Tumour-suppressive effect of oestrogen receptor β in colorectal cancer patients, colon cancer cells, and a zebrafish model

Geriolda Topi1, Shakti Ranjan Satapathy1, Pujarini Dash1, Syrina Fred Mehrabi1, Roy Ehmström2, Roger Olsson3, Marie-Louise Lydrup4 and Anita Sjölander1*

1 Division of Cell Pathology, Department of Translational Medicine, Lund University, Skåne University Hospital, Malmö, Sweden
2 Division of Pathology, Department of Translational Medicine, Lund University, Skåne University Hospital, Malmö, Sweden
3 The Chemical Biology and Therapeutics Division, Department of Experimental Medical Science, Lund University, Lund, Sweden
4 Division of Surgery, Department of Clinical Sciences, Lund University, Skåne University Hospital, Malmö, Sweden

Abstract

Oestrogen receptor β (ERβ) has been suggested to have anti-proliferative and anti-tumour effects in breast and prostate cancer cells, but other studies have indicated its tumour-promoting effects. Understanding the complex effects of this receptor in different contexts requires further study. We reported that high ERβ expression is independently associated with improved prognosis in female colorectal cancer (CRC) patients. Herein, we investigated the possible anti-tumour effect of ERβ and its selective agonist. CRC patients with high ERβ expression had significantly higher levels of membrane-associated β-catenin, cysteinyi leukotriene receptor 2 (CysLT2R), and 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which have anti-tumour effects, but lower levels of nuclear β-catenin, cysteinyi leukotriene receptor 1 (CysLT1R), and cyclooxygenase-2 (COX-2), which have tumour-promoting effects. These interesting findings were further supported by two different publicly available CRC mRNA datasets that showed a significant positive correlation between ERβ expression and 15-PGDH and CysLT2R expression and a negative correlation between ERβ expression and β-catenin, CysLT1R, and COX-2 expression. We next evaluated ERβ expression in three different colon cancer mouse models; ERβ expression was negatively correlated with tumourigenesis. Furthermore, treatment with the ERβ-agonist ERB-041 reduced CysLT1R, active β-catenin, and COX-2 levels but increased phospho-β-catenin, CysLT2R, and 15-PGDH levels in HCT-116, Caco-2, and SW-480 colon cancer cells compared to vehicle-treated cells. Interestingly, ERB-041-treated cells showed significantly decreased migration, survival, and colonosphere formation and increased apoptotic activity, as indicated by increased CASPASE-3 and apoptotic blebs. Finally, patients with low ERβ expression had significantly more distant metastasis at the time of diagnosis than patients with high ERβ expression. Therefore, we studied the effects of ERB-041-treated colon cancer cells in a zebrafish xenograft model. We found significantly less distant metastasis of ERB-041-treated cells compared to vehicle-treated cells. These results further support ERβ’s anti-tumour role in CRC and the possible use of its agonist in CRC patients.

Keywords: ERβ; colorectal cancer; CysLT1R; CysLT2R; metastasis

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Introduction

The link between colorectal cancer (CRC) and inflammation is now well established, and patients with inflammatory bowel disease have a two- to five-fold increased risk of developing CRC compared with the general population [1]. Pro-inflammatory lipid mediators, such as leukotrienes (LTs) and prostaglandins (PGs), play an important role in the CRC microenvironment and development and progression [2]. Cyclooxygenase-2 (COX-2), the enzyme responsible for PG production, is upregulated in CRC, and the use of COX-2 inhibitors, such as nonsteroidal anti-inflammatory drugs (NSAIDs), has been demonstrated to reduce the risk of CRC [3]. Furthermore, COX-2-mediated PGE2 synthesis is reported to have potent pro-tumour effects [4]. Additionally, downregulation of the cytoplasmic enzyme 15-hydroxyprostaglandin dehydrogenase (15-PGDH), which plays a key role in PGE2 degradation, has been reported in CRC [5]. The upregulation of 15-PGDH in CRC is thought to have tumour-suppressive activity by promoting cell differentiation, thus inhibiting improving disease progression [6–8].

Cysteinyi leukotrienes (CysLTs) are among the most studied LTs in CRC and act via two different G
protein-coupled receptors, CysLT1R and CysLT2R [9]. Patients with low levels of CysLT1R and high levels of CysLT2R have a better survival than those with high CysLT1R levels and low CysLT2R levels [10]. In addition, a recent report showed that upregulation of the tumour suppressor 15-PGDH in human colon cancer cells could be mediated via CysLT2R [11].

The occurrence of CRC is mostly sporadic. Somatic loss of the function of the tumour suppressor gene adenomatous polyposis coli (APC), leading to an abnormal Wnt/β-catenin pathway, which is crucial for the normal function of intestinal epithelial cells, occurs in approximately 85% of sporadic CRC cases [12,13]. Furthermore, sex differences in CRC clinical patterns and prognosis have been reported [14]. Previous evidence suggests a protective effect of oestrogen in CRC mediated via oestrogen receptor beta (ERβ) [15,16]. We recently reported that high ERβ expression in cancer tissue is associated with a better overall and disease-free survival (DFS) in female CRC patients [17].

Materials and methods

Ethical statement

All participating patients gave their written informed consent, and sample collection was made with the approval of the regional research ethics board of Lund University, Sweden (Dnr 3/2006). The studies were performed in compliance with the 1975 Declaration of Helsinki, as revised in 1983. All mouse models are approved by the regional ethical committee for animal research at Lund University, who approved the animal experiments (M-262-12 and M-263-12).

Patients, tissue microarray (TMA), and immunohistochemistry (IHC)

Female patients (n = 314) who underwent surgery for primary CRC between 1 January 2008 and 30 June 2012 were randomly selected for the study. Details on the study design, patient follow-up, and data collection are provided elsewhere [17]. The study was approved by the Ethical Committee at Lund University. The tumour samples were incorporated into a TMA; sections were immunostained for ERβ; and ERβ expression was scored following protocols described previously [17]. Staining immunoreactivity was assessed by two blinded independent investigators (GT and RE) using the immunoreactive score (IRS) as previously described [17]. A list of the antibodies used for IHC can be found in supplementary material, Supplementary materials and methods, and negative IgG control staining is shown in supplementary material, Figure S1A.

Cell lines

SW-480, HCT-116, HT-29, and Caco-2 human colon cancer cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured as described previously [11,18]. Treatment was performed with the ERβ-selective agonist ERB-041 (Sigma-Aldrich, St Louis, MO, USA) at 60 nM for 24 or 72 h, and the ERβ-selective antagonist PHTPP (Tocris Bioscience, Bristol, UK) at 1 μM for 30 min before ERB-041 stimulation, depending on the experiment.

siRNA transfection

ERβ-specific siRNA transfection was performed as described for siRNA transfection in a previous report [11]. siRNA: ERβ (SC-35325), control-A (SC-37007), -B (SC-44230), and -C (SC-44231) were from Santa Cruz Biotechnology (Dallas, TX, USA).

Reverse transcription-quantitative PCR (RT-qPCR)

Total RNA was isolated from cells using an RNeasy Mini Kit (Qiagen, Hilden, Germany), and first-strand cDNA synthesis was completed using a cDNA synthesis kit (Invitrogen, Carlsbad, CA, USA), as described previously [11]. The TaqMan probes used are shown in supplementary material, Supplementary materials and methods. Fold-changes were quantified with the 2−ΔΔCt method using MxPro software (Agilent Technologies, Santa Clara, CA, USA).

Migration assay

Untreated or ERB-041-treated cells with or without 1 μM PHTPP pretreatment for 30 min were seeded in the upper chambers of 24-well migration chambers in serum-free medium and allowed to migrate for 18 h, as described in supplementary material, Supplementary materials and methods.

Wound healing assay

An insert-based wound healing assay (Culture-Insert 2-Well, Ibidi, Martinsried, Germany) was performed over 24 h. After insert removal, cells were treated with ERB-041 with or without 1 μM PHTPP pretreatment for 30 min in 1.5% FBS medium.

Clonogenic cell survival assay

To assess the effect of ERB-041 on the colony-forming ability of colon cancer cells, a clonogenic cell survival assay was performed as described previously [19].

Cell survival assay

Cells were seeded into 96-well plates in medium containing 10% FBS. A WST-1-based cell survival assay (WST-1 cell proliferation reagent; Abcam, Cambridge, UK) was performed following the manufacturer’s instructions on cells with or without ERB-041 treatment for 72 h.
Colonoscopy formation
Colonospheres were formed from cells with or without ERB-041 treatment, and the formation of apoptotic blebs was detected using a protocol described previously [18].

Doxycycline-inducible CYSLTR2 stable knockdown cell line
Cell lines with doxycycline-inducible stable knockdown of CYSLTR2 were established from HCT-116 cells following a protocol previously described [20]. In brief, HEK293T cells were transfected using a CaPO4 method with plKO.1 plasmid containing shRNA against CYSLTR2 and a puromycin control gene in addition to the helper plasmids pHR 8.92 and 8.93 and PMDG2 (VSV envelope protein). After transfection, the supramolecular containing lentiviral particles was harvested and the viral particles were used to infect HCT-116 cells for 48 h. Viability of cells was monitored post-infection. The GFP-positive populations of HCT-116 cells were sorted by FACS (data not shown), and the efficiency of the knockdown was determined after treatment with 1 μM doxycycline (Dox) for 24–72 h. Prior to ERB-041 stimulation, the cells were cultured in the presence or absence of 1 μM Dox for 48 h.

Immunofluorescence analysis
Immunofluorescence analysis was performed on untreated or ERB-041-treated cells to assess the expression of ERβ, non-phospho (active) β-catenin, total β-catenin, COX-2, dilution 1:500; and 15-PGDH and CASPASE-3, dilution 1:400, as described previously [11].

Western blotting
Immunoblotting was performed as described previously [18]. Protein expression was visualised by enhanced chemiluminescence (ECL; Millipore, Burlington, MA, USA) using the Bio-Rad ChemiDoc™ Image system (Bio-Rad, Hercules, CA, USA). Bio-Rad Image Lab software was used for densitometric analysis. Antibodies used for immunoblotting are listed in supplementary material, Supplementary materials and methods.

Mouse colitis-associated colon cancer (CAC) model, ApcMin/+ mice, and tissues
We evaluated the expression of ERβ in three different CC mouse models: C57BL/6J-ApcMin/+ mice [21] and a CAC model using both wild-type (WT) mice and mice with disruption of the Cysltr1 or Cysltr2 gene on a C57BL/6N background [22]. The experimental design for the CAC model has been described in detail elsewhere [23]. Tissues were stained with anti-ERβ antibody (14C8, 1:100). The negative control is shown in supplementary material, Figure S1B.

Zebrafish xenograft model
The role of ERβ in HT-29 cell metastasis was validated using a zebrafish xenograft model [24]. The Tg(fl/fl: eGFP) zebrafish line (SciLifeLab, Uppsala, Sweden) was maintained and staged according to European animal welfare regulations and standard protocols [24,25]. In brief, cells were treated with or without ERB-041 for 48 h, labelled with Vybrant-DiI, and microinjected into the perivitelline space of an embryo 2 days post-fertilization. Details can be found in supplementary material, Supplementary materials and methods.

Statistical analysis
Continuous variables were compared using the Mann–Whitney U-test or t-test, and categorical variables using the χ2-test or Fisher’s exact test as indicated. Survival curves, adjusted for age and tumour–node–metastasis stage, were generated using the Kaplan–Meier method and differences assessed using a log-rank test. Correlations were assessed using Spearman’s correlation coefficient. Statistical analyses were performed using SPSS version 23.0 (IBM, Chicago, IL, USA) and GraphPad Prism version 7.0a (GraphPad Inc, San Diego, CA, USA). All tests were two-sided, and P values less than 0.05 were considered statistically significant.

Public databases
Publicly available mRNA data from the TCGA colon adenocarcinoma (COAD) database [26] and a CRC cohort with 688 patients [27] were used from the R2 Genomics Analysis and Visualization Platform (https://hgserver1.amc.nl).

Results
Correlation between ERβ expression and CysLT1 and CysLT2 receptor expression in CRC patients
CRC material from 314 patients was previously evaluated for ERβ expression, and patients were grouped into two categories: low ERβ expression and high ERβ expression [17]. Herein, the tissues were evaluated for the expression of CysLT1R and CysLT2R. As shown in Figure 1A, patients with high ERβ expression had lower CysLT1R and higher CysLT2R expression than those with low ERβ expression. Furthermore, patients with high ERβ expression had slightly lower IRSs for CysLT1R than patients with low ERβ expression [mean ± standard error of the mean (SEM) 5.94 ± 0.14 and 6.43 ± 0.12, respectively; p = 0.011; Figure 1B]. On the other hand, the mean IRS for CysLT2R expression in patients with high ERβ expression was significantly higher than that for patients with low ERβ expression (mean ± SEM 6.71 ± 0.14 and 4.81 ± 0.11, respectively; p < 0.0001; Figure 1B). There was a weak negative correlation between ERβ...
and CysLT₁R expression, and a strong positive correlation between ERβ and CysLT₂R expression (Figure 1C).

To validate these findings, we used mRNA gene expression data from the TCGA COAD database [26] and observed a significant positive correlation between ESR2 (ERβ) and CYSLTR2 (CysLT₂R) mRNA levels in 286 CRC patients determined from the public TCGA COAD database. (D) XY scatter plot of ESR2 (ERβ) and CYSLTR2 (CysLT₂R) mRNA levels in 286 CRC patients determined from the public TCGA COAD database. (E, F) Five-year disease-free survival (DFS) curves adjusted for age and TNM stage for subgroups of patients with both ERβ and CysLT₁R (E) or CysLT₂R (F) expression compared by a log-rank test. The last patient group was used as the reference category. Mean ± SEM. *p < 0.05, ***p < 0.001, Mann–Whitney test.

Correlation between ERβ expression and β-catenin, COX-2, and 15-PGDH expression in CRC patients

IHC analysis showed that CRC patients with high ERβ expression had higher levels of membrane β-catenin and 15-PGDH and lower levels of nuclear β-catenin and COX-2 than patients with low ERβ expression (Figure 2A,B). Likewise, a strong positive correlation was observed between ERβ expression and the expression of membrane β-catenin (r = 0.5, p < 0.0001) and 15-PGDH (r = 0.51, p < 0.0001, Figure 2C). In contrast, those with low ERβ expression. This result indicates that even patients who were defined as having a poor prognosis (patients with high CysLT₁R and low CysLT₂R expression) can significantly benefit from increased ERβ expression.
ERβ expression was negatively correlated with nuclear β-catenin and COX-2 expression (r = −0.28 and r = −0.32, respectively; Figure 2C). These findings are supported by public mRNA data, which show a significant negative correlation between ESR2 (ERβ) expression and the expression of both CTNNB1 (β-catenin) and PTGS2 (COX-2), and a significant positive correlation between ESR2 and HPGD (15-PGDH) gene expression (Figure 2D). We also found a stronger correlation in males between ESR2 and HPGD mRNA levels (supplementary material, Table S1). As shown in Figure 2E–G, even patients who were defined as having a poor prognosis (patients with high nuclear β-catenin and COX-2 expression and low 15-PGDH expression) had a significantly better DFS if they had high ERβ expression. These results further highlight the importance of ERβ expression for disease outcome.
Effect of ER\(\beta\) induction on colon cancer cells

To further explore the results in patients, we investigated the effects of ER\(\beta\) signalling on colon cancer cells. First, we determined the levels of endogenous ER\(\beta\) expression in two different colon cancer cell lines, SW-480 and HCT-116 cells. As shown in Figure 3A, both cell lines expressed ESR2 mRNA and ER\(\beta\) protein. Next, we stimulated HCT-116 and Caco-2 cells with ERB-041 and found a significant increase in the mRNA levels of ESR2, CYSLTR2, and HPGD, and a significant decrease of CYSLTR1 and PTGS2 mRNA levels compared with untreated cells (Figure 3B). We also investigated the effects of ERB-041 treatment on \(\beta\)-catenin and its target genes CCND1 (cyclin D1) and MYC (c-Myc). We observed that ERB-041 treatment significantly reduced the mRNA levels of CTNNB1 and MYC in both HCT-116 and Caco-2 cells, and CCND1 mRNA levels in Caco-2, but not in HCT-116 cells (Figure 3C). The fact that ERB-041 failed to reduce cyclin D1 levels in HCT-116 cells is probably due to a CTNNB1 (\(\beta\)-catenin) mutation that this cell line has. Likewise, immunofluorescence analysis showed that ERB-041 treatment increased the nuclear expression of ER\(\beta\) in both SW-480 and HCT-116 cells compared with untreated cells (Figure 3E). Interestingly, ERB-041 treatment induced phosphorylation of \(\beta\)-catenin (Figure 3D), a finding that suggests that ERB-041 treatment induces \(\beta\)-catenin degradation in these cells. In support of this finding, immunofluorescence analysis also revealed a significant reduction in active \(\beta\)-catenin (non-phospho-\(\beta\)-catenin, which translocates into the nucleus) and COX-2 expression levels after ERB-041 treatment and the concurrent induction of 15-PGDH expression in SW-480 cells compared with untreated cells (Figure 3F). Similar results were observed in HCT-116 cells, in which we examined the expression of only total \(\beta\)-catenin due to a \(\beta\)-catenin mutation and observed that ERB-041 treatment increased total \(\beta\)-catenin membrane levels (supplementary material, Figure S1D).

Effects of ER\(\beta\) induction on cell migration and survival

Next, we investigated the effect of ER\(\beta\) induction on cell migration and colony formation. ERB-041 treatment significantly reduced the migration of both SW-480 and HCT-116 cells compared with untreated cells in Transwell and wound healing experiments, but this effect was lost when cells were treated before with an ER\(\beta\)-selective antagonist, PHTPP (Figure 4A,C). To prove the specificity of ERB-041, we knocked down ER\(\beta\) in cells by using an ER\(\beta\)-specific siRNA (supplementary material, Figure 1D). ERB-041 failed to reduce cell migration and wound closure in both ER\(\beta\) siESR2 knockdown cell lines (Figure 4B,D). Additionally, ERB-041 treatment significantly reduced colony numbers in both cell lines (Figure 4E), with no effect in ER\(\beta\) siESR2 knockdown cells (Figure 4F). Furthermore, ERB-041 decreased cell survival after 72 h of treatment, with no effect in siESR2 cells (Figure 5A,B). To determine whether this change was due to ERB-041-induced apoptosis, we evaluated the pro-apoptotic marker CASPASE-3 and found that CASPASE-3 activation was increased 72 h after ERB-041 treatment compared with the untreated control (Figure 5C). Furthermore, we used a cell-derived colonosphere-based model to detect the formation of apoptotic blebs. Both ERB-041-treated SW-480 and HCT-116 colonospheres formed apoptotic blebs (Figure 5D), and the spheroid viability was significantly lower in the treated group than in the untreated control (Figure 5E). These results clearly showed the effects of ER\(\beta\) signalling on cell functions, including decreased cell migration and survival, where the latter is most probably due to the observed increase in apoptosis.

Contribution of CysLT2R in ERB-041-mediated effects in colon cancer cells

Our patients’ results suggested a strong correlation between ER\(\beta\) and CysLT2R expression levels. Furthermore, CYSLTR2 mRNA levels were the most affected among the ERB-041-upregulated genes that we investigated. Our group has previously studied the anti-tumour effect of CysLT2R in CRC [11]. Our next step was to investigate whether some of the ERB-041 effects on cells are mediated via CysLT2R. For this purpose, we used the HCT-116 cell line with a Dox-inducible stable knockdown of CYSLTR2. ERB-041 induced a two-fold increase of CysLT2-R protein expression levels in the absence of Dox (Figure 5F). When CYSLTR2 knockdown was induced in the presence of Dox, ERB-041 was incapable of upregulating its levels. Likewise, ERB-041 could not reduce cell migration, colony formation, or cell survival in the presence of Dox (Figure 5G–I). These results suggest that some of the effects of ERB-041 in functional assays of cells are mediated via upregulation of CysLT2R.

ER\(\beta\) expression in tissues of mice from three different colon cancer models

To further validate our results, we evaluated ER\(\beta\) expression in three different mouse models. In the first, a CAC model with a disruption in the Cysltr2 gene that gives a more aggressive phenotype, Cysltr2\(^{-/-}\) mice expressed less ER\(\beta\) than WT mice (Cysltr2\(^{+/-}\)) (Figure 6A). In contrast, the Cysltr1\(^{-/-}\) CAC mice, which exhibited a less aggressive phenotype [9,23], expressed more ER\(\beta\) than WT mice (Cysltr1\(^{+/-}\)) (Figure 6B). In the Apc\(^{Min/+}\) spontaneous colon cancer model, where Wnt–\(\beta\)-catenin signalling is ‘on’ due to an Apc mutation, preventing the phosphorylation and degradation of \(\beta\)-catenin and promoting its translocation into the nucleus [13], Apc\(^{Min/+}\) mice expressed significantly less ER\(\beta\) in their colon tissues than WT mice (Figure 6C). Images of mouse colon section 1 (S1) for each genotype (Figure 6A–C) and for the whole colon and sections 2–4 are presented in supplementary material, Figure S2.
Figure 3. ERB-041 treatment upregulates anti-tumourigenic proteins and downregulates pro-tumourigenic proteins. (A) Baseline mRNA and protein expression levels of ESR2 (ERβ) in SW-480 and HCT-116 human cells. (B) RT-qPCR analysis of the mRNA levels of ESR2 (ERβ), CYSLTR1 (CysLT1R), CYSLTR2 (CysLT2R), PTGS2 (COX-2), and HPGD (15-PGDH) in untreated and ERB-041-treated (60 nM, 48 h) HCT-116 and Caco-2 cells. (C) RT-qPCR analysis of the mRNA levels of CTNNB1 (β-catenin), CCND1 (cyclin D1), and MYC (c-Myc) in untreated and ERB-041-treated HCT-116 and Caco-2 cells. The samples were analysed and normalised against endogenous levels of the housekeeping gene HPRT1. (D) Western blot analysis showing the phosphorylation of β-catenin after treatment with ERB-041 in HT-29 and Caco-2 cells. (E) Induction of nuclear ERβ expression in SW-480 and HCT-116 cells after treatment with ERB-041 (60 nM, 48 h) as assessed by immunofluorescence analysis. (F) Expression of active β-catenin, COX-2, and 15-PGDH in untreated and ERB-041-treated (60 nM, 48 h) SW-480 cells as assessed by immunofluorescence analysis. ERB-041 treatment reduced the expression of active β-catenin (non-phospho-β-catenin, which is translocated into the nucleus) and COX-2, and increased the expression of 15-PGDH compared with untreated cells. The scale bar represents 10 μm. Mean ± SEM of at least three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, unpaired t-test.
Figure 4. ERB-041 treatment reduces cell migration and colony formation in human colon cancer cells. (A) Migration of untreated, ERB-041- (60 nM), and PHTPP (1 μM) + ERB-041-treated SW-480 and HCT-116 cells. In total, 5 × 10^4 cells per well in serum-free medium were seeded into the upper chambers of 24-well migration chambers and allowed to migrate to the lower chambers containing medium with 10% FBS for 18 h. Migrated cells were stained with crystal violet and quantified using a spectrophotometer. The graph shows the percentage of migrated cells in each group. When the effect of ERB-041 was blocked by using the ERβ-selective antagonist PHTPP, the migration of cells was increased to the same level as untreated cells. (B) Migration of SW-480 and HCT-116 cells transfected with control siRNA (siControl) or ERβ-specific siRNA (siESR2) with or without ERB-041 treatment. ERB-041 treatment had no effect on siESR2 cells. (C) Wound healing assay of untreated, ERB-041- (60 nM, 24 h), and PHTPP (1 μM) + ERB-041-treated SW-480 and HCT-116 cells. A two-well insert was used to create the wound, and 7 × 10^5 cells/ml were seeded into each well of the insert. Images were taken at 0 and 24 h. The graph shows the percentage of wound closure in each treated group respective to the control group. The wound area was measured using ImageJ software (NIH, MD, USA). (D) Wound healing assay of untreated or ERB-041-treated SW-480 and HCT-116 cells transfected with siControl or siESR2. (E) Clonogenic cell survival assay of untreated and ERB-041-treated (60 nM, 48 h) SW-480 and HCT-116 cells. The graph shows the percentage of colony survival in each group. (F) Clonogenic cell survival assay in cells transfected with siControl or siESR2. ERB-041 treatment drastically reduced colony formation, especially in HCT-116 cells where only two microcolonies were formed (indicated by arrows). The effect of ERB-041 treatment was lost in siESR2 cells. Scale bars = 100 μm. Mean ± SEM of at least three independent experiments. *p < 0.05, **p < 0.01, ***p < 0.001, unpaired t-test.
Effect of ERβ induction on colon cancer cell metastasis

We previously reported that high ERβ expression was significantly correlated with decreased metastasis in regional lymph nodes and distant organs [17]. The majority of CRC patients with distant metastasis at the time of diagnosis (M1) had lower ERβ expression than patients without distant metastasis (M0) at the time of diagnosis.
Therefore, we used an in vivo zebrafish xenograft model to investigate whether an ERβ-selective agonist would have any effect on cell metastasis (Figure 6E, F and supplementary Figure S3). HT-29 and HCT-116 cells treated with or without ERB-041 were injected into the perivitelline space of zebrafish. Images of the tail, i.e. the distant metastatic site, were taken at 48 h post-injection. The number of embryos that developed tail vein metastasis in the experimental group (in which embryos were injected with ERB-041-treated HT-29 cells) showed no tail metastasis, while untreated HT-29 cells metastasised to the zebrafish tail (indicated by arrowheads). After 48 h, the metastatic spread of cells was monitored and photographed. Scale bar = 50 μm. Data shown: (A–C)mean ± SEM of five mice per genotype. **p < 0.01, ***p < 0.001, unpaired t-test; (D) the percentage of patients, p = 0.014, χ²-test; (E) the number of zebrafish embryos with tail vein metastasis in the control and ERB-041-treated groups, p < 0.001, Fisher’s exact test. (G) Schematic representation of the effects of the ERβ-selective agonist ERB-041.

Representative fluorescence microscopic images of both the experimental and the control groups are presented in Figure 6F. Similar results were also observed with untreated or ERB-041-treated HCT-116 cells (supplementary material, Figure S3B,C). These results clearly show the suppressive effect of ERβ signalling on cell metastasis.

**Discussion**

Despite its slow progression and increased capacity for early diagnosis, cancer recurrence remains a major risk...
for CRC patients [28], creating the need for prognostic markers and targeted therapies to help establish individualised treatment for patients with CRC, for which ERβ might be a promising candidate [29].

Herein, we showed that ERβ expression is negatively correlated with CysLT1R expression and positively with CysLT2R expression in CRC tissues. Earlier, we reported that patients with low levels of CysLT1R and high levels of CysLT2R had a better prognosis than other patient subgroups [10], and used the CysLT1R antagonist montelukast to further demonstrate the important role of CysLT1R signalling in colon cancer progression [18,30]. Here, we demonstrated that ERB-041 treatment significantly reduced CYSLTR1 mRNA levels in cells. Furthermore, CysLT1R was reported to facilitate polyp formation and tumourigenesis in a CAC mouse model [23]. Additionally, in the ApcMin/+ mouse model, the pro-tumour effect of CysLT1R was shown to be sex-specific, with reduced tumour burdens observed for double-mutant female mice (Cysltr1−/−ApcMin/+). Interestingly, we observed increased ERβ expression in the Cysltr1−/−CAC mouse model compared with WT mice. To the best of our knowledge, this is the first study to report a significant negative correlation between ERβ and CysLT1R expression in CRC patients, human colon cancer cell lines, and mice models.

Unlike CysLT1R activation, the activation of CysLT2R was shown to induce 15-PGDH expression, leading to the redifferentiation of colon cancer cells [6]. We found strong positive correlations between ERβ expression and both CysLT1R and 15-PGDH expression. ERB-041 treatment significantly increased the mRNA and protein expression levels of CysLT1R and 15-PGDH in cells. Likewise, ERβ expression was significantly reduced in the colons of Cysltr2−/−CAC mice model compared with control littermates. These positive correlations between ERβ, CysLT1R, and 15-PGDH expressions were also supported by publicly available mRNA data [26,27]. Taken together, this evidence supports our finding that some of the ERB-041 effects are mediated via CysLT1R. In the presence of Dox+, where CYSLTR2 is suppressed, ERB-041 was incapable of reducing cell migration, survival, and colony formation. In support of this finding, Jiang et al have shown that knockdown of CysLT1R increased CysLT1R surface expression in mast cells [31].

Previous research has shown that COX-2 is upregulated in CRC patients [32] and that ERα expression is decreased during tumour progression [33]. We observed a significant negative correlation between ERβ and COX-2 expression in patients' cancerous tissues, which is consistent with publicly available mRNA data from CRC patients [27]. Moreover, the treatment of cells with ERB-041 decreased COX-2 (PTGS2) mRNA and protein levels. Previous studies have reported that treatment with 17β-oestradiol inhibited PGE2-induced COX-2 expression via Akt and ERK1/2 pathways [34]. In addition, Song et al showed that the treatment of an ovariectomised CAC mouse model with 17β-oestradiol decreased the protein and mRNA levels of the pro-tumourigenic inflammatory mediators COX-2 and interleukin 6 (IL-6) by enhancing the expression of ERβ [35].

Mutation of the APC gene leads to the hyperactivation of β-catenin signalling and increased translocation of β-catenin to the nucleus, increasing cell proliferation, survival, and migration [12,13]. We showed that CRC patients with high ERβ expression had lower nuclear β-catenin expression levels but higher membrane β-catenin expression levels than patients with low ERβ expression in cancerous tissues. A similar correlation was found when publicly available mRNA data from CRC patients were assessed [27]. We found that ERβ expression levels in the colons of ApcMin/+ mice were significantly reduced compared with WT control littermates. In support of our finding, Giroux et al have shown that ERβ-selective agonist diarylpropionitrile (DPN) treatment reduced the number of polyps in the small intestines of both male and female ApcMin/+ mice [36]. Furthermore, ERB-041 treatment reduced β-catenin mRNA levels, as well as its target genes NDDN1 (cyclin D1) and MYC (c-Myc); nuclear active β-catenin expression in SW-480 cells; increased the expression of membrane β-catenin in HCT-116 cells; and induced the phosphorylation of β-catenin in both cell lines.

Substantial evidence has shown that ERβ reduces the proliferation of cells by modulating the G1–S cell-cycle phase transition [37,38]. We also observed that ERB-041 treatment reduced cell survival as well as significantly reducing migration and clonogenic cell survival, induced the formation of apoptotic blebs, and increased CASPASE-3 expression. To prove the specificity of ERB-041 treatment, we used an ERβ-selective antagonist, PHTPP, and also knocked down ERβ using a specific siRNA. In both cases, ERB-041 had no effect on cell migration, proliferation, or survival. While ERβ was overexpressed in previous studies to demonstrate its repressive functions in cells, we induced basal levels of the receptor using the ERβ-selective agonist ERB-041, which has a 200-fold stronger affinity for ERβ than ERα. A recent study has shown that ERB-041 treatment significantly reduces the number and volume of UVB-induced skin tumours in SKH-1 hairless female mice [39]. Edvardsson et al reported the strong ERβ-mediated downregulation of IL-6 and downstream networks, which had significant implications for inflammatory mechanisms involved in colon carcinogenesis [40]. Excitingly, our report is the first to show a direct correlation between ERβ and CysLT receptors, β-catenin, COX-2, and 15-PGDH in CRC patients, colon cancer mouse models, and cell lines. Additionally, we showed that even patients who were defined as having a poor prognosis still had a significantly better DFS when they exhibited high ERβ expression.

We showed that ERB-041 treatment significantly reduced cell migration and strengthened our hypothesis by utilising an in vivo zebrafish xenograft metastasis model. We observed that zebrafish embryos injected
with ERB-041-treated cells exhibited significantly less distant metastasis than those injected with untreated cells.

In conclusion, our findings suggest that the possible mechanisms by which ERB-041 acts in cells are reduction of cell proliferation and migration by increasing the phosphorylation of β-catenin, which reduces translocation of active β-catenin in the nucleus and downregulates β-catenin signalling and its target genes cyclin D1 and c-Myc; reduction of cell survival by increasing the CASPASE-3 activity and formation of apoptotic blebs; and reduction of migration, survival, and colony formation by upregulating CysLT2R levels. Our results, summarised in Figure 6G, indicate that ERB-041 acts on the transcriptional level to upregulate or downregulate the genes of interest. These multi-target effects of ERB-041 are complex and require further mechanistic studies.

Taken together, our findings have translational relevance and emphasise the pivotal role of ERβ induction as a potential targeted therapy for the treatment and management of CRC. A recent report in which 26 cancer-affected males and females compared with matched normal patients with CRC.

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Author contributions statement

GT and AS conceived and designed the study. M-LL, RO, SRS, and GT were responsible for development of methodology. GT, SRS, PD, SM, RE, and AS analysed and interpreted the data. M-LL and RO contributed to administrative and/or material support. GT, SRS, and AS wrote and reviewed the manuscript.

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SUPPLEMENTARY MATERIAL ONLINE

Supplementary materials and methods

Figure S1. Representative images of negative controls stained with only a secondary antibody to test for unspecific binding

Figure S2. ERβ expression in the tissues of mice from three different CC models

Figure S3. ERB-041 treatment reduces the metastatic ability of CC cells in an in vivo zebrafish xenograft model

Table S1. Correlation coefficient (r) between mRNA levels of the indicated genes and ERβ (ESR2) taken from public databases in both gender groups.