells, focusing on cell cycle regulation. SW480 colon cancer cells have been lentivirus transduced with ER β expression construct with or without mutated DNA-binding domain or an empty control vector. Expression of ER β resulted in inhibition of proliferation and G₁ phase cell cycle arrest and this effect was dependent on a functional DNA-binding region. *c-Myc* is overexpressed in an overwhelming majority of colorectal tumors. By Western blot and real-time PCR, we found *c-Myc* to be down-regulated in the ER β -expressing cells. Furthermore, the *c-Myc* target gene p21^(Waf1/Cip1) was induced and *Cdc25A* was reduced by ER β at the transcriptional level. The second cdk2-inhibitor, p27^{Kip1}, was induced by ER β , but this regulation occurred at the posttranscriptional level, probably through ER β -mediated repression of the F-box protein p45^{Skp2}. Expression of the ER β -variant with mutated DNA binding domain resulted in completely different cell cycle gene regulation. We performed *in vivo* studies with SW480 cells \pm ER β transplanted into severe combined immunodeficient/beige mice; after three weeks of ER β -expression, a 70% reduction of tumor volume was seen. Our results show that ER β inhibits proliferation as well as colon cancer xenograft growth, probably as a consequence of ER β -mediated inhibition of cell-cycle pathways. Furthermore, this ER β -mediated cell cycle repression is dependent on functional ERE binding. [Cancer Res 2009;69(15):6100–6]

Introduction

The majority of sporadic colon cancers are believed to develop through a gradual process of mutations including inactivation of the APC gene. This molecular process corresponds to development of polyps and subsequently adenomas of the colonic epithelium (1). Randomized, placebo-controlled clinical trials, designed to investigate the risk of hormone replacement therapy, indicates that progestin-estrogen hormone replacement therapy reduces the

incidence of colorectal carcinoma (2, 3). Interestingly, there are also indications that use of oral contraceptives is associated with a lower incidence of colorectal cancer (4). Several possible explanations have been presented. Estrogens affect the metabolism of bile acids, reduce insulin-like growth factor (IGF-I) levels in the bloodstream, and have direct effects on the colonic mucosa (5–7). In the human colonic epithelium, estrogen receptor β (ER β) is the predominant ER (8, 9). The ERs mediate their effects by binding to estrogen response elements (ERE) in gene promoters but also indirectly through activator protein (AP-1) and SP-1 sites (10).

A striking reduction of ER β expression occurs in colon cancer compared with normal colonic epithelium, and furthermore, the level of ER β seems to be inversely correlated to differentiation grade and Dukes score (11, 12). This raises the question whether ERs in the colonic epithelium could have a function as tumor suppressors in early colonic neoplasms (13). To investigate the function of ER β in colon cancer, we have generated ER β -expressing SW480 colon cancer cells by lentiviral transduction of an ER β expression cassette. Also, we have used an ER β with mutated DNA-binding domain (DBD-mutation) to investigate if effects of ER β on gene transcription are dependent on ERE-binding or not. The transformation of normal colonic epithelial cells to colon cancer involves a sequential alteration in gene expression profile, involving induced expression of several cell cycle genes (1, 14). The cell cycle is a central pathway in carcinogenesis and development of a malignant disease. *c-Myc* is overexpressed in a majority of colon cancers and is believed to be an important factor in the transformation process (15, 16). *c-Myc* is one of the first genes to be expressed in the cell cycle, in turn regulating the expression of secondary cell cycle genes. However, there are other more or less *c-Myc*-independent cell cycle factors of importance in colon cancer growth and metastasis. Recently, several studies have shown a correlation of decreased p27^{Kip1} protein levels (p27) with poor overall survival compared with patients with high levels of p27 in their tumor (17). The main regulator of p27 protein level is the F-box protein p45^{Skp2}, which targets the protein for degradation through the proteasome complex. In addition, a correlation of increasing p45^{Skp2} levels (p45) with increased number of lymph node metastases has been found (18). Interestingly, both *c-Myc* and p27 are regulated by ER α in breast cancer cells and we have earlier shown that ER β induces p27 in breast cancer cells (19). Consequently, we are now investigating the effects of ER β on the *c-Myc* and p27 pathways in colon cancer cells.

Materials and Methods

Cell cultures and generation of stably ER β -expressing cells. Because colon cancer cell lines do not contain endogenous ER β , SW480, HCT-116, and HT29 colon cancer cells were lentivirus-transduced with ER β -construct

Note: Supplementary data for this article are available at Cancer Research Online (<http://cancerres.aacrjournals.org/>).

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(SW480-ER β), (HCT-116-ER β), and (HT29-ER β) or with empty vector as control (SW480-Control), (HCT-116-control), and (HT29-control). SW480 cells were also transduced with ER β -construct with mutated DBD-domain (SW480-ER β -DBDmut) to investigate the importance of ERE binding in ER β signaling. Both ER β constructs were expressed at similar levels in SW480 cells. The plasmid pcDNA3-FLAG ER β was used as a template for TOPO cloning into pLenti6/V5-D-TOPO according to the manufacturer's instructions (Invitrogen). The pLenti6/V5-D-FLAG ER β DBDmut was constructed from pLenti6/V5-D-FLAG ER β using Quick-Change (Stratagene) according to the manufacturer's instructions. Forward oligo ATCACTATGGAG TCTGGTCGTTG CA GCATGTAAGGCCTTTTAAAA GA changing amino acid residues E167 and G168, glutamic acid and glycine, respectively, to alanine. Lentivirus was then produced with the ViraPower Lentivirus expression system and infection was performed as described earlier (20). The lentivirus-treated cells were then incubated with 5 μ g/mL Blasticidine, resulting in death of nontransduced cells. The corresponding ER α DBD mutant has been characterized earlier. The ER β DBD mutant was further characterized in an ERE luciferase activation assay where wild-type ER β , but not the DBD-mutated ER β , activated ERE-luciferase transcription. The lentivirus transduction was made in independent triplicates for each construct (ER β , ER β -DBDmut, and control). The cells were cultured in RPMI 1640 (Life Technologies/Invitrogen) with 5% fetal bovine serum (FBS). Medium was changed to phenol red-free RPMI 1640 with 5% dextran/charcoal-treated FBS (stripped medium) 24 h before start of experiment. The cells were then incubated with 10 nmol/L E₂ or ethanol as control for 24 h in stripped medium.

Proliferation assay. To investigate the ER β -mediated effects on cellular proliferation, 2×10^5 SW480, HCT-116, and HT29 cells, respectively, were virally transduced with either ER β -construct or empty control vector and were plated onto 6-cm cell culture dishes in RPMI 1640. The plating efficiency was similar in the different plates. After 72 h, the cells were counted manually with a Bürkner chamber. Experiments were performed with three different clones, each in three separate dishes.

Flow cytometry. Flow cytometry was performed to observe different cell cycle distributions of the SW480 clones. Cells were treated with mevinolin at 40 μ mol/L (Sigma) for 36 h before the start of the experiment to ensure cell synchronization. Cells were then released into media containing 5% serum, harvested at 0, 16, 24, and 36 h by trypsinization and fixed in 70% cold ethanol for 30 min on ice. The fixed cells were washed in PBS and stained with 50 μ g/mL propidium iodide (Sigma) supplemented with RNase A (50 μ g/mL; Sigma) for 30 min at 37°C. Fluorescence-activated cell sorting (FACS) was performed using a FACS Calibur flow cytometer (Becton Dickinson). Cell cycle analysis was performed using CELLQuest program (Becton Dickinson).

Western immunoblotting and antibodies. SDS-PAGE was performed as described earlier (21), with the primary intention to investigate regulation of G₁-phase cell-cycle factors. The following primary antibodies were used for incubations: against ER β (Abcam), β -actin (Sigma), c-Myc sc-764, Cyclin A sc-751, Cyclin E sc-247, p27^{Kip1} sc-1641, p21^{Waf1/Cip1} sc-19, p45^{Skip2} sc-7164, p15 sc-612, p16 sc-468, and p14^{ARF} sc-8613 (all from Santa Cruz Biotechnology); pRb-antibody sampler kit (Cell Signaling); and Cdc25A Ab-3 (from NeoMarkers).

RNA-extraction and real-time PCR. RNA extraction with TRIzol (Invitrogen) was done according to standard protocol. RNA was purified with Qiagen RNeasy spin columns (Qiagen) with on-column DNase I digestion (for subsequent Microarray-experiment). cDNA was synthesized with NBS First Strand System (Nordic Bioservice).

Real-time PCR was performed with NBS Probe or NBS SYBR Mastermixes (Nordic Bioservice). Primer/probe sequences will be provided on request. The real-time PCR reactions were performed in an ABI PRISM 7500 (Applied Biosystems) under the following conditions: 10 min hot start at 95°C, followed by 15 s at 95°C, and 60°C for 50 s in totally 40 cycles. The optimum concentration of primers was determined in preliminary experiments and all SYBR-Green primer pairs were checked with melting curve analysis.

Immunohistochemistry. To examine the number of proliferating cells in \pm ER β -expressing tumor xenografts, immunohistochemistry with a Ki-67 antibody was performed. Antigen retrieval was done by microwave boiling

in 0.01 mol/L citric acid (pH 6.0) for 120 s and then slides were left undisturbed for 20 min. Endogenous peroxidase activity was blocked by incubating sections shaking in 3% hydrogen peroxide in methanol for 10 min and then blocked in 2% Bovine Serum Albumin (BSA) and 0.1% NP40 for 1 h at room temperature. Primary antibody Ki-67 (DakoCytomation) was diluted 1:100 in 2% BSA and 0.1% NP40 and incubated overnight at 4°C. Sections were then washed consecutively in PBS, 0.1% NP40 for 3 \times 5 min, followed by incubation with a biotinylated antibody BA-9200 1:200 in 0.1% NP40 for 1 h (Vector Laboratories). After this, sections were incubated in the streptavidin-horseradish peroxidase ABC complex (Vectastain Elite; Vector Laboratories) for 1 h, stained in 3,3'-diaminobenzidine and counterstained with Mayer hematoxylin (Sigma) before dehydration through ethanol, and mounted in Pertex (Histolab). The number of Ki-67-positive cells was counted on two independent fields per slide with $\times 20$ objective. The average number of positive cells and SD were calculated for each group.

Experimental animals and xenograft model. SW480 colon cancer cells \pm ER β expression were implanted into immunodeficient mice to investigate tumor growth. SW480-ER β /control cells (5×10^6) were diluted in 200 μ L normal medium + 200 μ L Matrigel (BD Biosciences) and injected into the abdominal fat close to the mammary tissue of 9- to 12-wk-old pathogen-free severe combined immunodeficient (SCID)/beige mice (Taconic) on day 0. E₂ pellets, 0.72 mg/pellet (IRA), were inserted s.c. into the neck with a pellet trochar (IRA). After 14 d, the mice were sacrificed and the tumor weight was measured. All tumors were fixed in 4% paraformaldehyde and stored in 75% ethanol. After this, tissues were paraffin embedded and subsequently sliced into 4.5- μ m sections according to standard protocol.

Statistics. Values are expressed as means with 95% confidence intervals. For real-time PCR analysis, unpaired two-tailed *t* test was used to compare differences between two parallel treatment groups of identical origin. Mann-Whitney *U* test was used to test differences between two groups of nonparametric data. Significance is presented as *P* value of <0.05 (*), <0.01 (**), and <0.001 (***); nonsignificant differences are presented as NS.

Results

Colon cancer epithelial cells do not contain ER α but varying amounts of ER β . The SW480 as well as most other colon cancer cell lines grown *in vitro* contain very low levels of ER β . To confirm this, we performed real-time PCR with primers/probe specific for ER α and ER β mRNA, respectively. As expected, we found no ER α and very low levels of ER β expression in SW480 cells (data not shown). To be able to study the effect of ER β in these cells, we used lentiviral transduction of an ER β expression cassette. In Fig. 1, we show the expression of ER β mRNA and protein in these cells (A). The level of ER β was not affected by E₂ treatment. Expression of exogenous ER β decreased the proliferation compared with SW480 cells transduced with empty expression cassette (SW480-control) as shown in Fig. 1B. As a comparison, two other colon cancer cell lines, HCT-116 and HT29, were transduced with an ER β expression cassette (HCT-116-ER β and HT29-ER β). The proliferation of HCT-116 cells was reduced in presence of ER β expression. However, the proliferation of HT29 cells was not reduced to a significant level (Fig. 1C). We then performed flow cytometry to investigate the cell cycle distributions of the SW480-clones. Thirty-six hours after start of experiment, 75% of SW480-ER β cells were distributed in the G₁ phase, whereas 52% of SW480-control cells were in the G₁ phase (Fig. 1D). Mutation of the DNA-binding domain of ER β (SW480-ER β -DBDmut) resulted in a nearly complete removal of the ER β -mediated G₁ arrest, resulting in 58% of the cells distributed in the G₁ phase. In agreement with this, expression of ER β -DBDmut did not reduce the proliferation of SW480 cells as measured with proliferation assay (data not shown).

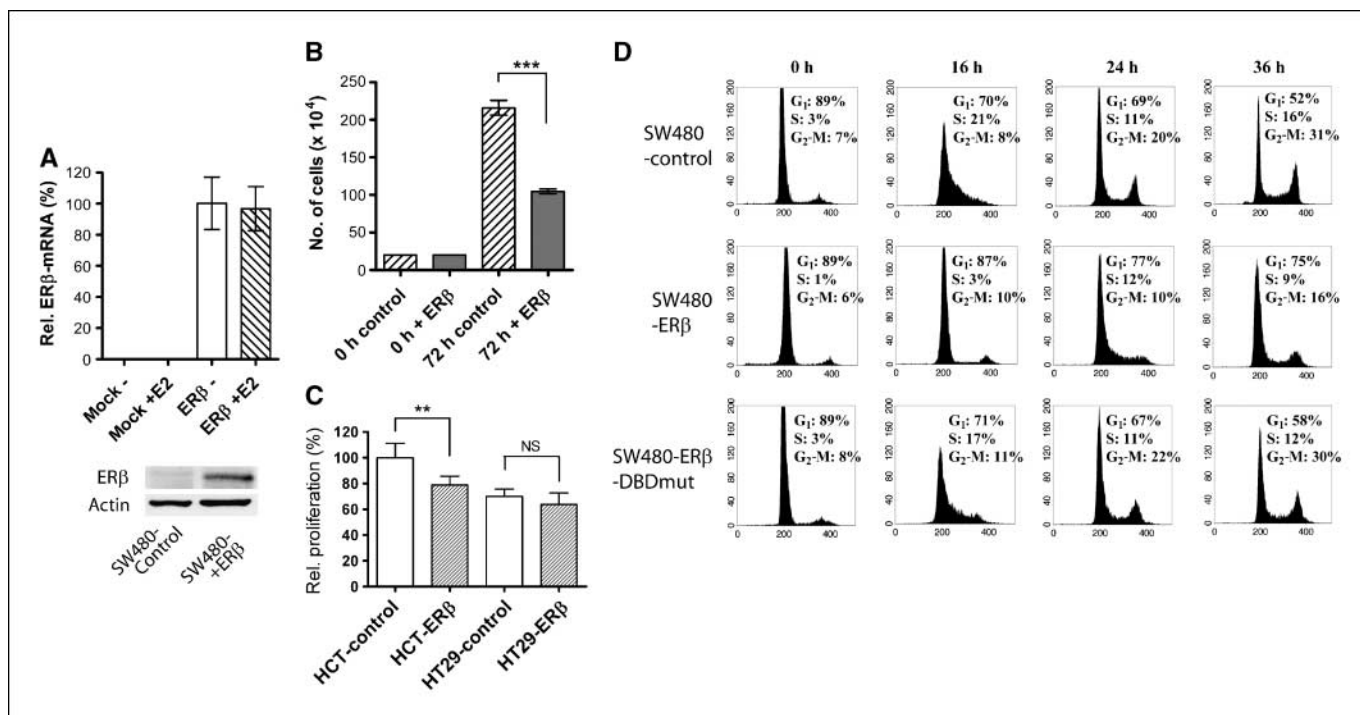


Figure 1. SW480 cells lentivirus-infected with ER β construct, empty control vector, or ER β construct with mutated DNA-binding domain. Both ER β constructs were expressed at similar levels. **A**, expression of ER β mRNA \pm E₂ measured by real-time PCR, and ER β protein in presence of E₂ measured by Western blot. **B**, proliferation measured by manual counting of SW480-ER β /control cells after 72 h incubation in presence of E₂. Columns, average values of triplicates; bars, SD. **C**, proliferation of HCT-116-ER β /control cells and HT29-ER β /control cells measured as in **B** in presence of E₂. **D**, flow cytometry analysis of SW480 cells to show cell cycle distribution. Synchronized cells were released into media containing 5% serum and E₂, harvested at 0, 16, 24, and 36 h. Y axis, cell count; X axis, DNA content. All experiments above were repeated twice with identical results. Statistics presented as $P < 0.01$ (**), $P < 0.001$ (***); NS, nonsignificant differences.

We went on to investigate the regulation of the most important genes governing proliferation in the G₁-S phase. Western blot was performed and the results are shown in Fig. 2A. The immediate early gene *c-Myc* was down-regulated by ER β expression and, as expected, the *c-Myc* target gene *Cdc25A* was also repressed by ER β . p14^{ARF}, a tumor suppressor involved in both cell-cycle arrest and apoptosis, was induced by ER β . p14^{ARF} functions by down-regulating MDM2 and, in this way, stabilizing p53-protein. As a consequence, we found p53 to be strongly induced by ER β .

The cdk4/6-inhibitors p15 and p16 were not affected by ER β at the protein level. Cyclin E and A, important in the late stage of the G₁ phase, were not affected significantly, whereas Cyclin D1 was up-regulated at the protein level. The cdk2-inhibitors p21^(Waf1/Cip1) (p21) and p27 showed strong induction following ER β expression and the expression of p45, which is the regulator of p27-degradation, was down-regulated by ER β . Forced expression of Retinoblastoma protein (pRb) efficiently blocks cell cycle transition and pRb is often mutated or inactivated in tumorigenesis resulting in free E2F1 (22). Interestingly, we found that total amount of pRb was increased in the ER β -expressing cells compared with the control. Phosphorylation status of pRb was changed as well, resulting in increased phospho-Ser780 and decreased phospho-Ser795 following ER β expression.

However, SW480 cells expressing ER β -DBDmut showed a completely different protein expression pattern (Fig. 2B). The DBD-domain is responsible for binding of ER β to EREs. Therefore, the DBD-mutant is crucial to investigate potential ERE-dependent activation/inactivation by ER β . *Cdc25A* was induced by ER β -DBDmut, whereas p21 and p27 were not induced to the same

extent as by wild-type ER β . Furthermore, *c-Myc* was not repressed to as large extent as in the SW480-ER β cells.

In HT29 cells (Fig. 2C), *c-Myc*, p21, p27, and p53 were all regulated by ER β expression in a similar fashion as in SW480 cells. However, ER β expression in HCT-116 cells caused a reduction of p53 and p21 levels but also of *c-Myc*, whereas p27 was not significantly changed.

To investigate the regulation of these proteins at the mRNA level, we performed real-time PCR (Fig. 3) on SW480-ER β , SW480-control, or SW480-ER β -DBDmut. We found that *c-Myc* mRNA was down-regulated >70% by ER β expression independently of E₂, and this effect was dependent on a functional DBD (Fig. 3A). *Cdc25A* mRNA expression was also reduced by ER β , but not by the ER β -DBDmut (Fig. 3C). There were no significant differences in mRNA expression of Cyclin E or Cyclin A by ER β (Fig. 3A), whereas Cyclin A expression was reduced by ER β -DBDmut. *Cyclin D1* mRNA expression was up-regulated in SW480-ER β in a DBD-dependent fashion. p21-mRNA was up-regulated in SW480-ER β independent of the DBD, indicating that the regulation of p21 by ER β occurs independently of ERE-binding. p27 mRNA was not regulated by ER β (Fig. 3B) but was induced by ER β -DBDmut; however, this induction was nonsignificant ($P = 0.32$, bars 2 versus 6). There was a strong reduction of p45-mRNA in SW480-ER β cells dependent on a functional DBD. As expected, pRb was not regulated at the mRNA-level by ER β but was strongly repressed by the ER β -DBDmut, an effect which we cannot explain at present (Fig. 3C). p14^{ARF} and p53 were not affected significantly at the mRNA-level (data not shown).

To obtain a more complete view of the antitumorigenic role of ER β , we investigated how ER β expression affected growth of SW480-xenografts (Fig. 4). SW480-control or SW480-ER β cells

were transplanted into the right flank of SCID/beige mice (Fig. 4A). At end point, the average weight of SW480-control xenografts was 704 mg, whereas average weight of SW480-ER β xenografts was 245 mg, a reduction of tumor weight by 65% following ER β expression (Fig. 4A and B). Immunohistochemistry with a Ki67-specific antibody was performed. The xenografts in the mice injected with ER β -expressing cells showed a significant down-regulation of the number of Ki67-positive cells (Fig. 4C and D). A TUNEL assay was performed on the xenografts to detect apoptosis, but there was no significant difference in apoptosis between SW480-ER β and SW480-control xenografts (data not shown).

Moreover, microarray studies were performed to investigate genome-wide transcriptional effects as a result of long-term expression (3 weeks) of ER β in SW480 cells. ER β expression resulted in dramatic changes of the transcriptome; 623 differentially regulated transcripts were detected and the effect was mainly repressive (Supplementary Data).

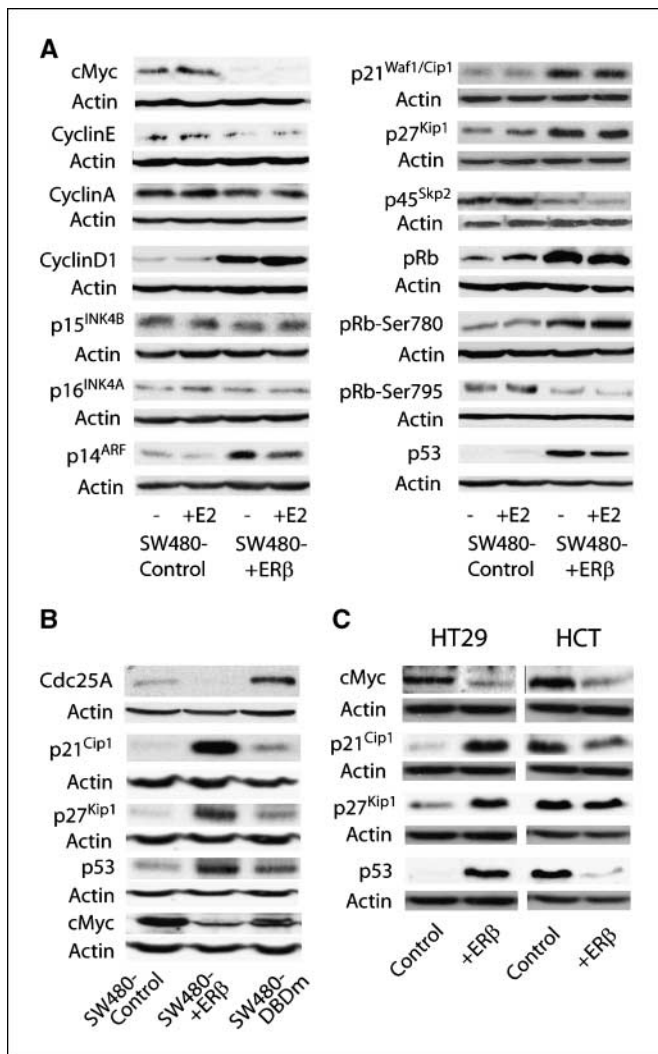


Figure 2. Protein expression of cell cycle factors in colon cancer cells. A, cell cycle proteins in SW480-ER β /control cells measured by Western blot \pm E₂ for 24 h. B, cell cycle proteins in SW480-ER β /control/ER β -DBDmut cells measured by Western blot after 24 h E₂ treatment. C, comparison of cell cycle proteins in HCT-116 and HT29 cells measured by Western blot after 24 h E₂ treatment. All experiments were repeated twice with identical results. β -Actin standard used as loading control for all proteins.

Discussion

As mentioned, preclinical studies have shown reduced expression of ER β in colon cancer tissue compared with normal colonic epithelium. ER β is expressed even in metastatic colon cancer but to a lower extent compared with normal colonic epithelium. This points toward a potential role of ER β as a protein with tumor suppressive function. Earlier studies by other researchers have shown effects of ER β on proliferation in another colon cancer cell line (23), but detailed analyses in combination with *in vivo* xenograft studies are lacking. Flow cytometry with propidium iodide staining revealed a clear difference in cell cycle distribution of SW480-ER β compared with SW480-control as well as SW480-ER β -DBDmut cells. There was a nearly 50% increase of cell proportion in the G₁ phase in presence of ER β expression compared with the control. The cell cycle distribution of SW480-ER β -DBDmut cells was very similar to that of SW480-control cells, pointing toward an ERE-dependent, antiproliferative effect of ER β . Furthermore, expression of ER β also reduced the proliferation of HCT-116 colon cancer cells but not that of HT-29 cells. The reason behind the lack of antiproliferative effect in HT-29 cells is not clear at present. However, all these cell lines express very low levels of endogenous ER β mRNA. Preliminary experiments and published data indicate that the relative ER β -mRNA level is higher in HCT-116 and SW480 compared with HT-29 cells (24). This finding might reflect alternative signaling pathways in proliferation of HT29 cells. Interestingly, this type of mechanism is well known in breast cancer cells, where ER α -cancers are frequently dependent on tyrosine-kinase signaling to proliferate (25). Moreover, the total ER β level in the transduced cells was lower in HT-29 cells compared with HCT-116 and SW480.

We performed both real-time PCR analysis of rate-limiting cell cycle factors in the G₁ phase as well as microarray analysis for the overall effects by ER β expression (Supplementary Data). We found several proliferation-regulating genes to be transcriptionally inhibited by ER β . *c-Myc* is an important immediate early gene, and an activator of numerous G₁-S-phase genes. Expression of *c-Myc* has been shown to counteract a dnTCF-4 expressed in the colon cancer cell line HT29 with respect to effects on proliferation. This points toward *c-Myc* as a central factor in colon cancer growth, neutralizing the effect of the APC tumor suppressor (16). We found that ER β was a strong inhibitor of *c-Myc* expression at both mRNA and protein levels, which could be an important step in the repression of proliferation by ER β . *c-Myc* expression affects the transcription of many proliferation-associated genes and Cdc25A is one of the most well-known targets (26). We found Cdc25A to be repressed at mRNA and protein-levels by ER β , most likely dependent on *c-Myc* repression. In SW480-ER β -DBDmut, *c-Myc* was induced at mRNA level but not as strongly regulated at protein level; therefore, it is likely that ER β -mediated repression occurs through ERE binding.

In response to *c-Myc* overexpression, normal cells undergo p53-dependent apoptosis and one pathway is through induction of p14^{ARF} (27). Interestingly, p14^{ARF} protein but not mRNA was induced in response to ER β , despite its strong inhibition of *c-Myc*. This indicates that ER β may induce p14^{ARF} through an alternative pathway. It is known that p14^{ARF} is regulated through proteasomal degradation (28). Accordingly, it is possible that ER β regulates p53 by affecting p14^{ARF} degradation. Induction of p53 by ER β through p14^{ARF} could be a novel pathway important for ER β -mediated apoptosis in premalignant lesions with wild-type p53.

p21 is an important downstream effector of p53-signaling in parallel to its proapoptotic effects and p21 was found to be

up-regulated in the ERβ-expressing cells. SW480 cells contain a mutated form of p53, presumably without proapoptotic potential but with some transcriptional activity such as the ability to induce p21 (29). In line with this, we could not find increased apoptosis in the ERβ-expressing tumor xenografts, but a strong induction of p21-protein *in vitro*.

We also analyzed the ERβ-mediated regulation of cell cycle genes in two other colon cancer cell lines: HCT-116 and HT29. Cell cycle regulation at protein level by ERβ was very similar in HT29 cells compared with SW480. Interestingly, in HCT-116 cells, which contain a nonmutated, wild-type p53, this protein was strongly reduced by ERβ, for unknown reasons. Furthermore, the p53 target gene p21 was reduced. Most interestingly, the expression of *c-Myc*

was reduced in both cell lines, further strengthening the role of *c-Myc* as a central ERβ-regulated cell cycle gene.

The other major cell cycle pathway regulated by ERβ was the p45-p27 pathway. p27-protein but not p27-mRNA was induced by ERβ expression. Therefore, we investigated whether p45, governing p27 degradation, was affected by ERβ. We found that p45 was strongly reduced at protein and mRNA-levels. We believe that the combined *c-Myc* reduction and p27/p21 induction efficiently inhibits cell cycle progression. We have earlier studied the effects of ERβ in breast cancer cells (19, 30) where *c-Myc* was reduced and p21 and p27 were induced. Remarkably, *Cyclin D1* was induced by ERβ in colon cancer cells and this was seen in breast cancer cells as well, but even under these conditions, proliferation was inhibited.

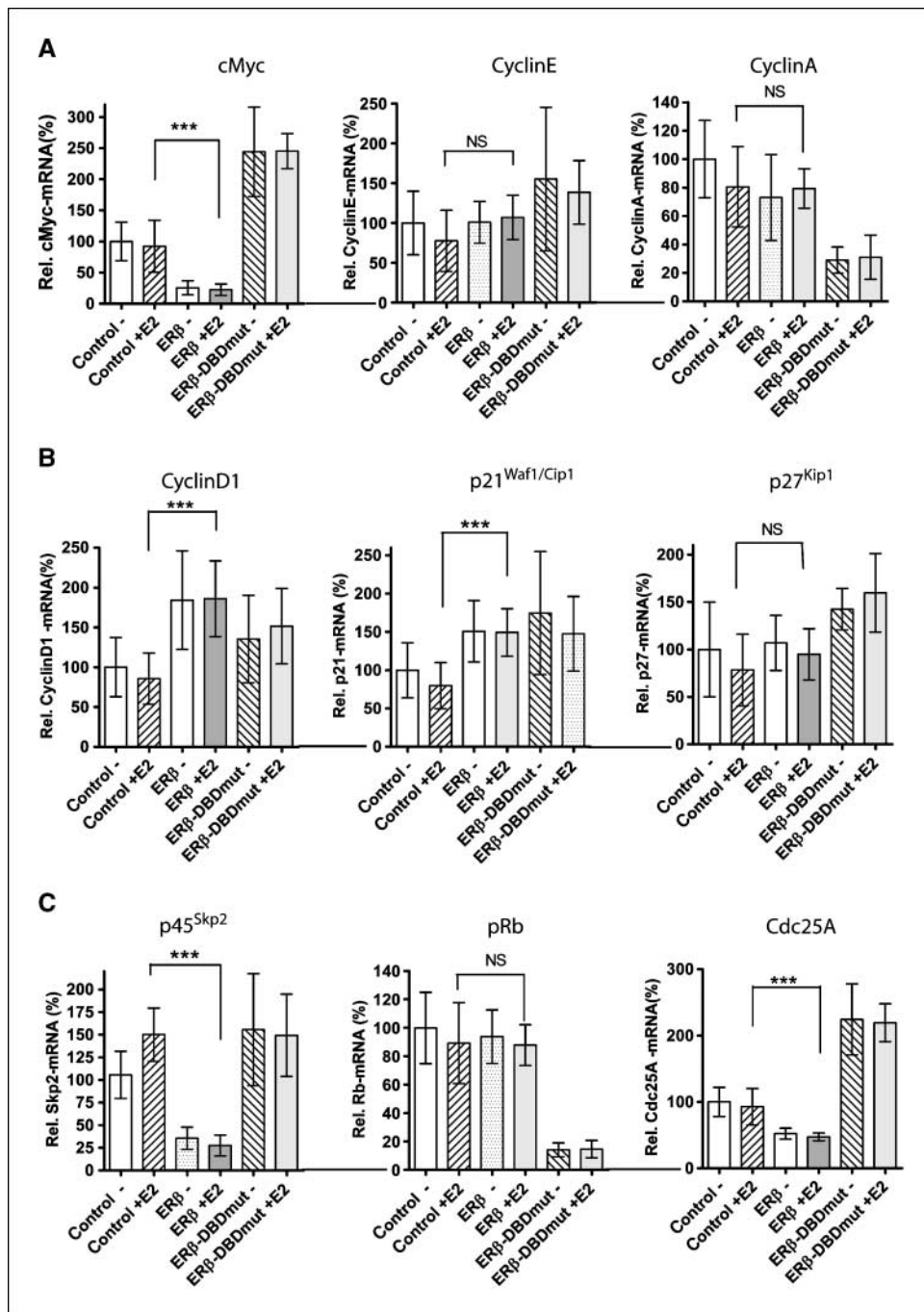
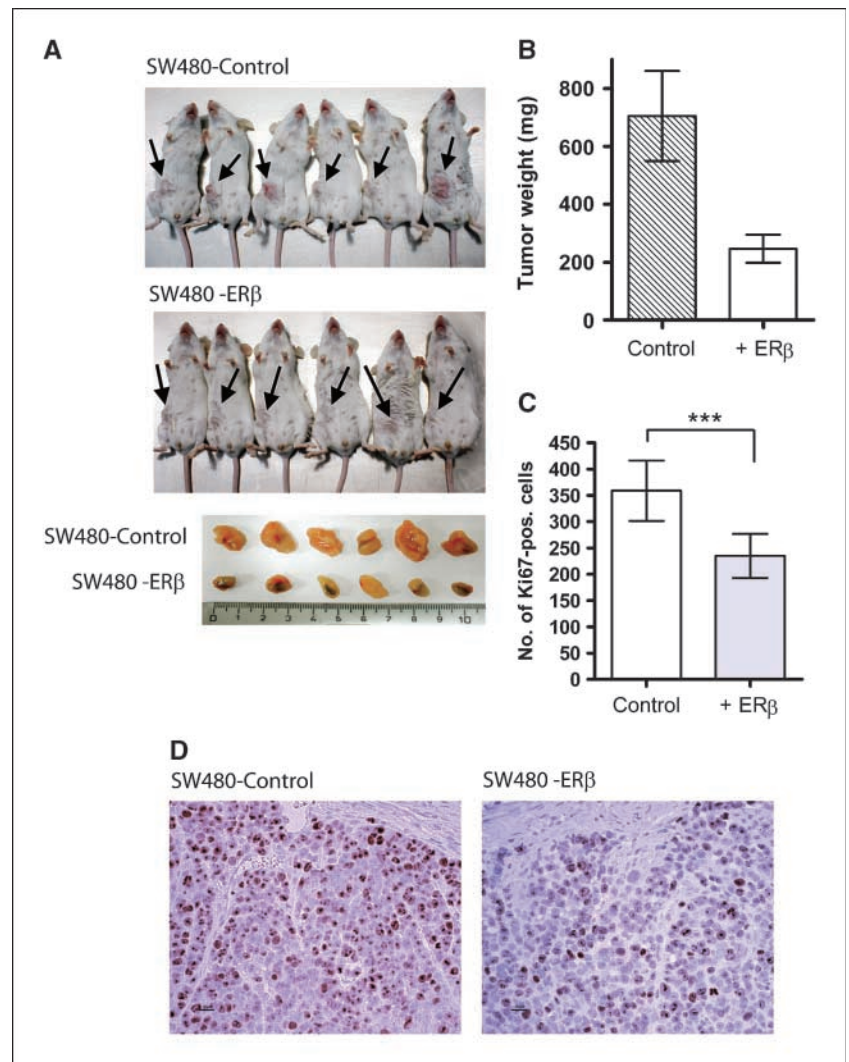


Figure 3. ERβ regulation of cell cycle genes, measured by Real-time PCR. SW480 cells lentivirus-infected with empty vector (columns 1–2), ERβ construct (columns 3–4), and ERβ construct with mutated DBD-domain (columns 5–6). In presence of 10 nmol/L E₂ treatment (bars 2, 4, and 6). A, *c-Myc* mRNA, Cyclin E mRNA and Cyclin A mRNA. B, Cyclin D1, p21^{Waf1/Cip1} mRNA and p27^{Kip1} mRNA. C, p45^{Skp2} mRNA, pRb mRNA, and Cdc25A mRNA. Columns, average values of triplicates and experiments were repeated twice with similar results; bars, SD. Statistics presented as *P* < 0.001 (***) ; NS, nonsignificant differences presented as NS.

Figure 4. SCID/beige mice transplanted with SW480 cells expressing ER β (SW480-ER β ; $n = 6$) or with empty vector (SW480-control; $n = 6$). The cells were mixed with Matrigel and implanted into mammary fat pad. All mice were subsequently implanted with slow-release E $_2$ pellet. After 3 wk, mice were sacrificed and photographed. **A**, SW480-control and SW480-ER β cell-transplanted mice, with arrows pointing at injection site and picture of the removed xenografts at end point. Scale in centimeters. **B**, tumor weight at end point. **C**, number of Ki67-positive cells in xenograft slides per visual field ($\times 20$ objective) identified by immunohistochemistry. **Bar 1**, SW480-control xenografts; **bar 2**, SW480-ER β xenografts. Number of positive cells was counted in two independent areas on each xenograft slide. **Columns**, average number; **bars**, SD. *******, $P < 0.001$. **D**, representative pictures of Ki67-immunohistochemically stained slides from SW480-control xenografts and SW480-ER β xenografts.



pRb is an important tumor suppressor, often mutated and inactivated in human malignancies. We found that total pRb level was up-regulated by ER β expression. Phosphorylation status of pRb was changed as well with increased phospho-Ser780, which requires Cyclin D-cdk4/6 activity (31) and decreased phospho-Ser795 following ER β expression. This indicates that the increase in *Cyclin D1*-level causes an activation of the Cyclin D1-cdk4/6-complexes, and a partial phosphorylation of pRb. Still, the antiproliferative and antitumorigenic effects of ER β expression are strong, which means that ER β probably has its major effect on a parallel or downstream target. It is believed that complete inactivation of pRb and dissociation of E2F1 requires sequential phosphorylation of pRb by the Cyclin E/A-cdk2 complexes as well (32). Therefore, we suggest that the combined action of increased cdk2-inhibitor levels (p21 and p27), decreased Cdc25A-level and, not least, increased pRb-levels results in strong inhibition of proliferation by inactivation of the Cyclin E/A-cdk2 complexes.

We have used an ER β construct with mutated DNA-binding domain (ER β -DBDmut) to investigate ER β effects in absence of functional, classic ERE binding. Several genes are regulated by ER α and ER β through nonclassic binding sites such as AP-1 and SP-1, independently of a functional DBD region. Therefore, the

ER β -DBDmut does not work as a negative control for ER β signaling. It is likely that in genes where the ER β effects are completely dependent on ERE binding, the effects can be reversed with the ER β -DBDmutant. On the other hand, when the ER β effects are mediated through other promoter elements, the expression of the ER β -DBDmut can result in both induction and repression of transcription. Based on the findings in this article, we conclude that the antiproliferative effect of ER β in colon cancer cells is dependent on ERE binding and, hence, a functional DBD region. Most interestingly, to our knowledge, this is the first study to indicate a ligand-independent, ERE-mediated regulation by ER β . However, we cannot rule out the possibility that the E $_2$ independence is a result of ER β overexpression. But today, no colon cancer cell lines with endogenous ER β are available.

Our *in vivo* xenograft experiments indicate that ER β efficiently blocks tumor growth. However, because all xenografts grew in presence of E $_2$, we do not know whether estrogens further strengthen the antiproliferative function of ER β . This should be investigated in future studies. Loss of ER β is seen in the progression of several types of malignancies, e.g., in breast, ovarian (33), and colon cancer. Konstantinopoulos and colleagues (11), among many authors, showed that reduction in ER β expression

was correlated with the degree of tumor differentiation, poorly differentiated tumors exhibiting a larger loss of ER β compared with moderately or well-differentiated tumors. It is interesting to note that reduced p27 and increased p45 are correlated to higher aggressiveness in many types of cancer (34, 35) in line with a regulation of these factors by ER β .

In conclusion, ER β inhibits proliferation and tumor growth of colon cancer cells by regulating G₁-phase cell cycle genes. Moreover, this regulation is dependent on a functional DBD-domain of ER β .

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