ERβ is a potent inhibitor of cell proliferation in the HCT8 human colon cancer cell line through regulation of cell cycle components

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Abstract

Several strands of evidence indicate that oestrogens exert a protective role against the development of colon cancer through indirect and direct effects on colonic epithelium. Oestrogen receptor β (ERβ), the predominant ER subtype in human colon, is significantly decreased in colonic tumours compared with normal mucosa suggesting a potential role in the regulation of colon tumour growth.

To investigate this hypothesis we engineered human colon cancer ERα-negative HCT8 cells in order to obtain ERβ protein over-expression. Stably transfected cells were cloned and ERβ expression and functionality were monitored by RT-PCR, Western blotting and transactivation in an assay using oestrogen-responsive reporter constructs.

Over-expression of ERβ inhibited cell proliferation and increased cell adhesion in a ligand-independent manner. Its constitutive activation is possibly due to cross-talk with intracellular signalling pathways, as epidermal growth factor and IGF-I were able to induce ERβ transactivation.

A possible mechanism by which ERβ over-expression inhibits proliferation in HCT8 cells is by modulation of some key regulators of the cell cycle; there is a decrease in cyclin E and an increase in the cdk inhibitor p21CIP1. In fact, flow cytometry analysis provided evidence for blocking of the G1-S phase progression induced by ERβ over-expression. The magnitude of this effect was affected by the level of ERβ expression.

These results provide the first direct evidence that ERβ plays an important role in colon cancer as a regulator of cell proliferation through the control of key cell cycle modulators and arrest in G1-S phase transition. These findings are compatible with the hypothesis that the loss of ERβ expression could be one of the events involved in the development or progression of colon cancer.

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Introduction

Oestrogens seem to exert a protective role against development of colon cancer with a reduced relative risk (up to 0.56) in women currently receiving hormone replacement therapy (HRT) (Grodstein et al. 1998, Chlebowski et al. 2004) and a reduced mortality from this pathology (relative risk 0.71 in women who used HRT and up to 0.55 in current users) (Calle et al. 1995).

Reasons for this protective effect include an oestrogen-induced decrease of both secondary bile acids and insulin-like growth factor I (IGF-I), a direct effect on the proliferation of colorectal epithelium, or a combination of these (Pasi & Robert 2000). Several in vivo and in vitro studies support a direct effect of oestrogens...
on colonic epithelium. In fact, the expression of both oestrogen receptor (ER) subtypes, ERα and ERβ, has been demonstrated in colon cancer cell lines (Fiorelli et al. 1999, Arai et al. 2000, Nakayama et al. 2000, Campbell-Thompson et al. 2001) and in normal and cancerous colonic specimens (Foley et al. 2000, Campbell-Thompson et al. 2001, Weyant et al. 2001). In particular, ERβ, identified as the predominant ER subtype expressed in human colon, has been observed to be significantly decreased in colonic tumours compared with normal mucosa, while ERα expression appeared to be substantially unchanged (Foley et al. 2000, Campbell-Thompson et al. 2001).

Overall, the physiological role of ERs in normal colon and their possible involvement in the aetiology of colon cancer is not well understood. This situation is further complicated by the complexity of species and isoform distribution of ERs, with consequent ligand tissue-specificity and by their low expression levels in colonic tissues with respect to classical oestrogen-dependent tissues.

The ERα: ERβ ratio has been identified as a possible determinant of the susceptibility of a tissue to oestrogen-induced carcinogenesis, leading to the theory that binding of oestrogens to ERα induces a cancer promoting response, whereas binding to ERβ seems to exert a protective action (Weyant et al. 2001). A potential mechanism through which the two ERs exert such opposing action is by regulation of cyclin D1 gene transcription (Liu et al. 2002, Ström et al. 2004). Indeed, progress in the study of the molecular mechanism(s) of action of oestrogens has revealed why different cells can respond to the same hormone in a different manner. Reasons for this can reside in the different expression patterns of ERα and ERβ in vivo and in the need for them to interact with cellular transcription cofactors (coactivators and corepressors) which are not functionally equivalent and ubiquitously expressed in all cells (McDonnel & Norris 2002).

In order better to understand and clarify the role of ERβ in proliferation and development of colon cancer cells, we selected a human colon adenocarcinoma cell line (HCT8), expressing only the ERβ subtype (Fiorelli et al. 1999), to be transfected with an appropriate plasmid for the over-expression of ERβ. The present results demonstrate that over-expression of ERβ has an anti-proliferative effect, mostly due to ligand-independent activation of the receptor, and that this effect can be partially due to the modulation of some key regulators of the cell cycle with consequent cell cycle arrest in the G1-S phase transition.

Materials and methods

Cell culture

The human colon cancer HCT8 cell line (Tomkins et al. 1974, Picariello et al. 1997) was obtained from the American Type Cultures Collection (ATCC, Rockville, MD, USA). Cells were cultured in RPMI (Bio Whittaker, Walkersville, MD, USA) supplemented with 10% fetal calf serum (FCS; Biological Industries, Kibbutz Beit Haemek, Israel) until confluence, then detached with trypsin/ethylenediaminetetraacetic acid (EDTA) solution and plated at the desired density in the appropriate medium.

Hormones and chemicals

17β-Oestradiol (17βE2) and tamoxifen were purchased from Sigma (St Louis, MO, USA) and [3H]17βE2 (specific activity 84.1 Ci/mmol) was obtained from Dupont-New England Nuclear (Milan, Italy). ICI 164,384 was kindly provided by AstraZeneca (Macclesfield Cheshire, UK) and raloxifene was kindly provided by Lilly Research Laboratories (Indianapolis, IN, USA).

Plasmids

The control vector for β-galactosidase expression (pBLUE-TOPO) was purchased from Invitrogen (Life Technologies, Inc.) and pSV2neo was purchased from the ATCC. The pCXN2-hERβ mammalian expression vector for human ERβ protein was kindly provided by Dr S Inoue (Niwa et al. 1991) and pEREtkLUC reporter gene vector was kindly provided by Dr M G Parker (Cowley & Parker 1999).

Stable and transient transfection

HCT8 cells were seeded in RPMI medium supplemented with 10% FCS, without antibiotics, and cultured at 37°C in 5% CO₂ in air. After 24–48 h, at 90–95% confluence, cells were transfected with 0.2 μg plasmid (pCXN2-hERβ or pSV2neo) and 0.5 μl Lipopectamine 2000 (Life Technologies, Inc.) per cm² of surface area according to the manufacturer’s instructions. After 5 h, medium was replaced and after an additional 48 h, cells were split 1:10 in complete medium containing 800 μg geneticin (G418, Gibco, Life Technologies, Inc.)/ml. Selection of stable transfected cells was carried out within 10–20 days of culture. Selected cells were finally cloned in 96-well plates by the dilution limit method. Stable transfected clones were then cultured and amplified in complete medium containing 200 μg G418/ml. Transient co-transfection with the pEREtk-LUC plasmid and with a control vector for
β-galactosidase expression was performed following the above described protocol with minor modifications (and without G418 selection). Before transfection, cells were cultured in phenol red-free RPMI supplemented with 10% charcoal-stripped FCS for at least 2 days. After transfection, cells were starved in phenol red- and FCS-free RPMI and then tested for luciferase activity after 24 h stimulation.

RT-PCR
Total RNA was obtained from 2 × 10⁶ cells using the RNAwiz isolation reagent (Ambion, Austin, TX, USA) according to the manufacturer’s instructions. ERα, ERβ, epidermal growth factor (EGF), IGF-I, the corresponding receptors (EGFr and IGF-Ir) and β-actin (control) mRNAs were retrotranscribed from HCT8 cells, from clones of ERβ-transfected HCT8 cells and from control tissue total RNA using pairs of appropriate oligonucleotides (Table 1). RT-PCR was carried out in a 50 μl reaction volume with the one-step system Titanium One-Step RT-PCR kit (Clontech, Palo Alto, CA, USA) according to the manufacturer’s instructions. RT was performed for 1 h at 50 °C and PCR for 35 cycles at specific annealing temperatures for each pair of primers as described in Table 1. Transcripts were electrophoresed through a 2% agarose gel and visualized by ethidium bromide staining of the gel.

Table 1 Sequences and annealing temperatures for oligonucleotides used in RT-PCR experiments

<table>
<thead>
<tr>
<th>Gene</th>
<th>Upper primer (5’ – 3’)</th>
<th>Down primer (5’ – 3’)</th>
<th>Annealing temperature (°C)</th>
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<tr>
<td>ERα</td>
<td>GGCCTCTTCTCAAGAGAATTTTC</td>
<td>TCTGGCGCCTTTGTTTTCACATT</td>
<td>58</td>
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<tr>
<td>ERβ</td>
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<td>TTGCCGCCGTTTATCGATTG</td>
<td>58</td>
</tr>
<tr>
<td>EGF</td>
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<td>60</td>
</tr>
<tr>
<td>EGFr</td>
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<td>CAGAGGAGAAGATAGTTGGTGA</td>
<td>56</td>
</tr>
<tr>
<td>IGF-I</td>
<td>GGACCGGGACGCCTCCTGGCAGG</td>
<td>GCGAGCTGACTTGGCAGGCTTG</td>
<td>60</td>
</tr>
<tr>
<td>IGF-Ir</td>
<td>CCACACTAAGCGCCAGAGAAG</td>
<td>ATCGATGCAGTACAACTGTA</td>
<td>56</td>
</tr>
</tbody>
</table>

Oestrogen binding studies
Colon cancer cells were plated in 24-well plates in the appropriate growth media. After 24 h, cells were plated in serum- and phenol red-free culture media and maintained overnight. Cells were then replaced in serum- and phenol red-free media supplemented with 10 mM HEPES, pH 7.4, 0.1% ethanol, 0.1% BSA (binding buffer, BB) and incubated with increasing concentrations (1–10 pM) of [³H]17βE2 in the absence or presence of a 500-fold molar excess of unlabelled 17βE2. After 1 h incubation at 37°C, cells were washed three times with BB, lysed with 1 M NaOH, and neutralized with 1 M HCl. The radioactivity present in the cellular lysate was measured in a β-counter. Each experiment was carried out in triplicate and binding data were analysed by Scatchard analysis.

Cell morphology and doubling time
General morphological and growth characteristics of transfected and untransfected HCT8 cells were studied by light microscopy and the evaluation of doubling time. For these studies cultured cells were used between the 29th and 31st passage. Aliquots of 5 × 10⁵ cells were cultured in 60-mm dishes in RPMI supplemented with 10% FCS with or without 200 μg G418/ml for seven days and then colonies were photographed. Doubling time was evaluated by culturing 10⁷ cells in 6-well plates in RPMI supplemented with 10% FCS with or without 200 μg G418/ml. Cells were counted every day, after trypsinization, for a seven-day period. Cell counts on triplicate cultures were performed using a Coulter counter.

Luciferase assay
After transient transfection with the pEREtkLUC reporter plasmid and pBLUE-TOPO, untransfected or ERβ-transfected HCT8 cells were starved in phenol red- and FCS-free RPMI medium for 24 h. Then, cells were stimulated in the same medium containing 0.1% ethanol with 10 nM 17βE2, 1 μM ICI 164,384 or both for 24 h. When growth factors were tested, cells were stimulated in the same medium containing 0.1% BSA with EGF (50 ng/ml), IGF-I (20 ng/ml) or the combined agents, with or without 1 μM ICI 164,384. Whole cell extract was obtained with the luciferase assay system (Promega, Madison, WI, USA) and luciferase activity was determined using an LKB luminometer (LKB Instruments, Rockville, MD, USA). Luciferase activity was normalized to β-galactosidase expression.
activity, measured by a β-gal assay kit (Invitrogen, Life Technologies, Inc.), and to total protein concentration.

**Cell growth and viability**

Cells were plated on 6-well plates at a density of approximately $8 \times 10^4$ cells/well in complete medium. After 24 h, cells were stimulated with different concentrations of 17βE2 (0.1–100 nM), raloxifene, tamoxifen or ICI 164,384 (100 nM or 1 μM), IGF-I (20 or 10 ng/ml) or EGF (50 or 25 ng/ml) in phenol red-free medium supplemented with 1% charcoal-stripped FCS and 0.1% ethanol in the case of steroid stimuli. Treatment was repeated after 3 days and cells were detached with trypsin/EDTA after an additional 3 days. Cell number was evaluated by a Coulter counter in quadruplicate samples. On day 6 cells were also counted by the Trypan blue dye-exclusion test for evaluation of viability. Alternatively, cell proliferation was evaluated by use of an 3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide (MTT) assay performed with CellTiter 96 Aqueous One solution cell proliferation assay (Promega), a colorimetric method for the determination of the number of viable cells in proliferation or cytotoxicity assays. The assay was performed according to the manufacturer's instructions. Briefly, 5 × 10^5 cells/well were cultured in 96-well plates in complete medium with and without stimulants for one week. CellTiter solution (20 μl) was added to each well in 100 μl culture medium and the plate incubated for 1–4 h at 37°C in a humidified atmosphere, 5% CO2 in air. The amount of soluble formazan produced by cellular reduction of MTT was determined, reading the absorbance at 490 nm.

**Chromosome ladder**

Apoptosis was evaluated by lysing 5 × 10^6 cells cultured for one week in appropriate media, and visualizing the 180 bp DNA fragmentation on 2% agarose gel using ApopLadder Ex kit (Takara Bio, Inc., Otsu, Shiga, Japan) according to the manufacturer’s instructions.

**Western blotting**

In experiments to determine the effect of EGF and IGF-I stimulation on ERK phosphorylation, cells were pre-incubated with the MAP kinase inhibitor PD-98059 (25 μM; Sigma, Basel, Switzerland) for 30 min and then stimulated with EGF (25 or 50 ng/ml) or IGF-I (10 or 20 ng/ml) for 15 min. Cytoplasmic and nuclear proteins were extracted from cells with an appropriate volume of M-PER mammalian protein extraction reagent (Pierce, Rockford, IL, USA), with the addition of proteases inhibitors (Complete, Mini from Roche, Basel, Switzerland) and phosphatase inhibitors (phosphatase inhibitor cocktail 1 and 2 from Sigma, USA), according to the manufacturer’s instructions (protein extraction buffer). Proteins (25 μg) were subjected to SDS-PAGE on a 10% (cyclin E, ERβ), 12% (cyclin D1, cdk2, cdk4, ERK1/2 and phospho-ERK1/2) or a 15% (p21CIP1 and p27KIP1) polyacrylamide gel and electrotransferred onto nitrocellulose membranes. After blotting, membranes were blocked with 1% Blocking reagent (BM Chemiluminescence Bloting System, Roche) when probed with monoclonal anti-ERβ N-19 (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) or with SuperBlock blocking buffer (Pierce) for anti-p27 (F-8), anti-p21 (F-5), anti-cyclin D1 (DCS-6), anti-cyclin E (M-20), anti-cdk2 (D-12), anti-cdk4 (H-303), anti-β-actin (C-2), anti-ERK1/2 and anti-phospho-ERK1(Thr202/Tyr204)/ERK2(Thr185/Tyr187) antibodies. All primary antibodies were purchased from Santa Cruz Biotechnology Inc, except for anti ERK1/2 antibodies which were purchased from Sigma (Switzerland). Incubation with primary antibodies was performed for 1 h under gentle agitation at room temperature in the appropriate blocking buffer with different Tween-20 concentrations depending on the antibody. Application of the horseradish peroxidase-conjugated secondary antibody (1:1000), anti-mouse IgG, anti-rabbit IgG or anti-goat IgG (Sigma, Switzerland), was carried out for 45 min at room temperature and subsequent signal detection, using enzyme-linked chemiluminescence (Supersignal chemiluminescent substrate' from Pierce), was performed according to the manufacturer's instructions. Membranes were then stripped in Restore Western Blot stripping buffer (Pierce) for 10 min at 37°C and re-probed with monoclonal anti β-actin for quantitative analysis. This was performed by acquiring images from X-ray films with a Kodak (Rochester, NY, USA) imaging system to obtain band intensities. In Western blot with anti-phospho-ERK1(pThr202/pTyr204)/ERK2(pThr185/pTyr187) antibody, after stripping, the membrane was re-incubated with secondary antibody and the presence of residual signal was detected by chemiluminescence as previously described. Then the membrane was re-probed with anti-ERK1/2 antibody as described.

**Immunoprecipitation and kinase assay**

For immunoprecipitation and H1 kinase assays, 400 μg cell extracts were used per immunoprecipitation with antibody to cyclin E (2 μg). The antibody binding reaction was carried out in protein extraction at
4 °C overnight. The protein/antibody mixture was incubated with Protein A/G PLUS-Agarose beads (Santa Cruz Biotechnology, Inc.) for 1 h and then washed twice with protein extraction buffer and twice with histone wash buffer (50 nM Tris–HCl pH 7.4, 30 μM ATP, 10 mM MgCl₂, 1 nM dithiothreitol). Immunoprecipitates were then analysed by either Western blot analysis or H1 kinase assay. For Western blot analysis, Protein A/G PLUS-Agarose beads from the immunoprecipitation were boiled in Laemmli sample buffer (Bio-Rad Laboratories) and proteins were separated on a 12% SDS-PAGE gel. Western blotting was performed with monoclonal anti-CDK2 as previously described. For the kinase assay, immunoprecipitates were incubated with histone assay solution (200 μg/ml histone H1, 100 μCi/ml [³²P]ATP in histone wash buffer) in a final volume of 50 μl at 30 °C for 20 min. H1 kinase reactions were analysed on a 12% SDS-PAGE gel. The gel was then dried and exposed to X-ray film.

Flow cytometric analysis
For flow cytometric analysis, HCT8, HCT8pSV2neo, and all transfectant clones were harvested in saline-EDTA, fixed in cold 70% ethanol, and stored at −20 °C for 30 min. Fixed cells were subsequently stained with propidium iodide (Sigma) for 30 min in the dark and analysed in a FACScan flow cytometer (Becton Dickinson Labware, Lincoln Park, NJ, USA) with an excitation wavelength of 488 nm. The resulting histograms were analysed by MODFIT software (Becton Dickinson Labware) for cell distribution in the cell cycle phase. The proliferative fraction in the culture was determined as cells in the S and G2/M phases of the cell cycle based on DNA content.

Statistics
Data were expressed as means ± S.D. Statistical differences among mean values were analysed using Scheffe’s test (Statistica 5.1; Statsoft Inc, Tulsa, OK, USA).

Results
Human colon cancer cell model stably expressing ERβ
Among twelve clones resistant to G418, the one that showed maximal expression of ERβ, as verified by RT-PCR, quantitative Western blotting and binding experiments (about 35 000 binding sites compared with 3500 detected in wild type HCT8), was named HCT8-β8 and used for subsequent experiments (Fig. 1A–C). However, experiments regarding key features of the cellular model such as growth curves and morphological analysis have been performed even on clones expressing intermediate levels of ERβ protein to confirm that the HCT8-β8 phenotype was really due to the over-expression of the receptor (data not shown).

Functionality of the over-expressed ERβ protein
As shown in Fig. 1D, 17βE2 induced a 10-fold increase in luciferase expression compared with that of control cells. On the other hand, ICI 164,384 alone did not affect transactivation, but almost abolished the effect of 17βE2. As shown in the inset panel of the figure, endogenous ERβ expression was not detectable by this method.

Effect of liganded and unliganded over-expressed ERβ on cell proliferation
In basal conditions, proliferation of HCT8-β8 cells was significantly (~fivefold) lower than for HCT8pSV2neo cells. Exposure to 17βE2 (0.1–100 nM) produced a biphasic, although not significant, stimulatory effect on HCT8pSV2neo cell growth, while 100 nM 17βE2 significantly inhibited HCT8-β8 cell proliferation (Fig. 2A). 17βE2 at doses of 0.1–100 nM did not influence cell viability of HCT8-β8 and HCT8pSV2neo cells (~95% vs ~90% viability respectively). We tested the effect of two doses (100 nM and 1 μM) of the antagonists ICI 164,384, tamoxifen and raloxifene on HCT8 cell growth in the presence or absence of over-expressed ERβ. As reported in Fig. 2C only the 1 μM concentration of all chemicals was shown to be effective on HCT8pSV2neo and on HCT8-β8 cell proliferation by significantly inhibiting cell growth, but the percentage reduction induced by all three chemicals was similar in HCT8pSV2neo and HCT8-β8 while the different absolute cell numbers reached both in control and stimulated cells was lower in transfected cells.

Effect of ERβ over-expression on cell morphology
Morphological and growth characteristics of the HCT8-β8 cell line compared with HCT8pSV2neo cells were evaluated by contrast phase microscopy and by determination of lag and doubling times. HCT8pSV2neo cells grew in multilayered large colonies, assuming a spindle-shaped morphology while HCT8-β8 cells showed a polygonal shape growing in small colonies and never in multilayers (Fig. 3A). These observations were confirmed by measuring lag and doubling times of the two cell populations. The lag
times of HCT8-β8 cells and of HCT8pSV2neo cells were respectively 58 h and 38 h, and the doubling times were 59 h and 31 h respectively (Fig. 3B). Measurement of the same parameters in the HCT8-β6 cell line, a clone showing a twofold increase in ERβ overexpression (Fig. 1B), revealed intermediate values (lag time: 48 h and doubling time: 39 h).

**Effect of EGF and IGF-I on ERβ transactivation and on cell proliferation**

In order to verify potential actions of growth factors on over-expressed ERβ protein, a luciferase transactivation assay was performed on HCT8-β8 cells transiently co-transfected with the pEREtkLUC plasmid or with the internal control plasmid pBLUE-TOPO. After 24 h in phenol red- and FCS-free RPMI, HCT8-β8 cells were treated with vehicle (control), 10 nM 17βE2, 1 μM ICI 164,384, 50 ng EGF/ml, 20 ng IGF-I/ml, and with the combined agents for 24 h. EGF and IGF-I induced respectively a 2.5-fold and a 4-fold increase in luciferase expression compared with that of controls (Fig. 4A). The two combined growth factors induced an even higher expression of luciferase (6-fold) and this effect was only partially reversed by the addition of ICI 164,384 (Fig. 4A), which in turn almost completely abolished the effect of 17βE2 (Fig. 1D).

As EGF and IGF-I proved to be potent activators of ERβ in this cell model, we investigated if the anti-proliferative effect of the receptor could be associated with its stimulation by these growth factors by analysing their effects on untransfected and HCT8-β8 cell lines. In HCT8pSV2neo neither IGF-I (10 and
Figure 2 Effect of 17βE2 and of different ER antagonists on colon cancer cell proliferation. (A) Effect of 17βE2 on growth of HCT8pSV2neo and HCT8-β8 cells. Cells were treated with vehicle or 17βE2 at different concentrations (0.01, 1 and 100 nM) in phenol red-free medium supplemented with 1% charcoal-stripped FCS. Treatments were repeated after 3 days in fresh medium. After 6 days, cell number was evaluated using a Coulter counter. Results are expressed as the mean value of four separate experiments carried out in triplicate and are expressed as relative cell growth in the left panel (percentage of the control, untreated HCT8pSV2neo cells) and as cell number/well in the right panel. * P<0.05 vs untreated cells. (B) Effects of 17βE2 on HCT8, HCT8pSV2neo and HCT8-β8 cell growth in the absence or presence of ICI 164,384 (ICI). Cells were treated with vehicle, 17βE2 (100 nM), ICI (1 μM) or with the combined treatments in phenol red free-medium supplemented with 1% charcoal-stripped FCS. After 3 days, medium was replaced with fresh medium containing the appropriate stimulants and, on day 6, cell number was evaluated using a Coulter counter. Results are expressed as the mean value ± s.d. of four separate experiments carried out in triplicate. * P<0.05 vs untreated cells. (C) Cells were treated with vehicle, ICI 164,384 (I), tamoxifen (T) or raloxifene (R) at two different concentrations (100 nM and 1 μM) in phenol red-free medium supplemented with 1% charcoal-stripped FCS. Treatments were repeated after 3 days in fresh medium. After 6 days cell number was evaluated by the MTT test. Results are expressed as the mean value ± s.d. of four separate experiments carried out in triplicate. * P<0.05 vs untreated cells.
20 ng/ml) nor EGF (25 and 50 ng/ml) had significant effects on cell proliferation (Fig. 4B).

We analysed EGF, EGFr, IGF-I and IGF-Ir mRNA expression in HCT8pSV2neo and HCT8-β8 cell lines by RT-PCR and showed that both cell lines express the corresponding mRNA (Fig. 5A). Moreover, EGF and IGF-I stimulate their classical signal transduction cascade through activation of MAP kinases as demonstrated for HCT8-β8 cells in Fig. 5B where a significant increase in phospho-ERK1 and phospho-ERK2 (upper panel) proteins is observed in respect to their total content (lower panel) after cell stimulation with different doses of EGF and IGF-I.

**Effect of ERβ over-expression on apoptosis**

We performed chromosome ladder analysis on HCT8pSV2neo cells and on three clones expressing different levels of transfected ERβ protein in order to investigate the role of apoptosis in mediating the anti-proliferative effect of the over-expression of the receptor in this cell model. No evidence of 180 bp DNA fragmentation was present in untransfected cells or in three ERβ-over-expressing clones (data not shown).

**Unliganded ERβ modulates some cell cycle components causing HCT8 cells arrest in G1-S**

Western blot analysis of cdk4, cyclin D1, cdk2, cyclin E and of their direct inhibitors p21CIP1 and p27KIP1 was performed on cell extracts of HCT8pSV2neo and of HCT8-β8 and -β6 clones showing seven- and twofold higher ERβ expression levels respectively compared with HCT8pSV2neo cells (Fig. 1B). Expression of cdk4 was up-regulated in both HCT8-β6 and HCT8-β8 clones (Fig. 6A). Conversely, cyclin D1, the major partner in the active cdk4 complex, was unaffected by ERβ over-expression (Fig. 6B).

Regarding the cdk2 complex, the other main cell cycle promoter complex, ERβ over-expression did not
affect expression level of the kinase protein (Fig. 6C) but drastically (6 times) reduced cyclin E protein (Fig. 6D) in the HCT8-β6 clone. Finally, ERβ over-expression did not affect p27KIP1 expression level (Fig. 6E) but doubled that of p21CIP1 (Fig. 6F), which is a common inhibitor of both active cdk4 and cdk2 complexes.

Activity of the cdk2/cyclin E complexes immunoprecipitated from cells was markedly decreased in the HCT8-β clone relative to those from the HCT8pSV2neo, causing a decrease of cells in the proliferative fraction S+G2/M. Immunoprecipitates from HCT8-β6 cells showed an intermediate level of activity corresponding to an intermediate fraction of non-resting cells (Fig. 7).

Discussion
The presence of functional oestrogen receptors has been described in normal and cancerous colon tissues...
as well as in various colon cancer cell lines supporting a physiological role of oestrogens in the biology of colonic mucosa. Expression of the sole ERβ subtype has been demonstrated in HCT8, LoVo (Fiorelli et al., 1999), DLD-1 (Fiorelli et al., 1999, Nakayama et al., 2000), HCT116 (Fiorelli et al., 1999, Arai et al., 2000), Colo320, SW480 (Arai et al., 2000), HT29 (Arai et al., 2000, Campbell-Thompson et al., 2001), SW1116, SW48, and T84 (Campbell-Thompson et al., 2001) cell lines. Moreover, studies from different laboratories on paired samples of normal and neoplastic mucosa revealed the presence of higher levels of ERβ compared with ERα in normal mucosa and a selective loss of ERβ protein in malignant colon cancer (Foley et al., 2000, Campbell-Thompson et al., 2001). These findings, together with a lower colon cancer incidence and mortality in females (Levi et al., 1991, deCosse et al., 1993, Ries et al. 2000) and a reduced cancer risk in postmenopausal women taking HRT (Calle et al. 1995, Campbell et al., 1999, Grodstein et al. 1998, 1999, Nanda et al. 1999, Chlebowski et al. 2004), suggested a role for oestrogens in colon tumorigenesis through an effect on ERβ.

Moreover, in vitro studies on the effects of oestrogens on proliferation of human colon cancer cell lines produced conflicting results (Fiorelli et al. 1999, Arai et al. 2000). These discrepancies could be explained by the low level of expression of the two ERs and/or by their different transcriptional activities in different cell models. Over-expression of ERβ in colon cancer cells expressing solely ERβ was, therefore, selected as a possible strategy to elucidate the action of ERβ on in vitro growth of colon cancer cells. In agreement with previous findings in other cellular models (Lazennec et al. 2001, Ström et al. 2004, Paruthiyil et al. 2004), transfected ERβ protein appears to be fully functional with stimulation of the estrogen responsive element (ERE)-containing reporter gene by 17βE2 and inhibition by the pure antioestrogen ICI 164,384.

Over-expression of ERβ receptor was able to induce a fivefold reduction in HCT8-β8 cell proliferation. Moreover, HCT8-β8 cells exhibited morphological features different from the parent cell line, with a flattened polygonal shape, lack of growth in multilayers and resistance to trypsin-induced detachment. These findings clearly showed that ERβ plays an important role in colon cancer cell proliferation and morphological features and that its action is almost completely disengaged from its binding to oestrogens. In agreement with this interpretation, cells with an

Figure 5 Presence of an EGF and IGF-I stimulating autocrine loop in HCT8 cells. (A) Expression of endogenous EGF, EGFr, IGF-I and IGF-Ir mRNAs in HCT8 (left lane), HCT8-β8 (central lane) and control tissues (right lane) analysed through RT-PCR. Control tissues are normal colonic mucosa for EGF, fetal liver for EGFr and IGF-I, and lymphocytes for IGF-Ir. (B) EGF and IGF-I induction of ERK1/2 phosphorylation in HCT8-β8 cells. Cells were pre-incubated with the MAP kinase inhibitor PD-98059 for 30 min and then stimulated with EGF (25 or 50 ng/ml) or IGF-I (10 or 20 ng/ml) for 15 min. Phosphorylated ERK1 and ERK2 (upper panel) were analysed by Western blotting versus total amount of ERK1/2 (lower panel).
**Figure 6** Western blot analysis of the effects of ERβ over-expression on cell cycle components expression: (A) cdk4, (B) cyclin D1, (C) cdk2, (D) cyclin E, (E) p27KIP1 and (F) p21CIP1 expression in HCT8pSV2neo (left lane), HCT8-β6 (middle lane) and HCT8-β8 (right lane) cell clones. Expression of β-actin in each sample is shown in the lower panel of A–F. When protein modulation is present the quantitative analysis of the expression of the corresponding protein, normalized against β-actin, is reported close to each Western-blotting panel. Results are expressed as percentage of control (HCT8pSV2neo cells) and are representative for one additional experiment.
intermediate ERβ expression level, between that of untransfected cells and of the HCT8-β8 clone, showed intermediate proliferative and morphological features. Our observation is in agreement with what observed by Lazennec et al. (2001) in breast cancer cells (MDA-MB-231), where ERβ inhibition of cell proliferation was ligand-independent, whereas ERβ was able to regulate reporter genes in a ligand-dependent manner. Moreover, these results reinforce the interpretation of a role for ERβ as a negative regulator of colon tumour growth based on reduced ERβ expression in normal compared with cancerous colonic mucosa that, conversely, showed a greater abundance of ERα mRNA (Enmark et al. 1997, Moore et al. 1998, Foley et al. 2000, Campbell-Thompson et al. 2001, Weyant et al. 2001). Altogether, these observations point to ERβ expression in colon cancer as a potential prognostic indicator of tumour progression but this requires confirmation in prospective clinical trials.

An important question to be answered is the mechanism through which over-expression of ERβ affects colon cancer cell proliferation by a ligand-independent pathway. Indeed, steroid receptors could be activated in the absence of their ligands, depending on the cell type and on promoters and activators (Weigel & Zhang 1998, Tremblay & Giguère 2001). In human colon cancer cells, ERβ is modulated by EGF and by IGF-I depending on endogenous ERβ expression with a cumulative effect of these growth factors exceeding that of 17βE2. ERβ activation by these two growth factors was only partially abolished by treatment with ICI 164,384. This result is in accordance with previous observations on the different effect of the antioestrogenic molecule on ERα and ERβ (Pace et al. 1997, Van Den Bemd et al. 1999).

The assumption that growth factors can modulate proliferation regulating ERβ activity was not supported by the results obtained on the effects of the two growth factors on cell proliferation in untransfected and HCT8-β8 cells, where no statistical differences emerged. However, effects of exogenous doses of EGF and IGF-I used in proliferation assays could be masked by expression of endogenous growth factors. Indeed, both HCT8 cells and ERβ-over-expressing derived clones produce both EGF and IGF-I and their cognate receptor mRNAs, becoming, therefore, prone to an autocrine loop of these growth factors (O’dwyer & Benson 2002, Wang & Sun 2002). Moreover EGF and IGF-I stimulate MAP kinase causing ERK1/2 phosphorylation and then altering directly the cell cycle. These results can explain both ligand-independent ERβ activation (Tremblay & Giguère 2001) and perhaps the limited effect exerted by 17βE2 on cell proliferation, because ERβ is constitutively activated through ligand-independent mechanisms. ERβ expression could, therefore, be interpreted as a regulator of the growth stimulatory or inhibitory actions of molecules (Platet et al. 2000), such as EGF (Lopez et al. 1995, Mendelsohn 1997, 2000) and IGF-I (Giovannucci 2001), on cancer-derived cell proliferation. In fact, possible proliferative effects by these growth factors on cancer cell lines could be reduced by

Figure 7 CycinE/ck2 complex kinase activity on histone H1 substrate. Total cell lysates from control cells and from two clones expressing an intermediate (HCT8-β6) or the maximal quantity (HCT8-β8) of ERβ protein were immunoprecipitated (IP) with anti-cyclin E antibody and immunoprecipitates were subjected to Western blot analysis (W) with anti-ck2 antibody (A) or used for H1 kinase assay (B). The proliferative fraction in the culture is reported as S + G2/M. The results are expressed as the mean of three experiments ± s.d. * P<0.01 vs HCT8pSV2neo cells.
highly expressed ERβ protein, while in cells expressing low levels of ERβ stimulatory actions of growth factor could become more dominant.

Another possible mechanism through which ERβ over-expression could inhibit colon cancer cell proliferation is by an increase in programmed cell death. The presence of the chromosome ladder phenomenon has already been described in the HCT8 cell line (Picariello et al. 1998). However, the absence of 180 bp DNA fragmentation in untransfected cells and in three clones obtained from transfected cells did not support this hypothesis. This is in good agreement with the overall observations regarding the morphological features of ERβ over-expressing cells, exhibiting reduced cell growth without changes in cell death rate.

Finally, altered expression of cell cycle regulators and the subsequent deregulation of the cell cycle may be important steps in tumour promotion and are the most consistently found events in human malignancies including colorectal cancer (Hartwell & Kastan 1994). Changes in cyclin D1, cyclin E, p21CIP1, and p27KIP1 expression have been described in various stages of colorectal cancer in animal and human tissues and in various cell lines (Sutter et al. 1997, Yamamoto et al. 1999, Hur et al. 2000). Moreover, c-Myc, cyclin D1, p21CIP1, and the cyclin E/cdk2 complex have been identified as central components of oestrogen regulation of cell cycle progression and hence as potential downstream targets that contribute to the role of oestrogen-induced carcinogenesis (reviewed in Foster et al. 2001, Doisneau-Sixou et al. 2003). However, most of the information regarding ERs and cell cycle regulation refers to ERα and derives from studies on breast cancer cell lines and engineered cell models (reviewed in McDonnel & Norris 2002). Moreover, several human tissues co-express both ERs and this is a confounding factor in interpreting the effects of their agonist and antagonist ligands. In HCT8 cells, ERβ over-expression induced an increase in cdk4 protein of up to fourfold. This finding is in apparent contradiction with data on cell proliferation as active cdk4-complex is a central key regulator of DNA synthesis initiation (Weinberg 1995). However, in HCT8 cells this does not seem to represent a mitogenic stimulus per se, since it is not associated with a contemporary increase in cyclin D1, the essential modulator for entry into the proliferative stage of the cell cycle (Prall et al. 1998). Indeed, in another cell system a similar effect was observed on cyclin D1 but not on cdk4 contemporary to the inhibition of cell growth (Ström et al. 2004). More importantly, ERβ induced a 4-fold reduction and a 4.5-fold induction in cyclin E and p21CIP1 expression respectively. Cyclin E is a critical component of the active cdk2 complex which drives the passage from G1 into S phase, and over-expression is usually present in a significant fraction of human colon carcinomas (Sutter et al. 1997). In the T47D breast cancer cell line, over-expression of ERβ induced a decrease of both cyclins E and A in a ligand-dependent manner (Ström et al. 2004). This is clearly different from what was observed in HCT8 cells where ERβ action on cyclin E regulation is ligand independent. Moreover, the use of a model that lacks ERα expression makes it possible to state that cyclin E regulation is due to a direct action of ERβ repressing the activity of the cyclin E promoter. Furthermore, induction of p21CIP1 expression represents an additional mechanism through which ERβ controls the colon cancer cell cycle, as the protein is essential in mammary cancer cells for growth and cell cycle arrest (Carroll et al. 2002, Licznar et al. 2003). Indeed, alteration in the expression of these proteins is sufficient to induce a G1-S cell cycle arrest in this cell model. These data clearly demonstrate the existence of an ERβ-induced regulation of cell cycle components, supporting its anti-proliferative effect, and this action seems to be dose dependent. In fact, a doubled quantity of ERβ protein is sufficient to induce cdk4 expression whereas for cyclin E and p21CIP1 induction and, most importantly, to provide a clear effect on the cell cycle, a quadruple quantity is needed. A similar anti-proliferative effect was observed in breast cancer MCF-7 cells infected with adenovirus-ERβ. In this cell model ERβ inhibited cell proliferation and tumour formation by causing G2 cell cycle arrest mainly due to inhibition of cyclin A and induction of p21CIP1, p27KIP1 and c-myc mRNAs expression. These effects were almost linked due to a ligand-independent action of ERβ as 17βE2 produced only a modest G2 arrest enhancement (Paruthiyil et al. 2004).

In conclusion, ERβ plays an anti-proliferative role in colon cancer cells in a ligand-independent fashion, directly regulating some cell cycle components, namely cyclin E and p21CIP1 and then causing a G1 cell cycle arrest. Altogether, these observations point to ERβ expression in colon cancer as a potential prognostic indicator of tumour progression and a potential target for chemotherapy with ERβ-selective oestrogenic molecules.

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