Intestinal estrogen receptor beta suppresses colon inflammation and tumorigenesis in both sexes

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A B S T R A C T

Estrogen hormones protect against colorectal cancer (CRC) and a preventative role of estrogen receptor beta (ERβ) on CRC has been supported using full knockout animals. However, it is unclear through which cells or organ ERβ mediates this effect. To investigate the functional role of intestinal ERβ during colitis-associated CRC we used intestine-specific ERβ knockout mice treated with azoxymethane and dextran sodium sulfate, followed by ex vivo organoid culture to corroborate intrinsic effects. We explored genome-wide impact on TNFα signaling using human CRC cell lines and chromatin immunoprecipitation assay to mechanistically characterize the regulation of ERβ. Increased tumor formation in males and tumor size in females was noted upon intestine-specific ERβ knockout, accompanied by enhanced local expression of TNFα, deregulation of key NFκB targets, and increased colon ulceration. Unexpectedly, we noted especially strong effects in males. We corroborated that intestinal ERβ protects against TNFα-induced damage intrinsically, and characterized an underlying genome-wide signaling mechanism in CRC cell lines whereby ERβ binds to cis-regulatory chromatin areas of key NFκB regulators. Our results support a protective role of intestinal ERβ against colitis-associated CRC, proposing new therapeutic strategies.

1. Introduction

Chronic inflammation is one of the hallmarks of colorectal cancer (CRC) promotion and correlates with poor prognosis [1–3]. A variety of pro-inflammatory mediators, such as tumor necrosis factor alpha (TNFα/TNF) trigger inflammatory responses [4]. Consequently, anti-inflammatory treatment with acetylsalicylic acid has been shown to reduce the CRC incidence in clinical trials, but the riskbenefit balance remains poor [5,6]. Development of a preventive therapy with less adverse effects could significantly reduce CRC incidence.

Men have an earlier onset and higher incidence of CRC [7] and a role for hormones is supported by findings that oral contraceptives, hormone-replacement therapy, phytoestrogens, and endogenous estrogens lower the incidence [8–12]. Hormone-replacement therapy reduces the CRC incidence by approximately 20% [8,13,14]. Interestingly, estrogen alone renders the largest preventive effects [15]. Estrogen therapy is, however, not suitable as a preventive approach due to adverse effects.

Studies have indicated CRC-preventive effect of the estrogen receptor (ER)-β, which is expressed at low levels in normal intestinal epithelial cells and declines during CRC progression [16]. Polymorphism in the promoter region correlates with CRC risk/survival [17,18], and in vivo studies, using full-body ERβ knockout female mice or ERβ-selective agonists, support that estrogen through ERβ mediates...
CRC-protective effect [19–23]. Although an impact of ERβ on CRC has been supported, it is unclear through which cells or organ ERβ mediates this effect. ERβ is expressed in several immune-related organs and in intestinal immune cells [24]. Whether or how intestinal epithelia ERβ modulates CRC development has not been investigated. Understanding this mechanism is critical since selective activation of ERβ could be an ideal approach for therapeutic prevention of CRC.

In order to characterize the role of intestinal ERβ during inflammation-driven CRC, we generated mice lacking ERβ specifically in the intestinal epithelial cells (ERβKO) and induced colitis and CRC using azoxymethane (AOM) and dextran sodium sulfate (DSS). In the present study, we demonstrate for the first time that intestinal ERβ is protective against colitis-associated adenomas in both sexes, but with sex differences. We show that lack of ERβ enhances inflammatory signaling in vivo, and corroborate that activation of intestinal ERβ can oppose TNFα-induced epithelial cell damage ex vivo. We show that male mice are especially sensitive to a lack of ERβ in terms of inflammatory signaling and tumor number and our results support a plausible mechanism in which the protective effect of ERβ is mediated by inhibition of the inflammatory and carcinogenic effects by TNFα/NFκB signaling.

2. Materials and methods

2.1. CRC clinical specimens

Clinical samples (colon tumors and non-tumor adjacent tissue) were collected from patients (16 women and 6 men) undergoing surgery in Stockholm. The study was approved by the regional ethical review board in Stockholm (2016/957-31). Patients or the public were not involved in the design, conduct, reporting or plans of our research. A detailed description of the qPCR and statistical analysis is provided (SI Appendix, Material and Methods).

2.2. Animal experiment

ERβKO mice lacking ERβ specifically in the intestinal epithelial cells were generated by crossing ERβfloox/mice (B6.129X1-Esr2tm1.Gum/J, Jackson Laboratory, Bar Harbor, ME, JAX stock #004586). Mice were maintained on a C57BL/6J background. Littermates (ERβfloox/mice lacking the Cre allele were used as controls (WT). The genotype was confirmed by genotyping and qPCR. Intestinal ERβ expression was assessed in vivo by qPCR and immunohistochemistry, and qPCR demonstrated ERβ expression in colonic epithelium of mice, and lack of expression in adenomas (Fig. 1B, S1, 1D). No difference in ERα expression between WT and ERβKO mice was noted in either normal or tumor tissue (Fig. S1C). However, we note that, contrary to in human CRC (HPA and The Cancer Genome Atlas (TCGA) dataset, Figs. S1D and S1E), ERα expression was significantly increased in the adenomas of mice (Fig. S1B). We conclude that ERβ is expressed in colon epithelial cells in human and mice.

2.3. Males are more sensitive to colitis

AOM/DSS treatment induced colitis as characterized by body weight (BW) loss, diarrhea and rectal bleeding (Fig. S2), and generated tumors in all mice within 15 weeks. We note that WT males had a significantly more severe response compared to females, in terms of disease activity index (DAI) after the first DSS cycle (Fig. 2A). Male and female WT mice developed the same number of tumors, but males developed larger tumors (Fig. 2G). Thus, we noted sex differences in colitis and tumor size.

3. Results

3.1. ERβ is expressed in human and mouse colon and lost in tumors

The expression of ERβ in the intestine has been unclear, due to relatively low mRNA levels and unspecific antibodies. Immunohistochemistry from the Human Protein Atlas [27,28], with a validated antibody [24], support ERβ expression in colonic epithelial cells and lack of expression in CRC (Fig. 1A). We verified this by qPCR in clinical specimens (Fig. 1B, 1C). Further, RNA in situ hybridization and qPCR demonstrated ERβ expression in colonic epithelium of mice, and lack of expression in adenomas (Fig. 1B, S1, 1D). No difference in ERα expression between WT and ERβKO mice was noted in either normal or tumor tissue (Fig. S1C). However, we note that, contrary to in human CRC (HPA and The Cancer Genome Atlas (TCGA) dataset, Figs. S1D and S1E), ERα expression was significantly increased in the adenomas of mice (Fig. S1B). We conclude that ERβ is expressed in colon epithelial cells in human and mice.

3.3. Loss of intestinal ERβ enhances tumor formation in a sex-dependent manner

To test our hypothesis that intestinal ERβ mediates CRC-protective effects, we generated mice lacking ERβ in intestinal epithelial cells (ERβKO, Fig. S1). There were no significant differences between WT and ERβKO mice in terms of BW loss and DAI (Fig. S2, 2C-D). However, ERβKO male mice showed a trend of higher DAI (Fig. 2D), thereby exacerbating sex differences in response to treatment (Fig. 2B). Additionally, after 9-weeks treatment, ERβKO females had a higher colitis score and displayed more ulcerated areas compared to WT females and after 15-weeks treatment ERβKO males and females presented ulcers, which was not observed in WT mice (Figs. S2 and 2H).

Importantly, after 15-weeks treatment, ERβKO males developed significantly more tumors compared to WT males (Fig. 2E-F, S2). In females, the tumor number did not change, resulting in sex differences (Fig. 1F). However, the tumor size was significantly larger in ERβKO females compared to WT (Fig. 2E-G). We conclude that deletion of intestinal ERβ had significant effects in both sexes, enhancing sex differences related to colitis (DAI) and tumor numbers. Moreover, lack of intestinal ERβ had an impact on ulceration in both sexes. Our results demonstrate that loss of intestinal ERβ enhances tumor formation in both sexes, but with sex differences.
3.4. Altered epithelial cell proliferation upon intestinal loss of ERβ

In the applied mouse model, DSS initiated more ulcerated areas in ERβKO males compared to WT females. DSS promotes a wound-healing response marked by enhanced cell proliferation. In order to explore whether and how ERβ impacts this, epithelial cell proliferation was assessed using immunohistochemistry of the cell proliferation marker Ki67 in colon tissue sections from WT and ERβKO males. The cell proliferation at 9-weeks was significantly increased by the treatment in WT but not in ERβ KO males (Fig. 3A and B). Further, the larger tumors in ERβ KO females were not accompanied by increased cell proliferation in the adenomas (Fig. S3). Thus, knockout of ERβ appeared to blunt the colitis-induced proliferation in females.

3.5. Intestinal loss of ERβ increases inflammatory signaling

Above, we noted increases in DAI and ulceration after DSS treatments in ERβKO males, indicating increased inflammation after loss of ERβ. In order to explore if and how inflammatory signaling is affected by ERβ, we examined several inflammatory markers in the whole colon tissue. WT males responded stronger to the treatment (9-weeks) with significantly higher levels of Il1b compared to females (Fig. S3). Further, ERβ KO males presented significantly higher Tnfα expression compared to WT males after 9-weeks treatment (Fig. 3C), followed by significantly elevated Il6, Ccl2 and Ccl4 levels after 15-weeks. In females, Il1b expression was enhanced by the knockout at 9-weeks treatment, and at 15 weeks both Tnfα and Il6 were significantly increased compared to WT females (Fig. 3C). Thus, absence of ERβ enhanced the inflammatory response to treatment in both sexes, most evident in males.

3.6. Intestinal ERβ activation protects against TNFα-induced epithelial cell damage ex vivo

Cytokines are primarily secreted by immune cells, but can also be produced by e.g. tumor cells. During this study, a subset of the mice was analyzed for gut microbiota, which we found was modulated by ERβ [29]. Based on this, it is possible that the altered microbiota would impact inflammation and corresponding TNFα production by immune cells in the microenvironment. Alternatively, or in addition, ERβ might directly impact NFκB and inflammatory signaling in the epithelial cells. In order to separate these events, we tested whether ERβ could protect against TNFα-induced epithelial cell damage in isolated tissue. TNFα treatment significantly decreased crypt formation, which is a measure of regenerative growth and differentiation capacity [30,31], in both WT and ERβ KO mice of both sexes. Selective ERβ activation with DPN could significantly counteract the TNFα-induced cell damage in WT mice (Fig. 3D–E, S3). This demonstrates that intestinal ERβ can protect against TNFα-induced epithelial cell damage intrinsically.

3.7. TNFα-induced colonic cell transcriptome and NFκB signaling are modified by ERβ

Our in vivo data demonstrate that lack of intestinal ERβ increases TNFα expression and inflammatory signaling. To explore if intestinal ERβ opposes TNFα-signaling in a human in vitro setting, we characterized the effect of TNFα in two human CRC cell lines in presence and absence of ERβ. We defined a core set of 122 genes that was significantly regulated by TNFα in both cell lines, with the most overrepresented biological process being related to NFκB-signaling (Fig. 4A, S4, Table S3). The majority of TNFα-modulated genes were, overall,
Fig. 2. Intestinal epithelial ERβ expression protects against tumor development with clear sex differences. (A) Assessment of disease activity index (DAI) during DSS treatment shows that WT males ($n = 26$) are more affected by the treatment compared to WT females ($n = 28$), significance calculated for area under curve, and that (B) this sex difference is enhanced in knockouts (males = 37 and females = 37). Assessment of DAI in (C) females and (D) males shows no significant differences between WT and ERβKOVII mice (area under curve). (E) Representative images of male and female colons from WT and ERβKOVII mice, after 15-week treatment. (F) Knockout males ($n = 19$) had significantly more tumors than WT males ($n = 16$) and knockout females ($n = 20$) at 15 weeks (two-way ANOVA with uncorrected fisher’s LSD test). (G) WT males ($n = 15$) developed significantly larger tumors compared to WT females ($n = 16$), whereas the knockout females ($n = 20$) developed significantly larger tumors compared to WT females, which abolished this sex difference (two-way ANOVA with uncorrected fisher’s LSD test). (H) Assessment of H&E staining of females after 9 weeks treatment and males after 15 weeks treatment shows more ulceration in knockout mice (arrows in representative figure). Bars in magnified images of ulceration represent 200 μm. Stars highlight tumor areas. # indicate significant sex differences.
Fig. 3. Knockout of ERβ enhances inflammatory signaling in the colon and modulates colitis-driven proliferation. (A) Representative Ki67 immunohistochemistry (IHC) staining of AOM/DSS-treated WT and ERβKOVI female mice, and respective untreated control groups. (B) Fraction of Ki67 positive cells per crypt epithelial cells. The cell proliferation increased significantly in WT treated female mice (n = 5) but not in ERβKOVI females (n = 8, two-way ANOVA with uncorrected Fisher’s LSD test). (C) Quantification of relative mRNA expression levels of inflammatory genes Tnfα, Il6, Il1β, Ccl2 (MCP1) and Ccl4 (MIP-1β) by qPCR in colon tissue indicates elevated AOM/DSS-induced inflammation in knockout mice (n = 4–12, # indicate significant sex differences, two-way ANOVA with uncorrected Fisher’s LSD test). (D) Representative images of intestinal organoids from WT and ERβKOVI male mice treated with vehicle, Tnfα, DPN and a combination of Tnfα and DPN. (E) The number of crypts per organoid was quantified in WT (n = 7) and ERβKOVI (n = 7, two-way ANOVA with Tukey’s multiple comparison test) mice of both sexes. Tnfα reduced the crypt formation in both genotypes and sexes and activation of ERβ with DPN significantly counteracted the effect of Tnfα in WT mice but not in ERβKOVI.
inhibited or attenuated by ERβ, and NFκB signaling was impacted by ERβ (Fig. 4B–D, Table S1). Overall, ERβ can modulate the TNFα-activated gene regulation in both directions, but represses a major part of TNFα-induced NFκB signaling in colon cells.

The top TNFα-regulated genes included multiple known NFκB targets, which were attenuated by ERβ (Fig. 5A, S4). Additionally, the positive regulator of the canonical NFκB signaling BIRC3 was attenuated by ERβ, whereas a negative regulator of NFκB, ATF3, was upregulated by TNFα only in presence of ERβ (SW480 cells). We could translate this findings in vivo and found that absence of ERβ in the mouse intestine significantly increased the treatment-induced expression of Ccl1 in both sexes, and of Nfkbia, Ccl20, and Bcl3 in male mice after 15-weeks treatment (Fig. S3). Further, the negative NFκB regulator Atp3 was upregulated by treatment only in WT and not in ERβ-deficient females (Fig. 5B, S3). Altogether, ERβ can modulate TNFα response, including key NFκB related genes and this mechanism is conserved between species (human-mice), and exhibits sex differences.

3.8. TNFα enhances ERβ transactivation in CRC cells

Our results indicate a potential crosstalk between ERβ and TNFα-NFκB signaling, hence, we tested whether ERβ could impact NFκB transactivation, or vice versa. We found that ERβ slightly reduced TNFα-mediated p65 nuclear translocation, but did not impact NFκB transactivation at its consensus response element (Fig. S5A-B, S4E). However, the ligand-mediated transactivation of ERβ at consensus estrogen response elements (ERE) was significantly enhanced by TNFα (Fig. 5C), and this was not due to increased expression of ERβ (Fig. S4F).

Our mechanistic experiments thus suggest an intricate crosstalk in CRC cells where TNFα enhances ERβ transactivation, and ERβ impacts a proportion of the TNFα-regulated transcriptome (attenuating a majority and enhancing a fraction) without modifying p65 transactivation.

3.9. ERβ directly binds to cis-regulatory chromatin regions of NFκB regulators

Our results corroborate a strong modulation of NFκB targets by ERβ, but did not reveal a major and consistent impact through a general mechanism (did not significantly impact p65 nuclear translocation nor transactivation). We next hypothesized that gene-specific elements determine chromatin binding of ERβ to NFκB target genes and indeed we found that ERβ binds to cis-regulatory chromatin areas of the key NFκB regulators ATF3, BCL3 and BIRC3 (Fig. 5D). ERβ attenuated the expression of the positive NFκB regulators, BCL3 and BIRC3 by binding near the promoter, and enhanced the expression of ATF3, a negative regulator of NFκB, by binding to an assumed enhancer approximately 7 kb upstream of its TSS. We thus propose that ERβ attenuates the effect of TNFα including direct ERβ-mediated repression of NFκB-regulated genes.

3.10. TNFα induces genome-wide response in human CRC cells and is modified by ERβ

Fig. 4. TNFα induces genome-wide response in human CRC cells and is modified by ERβ. (A) Venn diagram comparing differentially expressed genes upon TNFα (10 ng/ml, 2 h) stimulation in SW480 and HT29 cells (Illumina bead array). (B) Venn diagrams comparing TNFα-regulated genes in SW480 and HT29 cell lines with and without ERβ indicate that ERβ modulates the response to TNFα treatment. (C) Heatmap of TNFα regulation in cells with and without ERβ illustrates that ERβ attenuates both TNFα-induced and TNFα-repressed genes. (D) KEGG pathway analysis shows that ERβ primarily modulate TNFα-induction of genes related to NFκB signaling.
inflammatory genes, through binding to cis-regulatory chromatin regions of NFκB regulators.

4. Discussion

Our objective in this study was to determine whether intestinal epithelial ERβ is responsible for the CRC-protective effects of estrogen and to explore this mechanism. Several studies support CRC protective effects of ERβ (reviewed in Williams et al., 2016), but the general expression and function are controversial. Its mRNA expression in colon is low [24], and it is unknown if this is sufficient for a functional role. To test this, we performed a conclusive experiment, which demonstrated that intestinal ERβ is protective against experimental colon adenoma formation.

Our findings include identification of several significant sex differences. While ERβ was protective against tumor development in both sexes, males lacking intestinal ERβ showed an increase in tumor number, whereas the NFκB regulator Atf3 (colon epithelium) was increased by treatment in WT female mice (n = 4–12/group, one- and two-way ANOVA with uncorrected fisher’s LSD test, # indicate significant sex differences). (C) SW480 cells with and without (mock) ERβ, transfected with TATA-ERE luciferase reporter construct, and treated with E2 or DPN and/or TNFα. TNFα significantly enhanced ERβ-E2 and ERβ-DPN transactivation. Figure illustrates the average of three independent experiments for E2 and one for DPN (two-way ANOVA with Tukey’s multiple comparison test), # indicate significant differences with and without ERβ. (D) ChIP-qPCR (three-independent experiments, two-way ANOVA with uncorrected fisher’s LSD test) confirms recruitment of ERβ to NFκB targets ATF3, BCL3 and BIRC3. ERβ binds upstream of the transcription start site (TSS) of BIRC3 (~264/290 bp) in both cell lines, and of BCL3 (~973 bp) in HT29 cells and of the enhancer region of ATF3 (~7193 bp) in SW480 cells.

Fig. 5. ERβ modulates NFκB signaling. (A) Heatmap illustrating ERβ repression of NFκB target genes induced by TNFα treatment in both cell lines, per Illumina bead-array. (B) qPCR analysis of NFκB related genes Cxcl1, Ccl20 (colon), Nfkb1, Nfkb2 and Bcl23 (colon epithelium) 15 weeks after initiation of AOM/DSS treatment demonstrates significant increases upon loss of intestinal ERβ, especially in males, whereas the NFκB regulator Atf3 (colon epithelium) was increased by treatment in WT female mice (n = 4–12/group, one- and two-way ANOVA with uncorrected fisher’s LSD test, # indicate significant sex differences). (C) SW480 cells with and without (mock) ERβ, transfected with TATA-ERE luciferase reporter construct, and treated with E2 or DPN and/or TNFα. TNFα significantly enhanced ERβ-E2 and ERβ-DPN transactivation. Figure illustrates the average of three independent experiments for E2 and one for DPN (two-way ANOVA with Tukey’s multiple comparison test), # indicate significant differences with and without ERβ. (D) ChIP-qPCR (three-independent experiments, two-way ANOVA with uncorrected fisher’s LSD test) confirms recruitment of ERβ to NFκB targets ATF3, BCL3 and BIRC3. ERβ binds upstream of the transcription start site (TSS) of BIRC3 (~264/290 bp) in both cell lines, and of BCL3 (~973 bp) in HT29 cells and of the enhancer region of ATF3 (~7193 bp) in SW480 cells.
agreement with previous findings [33], and that men with inflammatory bowel disease have a 60% higher risk to develop CRC compared to women [34]. Perhaps more surprising, is the novel finding that males responded stronger to absence of ERβ compared to females, both in terms of tumor development and inflammatory signaling. Estrogen is not thought of as a male hormone but several estrogens, such as estrone and the steroid origin (phytoestrogens) activate ERα, and the animals were fed regular chow diet, which is based on phytoestrogens-rich soy. There is thus a physiologic context where ERβ could be active in both male and female colon. Our study is the first to identify that the protective effect of β estrogen is mediated by intestinal epithelial ERβ.

Dietary origin (phytoestrogens) activate ERβ, and responded stronger to absence of ERβ. This phenomenon has been shown in various bowel disease have a 60% higher risk to develop CRC compared to females, both in terms of tumor development and inflammatory signaling. Estrogen is not thought of as a male hormone but several estrogens, such as estrone and the steroid origin (phytoestrogens) activate ERα, and the animals were fed regular chow diet, which is based on phytoestrogens-rich soy. There is thus a physiologic context where ERβ could be active in both male and female colon. Our study is the first to identify that the protective effect of β estrogen is mediated by intestinal epithelial ERβ.

As we found that TNFα was elevated in intestinal-specific ERβ knockout animals of both sexes, along with numerous NFκB-related markers, we explored and characterized a crosstalk between TNFα/NFκB. We demonstrated using an organoid model that activation of intestinal ERβ protects against TNFα-induced cell damage. We could thus dissociate this effect from the impact of microbiota or systemic immune effects, and demonstrated a local interaction between epithelial ERβ and TNFα-signaling. Moreover, our mechanistic experiments demonstrated that TNFα enhances ERβ transactivation at ERE, and ERβ, in turn, suppresses a fraction of key NFκB-related genes. We demonstrate that ERβ binds nearby cis-regulatory chromatin areas of key NFκB regulators ATF3, BCL3 and BIRC3 and confirm the ERβ regulation of Atf3 (females) and Bcl3 (males) in vivo. We thus detail how ERβ modulates TNFα-induced NFκB inflammatory signaling in colon, which reduces the risk for tumor development. We propose that ERβ reduces inflammatory signaling, leading to reduction in immune activity and, correspondingly, less immune cell-secretion of TNFα in vivo, which generates a de facto feed-forward loop (see Graphical abstract). Together, this may explain the protective effects of intestinal ERβ.

The detected levels of ERβ in the colon are low relative its strong impact on CRC in vivo. Since we found that TNFα significantly increases transactivation of ERβ, we propose an inhibitory feedback mechanism: The inflammatory state amplifies ERβ activation, which in turn inhibits TNFα-mediated signaling and reduces the inflammatory state. The higher inflammatory state in males may thus explain the enhanced impact of ERβ knockout in males.

In conclusion, our data demonstrate for the first time that intestinal epithelial ERβ can modulate the outcome of colitis-induced CRC in vivo, in both sexes. Our results demonstrate a plausible mechanism, which involves an intricate crosstalk between ERβ and the TNFα/NFκB signaling pathway. Our data support that intestinal ERβ is activated by TNFα, and attenuates colon adenomas by inhibiting the TNFα/NFκB signaling. We show that ERβ directly binds and represses the NFκB activators, BIRC3 and BCL3, while upregulating the NFκB inhibitor ATF3. The resulting reduction of inflammatory signaling, leads to less secretion of pro-inflammatory TNFα-secreting macrophages. Thus, we propose that the protective effects are mediated in a dual manner: reducing the intrinsic signaling will, in turn, reduce surrounding immune cell secretion of TNFα. Functionally, this manifests as a reduction of ulceration and tumor development. Our data clearly support the notion that an ERβ agonist can be a suitable preventive approach for colitis-induced CRC in both sexes.

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Data and materials availability
Gene expression data are deposited in the NCBI Gene Expression Omnibus database [GSE65979].

Declaration of competing interest
The authors declare no conflicts of interest.

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Appendix A. Supplementary data
Supplementary data to this article can be found online at https://doi.org/10.1016/j.canlet.2020.06.021.

References


