UNIVERSITY OF BIRMINGHAM | IMSR INSTITUTE OF METABOLISM AND SYSTEMS RESEARCH

Oestrogens Regulate Proliferation in Colorectal Cancer via GPER and the Hippo signalling pathway

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Abstract

The concentration of circulating oestrogens is associated with the incidence and outcomes of colorectal cancer (CRC). Both the physiological and pathological effects of oestrogens are mediated by oestrogen receptors. This project aims to investigate the potential role of G protein-coupled oestrogen receptor 1 (GPER) as an oestrogen-induced mediator in CRC proliferation. To achieve this, colorectal adenoma and carcinoma cell lines were examined for protein expression of oestrogen receptors. Immunoblotting did not report ERα and ERb expression, although GPER was detected. Following this, the GPER-associated gene connective tissue growth factor *(CTGF)* expression was measured after 17β-estradiol (E2) and GPER agonist (G1) treatment. qRT-PCR results showed no significant increase in *CTGF* expression levels, 24 and 48 hours after treatment. In addition, GPER interaction with the Hippo pathway in CRC was examined by treating cells with E2 and G1 for 0, 15, 30 minutes, 1, and 2 hours. Alterations in P-YAP1 expression were unclear after treatment in the cell lines examined. However, addition of GPER antagonist, G15, resulted in significant inhibition in YAP1 phosphorylation in HCT116 cells, 15 and 30 minutes of treatment. Consequently, our data supports that oestrogens and G1 treatment leads to increase in YAP1 phosphorylation and nucleus-cytoplasmic shuttling via GPER stimulation. YAP1 knock-down studies and pharmacological inhibition followed by proliferation assays established that this early metabolic effect translates into increased cellular proliferation. Collectively, our data propose a novel oestrogen-driven pro-proliferative pathway via GPER through Hippo pathway's key downstream effector, YAP1, in CRC. Further studies are required to reveal the detailed signalling cascade by which GPER mediates the increased YAP1 phosphorylation.

Nothing in this world is to be feared...

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Publications/Abstracts

1. Gilligan L.C., Rahman H.P., Hewitt A., Sitch A.J., Gondal A., **Arvaniti A.,** Taylor A.E., Read M.L, Morton D., Foster P.A. Estrogen Activation by Steroid Sulfatase increases Colorectal Cancer proliferation via GPER. J Clin Endocrinol Metab. 2017; 2016-3716. doi: 10.1210/jc.2016-3716

2. Gilligan L.C, Gondal A., Tang V., Hussain M.T, **Arvaniti A.,** Hewitt A.M, Foster P.A. Estrone Sulfate Transport and Steroid Sulfatase Activity in Colorectal Cancer: Implications for Hormone Replacement Therapy. Front Pharmacol. 2017; 8: 103. doi: 10.3389/fphar.2017.00103

3. **Arvaniti A**., Giligan L.C., Rahman H.P., Gondal A., Foster P.A. Oestrogens Stimulate Proliferation in Colorectal Cancer via GPER and the Hippo signaling pathway. Endocrine Abstracts (2016) 44 OC3.6 DOI:10.1530/endoabs.44.OC3.6. Oral communication.

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List of Abbreviations

AA/C1-	Human colonic adenoma cells
ABL-	Abelson murine leukemia viral oncogene homolog
APC-	Adenomatous polyposis coli
Akt-	Protein kinase B (PKB)
BCA-	Bicinchoninic Acid
BSA-	Bovine serum albumin
BrdU-	Bromodexyuridine
CACO2-	Human epithelial colorectal adenocarcinoma cells
CDK1-	Cyclin-dependent kinase 1
CIMP+-	CpG island methylator phenotype
CIN-	Chromosomal instability
CO2-	Carbon dioxide
CRC-	Colorectal Cancer
CTGF-	Connective Tissue Growth Factor
Ca+-	Calcium ATPase
DMEM-	Dulbecco's Modified Eagle Media
DMSO-	Dimethyl Sulphoxide
Ds-	Dachsous atypical cadherin
E1-	Oestrone
E1S-	Oestrone Sulfate
E2-	17β-oestradiol
E3-	Oestriol
E4-	Oestetrol
ECL-	Electrochemiluminescence
EGF-	Epidermal Growth Factor
EGFR-	Epidermal growth factor receptor
ELISA-	Enzyme-Linked Immunosorbent Assay
EMT-	Epithelial-mesenchymal transition
ER-	Oestrogen Receptor
ERE-	Oestrogen response elements
ERK1/2-	Extracellular signal–regulated kinases or classical MAP kinases
ESR1-	Oestrogen receptor 1 gene
ESR2-	Oestrogen receptor 2 gene
EtOH-	Ethanol
FAP-	Familial Adenomatous Polyposis
FASN-	Fatty acid synthase gene
FAT4-	Atypical cadherin 4
FBS-	Foetal Bovine Serum
FN-	Fibronectin
FOB-	Faecal occult blood
G1-	GPER specific agonist
G15-	GPER specific antagonist
G2/M-	Damage checkpoint, prevents entering mitosis (M) with DNA damage

GPER-	G Protein-coupled Oestrogen Receptor
HCT116-	Human epithelial colorectal carcinoma cells
HIPK-	Homeodomain-interacting protein kinase
HNPCC-	Hereditary nonpolyposis colorectal cancer
HRP-	Horseradish Peroxidase
HRT-	Hormone Replacement Therapy
HSD-	Hydroxysteroid Dehydrogenase
HT29-	Human epithelial colorectal adenocarcinoma cells
IBS-	Irritable Bowel Syndrome
IGF-	Insulin-like Growth Factor
IL6-	Interleukin 6
ILK-	Integrin-linked kinas
JNK-	c-Jun N-terminal kinases
KIBRA-	Bone fide human tumour suppressor
KRAS-	Proto-oncogene
LATS1/2-	Large tumour suppressor kinase ½
MAP-	Mitogen-activated protein kinase
MEM-	Minimum Essential Media
MISS-	Membrane-initiated steroid signalling
MMR-	DNA mismatch repair
MOB1-	Activator 1 A/B
MSI-	Microsatellite instability
MST1/2-	Mammalian sterile like kinase
NF2-	Neurofibromin 2
OATP-	Organic anion transporter polypeptide
OHT-	4-hydroxytamoxifen, anti-oestrogen
P-YAP1-	Phosphorylated Yes-associated protein 1
P21-	Cyclin-dependent kinase inhibitor (CKI)
P38-	Mitogen-activated protein kinases (MAPK)
P53-	Tumor suppressor protein
PBS-	Phosphate Buffered Saline
PIK3CA-	Phosphatidylinositol 4,5-bisphosphate 3-kinase catalytic subunit alpha isoform
PVDF-	Polyvinylidene Fluoride
RG/C2	Human colonic adenoma cells
ROS-	Reactive oxygen species
RPLPO-	Large Ribosomal Protein
SAV-	Salvador protein kinase
SERM-	Selective Oestrogen Receptor Modulator
SF1-	Splicing factor 1 or zinc finger protein 162
SKBR3-	Mammary gland/breast cells, derived from metastatic site
STE20-	Serine/threonine-protein kinase family member
STS-	Steroid Sulfatase
SULT1E1-	Oestrogen Sulfotransferase
SW480-	Human epithelial colorectal adenocarcinoma cells
Shc-	Shc-transforming protein 1
Src-	Proto-oncogene tyrosine-protein kinase
TAZ-	PDZ-binding motif
TBS-	Tris-Buffered Saline

TEAD1-4-	TEAD family transcription factors
TEMED-	Tetramethylethylenediamine
TGFβ-	Transforming Growth Factor β
TNM-	Tumour Node Metastasis
V-	Voltage
VEGF-	Vascular Endothelial Growth Factor
VGLL4-	Vestigial-like protein 4
WHI-	Women's Health Initiative
YAP1-	Yes-associated protein 1
c-Fos	Proto-oncogene
cAMP-	Cyclic adenosine monophosphate
dsFBS-	Double Stripped Foetal Bovine Serum
mRNA-	Messenger RNA
qRT-PCR-	Quantitative Real Time Polymerase Chain Reaction
sFBS-	Stripped Foetal Bovine Serum
siRNA-	Small interfering RNA
°C-	Celsius scale

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1. Introduction

1.1 Cancer Epidemiology

For many years, cancer has remained one of the global primary causes of death following cardiovascular diseases. Cancer is the outcome of a vast and diverse spectrum of diseases, all of which are characterised by the uncontrolled growth of cells, often involving invasion into surrounding tissues and possibly metastasis into distant organs. The prevalence rates of individual cancers differ between men and women. In men, the most common cancers include lung, prostate and colorectal cancer, while in women the most common cancer is breast cancer, followed by colorectal and cervical cancer [1].

1.1 Colorectal Cancer

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in both sexes and the second cause of cancer-related death in Europe [2]. Worldwide CRC incidence is expected to increase by 60% by the year 2030 and more than 2.2 million new cases are estimated to occur [3,4]. Despite CRC developing in a wide geographic area, its burden is positively influenced by development and westernisation. As a result CRC is most commonly seen in industrialized countries such as in Western Europe, North America, New Zealand and Australia [5].

Hereditary nonpolyposis colorectal cancer (HNPCC), also known as Lynch Syndrome, is a genetic condition predisposing patients to CRC. It accounts for about 3% of all CRCs and is

associated with the development of up to 100 colonic polyps, usually in the right colon [6]. Familial Adenomatous Polyposis (FAP), the most common adenomatous polyposis syndrome, accounts for about 1% of CRC and the key feature of FAP is the presence of thousands of colonic polyps which usually increase with age [7].

As expected, increasing age is strongly associated with CRC incidence. More specifically, the incidence of colorectal cancer increases dramatically above the age of 45 for both men and women. However, in most countries, the age-standardized incidence rates are considerably lower for women than for men [8].

In England from July 2006 NHS has introduced NHS Bowel Cancer Screening Programme (NHS BCSP). The members are offered two types of screening:

a) A faecal occult blood (FOB) test every two years between the ages of 60-74. The positive samples for blood in the stools are re-tested and biopsy, or a radiological test is offered [9].
b) A bowel scope screening to men and women at the age of 55 that involves examination of the lower part of the bowel.

Considering the growth in health service capacity for early detection through screening, there is an increasing likelihood there will be a need for preventative therapies for CRC. The current known therapeutic approaches, such as surgery and anti-inflammatory drugs [10,11], have limited efficacy, imposing the necessity for development of new preventative or targeted therapies [12].

1.1.2 Classification and Prognosis of Colorectal cancer

The treatment strategies and the prognostic prediction of patients vary according to the stage of CRC diagnosis[13]. In 1932 Cuthbert E. Dukes examined approximately 2,000 samples of CRC and developed the first classification system, highlighting the importance of the lymphatic metastasis and local tumour invasion in progression. This classification system has since been modified and in 1954 the Tumour, Node and Metastasis Classification (TNM) was proposed by Pierre Denoix. TNM is now the only classification system used for CRC [14]. According to TNM staging colorectal tumours are classified into three categories with "T" (tumour) to denote the degree of invasion of the bowel wall, "N" (node) the degree of lymphatic node involvement, and "M" (Metastasis) the degree of metastasis (Table 1).

Table 1: Dukes and TNM classification [5]

	Dukes	**TNM**
Tumour invasion confined to the mucosa	A	Tis, N0
Tumour invasion limited to the submucosa, no lymph node involvement	A	T1, N0
Tumour invasion limited to the submucosa, lymph node involvement	C	T1, N1-2
Limited tumour invasion into the muscle layer, no lymph node involvement	A	T2, N0
Limited tumour invasion into the muscle layer, lymph node involvement	C	T2, N1-2
During the whole muscle layer tumour involvement, no lymph node involvement	B	T3, N0
During the whole muscle layer tumour involvement, lymph node involvement	C	T3, N1-2
Tumours have kept the neighbouring organs, no lymph node involvement	B	T4, N0
Tumours have kept the neighbouring organs, lymph node involvement	C	T4, N1-2
Other factors notwithstanding distant metastases	D	T1-4,N0-2, M1

1.1.3 Colorectal carcinogenesis

Most CRCs develop as an outcome of accumulation of genetic and epigenetic changes in cancer-causing genes. These changes promote the formation of benign neoplastic adenomatous polyps in mucous-secreting epithelial cells. In the following stages of the disease the adenomatous polyps can evolve into late adenoma polyps which later progress to adenocarcinoma, and ultimately to invasive and metastatic colorectal carcinoma [15]. This transition was described by the Vogelstein *et al* as the adenoma-carcinoma sequence (Figure 1) [16]. The pathogenesis of CRC differs according to alterations in cancer-causing genes that contribute to tumour initiation and progression [17,18]. The Global genome sequencing of metastatic lesions and primary CRCs revealed hardly any metastasis-specific mutation [19]. The cellular environment plays a pivotal role to the occurrence of genomic instability that enables mutations and inherited factors to develop cancer [20,21]. In general, the most common genomic instability in CRC is chromosomal instability (CIN) which results in chromosomal abnormalities such as aneuploidy. Furthermore failure of the DNA repair system to detect and repair mismatched bases, promotes microsatellite instability (MSI) which also contributes to CRC along with epigenetic factors such as CpG island methylation phenotype ($CIMP^+$) [18].

In CIN and MSI the changes occur basically in two classes of genes, oncogenes and tumour suppressor genes[22]. The tumour suppressor gene Adenomatous polyposis coli *(APC),* the tumour suppressor protein p53 along with the proto-oncogene *KRAS* and *SMAD4* are the most thoroughly examined genes in CRC [18]. *APC* gene is located at chromosome 5q.22.2, mediates many primary cellular functions of intestinal cells including proliferation, stimulates the deactivation of β-catenin and restrains transcription of Wnt pathway target genes involved

in the cell cycle [23]. *P53* is located at chromosome 17p and is involved in the cell cycle, apoptosis and cellular senescence [24]. *KRAS* is located at 12p12.1 and various genetic alterations in the gene have been associated with poor prognosis for patients on epidermal growth factor receptor (EGFR) inhibitors [18]. *SMAD4* is located at 18q, and its low expression has been associated with poor prognosis and limited response to 5-Fluorouracil [18].

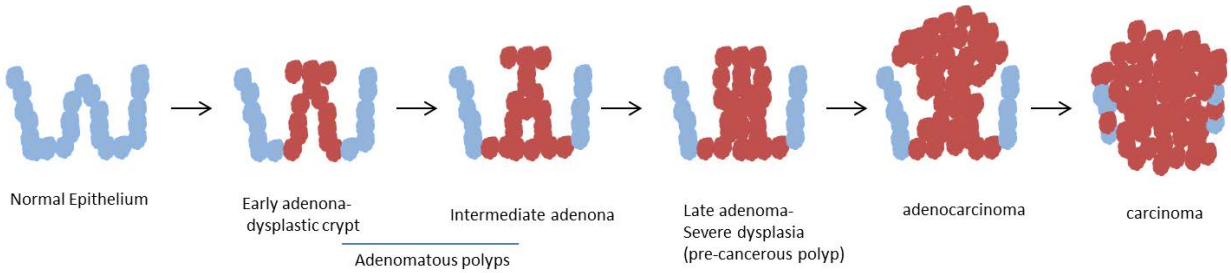

Figure 1: CRC initiation and progression. The adenoma-carcinoma sequence initiates with loss of the *APC* gene. Progression from adenomatous polyp to carcinoma is associated with a step-wise accumulation of genetic mutations in *KRAS*, *SMAD4*, loss of chromosome 18q and ultimately p53 inactivation (adapted figure) [18].

Taking into consideration the male preponderance, a person's lifetime risk of developing CRC is about 5% [25]. Smoking, obesity, alcohol consumption and increased consumption of red meat and are proven risk factors for CRC initiation in 70-80% of the cases. Genetic predisposition accounts for 35% of the risk of CRC initiation [26]. The presence of a first degree relative with CRC doubles compared to the average risk [27].

1.4 Oestrogen and Colorectal Cancer

Observational and clinical evidence has shown an association between hormones and mammalian neoplasia [28]. The first indication of this association in colorectal cancer was recorded in 1969 when a groundbreaking study about cancer and marital status took place. The data reported that nuns, who have no disruption in their menstrual cycle due to being nulliparous, thus greater lifetime exposure to oestrogen, were more likely to develop CRC [29]. The hypothesis that endogenous oestrogens act in a pro-proliferative manner in women was enhanced in 1980 when hormonal changes associated with pregnancy were correlated with lower CRC risk [30]. The study of Weiss and colleagues provided further evidence in support of this hypothesis, finding that, on average, women with CRC had given birth to fewer children [31]. In the following years many studies demonstrated that carcinogenesis in the colon is at least partially mediated by endogenous oestrogens [32,33]. Few studies observed no substantial effect or correlation between increasing parity and the risk of CRC [34,35].

A big controversy came in 1994 after a case-controlled study implicating Hormone Replacement Therapy (HRT) (conjugated oestrogen [oestrone sulfate (E1S)] plus medroxyprogesterone) as protective against CRC development in postmenopausal women [36,37]. In the following years, further human studies were warranted from the Women's Health Initiative (WHI) Clinical Trial and academic research groups highlighting the positive role of exogenous oestrogen such as HRT or oral contraceptives, in reducing CRC risk in postmenopausal women [38–43]. These findings suggested that oestrogens might be the key to the observed decline in CRC incidence in women but not in men in developed countries [44].

1.5 Oestrogen Biosynthesis and Metabolism Pathway

Oestrogens are a diverse group of cholesterol-derived steroid hormones including oestrone (E1), oestriol (E3), oestetrol (E4), and the biologically active with the highest affinity for oestrogen receptors, 17β-oestradiol (E2). In terms of serum levels and oestrogenic activity, E2 is dominant between the menarche and menopause stages of life with levels highest before ovulation. Oestriol (E1) and oestetrol (E4) are intensively produced during pregnancy and when menopause is achieved E1 becomes the primary circulating oestrogen [45,46].

In general, oestrogen metabolism is regulated by three key enzymes, 17β-hydroxysteroid dehydrogenases (17β-HSD), aromatase and steroid sulfate (STS) [47]. The *de novo* synthesis of oestrogen initiates in the ovaries, adrenal gland and testis by the synthesis of androstenedione from cholesterol. Androstenedione has weak androgenic activity and can be converted directly to E1 by aromatase or to testosterone by 17β-HSD3 and finally to estradiol by aromatase [48]. In the additional steps of steroidogenesis 17β-HSD 1 and 2 catalyze the reduction of E1 to E2 and oxidation of E2 to E1 respectively. Eventually oestrogen is transformed to hydrophilic glucuronide or sulphate conjugates by glucuronidation and the sulphatase pathway, respectively. These modifications make the steroids more soluble and therefore easily transported in the blood. [49].

Briefly, oestrogen metabolism through the sulphatase pathway is based on steroid sulphatase (STS) action. STS can be found in a range of peripheral tissues allowing local synthesis of E1 from oestrone sulphate (E1S), the most abundant oestrogen transported by the circulatory system to target distant organs [50]. STS reduces the hydrophilic E1S to hydrophobic E1, which

is further reduced via 17β-HSD1 to the active oestrogen E2 [28,47]. E1S and all conjugated oestrogens are biologically inactive because of their very low affinity for the oestrogen receptor. However, they are of great importance taking in consideration their ability to act as an oestogen reservoir with twice half-life compared to E1 and E2 [51] (Figure 2).

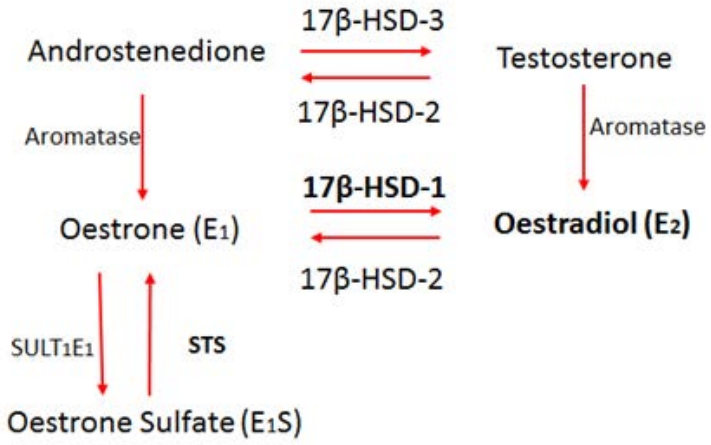

Figure 2: Oestrogen metabolism pathway. 17β-HSD-1 and STS are responsible for the reduction of E1 to biologically active E2. 17β-HSD-2 and SULT1E1 oxidize E2 to E1 followed by E1 sulphation to E1S [28].

Oestrogens play an important role in a plethora of physiological processes in humans and they have been implicated in the initiation and development of many diseases such as cancer. Oestrogen production and metabolism are tissue specific. In the normal colon, 17β-HSD 2 and 4 efficiently convert E2 to E1 providing anti-proliferative effects [52,53]. However, in colorectal cancer these enzymes are downregulated and oestrogens are shown to be locally produced mainly by the sulfatase pathway [47].

1.6 Oestrogen Receptors

Oestrogens mediate their intracellular effects through their receptors (ERs) via genomic and non-genomic pathways. The genomic effects regulate transcription of genes associated with angiogenesis (VEGF), cellular adhesion (cadherins), proliferation and apoptosis by direct interaction with specific DNA sequences, known as oestrogen response elements (ERE) and transcription factors (SP1, AP1, NFkB). The non-genomic effects regulate rapid indirect gene transcription via pathways such as protein kinase C, calcium (Ca+), cyclic adenosine monophosphate (cAMP) and mitogen-activated protein kinases (MARK) [54]. The term 'membrane-initiated steroid signalling' (MISS) has been suggested as an alternative to the 'non-genomic action'. However, this proposed term is not completely correct taking into consideration that non-genomic action do not always originate at the membrane [55,56].

The two classic ERs, ERα and ERβ, are encoded by *ESR1* and *ESR2*, respectively and are able to regulate gene expression after interacting with high affinity ligands. ERα regulates the oestrogenic signalling mainly in uterus, mammary gland and skeleton, whereas ERβ is mainly found in cells of the immune system, the prostate, the gastrointestinal tract, and the hypothalamus. Alternative splicing, during gene expression, produces different isoforms of each gene, resulting in three ERα and five ERβ isoforms, with differential tissue distribution, selective binding affinity, potency and efficacy for various compounds [57,54]. Their mechanism of action can vary when it comes to ligand interaction and signaling. ERs are inactive in the cytoplasm where they are associated with heat shock proteins. After ligand binding, they undergo conformational changes that enables them to shuttle from the cytoplasm to the nucleus and initiate expression of the target genes, mediating the genomic effects of

oestrogen [55]. In almost every tissue the two ERs overlap functionally [58] and specifically in colon ERβ is reported to diminish the transcriptional activity of ERα [54].

The amount of available oestrogen has been reported to regulate the expression levels of both ERs [59]. ERα and ERβ have different biological functions that depend on nuclear and cytoplasmic signaling. More specifically in colonic mucosa, although ERα has limited or no expression, evidence demonstrates a pro-proliferative role by regulating expression of proteins, such as cyclin D1, and promoting cell cycle transition. Other studies suggest that ERα activates the Wnt / β-catenin signalling pathway in SW480 and HCT116 CRC cell lines [60].

On the other hand, ERβ, the dominant ER isoform in CRC, is documented to be an anti-proliferative regulator in vivo and in vitro causing cell cycle arrest via the p38/MAPK pathway [54,61]. Furthermore, ERβ is shown to regulate TGFβ signalling pathway and decrease inflammation regulating IL6 [54]. However, evidence proposes that in CRC ERβ is downregulated compared to normal tissue, leading to a positive correlation between the receptor's expression and the stage of the disease [62]. Hence, the loss of ERβ is seen by some as the oestrogenic switch in CRC that leads to the loss of the anti-proliferative effects of oestrogen.

Despite the established role of the two classic ERs, the effect of oestrogen in CRC through the latest discovered ER, GPER, remains unclear. GPER is a typical seven-transmembrane G protein-coupled receptor, widely expressed in the body, with high affinity to E2 at physiological concentrations [37]. It emerged at the forefront of the scientific interest after

Filardo and colleagues revealed that it promotes rapid oestrogen-induced activation of ERK1/ERK2 independently of ERα and ERβ action in breast cancer cells [63]. The mechanism was elucidated in 2004 when Maggiolini *et al.* using SKBR3 cells as a model system, determined that GPER, via ERK1/ERK2 activation, mediates an early *c-Fos* upregulation [64]. The importance of GPER was elaborated after ERα positive breast tumors treated with hydroxytamoxifen (OHT) had intensive disease progression due to agonistic action of the drug to the novel receptor [65]. Poola and colleagues in 2008 observed that the expression of *GPER* was downregulated in most of the breast cancer tissues in comparison to respective normal tissues, hypothesizing that proliferation may arise partially due to receptors loss [66]. Furthermore, GPER has been identified as the receptor responsible for the inhibitory effect of oestrogens on the divergent TGFβ signalling and function [67], the oestrogen-mediator of FN matrix assembly and growth factor release via a Shc-dependent signaling mechanism [68] and the basic regulator of oestrogen induced expression and function of vascular endothelial growth factor (VEGF) [69] in ER-negative breast cancer cells.

After the discovery that hydroxytamoxifen (OHT) is a GPER agonist, experiments were conducted in endometrial cancer cells, reporting increased *c-Fos* expression after E2 and OHT treatment, independently of ERs [70]. In addition, GPER stimulation by tamoxifen, and genistein, has been reported to increase *SF1* transcription, promote endometrial cell proliferation, and induce the *SF1* target gene aromatase [71].

G1, the first GPER-selective ligand and a key indicator of GPERs action, was used to identify the receptors role in ovarian cancer. The data showed G1 implication in ERα-dependent proliferation through *c-Fos* stimulation and ERK activation, suggesting that in ovarian cancer

cells GPER/EGFR signaling is ER-dependent [72]. The expression of *GPER* is associated with a poor outcome in ovarian cancer, taking into consideration the data showing that GPER increases Akt phosphorylation via the EGFR [73]. In prostate cancer cells Chan and colleagues reported reduced proliferation rate after G1-induced GPER mediation of Erk1, Erk2 and p21 [74]. In addition, Wei and colleagues demonstrated that activation of GPER by G1 can inhibit proliferation of ER negative breast cancer cells *in vitro* by upregulation of *p53*, *p21* and downregulation of *cyclin B* [75].

In various studies GPER has been reported to mediate cell biological responses in reproductive tissue, however the receptors deletion in vivo have shown little reproductive anomalies compared to ERα knockouts [76]. GPER localization varies between tissues and evidence proposes a possible membrane-cytoplasmic shuttling of the receptor [77]. It is reported to be located on the plasma membrane as a typical ER in uterine, myometrium and renal epithelia and intracellularly, possible in the endoplasmic reticulum, in normal and cancerous epithelia in ovaries and endometrium[78]. The intracellular localisation can be associated with slow release from the endoplasmic reticulum during receptor biosynthesis as well as receptor downmodulation during reuptake [79].

According to studies in samples from patients with reproductive tumours, GPER action is independent from the other ERs [78] and it is reported to mediate various target genes and have a broad of biological and pathological functions [80]. Gene expression profiling of *GPER* signaling in breast cancer has shown mediation of various genes, such as *c-Fos*, *cyclins A*, *D1* and *E*, *VEGF* and *CTGF* [81]. *CTGF*, the higher GPER-induced gene, is a member of the CCN family of matricellular proteins [82], is implicated in cell division, apoptosis, adhesion, motility,

angiogenesis [83], tumor development [82] and along with the Hippo pathway constitute the two basic proposed downstream signaling targets of GPER in cancer.

1.7 Hippo signaling pathway

As a highly conserved pathway in mammals and a basic regulator of organ size and tissue regeneration, the Hippo pathway has been attracting considerable attention in the last decade (Yu et al. 2015). The pathway's basic mediators are demonstrated to be the yes-associated protein 1 (YAP1) and its transcriptional co-activator PDZ-binding motif (TAZ). These co-activators compete with the transcription co-factor vestigial-like protein 4 (VGLL4) for binding with the TEAD family transcription factors (TEAD1–4) and promote target gene expression [85,86]. YAP/TAZ activity is regulated by a core kinase cascade, mammalian sterile like kinase 1/2 (MST1/2). MST1/2 phosphorylates Mob kinase activator 1A/B (MOB1) and Salvador protein (SAV1) and activates protein kinase, large tumor suppressor kinase 1/2 (LATS1/2) which directly phosphorylates YAP/TAZ. Depending on the position of the phosphorylation site, a 14-3-3 binding region is generated, allowing YAP1 sequestration in the cytoplasm. Alternatively, ubiquitin is activated, leading to proteasomal degradation of YAP1 (Figure 3) [84].

The upstream regulators of Hippo pathway remain poorly understood. Up to date, tumor suppressor homolog 4 (FAT4), neurofibromin 2 (NF2) and Bona fide human tumor suppressor (KIBRA) have been suggested as upstream components. FAT4 interacts with atypical cadherin, Dachsous (Ds) and regulates membrane's organization with a low affinity

to the pathway's progression. NF2 and KIBRA, sequesters LATS1/2 to the plasma membrane for activation by MST1/2 and stabilizes LATS2 by inhibiting ubiquitination (figure 3) [84,87].

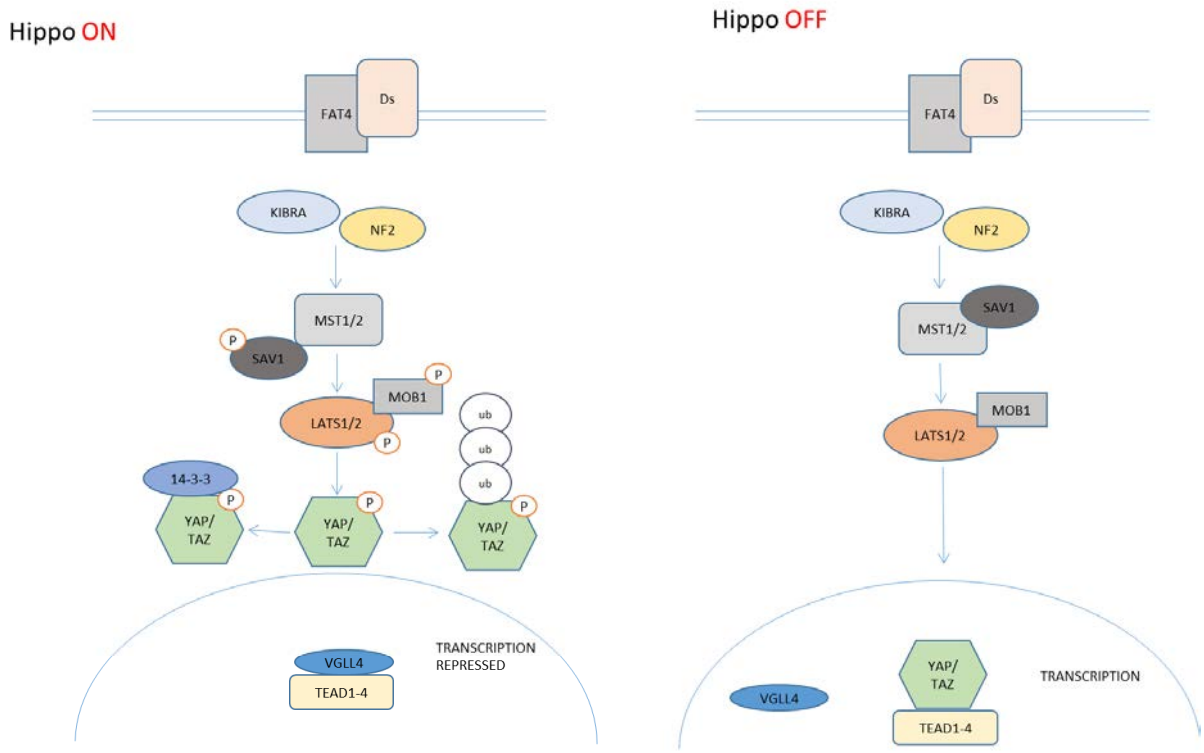

Figure 3: Hippo pathway: Main regulators and signalling. (Left) Hippo signalling is on, YAP/TAZ are phosphorylated on multiple sites and remain in the cytoplasm bind to 14-3-3 or face protein degradation by the ubiquitin system. (Right) Hippo signalling is off, YAP/TAZ enter the nucleus, interact with TEAD1-4 and activate target genes.

Evidence proposes the existence of an additional Hippo-like signaling pathway where MST1/2 is not necessary for LATS1/2 activation [88] and where YAP/TAZ are phosphorylated by other kinases [89]. Mitogen-activated protein 4 kinase (MAP4K) family and Serine/threonine-protein kinase (STE20) family members have been reported to facilitate LATS1/2 activity as a response to tissue-specific upstream signals [84,90,91]. YAP/TAZ have

been demonstrated to be deactivated by cyclin-dependent kinase 1 (CDK1), Jun N-terminal kinases (JNK), homeodomain-interacting protein kinases (HIPK), and Src family tyrosine kinases [89].

Literature reports that the Hippo pathway is dysregulated in many human neoplasms, including CRC, playing a paramount role in cancer development and progression. YAP/TAZ co-activators in their non-phosphorylated form increase proliferation [92], *YAP1* as an oncogene and *TAZ* as a mediator of the epithelial-mesenchymal transition (EMT) and the cell migration-invasion [93]. The evidence suggests a synergistic action between YAP1 and TAZ depending on the common TEAD-mediated transcription pathway but also an independent less efficient anti-apoptotic action though different mechanisms [94]. The mechanisms underlying these effects have not been fully elucidated.

Research has been devoted to identifying the unknown upstream moderators as the key to the pathways proliferative action, with G-protein-coupled receptors repeatedly noted as mediators through Rho GTPase and cytoskeleton remodelling [95,96]. In breast cancer, published data have demonstrated that oestrogens can activate GPER, which forward controls part of the Hippo Pathway and increase proliferation by deactivating anti-tumour regulators [96].

1.8 Aims

There is evidence in the literature that suggests an association between oestrogen and colorectal cancer. Epidemiological studies of the Women's Health Initiative (WHI) indicate exogenous HRT as protective against CRC development. However, how oestrogens impact CRC once it has developed remains poorly understood.

A recent paper by our lab revealed some important information about oestrogens role in CRC. Our data suggest, CRC cell lines (Caco2, HCT116 and HT29) express high amount of steroid sulfatase (STS), and are able, through OATP4A1 transporter, to internalise E1S and E2S and hydrolyse them to their active forms [49]. Moreover, our data suggest that the increased STS activity is mediated by oestrogenic GPER-stimulation and is associated with increased tumour burden in cell and animal models [37]. Therefore, the aim of this project was to verify GPER's pro-proliferative role and investigate the possible downstream mechanism of action of GPER stimulation. Furthermore, the project will examine whether E2-GPER interaction induces CTGF, a known regulator of GPER action, and/or alters the Hippo signalling pathway.

2. Methods and Materials

2.1 Health and Safety

Good Laboratory Practice was employed; a lab coat and gloves were worn when performing all the procedures. Sterile conditions were utilised for the prevention of contamination of cell cultures and RT-PCR experiments. To secure aseptic environment, separate coats were used for cell culture and other labs, gloves were frequently changed and sanitised with 70% ethanol, the reagents were always kept up to date and the labs where cleaned fortnightly.

2.2 Cell culture techniques

2.2.1 Human colon cancer cell lines

HCT116, HT29, CACO2 (Table 2) are immortalised, adherent, epithelial colon cancer cell lines kindly provided by Professor C. McCabe (University of Birmingham) and Professor C. Tselepis (University of Birmingham). Culture media McCoy's 5A Medium (Modified) (Sigma-Aldrich,UK) and Minimum Essential Medium (MEM) (Sigma-Aldrich,UK) were used with 2.0 mM L-glutamine, sodium bicarbonate, 10% v/v heat inactivated fetal bovine serum (FBS) (Thermofischer Scientific,UK), 100 U/ml and 100 μg/ml penicillin/streptomycin (Thermofischer Scientific, UK). Cells were grown at 37°C in 5% CO_2 with saturating humidity and split after reaching 90% confluency. During splitting, growth medium was aspirated, cells were washed with phosphate buffered saline (PBS) (Thermo

Scientific, UK) and detached by TrypLE™ Express Enzyme (1X) (Thermofischer Scientific, UK). Cells were resuspended into T-75 flasks (Sigma-Aldrich, UK) containing fresh, pre-warmed medium at required density. The cells were microscopically examined for degree of confluence, bacterial or fungal contaminants and the growth medium was renewed 2 – 3 times per week.

Table 2: Description of cell lines

Cell line	Organism	Disease	Culture media	Formation	Sub-cultivation ratio
CACO2	Homo sapiens	Colorectal adenocarcinoma	MEM	Monolayer	1:3
HCT116		Colorectal carcinoma	McCoy's 5A	Multilayer	1:20
HT29		Colorectal adenocarcinoma	McCoy's 5A	Multilayer	1:10
AA/C1		Colonic adenoma	DMEM	Multilayer	1:3
RG/C2		Colonic adenoma	DMEM	Multilayer	1:5

2.2.2 Human colonic adenoma cell lines

Two human non-tumorigenic adenoma cell lines were part of this study; AA/C1 and RG/C2 (Table 2). These cells were kindly provided by Professor C. Paraskeva, CRC Laboratories, University of Bristol, UK. The cell lines were cultured in Dulbecco's Modified Eagle's Medium - high glucose (DMEM) (Sigma-Aldrich, UK), with 4500 mg/L glucose, 2.0 mM L-glutamine, sodium bicarbonate, without sodium pyruvate, containing 20% v/v heat inactivated fetal bovine serum (FBS) (Thermofischer Scientific, UK), 0.2 Units ml^{-1} insulin (Sigma-Aldrich, UK), 1 $\mu g\, ml^{-1}$ hydrocortisone (Sigma-Aldrich, UK) and maintained at 37°C in humidified conditions with 5% CO_2.

2.2.3 Oestrogen starvation

Studies influenced by steroid hormones, such as oestrogen action, were carried out in cells grown in media with very low concentration of steroids. Components with oestrogenic action such as Phenol red were excluded and FBS was mandatory stripped of hormones with charcoal without loss of necessary serum nutrients. During the experiments the cells were grown in Phenol-red free Hyclone McCoy's 5A modified medium with L-glutamine (Thermo Scientific, USA) and 10% HyClone ^{TM} Charcoal/Dextran Treated Fetal Bovine Serum (GE Healthcare, US). The duration of oestrogen starvation varied for 24-96 hours according to experiments (Table 3). No antibiotics were used.

Table 3: Experiments conditions

	Duration			
Experiment	Starvation	Treatment	Starting Cell density	Treatment solutions
CTGF regulation	24 h	24 ,48 h	250.000/well (6 well plate)	E2 , G1 (100nM)
YAP1 regulation	24 h	0, 15, 30 min 1, 2 h	250.000/well (6 well plate)	E2 , G1 (100nM)
P-YAP1 regulation	24 h	15, 30 min	250.000/well (6 well plate)	E2 , G1 (100nM), G15 (1 μM)
BrdU proliferation assay	96 h	48 h	500/well (96 well plate)	E2 , G1 (100nM), G15 (1μM), VP (10nM)

2.2.4 Treatment solutions

Oestradiol (E2) (Figure 4) was maintained as lyophilized powder (Sigma-Aldrich, USA) covered with foil for minimum light degradation. A 10mM stock solution was prepared in ethanol prior to experiments and stored in -20°C for up to 1 month. G1 and G15 (Figure 4) (Tocris, UK) were also supplied in powder form and immediately after arrival were dissolved in DMSO and stored in -20°C as 10mM stock. All the E2 stock solutions were prepared in glass vials to minimise interaction with plastic and loss of oestrogens during storage. Sterile Eppendorf tubes were used for G1 and G15 stocks and for all treatment solutions for immediate use.

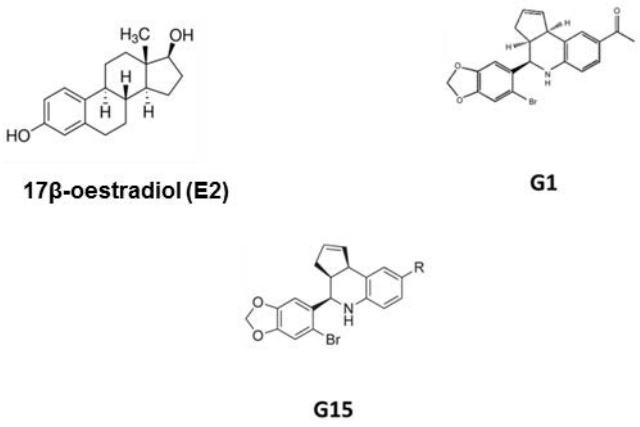

Figure 4: Molecular structures of 17β-oestradiol, GPER specific agonist G1 and antagonist G15

For treatments in 6 and 96 well plates the concentrations of all substrates were calculated on basis of 1mL and 100 μl per well respectively. For the dilution the formula $C_{initial}V_{initial}=C_{final}V_{final}$

was used and solutions were always mixed with vortex mixer to ensure homogeneous distribution prior use.

2.3 Gene expression Analysis

2.3.1 mRNA isolation and quantification

mRNA extraction was performed with the RNeasy Mini kit (Qiagen, UK). Following treatment with oestrogens, the media was removed and cells were scraped while incubated with RLT lysis buffer. Lysates were collected and transferred to labelled 1.5 ml tubes. An equal volume of 70% ethanol was added to each sample, and after homogenization with needle and syringe they were transferred to an RNeasy Mini spin column and centrifuged. Samples were washed once with 700µl RWI buffer and twice with 500 µl RPE buffer. RNA was eluted in a RNA free 1.5ml tube by pipetting 30 µl of RNAse-free water directly onto the spin column membrane and centrifuging for 1 min at >8000 x g. A NanoDrop spectrophotometer (Labtech International, UK) was used to quantify mRNA concentration by absorbance measurement at 260 nm. The 260/280 ratio was used to verify the samples purity; hence samples with ratios above 2 were only accepted.

2.3.2 Reverse Transcription

cDNA was generated from mRNA with the Tetro cDNA synthesis Kit (Bioline, Uk). For all samples, 1000ng of mRNA were used. All solutions and mRNA samples were vortexed and

centrifuged briefly before use. The priming premix was prepared on ice in RNase-free reaction tube and was mixed gently by pipetting before being aliquoted to tubes with the corresponding mRNA amount and DEPC-treated water (Table 4). The samples were incubated at 45 °C for 30 min and the reaction was terminated at 85 °C for 5 min. For long term storage the cDNA was kept at -20 °C.

Table 4: Quantities used to prepare the cDNA synthesis reaction per sample.

Total mRNA	*n*µl
Oligo $(dT)_{18}$	1 µl
10mM dNTP mix	1 µl
5x RT Buffer	4 µl
RiboSafe Rnase Inhibitor	1 µl
Tetro Reverse Transcriptase (200u/ul)	1 µl
DEPC-treated water	to 20 µl

2.3.3 Real-Time Quantitative PCR

To assay induction of *CTGF* mRNA expression, serum-starved cells received E2 and G1 treatment for 24 and 48 h at a concentration of 100 nM. qRT-PCR was performed to measure the mRNA levels of target genes (Table 5, Figure 5).

Table 5: qRT-PCR Master Mix preparation per 1ul of cDNA

TaqMan Gene Expression Master mix (2x)	5 µl
Probe for Gene of Interest	0.5 µl
RPLPO Primers/probe	0.5 µl
Nuclease-free water	3 µl

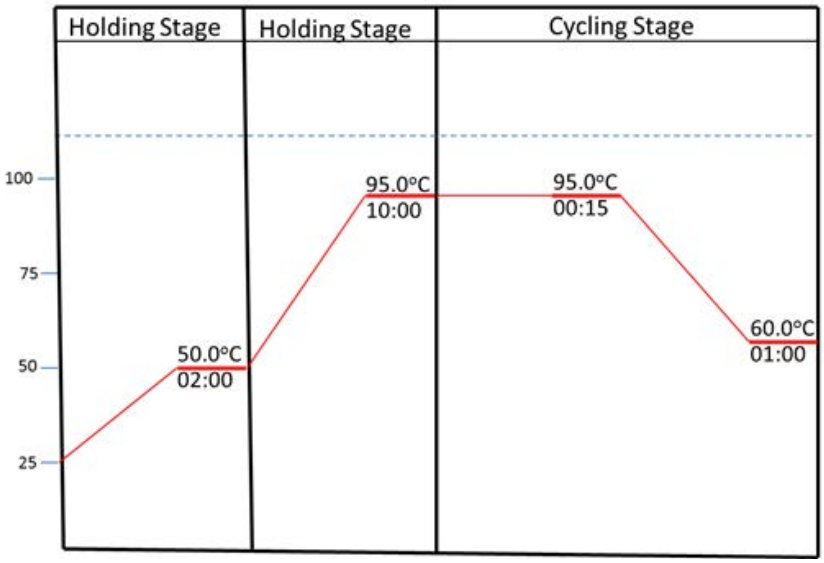

Figure 5: PCR program

Taqman assay and dual-labelled fluorogenic hybridization probes were used (*CTGF*, Hs01026927_g1, Thermo Scientific™,UK). Large Ribosomal Protein gene (*RPLPO*, 4310879E, Thermo Scientific™,UK) was used as housekeeping-reference gene for data normalisation (Figure 6). Threshold cycle number (Ct) using the equation 1000*2^-ΔCt was converted into arbitrary units of transcript molecules. Real Time PCR 7500 software was used for the data analysis.

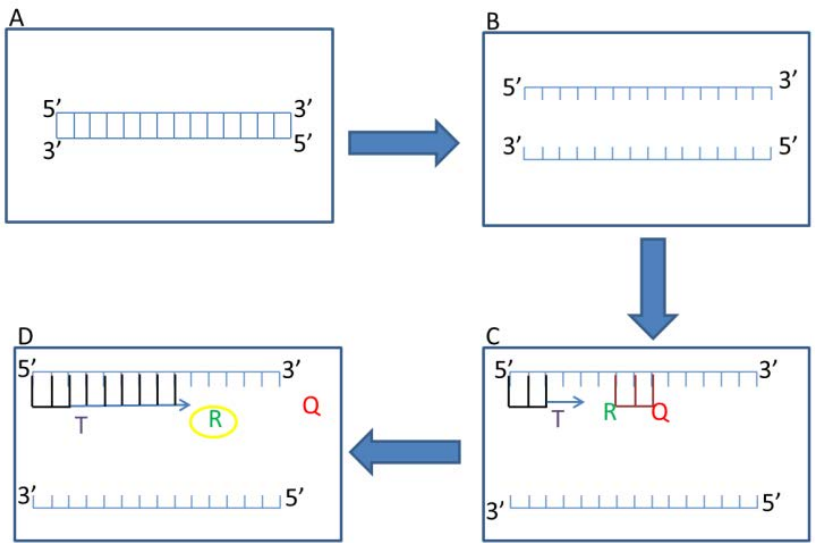

Figure 6: qRT-PCR mechanism. A)Target gene B)Denaturation of the template at 95 degrees. C)Primer in black and dual-labeled fluorogenic hybridization probe in red bind on DNA and extension face of the PCR starts. The probes are attached to a fluorescent dye serving as a Reporter (R, FAM or VIC) and a Quencher (Q,TAMRA) which absorbers emission when close to the reporter. D)The method exploits the 5' endonuclease activity of Taq DNA polymerase to expand the primers and the exonuclease activity of the enzyme to cleave the oligonucleotide probe during PCR, generating a detectable signal monitored in real time.

2.4 Determination of protein concentration

Protein quantification was performed with Pierce® Bicinchoninic Acid (BCA) Protein Assay Kit. Serial dilutions (Table 6), of stock solution bovine serum albumin (BSA) were used to construct a standard curve. The BCA Reagent consists of 98% Reagent A and 2% Reagent B (50:1 dilution of A to B). Protein lysate and BSA standards were placed in duplicates in 96-well plate and diluted in a ratio 1:20 using the BCA Reagent. After 30 minute incubation at 37 °C, the absorbance of the standard and samples was measured at 562 nm. The mean absorbance measurement of the samples was calculated and the mean value of the blank standard was subtracted from the mean absorbance of all the other samples. This final

absorbance of each BSA standard with its matched concentration in µg/mL was used to construct a standard curve. The chart was used only when the R-Squared value was above 95% and the concentration of the samples was calculated according to the equation "y = mx + c", where "y" stands for absorbance at 562 nm and "x" for protein amount.

Table 6: Preparation of diluted bovine serum albumin (BSA) standards

Diluent Volume Ripa Buffer (µl)	BSA Source and Volume (µl)	Concentration (µl /ml)
0	200 of stock (2mg/ml)	2,000
66	200 of stock (2mg/ml)	1,500
100	100 of vial A	1,000
100	100 of vial B	750
100	100 of vial C	500
100	100 of vial E	250
100	100 of vial F	125
100	0	0

2.5 Western Blotting

2.5.1 Polyacrylamide Gel Electrophoresis

Quantity of cell lysate containing 25 µg of protein was diluted with Laemmli Buffer 2x (Bio-Rad, UK) and boiled at 100 °C for 5 minutes. The samples were loaded into wells in Tris-glycine SDS-polyacrylamide 10% gel (Table 7, 8), polymerised in a gel caster consisted of a short glass plate (Bio-Rad, UK), a 1.5 mm spacer plate (Bio-Rad, UK), and a 1.5 mm loading comb (Bio-Rad, UK) with 8 µl of Precision Plus Protein Dual Color Standards (Bio-Rad,

UK). The gel was placed into a Mini-PROTEAN® 3 Cell Assembly apparatus (Bio-Rad, UK) containing 10X Tris/glycine/SDS running buffer (Bio-Rad, UK) diluted ten times with distilled water. The gel at first was run at 70 volts (V) for 20 minutes and after the proteins enter the resolving gel the voltage was raised to 140 V for 1 hour and half.

Table 7: Resolving Gel preparation quantities

Resolving Gel				
Protogel 30%	3.33 ml	6.66 ml	9.99 ml	13.32 ml
Resolving Buffer	2.5 ml	5 ml	7.5 ml	10 ml
Deionised water	4.17 ml	8.34 ml	12.51 ml	16.68 ml
APS 10%	100 µl	200 µl	300 µl	400 µl
TEMED	10 µl	20 µl	30 µl	40 µl

Table 8: Stacking Gel preparation quantities

Stacking Gel				
Protogel 30%	0.325 ml	0.65 ml	0.975 ml	1.3 ml
Resolving Buffer	0.625 ml	1.25 ml	1.875 ml	2.5 ml
Deionised water	1.525 ml	3.05 ml	4.575 ml	6.1 ml
APS 10%	12.5 µl	25 µl	37.5 µl	50 µl
TEMED	2.5 µl	5 µl	7.5 µl	10 µl

2.5.2 Immunoblotting transfer

The gel was equilibrated in Transfer Buffer for 15 minutes. The proteins were transferred to Nitrocellulose membrane by wet transfer using protein blotting cassette (Figure 7). The cassette was placed in the transfer apparatus with 1x transfer buffer (Appendix: Table 10). The black side of the cassette was facing the black side of the apparatus. The transfer process ran for 1 hour and a half at 360 mA at room temperature.

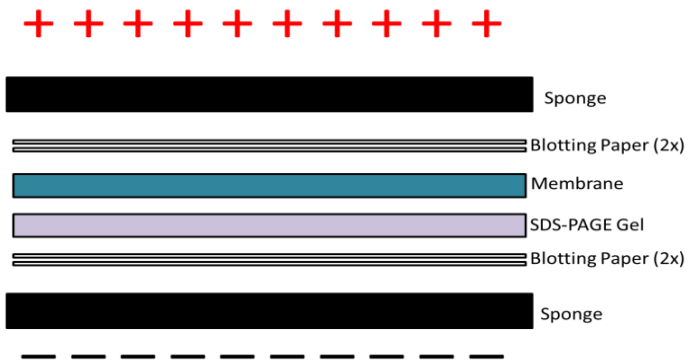

Figure 7: Structure of blotting cassette

2.5.3 Antibody Staining and Film Exposure

To prevent the non-specific binding, the membrane was soaked in blocking solution for 1 hour to overnight. After blocking, the membrane was rinsed 3 times for 5 minutes with PBS-T or TBS-T depending on the used primary antibody. The membrane was incubated with a corresponding antibody (primary) for the protein of interest followed by a secondary antibody that binds to the primary. The primary antibodies and their working dilutions are shown in Table 9. The secondary antibody conjugated to Horse Radish Peroxidase (HRP) was then

activated by ECL (RPN2106, GE Healthcare Life science) that was applied to the membrane. The emitted light was captured on X-ray film. The membranes were stripped to remove the antibodies and incubated with β–actin antibody (A5441, ThermoFisher Scientific), a 'housekeeping' gene to determine the same amount of protein has been loaded for each sample.

Table 9: Concentrations of the primary and secondary antibodies

Antibody	Supplier	Blocking	Primary Concentration	Secondary Concentration
β-actin (A5441)	Sigma	5% milk in TBST incubated at room temperature for 1 hour	1:5,000 in TBST incubated at room temperature for 1 hour	Anti-mouse 1:10,000 in TBST incubated at room temperature for 1 hour
YAP1 (ab52771)	Abcam	5% BSA in TBST incubated at room temperature for 3 hour	1:20,000 in 5% BSA in TBST incubated over night at 4^{o}C	Anti-rabbit 1:4,000 in 5% BSA in TBST at room temperature for 1 hour
CTGF (sc-14939)	Santa Cruz™	5% milk in TBST incubated at room temperature for 2 hour	1:500 in TBST incubated over night at 4^{o}C	Anti-goat 1:2,000 in TBST at room temperature for 90 min
Phospho-YAP1 (Ser127) (#4911)	Cell signalling	5% BSA in TBST incubated at room temperature for 3 hour	1:2,000 in 5% BSA in TBST incubated over night at 4^{o}C	Anti-rabbit 1:4,000 in 5% BSA in TBST at room temperature for 1 hour
GPER (sc-48525-R)	Santa Cruz™	5% milk in TBST incubated at room temperature for 2 hour	1:500 in 1% milk in TBST incubated over night at 4^{o}C	Anti-rabbit 1:2000 in TBST at room temperature for 1 hour

ERa (sc-130072)	Santa Cruz™	5% BSA in PBST incubated over night at 4°C	1:200 in 5% BSA in PBST incubated over night at 4°C	Anti-mouse1:2000 200 in 5% BSA at room temperature for 1 hour
ERb (14C8)	Abcam	5% BSA in PBST incubated over night at 4°C	2.5ug/ml in PBST room temperature for 1 hour (1:500)	Anti-mouse1:2000 in TBST at room temperature for 1 hour

2.6 Immunocytochemistry: Cell Fixation, Permeabilisation and Staining

Glass coverslips were sterilized in 90% ethanol and coated with Poly-l-lysine (P4832, Sigma-Aldrich) on 6-well tissue culture plates (CLS3506, Sigma-Aldrich) for 10 minutes in a sterile tissue culture hood. Cell suspension was added over each coverslip to a finally density of 200,000-350,000 cells per well. After 24 hours incubation, the medium was removed and the cover-slips were rinsed with PBS twice (1 ml per well) at room temperature. The cells were fixed with 4% (w/v) paraformaldehyde in PBS for 15 minutes, quenched with 1ml of 0.1 M glycine-PBS for 5 min and permeabilized with 1ml 0.1% Triton-X-100-PBS for 5 min at room temperature. The coverslips were incubated in diluted primary antibody (1:100 in 5% BSA-PBST, YAP1) for 1 hr 30 mins and then in secondary Alexa® Fluor 488 antibody (1:100, anti-rabbit) for 1hr in the dark at room temperature. A control was prepared for every antibody without adding the primary antibody. Any fluorescence that was seen was due to the secondary antibody binding non-specifically to the sample. To stain the nuclei, Hoechst 33342 fixed cell stain was used for 10 min in the same conditions. For the coverslip mounting, Mowiol mounting medium (81381, Sigma-Aldrich) was used and the slides were left to dry overnight at room temperature protected from light. The next day, they were

transferred to a fridge or in -20 °C freezer for long term storage. Imaging was done using a Zeiss LSM 510 META confocal microscope (Carl Zeiss; Oberkochen, Germany).

2.7 Proliferation

2.7.1 Cell counting

Cells grown in stripped media for 72h were trypsinised. The suspension was centrifuged at 1500 rpm for 5min, the supernatant was aspirated to remove the trypsin and the cell pellet was washed with PBS. After centrifuged again the cells were re-suspended in stripped media and 10 μl of the suspension were visually counted with a disposable haemocytometer. The average number of cells (from 3 boxes) was multiplied by 10 times to give the final concentration. The initial seeding density for the cells treated had been previously optimised by members of the lab at 500 cells per 100μl per well, subsequently ~500.000 cells in a 96 well plate. The fraction of cell number per plate over the concentration was calculated and 10 ml of suspended cells were prepared accordingly.

2.7.2 BrdU proliferation assay

Proliferation activity was measured using the BrdU (Colorimetric) kit (Roche, Germany). This rapid non-radioactive assay is based in the incorporation of bromodeoxyuridine (BrdU), a synthetic nucleoside analogue of thymidine, into the replicating DNA, localisation of

labelled cells using monoclonal antibody and measurement of the fluorescent immune complex by an ELISA multi-label reader (Wallac, Victor3 1420) at wavelength of 405nM [97].

Proliferation assays were carried out in flat-bottomed 96-well plates, seeded as described, following the manufacturer's protocol. A total of 6-well repeats were carried out for all assays. After incubation in the 96 well plate for 24 hours, the media was carefully aspirated and 100μL of warmed treatment solution was added into each well using a single channel pipette. The cells were retreated the next day and after 24 h incubation BrdU labelling solution was added. Four hours later the cells were fixed for 30 minutes and immersed in anti-BrdU solution. The antibody solution was removed after 90 minutes and after 3 washes with PBS an immune complex detection reagent was added. The plate was placed on a rocker for 25 minutes before being read by the ELISA multi-label reader. The averages of the well repeats was used to find a mean absorption value and the final mean absorption was calculated after subtracting from the average absorption the average absorption values of blanks (media without cells). Optimization of the results was applied with values greater than two times the SEM to be excluded prior averaging.

2.6 YAP1 SiRNA silencing

Knockdown of *YAP1* was carried out with small interfering RNA (siRNA). RNA silencing refers to negative regulation of gene expression triggered by sequence-specific double-stranded RNA (siRNA) that operate within the RNA interference (RNAi) pathway by activating a degradation process [98,99]

Silencing was carried out on colorectal carcinoma HCT116 cells while optimising the technique. *YAP1* siRNA (Dharmacon, UK) was delivered into the cells using DharmaFECT 4 Transfection Reagent. DharmaFECT 4 Transfection reagent forms liposomes in serum free media (Opti-Mem, Life Technologies) which entraps the siRNA within. Cells were incubated in 6-well plates at a density of 200.000 cells per well in dsFBS media. 24 hours later a treatment solution of 25 nM of siRNA and 2 μl of DharmaFECT 4 Transfection Reagent was made up in 2 mls per well of Opti-Mem as protocol describes. The dsFBS media was carefully aspirated and replaced with 2mLs of the siRNA solution and incubated in a humidified incubator at 37°C, 5% CO2. After 24 hours, the treatment solution was aspirated and replaced with dsFBS stripped media. Opti-Mem -only control were carried out to ensure they were not confounding factors. Silencing was confirmed by RT PCR and western blot, from RNA and protein extracted 24, 48, 72 and 96 hours post transfection.

2.7 Data analysis

The results were analysed using GraphPad Prism software. All the values were expressed as mean ± S.E.M. For the statistics unpaired 2-tailed Student's t test was used and p value <0.05 was taken as the level of significance. The western blots were analysed with IMAGEJ software. In detail the western blots were scanned and saved as pictures. The files were open with IMAGEJ and the mean intensity of each couple of bands P-YAP1 and YAP1 were measured using Analyse -> Gels -> Select first line (P-YAP1) -> Select second line (YAP1). The values were exported to excel and the ratio of P-YAP1/YAP1 was calculated to express the amount of YAP1 that is phosphorylated in every time point.

3. Results

3.1 GPER is expressed in colon cancer and colon adenoma cells

Oestrogens affect the incidence and outcomes of colorectal cancer (CRC) [28]. Previous projects in the lab have shown that steroid sulphatase (STS), the enzyme that converts conjugated oestrogens to their active forms [50], is significantly upregulated in human CRC tissue leading to increased availability of active oestrogen. Furthermore, the increase in STS activity substantiated greater CRC tumour burden in mouse models [37]. However, the role of oestrogen is regulated apart from their amount and the presence or absence of oestrogen receptors that mediate their action. Thus, we first determined the oestrogen receptor status of our CRC cell lines.

qRT-PCR showed very low expression of the oestrogen receptors genes with general AU<0.8 (Figure 8A,B,C). Immunoblotting showed GPER as the only receptor expressed in all the cell lines examined. The colon cancer cell lines HCT116, HT29 and colon adenoma cell line RG/C2 assembled only this receptor. JEG3, Caco2 and Colo205 exhibited some long isoform ERα immunoreactivity. MCF7 were used as control for ERα and ERβ expression (Figure 8D). Immunoblotting for ERα showed no band for MCF7 at 66kDa. However, bands appeared at approximately 48kDa and 36kDa (Appendix: Figure 21) verifying that other isoforms of ERα are present (ER46 and ER36, respectively).

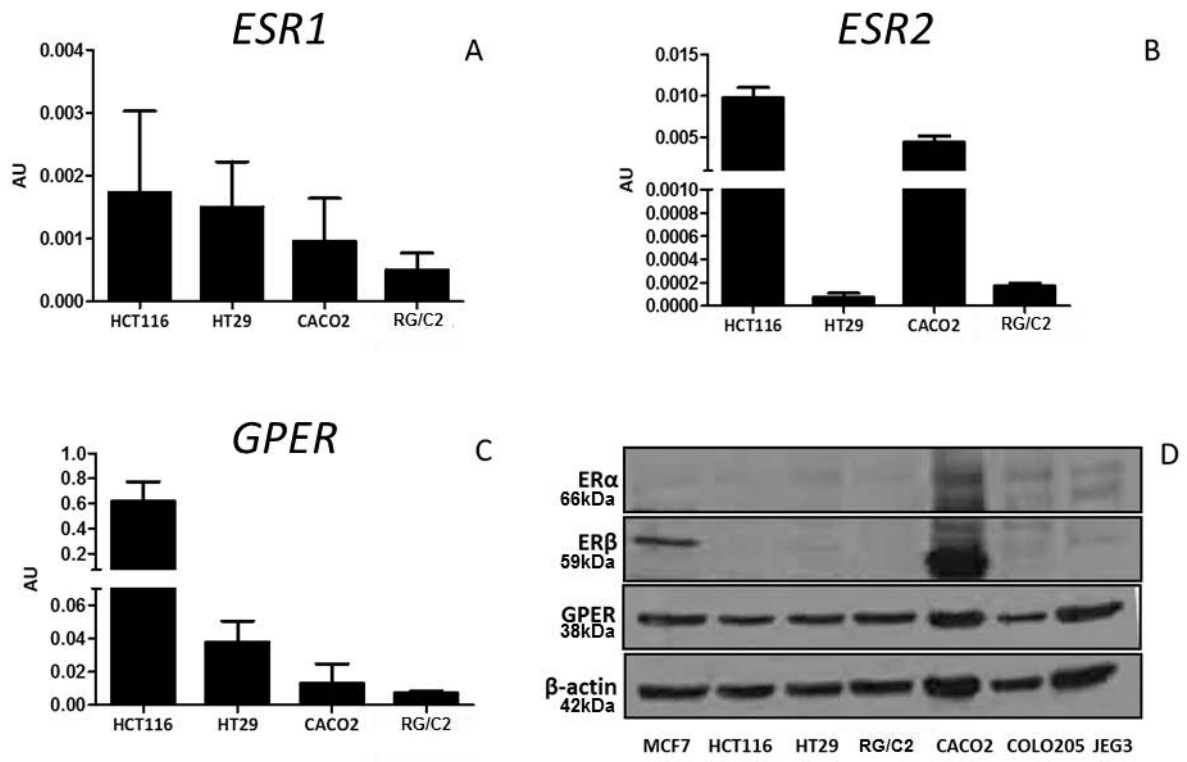

Figure 8: GPER is the dominant oestrogen receptor in CRC cell lines. **A-C)** Mean (± SEM) total relative *ESR1* **(A),** *ESR2* **(B)** and *GPER* **(C)** expression in HCT116, HT29, CACO2 and RG/C2 cells obtained from individual passages (n = 3), grown in full media withought treatment. **D)** Immunodetection of ERα (66 kDa), ERβ (59 kDa), GPER (38 kDa) in human colon adenoma and adenocarcinoma cell lines. Cell lysates from AA/C1, RG/C2, CACO2, HT29 and HCT116 cells were resolved by 10% SDS–PAGE with B-actin as the loading control.

3.2 Oestrogenic stimulation of GPER increased proliferation in CRC cell lines

The absence of ERα/ERβ in combination with GPER's expression led us to focus on the role of this receptor in CRC. To assess the role of GPER in CRC we employed BrdU proliferation assays. HCT116 cells were used as the most suitable as they did not express ERα and ERβ. Cells grown in stripped media for 24 hours were treated with E2, or G1, a GPER specific agonist, for 48 hours. In the same experiments cells were also treated with E2 or G1 with the addition of G15, a GPER antagonist (Figure 9).

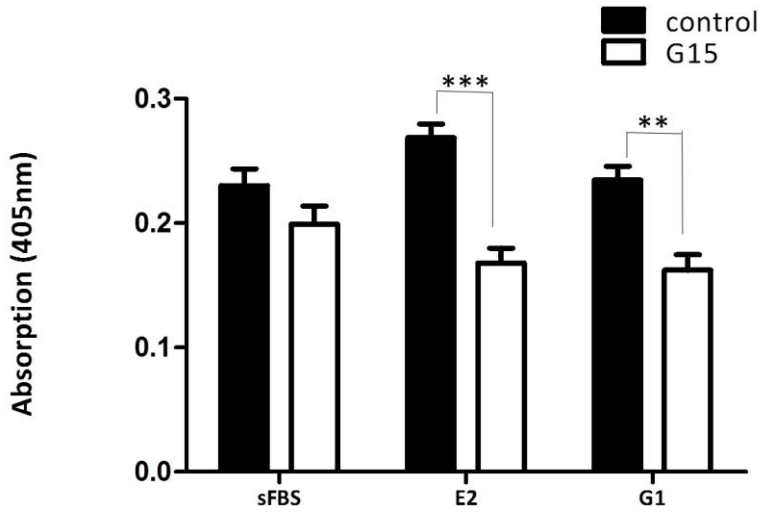

Figure 9: GPER stimulation with E2 and G1 increased proliferation in HCT116 cells. The effected was inhibited by GPER antagonist G15. HCT116 cells were treated with E2 (100nM) and G1 (100nM) for 48 hours with and without the addition of G15 (1 uM). Viable cells were analyzed using BrdU assay, whose results are expressed as absortion in 405nm. All conditions are normalised to the media with charcoal stripped FBS (sFBS) without cells (blank). 2-tailed Student's t test showed significant difference among E2 and G1 treatments after G15 addition ** $p < 0.01$, *** $p < 0.001$. ($n = 6$, ± SEM).

The results show a trend towards increase in the proliferation rate of HCT116 cells after G1 and E2 treatment. Furthermore, the proliferation rate of the cells is significantly decreased after the addition of G15, at approximately 35%, suggesting GPER acts pro-proliferative in CRC.

3.3 Mechanism of GPER signalling

3.3.1 *CTGF* is not oestrogen-induced in CRC via GPER

Previous data in breast cancer have shown GPER to increase proliferation through connective tissue growth factor *(CTGF)* [81]. Thus, we next examined *CTGF* expression after E2 and G1

treatment in CRC cell lines. Before examining the possible upregulation of *CTGF* through GPER, *CTGF* expression was confirmed (Appendix: Figure 22). The cells treated with E2 and G1 for these experiments were HCT116 and HT29. CACO2 cells were also examined to investigate whether ERα presence would lead to any differences in the result. The arbitrary expression of *CTGF* mRNA in the cells relative to housekeeping gene *RPLPO* showed no significant difference or trend between the treatments and the control (Figure 10).

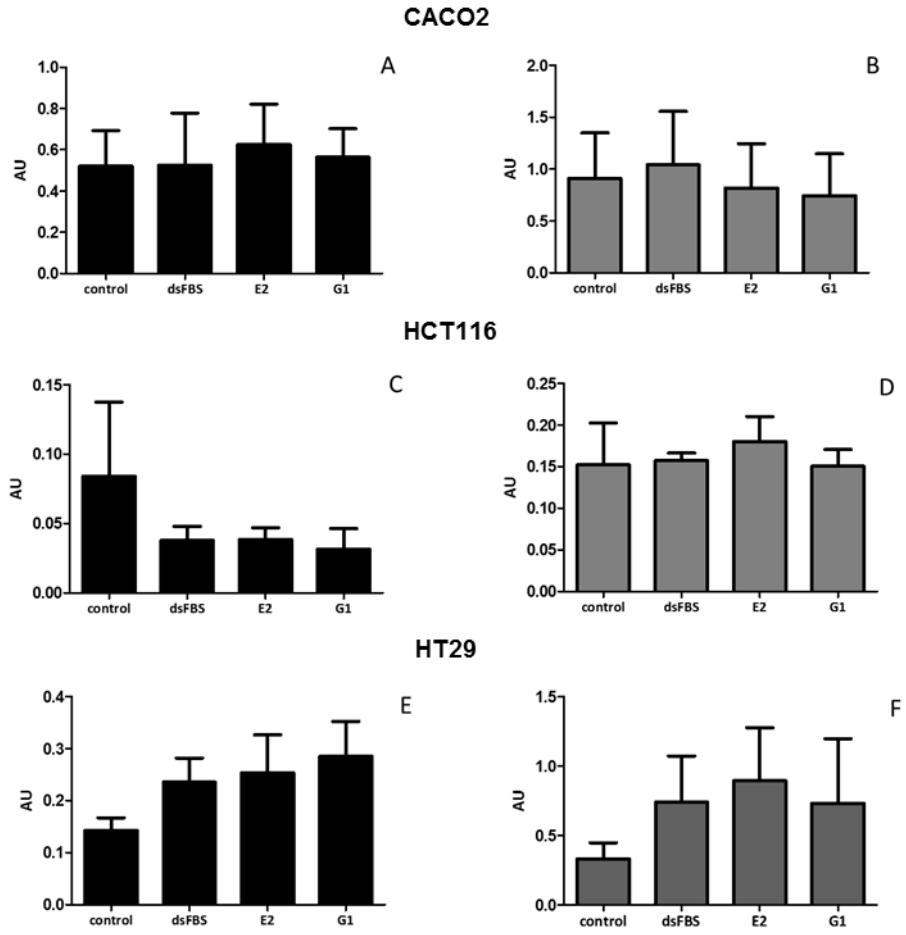

Figure 10: *CTGF* mediation is independent of GPER oestrogenic stimulation. Mean (± SEM) total relative *CTGF* expression of CACO2 (**A,B**), HCT116 (**C,D**) and HT29 (**E,F**) cells obtained from individual passages (n = 3) after consecutive treatments with oestrogens (E2) and G1, in stripped media after 24 (**A,C,D**) and 48 (**B,D,F**) hour with normal media as control. 2-tailed Student's t test showed no significant difference among treatments.

3.3.2 Oestrogen increased P-YAP1 expression in HCT116 but not in colon adenoma cells

GPER is suggested to mediate its effects via the Hippo Pathway in breast cancer [96]. To investigate whether YAP1 is oestrogen induced through GPER in CRC, HT29 (figure 11), RG/C2 (Figure 12) and HCT116 (Figure 13) cells were treated with E2 and G1 for up to 2 hours. Cell lysates were extracted and used for immunoblotting (Figure 11A, 12A, 13A). To furtherly examine possible mediation of the Hippo Pathway through the GPER, we repeated the experiment with the addition of G15. Proteins were extracted 15 and 30 minutes after treatment. P-YAP1 and total YAP1 was immunodetected with β-actin as loading control (Figure 11B, 12B, 13B) and the ratio of P-YAP1 to the total YAP1 was calculated with the use of ImageJ (Figure 11C, 12C, 13C). In all the cell lines, no trend in the alterations of P-YAP1 expression was observed following E2 and G1 treatment in the 2-hour period. However, in HCT116 cells inhibition of GPER action with G15 led to a decrease in p-YAP1, 15 and 30 min after treatment (Figure 13C).

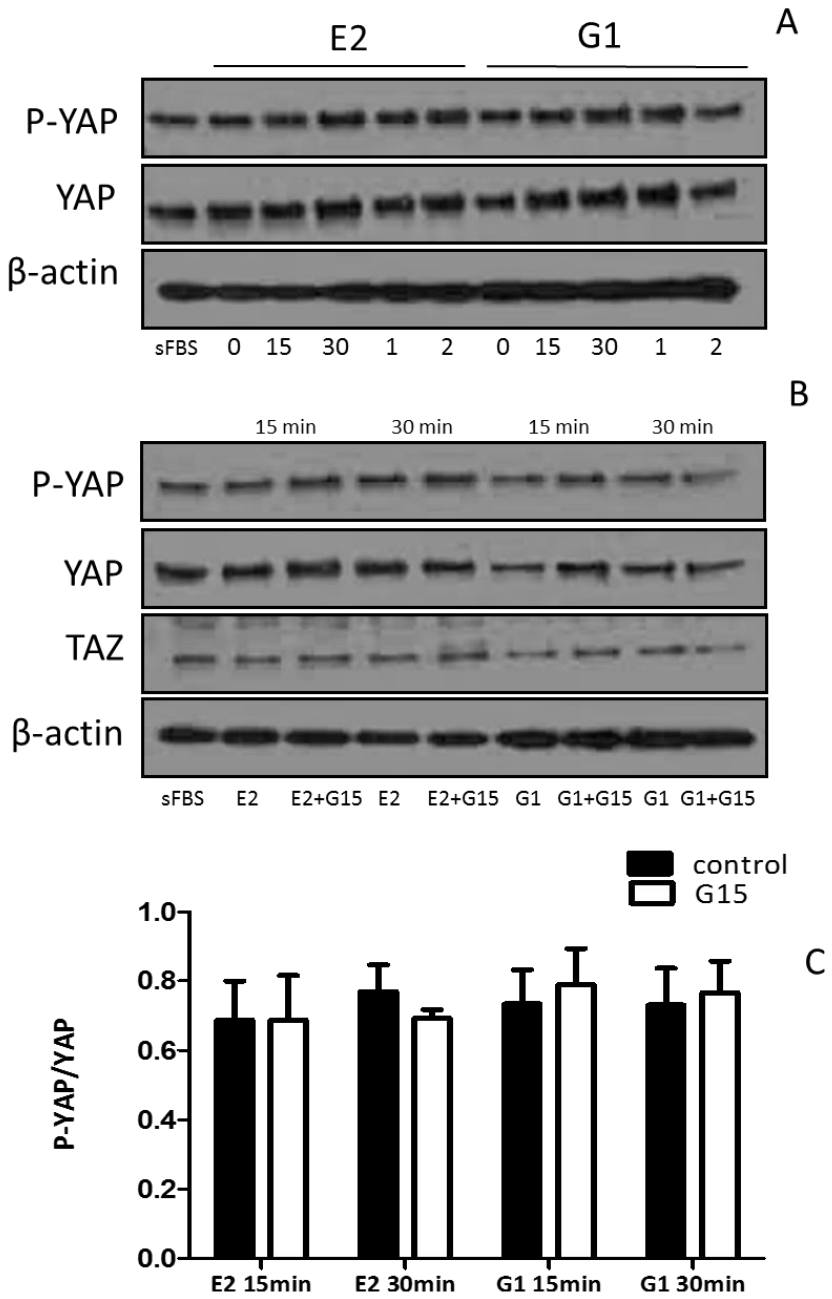

Figure 11: YAP1 is not regulated by GPER in HT29 cells. **A**) Cell lysates from HT29 cells were resolved by 10% SDS–PAGE with B-actin as the loading control after 0, 15, 30 minutes, 1 and 2 hours of E2 and G1 treatment. **B**) Cell lysates from HT29 cells were resolved by 10% SDS–PAGE with B-actin as the loading control after 15 and 30 minutes of E2 and G1 treatment with the addition or absence of G15. **C**) The ratio of the relative intensity of P-YAP1 to YAP1 measured by ImageJ. 2-tailed Student's t test showed no significantly different among treatments.

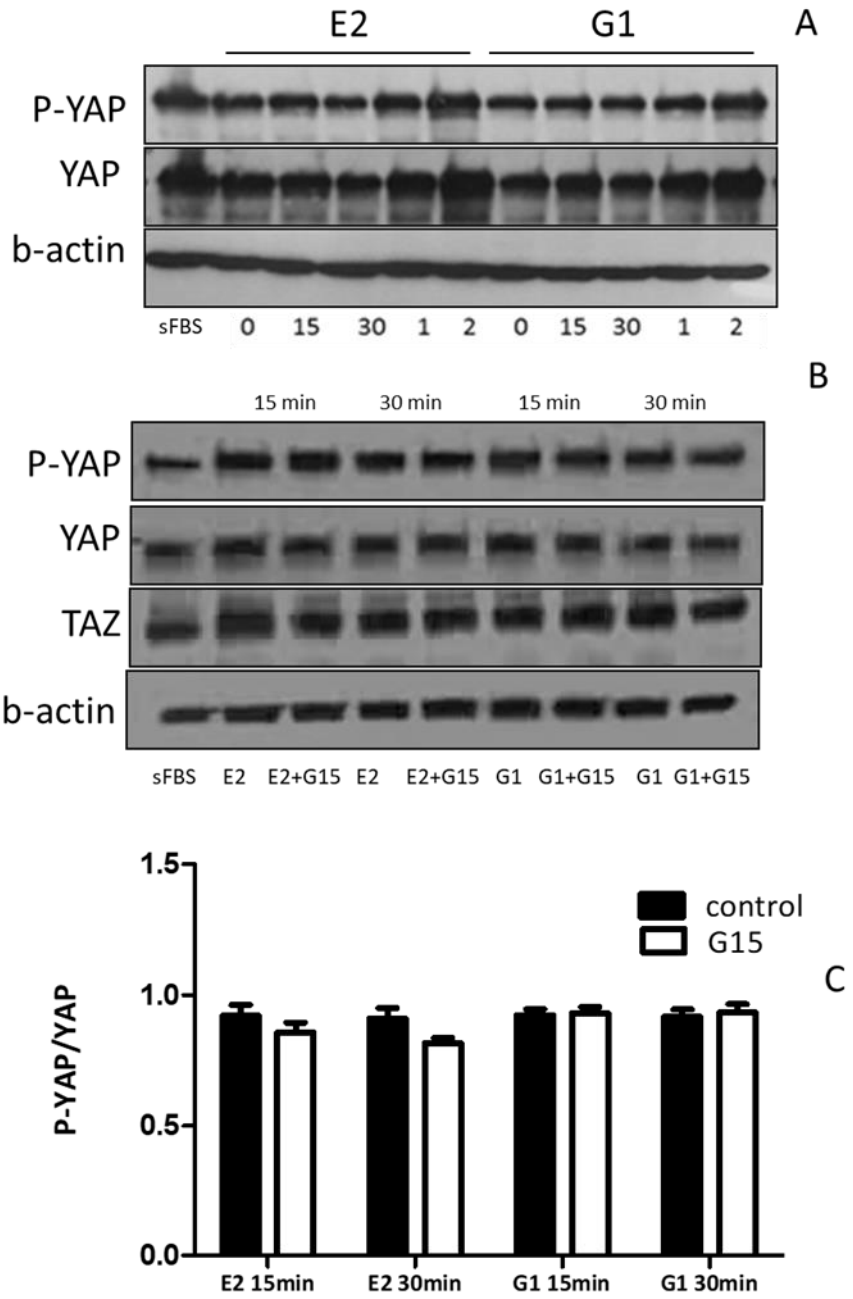

Figure 12: YAP1 is not regulated by GPER in RG/C2 adenoma cells. **A**) Cell lysates from RG/C2 cells were resolved by 10% SDS–PAGE with B-actin as the loading control after 0, 15, 30 minutes, 1 and 2 hours of E2 and G1 treatment. **B**) Cell lysates from RG/C2 cells were resolved by 10% SDS–PAGE with B-actin as the loading control after 15 and 30 minutes of E2 and G1 treatment with the addition or absence of G15. **C**) The ratio of the relative intensity of P-YAP1 to YAP1 measured by ImageJ. 2-tailed Student's t test showed no significantly different among treatments.

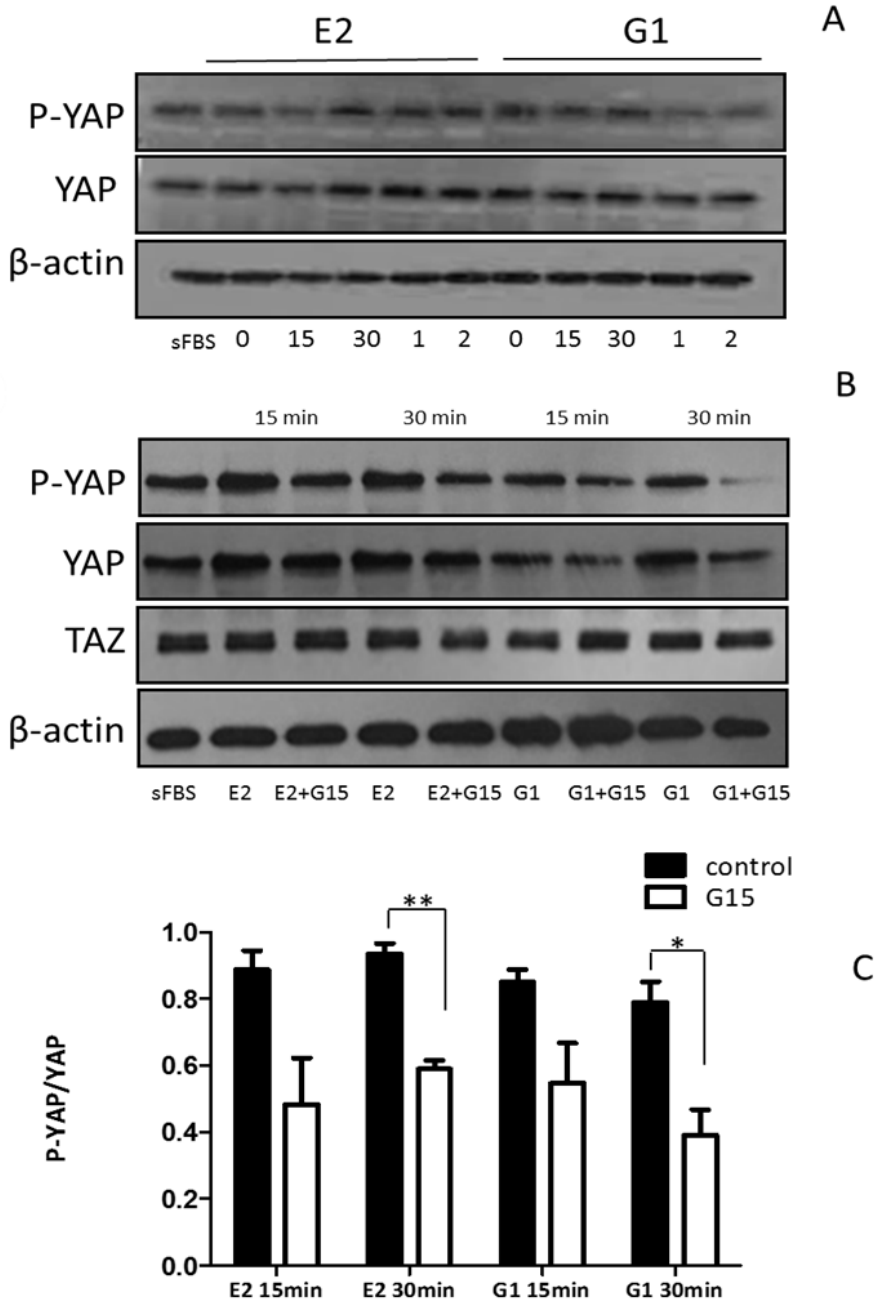

Figure 13: YAP1 is regulated by GPER in HCT116 cells. **A**) Cell lysates from HCT116 cells were resolved by 10% SDS–PAGE with B-actin as the loading control after 0, 15, 30 minutes, 1 and 2 hours of E2 and G1 treatment. **B**) Cell lysates from HCT116 cells were resolved by 10% SDS–PAGE with B-actin as the loading control after 15 and 30 minutes of E2 and G1 treatment with the addition or absence of G15. **C**) The ratio of the relative intensity of P-YAP1 to YAP1 measured by ImageJ. 2-tailed Student's t test showed significantly different among treatments after G15 addition, 30 minutes after treatment. * $p < 0.05$, ** $p < 0.01$.

Immunofluorescence was also used to verify YAP1's mediation through GPER after G1 treatment in HCT116 cells (Figure 14). The use of total YAP1 antibody revealed that in the untreated HCT116 cells (control) YAP1 is mainly located inside the nucleus (YAP1 form). G1 treatment led to rapid translocation of the protein from the nucleus to the cytoplasm (P-YAP1 form) and inhibition of GPER action with G15, blocked this effect.

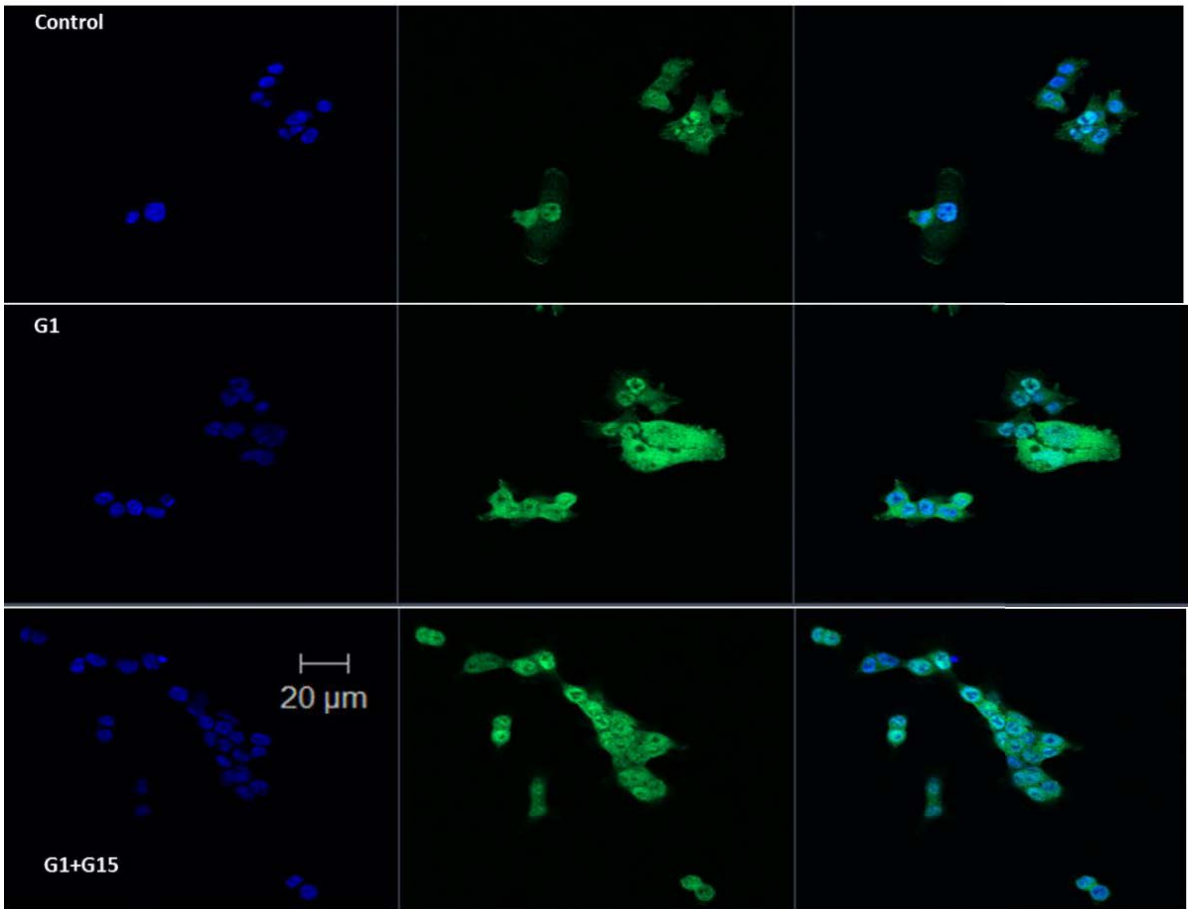

Figure 14: Immunocytochemistry of total YAP1 (green) in human cancer colon cell line HCT116. The Nuclear DNA labelling (blue) was done with Hoechst 33342. YAP1 was detected both in the nuclei and the cytoplasm following 30 min of G1 treatment. After inhibiting the action of GPER with G15, the protein was localized mainly in the nuclei similarly to the control.

To confirm GPER stimulation increases P-YAP1 expression in HCT116 cells we repeat the experimental set up (E2 and G1 treatment with extractions after 0, 15, 30 minutes, 1 and 2 hours) with cells starved of oestrogen for 48 hours (Figure 15). The results showed a trend in both E2 and G1 treatment toward increase in P-YAP1 expression immediately after treatment compare to untreated control.

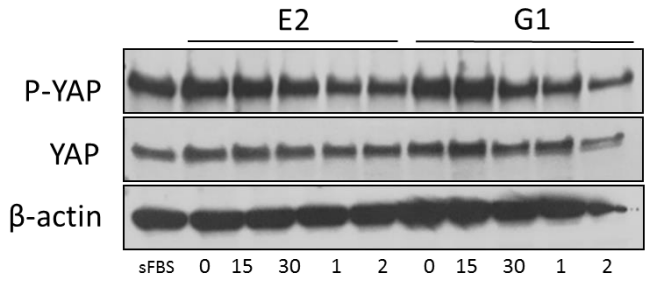

Figure 15: Immunodetection of P-YAP1 and YAP1 (75 kDA). Cell lysates from HCT116 cells, starved for 48 hours, were resolved by 10% SDS–PAGE with B-actin as the loading control. E2 and G1 caused an increase in P-YAP1 for 30 minutes after treatment and the response was decreased after 1 hour.

3.3.3 Verteporfin inhibits the proliferative effect of GPER in HCT116 cells

To investigate the function of YAP1 in GPER-induced tumour cell proliferation we employed a pharmacological inhibition of YAP1, Verteporfin. Various lower concentrations of Verteporfin were tested (Appendix: Figure 26) with the addition of G1 (Figure 16). The inhibitor was shown to be very efficient even in very low doses of the grade of 10nM which was chosen as the optimum.

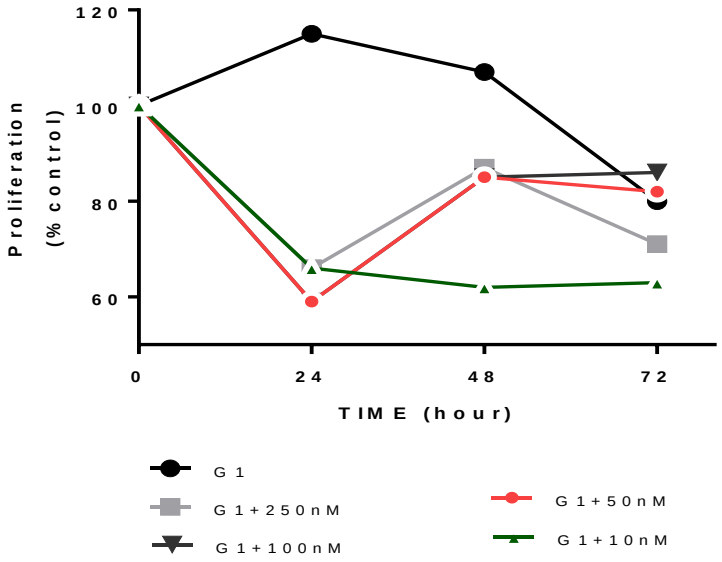

Figure 16: Verteporfin (VP) treatment attenuates proliferation capacities of HCT116 cell line. HCT116 cells were incubated with VP at various concentrations (250, 100, 50 and 10 nM,) for different durations (24, 48, 72 hours), respectively with and without the addition of G1 (100nM). Viable cells were analysed using BrdU assay, whose results are expressed as percentages of inhibited cells. All conditions are normalised to the dFBS control (100%).

Verteporfin effect on growth was further verified with more proliferation assays with G1 and E2 treatment for 48 hours. All results showed a trend towards increase in the proliferation rate after E2 treatment and a statistical significant inhibition ($p<0.05$) in the proliferation rate after G1 treatment with addition of Verteporfin (Figure 17).

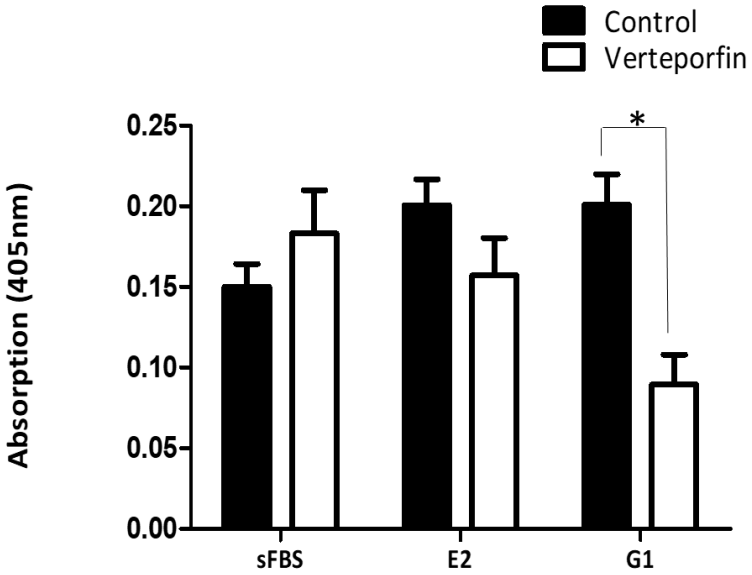

Figure 17: Verteporfin blocked GPER-driven cell proliferation in HCT116 cells. Cells were treated with E2 (100nM) and G1 (100nM) for 48 hours with and without the addition of Verteporfin (10 nM). Viable cells were analyzed using BrdU assay, whose results are expressed as absortion in 405nm. All conditions are normalised to the dFBS control without cells. 2-tailed Student's t test showed significantly different among G1 treatments after Verteporfin addition *$p < 0.05$.

3.3.4 GPER-induced cell proliferation was blocked by YAP1 transient knockdown in HCT116 cells.

We next silenced *YAP1* in HCT116 with the use of siRNA. The efficiency of the knockdown was very high since the mRNA and protein levels were almost diminished, compared to the untreated control, from the first 24 hours after transfection (Figure 18).

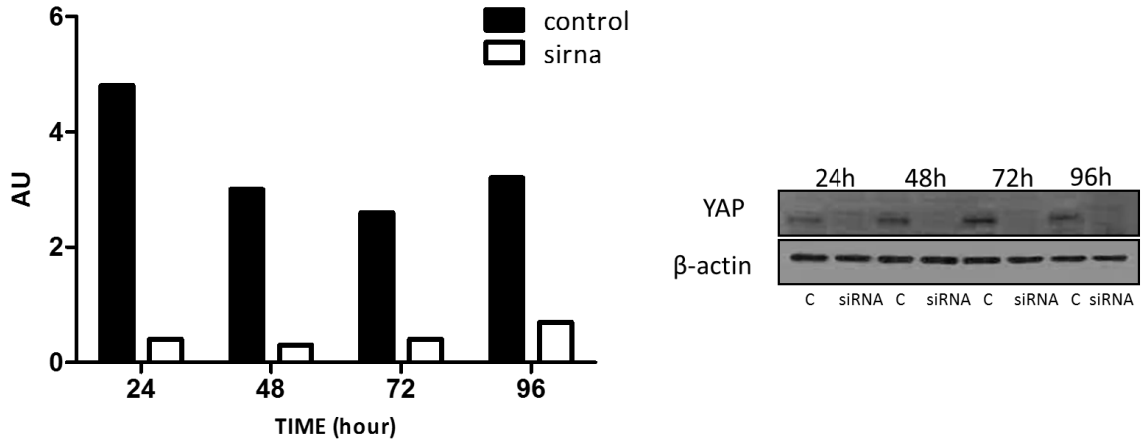

Figure 18: *YAP1* siRNA optimization in HCT116 cells. mRNA and protein was collected 24, 48, 72 and 96 hours after transfection. The relative YAP1 expression was diminished after 24 hour of transfection and the silencing was stable for up to 96 hours. Cell lysates from HCT116 cells, 24, 48, 72 and 96 hours after transfection, were resolved by 10% SDS–PAGE with B-actin as the loading control. YAP1 (75 kDa) protein expression was highly decreased for the first 24 hours and the knockdown was stable for up to 96 hours.

To further delineate YAP1 action in CRC, we employed proliferation assays with the *YAP1* knockdown cells and non-target siRNA transfected cells as control (Figure 19). The results in figure 19 suggested that HCT116 *YAP1* knockdown cells loss their ability to respond to the proliferative effect of E2 treatment (as shown in Figure 9). Furthermore, G1 effect in *YAP1* knockdown cells was cytotoxic compare to the no target control suggesting clearly that deletion of *YAP1* inhibits GPER induced proliferation and possibly increases apoptosis.

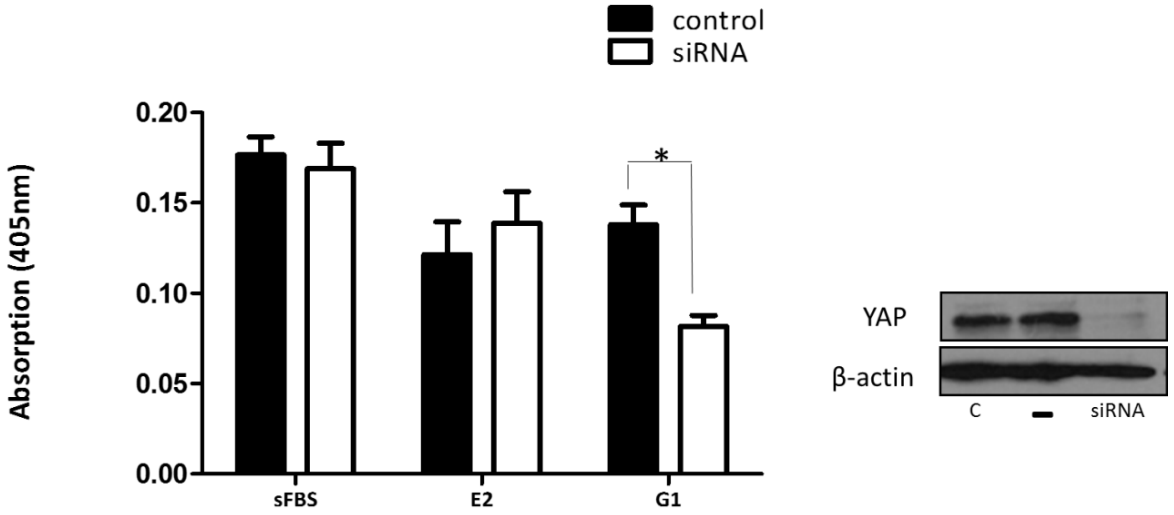

Figure 19: GPER promotes cell proliferation through YAP1. HCT116 expressing *YAP1* and HCT116 *YAP1* knockdowns were treated with E2 (100nM) and G1 (100nM) for 48 hours. Viable cells were analysed using BrdU assay, whose results are expressed as absorption in 405nm. All conditions are normalised to the dFBS control without cells. *YAP1* knockdown HCT116 cells do not respond to E2 and G1 treatment. Compared to the non-targeting negative control G1 is shown to be cytotoxic for the *YAP1* knockdown cells 2-tailed Student's t test showed significantly different among G1 treatments after Verteporfin addition *$p < 0.05$.

4. Discussion

Colorectal cancer is the third most commonly diagnosed cancer in both sexes and the second cause of cancer-related death in Europe [2]. Here we investigated whether oestrogens effect CRC proliferation via GPER and Hippo Pathway signalling.

In CRC the research in oestrogen induced action through GPER is limited and controversial. Liu and colleagues study shows that *GPER* mRNA and protein expression is downregulated in CRC compared to matched normal tissue and this downregulation is associated with poorer prognosis [100]. Published data from our lab support the fact that *GPER* mRNA levels are decreased in human CRC tissue compared to matched normal tissue. However, GPER protein levels were reported to be raised in human CRC tissue compared to matched normal control implying that GPER protein degradation pathways possibly alter in CRC, permitting GPER protein retention [37].

Liu and colleagues study also proposed that GPER stimulation by G1 leads to G2/M phase arrest, elevated ER stress, and increased apoptosis in CRC cells via the Ras/ERK1/2 and NFκB pathway [100]. However, our results here demonstrate that ER negative but GPER positive HCT116 present increased proliferation rate after E2 and G1 treatment with the effect inhibited by 35% after use of the specific GPER antagonist G15. The contrast to our findings is possible due to use of different experimental conditions. In detail, Liu and colleagues employed in their experiments doses of G1 up to 10 μM where we used 100 nM. Interestingly, previous projects in our lab have proven this biphasic response to G1 where

low doses increase the proliferation rate of CRC cells and high doses induce apoptosis, alike ERα biphasic stimulation in breast cancer cells [37,101].

Santolla and colleagues propose that GPER stimulation by E2 is associated with increased proliferation in Lovo cells via a FASN-mediated mechanism[102]. Taking into consideration that the GPER-FASN mechanism was common in colon and breast cancer we decided to examine if known signalling pathways of GPER in breast apply to CRC. Pandey and colleagues examined the expression profiling of GPER signalling in ERα and ERβ negative SKBr3 breast cancer cells. They identified *CTGF* as the gene most induced by E2 and OHT [81]. Thus, we examined *CTGF* gene expression levels in HCT116, HT29 and CACO2 CRC cells after E2 and G1 treatment. The results showed no significant alteration in *CTGF* gene expression levels (Figure 10). However, previous experiments in our lab had evidenced that GPER stimulation after E2 and G1 (100nM) treatment leads to *CTGF* upregulation and elevated protein expression in HCT116 and HT29 cells [37]. The controversy between the results may be due to different passage number of the cells examined.

One of the hallmarks of cancer is silencing of tumour suppressor genes and pathways. Previous studies have demonstrated that aberrant modifications in the key components of the Hippo pathway may be associated with increased proliferation and possible carcinogenesis [84,92,103]. The Hippo pathway has been reported to be cross talked with many well-known signaling pathways in malignancy. Previous data shows that in skin cancer YAP/TAZ are associated with TGFβ signalling pathway (*SMAD2/3*) [104]. In colon cancer, Hippo signalling and Akt pathway has been reported to interact and control cell growth [105] and YAP/TAZ have been suggested to mediate GPCR proliferative effects in intestinal epithelial cells through the

PKD gene family [106]. In breast, prostate and colon cancerous cells inhibition of ILK resulted in regulation of MST1 and LATS1 followed by YAP/TAZ inactivation [107].

Hormones have been implicated in the Hippo pathway in the evolutional study of Yu and colleagues in which a large amount of GPCR ligands where tested for possible association with YAP/TAZ action, such as ERα and thyroid stimulating hormone receptor [95]. In a more recent study of Zhou and colleagues, treatment with E2, OHT and G1 induced rapid and significant YAP1 dephosphorylation and TAZ accumulation in different breast cancer cells. The responses were blocked with GPERs antagonist G15 and with GPER knockdown, suggesting a cell line–independent roles of GPER in YAP/TAZ activation [96].

To examine whether GPER is associated with the Hippo pathway in CRC, we treated CRC cells in stripped media with E2, G1 and G15. Immunoblotting (figure 11-13) and Immunostaining (figure 14) revealed rapid increase in YAP1's phosphorylation, with G15 to attenuate YAP1 nuclear-cytoplasmic shuttling, in HCT116 cells. HT29 and adenoma cells did not respond to treatment. In contrast with the study of Yuen *and colleagues,* in which TAZ mRNA expression is suggested as a prognostic indicator for colon cancer progression, we witnessed no change to total TAZ protein levels after oestrogenic treatment in any of the cell lines examined [108].

In normal tissue this early metabolic impact (rapid increase in YAP1's phosphorylation) would be translated into impaired cellular proliferation. However, pharmacological inhibition and gene silencing revealed that YAP1 has a pro-proliferative role in HCT116 cells suggesting that in malignancy the well elucidated pathway is dysregulated in favour of the

neoplasia. In addition to that the differences between the cell lines can be explained by the fact that HCT116 cells are highly aggressive and non-differentiating cells in contrast to the HT29 which retain some capacity to differentiate [109].

According to literature HCT116 YAP1 and TAZ knockdowns have been demonstrated to reduce proliferation independently and synergistic [93]. The cytoplasmic YAP1 expression is suggested to be significantly higher in colon adenocarcinoma, compared to normal tissues and YAP1 has been reported to be upregulated and mounted in the cytoplasm (P-YAP1) [85]. Taking into consideration that rarely cytoplasmic expression occurs without nuclear expression, the inactive P-YAP1 can possible act as a constant reservoir available when needed to be dephosphorylated and translocated into the nucleus [85]. Furthermore, recent evidence points out that mitogenic GPCR stimulation in intestinal epithelia cells, can possible be associated with biphasic regulation of YAP1 and rapid cytoplasmic-nuclear shuttling [106]. The alteration of YAP1 expression in CRC is of great interest as a therapeutic approach, considering the role it displays in normal colonic tissue unlike the Wnt and Notch pathways whose outputs are fundamental for normal intestinal homeostasis [88].

5. Conclusions

Taken together, our results propose a new oestrogen-driven pro-proliferative GPER-stimulated pathway through the Hippo signalling pathway (YAP1) in CRC.

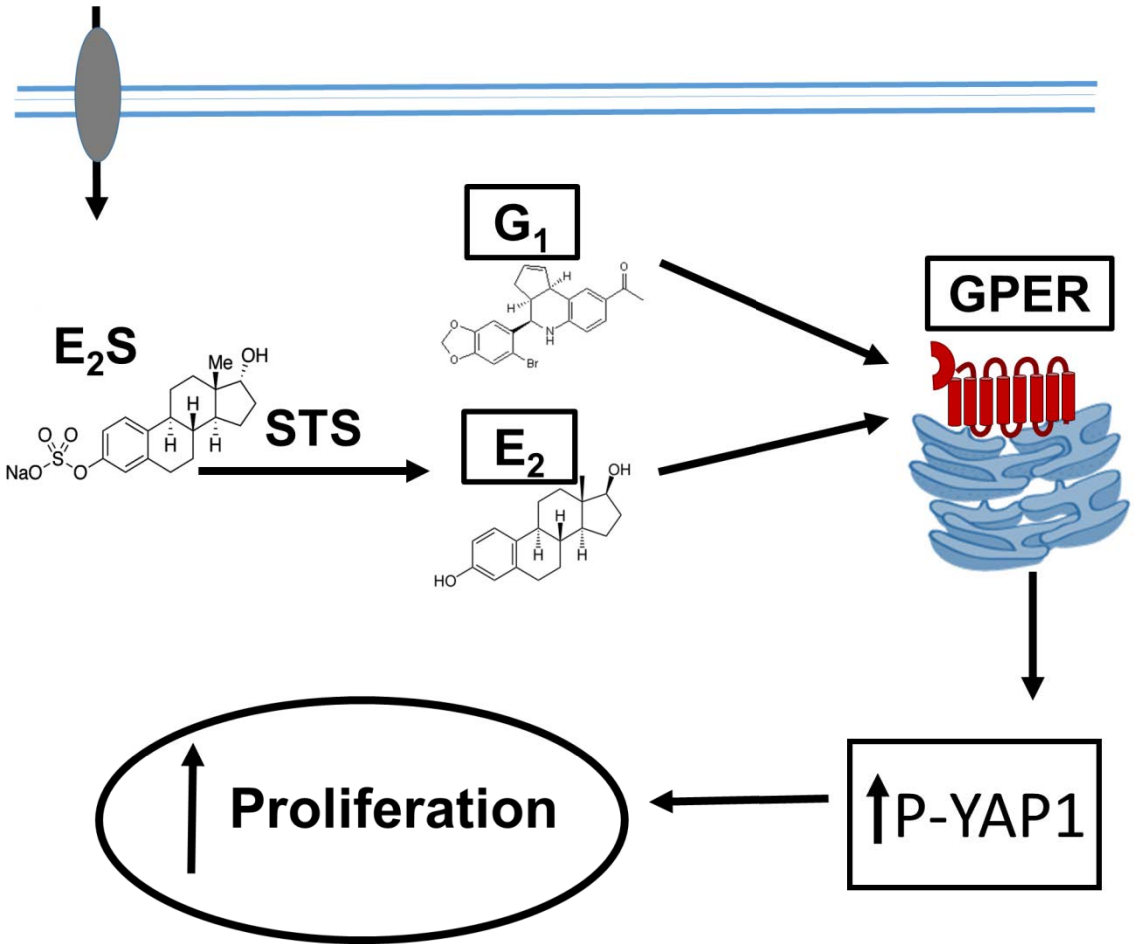

Figure 20: CRC cells internalise E1S and E2S via the OATP4A1 transporter. Intracellularly STS hydrolyses the conjugates to their active forms. GPER shuttles between the membrane and the endoplasmic reticulum where it's stimulated by the high affinity E2 and its agonist G1. The receptor activation leads to phosphorylation of YAP1, translocation from the nucleus to the cytoplasm and is associated to increase proliferation via an mechanism which is yet to be elucidated.

6. Futures Aims

This project aim was to provide a basic assessment of the potential role of GPER as oestrogen-induced mediator in CRC via the Hippo signalling pathway. Taking into consideration the limited duration of the project, all the experiments were performed in human immortalised cell lines. However, to verify the physiological relevance of the results the experiments should be repeated with the use of CRC and match normal primary cells. Furthermore, *TAZ* and *YAP1* gene expression levels would be interesting to be measured in paired cancerous and normal human tissue samples, to investigate a possible association between YAP1 expression and survival outcome [110].

For our proliferation studies we employed the widely used BrdU incorporation assay which measures DNA replication. However, our results between experiments had variations which might be overcomed by the use of another cell viability assay, such as MTT that evaluates the number of the living cells based on their ability to metabolise MTT into formazan [111]. In the same time, it would be very interesting to evaluate the apoptosis and cytotoxicity in the cells after our oestrogenic treatment with the use of Caspase 3 assay and lactate dehydrogenase (LHD) assay respectively [111,112].

The extent of our imaging studies in this project was limited. Due to lack of time live cell imaging after oestrogen treatment was not attempted. In a future project it would be very interesting to use confocal time series application in order to record GFP-tagged YAP1 shuttling from the nucleus, where it has been reported to be mainly localised in CRC cell

lines [113], to the cytoplasm after oestrogen treatment with the use of G15 and verteporfin. However, the use of a GFP-tag can influence localisation and the experimental set up can be very challenging. The controversy regarding GPER localisation was promptly investigated GPER (Appendix: Figure 24). Immunostaining showed GPER localisation both in the membrane and in the cytoplasm of CACO2 cells. Nevertheless, further studies have to be done in order to clarify the receptors location and possible shuttling between the membrane and the endoplasmic reticulum [79].

The siRNA against *YAP1* was very efficient but the non-target siRNA used as control had off target effects when treated with oestrogens. The transfection reagent did not appear to have off target effects (Appendix: Figure 28). CRC cells were also transfected with siRNA against *GPER* (Appendix: Figure 30) and *CTGF* (Appendix: Figure 31). The transient knockdown of *GPER* was not efficient and *CTGF*'s was controversial. This points out on the one hand that the experiments should be repeated with an alternative non-target siRNA and on the other hand that stable knockdown cell line would be the appropriate tool in order to fully investigate the role of YAP1 as well as GPER and possible CTFG. Also, stable *GPER* and *YAP1* knockdowns *in vivo* would elucidate the non-genomic action of GPER and the Hippo signaling pathway as well [96].

Further studies are finally required to reveal the detailed signalling cascade by which GPER mediates the increased YAP1 phosphorylation. LATS1/2 and MIST1/2 should be the next proteins whose phosphorylation and thus action should be investigated with immunoblotting after oestrogen treatment. MIST1/2 anti-proliferative effect in normal colon tissue has been reported to be suppressed by *YAP1* overexpression in CRC [114], consequently it would be very

important to examine whether the increased phosphorylation of YAP1 we reported is regulated by deactivation of these modulator and investigate the unclear role of LATS1/2. Furthermore, it would be very interesting to examine TEAD activation upon oestrogen treatment and GPER inhibition with Luciferase-based genetic reporter assays and investigate the role of Gαq/11 and PLCβ/PKC in the upregulation of YAP1 via GPER which has been reported in breast cancer to be of paramount importance [96].

8. References

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7. Appendix

Table 10: Western blot buffers and their components

TBS-T 1X	
1M TRIS pH 7,6	50 ml
NACI	20 g
Tween80	0.625 ml
dWater	2447 ml

Transfer Buffer 1X	
MEOH	400 ml
Glycine	28.8 g
TRISMA BASE	6 g
dWater	1600 ml

Running Buffer 1X	
10x Tris/Glycine/SDS	100 ml
dWater	900 ml

Mild stripping	
Glycine	15 g
SDS	1 g
Tween20/80	10/2.5ml

Adjust pH to 2.2. Bring volume up to 1 L with ultrapure water

Figure 21: Full blots of ERα (66 kDa), ERβ (59 kDa), GPER (38 kDa) in human colon adenoma and adenocarcinoma cell lines. Cell lysates from AA/C1, RG/C2, CACO2, HT29 and HCT116 cells were resolved by 10% SDS–PAGE with B-actin as the loading control.

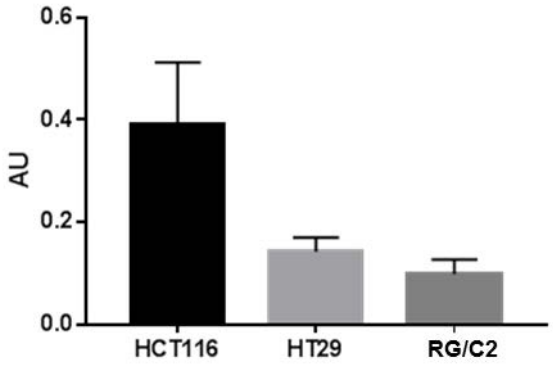

Figure 22: *CTGF* is expressed in CRC cell lines. qRT-PCR was performed in untreated human CRC cell lines HCT116, HT29 and RG/C2.

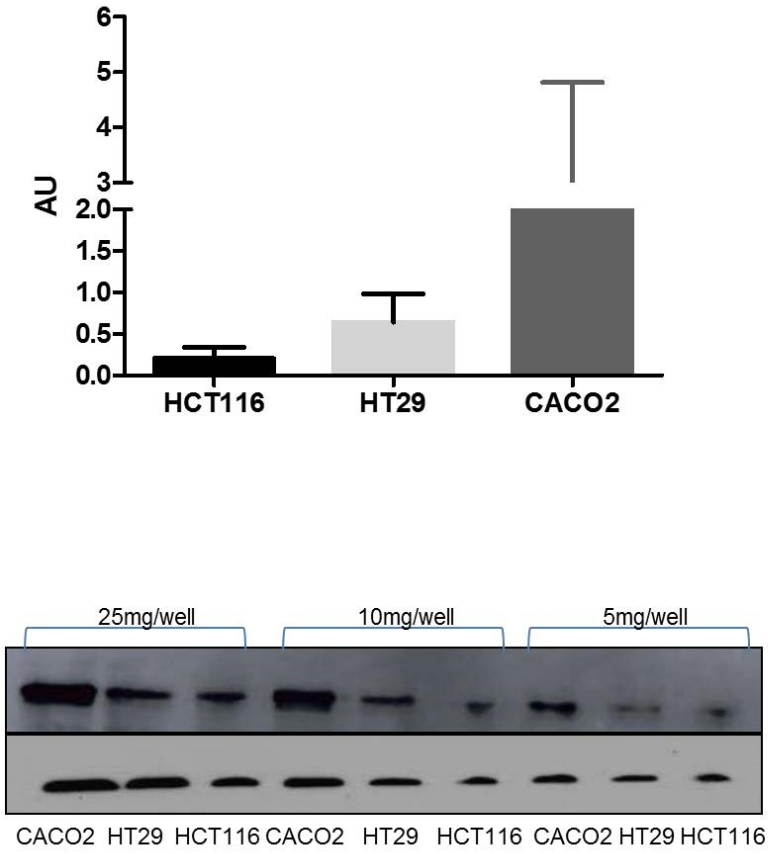

Figure 23: YAP1 is expressed in CRC cell lines. qRT-PCR and Immunodetection was performed in untreated human CRC cell lines CACO2, HT29 and HCT116. Various concentrations of cell lysates were resolved by 10% SDS–PAGE with B-actin as the loading control. The results suggested that the optimal concentration was 25mg/ml. This concentration was used in all our experiments.

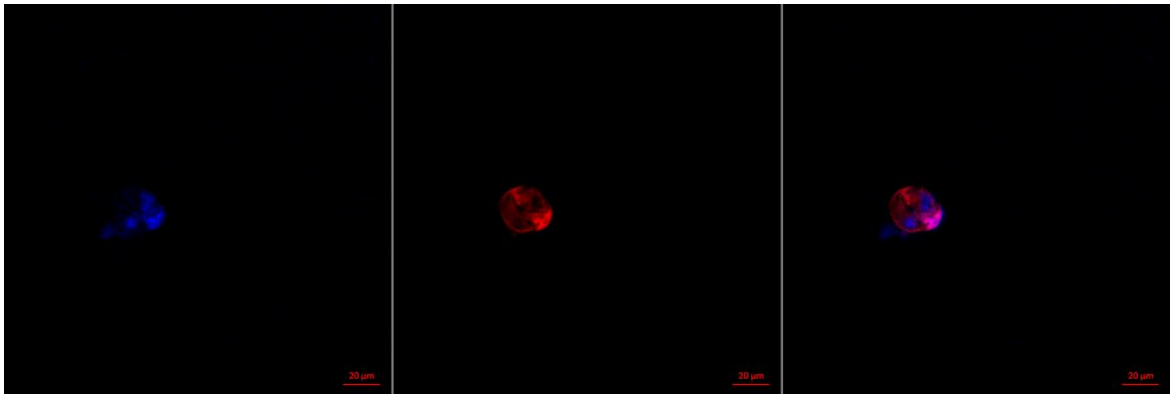

Figure 24: Immunocytochemistry of GPER (red) in human cancer colon cell line. CACO2 cells were stained and the Nuclear DNA labelling (blue) was done with Hoechst 33342. GPER is reported to be localised on the cytoplasmic membrane and intracellulary.

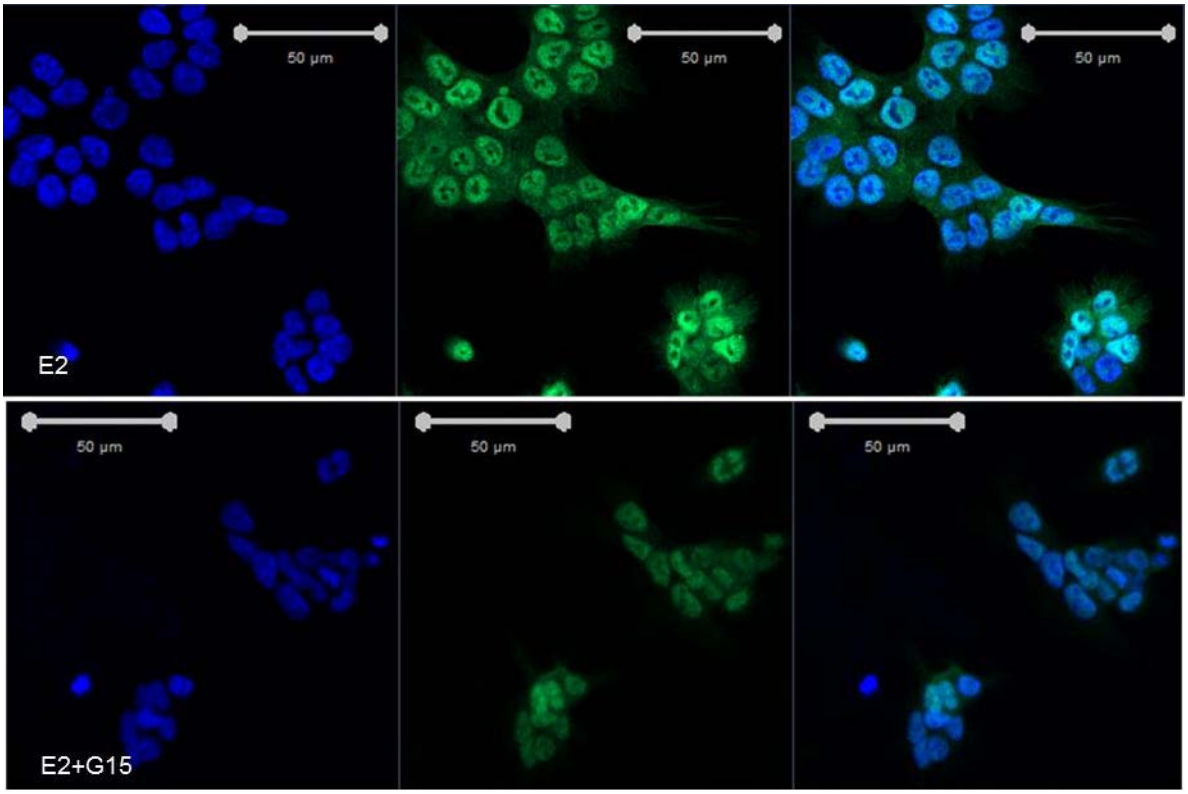

Figure 25: Immunocytochemistry of YAP (green) in human cancer colon cell line. HCT116 cells were stained and the Nuclear DNA labelling (blue) was done with Hoechst 33342. The inhibition of action is clean with the use of G15. Oestrogens (E2) appear to phosphorylate YAP and the dephosphorylated form is appear to be increased after GPER inhibition with G15.

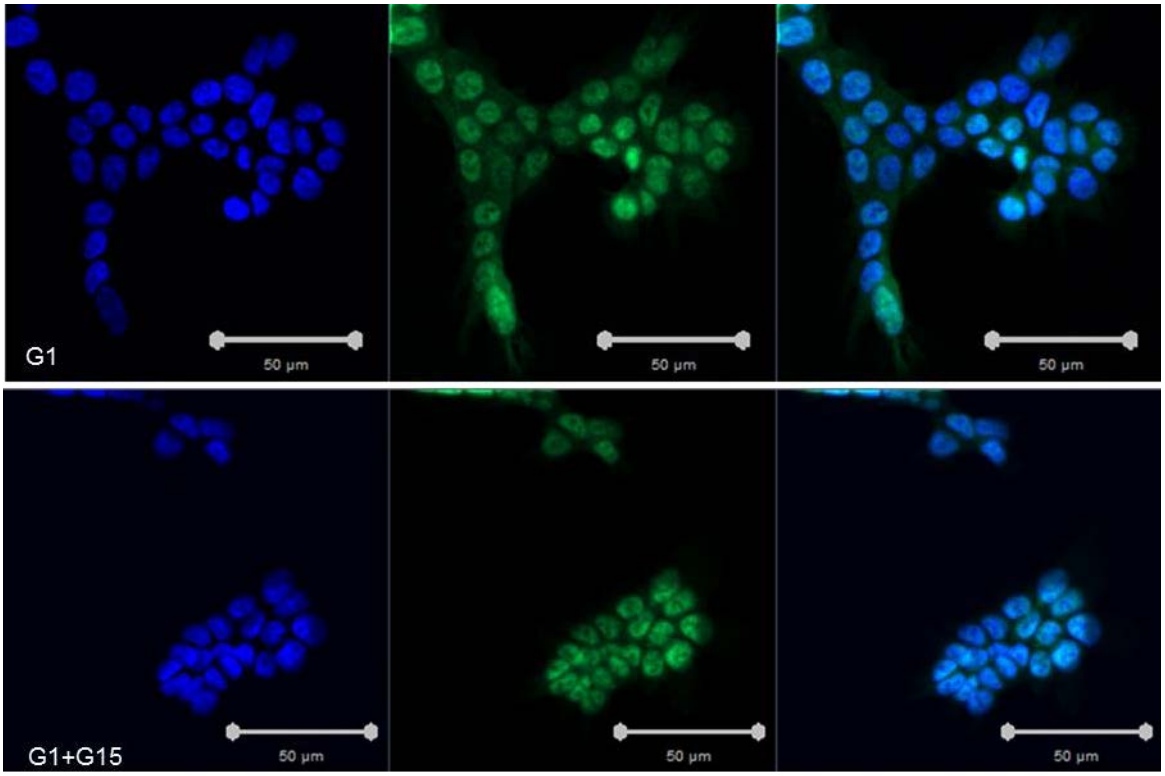

Figure 26: Immunocytochemistry of YAP (green) in human cancer colon cell line. HCT116 cells were stained and the Nuclear DNA labelling (blue) was done with Hoechst 33342. The inhibition of action is clean with the use of G15. G1 appears to phosphorylate YAP and the dephosphorylated form is appear to be increased after GPER inhibition with G15.

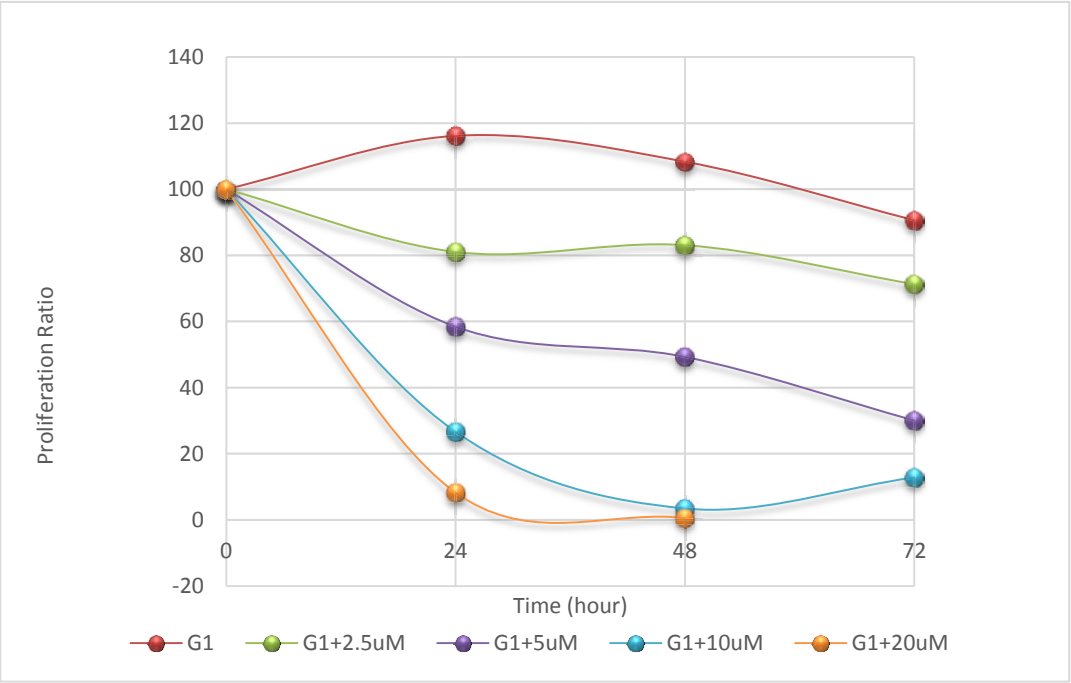

Figure 27: (VP) treatment attenuates proliferation capacities of HCT116 cell line. HCT116 cells were incubated with VP at various concentrations (2.5, 5, 10, and 20 µM) for different durations (24, 48, 72 hours), respectively with and without the addition of G1 (100nM). Viable cells were analysed using BrdU assay, whose results are expressed as percentages of inhibited cells. All conditions are normalised to the dFBS control (100%).

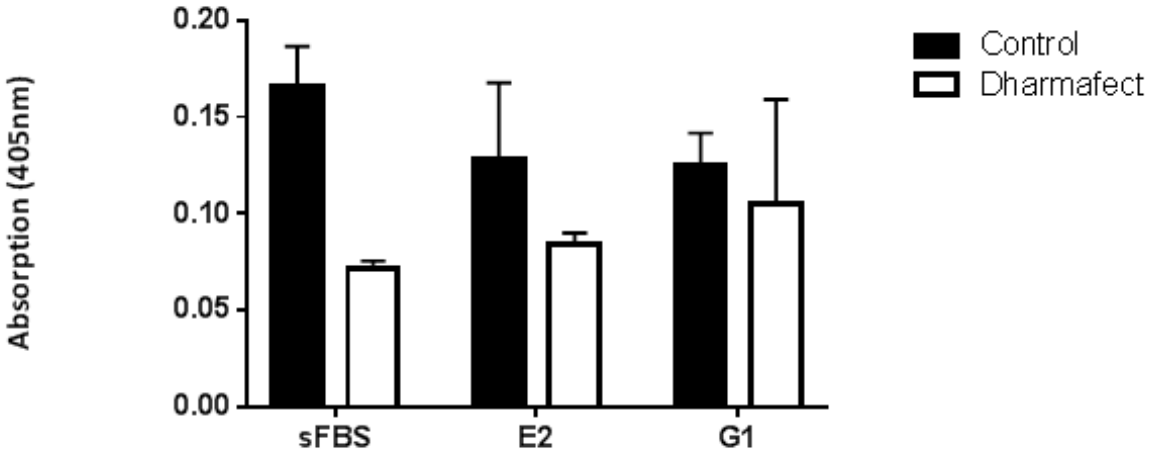

Figure 28: HCT116 incubated with non-target siRNA and HCT116 cell incubated with the transfection reagent Dharmafect 4 were treated with E2 (100nM) and G1 (100nM) for 48 hours. Viable cells were analysed using BrdU assay, whose results are expressed as absorption in 405nm. All conditions are normalised to the dFBS control without cells. HCT116 cell incubated with the transfection reagent Dharmafect have a trend towards increase in proliferation after E2 and G1 treatment. HCT116 incubated with non-target siRNA do not respond to treatment. 2-tailed Student's t test showed significantly different among G1 treatments after Verteporfin addition ** $p < 0.01$, *** $p < 0.001$

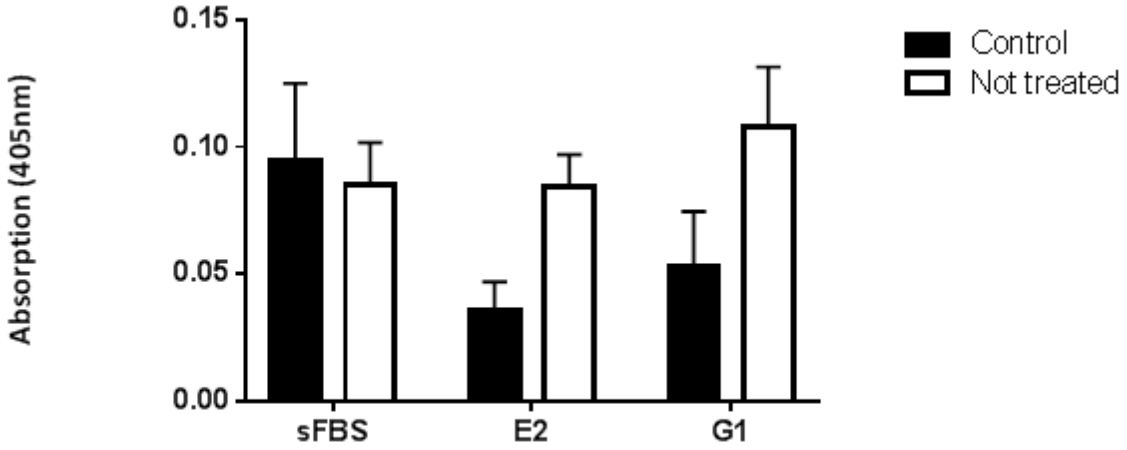

Figure 29: HCT116 incubated with non-target siRNA and HCT116 cell grown in double stripped media were treated with E2 (100nM) and G1 (100nM) for 48 hours. Viable cells were analysed using BrdU assay, whose results are expressed as absorption in 405nm. All conditions are normalised to the dFBS control without cells. HCT116 cell grown in double stripped media have a trend towards increase in proliferation after E2 and G1 treatment. HCT116 incubated with non-target siRNA do not respond to treatment. 2-tailed Student's t test showed significantly different among G1 treatments after Verteporfin addition ** $p < 0.01$, *** $p < 0.001$.

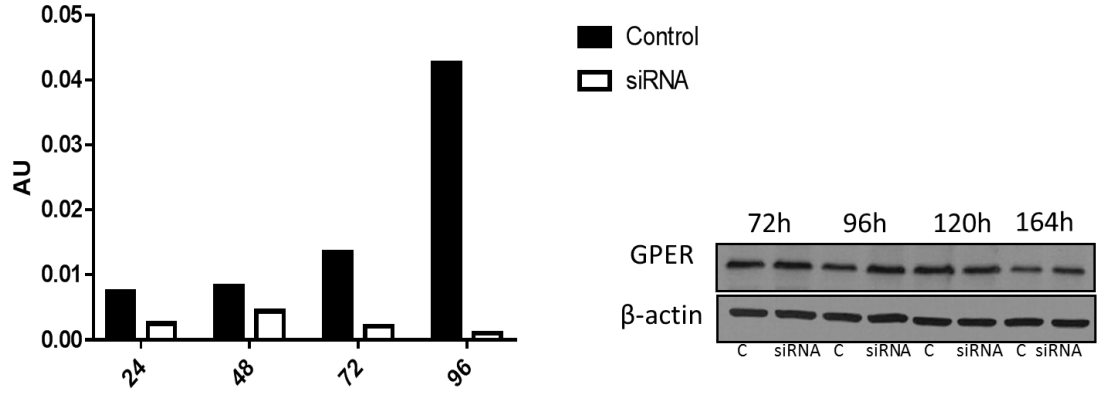

Figure 30: *GPER* siRNA optimization in HCT116 cells. Cells were transfected with 25nM of siRNA. After 24 hours the stripped media was replaced with fresh double stripped media. mRNA and protein was collected 24, 48, 72 and 96 hours after transfection. Cell lysates from HCT116 cells, 24, 48, 72 and 96 hours after transfection, were resolved by 10% SDS–PAGE with B-actin as the loading control. GPER (38 kDa) protein expression was not reduced after gene silencing.

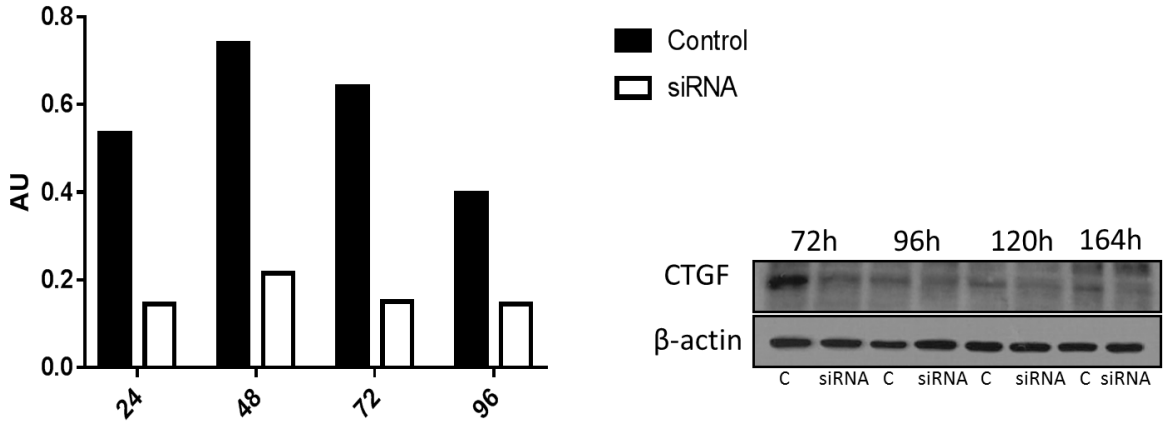

Figure 31: *CTGF* siRNA optimization in HCT116 cells. Cells were transfected with 25nM of siRNA. After 24 hours the stripped media was replaced with fresh double stripped media. mRNA and protein was collected 24, 48, 72 and 96 hours after transfection. Cell lysates from HCT116 cells, 24, 48, 72 and 96 hours after transfection, were resolved by 10% SDS–PAGE with B-actin as the loading control. CTGF (38 kDa) protein expression was reduced after 72 hour of transfection