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Estrogen Activation by Steroid Sulfatase increases Colorectal Cancer proliferation via GPER

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Abstract

Context: Estrogens impact the incidence and progression of colorectal cancer (CRC) although precise molecular mechanisms remain ill-defined.

Objective: Pre-receptor estrogen metabolism through steroid sulphatase (STS) and 17 β -hydroxysteroid dehydrogenase activity and subsequent non-genomic estrogen signaling in human CRC tissue, in the TCGA COAD dataset, and in *in vitro* and *in vivo* CRC models was investigated. The study aimed to define and therapeutically target pathways through which estrogens alter CRC proliferation and progression.

Design, Setting, Patients, and Interventions: Human CRC samples with normal tissue matched-controls were collected from post-menopausal female and age-matched male patients. Estrogen metabolism enzymes and non-genomic downstream signaling pathways were determined. CRC cell lines were transfected with STS and cultured for *in vitro* and *in vivo* analysis. Estrogen metabolism was determined through a novel uHPLC-MS/MS method.

Primary Outcome Measure: The proliferative effects of estrogen metabolism were evaluated using BRdU assays and in CRC mouse xenograft studies.

Results: Human CRC exhibits dysregulated estrogen metabolism favoring estradiol synthesis. The activity of steroid sulfatase (STS), the fundamental enzyme that activates conjugated estrogens, is significantly ($p < 0.001$) elevated in human CRC compared to matched controls. STS over-expression accelerates CRC proliferation in *in vitro* and *in vivo* models, with STS inhibition an effective treatment. Uniquely we define a G-protein coupled estrogen receptor (GPER) pro-proliferative pathway potentially through connective tissue growth factor (CTGF) in CRC.

Conclusion: Human CRC favors estradiol synthesis to augment proliferation via GPER-stimulation. Further research is required on whether estrogen replacement therapy should be used with caution on patients at high-risk of developing CRC.

Introduction

Controversy surrounds the role estrogens play in CRC (1). Observational studies from the Women's Health Initiative (WHI) suggest pre-menopausal women have a 20% reduction in CRC compared to age-matched men (2). These gender differences plateau as women become post-menopausal. However, women on exogenous hormone-replacement therapy (HRT = conjugated estrogen (estrone sulfate (E_1S)) plus medroxyprogesterone), maintain protection against CRC (3), and elevated endogenous plasma estrogen concentrations also protect against CRC incidence (4). Conversely, other studies suggest greater endogenous plasma estrone (E_1) concentrations in post-menopausal women increase CRC risk (5); similarly, women with estrogen-dependent breast cancer have a higher risk of developing CRC (6). Importantly, women taking HRT at the time of CRC diagnoses are more likely to present with advanced-stage disease (7), suggesting either the symptoms associated with HRT use leads to delayed clinical diagnosis, or that HRT increases CRC development and proliferative rates.

As HRT, and thus estrogens, may influence CRC proliferation, the local colonic tissue activation of estrogens via steroid sulfatase (STS) and 17β -hydroxysteroid dehydrogenases (HSD17B) must be important (8). The expression of STS, the fundamental enzyme desulfating circulating estrogens to their active forms (Figure 1A), is prognostic for CRC survival (9), and mRNA expression of HSD17B2, which catalyzes estradiol (E_2) to E_1 , is down-regulated in human CRC tissue (10) suggesting estrogen metabolism as important in CRC progression. However, little is known about HSD17B1, HSD17B7, and HSD17B12 expression, all of which activate E_1 to E_2 (11,12).

Questions also remain regarding how estrogens act in CRC. ER α has either low (13) or no (14) expression in both normal colon and CRC, although splice variants do exist (15). Furthermore, loss of the pro-apoptotic ER β , which implies subsequent dominance of other ERs, defines CRC progression (16). Indeed, a recent meta-analysis has confirmed the loss of ER β expression as CRC develops (17). However, no human CRC studies have examined the G-protein coupled estrogen receptor (GPER), an endoplasmic reticulum membrane-bound receptor with high E₂-binding affinity (18) with known pro-proliferative actions in breast (19) and endometrial cancer (20).

Here we aimed to determine how estrogen metabolism and action impacts CRC. By examining key estrogen metabolizing enzymes in matched normal and cancerous human colorectal tissue, and then translating findings to *in vitro* and *in vivo* systems, we demonstrate for the first time that CRC exhibits dysregulated estrogen metabolism with STS activity and estrogen reductase pathways elevated in CRC. We go on to show greater STS activity increases estrogen-stimulated CRC proliferation *in vitro* and *in vivo* through GPER-activation via increased expression of connective tissue growth factor (CTGF), a known modulator of GPER action (21). Finally, we demonstrate that GPER expression is elevated in human CRC tissue, with this significantly correlating to increased CTGF expression. Thus, both STS and GPER inhibition may represent novel therapeutic targets for patients with CRC.

Results

Estrogenic enzymes favor E₂ metabolism in human CRC.

Immunohistochemical studies show STS expression is increased in human CRC (9). However, as STS expression does not correlate with enzyme activity, and no data exists on STS activity in human colon, we determined STS activity in human CRC and histopathologically unchanged colonic mucosa located at least 10-20 cm away from cancerous lesions (see patient characteristics for 64 participants: Supplementary Table 1). Post-menopausal female and aged-matched male CRC STS activity was significantly increased in CRC tissue compared to matched tissue (percentage change (95% CI) 24.6 (10.3, 38.8); p=0.001) (Table 1, Figure 1B). Although not formally tested, plotting the data suggests a more pronounced effect in females (Figure 1B). Increased STS activity does not correlate with increasing STS mRNA expression (STS dCT) in either normal or cancerous tissues (Figure 1C) (calculated correlation coefficient (p-value) g 0.27 (0.07) and 0.04 (0.77) for normal and cancerous samples, respectively). Indeed, RNASeq data (RNASeq V2) analyzed from The Cancer Genome Atlas (TCGA) colon adenocarcinoma (COAD) dataset showed no significant change in STS expression from normal to cancerous tissue (Supplementary Figure 1). As STS activity is altered by various post-translational modifications (22) this suggests determining only STS expression does not represent *in situ* colon activity. Furthermore, STS activity does not correlate to Duke's or T-staging (Table 1), indicating increased STS activity is most likely an early event in tumor formation.

As STS desulfates circulating and peripheral E₁S to E₁, we next determined the expression of enzymes that oxidize E₂ to E₁ (HSD17B2) and reduce E₁ to E₂ (HSD17B1, HSD17B7, and HSD17B12) in the same human CRC samples. HSD17B2 mRNA significantly (p<0.01)

decreased in CRC tissue compared to matched controls (Figure 1D, Supplementary Table 2, Supplementary Table 3 for raw dCT values). HSD17B1 mRNA was not detectable (data not shown). HSD17B7 and HSD17B12 mRNA were significantly increased in female and male CRC tissue compared to matched controls (Supplementary Table 2, Supplementary Table 3 for raw dCT values, and Figure 1E, 1F). This data was supported by further analysis of the TCGA COAD data, which demonstrated a significant decrease in mRNA expression of HSD17B2, and increased expression of HSD17B7 and HSD17B12, in colon cancer (Supplementary Figure 1). Immunoblotting (Figure 1G) and subsequent densitometry analysis (Figure 1H) of normal and cancerous tissue confirmed lack of HSD17B1 and increased HSD17B12 expression. HSD17B7 protein expression showed a trend towards increased expression in CRC. In contrast to mRNA data (Figure 1F), HSD17B2 protein was not decreased in CRC compared to controls (Figure 1H). HSD17B4, which oxidizes E₁ to E₂, expression was not determined as previous studies have shown this as significantly down-regulated in human CRC (23). Taken together our data suggests CRC up-regulates pathways favoring E₁S hydrolysis and subsequent E₂ synthesis.

Estrogen metabolizing enzyme expression defines CRC estrogenic proliferative response.

As human CRC exhibited dysregulated estrogen metabolism we hypothesized CRC cell lines expressing E₂ synthesis pathways may be more responsive to estrogen signaling. Thus, we determined expression patterns of key estrogen metabolizing enzymes in selected CRC cells. Compared to human CRC tissue, HCT116 and HT-29 cells exhibited similar HSD17B mRNA (data not shown) and protein (Figure 2A) expression (i.e. lack of HSD17B1, presence of HSD17B7 and HSD17B12, limited HSD17B2 expression). In contrast, Caco2 cells have low

HSD17B7 and HSD17B12, and higher HSD17B2 expression. Colo205 cells had low or no HSD17Bs mRNA (not shown) and protein expression and thus these cells were not used in further testing. When incubated for 72h with E₁ (Figure 2B, raw absorbance data Supplementary Figure 2A) or E₂ (Figure 2C, raw absorbance data Supplementary Figure 2B) in charcoal-stripped FBS (sFBS) media, HCT116 and HT-29 cells had increasing dose-dependent proliferative rates compared to sFBS media controls. Caco2 cells failed to respond to E₁ or E₂ stimulation.

Using LC-MS/MS we next examined how CRC cells metabolized estrogens over 24h. HCT116 cells did not significantly metabolize E₂ to other oestrogen metabolites, HT-29 cells metabolized E₂ to unknown metabolites, and Caco2 cells rapidly oxidized E₂ to E₁ (Figure 2D and 2E) indicative of its high HSD17B2 reductase expression. This suggests oxidation of E₂ via HSD17B2, expressed in Caco2 but not HCT116 and HT-29 cells, impacts local E₂ availability and consequently the ability of Caco2 cells proliferation to E₂. This further implies that peripheral estrogen metabolism in CRC may define the tumors responsiveness to estrogen action.

STS over-expression augments E₁S- and E₂S-stimulated proliferation in CRC.

As STS activity was significantly increased in human CRC samples (Fig. 1B), we examined how over-expression of STS affects CRC proliferation. Firstly, the STS activity of CRC cells was determined (Supplementary Figure 3A). Caco2 cells had the highest STS activity (165.4±4.7pmol/mg/h) with HCT116 cells exhibiting very low activity (1.65±0.1pmol/mg/h). Thus, we selected HCT116 cells to stably transduce with STS (HCT116[sts]) or vector only

(HCT116[vo]). Stable over-expression increased enzyme activity to 200.42 ± 5.91 pmol/mg/h compared to vector only expressing controls at 10.58 ± 1.37 pmol/mg/h (Supplementary Figure 3B).

In full media, HCT116[sts] proliferation significantly increased compared to HCT116[vo] cells (Figure 3A), with this augmented growth blocked by the non-cytotoxic, specific STS inhibitor STX64. Incubation of these same cells in sFBS media supplemented with E₁, E₂, or E₁S (at 100nM) for 72h, a significant ($p < 0.001$) growth difference was observed between HCT116[sts] and HCT116[vo] cells treated with E₁S only (Supplementary Figure 3C). This demonstrates greater STS desulfation of E₁S, leading to increased E₁ liberation driving proliferation. When these cells were grown for 72h in sFBS supplemented with E₂S (100nM), all proliferated in response to E₂S, with the greatest increase seen in HCT116[sts] cells compared to sFBS controls (Figure 3B). STX64 blocked this increased growth suggesting estrogen desulfation as an important regulator in CRC proliferation.

STS over-expression increases CRC xenograft growth.

As HCT116[sts] cells exhibited increased proliferation *in vitro*, we next examined whether this effect was evident in an intact, female mouse CRC xenograft model. HCT116[sts] or HCT116[vo] cells (1×10^6) were subcutaneous implanted into the flanks of female MF-1 nude mice. Intact adult female mice were chosen as they have circulating E₁S and E₂S available for hydrolysis. Over 21 days, HCT116[sts] xenograft growth was significantly ($p < 0.01$) greater compared to HCT116[vo] controls (Figure 3C), leading to a greater tumor burden by day 21 post implantation (Figure 3D and Figure 3E). Dosing of STX64 (20mg/kg, p.o., thrice weekly) initially completely stagnated (days 3–18) HCT116[sts] growth (Figure 3C), although tumors were proliferating by day 24. Despite tumor STS activity being almost

completely ablated by STX64 treatment (Figure 3F), HCT116[vo] xenograft growth was not affected by STS inhibition. This suggests once STS is over-expressed, CRC may rely more heavily on estrogen desulfation as pro-proliferative.

Estrogens increase proliferation through GPER signaling in CRC.

As controversy surrounds how estrogens elicit their effects in CRC (24), we investigated whether GPER was expressed in human CRC and if GPER-stimulation augmented CRC proliferation. In contrast to HCT116 and HT-29 cells, Caco2 and Colo205 cells express ER α . None of the CRC cell lines tested expressed ER β , but all expressed GPER (Figure 4A). Others have shown (21) in breast cancer that GPER stimulation with E₂ can increase proliferation and increase the expression of various downstream regulators of survival and migration (Figure 4B). Thus, we next examined whether the specific GPER agonist G1 (72h treatment) stimulated HCT116, HT-29 and Caco2 cell proliferation, as measured by BRdU incorporation, compared to sFBS controls (Figure 4C). G1 induced a dose-dependent stimulation in proliferation, with this effect more pronounced in HCT116 and HT-29. These results mimicked proliferative effects by E₁ and E₂ (see Figure 2B and Figure 2C). Intriguingly, Caco2 cells modestly responded to G1 treatment in contrast to their lack of increased proliferation in response to E₂ (Figure 2B) supporting the notion that rapid E₂ oxidization in Caco2 limits estrogenic effects. However, when GPER is stimulated by G1, Caco2 cells can increase proliferation through this pathway. In HCT116 and HT-29 cells, the GPER antagonist G15 (1 μ M) blocked both E₂- and G1-stimulated proliferation over 72h compared to controls (Supplementary Figure 4A).

To further delineate GPER action in CRC, we also determined how E₂ and G1 affected downstream molecular regulators of GPER action (21). Figure 4B illustrates the key genes that we examined in CRC cells, namely FOS, EGR1, ATF3, CTGF, DUSP1, and TNF α . All these genes are upregulated in response to GPER stimulation in breast cancer cell lines (21). In HT-29 cells EGR1 (Supplementary Figure 5A), ATF3 (Supplementary Figure 5B), DUSP1 (Supplementary Figure 5C), and CTGF (Supplementary Figure 4D) but not FOS (Supplementary Figure 4E) and TNF α (Supplementary Figure 4F), were significantly elevated in response to E₂ (100nM for 24h) and G1 (100nM for 24h) compared to sFBS treated cells. In HCT116 cells, EGR1, ATF3 and CTGF were significantly elevated in response to treatment. As CTGF gave the largest response to E₂ and G1 stimulation, we further examined its protein expression in response to treatment. In HCT116 and HT-29 cells, E₂ (100nM) and G1 (100nM) increased CTGF protein expression after 24h as measured by immunoblotting (Figure 4D).

To confirm the importance of GPER or CTGF in mediating the pro-proliferative effects of E₂, we next did transient knockdown of these two proteins using siRNA and determined their response to E₂ treatment. In HCT-116 cells, siRNA of GPER and CTGF provided protein knockdown for 96h, the length of time required for subsequent proliferation studies (Figure 4E). Knockdown of GPER and CTGF significantly ($p < 0.001$ and $p < 0.05$, respectively) inhibited the proliferation driven by E₂ and G1 in HCT-116 (Figure 4F). Intriguingly, when we again moved into an *in vivo* model of CRC, the use of the GPER antagonist G15 (at 50 μ g/kg i.p. thrice weekly) also significantly ($p < 0.01$) inhibited HCT116[sts] xenograft growth implanted into female nude mice (Figure 4G). Indeed, interrogation of the TCGA COAD data-set indicated that although all estrogen receptors (ER α , ER β , and GPER) were

significantly ($p < 0.0001$) down-regulated in CRC compared to normal controls (Supplementary Figure 6A), GPER still has the highest expression in CRC. Further analysis of the TCGA dataset demonstrated that patients with CRC tumors expressing high GPER had significantly ($p = 0.0431$) poorer outcomes compared to low to mid expression levels (Figure 4H). Indeed, CRC with high ER α also had significantly ($p = 0.0265$) worse outcomes (Supplementary Figure 6C), suggesting the importance of these pro-proliferative pathways in CRC. High ER β expression did not affect CRC patient outcomes (Supplementary Figure 6D). With regards to CTGF expression, analysis of the TCGA COAD dataset showed increased expression in CRC compared to normal colon (Supplementary Figure 6B). CRC with high mRNA expression of CTGF resulted in a significantly ($p = 0.0272$) poorer outcome (Supplementary Figure 6E).

However, as mRNA and protein expression correlation is notoriously poor, hovering around 40% explanatory power across many studies (25) and GPER protein expression was present in CRC cell lines (Figure 4A), we determined GPER protein expression in our human CRC samples and demonstrated an almost significant ($p = 0.054$) increase in expression in CRC (Figure 5A, Figure 5B, and Supplementary Figure 7 shows all original immunoblots). CTGF mRNA (Supplementary Figure 6B) and protein was significantly ($p < 0.001$) increased in CRC as determined by relative densitometry (Figure 5B). Relative intensity of immunoblots for GPER and CTGF highlighted a significant ($p = 0.0042$) positive correlation between GPER and CTGF expression in cancerous tissue but not matched normal controls (Figure 5C). As GPER stimulation increases CTGF expression our results indicate greater estrogen availability through STS activity in these tumors may lead to increased GPER stimulation and CTGF expression (Figure 5D).

Discussion

Here we demonstrate a critical role for pre-receptor local estrogen metabolism and action in the proliferation of CRC. For the first time we show estrogen synthesis pathways, via STS, HSD17B7 and HSD17B12, are elevated in CRC and estrogens stimulate CRC growth through a GPER-mediated mechanism. Of particular importance is STS, a key regulator in estrogen activation. When over-expressed in HCT116 cells STS drives greater tumor proliferation in *in vitro* and *in vivo* models. Finally, we demonstrate E₂ acts through GPER signaling, most likely via CTGF, in CRC, and that both GPER and CTGF are increased in human CRC. Our results suggest that inhibiting GPER or estrogen metabolism may be a novel therapeutic option for this malignancy.

Controversy exists on estrogens role in CRC development and progression. The Women's Health Initiative (26) has highlighted various questions on how estrogens and progestins impact cancer. Epidemiological studies indicate estrogens as protective against CRC development. However, how estrogens impact CRC once it has developed is poorly defined. It has been suggested that whilst initially protective, estrogens may be mitogenic in CRC (24) through changes in local estrogen metabolism and receptor availability. Indeed, estrogens promote tumorigenesis in colitis-associated CRC (27), and E₂ increases LoVo cell line proliferation via up-regulation of fatty acid synthesis (28). However, few studies have investigated enzymes involved in estrogen metabolism in CRC, and the ones that have overlooked key 17 β HSDs and STS activity. Furthermore, although evidence strongly suggests that ER β down regulation, and thus the loss of this pro-apoptotic pathway, is an important turning-point in CRC development (16), whether GPER expression or stimulation impacts CRC has not previously been determined.

We show that STS activity is significantly elevated in human CRC and that STS over-expression stimulates CRC cell proliferation. Previous findings had indicated increased STS expression as prognostic for CRC survival (9), however this study did not measure STS activity. This is an important distinction. STS is subject to post-translational modifications affecting activity and we show here colon STS activity and expression do not correlate. Furthermore, analysis of the TCGA COAD database demonstrated no significant changes in STS expression in colon cancer compared to normal controls. Although eventual patient outcomes have not been determined, we show STS activity does not correlate to Duke's staging or tumor T-stage implying increased STS activity is most likely an early event in CRC development, and thus its prognostic significance is questionable (9). Along with increased aromatase expression, elevated STS activity is a hallmark of estrogen-dependent cancers (29). STS inhibition is currently in Phase II clinical trials in patients with hormone-dependent breast cancer, after it had shown promise in pre-clinical studies against E_2 S-stimulated breast cancer *in vivo* as well as in Phase I trials (30). As aromatase expression is not detectable in the human colon (9), local desulfation of circulating E_1 S may act as the primary route for estrogen availability in CRC.

Once desulfated, HSD17B1, HSD17B7, and HSD17B12 reduce E_1 to E_2 , with HSD17B2 catalyzing reverse oxidation. Supporting our findings, TCGA COAD analysis and others (23) have shown that HSD17B2 expression is down-regulated in CRC, however our data here indicates no change in HSD17B2 protein expression suggesting this pathway may remain active. Although HSD17B1 is the prime reducer of E_1 (31) we demonstrate this enzyme is absent in CRC. Interestingly, HSD17B7 and HSD17B12 expression are significantly up-

regulated in CRC compared to matched normal controls, with this effect mimicked at the protein level, and our findings are supported by TCGA COAD data analysis. Thus, CRC may favor E₂ synthesis. Recently, pre-clinical studies show inhibition of HSD17B7 in hormone-dependent breast cancer blocks E₁ to E₂ synthesis and thus has therapeutic potential (32). As intratumoral E₁ and E₂ concentrations in CRC tissue pertains to a poor prognosis (9), inhibiting these enzymes in CRC may be therapeutically beneficial.

As ER α and ER β are not present in the CRC cell lines tested, a question arose, how do estrogens act in CRC? Limited data on colonic GPER expression exists: GPER stimulation may affect colonic motility in mice (33) and its expression may influence abdominal pain severity in IBD (34). We demonstrate GPER protein, but only limited mRNA, is expressed in human CRC tissue and cell lines. GPER protein expression is elevated in human CRC tissue compared to matched normal controls, in contrast to mRNA which is decreased. This may imply that GPER protein degradation pathways may be altered in CRC, effectively allowing for GPER protein retention. Stimulation of GPER by E₂ or the specific agonist G1 increased CRC proliferation *in vitro*, with this effect blocked by GPER inhibition in *in vitro* and *in vivo* CRC models. In contrast to our findings, recent research has shown GPER stimulation by G1 decreases proliferation of various CRC cell lines, including HCT116 (35). However, these studies used higher doses of G1 (up to 10 μ M) compared to our 100 nM dose, and, unlike the work presented here, the studies are not performed in stripped-media (i.e. no/low estrogen) conditions. Thus, this suggests there may be a biphasic response to G1 and estrogens with regards GPER stimulation in CRC, with low doses increasing proliferation and high doses inducing apoptosis. This biphasic response is also evident with ER α stimulation in breast cancer (36).

GPER deficiency results in multiple physiological alterations including obesity, cardiovascular dysfunction, insulin resistance and glucose intolerance (37), and there is much interest in its pro-proliferative effects in breast cancer. In breast cancer patients, GPER expression is associated with increased primary tumor size and the prevalence of distant metastasis (38). Indeed, GPER-stimulation by tamoxifen is a potential pathway of tamoxifen-resistant hormone-dependent breast cancer (39) and intriguingly, breast cancer patients treated with tamoxifen are more likely to develop CRC (40). Our results strongly implicate E₂-GPER-mediated action, through CTGF, in CRC proliferation. As the loss of ER β defines CRC development (16), it will be of interest to further examine GPER action in the context of ER β expression to determine whether an estrogen receptor “switch” occurs during CRC progression.

Furthermore, in CRC cell lines, the expression of CTGF, a known downstream regulator of GPER action (21), was elevated by E₂ and G1 treatment. A correlation is evident between GPER and CTGF expression in human CRC tissue. CTGF is up-regulated in some CRC patients (41), although its expression reduces in latter-stage disease (42). Analysis of the TCGA COAD dataset also suggests that high CTGF is related to poor patient survival, although others have shown high CTGF expression correlates to improved CRC survival rates (41). This implies a complicated relationship between E₂-stimulation of GPER, increased proliferation, CTGF-mediated effects, and patient outcomes. However, in general, dysregulation of CTGF expression is linked to poor outcomes in many human cancers (43).

In conclusion, we have identified a new estrogen-driven proliferative pathway in CRC. Increased STS activity leads to greater estrogen desulfation, thereby increasing HSD17B substrate availability for subsequent E₂ synthesis, followed by GPER activation and CTGF up-regulation. These findings identify STS, 17BHS7, 17BHS12, and GPER as potential new therapeutic targets for CRC.

Experimental Procedures

Compounds

STX64 (Irosustat) was from Sigma-Aldrich Ltd. (Dorset, UK). G1 and G15 were from Torcis Bioscience (Abingdon, UK). E₁S, E₁, E₂S, and E₂ were from Sigma-Aldrich.

Human tissue and cell culture

Matched normal and cancerous human colorectal tissue was obtained with local ethics committee approval and informed patient consent. CRC samples from patients with genetic pre-disposition to CRC, such as familial adenomatous polyposis (FAP) and hereditary non-polyposis colorectal cancer (HNPCC) were excluded. Patients currently on HRT were also excluded. Patient characteristics and disease stage are outlined in Supplementary Table 1.

HCT116 and HT-29 cells were cultured in McCoy's 5a modified medium (Life Technologies). Caco2 cells were cultured in MEM and JEG-3 cells in DM-F12 (Life Technologies). All medium was supplemented with 10% FBS (Sigma-Aldrich) and 2mM L-glutamine (Sigma-Aldrich). All cell lines were authenticated (March 2014) by short tandem

repeat profiling and regularly mycoplasma tested (every 6 months). After 20 passages cells were discarded and fresh cells obtained. For all estrogen and GPER antagonist/agonist experiments, charcoal stripped FBS was used in phenol-free media. Charcoal-stripping of FBS is known to reduce estrogen concentrations to undetectable levels.

Data sets

Normalized gene expression data generated using the Illumina RNA-seq platform (accessed Jan. 2017) and clinical information was downloaded from cBioPortal (44). Gene expression values were transformed as $X = \log_2(X + 1)$ where X represents the normalized fragments per kilobase transcript per million mapped reads (FPKM) values. Transcriptomic and clinical information was analyzed for 284 patients with colon cancer.

Generation of STS over-expressing HCT116 cells

HCT116 cells were transfected using Lipofectamine (Invitrogen, Paisley, UK) with a pCl-neo (Promega) construct containing either vector only [vo] or complete coding sequence for the human STS [sts] gene. Cells were subsequently grown in 1 mg/mL G418 (Promega). STS activity was routinely measured to determine STS transfection stability.

STS activity assay

STS activities of human CRC tissue samples and cell lines were measured as previously described (45). Briefly, tissue and cell supernatants were incubated with [6,7-³H] E₁S (4 x 10⁵ dpm, Perkin-Elmer) adjusted to a final concentration of 20 μM with unlabeled E₁S (Sigma).

[4-¹⁴C] E₁ (1 x 10⁴ dpm, Perkin-Elmer) was included to monitor procedural losses. Samples were incubated at 37°C after which the product, E₁, was separated from E₁S by partition with toluene. ³H and ¹⁴C radioactivity was measured by liquid scintillation spectrometry. Mass of E₁S hydrolysed was calculated from ³H counts detected corrected for procedural losses. Results were determined as pmol product formed/h/mg protein.

qRT-PCR analysis

From human samples 30mg tissue was homogenised in RLT buffer containing β-mercaptoethanol. cDNA was manufactured with SENSIFast kit (Bioline) using 1 μg mRNA as per the manufacturers' instructions. From cell lines mRNA was purified using RNeasy kits (QIAGEN) as per the manufacturers' instructions. mRNA samples were reverse transcribed to form cDNA using Tetro cDNA Synthesis Kit (Bioline Reagents Ltd.).

Expression of specific mRNAs was determined on a 7500 Real-time PCR system (Applied Biosystems) using the QuantiTect Probe RT-PCR kit (Qiagen). Relative expression was determined using the 2^{-ΔΔCt} method. Taqman assays are described (Supplementary Table 4)

Immunoblotting

Blots were probed as outlined in Supplementary Table 5. Secondary antibodies were from Santa Cruz, goat anti-mouse (sc-2005) and goat anti-rabbit (sc-2004). Bound antibody was detected with horseradish peroxidase-conjugated secondary antibody and chemiluminescence. Bands were quantified using Image J software from NCBI (<http://rsbweb.nih.gov/ij/>). Images were converted into binary mode and ratios derived by comparing protein of interest bands to β-actin.

LC-MS/MS

Estrogens were measured by uHPLC-MS/MS. After addition of internal standard steroids (E_1S -d4, E_2S -d4 (Cambridge Isotopes) and ^{13}C E_2 (Sigma)) samples were extracted using solid phase extraction (C18 Isolute SPE columns 500mg, Biotage). Estrogens were quantified relative to a calibration series (0.5-500ng/L) via tandem mass spectrometry. A Waters Xevo mass spectrometer with an electrospray ionization source was used with an attached Acquity liquid chromatography system. Estrogens were eluted from a HSS C18 SB 1.8 μ m, 2.1 x 30mm column using a methanol/water gradient system with 0.3mM ammonium fluoride added to the aqueous phase. The coefficient of variation for all assays was less than 20%.

siRNA Design and Transfection

The siRNA oligonucleotides and transfection reagents were purchased from Dharmacon, Inc. (Lafayette, CO). The predesigned ON-TARGETplus SMARTpool for human GPER and human CTGF genes, containing a mixture of four-targeting siRNA oligonucleotides, was used for knockdown. An ON-TARGETplus Non-Targeting pool, containing four nonspecific siRNA oligonucleotides, was used for control. For siRNA transfection, HCT116 cell were cultured overnight and subsequently transfected with control or GPER or CTGF siRNA oligonucleotides using DharmaFECT transfection reagent according to the manufacturer's instructions. The medium was changed to sFBS media every 24h, and in certain experiments included E2 (100 nM) or G1 (100 nM). Proliferation assays were started 48 h after siRNA transfection.

In vivo xenograft studies

Six week old athymic, female CD-1 nude mice (*nu-/-nu-*) were purchased from Charles River. All experiments were carried out under conditions that complied with institutional guidelines. Five million HCT116 cells were injected s.c. into the right flanks of the animal. For STS inhibition studies, when tumors reached 70 to 100mm³, mice were randomly divided into two treatment groups: oral vehicle (10% ethanol:90% propylene glycol thrice weekly) or oral STX64 (20 mg/kg/thrice weekly). For GPER inhibition studies HCT116[sts] bearing animals were randomly divided into two treatment groups: i.p. vehicle (0.9% NaCl, 0.1% Tween80, 1% EtOH, thrice weekly) and G15 (50 µg/kg, i.p. thrice weekly). Mice were weighed and tumor measurements taken thrice weekly with the researcher blinded to groups. Tumor volumes were calculated using the formula (length × width²/2). At the conclusion of dosing, animals were terminated and their tumors removed, weighed, and stored at -80°C.

Proliferation assays

Cell proliferation was measured by the CyQuant cell proliferation (Thermo Fisher Scientific) and BrdU incorporation assays (Roche Applied Science) as per the manufacturer instructions. Prior to experiments, cells were placed into stripped-FBS phenol-red free medium (Thermo Scientific) with 5mM L-glutamine for 72h to clear any remaining estrogens in the media. Cells were cultured in flat-bottom 96-well plates in either complete or stripped FBS phenol-free growth media containing estrogens and subsequent assays performed.

Statistics

For human data the population analyzed was described using summary statistics and relationships between STS activity and STS expression investigated by plotting the data and

calculating correlation coefficients. Further analyses used random effects linear regression modeling (with outcomes transformed to reduce the impact of outliers where appropriate) to allow for normal and cancer samples being patient matched. Models were fitted to investigate differences in STS activity, HSD17B7 mRNA expression, HSD17B12 mRNA expression and HSD17B2 mRNA expression between normal and cancer cohorts. For the primary analysis (investigation of differences in STS activity) models were fitted with and without adjustment for patient characteristics (sex, age and BMI) and stage (T and Dukes). For other models, adjustment was made for sex and age. Where model outcomes required log transformation the estimates obtained are interpreted as approximate percentage differences.

For *in vivo* experiments involving multiple treatment groups, a one-way ANOVA followed by a Tukey multiple comparison test was done to determine statistical significance. Where only two groups are compared, a Student's *t* test was applied. All analysis related to TCGA COAD patient survival curves were testified by Kaplan-Meier survival analysis (Log-rank method). All statistics were performed on Prism 5.0 software.

Author Contribution

LCG, DM, and PAF designed and conceived the research studies. LCG, HPR, AMH, AG, AA, and PAF performed experiments, interpreted and analyzed data. AJS analyzed the data. MLR helped with analysis of the TCGA database. LCG and AET designed novel LC/MSMS methods. PAF created the figures and wrote the manuscript

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Figure Legends

Figure 1: Estradiol synthesis pathways are up-regulated in human CRC. (A) Estrogen metabolism pathways demonstrating the importance of STS and HSD17Bs in estrogen

synthesis. **(B)** STS activity is increased in female (n = 29) and male (n = 31) CRC compared to matched normal colon tissue. * p < 0.05, ** p < 0.01, *** p < 0.001 using random effects linear regression modeling. **(C)** STS activity does not correlate to STS expression (dCT) in CRC or normal colon tissue (n = 62). **(D)** HSD17B2 (female, n = 19; male, n = 28) expression is down-regulated in CRC. * p < 0.05, ** p < 0.01, *** p < 0.001. (2-tailed paired Student's *t* test was used). **(E)** and **(F)** HSD17B7 (female, n = 21; male, n = 22) and HSD17B12 (female, n = 21; male, n = 22) expression is up-regulated in CRC. * p < 0.05, ** p < 0.01, *** p < 0.001. (2-tailed paired Student's *t* test was used). **(G)** and **(H)** representative blots and relative intensity (AU) of HSD17B enzymes in normal and matched cancerous human colon tissue. HSD17B1 (n = 16) protein expression is not present in human CRC, whereas HSD17B7 is expressed, and HSD17B12 expression is increased (n = 16), with little change in HSD17B2 expression (n = 16). For relative intensity data, a 2-tailed Student's *t* test was used. All data represents mean ± s.d.

Figure 2: Estrogens increase proliferation in CRC cell lines. **(A)** Expression profile of HSD17B1, HSD17B2, HSD17B7, and HSD17B12 in HCT116, HT29, Caco-2, and Colo205 cells. β -actin was used as a loading control. 1 representative blot from 3 independent experiments. **(B)** and **(C)** Estrone (E_1) and estradiol (E_2) increase proliferation rates in a dose-dependent manner in HCT116 and HT-29 cells. Caco-2 cells did not respond to E_1 or E_2 treatment. (n = 4 independent experiments). **(D)** and **(E)** HCT116 cells do not readily metabolize E_1 , E_2 , and E_1S . HT-29 cells metabolize E_1 and E_2 to an unknown metabolite. Caco-2 cells rapidly metabolized E_2 to E_1 . (n = 3 independent experiments). All data represents mean ± s.d.

Figure 3: Over-expression of STS in HCT116 cells increases estrogen-dependent proliferation *in vitro* and *in vivo*. **(A)** HCT116[sts] cells proliferate at a greater rate compared to HCT116[vo] cells. This proliferation is significantly inhibited by STX64 (1 mM). ** $p < 0.01$, *** $p < 0.001$. (n= 3 independent experiments, One-way ANOVA followed by a Tukey multiple comparison post test). **(B)** HCT116[sts] cells had increased proliferation when stimulated with E₂S (100 nM for 72h) compared to HCT116[wt] and HCT116[vo] cells. This increased proliferation was blocked by STS inhibition using STX64 (1 mM). *** $p < 0.001$ compared to control; ## $p < 0.01$, ### $p < 0.001$ compared to E₂S treatment; a $p < 0.001$ compared to HCT116[vo]. (2-tailed Student's *t* test was used, n = 4 independent experiments). **(C)** HCT116[sts] xenografts grow at an increased rate compared to HCT116[vo] xenografts. This increased proliferation was inhibited by STX64 (20 mg/kg/thrice weekly, p.o.). ** $p < 0.01$, *** $p < 0.001$. (One-way ANOVA followed by a Tukey multiple comparison post test). **(D)** Five randomly taken tumors imaged after removal. **(E)** Wet tumor weights at 21 days post HCT116 cell inoculation. HCT116[sts] resulted in increased tumor burden, which was inhibited by STX64. * $p < 0.05$, *** $p < 0.001$. (2-tailed Student's *t* test was used). **(F)** STS activity in HCT116[vo] and HCT116[sts] xenografts at day 21. HCT116[sts] xenograft maintained elevated STS activity compared to HCT116[vo]. STX64 treatment significantly inhibited HCT116[vo] and HCT116[sts] activity. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. (n = 5 – 14, 2-tailed Student's *t* test was used). All data represents mean \pm s.d.

Figure 4: E₂ acts through GPER signaling to increase CRC proliferation. **(A)** ER α and ER β are not expressed in HCT116 or HT-29, but are present in Caco2 and Colo205 cells. GPER is expressed in all cell lines tested. β -actin was used as a loading control. 1 representative blot expressed in all cell lines tested. **(B)** Schematic of the downstream molecular signaling

factors stimulated by GPER action as defined in breast cancer. (C) The GPER agonist G1 increases proliferation rates in a dose dependent manner compared to cells grown only in media with SFBS. (2-tailed Student's *t* test was used, n = 4 independent experiments) (C) The GPER antagonist G15 (1 mM) inhibits the increased proliferation induced by E₂ (100 nM for 72 h) and G1 (100 nM for 72 h) in HCT116 and HT-29 cells. ** p < 0.01, *** p < 0.001 compared to controls. (2-tailed Student's *t* test, n = 4 independent experiments) (D) E₂ (100 nM) and G1 (100 nM) treatment increases CTGF protein expression in HCT116 and HT-29 cells. β-actin was used as a loading control. 1 representative blot from 3 independent experiments. (E) siRNA knockdown of GPER and CTGF in HCT116 cells was achieved for 96h post siRNA treatment. (F) siRNA knockdown of GPER and CTGF inhibits E₂ (100 nM) and G1 (100 nM) stimulation of HCT116 proliferation. * p < 0.05, *** p < 0.001 compared to controls. (Two-way ANOVA followed by a Bonferroni post-test, n = 3). (G) G15 (50 mg/kg/thrice weekly, i.p.) significantly attenuates HCT116[sts] xenograft tumor growth in female nude mice. ** p < 0.01 (Two-way ANOVA, n = 10). (H) Patients with high GPER expression (n = 110) had a significantly worse survival outcome compared to mid-low GPER expressing (n = 330) CRC tumors, as shown from analysis of the TCGA COAD dataset. (Kaplan-Meier survival analysis (Log-rank method). All data represents mean ± s.d.

Figure 5: CTGF and GPER expression correlate in human CRC. (A) Immunoblotting of GPER and CTGF expression in normal (N) and cancerous (C) human colon tissue. β-actin was used as a loading control. 1 representative blot from 3 independent experiments. (B) GPER and CTGF expression are increased in human CRC, as measured by immunoblotting relative intensity to β-actin * p < 0.05, *** p < 0.001. (2-tailed Student's *t* test was used, n = 17) (C) Correlation between GPER and CTGF relative intensity in normal and cancerous human colon tissue. n = 17. (D) Schematic showing proposed novel pathway through which estrogens act, via GPER, to augment proliferation in CRC.

