The G-protein coupled estrogen receptor in breast tumors positively associates with ERα, and constitutes a clinically significant genomic target of estrogen in breast cancer cells

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By

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DECLARATION

The thesis titled **"The G-protein coupled estrogen receptor in breast tumors positively associates with ERα, and constitutes a clinically significant genomic target of estrogen in breast cancer cells"** represents my original research work carried out in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India, under the supervision of Dr. Anil Mukund Limaye.

Sincere efforts have been made to acknowledge contributions from other investigators that helped in conceptualizing and executing the research work. Those who have provided suggestions and technical help have been duly acknowledged. All the research articles and resources used have been cited in the reference section.

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CERTIFICATE

This is to certify that the work described in the thesis titled, **"The G-protein coupled estrogen receptor in breast tumors positively associates with ERα, and constitutes a clinically significant genomic target of estrogen in breast cancer cells"** submitted by **Uttariya Pal** (Roll No. 166106001) to the Indian Institute of Technology Guwahati, India, for the award of the degree of Doctor of Philosophy is an authentic record of the research work carried out under my supervision in the Department of Biosciences and Bioengineering, Indian Institute of Technology Guwahati, India.

This thesis or any part thereof has not been submitted elsewhere for the award of any other degree or diploma.

30.06.2023

Dr. Anil Mukund Limaye
Thesis Supervisor

To my beloved family

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CHAPTER 1

Introduction

1.1. Introduction

Estrogen exerts its effects on target cells, and tissues via genomic, and non-genomic pathways. The genomic effects of estrogen are mediated by the canonical estrogen receptors, namely ERα and ERβ. These are ligand-dependent transcription factors encoded by the ESR1 and ESR2 genes, respectively[1]. The non-genomic effects of estrogen are mediated by membrane-tethered canonical estrogen receptors[2–5], ERα36[6,7], a splice variant of ERα, or the non-canonical G-protein coupled estrogen receptor (GPER)[8].

GPER is the most recent entry into the list of membrane-associated ERs (mER). It was originally cloned by independent investigating groups with unrelated research interests[9–15]. Sequence analysis using bioinformatic tools revealed that GPER codes for a G-protein coupled receptor with a predicted molecular weight of 42.2 kDa, which shares 28% sequence identity with angiotensin II 1A and interleukin 8A receptors[14]. An orphan receptor then, this protein was called GPR30. Its recognition as an estrogen receptor has its origin in the substantial works of Filardo et al. (2000)[16] and others[17,18], which demonstrated specific estrogen-GPER interaction, and subsequent downstream effects. Both short-term non-genomic, and long-term genomic effects on gene expression were demonstrated[16,18]. A large body of work indicates that GPER activation leads to increased cyclic adenosine monophosphate (cAMP) levels[19,20]. However, with contradictory reports, this issue is debatable[21,22]. Recently, the role of GPER as an estrogen receptor has also been questioned[23]. Nevertheless, GPER is relevant in different physiological systems, such as immune, reproductive, cardiovascular, neuroendocrine, urinary, and musculoskeletal systems[24,25]. GPER is aberrantly expressed in endocrine and non-endocrine tumors, and significantly associated with various clinicopathological markers[26,27].

The rising popularity of GPER in the field of breast cancer research stems from the large volumes of data that have revealed- a) its association with clinicopathological variables, b) its role in epidermal growth factor (EGF) - like effects of estrogen, c) its potential as a therapeutic target or a prognostic marker, and d) tamoxifen agonism and endocrine resistance. GPER cross-talks with ERα[28]. However, the literature portrays ambivalence in the nature of their association. Besides, the significance of their association in mammary epithelial cells, or breast tumors, is not clear. Despite the known regulation of GPER by hormones, the mechanism of estrogen-mediated regulation of GPER in breast cancer cells is not completely understood.

1.2. Aims and scope

The estrogen receptor α (ERα) is a decisive variable that governs the mode of breast cancer treatment. It influences breast cancer prognosis, and tumor phenotype. ERα expression in breast tumors has been consistently linked to indolence, favorable prognosis, and prediction of response to endocrine therapy, while its absence suggests a more aggressive tumor behavior. However, the underlying genetic and molecular determinants of these differences and their relationship with ERα expression remain poorly understood. A potential avenue for exploration lies within the ERα co-expression network, involving genes that interact with or are regulated by ERα. Unraveling the intricate interactions and functions of these genes may provide insights into the mechanisms underlying ERα-associated outcomes.

In line with this supposition, Carmeci et al. (1997) employed differential screening of cDNA libraries from two breast cancer cell lines: MCF-7 (ERα-positive) and MDA-MB-231 (ERα-negative). Their objective was to identify genes associated with ERα expression. They identified a gene called GPCR-Br, which encodes an orphan G-protein-coupled receptor (GPCR). They found that GPCR-Br was abundantly expressed in ERα-positive MCF-7 cells but not in ERα-negative MDA-MB-231 cells. This finding suggests a potential involvement of GPCR-Br with ERα signaling or function. However, the precise role of GPCR-Br (now known as GPER) and its relationship with ERα expression in breast cancer is yet to be fully elucidated[14].

The clinical import of ERα-GPER co-expression in the aforementioned cell culture models is of value, but warrants due attention in the face of the inconsistencies and knowledge gaps. Since its discovery, the co-expression, or association of GPER with ERα in breast tissue specimen was examined by several investigators. While a positive association was reported by some[29,30]; negative[31–33], or no association[34–36] was reported by others. More independent investigations across different cohorts are needed to better understand the relationship between GPER-ERα co-expression in breast tumors. Furthermore, the clinical significance of GPER-ERα co-expression has remained elusive. The prognostic implications of GPER expression in the presence, or absence, of ERα, if any, would be clinically valuable.

Clinical investigations have indicated lower GPER expression in breast tumors compared to normal tissues, and its expression was found to be negatively associated with tumorigenesis[34,37,38]. Based on these findings, GPER is predicted as a potential tumor

suppressor. The epigenetic silencing of tumor suppressors, and its association with tumorigenesis, is a well known[39]. Recent studies show that DNA methylation is associated with the loss of GPER expression in breast and colorectal cancer cells[37,40]. In a study on breast cancer cell lines, GPER expression showed an inverse relationship with methylation in the upstream CpG island (upCpGi) in the GPER locus[37]. Given either negative[31-33] or positive[29,30] association between GPER and ERα expression in breast cancer, the current investigation examines the association between upCpGi methylation and ERα expression in breast tumors.

The mechanistic basis of GPER-ERα co-expression is unknown. Hormonal regulation of GPER, particularly by estrogen, has been touched upon in several published studies[41–43]. However, the data are confusing, and do not address the role of hormone receptors, particularly ERα. The impact of hormonal regulation of GPER on cells' responsiveness towards GPER activating ligands, and its clinical import, is yet to be determined.

1.3. Objectives

The work embodied in the thesis is based on the following objectives,

1. To investigate the correlation between GPER and ERα in breast tumor samples.
2. To study the estrogen regulation of GPER in breast cancer cells.
3. To study the impact of estrogen stimulation on the cellular response to G1, a GPER activating ligand.

CHAPTER 2

Review of literature

2.1. Breast cancer

Breast cancer has emerged as the most common malignancy in women worldwide. It is the second leading cause of cancer-related deaths among women after lung cancer. In India, it accounts for 27% of all newly diagnosed cancers among women. The mortality rate of patients with breast cancer is very high in India compared to other countries due to factors, such as lack of awareness, delayed diagnosis, and socioeconomic status[44].

Breast cancer is classified into two types: ductal carcinoma and lobular carcinoma. Ductal carcinoma begins in the lining of the milk ducts, and lobular carcinoma arises in the milk-producing lobules. Based on the pathological conditions, breast cancer is classified into three main categories: invasive, non-invasive, and metastatic. Invasive are those that have migrated into the surrounding breast tissues. Invasive ductal carcinoma and lobular carcinoma are the two most prevalent types of the invasive type, among which invasive ductal carcinoma (IDC) is the predominant[45]. It accounts for approximately 8 out of 10 invasive cases. Non-invasive are those that remain within the milk ducts or lobules. They do not metastasize into surrounding tissues and are also known as *in-situ* breast cancers. Breast cancer that has spread to other parts of the body, such as the lungs, brain, lymph nodes, and heart, is referred to as metastatic or stage IV[20].

Immunohistochemically, breast cancer can be classified into three main categories: hormone receptor-positive, HER2-positive, and triple-negative[46]. The intrinsic molecular subtypes of breast cancers were redefined by an advisory committee at the St. Gallen International Breast Cancer Conference in 2011[47]. On the basis molecular signatures, breast tumors are classified into five major molecular subtypes (Table 2.1).

Table 2.1. Molecular classification of breast tumors

Molecular subtypes	Molecular signatures
Luminal A	ERα+ and/or PR+, HER2-, Ki-67^{low}
Luminal B (HER2-)	ERα+ and/or PR+, HER2-, Ki-67^{high}
Luminal B (HER2+)	ERα+ and/or PR+, HER2 over-expressed or amplified, Ki-67$^{high/low}$
HER2+	ERα- and PR-, HER2 over-expressed or amplified
Basal-like	ERα- and PR-, HER2-

Breast cancer is linked to several risk factors, including age, mutations in genes such as BRCA-1 and BRCA-2, reproductive history, body weight, alcohol consumption, long-term post-menopausal hormone therapy, family history, and smoking[48]. Long-term exposure to endogenous estrogen is also linked to breast cancer initiation and development, involving three major mechanisms, which include induction of aneuploidy, receptor-mediated induced cellular proliferation, and increased mutation rates via metabolic activation mediated by cytochrome P450[49].

2.2. Breast cancer treatments

Breast cancer treatment depends on a number of factors, including the patient's age, type of breast cancer and the extent to which it has spread[48]. Treatment options include surgery, radiation therapy, chemotherapy, immunotherapy, and endocrine therapy.

2.2.1. Surgery includes lumpectomy or mastectomy. Lumpectomy is the surgical excision of a tumor as well as a small, cancer-free margin of healthy tissue surrounding the tumour. Mastectomy involves the surgical removal of the entire breast[50].

2.2.2. Radiation therapy destroys cancer cells using high-energy X-rays or gamma rays[51].

2.2.3. Chemotherapy involves using drugs to destroy cancer cells by preventing them from growing, dividing, and proliferating[52].

2.2.4. Immunotherapy enhances a person's immune response by targeting specific proteins, and efficiently identifying and eliminating cancer cells[53].

2.2.5. Endocrine therapy is used for the treatment of hormone receptor-positive breast cancer. Three different strategies are used in endocrine therapy, which include treatment with aromatase inhibitors, such as anastrozole to block the production of estrogen[54]**,** treatment with tamoxifen and other selective estrogen receptor modulators (SERMs) to prevent estrogen from binding to estrogen receptors[55], and treatment with selective estrogen receptor degraders (SERDs) such as fulvestrant to lower the levels of estrogen receptors[56]**.**

2.2.6. Neoadjuvant and adjuvant therapy. Neoadjuvant therapy is administered before surgery to decrease the size of a tumor and/or lower the risk of recurrence[57]. Adjuvant therapy is used after surgery to lower the risk of recurrence. Adjuvant therapy includes chemotherapy, radiation, and endocrine therapy[58].

2.3. Endocrine resistance

Patients with early-stage ERα+ breast cancer benefit from adjuvant treatment with antiestrogens that target estrogen receptors, inhibiting the activation of estrogen receptors, and thereby preventing disease recurrence. However, *de novo* or acquired endocrine resistance is seen in a significant number of cases[59]. Mechanisms for endocrine resistance include aberrations in the ERα/PR pathway (deregulation of ERα expression, co-activators, and co-repressors), genomic and epigenetic alterations, truncated ERα-isoform expression, post-translational modification of proteins, upregulated receptor tyrosine kinase signaling, and altered cell cycle regulation[60].

2.4. Estrogen

Estrogens are the steroid hormones that are primarily involved in developing primary and secondary sexual characteristics in women. Estrogens also play a vital role in maintaining physiological functions of the central nervous, cardiovascular, immune, and musculoskeletal systems[61]. In pre-menopausal women, estrogen is produced by the ovaries, corpus luteum and placenta. Females have three major types of physiological estrogens: estrone (E1), estradiol (17β-estradiol; E2), and estriol (E3)[62]. E2 is the most potent form of estrogen, which is synthesized during the pre-menopausal stage[61]. Uterus, ovary, vagina, and mammary glands, are the primary target organs of estrogen in females.

2.5. Estrogen receptors

Estrogen exerts its effects by binding to its cognate receptors. Estrogen receptors (ERs) are classified as nuclear hormone receptors, which include ERα and ERβ, or membrane estrogen receptors, the prominent of which is the G-protein-coupled estrogen receptor (GPER). Genomic actions of estrogen signaling are mediated by nuclear estrogen receptors, and the rapid non-genomic estrogen signaling, which includes activation of secondary messenger and receptor tyrosine kinases, is mediated via GPER[16].

2.5.1. Nuclear estrogen receptors

Nuclear estrogen receptors (nERs) function as ligand-activated transcription factors, that bind to the estrogen response elements (EREs) in the promoter regions of estrogen-regulated genes[63]. ERα and ERβ, are encoded by the genes ESR1 and ESR2, respectively[64].

They encode 66 and 60 kDa proteins respectively, and are located on chromosomes 6q24-27 and 14q22-24, respectively[65].

2.5.2. Nuclear estrogen receptor signaling

Upon binding to E2, ERα undergoes conformational changes, resulting in its dissociation from the heat shock proteins, followed by phosphorylation. The phosphorylated ERα dimerizes and translocates to the nucleus, where it binds to specific sequences in the E2 target genes known as EREs. The ERE comprises the DNA palindrome 5'-GGTCAnnnTGACC-3', where n is any nucleotide. The ER-ERE DNA-protein complex recruits several cofactors (co-activators or co-repressors), which regulate gene expression[66].

The ligand-activated ERα can also interact with transcription factors such as activator protein (AP1) or stimulatory protein (SP1) and regulates the transcription of E2 target genes via an ERE-independent mechanism[67]. Ligand-activated ERα forms protein-protein complexes with other transcription factors, including Fos and Jun. These complexes recognize and bind to AP1 or SP1 sites, modulating the expression of E2 target genes such as cyclin D[68] and cathepsin D[69] (Figure 2.1).

The phosphorylation of serine and tyrosine residues in the AF-1 domain can also activate ERα in a ligand-independent manner. Epidermal growth factor (EGF) and insulin-like growth factor (IGF) bind to their respective receptors, triggering a number of signaling cascades, including the activation of mitogen-activated protein kinase[70,71]. Through MAPK signaling, the activated kinases phosphorylate the ERα at serine-118, and the phosphorylated ERα then regulates the estrogen-responsive genes[71].

2.6. GPER

The gene encoding GPER is located on the short arm of chromosome 7 in the p22.3 region. GPER is a seven-transmembrane GPCR, 375 amino acids long, with an estimated molecular weight of 42.2 kDa[14]. The localization of GPER is controversial. Its localization has been reported in the plasma membrane and endoplasmic reticulum[25]. Interestingly, independent clinical studies have also reported nuclear localization of GPER[27]**.** Its activation leads to rapid short-term non-genomic effects, which occur within seconds to minutes[16]. GPER is an emerging prognostic marker and a potential therapeutic target in endocrine cancers[24,72].

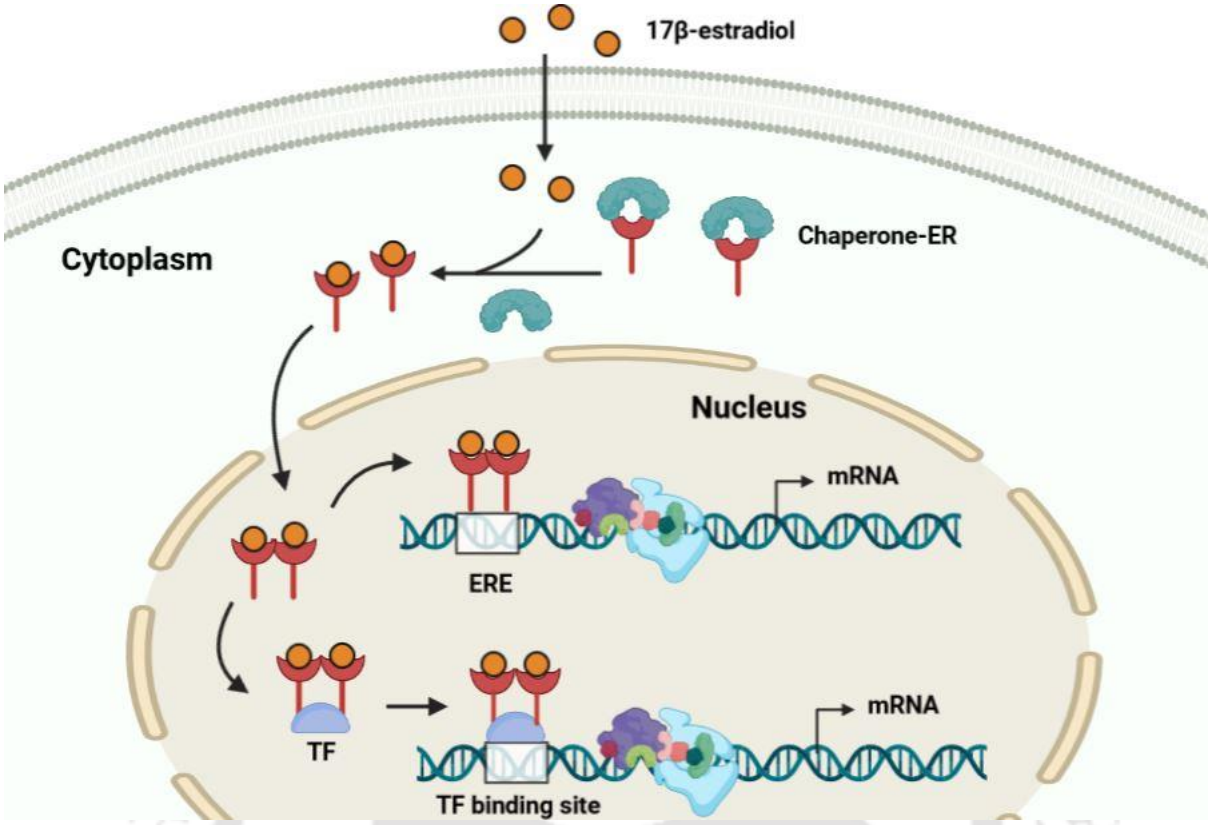

Figure 2.1: **Schematic representation of genomic signaling pathway of estrogen.** Upon binding of estrogen to its cognate estrogen receptor, the estrogen receptor dimerizes and translocates to the nucleus. The ligand-activated ERα binds to the estrogen response element (ERE) either directly or indirectly via interaction with transcription factors (eg, AP1 or SP1) at their cognate transcription factor binding sites, regulating the transcriptional activity of a gene. Created with BioRender.com.

2.6.1. GPER mRNA variants

According to the NCBI nucleotide database, GPER has three curated mRNA variants: NM_001505.2 (GPER-v2), NM_001039966.1 (GPER-v3), and NM_001098201.1 (GPER-v4). They have an identical coding region (encoding a 375 amino acid protein) and 3′ UTRs, but differ in their sequence lengths, exon-intron organization, and 5′ UTRs (Figure 2.2 A). The majority of studies on GPER expression have reported total GPER mRNA using primers designed to amplify a region common to all the variants. Only a few studies have shown the expression of GPER at the variant level. GPER mRNA expression was examined at the variant level in the hamster ovary[73], human endometrium[74], breast[37], and colon cancer cell lines[40]. We recently showed the expression of an uncharacterized and novel transcript annotated as GPER in the UCSC Genome Browser (uc010ksd.1, GENCODE Transcript ID - ENST00000401670.1), and referred to it as GPER-v5[75]**.** This transcript, has two exons and a long intron. It is similar to the known variants in terms of the open reading frame and 3′ UTR,

which are present in the second exon. The exon 1, located about 5 kb upstream relative to the first exon of GPER-v2 and -v3, is unique to this variant.

2.6.2. GPER protein

All GPER mRNA variants code for the same protein, with an estimated molecular weight of 42.2 kDa. Carmeci and co-workers were the first to investigate the primary structure of GPER. In-silico analysis of GPER indicated that it has high similarity with other GPCRs. It shares 28% identity with the angiotensin II 1A receptor, 28% with the interleukin 8A receptor, and 24% with chemokine receptor type I. The highly conserved regions of GPER are found in the seven transmembrane (TM) domains. Three N-glycosylation sites (Asn-X-Ser/Thr) are present in the N-terminus of the extracellular domain. There are 11 cysteine residues in the GPER protein, four of which are conserved. GPER protein also has the highly conserved DRY (Aspartate-Arginine-Tyrosine) sequence, which regulates the conformational state of GPCRs[14] (Figure 2.2B).

2.7. Estrogen as a GPER ligand

In 2005, two independent studies revealed that 17β-estradiol (E2) binds to GPER with high affinity[17,18]. Thomas et al. (2005) reported that tritiated estradiol binds to GPER with an affinity of 3 nM[17]**.** On the other hand, using a flurophore-tagged estrogen, Revankar et al. (2005) demonstrated the selective binding of estrogen to several cancer cell lines, and GPER-transfected COS cells with an affinity of 6 nM[18]**.** These studies led to the classification of GPER as a membrane estrogen receptor (mER) by the International Union of Pharmacology.

2.8. GPER signaling

GPER signaling is similar to that of other GPCR family members. On activation, the heterotrimeric G-protein is dissociated into its constituent G_{α} and $G_{\beta\gamma}$ subunits. These subunits produce different effects by interacting with different molecules as illustrated in Figure 2.3. Upon GPER activation, the $G_{\beta\gamma}$ subunit activates Src-like tyrosine kinase, which in turn activates cell surface MMPs[76]. The MMPs cleave the surface-bound pro-HB-EGF. The released HB-EGF binds to the EGF receptor, which results in the activation of the EGFR/MAPK pathway.

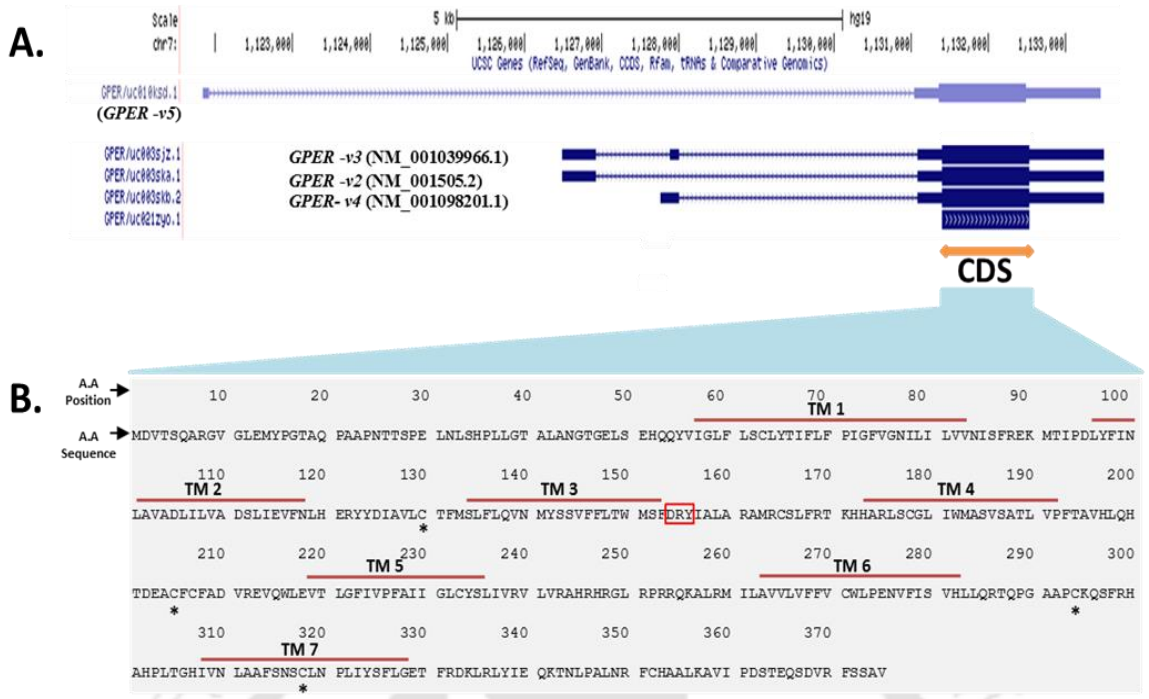

Figure 2.2: GPER transcript variants visualized in the UCSC Genome Browser. A. snapshot of GPER locus in the human genome, as viewed from the UCSC genome browser, along with all the GPER variants. NM_001505.2: GPER-v2), NM_001039966.1: GPER-v3, NM_001098201.1: GPER-v4, transcript in light blue colour: GPER-v5 (uc010ksd.1). B. Amino acid sequence of GPER. The seven hydrophobic transmembrane domains are indicated by red lines above the amino acid sequence. The four conserved cysteine residues and the characteristic DRY residues are indicated with * and red coloured box, respectively.

Activation of the EGFR/MAPK pathway phosphorylates extracellular signal-regulated kinase (ERK) via Raf-1 phosphorylation. This leads to the activation of various transcription factors such as c-Myc, c-fos, and c-jun[8]. EGFR/MAPK pathway also triggers the activation of the AKT pathway[76]. Downstream signaling events of the $G_{\beta\gamma}$ subunit result in the proliferation of cells. These effects of $G_{\beta\gamma}$ activation have also been reported in ERα-negative SkBr3 cells[76].

The release of G_{α} post GPER activation leads to increased cAMP levels via activation of the enzyme adenylate cyclase[76]. cAMP stimulates the protein kinase A-mediated deactivation of Raf-1, which inhibits the MAPK/ERK pathway. GPER-mediated cAMP production inhibits the proliferative effect of the $G_{\beta\gamma}$ subunit[17,76]. Furthermore, the G_{α} subunit facilitates the membrane-localized accumulation of PIP3, which subsequently increases intracellular calcium levels[18,77]. GPER signaling is known to cross-talk with other signaling pathways, leading to various physiological effects, summarised in Table 2.2.

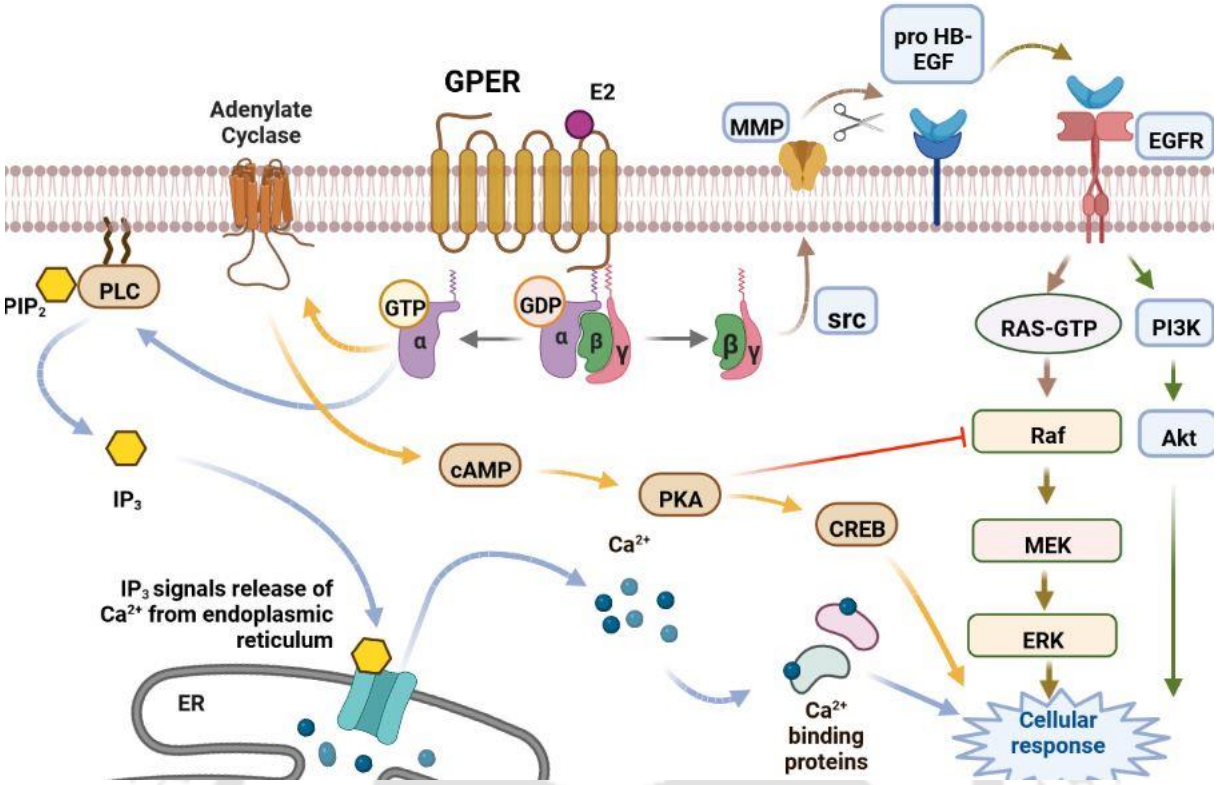

Figure 2.3: Schematic representation of GPER signaling pathway. GPER produces short-term non-genomic effects upon ligand binding. GPER activation dissociates the hetero-trimeric G-protein into its constituents G_{α} and $G_{\beta\gamma}$ subunits. The release of the G_{α} subunit led to increased cAMP and the production of cytoplasmic calcium. In contrast, the release of the $G_{\beta\gamma}$ subunit led to the activation of the PI3K and MAPK pathways. Created with BioRender.com.

Table 2.2. Cross-talk of GPER signaling with other signaling pathways

Signaling Pathway	Model system	Effect	Reference
Notch signaling pathway	SkBr3 cells, MCF-7 cells	Enhanced cell migration	78
Hypoxia-inducible factor (HIF) induced pathway	SkBr3 cells	Enhanced CTGF, VEGF, IL-6 expression to up regulate hypoxia-related vasculogenesis	79
Insulin-like growth factor receptor (IGFR) pathway	MCF-7 Cells	IGF-I-mediated cell migration and suppression of apoptosis	80
NF-κB pathway	SkBr3 Cells	Chemokine-induced cell proliferation and invasion	81
Hippo/YAP pathway	Primary tumors, ZR-75-30 cells	Epigenetic silencing of pro-apoptotic factors, activation of pro-proliferative transcription factors such as YAP/TAZ	82

2.9. Regulation of GPER

In several cancer cell lines and Cancer associated fibroblasts (CAFs), GPER is regulated by growth factors such as epidermal growth factor (EGF), transforming growth factor (TGF), insulin-like growth factor-I (IGF-I), and insulin[43,83]. The putative function of AP1 in GPER regulation was initially proposed by Carmeci et al. (1997)[14]. In response to EGF and TGFα, recruitment of c-fos to the AP1 site of the GPER locus upregulates the expression of GPER in Ishikawa and TAM-R MCF-7 cell lines[43]. Demarco et al. (2013)[83] reported that the IGF-IR/PKC-δ/ERK/c-fos/AP1 transduction pathway is involved in the IGF-mediated stimulation of GPER expression. They also demonstrated that phospho-ERα is recruited to the AP1 site located within the GPER promoter sequence, and is involved in the IGF-I-dependent regulation of GPER expression[83]. Collectively, these reports highlighted the importance of the AP-1 site in the intronic region of the GPER locus. Hypoxia transcriptionally induces the expression of GPER via HIF-1α binding to the hypoxia-responsive element (HRE) of GPER. Its expression in triple-negative breast cancer cells is elevated by heregulin-ß1 (HRG-ß1) via ERBB2/ERBB3 heterodimers, and the MAPK/ERK pathway[84].

2.10. Role of DNA methylation in the regulation of GPER expression

Hypermethylation of the promoter region is a known mechanism for silencing tumor suppressors. Recent studies, including those reported from our laboratory, show that DNA methylation is associated with the loss of GPER expression in breast and colorectal cancer[37,40,85,86]. An inverse relationship between GPER expression and methylation of the upCpGi in the GPER locus was found in breast and colon cancer cell lines[37,40]. The upCpGi aligns with the first exon of GPER-v2 and GPER-v3 and is near the transcription start site. A differentially methylated region (DMR) comprising terminal eight CpG dinucleotides in the upCpGi was also identified in breast, and colon cancer cell lines. Although higher GPER expression is associated with hypomethylation in the DMR[37,40] in breast and colon cancer cell lines, the association has not been examined in the tumors.

2.11. GPER ligands

E2 is a prominent ligand of GPER[87]. Besides estrogen, other steroids such as E3, and estrone also bind to GPER. Estriol and estrone are antagonists to GPER, and exert inhibitory effects on GPER-mediated signaling[88]. Additionally, phytoestrogens (e.g. genistein, zearalenone) and xenoestrogens (e.g. bisphenol A, methoxychlor, dichloro diphenyl-trichloro-ethane (DDT), dioxins) can also activate GPER[89]. Fulvestrant (a SERD), and tamoxifen (a SERM), are GPER agonists[90,91]. Gros et al. (2013) demonstrated aldosterone mediated activation of GPER[92]. MIBE (ethyl 3-[5-(2-ethoxycarbonyl-1-methylvinyloxy)-1-methyl-1H-indol-3-yl]but-2-enoate) a novel compound, was shown to function as an antagonist for GPER in breast cancer cells, inhibiting the GPER-mediated EGFR and ERK activation[93].

2.12. G1, a GPER-specific agonist

In order to investigate the specific effects of GPER activation, GPER-specific agonists and antagonists have been developed in recent years. A combination of virtual and biomolecular screens was used to identify GPER-specific pharmacological agents, which resulted in the identification of G1 (1-[4-(6-bromobenzo[1,3]dioxol-5-yl)-3a,4,5,9b-tetrahydro-3H-cyclopenta[c]quinolin-8-yl]-ethanone), a non-steroidal compound with high affinity and good selectivity[94]. Soon after the identification of G1, Prossnitz and colleagues developed G15, 4-(6-bromo-benzo[1,3]dioxol-5-yl)-3a,4,5,9btetrahydro-3H-cyclopenta[c]quinoline) that functions as a GPER antagonist capable of inhibiting uterine and neurological responses *in vivo*[95]. Later, Dennis et al. (2011) developed G36, a GPER antagonist with enhanced selectivity. G36 specifically inhibits estrogen-mediated PI3K activation via GPER but not by ERα[96].

G1 shows no measurable binding for the ERs at concentrations as high as 1 μM. Another study has reported that G1 does not significantly interact with 25 other G-protein-coupled receptors, further confirming its specificity[97]. Fluorescence-based assays showed that G1 could mobilize intracellular calcium in transiently GPER-expressing COS-7 cells and activate PI3K pathways in SKBr3 (ER-, GPER+) and MCF7 (ER+, GPER+) breast cancer cells[94]. G1 also inhibits the chemo-attractant-induced migration of SKBr3 or MCF7 cells[94,95]. Such characteristics make G1 a popular ligand for exploring the pathophysiological functions of GPER[87].

2.13. Controversies regarding G1

G1 induces the expression of genes, and activates pathways that stimulate the proliferation of ovarian[98], testicular[99], endometrial[100,101], fibroblast[102], and breast cancer cells[103]. In 2007, Albanito et al. (2007) showed that G1 induces SKBr3 cell proliferation[98]. In contrast, Chan et al. (2010) demonstrated that G1-mediated activation of GPER led to the sustained activation of ERK1/2 and upregulation of p21CIP1, resulting in the arrest of PC-3 prostate cancer cells in the G2 phase of the cell cycle[104]. Similar effects of G1 were also observed in ERα-positive breast cancer cells[41]. Wang and colleagues (2012) demonstrated that the effect of G1 on cell proliferation was GPER-independent. G1 blocked the tubulin polymerization, thereby leading to cell cycle arrest and suppression of the proliferation of ovarian cancer cells[105]. In agreement with this, another study revealed that G1, at micromolar concentrations, induces mitotic arrest, blocks tubulin polymerization, and mitotic spindle disruption, which leads to apoptosis in human vascular smooth muscle cells. Treatment with G15, or GPER siRNAs, did not reverse these effects, implying that these effects of G1 are GPER-independent[106].

2.14. GPER expression in breast cancer

60% of the breast tumor samples are GPER positive[35,36]. It is found to be associated with known histopathological markers such as ER, PR, and HER2 in breast tumors. The association between GPER and ER in breast cancer is debatable. While Filardo et al. (2006) and some researchers reported a positive association between GPER and ERα[29,30]; others observed a negative[31-33] or no association[34-36]. The reduced expression of GPER in tumor samples compared to normal tissues, as well as the findings of survival analysis performed by Ignatov et al. (2013) and Manjegowda et al. (2017) not only support the hypothesis that GPER is a tumor suppressor but also indicate the clinical value of GPER expression in breast cancer[37,38]. GPER is highly expressed in TNBC cell lines[107]. Immunohistochemical analysis of the TNBC tumors revealed that the GPER-positive tumors stained much more strongly for p-ERK1/2 than the GPER-negative tumors. The positive correlation between GPER expression and p-ERK1/2 is linked with tumor size and advanced stage, indicating that GPER/ERK signaling may contribute to tumor growth in TNBC patients, which correlates with the *in vitro* experimental data[107]. It is also expressed in the stromal fibroblasts of primary breast tumors. Through a positive feedback loop involving the GPER/EGFR/ERK signaling pathway, GPER in the tumor-associated stroma likely contributes to breast cancer

progression, particularly in the case of tamoxifen resistance[108]. The correlation between GPER expression and clinicopathological factors associated with breast cancer development, such as patient survival, size of the tumor, lymph node positivity, and vascular invasion is inconsistent, and the clinical significance of this relationship has remained elusive[29,30 31–3334–36]. More independent investigations across different cohorts are needed to better understand the association between GPER and clinicopathological factors associated with breast cancer development.

2.15. GPER in endocrine resistance of breast cancer

Tamoxifen, a selective estrogen receptor modulator and a preferred drug for treating ER-positive breast cancer, functions as a GPER agonist[17]. The activation of the EGFR-MAPK pathway by activated GPER, together with its tamoxifen-mediated agonism, implicate this receptor in endocrine resistance[33]. Ignatov et al. (2011) determined GPER expression in 323 samples of primary invasive breast tumors using immunohistochemistry. They found 183 (56.7%) samples positive for GPER. On the contrary all normal tissues in the margins of the tumor samples were GPER-positive. The study also reported that 78.8% of the recorded relapses were found in patients undergoing endocrine therapy. While GPER expression did not affect overall survival, it significantly decreased relapse-free survival. Notably, relapse free survival was significantly decreased in patients with GPER-positive tumors, who received tamoxifen with or without chemotherapy. They also compared relapse-free survival in two groups of patients with GPER-positive tumors; those treated with tamoxifen and those treated with tamoxifen followed by an aromatase inhibitor. The 5-year relapse-free survival was significantly reduced in former, compared to the latter. Conversely, survival analysis in patients administered with tamoxifen showed that GPER positivity was significantly associated with reduced 5-year relapse free survival. GPER expression in relapsed tumors from patients receiving tamoxifen-treatment was significantly higher than in the matched primary tumors. These data along with the results of univariate and multivariate analysis uphold the utility of GPER as a predictor of unfavourable response to tamoxifen-treatment[35].

Sjostrom et al. (2014) studied total (cellular) GPER and plasma membrane GPER (PM-GPER) expression in 742 breast tumor samples from a Swedish cohort. They did not observe any association between total GPER expression and distant disease-free survival; even when the patients were stratified on the basis of ERα expression and tamoxifen-treatment. However, they reported a strong correlation between PM-GPER expression and

reduced distant disease-free survival (DDFS), not only in all patients but also in tamoxifen-treated subgroup. The negative correlation between PM-GPER and DDFS was also observed in the ERα-positive subgroup of tamoxifen-treated patients. Applying multivariate statistics, which included variables such as ERα, histological grade, HER2, tumor size and tamoxifen, they showed that PM-GPER was an independent prognostic factor for DDFS. Furthermore, PM-GPER positivity and negativity correlated with reduced and increased DDFS, respectively, in tamoxifen-treated patients with ERα- and PR-positive breast tumors. Their analysis did not reveal any predictive value of GPER in terms of response to tamoxifen-treatment[109].

Ignatov et al. (2020) reported GPER positivity in 80.9% (352/435) of primary non-metastatic breast tumors. In this cohort, they found an overall association between GPER expression and longer disease-free survival. They considered a subset of patients with GPER-positive tumors within this cohort and analyzed the effect of tamoxifen- or aromatase inhibitor- treatment on disease-free survival. No difference in median survival was found. However, when selection bias was addressed by matching tumor size, histology, and grade, tamoxifen-treatment was associated with significantly reduced disease-free survival compared to aromatase inhibitors[110].

2.16. GPER signaling in endocrine resistance of breast cancer

The development of endocrine resistance is a gradual and complex process. GPER signaling is considered a contributing factor in the development of endocrine resistance. Several signaling pathways activated by GPER signaling, including GPER-EGFR cross-talk, upregulation in aromatase expression, β1-integrin, and activated PI3K/AKT signaling, may contribute to endocrine resistance in breast cancer, as illustrated in Figure 2.4.

2.16.1. GPER and EGFR crosstalk

GPER-EGFR crosstalk facilitates the progression of endocrine-resistant breast tumors. Treatment with E2 in TAM-R MCF-7 cells facilitates GPER translocation from the endoplasmic reticulum to the cell surface. Activation of surface GPER induces the activation of c-Src and matrix metalloproteinases (MMPs), which further results in the activation of EGFR. The GPER-EGFR crosstalk leads to increased proliferation of TAM-R cells upon E2 or G1 treatment with enhanced MAPK signaling[33]. In TAM-R cells, the ability of the G_{α}

subunit to promote cAMP generation is restricted, thereby attenuating cAMP-mediated inhibition of ERK1/2 activation[33].

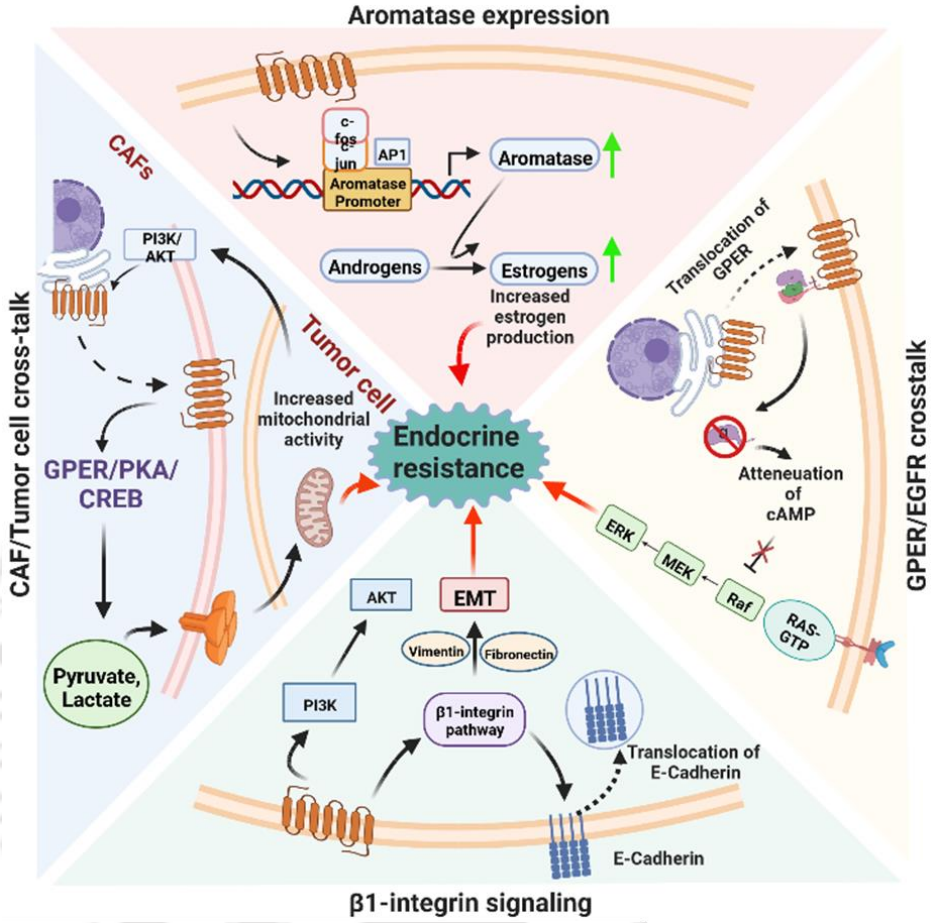

Figure 2.4: Illustration of GPER mechanisms driving endocrine resistance in breast cancer. GPER-EGFR cross-talk, GPER-mediated upregulation of aromatase expression and β1-integrin signaling, and CAF/tumor cell cross-talk have been detected in tamoxifen-resistant breast cancer cells**.** Alterations in any of these signaling elements can mediate resistance to endocrine therapy to provide alternative proliferation and survival stimuli. Created in BioRender.com.

2.16.2. Aromatase expression

Aromatase catalyzes the conversion of androgens to estrogen[111]. GPER confers endocrine resistance in MCF-7 cells by up-regulating aromatase expression. Catalano and colleagues have shown that treatment with G15 and GPER siRNA reduces aromatase expression in tamoxifen-resistant MCF7 cells, which are otherwise known to exhibit high aromatase expression. The mechanism of GPER-mediated upregulation of aromatase expression was also demonstrated in SkBr3 cells[91]. The activation of GPER facilitates the recruitment of cfos/c-jun complex to AP1-responsive elements within the aromatase promoter

region, which ultimately leads to increased aromatase promoter activity. These observations suggest a potential involvement of GPER in promoting the proliferation of breast tumors by up-regulating aromatase expression in response to tamoxifen treatment.

2.16.3. β1-integrin

β1-integrin is involved in inflammation, proliferation, adhesion, invasion, and signal transduction between the tumor cell and its microenvironment[112]. Recent studies have shown the GPER-mediated up-regulation of β1-integrin in tamoxifen-resistant MCF-7 cells. This effect is reversed upon treating the cells with G15. Enhanced β1-integrin signaling via GPER activation has pronounced downstream effects, such as activation of the pAKT pathway and improved migration of CAFs. GPER activation by tamoxifen increases the expression of mesenchymal markers such as vimentin and fibronectin via the β1-integrin signaling pathway. It also promotes the translocation of E-cadherin from the cell membrane to the cytoplasm. This is relevant because of the important role of E-cadherin in maintaining cell-cell interaction. These data suggest the critical role played by GPER in activating β1-integrin signaling in EMT[113].

2.16.4. Activated PI3K/AKT signaling

Crosstalk between CAFs and tumor cells is a potential mechanism that directly or indirectly activates GPER translocation from the nucleus to the cytoplasm in CAFs. It is regulated by tumor cell-induced PI3K-AKT signaling in CAFs. The translocated GPER in the cytoplasm stimulates novel estrogen/GPER/PKA/CREB signaling, which leads to metabolic reprogramming and upregulates the production of pyruvate and lactate. These energy-rich biomolecules produced in CAFs are exported to cancer cells via lactate and monocarboxylate transporters (MCT), where they induce energy production from mitochondria. The increased mitochondrial activity in cancer cells confers them with resistance to multiple anti-cancer drugs[114].

CHAPTER 3

Materials and Methods

3.1. Materials

Details of the reagents and plasticware used in the present study are given in supplementary table 3.1.

3.2. TCGA-BRCA data

Publicly available breast cancer mRNA expression data from The Cancer Genome Atlas (TCGA), hereafter referred to as TCGA-BRCA data, were collected from the UCSC Xena Browser (xenabrowser.net). This data set contains genome-wide mRNA expression values in the form of $\log_2$(RPKM+1) on 114 normal breast tissues and 1097 primary breast tumors.

3.3. TCGA-BRCA data analysis

The ERα mRNA expression of the TCGA-BRCA data was modelled as a mixture of two Gaussian populations, which led to the estimation of mean ERα mRNA expression in ERα-low and ERα-high populations. Each tumor was classified as ERα-low or ERα-high, if ERα mRNA expression was within two standard deviations (sd) of the respective population means. The Shapiro-Wilk test revealed that GPER mRNA expression was not normally distributed in both the subgroups. Hence, the Mann-Whitney U test was employed to compare median GPER mRNA expression in ERα-low and ERα-high tumors.

3.4. Survival analysis

Survival was analysed using the Kaplan-Meier Plotter online tool (www.kmplot.com) [115]. Using the "auto select best cut-off" option, tumor samples were divided into GPER-high and GPER-low groups. The JetSet best probe "210640_s_at" for GPER was used for the analysis. The effect of GPER expression on overall survival (OS), relapse-free survival (RFS), and distant metastasis-free survival (DMFS) of patients with ERα-positive or ERα-negative tumors, was graphically represented as Kaplan-Meier plots. The log-rank test was used to examine statistical significance.

3.5. Breast tumor samples

Breast tumors were collected from patients registered at Dr. Bhubaneswar Borooah Cancer Institute (BBCI) located in Guwahati, India, from December 2018 to October 2019, after obtaining their informed consent. The mean age of the cohort was 47.3 years (range: 26-82 years). Pathology reports with ER, PR, and HER-2 expression data were available. The study was approved by the Ethics Committee of BBCI (Reference no. BBCI-TMC/SC/Appr/15/2019 and BBCI-TMC/Misc-119/3180/2018).

3.6. Cell culture

MCF-7 and T47D breast cancer cell lines were procured from the National Center for Cell Science, Pune, India. For routine culture, phenol red-containing DMEM was used for MCF-7 cells, whereas phenol red-containing RPMI-1640 was used for T47D cell line. Both media were supplemented with 10% heat-inactivated FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin (M1 medium). Cells were cultured under standard humidified conditions of 37 ºC and 5% CO_2. Once the cells were 70-80% confluent, they were trypsinized, sub-cultured, seeded for experiments, or cryopreserved.

3.7. Subculturing and seeding

Once the cells were 80-90% confluent, the cell monolayer was rinsed with DPBS, treated with trypsin-EDTA, and incubated at 37 ºC until the cells detached from the surface. The cells were resuspended in M1 medium. Viable cells were counted by the trypan blue dye exclusion method. Depending on the treatment duration of the experiment, cells were seeded in culture dishes at appropriate densities.

3.8. Cell counting using trypan blue dye exclusion method

Cells were trypsinized, and resuspended in 1 mL of media. 20 µL of cell suspension was mixed with 20 µL of 0.4% trypan blue solution, and 160 µL of medium. 10 µl of the mixture was loaded on a haemocytometer, and unstained live cells in 4 corner squares (1mm X 1mm) were counted. The number of cells per ml in each sample was determined three times, and the average was calculated. The following formula was used to calculate the number of cells per mL of suspension. The number of cells per ml in each sample was determined three times, and the average was calculated.

$$\text{Number of cells per mL} = \frac{(\text{Number of live cells counted})}{4} \text{X } 10^4 \text{ X dilution factor}$$

3.9. Treatments

2×10^5 MCF-7 or T47D cells were seeded in 35 mm dishes with M1 medium and incubated for 48 h. Cells were washed with DPBS followed by incubation in phenol red-free DMEM (for MCF-7) or RPMI-1640 (for T47D) supplemented with 10% heat-inactivated charcoal-stripped FBS, 100 units/ml penicillin, and 100 µg/ml streptomycin (M2 medium) for 24 h. Cell monolayers were then washed with DPBS, and fed with fresh M2 medium containing indicated concentrations of E2, PPT, TAM or combinations thereof, for the indicated period. Cells treated with vehicle (ethanol) served as control. In experiments involving longer treatment durations, the treatment medium was replenished every 48 h. After completion of the experiment, the cells were processed for total RNA and protein isolation.

3.10. siRNA transfection

2×10^5 MCF-7 or T47D cells were seeded in 6-well plates using M1 medium, and incubated for 24 h. The cell monolayer was washed with DPBS. Cells were transfected with siRNA using Lipofectamine RNAiMAX in M1 medium according to the manufacturer's instructions. Control scrambled siRNA or ERα-specific siRNA was mixed in Lipofectamine RNAiMAX, diluted in Opti-MEM, and incubated at room temperature for 10 minutes. Each well of the 6-well plate was treated with 25 pmol of siRNA. The siRNA-to-reagent ratio of 1:3 (v/v) was maintained in each well.

3.11. Primers

The primers for routine RT-PCR and qRT-PCR were designed using the following features: Length 18-23 bp, GC content 50-60%, and melting temperature (T_m) between 55 and 65 °C. The T_m of the primers were calculated manually using the formula $4 \times (G + C) + 2 \times (A + T)$. The annealing temperature (T_a) was calculated using the formula $T_m - 5$. The primer pairs were designed at different exons separated by a large intron to avoid genomic DNA amplification. The details of the primer pairs used in this study are given in supplementary table 3.2.

3.12. RNA isolation and cDNA synthesis

Total RNA was isolated from the breast cancer cell lines and tumor samples using the method described by Chomczynski and Sacchi with modifications [116]. 2 µg of total RNA was reverse transcribed using High Capacity cDNA Reverse Transcription kit (Invitrogen Corporation, Grand Island, NY, USA) as per manufacturer's instructions.

3.13. Routine RT-PCR

cDNA equivalent to 20 ng of total RNA was amplified with gene-specific primers (Appendix II). Cyclophilin A served as an internal control. The PCR products were analyzed on 8% TBE PAGE or 2% agarose gels, and stained with ethidium bromide (EtBr). The images of EtBr stained gels bands were captured with the Chemidoc XRS+ system (BioRad, USA).

3.14. RT-qPCR

The mRNA expression of GPER variants in breast tumor samples, and treated or untreated MCF-7 or T47D cells was analyzed by RT-qPCR. Total RNA was extracted and quantified using Biospectrometer (Eppendorf, Germany). 2 µg of total RNA was reverse-transcribed using the High Capacity cDNA Reverse Transcription kit (Invitrogen Corporation, Grand Island, NY, USA), according to the manufacturer's instructions. cDNA equivalent to 20 ng of total RNA was amplified with gene-specific primers (Supplementary table 3.2). RT-qPCR reactions were carried out in AriaMX (Agilent, CA, USA). Reactions were carried out with 2X PowerUP SYBR Green master mix. ROX dye served as a passive reference. RPL35a served as an internal control. Expression levels of genes in test samples relative to control were analyzed by ΔΔCt method[117].

3.15. Western Blotting

Total protein was isolated from the aqueous phase of Trizol lysate and quantified by Lowry's protein estimation method[118]. Proteins were fractionated on a 10% denaturing SDS page and transferred to nitrocellulose membranes. The membranes were blocked with 1% gelatin in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 2 h at room temperature, followed by overnight incubation with primary antibody at 4°C in 0.1% gelatin in TBST. The blots were then washed (6 X 5 min) with TBST, followed by incubation with goat anti-rabbit IgG HRP (secondary antibody) for 1 h. The blots were then washed (6 X 5

min) with TBST and developed using Clarity Western ECL Substrate (Bio-Rad Laboratories Inc., CA, USA). Colorimetric images at suitable exposures were captured using the Bio-Rad Gel Doc EZ Imager. For GPER western blots, peptide affinity-purified polyclonal antibody was used[119]. Histone (H3) served as an internal control.

3.16. ChIP-seq analysis

We searched the Sequence Read Archive (SRA) ERα-related ChIP-seq studies using MCF-7 cells. From the project ERP000380, a subset of files of MCF-7 cells treated with E2 (ID: ERR022026) or vehicle (ID: ERR022025) was selected. The raw data quality was assessed on the Galaxy web-based platform[120]. The quality of all the input read files was assessed by FASTQC[121] with the default settings. The quality scores were converted to Sanger quality type by FASTQ Groomer[122]. Reads were mapped to the reference human genome (hg19) using "Map with Bowtie for Illumina" tool[123]. Reads mapping to multiple locations were discarded and unmapped reads in the output SAM file were filtered using "Filter SAM or BAM, output SAM or BAM" tool[124]. The genomic regions enriched by reads were identified by MACS (Model-based analysis of ChIP-seq) tool[125]. The peaks were visualized in the UCSC genome browser[126] after converting Wig files to bigWig files using "Wig/BedGraph-to-bigWig" tool.

3.17. Chromatin immunoprecipitation (ChIP) assay

The cells were fixed with 0.75% (v/v) formaldehyde at room temperature for 10 mins followed adding 125 mM glycine to inhibit the cross-linking reaction. Cells were washed, and lysed with 1 ml of ChIP lysis buffer. The lysates were sonicated, to shear the DNA at an amplitude of 30% for 35 cycles, each cycle with a 10-sec pulse on and a 25-sec pulse off. Sonicated samples were centrifuged at 14000 rpm for 10 mins at 4^0C. The supernatant containing the sheared chromatin was pre-cleared with Protein G plus-Agarose beads, which were pre-coated with bovine serum albumin and herring sperm DNA. 5% of the pre-cleared chromatin samples were kept aside as input. The remaining portion of the chromatin was incubated with normal rabbit IgG antibody or ERα antibody for 4 h. Protein-antibody complexes were immunoprecipitated by incubating with 20 µl of pre-blocked Protein G plus-Agarose beads followed by centrifugation. After washing the beads extensively, the DNA was eluted in the elution buffer, column purified and used as a template in PCR reactions

with primers (Supplementary table 3.2) to amplify a specific region of GPER (test) or TFF1 (pS2) (positive control) locus[127].

3.18. MTT assay

MCF-7 or T47D cells were seeded (4000-5000 cells/well) in 96 well plates. After 48 h, the monolayer was washed with DPBS. The cells were then treated with indicated concentrations of drugs or ethanol (vehicle control) for 120 h before the MTT assay. The media containing drugs or ethanol were replenished after every 48 h. The spent medium was removed and the monolayer of cells was washed with DPBS and incubated with 100 µL MTT reagent (0.5 mg/ml) for 3 h at 37°C. After incubation, the MTT reagent was removed, and the formazan crystals were dissolved in DMSO. Absorbance was measured at 570 nm in the Infinite Pro M200 microplate reader (Tecan Life Sciences, Switzerland), with 690 nm as the reference wavelength. The difference in the absorbance (A_{570}-A_{690}) was considered as a measure of cell viability. The viability of ethanol-treated cells was assigned the value of 100, and those of drug-treated cells were expressed relative to the control. The inhibition concentration 50 (IC_{50}) were calculated by AAT Bioquest-calculator-https://www.aatbio.com/tools/ic50-calculator.

3.19. Targeted bisulfite sequencing

3.19.1. Sample preparation

The genomic DNA (gDNA) from the breast cancer cell lines and tumors was extracted using the PureLink Quick Gel Extraction Kit (Invitrogen, CA, USA) and subjected to bisulfite conversion using the EpiJET Bisulfite Conversion kit (Thermo Fisher Scientific, USA) as per manufacturer's instructions. The bisulfite-converted gDNA was used as a template to generate upCpGi containing amplicons using specific primers (Supplementary table 3.2), and the amplified products were eluted from agarose gels.

3.19.1. Library preparation

The library was constructed with the "Kappa Hyper Prep Kit" (Roche, Switzerland). End repair and A-tailing were performed to generate 5'-phosphorylated and 3'-dA-tailed dsDNA fragments. dsDNA adapters with 3'-dTMP overhangs are ligated to 3'-dA-tailed fragments. Final library amplification was performed to amplify library fragments with the

appropriate adapter sequences on both ends using high-fidelity, low-bias PCR. The library was sequenced with the Illumina Miseq 2 X 300bp paired-end technology.

3.19.2. Analysis Workflow

i. Quality assessment

The raw reads were generated in FASTQ format. The fastqc tool was used to analyze the quality of the paired-end raw sequence reads for base quality and contamination by sequencing artefacts.

ii. Trimming & Filtering

Trimming of adapters and poor-quality sequences was performed for paired sequence reads with Trim Galore.

iii. Read Alignment

Trimmed sequence reads were mapped to reference genome assembly using the BS aware alignment tool bwa-meth. The alignment was performed to the following reference genome.

Assembly: hg38, GRCh38.p12 (GCA_000001405.27), Dec. 2017,

Data source: UCSC GenomeBrowser,

Weblink:http://hgdownload.soe.ucsc.edu/goldenPath/hg38/bigZips/analysisSet/hg38.analysisSet.fa.gz.

iv. Methylation Extraction

The MethylDackel tool was used to extract methylation information from the bisulphite sequence alignment. The mean methylation was calculated by averaging the percentage methylation reported across all retrieved methylation positions.

3.20. RNA sequencing

3.20.1. Sample preparation

Treated cells were lysed in RNA extraction reagent prepared in-house as per Chomczynski and Sacchi[116] with modifications. Lysates from two replicate dishes were pooled for the purification of RNA samples. The RNA integrity number (RIN) values of the RNA samples used for sequencing were greater than 7. The RNA samples were then subjected to library preparation and sequencing on the Illumina platform.

3.20.2. Library preparation

1 µg of total RNA was used to enrich mRNA using NEB Magnetic mRNA Isolation Kit (NEB, USA). Using the fragmentation buffer, the enriched mRNA was fragmented (approximately 200 bp) and reverse transcribed into double-stranded cDNA using random primers. The purified cDNA was ligated to the adapter. The adapter-ligated DNA was purified using Ampure beads (Beckman Coulter, USA), and enriched with specific primers compatible with Illumina platforms. The transcriptome library was constructed with the NEB Ultra II RNA Library Prep Kit (NEB, USA). The final enriched library was purified, quantified by Qubit (Thermo Fisher Scientific, USA), and analyzed by 2100 Bioanalyzer (Agilent, USA). The library was sequenced with the Illumina NextSeq 500 paired-end technology.

3.20.3. Analysis workflow

i. Quality assessment of reads, and trimming

The raw reads were in FASTQ format. The quality of the reads was assessed using fastqc tool[121]. The adapters, and low-quality reads were filtered out from the FASTQ files using fastp tool[128]. Three criteria were set to filter out the low-quality reads: removing reads lower than Q30 phred score; discarded reads shorter than 15 bp; Illumina adapter clipping. The fastqc tool was used to re-assess the filtered reads prior to mapping. The FASTQ files after the quality trimming and assessment were used for mapping. The number of read pairs passed after quality trimming and assessment are shown in supplementary table 3.3.

ii. Alignment

The Ensemble Homo sapiens GRCh38 genome was used as the reference genome for mapping the clipped reads (https://asia.ensembl.org/Homo_sapiens/Info/Index). Prior to mapping, indexing of the reference genome was done using the HISAT2 indexing scheme based on the Burrows-Wheeler transform and the Ferragina-Manzini (FM) index. Subsequently, clean reads were mapped using the HISAT2 tool against the index file[129]. The mapped output files (sam files) were converted into binary files (bam files) using Samtools[124].

iii. Quantification of mapped reads

The Subread package with the featureCounts tool was used for the quantification of mapped reads[130]. Mapped reads were counted at the feature (gene) level with the help of Homo sapiens GRCh38 annotation file (gtf).

iv. Normalisation and differential gene expression analysis

The statistical analysis was performed on count data using the R/Bioconductor package DESeq2[131]. DESeq2 was carried out on count data for normalization, visualisation and differential gene expression analysis. DESeq2 uses a generalized linear model (GLM) to evaluate differential gene expression and also uses a Wald test to evaluate significance. Genes having counts below a threshold of 10 were removed from all samples. p-values were adjusted using the Benjamini-Hochberg adjustment.

v. Gene enrichment analysis

Enrichment analysis utilizing fGSEA tool in R with an FDR correction of 25% was used to identify the signature genesets enriched upon G1 stimulation[132]. Additional R packages were used to generate enrichment plots and normalized enrichment scores (NES) plots.

3.21. Statistical analysis

Two-group, non-normal data were analyzed by Mann-Whitney U test to examine significant differences in median. Else, Welch two-sample t-tests were used. Multiple group data were analyzed by one-way ANOVA, followed by Tukey's HSD post-hoc test. Statistical tests were carried out in R, considering 5% level of significance ($p < 0.05$).

CHAPTER 4

ERα-GPER association in breast cancer

4.1. Introduction

The majority of breast tumors (~70%) express ERα[133], a significant variable that governs the phenotype, mode of treatment, and prognosis of breast tumors. ERα-positive breast tumors have a better prognosis than ERα-negative tumors. Biologically and histopathologically, ERα-positive breast tumors differ significantly from ERα-negative breast tumors; the latter having a more aggressive phenotype[134]. The genetic or molecular determinants of these aforementioned phenotypic differences, and their relationship with ERα expression are not completely understood[135]. Presumably, the genes that are co-ordinately expressed with ERα, or those that are a part of the ERα target gene and co-expression network hold the key towards understanding the phenotypic difference. On this proposition, differential screening of MCF-7, and MDA-MB-231 cDNA libraries was employed by Carmeci et al. (1997) to identify genes associated with ERα. They cloned a novel cDNA that was abundantly expressed in the ERα positive MCF-7 cells but not in the ERα-negative MD-AMB-231 cells[14]. Bioinformatic analysis of the novel cDNA revealed that it encodes for GPCR-Br, an orphan G protein-coupled receptor (GPCR).

The clinical significance of GPER in breast cancer is not well understood, and reports addressing the association between ERα and GPER in clinical samples are contradictory. Independent investigations have found a significant positive correlation between GPER and ERα expression in breast tumors[29,30,41]. However, some reports have also suggested a negative[31–33], or no association[34–36] between the two receptors. Thus, independent studies across diverse cohorts are required to better understand the association between GPER-ERα co-expression in breast tumors.

The importance of GPER expression, or lack of it, in the etiology of breast cancer and its progression is ambiguous. Weissenborn et al. (2014) reported the role of GPER as a potential tumor suppressor in MCF-7 and SK-BR-3 breast cancer cells[85]. In most studies, GPER expression was found to be lower in tumors than in normal breast tissues[37,38]**.** On the contrary, overexpression of GPER in breast tumors compared to normal breast tissues is also reported[136]. Despite the contradiction, the association between altered GPER expression and breast cancer is apparent.

Tumor suppressors are often silenced epigenetically[137]. Given the relevance of GPER in the diagnosis and prognosis of cancer, the current investigation is based on the idea that

reduced expression of GPER in ERα-negative breast tumors is likely to be associated with DNA methylation. Recent studies, including those reported from our laboratory, show that DNA methylation is associated with the loss of GPER expression in breast cancer cells[37,86]. Manjegowda et al. (2017) described a differentially methylated region (DMR) comprising terminal eight CpG dinucleotides in the upCpGi, which was differentially methylated in two breast cancer cell lines with contrasting GPER expression levels[37]. However, the methylation status of the GPER upCpGi region in breast tumors has not been investigated.

This chapter presents an independent assessment of the association between ERα and GPER expression in breast cancer. Using an assortment of methods, we analyzed GPER and ERα expression in breast tumors from two independent cohorts. We have also examined the methylation of the GPER upCpGi in ERα-positive and -negative breast tumors using targeted bisulfite sequencing.

4.2. Results

4.2.1. GPER mRNA expression is positively associated with ERα mRNA expression in breast tumors of TCGA-BRCA cohort

The inconsistency among researchers on the association between ERα and GPER, motivated us to examine their correlation in primary breast tumors from the TCGA cohort. There was a significant correlation between the two variables ($r = 0.266$, $p < 0.0001$)[138]. However, the existence of two subgroups of ERα-positive tumors, namely ERα-low and ERα-high, was apparent. We determined the mean ERα expression in these subgroups by mixture modelling (described in Materials and method; section 3.3). ERα-low, and ERα-high tumors were considered as those that had ERα mRNA expression levels within two standard deviations on either side of their respective means (Figure 4.1A). The Shapiro-Wilk test revealed that GPER mRNA expression was not normally distributed in ERα-low, and ERα-high tumors. Hence, the Mann-Whitney U test was performed, which revealed a significant difference in the median GPER mRNA levels in the subgroups; the ERα-high tumors expressing higher median levels, as shown in Figure 4.1B.

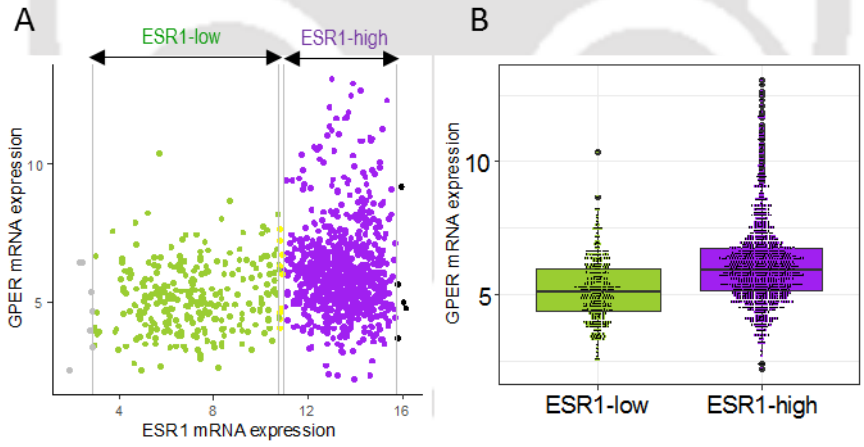

Figure 4.1: GPER expression in TCGA breast tumors stratified according to ERα expression. (A) A scatter-plot of GPER and ERα mRNA levels in primary breast tumors (n = 1097). The ERα expression was modelled as a mixture of two Gaussian populations. The green and blue colored clusters represent ERα-low and ERα-high tumors, respectively, which were grouped on the basis of expression level within two standard deviations on either side of the respective means. (B) A boxplot showing the distribution of GPER mRNA expression in ERα-low (n = 286) and ERα-high (n = 790) breast tumors identified in (A). The tumors that fall in the extreme of the bimodal distribution and those which have intermediate expression have not been accounted for analysis. ESR1- symbol for gene encoding human ERα. mRNA expression levels are in terms of $\log_2$(RPKM+1) values. Data in (B) was analyzed by Mann-Whitney U test (***$p < 0.0001$)

4.2.2. Expression levels of GPER or ERα mRNA with menopausal status or age

The association between GPER or ERα expression and the patients' age or menopausal status was examined in the TCGA-BRCA dataset. A significant difference was observed in ERα mRNA expression in breast tumors from younger (less than median age) versus older (greater than median age), as well as in pre-menopausal versus post-menopausal patients, as shown in Figure 4.2, A,C. However, as demonstrated in Figure 4.2, B,D, GPER mRNA expression did not show any significant difference with the age or menopausal status of the patients.

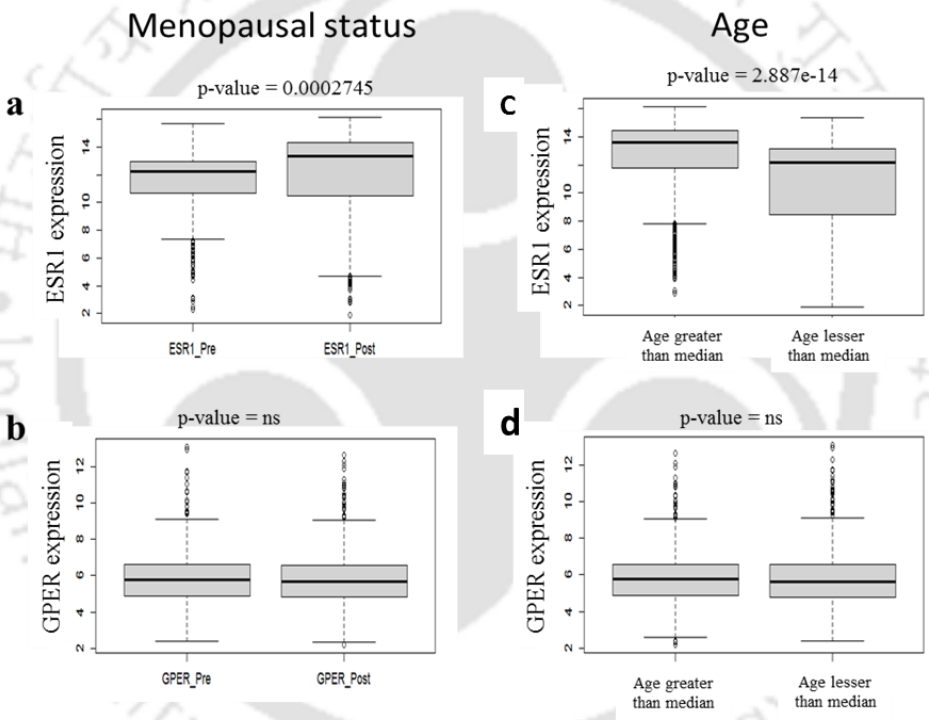

Figure 4.2: Expression levels of GPER or ERα mRNA in post- versus pre-menopausal or older versus younger patients. Box plots showing the distribution of ESR1 (A) and GPER (B) mRNA expression in Post- and Pre-menopausal breast tumor samples. Box plots showing the distribution of ESR1 (C) and GPER (D) mRNA expression in breast tumor samples from younger and older patients. The data were analyzed by Welch two-sample t-test. The p-value is mentioned above the box-plots. ns = not significant

4.2.3. Confirmation of ERα status in breast tumor samples from the BBCI cohort

The ERα status of the breast tumors was validated using RT-PCR in randomly selected ERα-positive (n = 10) and ERα-negative (n = 10) samples. The results (Figure 4.3) were in agreement with the histopathological reports on ERα expression in the breast tumor samples.

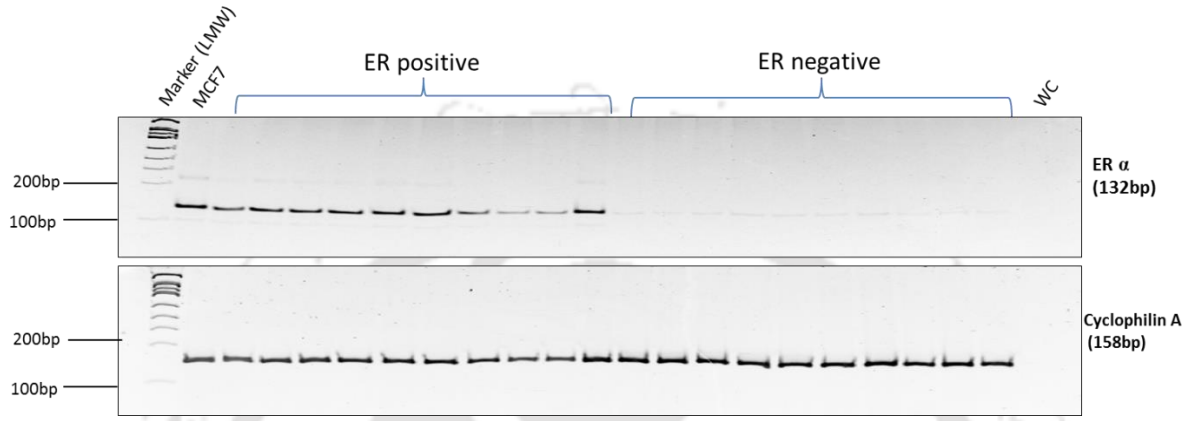

Figure 4.3: ERα status of breast tumor samples from the BBCI cohort. Total RNA was isolated from breast tumor samples. ERα mRNA expression in ERα-positive (n = 10) and ERα-negative (n = 10) breast tumor samples were analyzed by RT-PCR. Cyclophilin A served as the internal control. The PCR products were resolved on 8% TBE-PAGE. Primers used in the study are shown in supplementary table 3.2. MCF-7 cells were used as a positive control for ERα expression. Inverted images are shown. WC- water control. LMW-Low Molecular Weight ladder.

4.2.4. GPER is co-ordinately expressed with ERα at the protein and mRNA levels in breast tumor samples

We applied the western blotting technique to study GPER protein levels in immunohistochemically confirmed ERα-positive or ERα-negative breast tumor samples from BBCI. Western blots in Figure 4.4A show higher GPER protein levels in ERα-positive breast tumors compared to the ERα-negative tumors. We examined the GPER-v2, -v3, and -v4 mRNA variant expression in immunohistochemically confirmed ERα-positive (n = 25) and ERα-negative (n = 25) breast tumors of the BBCI cohort. As shown in Figure 4.4B, GPER-v2, -v3, and –v4 were significantly elevated in ERα-positive breast tumors compared to the negative ones. We were unable to detect the expression of GPER-v5 in these samples.

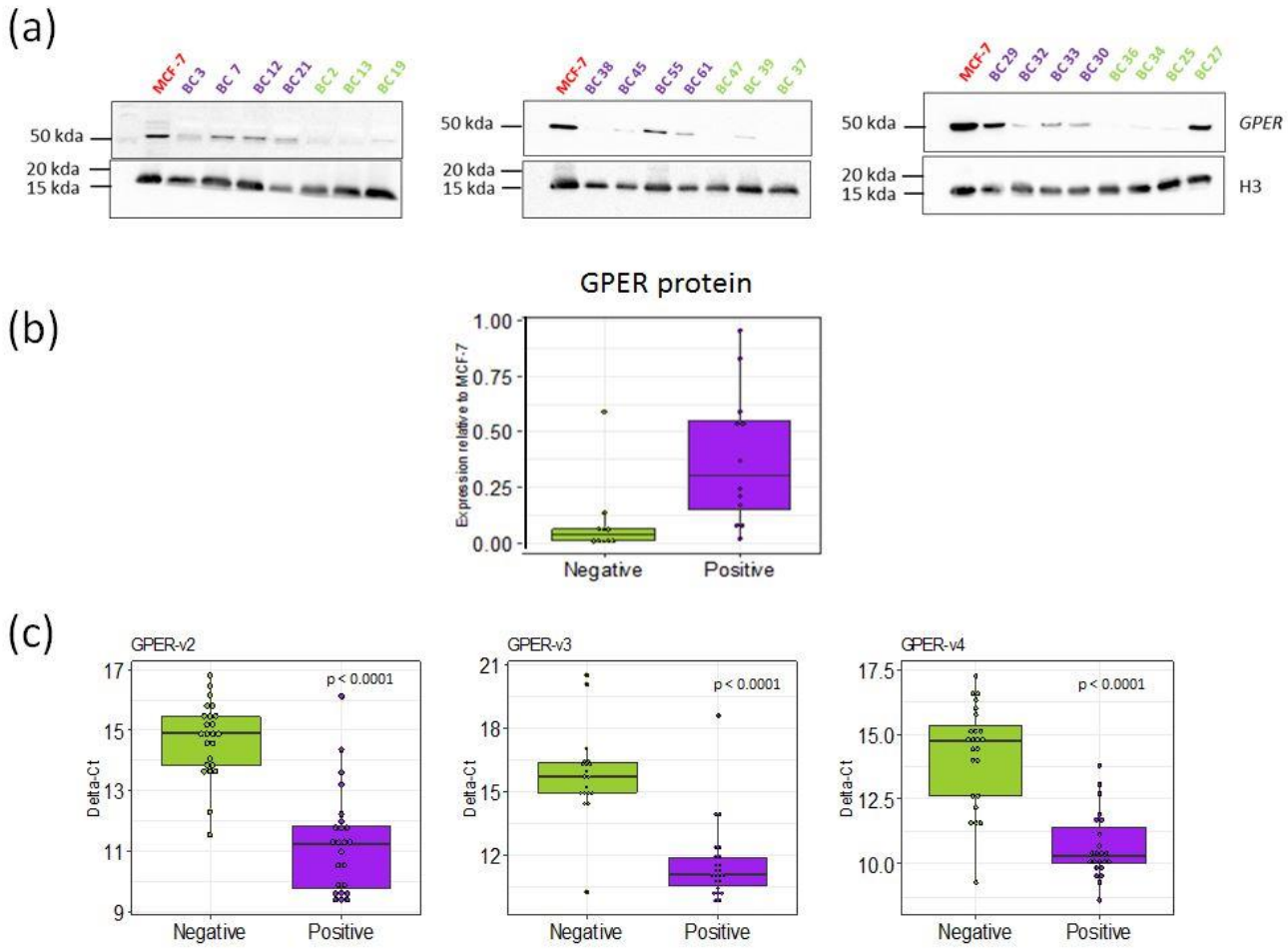

Figure 4.4. Co-ordinated protein and mRNA expression levels of GPER and ERα. (A) GPER protein expression levels in ERα-negative and ERα-positive breast tumors. Total protein from breast tumors were subjected to western blotting using anti-GPER polyclonal antibody. Chemiluminescence signals were processed using ImageJ. The background-subtracted integrated band intensity obtained for GPER for each sample was normalized against that obtained for Histone (H3). Normalized GPER protein expression in tumor samples was expressed relative to that in MCF-7 cells, which was set to 1. (B) Box plot showing the distribution of normalized GPER protein expression relative to MCF-7 control in ERα-positive (n = 12) and ERα-negative (n = 10) breast tumor samples. The data were analyzed by Mann-Whitney U test. *p < 0.05. The samples demarcated with purple and green are ERα-positive and ERα-negative, respectively. (C) GPER mRNA variant expression levels in ERα-negative and ERα-positive breast tumors. Total RNA isolated from ERα-positive (n = 25) and ERα-negative (n = 25) breast tumors were reverse transcribed, and the resultant cDNAs were subjected to RT-qPCR using variant specific primers. RPL35a was used as an internal control. For each sample the average Ct value for a given mRNA variant ($Ct^{variant}$), and RPL35a (Ct^{RPL35a}) obtained from duplicate reactions were determined. The difference $Ct^{variant}$ - Ct^{RPL35a}, was considered as a measure of the normalized variant expression. Samples with no Ct values in duplicate technical replicates were excluded, GPER-v2 (ERα-positive, n = 24; ERα-negative, n = 24), GPER-v3 (ERα-positive, n = 23; ERα-negative, n = 19) and GPER-v3 (ERα-positive, n = 23; ERα-negative, n = 24). Box plots show the normalized GPER variant mRNA expression in the BBCI cohort. Data were analyzed by Mann-Whitney U test. Note that the difference is significantly lower in ERα-positive tumors, indicating higher expression.

4.2.5. ERα knockdown reduces GPER protein expression in MCF-7 and T47D breast cancer cells

MCF-7 or T47D cells were transfected with ERα-specific siRNA or scrambled control siRNA (Scr) to examine the effect of ERα knockdown on GPER protein expression. In MCF-7 or T47D cells, ERα knockdown for 48 h caused significant downregulation of GPER protein (Figure 4.5).

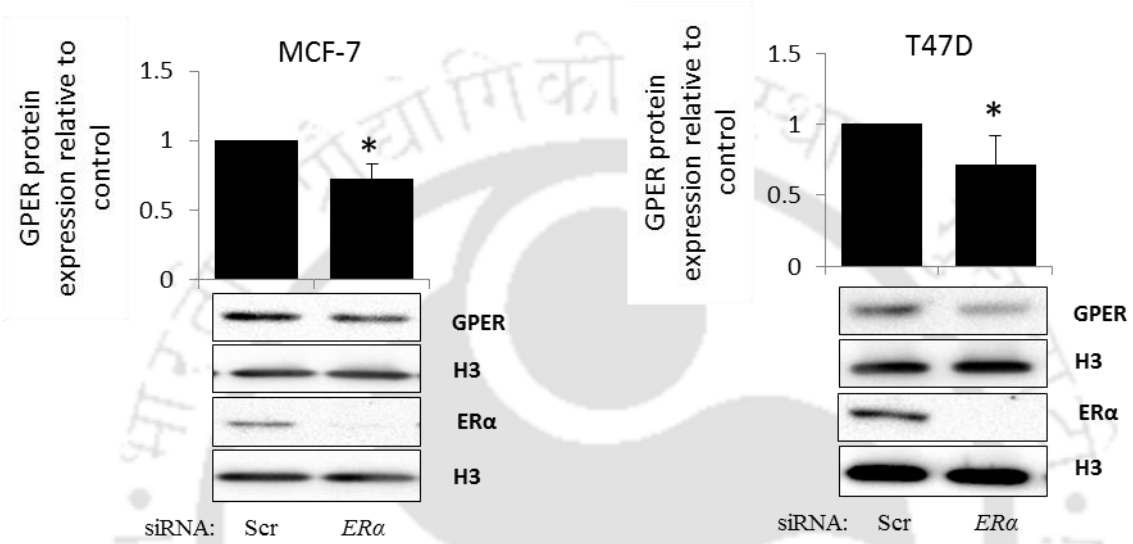

Figure 4.5: Effect of ERα knockdown on GPER expression in MCF-7 and T47D cells. MCF-7 and T47D cells were transfected with scrambled (Scr) or ERα-specific siRNA, and GPER protein expression was analyzed by using the western blotting method. Histone (H3) served as normalizing control. GPER expression in Scr-transfected cells (control) was set to 1, and that obtained in ERα-specific siRNA transfected cells was expressed relative to control. The experiment was done in M1 medium, with three biological replicates; each comprising of one dish each for both experimental groups. Bars represent mean relative expression ± sd (n = 3). Data were analyzed by Welch one-sample t-test. *p < 0.05, *** p < 0.001.

4.2.6. GPER expression in ERα-positive tumors is associated with prolonged survival.

Survival analysis was performed using Kaplan-Meier Plotter to investigate the clinical significance of GPER-ERα co-expression in breast cancer. We divided the patients into two groups; those with ERα-positive or ERα-negative breast tumors. In each of the groups, we analyzed the association of GPER expression with OS, RFS, and DMFS of patients. Figure 4.6 shows that high GPER expression was significantly associated with prolonged RFS (HR = 0.72, log-rank P < 0.0001) and OS (HR = 0.77, log-rank P = 0.035) in patients with ERα-positive tumors. In contrast, high GPER expression did not affect RFS (HR = 1.15, log-rank P = 0.17) and had an opposite effect on OS (HR = 1.4, log-rank P = 0.035) in patients with ERα-negative breast tumors. GPER expression did not have any effect on DMFS in patients

with ERα-positive (HR = 0.85, log-rank P = 0.08) or ERα-negative breast tumors (HR = 1.13, log-rank P = 0.05).

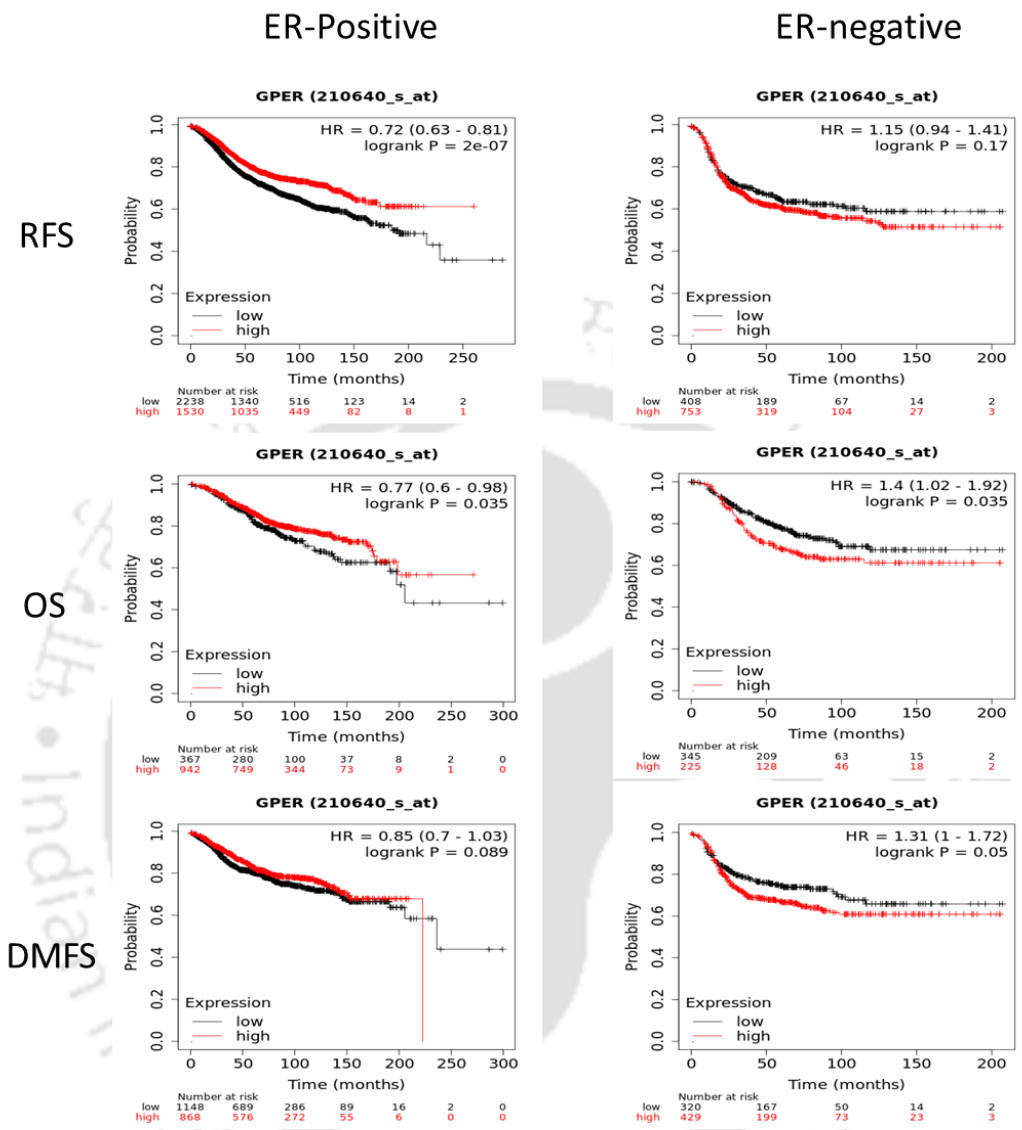

Figure 4.6: The influence of GPER expression on patient survival depends on ERα status of breast tumors. Survival analyzes were performed using Kaplan–Meier Plotter online tool. The Jetset best probe "210640_at" was considered for GPER expression. The breast tumors were classified as GPER-high or -low based on "auto select best cut-off" option. OS, RFS, and DMFS of patients stratified on the basis of high, or low GPER expression in breast tumors separately in patients with ERα-positive or ERα-negative breast tumors.

4.2.7. Differential methylation of the GPER upCpGi in ERα-positive and ERα-negative breast tumor samples

Targeted bisulfite sequencing was used to investigate the differential methylation at the upCpGi of the GPER locus in MCF-7, T47D, and MDA-MB-453 breast cancer cells. Percentage methylation was reported across all CpG positions, represented in the form of a

methylation matrix as shown in Figure 4.7A. The numbers (1-32) above the methylation matrix represent the position of CpGs in the upCpGi of the GPER locus. The eight 3′ terminal CpGs (referred to as the DMR) are highlighted in yellow color. Figure 4.7B represents the individual CpGs in the DMR region of the upCpGi as pie-charts, with the shaded region representing the percentage methylation. The percentage methylation of the indicated cell lines revealed that most of the methylation was observed in the DMR region. The DMR region was hyper-methylated in MDA-MB-453 cells compared to MCF-7 and T47D cells. This observation correlates with the finding that, unlike T47D and MCF-7 cells, MDA-MB-453 cells do not express the GPER protein, as shown in Figure 4.7C. The inverse relationship was already shown by us and others by different techniques[37,139,140].

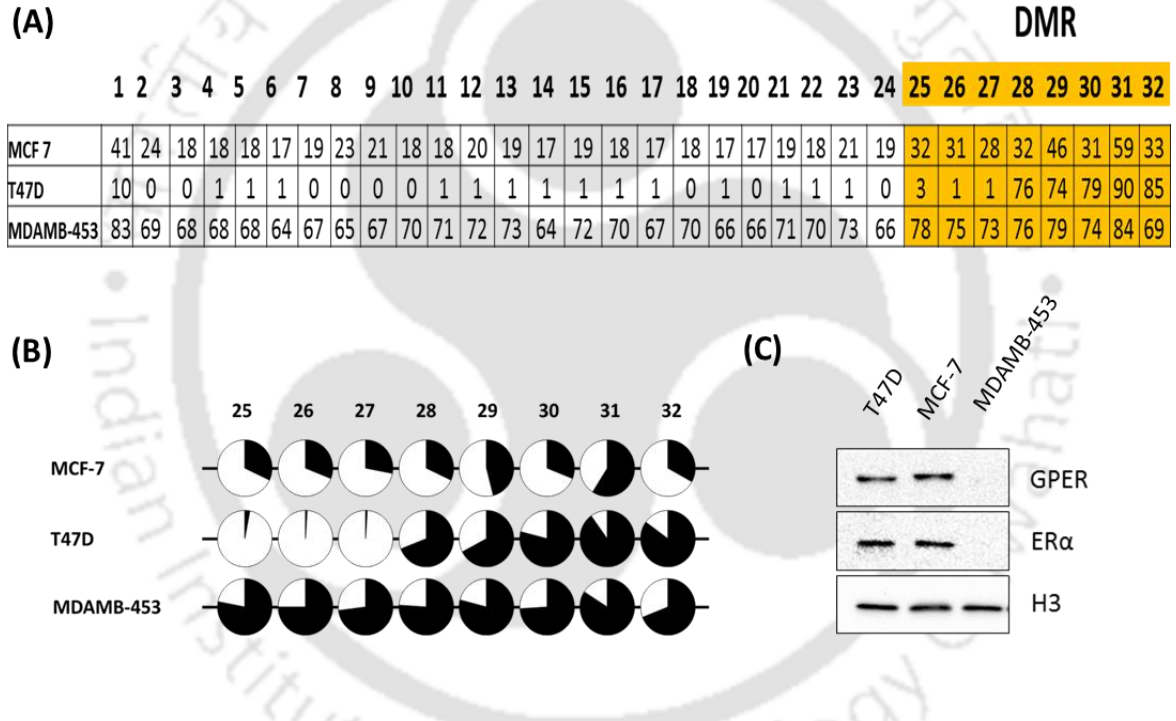

Figure 4.7: Differential methylation of the upCpGi in breast cancer cell lines: (A) Methylation matrix showing the targeted bisulfite sequencing results of indicated cell lines. The upCpGi region was amplified with primers flanking the upCpGi region using bisulfite converted gDNA as a template. The amplified PCR products were gel purified and sent for targeted bisulfite sequencing. Numbers (1-32) above the methylation matrix represents CpG sites. The values inside each box represent the percentage methylation of that particular CpG. The orange colour demarcates the differentially methylated region (DMR) of the upCpGi. (B) The percentage methylation of the DMR region of indicated breast cancer cell lines is represented as pie-charts. Each circle represents the percentage methylation of that particular CpG. (C) GPER expression in breast cancer cell lines. Total protein was isolated from the indicated breast cancer cell lines and subjected to western blot analysis using primary antibodies specific to GPER, ERα, and Histone (H3). Histone (H3) served as an internal control.

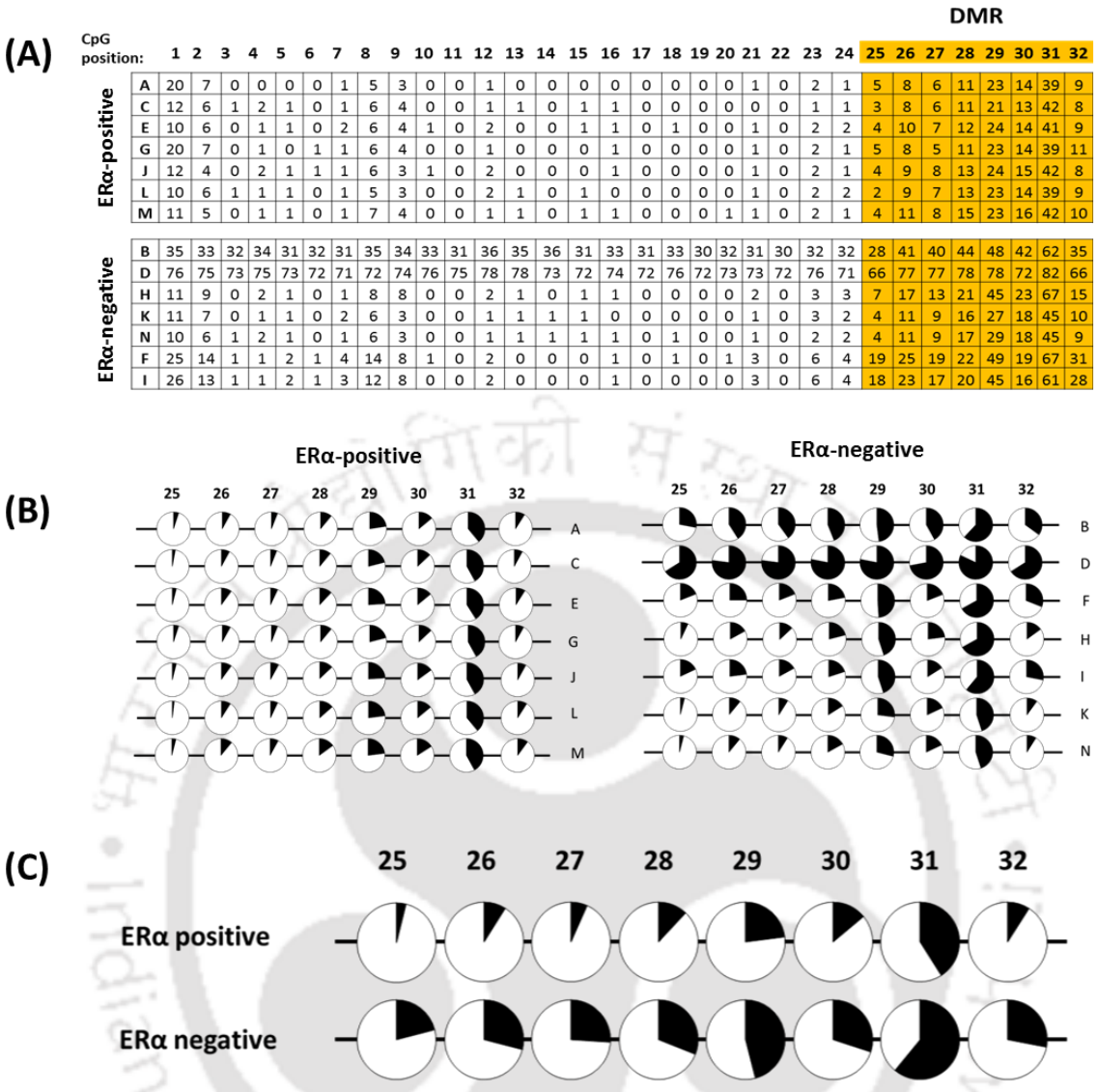

Figure 4.8: upCpGi methylation in the GPER locus of breast tumor samples: (A) Methylation matrix showing the targeted bisulfite sequencing results of breast tumor samples. The upCpGi region was amplified with primers flanking the upCpGi region using bisulfite converted gDNA of the ERα-positive and –negative breast tumor samples as a template. The amplified PCR products were gel purified and sent for targeted bisulfite sequencing. Numbers (1-32) above the methylation matrix represents CpG sites. The values inside each box represent the percentage methylation of that particular CpG. The orange colour demarcates the differentially methylated region (DMR) of the upCpGi. ERα-positive and ERα-negative samples are segregated in the methylation matrix. (B) The percentage methylation of the DMR region of the breast tumor samples is represented as pie-charts. Each circle represents the percentage methylation of that particular CpG. (C) Overall percentage methylation of CpGs in the DMR region of ERα-positive and ERα-negative breast tumor samples.

Based on our targeted bisulfite sequencing results of breast cancer cell lines, we decided to investigate the differential methylation of the GPER upCpGi in ERα-positive and ERα-negative breast tumor samples. Percentage methylation per CpG was represented as a methylation matrix for seven ERα-positive and seven ERα-negative tumor samples across 32 CpG sites of the upCpGi, as shown in Figure 4.8A. The cluster of 3' terminal eight CpGs in the upCpGi (DMR) are highlighted differently. Except for the two ERα-negative samples (B

and D), the percentage methylation of the first 24 CpGs was nearly the same in all the remaining samples. Figure 4.8B represents the percentage methylation in the DMR region in the form pie-charts.

Interestingly, as in breast cancer cell lines, the majority of the methylation was observed in the DMR region. As demonstrated in Figure 4.8C, methylation in the DMR region was higher in ERα-negative samples than in ERα-positive samples. Our observations indicate an association between methylation in the upCpGi of the GPER locus and ERα expression in breast tumors.

4.3. Discussion

Genes regulated by ERα, or those that constitute the ERα co-expression network are key to understanding phenotypic differences between ERα-positive, and ERα-negative breast tumors. GPER was identified as an ERα co-expressed marker in breast cancer cell lines and tumors[14]. Although the co-expression is confirmed by others[37], the clinical data on the association between ERα and GPER expression are inconsistent. In this chapter, using an assortment of methods, we determined, and analyzed GPER mRNA and protein expression in two independent cohorts of breast tumors. Our results suggest that GPER expression is positively associated with ERα expression in breast tumor samples. Downmodulation of GPER protein in MCF-7 or T47D cell lines following knockdown of ERα indicates that both are coordinately expressed in breast cancer cells.

A bimodal distribution of ERα in breast tumors from the TCGA cohort was clearly visible. Using mixture modelling, it was possible to classify the tumors into ERα-low, and ERα-high groups. ERα expression varies with patient age or menopausal status, and ERα positive tumors are more frequent in post-menopausal women[141]. This, difference in age or menopausal status, arguably could explain the existence of ERα-low and ERα-high subgroups. Consistent with this, a significant difference in ERα expression was observed in breast tumors from young (less than median age) versus older (greater than median age), or pre-menopausal versus post-menopausal women. On the contrary, GPER mRNA expression did not differ; inconsistent with the positive correlation between ERα and GPER. However, this can be explained by the estrogen-mediated induction of GPER expression, as discussed in Chapter 5 of this thesis. Despite low levels of ERα, the higher circulating estrogen levels

in young or pre-menopausal patients may raise the expression of GPER, thereby explaining similar levels of GPER in older and young, or pre-menopausal and post-menopausal patients.

The large body of work on the GPER-ERα crosstalk, asserts a functional association between these two structurally divergent forms of estrogen receptors. The molecular basis of GPER-ERα co-expression has remained elusive. Some signaling inputs likely coordinate their expression via the activation of transcription factors that have cognate binding sites on their regulatory sequences. Carmeci et al. (1997) have discussed the possible role of AP1 and AP2 transcription factors[14]. In response to EGF and TGFα, the expression of GPER is upregulated in Ishikawa and TAM-R MCF-7 cell lines via the EGFR/MAPK signaling pathway, which involves recruitment of c-fos to the AP1 site of the GPER locus[43]**.** Demarco et al. (2013) reported that the IGF-IR/PKCd/ERK/c-fos/AP1 transduction pathway is involved in the IGF-mediated stimulation of GPER expression[83]. GPER promoter also contains an AP2 binding site. AP1 and AP2 are also involved in the expression of ESR1, the gene encoding ERα. Thus, signaling inputs likely coordinate GPER and ERα expression via activation of common transcription factors that regulate their expression. GPER expression in about 50% of ERα-negative breast tumors[36] implies that co-ordinated expression may not be the only basis for co-expression.

The findings of this investigation lead us to explore a fundamental question. What is the biological explanation for the positive association between ERα and GPER in breast cancer? The mechanism underlying the coordinated expression is worth investigating since their coexpression appears to be related to the survival of breast cancer patients. The coordinated expression of GPER also sets the context for estrogen-mediated regulation of GPER in breast cancer.

Earlier, Manjegowda et al. (2017) reported that methylation in the upstream CpG island of GPER is likely to be associated with its expression in breast cancer cell[37]. They found differential expression of GPER in MCF-7 (ERα-positive) and MDA-MB-231 (ERα-negative) cells, which was in line with the observations by Carmeci et al. (1997)[14]. Bisulfite sequencing of MCF-7 and MD-AMB-231 revealed that the cluster of eight 3' terminal CpGs in the upCpGi region (DMR) is hypomethylated in MCF-7 cells, and hypermethylated in MDA-MB-231 cells. In order to determine the percentage methylation of each CpG in the upCpGi, we performed targeted bisulfite sequencing. We found that the DMR in the upCpGi

of the MD-AMB-453 (ERα-negative) cell line was hyper-methylated compared to the MCF-7 and T47D (ERα-positive) cell lines, which were hypo-methylated.

The observations from the targeted bisulfite sequencing of breast cancer cells motivated us to examine the association between methylation in the upCpGi and ERα expression in breast tumors. The targeted bisulfite sequencing of the tumor samples revealed that the DMR in the upCpGi of ERα-negative samples were hyper-methylated compared to that in the ERα-positive samples, which were hypo-methylated. This was reminiscent of the methylation pattern reported earlier in MCF-7 and MDA-MB-231 cells[37].

Tumor suppressors are frequently inactivated through the methylation of DNA in neoplastic conditions[137]**.** The biomarker and prognostic potential of methylated CpG islands in specific tumor suppressors are appreciated generally. Despite the ambiguity in the perceived role of GPER in the breast, its expression level in primary tumors appears to be an important clinical marker. The targeted bisulfite sequencing study examines the association between upCpGi methylation and ERα expression in breast tumors. Furthermore, it underlines the importance of the DMR that encompasses the terminal eight CpGs and its association with methylation-dependent reduced expression of GPER in ERα-negative breast tumors. GPER expression and methylation data from the same tumor samples would have been ideal. However, it was not possible to isolate protein, mRNA and gDNA from the same tumor samples. Hence, we could not simultaneously determine methylation and GPER expression in the same tumor samples. Nevertheless, the fact that methylation is lower in ERα-positive samples, and higher in negative samples, and that GPER expression is negatively associated with methylation in upCpGi is an indirect evidence that ERα and GPER expression are positively associated in breast tumors. More investigations are warranted to explore DMR hypermethylation as an indicator of GPER expression in breast cancer and its utility in diagnosis and prognosis.

CHAPTER 5

Estrogen-mediated regulation of GPER expression in breast cancer cells

5.1. Introduction

Investigations into the regulation of GPER expression are key to understanding the role of GPER in physiological and pathological conditions. Growth factors like epidermal growth factor (EGF), transforming growth factor (TGF), and insulin-like growth factor-I (IGF-I) have been shown to upregulate GPER expression in a variety of cell lines[43,83]. These observations highlighted the significance of the AP1 site in the GPER promoter region. A few studies have touched upon the hormonal regulation of GPER expression. Ahola et al. (2002) demonstrated that progestin stimulation upregulated the expression of GPER in breast cancer cells[142], consistent with the findings of Thomas et al. (2005)[17]. They demonstrated that progesterone-induced the expression of GPER mRNA and protein. However, the experiments conducted by them were in media containing 1 nM E2, masking the effect of estrogen.

Estrogen-mediated regulation of GPER expression has been touched upon in the literature, although the results are inconsistent. Estrogen-mediated suppression of GPER expression was demonstrated by Carmeci et al. (1997) in MCF-7 breast cancer cells[14]. In line with their observations, Ariazi et al. (2010) also showed estrogen-mediated downregulation of GPER mRNA expression in a dose-dependent manner[41]. These observations were contradicted by Ignatov et al. (2010) who demonstrated estrogen-mediated upregulation in the GPER protein expression in MCF-7 cells[42].

In the previous chapter, we investigated the positive association between GPER and ERα in breast tumors. However, the mechanistic basis of co-expression in breast cancer is unknown. Estrogen regulation of GPER could be a basis for this co-expression. The reports related to the estrogen regulation of GPER, on the other hand, are contradictory, and do not address the involvement of ERα.

The present study demonstrates higher GPER expression in estrogen-stimulated human ERα-positive MCF-7 and T47D breast cancer cell lines, and revealed GPER as a target of estrogen. ERα blockade, and knockdown experiments, together with *in silico* analyses and chromatin immunoprecipitation assays revealed the role of ERα in the estrogen-induced expression of GPER.

5.2. Results

5.2.1. E2-mediated upregulation of GPER mRNA in MCF-7 and T47D cells

There are three variants of GPER mRNA, namely GPER-v2, -v3, and -v4. They have the same open reading frame and 3'UTR, but have different exon-intron organization and 5'UTR. Using variant-specific primer combinations (Supplementary table 3.2), we studied the expression of GPER mRNA, and the effect of E2 treatment on MCF-7 and T47D breast cancer cell lines. To evaluate the dose-dependent effect of E2 on GPER mRNA expression, MCF-7 or T47D cells were treated with various concentrations of E2 (0.1 to 100 nM) or ethanol (vehicle control) for 72 h. In T47D cells, compared to the ethanol-treated group, all the indicated concentrations of E2 significantly induced the expression of all the GPER variants (Figure 5.1B). However, in MCF-7 cells, 10 nM E2 produced significant induction (Figure 5.1A). The time-dependent effect of E2 on the mRNA expression of GPER was analyzed by treating MCF-7 or T47D cells with 10 nM E2 or ethanol for 0 h to 96 h. In T47D cells, all variants were significantly induced at 6 h, with the expression peaking at 72 h (Figure 5.1D). However, in MCF-7 cells, all the variants were significantly induced at 24 h (Figure 5.1C).

5.2.2. E2 upregulates GPER mRNA via ERα in MCF-7 and T47D cells

To examine the involvement of ERα in the E2-mediated upregulation of GPER mRNA, MCF-7 and T47D cells were treated with PPT, a selective ERα agonist. Treatment with PPT significantly upregulated GPER -v2, -v3, and -v4 mRNA expression in both MCF-7 and T47D cells to the same extent as E2 (Figure 5.2, A,B). TAM, a selective estrogen receptor modulator alone, did not significantly affect GPER -v2, -v3, and -v4 mRNA expression (Figure 5.2, A,B). However, TAM treatment blocked E2 or PPT-mediated upregulation in both the cell lines. These observations indicated the involvement of ERα in the E2-mediated upregulation of GPER mRNA expression.

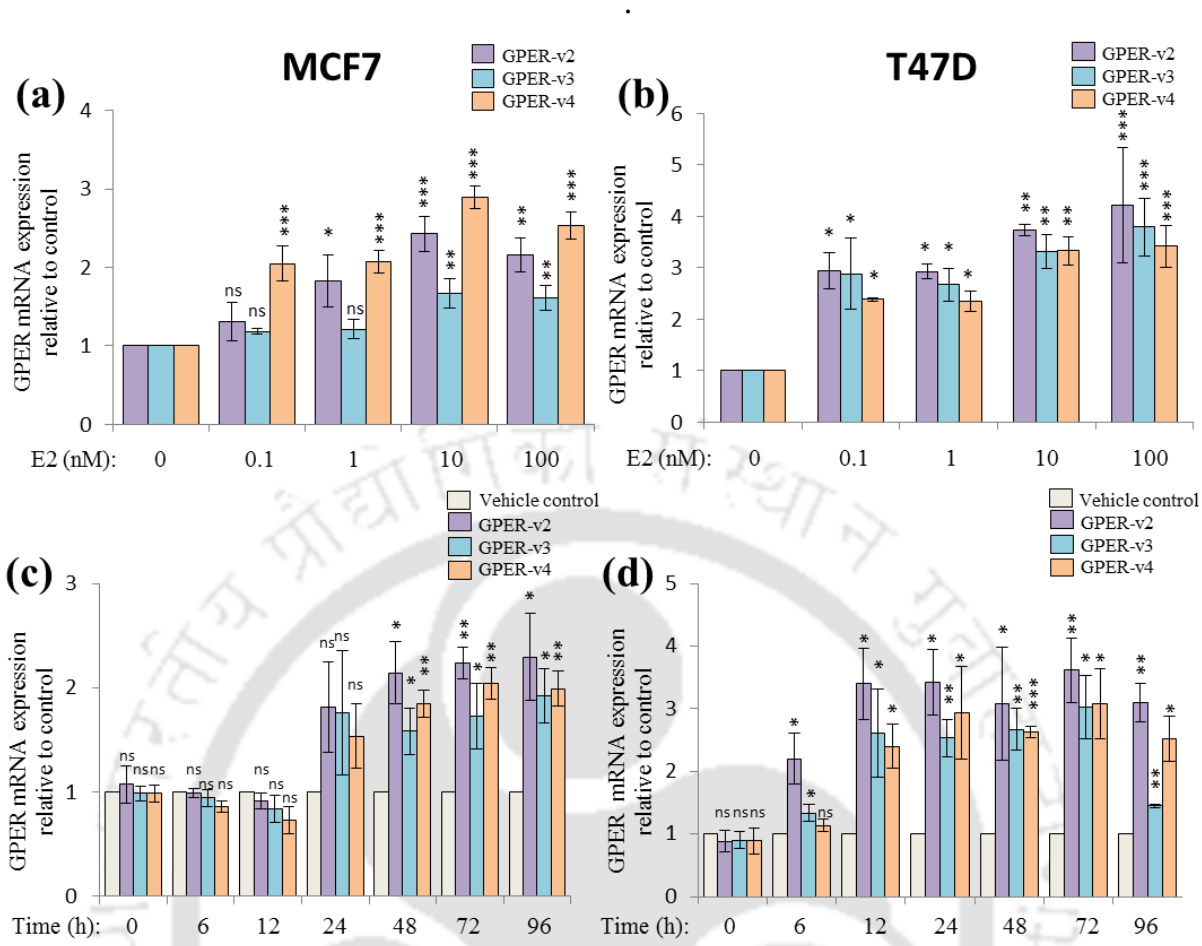

Figure 5.1: Estrogen positively affects GPER mRNA expression. Dose-response. MCF-7 (A) or T47D (B) cells were treated with indicated concentrations of E2 for 72 h. Expression of GPER mRNA variants relative to RPL35a was analyzed using RT-qPCR. Expression levels in vehicle controls (ethanol-treated cells) were set to 1 and those in E2-treated groups were expressed relative to control. Bars represent mean ± sd (n = 3). Data were subjected to ANOVA with Tukey's HSD. *p < 0.05, **p < 0.01, ***p < 0.001. Time-course**.** MCF-7 (C) or T47D (D) cells were treated with 10 nM E2 for indicated durations. Expression of GPER mRNA variants relative to RPL35a was analyzed using RT-qPCR. For each time-point the relative GPER variant expression in vehicle control samples (ethanol-treated, grey bars) were set to 1, and those in treated samples were expressed relative to controls (black bars). Bars represent mean ± sd (n = 3). For each time-point data were analyzed by Welch two-sample t-test. *p < 0.05, **p < 0.01, ***p < 0.001.

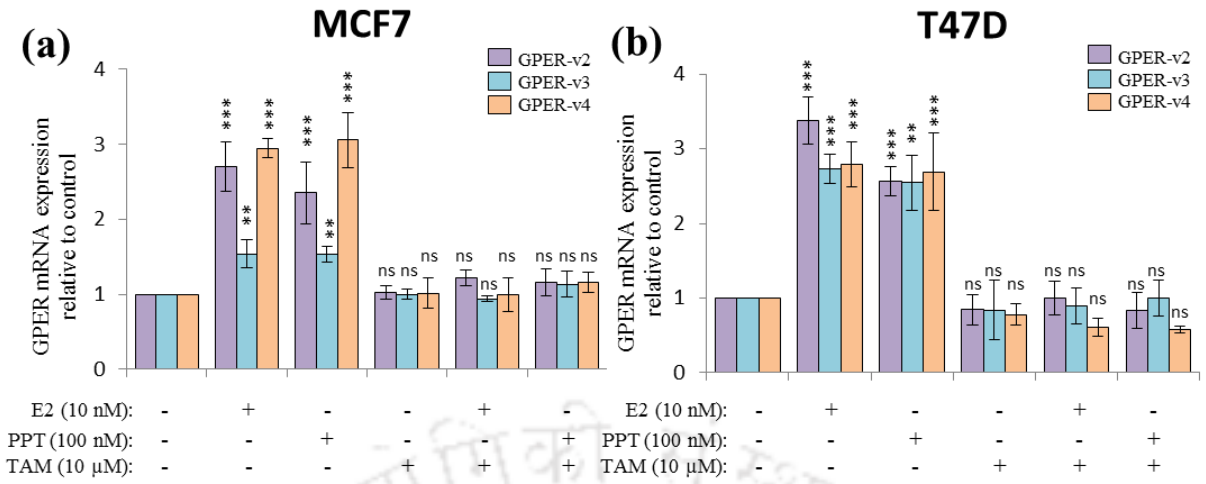

Figure 5.2. Tamoxifen blocks E2- or PPT- induced expression of GPER mRNA variants. MCF-7 (A) or T47D (B) cells were stimulated with 10 nM E2 or 100 nM PPT alone or in combination with 10 µM tamoxifen. Relative GPER mRNA variant expression levels were ascertained by RT-qPCR as in dose-response experiments. Cells treated with tamoxifen alone were also included in the experiment design. Expression levels in vehicle controls (ethanol treated cells) were set to 1 and those in E2-treated groups were expressed relative to control. Bars represent mean ± sd (n = 3). Data were subjected to ANOVA with Tukey's HSD. *p < 0.05, **p < 0.01, ***p < 0.001

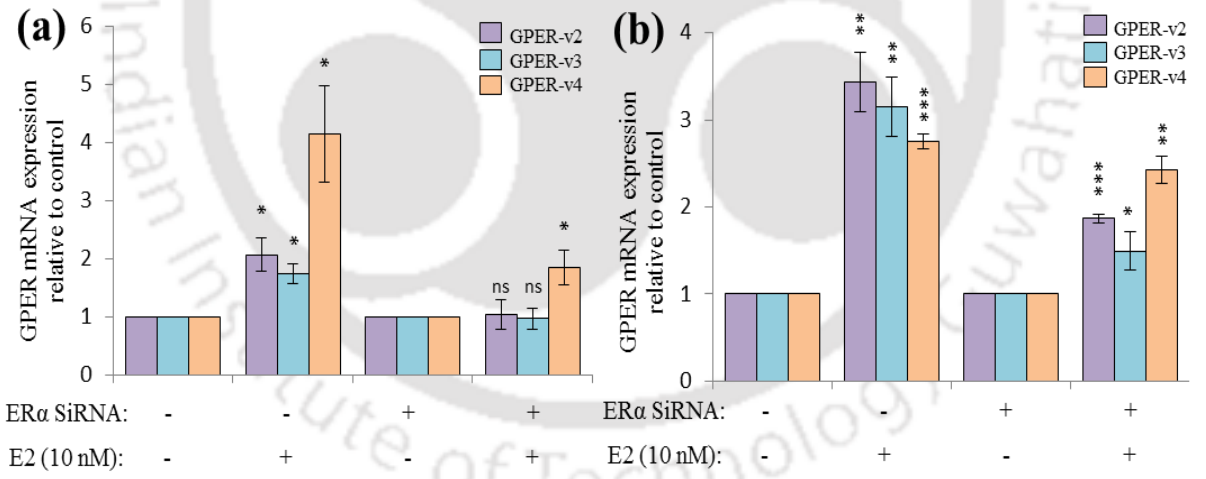

Figure 5.3: ERα-knockdown blocks E2-mediated induction of GPER mRNA variants. MCF-7 (A), or T47D (B) cells, transfected with scrambled or ERα-specific siRNA for 24 h were stimulated with vehicle or 10 nM E2 for 72 h. GPER mRNA variant expression relative to RPL35a was ascertained by RT-qPCR. For scrambled, or ERα-specific siRNA transfected groups, the relative GPER expression levels in E2-treated cells were expressed relative to vehicle control (ethanol-treated), and analyzed by Welch two-sample t-tests. Bars represent mean ± sd (n = 3). *p < 0.05, **p < 0.01, ***p < 0.001.

5.2.3. ERα-knockdown blocks E2-mediated induction of GPER mRNA

We investigated the effect of ERα-knockdown on E2-mediated upregulation of GPER. MCF-7 or T47D cells were treated with scrambled or ERα-specific siRNA for 24 h, and were stimulated with E2 or ethanol for 72 h. E2 significantly upregulated the expression of GPER mRNA in both the cell lines transfected with scrambled siRNA. However, the estrogen-mediated induction of GPER mRNA variants was abolished in the ERα-specific siRNA transfected group, confirming that ERα is involved in E2-mediated upregulation of GPER mRNA expression (Figure 5.3, A,B).

5.2.4. Enhanced ERα occupancy in the upstream region of GPER locus of MCF-7 and T47D cells treated with E2

Liganded ERα modulates gene expression by engaging with estrogen response elements in target gene promoters. We hypothesized that GPER is a transcriptional target of liganded ERα. To test this hypothesis, we analyzed ChIP-seq data of chromatin samples from MCF-7 cells treated with vehicle or 10 nM E2 for 24 h, immunoprecipipated with ERα-specific antibody. The data were retrieved from SRA and analyzed in GALAXY online platform, and the results were viewed in the UCSC genome browser. Several ERα enriched regions in the GPER locus were apparent (Figure 5.4A).

Furthermore, *in silico* analysis of the GPER locus using the JASPAR tool predicted an ERE in the region corresponding to the 5'-most peak in the ChIP-seq data (Figure 5.4A). A ChIP assay employing ERα-specific antibody, and primers designed to amplify this region, confirmed the enhanced ERα occupancy in MCF-7 or T47D cells exposed to 10 nM E2 for 24 h (Figure 5.4, B,C). These observations provide strong evidence that E2-mediated regulation of GPER expression occurs via the binding of ERα to the 5' of GPER locus.

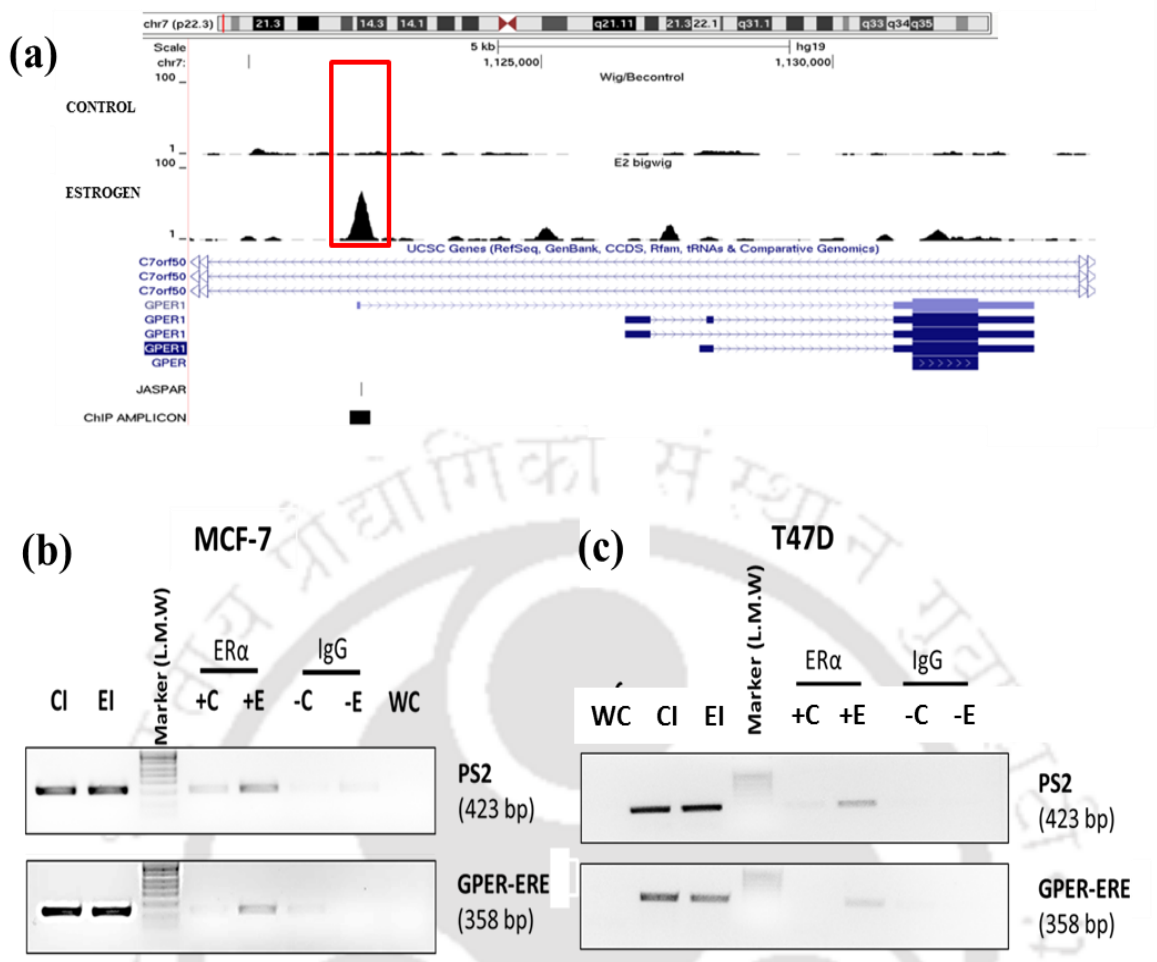

Figure 5.4: ERα binding sites at the GPER locus in the human genome. (A) E2 induces ERα occupancy in the upstream region of GPER. Analysis of ChIP-seq data. ChIP-seq data obtained from MCF-7 cells treated with vehicle, or 10 nM E2 (SRA accession no. ERP000380) was analyzed using Galaxy. ERα-enriched regions were visualized in the UCSC Genome Browser. Sonicated chromatin samples from the vehicle or 10 nM E2-treated MCF-7 (B) or T47D (C) cells were immunoprecipitated with non-specific IgG or ERα-specific antibody. Immunoprecipitated DNA was subjected to PCR using primer pairs designed to specifically amplify the region indicated by black rectangle (ChIP amplicon). CI and EI- Input samples, WC- Water control, LMW- Low molecular weight ladder

5.3. Discussion

Despite volumes of clinical and cell biological data, little is known about the estrogen-mediated transcriptional regulation of GPER, let alone the contribution of ERα in its expression and function. Reporting on the discovery of GPER cDNA (then referred to as GPCR-Br), Carmeci et al. (1997) have discussed the non-effect of serum stimulation on its expression in MCF-7 breast cancer cells[14]. On the contrary, higher expression of GPER mRNA and protein was found in MCF-7 cells cultured in M1 medium, compared to those cultured in M2 medium[143].

Carmeci et al. (1997) showed a decrease in GPER expression in MCF-7 cells exposed to estrogen. Similar to Carmeci et al. (1997), Ariazi et al. (2010) reported estrogen-mediated down-regulation of GPER expression via ERα in a time-dependent manner[41]. However, this finding was not in agreement with Ignatov et al. (2010) who demonstrated that GPER protein expression was upregulated following estrogen treatment in tamoxifen-resistant MCF-7 cells[42]. Estrogenic regulation is also indicated by higher GPER expression in diestrus hamster endometrium[73]. However, *in vivo* data shows higher expression of murine mammary GPER expression in the estrogen-dominant (estrus) phase of the reproductive cycle[138]. The present study demonstrates the positive influence of estrogen on GPER expression in breast cancer cell lines.

The ChIP-seq and chromatin immunoprecipitation data show that GPER is a genomic target of estrogen-ERα signaling. This is not the first instance of transcriptional activation or involvement of ERα in regulating GPER expression. Hypoxia transcriptionally induces the expression of GPER via HIF-1α binding to the HRE sequence of GPER[144]. Interestingly, the induction of GPER via IGF-IR/PKC δ/ERK/c-fos/AP1 transduction pathway involves the recruitment of phospo-ERα, to the AP1 site in the GPER promoter[83]. However, the regulatory regions operative in these instances are distinct from each other. This suggests that different signaling pathways co-opt different regulatory regions to alter GPER expression.

Our study provides strong evidences in favour of the role of liganded ERα in inducing GPER expression, thereby providing an alternative basis for their co-expression. Tumor cells are known to exhibit overt growth factor signaling which can cause ligand-independent activation of ERα. That GPER is a downstream target of ERα, explains their co-expression, even in the absence of the ligand. 15-20 % of ERα-positive breast tumors do not respond to endocrine therapy[145]. Thus, the ability to distinguish between endocrine responsive, and non-responsive ERα-positive breast tumors has therapeutic value. PR, a downstream target of ERα and a gene induced by estrogen-ERα signaling is a classical marker of estrogen responsiveness, and estrogen responsive breast tumors. Given that GPER is a downstream transcriptional target of estrogen- ERα signaling axis, GPER presents itself as a potential alternative marker of endocrine therapy response

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CHAPTER 6

Effect of E2 stimulation on the responsiveness of breast cancer cells to G1 treatment

6.1. Introduction

The highlight of GPER signaling is the activation of the EGFR/ERK/MAPK pathway[16], which not only induces proliferation[98], thereby mimicking EGF-like effects, but also enables cross-talk with ERα via Ser118 phosphorylation[28,146]. Depending upon cell type and context, activation of GPER leads to either cell cycle arrest or proliferation.

Since GPER, ERα, and ERβ bind to estrogen, assessment of the effect of GPER activation on cellular physiology has been challenging, particularly in cell types co-expressing the nuclear estrogen receptors. To distinguish between GPER- and classical ER-mediated activity, selective GPER agonists and antagonists, have been developed. The specific GPER agonist G1 has facilitated investigations on GPER signaling and its cellular effects. However, the specificity of G1 has been questioned by some researchers[105,106]. Much of our understanding of the role of GPER in cellular physiology is derived from experiments employing G1. But, the data are controversial in relation to its role in regulating cell proliferation or inhibition. It has been reported that the activation of GPER by G1 either promotes[108,147] or attenuates[148,149] cell proliferation. Despite significant advances in GPER signaling, the target transcriptome has not been thoroughly investigated. Pandey et al. (2009) investigated the early effects of estrogen treatment in the SkBr-3 cell line (ERα-negative, ERβ-negative, and GPER-positive) to understand GPER-mediated global transcriptome changes[103]. Later, Toprak et al. (2020) reported that long-term G1 treatment led to transcriptomic changes in ovarian cancer cells, which involved growth inhibition pathways[150]. However, at the global transcriptome level, the long-term effects of GPER activation, particularly in breast cancer cells, remain unknown. In this chapter we have examined the transcriptome response to long-term GPER activation upon G1 treatment in MCF-7 breast cancer cells using RNA-sequencing.

Ignatov et al. (2010) demonstrated estrogen-mediated upregulation of GPER expression in tamoxifen-resistant MCF-7 cells, which made the cells more responsive to the G1 treatment[42]. In the earlier chapters of this thesis, we have demonstrated the positive correlation between GPER and ERα expression in breast tumors and the upregulation of GPER expression by estrogen via ERα. On the premise of these observations, this chapter focuses on to clarify whether estrogenic stimulation of GPER renders cells more responsive toward G1.

6.2. Results

6.2.1. G1 treatment reduces the cell viability of breast cancer cells

To examine the effect of G1 on the viability of breast cancer cell lines, we designed an MTT assay to examine the change in the viability of MCF-7 or T47D cells following 120 h of treatment with varying concentrations of G1. Up to 100 nM G1 treatment, none of the cell lines had reduced viability. MCF-7 and T47D cell lines showed significantly reduced viability when treated with 1 µM or above the concentration of G1 (Figure 6.1). The two cell lines significantly differed in their response to 1 µM G1. At this concentration, the viability of MCF-7 was reduced by 55%, whereas reduced the viability of T47D by 20%. Notably, MCF-7 cells had significantly reduced viability with 500 nM G1, indicating that MCF-7 cells were more sensitive to G1 than T47D cells.

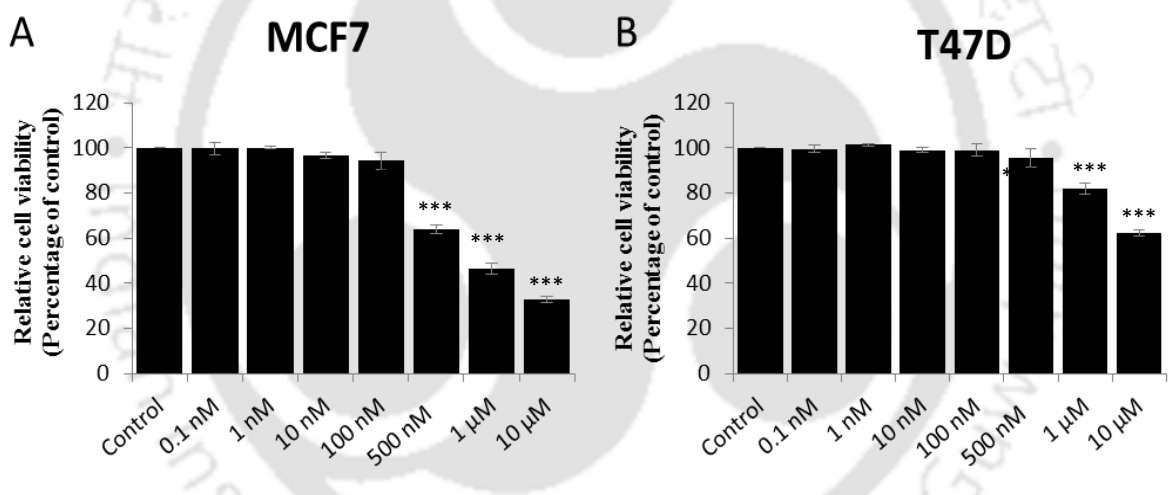

Figure 6.1: Effect of G1 treatment on viability of MCF-7 or T47D cell lines: MCF-7 (A) or T47D (B) cells were seeded into 96 well plates and allowed to grow for 48h. Cells were treated with indicated concentrations of G1 or ethanol (vehicle control) for 120 h in M1-medium. Treatment media containing G1 or ethanol were replenished after every 48 h. The treatment was terminated by removing the spent medium followed by a DPBS wash. The cells were incubated with MTT reagent for 3 h. Excess reagent was removed and the formazan crystals were dissolved in dimethyl sulfoxide (DMSO). Absorbance at 570 nm and 690 nm was taken and the mean of A_{570}-A_{690} was plotted in the bar graph along with the ±SD as error bars. * $p<0.05$, ** $p<0.01$ ***$p<0.001$versus EtOH, n=3. MCF-7 (A) and T47D (B) cells treated with different concentrations of G1, respectively.

6.2.2. G1 treatment alters the morphology of MCF-7 cell lines.

MCF-7 or T47D cells were treated with 100 nM or 1 μM G1 for 48 h. T47D cells treated with 100 nM or 1 μM G1, and MCF-7 cells treated with 100 nM G1, did not show any morphological differences compared to the ethanol (vehicle) treated group. Suggesting that the proliferation and growth of the cells were primarily unaffected, and cell adhesion remained appropriate. However, the 1 μM G1-treated MCF-7 cells were morphologically different from the ethanol-treated group (Figure 6.2). The cells were spherical, partly adhered to the surface, and cell proliferation was also affected.

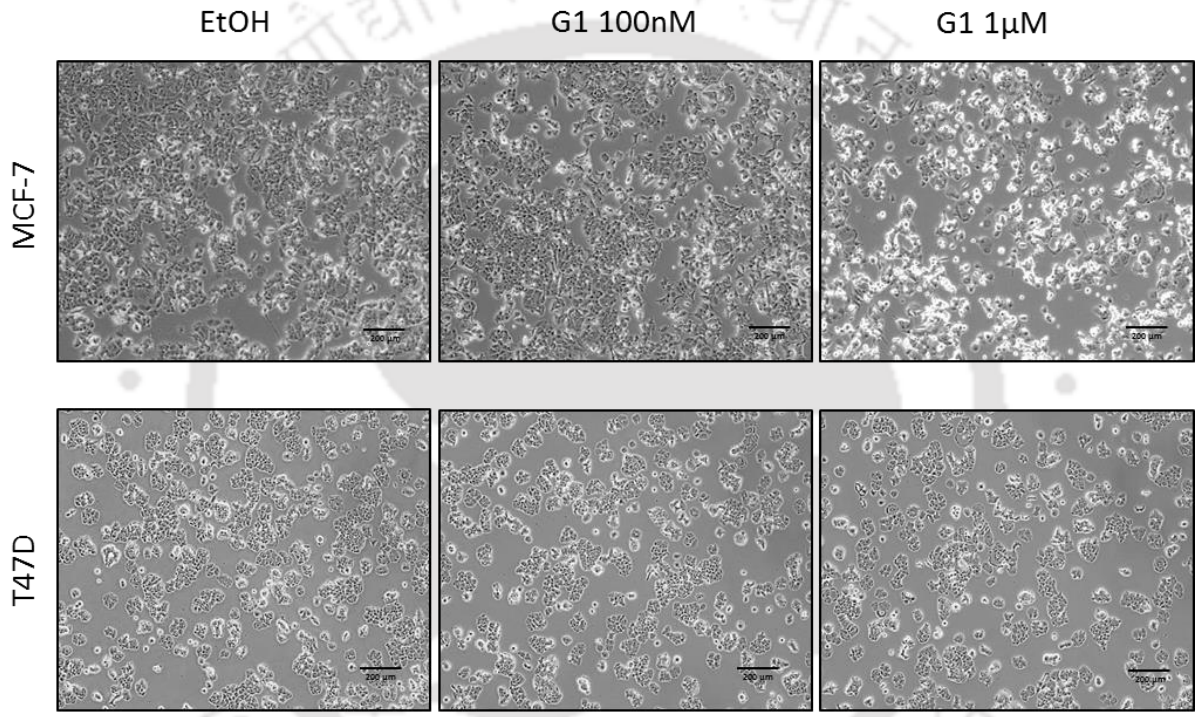

Figure 6.2: G1-induced morphological changes in MCF-7 and T47D cells: MCF-7 or T47D cells were seeded in 35 mm dishes in M1 medium. After 48 h, the spent M1 medium was removed. The monolayer of cells was washed with DPBS, and treated with M1 medium containing 0.1% ethanol (vehicle control), 100 nM G1, or 1 μM G1. After 48 h of treatment, the images were captured using a 20X objective on the EVOS XL core cell imaging system. Scale bar is 200 microns.

6.2.3. Transcriptome analysis of MCF-7 cells treated with G1

RNA sequencing was carried out to examine transcriptome changes triggered by G1 treatment based on our cell-based assays. A study of the genome-wide transcriptomic effects of GPER activation with G1 required identifying an optimal treatment concentration and duration. MCF-7 cells, when treated with G1 for 120 h, showed reduced cell viability in a dose-dependent manner, and showed morphological changes with 1 μM G1 treatment for 48 h. Based on our MTT assay, and morphological analysis, we investigated the genome-wide

changes in the MCF-7 cell transcriptome following 48 h treatment with 100 nM and 1 μM of G1.

6.2.4. Quality assessment of RNA-seq data

Unsupervised clustering analysis was performed using sample-to-sample heatmap and principal component analysis (PCA) in R statistical package. Figure 6.3 A shows the sample-to-sample heatmap. Replicates within the control and G1- treated groups clustered separately, indicating that the biological groups were different from each other. Figure 6.3 B shows the PCA plot for control or G1-treated samples. The count data from control, 100 nM G1, and 1 μM G1-treated samples were partitioned according to their expression pattern. The analyses revealed that 1 μM G1-treated (C1, C2, C4) samples appeared to form a distinct cluster along PC1 (axis of maximum variation). In contrast, the three control (A1, A2, and A3) and 100 nM G1 (B1, B2, and B3) treated samples were clustered together, indicating that the 1 μM G1-treated samples were indeed different from the control or 100 nM G1-treated samples.

6.2.5. GPER-regulated genes in MCF-7 cells

MCF-7 cells were treated for 48 h in M1 medium in the presence of ethanol, 100 nM or 1 μM G1, and the transcriptome profiles were analyzed using RNA-seq. We identified G1 modulated genes by interrogating the read count data with the DESeq2 package in R. The volcano plot in Figure 6.4A shows that 2676 genes (blue dots) were modulated by 1 μM G1 based on a threshold of 0 for LFC, and $p < 0.05$ for Wald statistic and FDR. 1705 genes were repressed, and 971 genes were induced upon G1 treatment, as illustrated in Figure 6.4B. The upregulated genes ($logFC > 0$) are represented in shades of red, downregulated ($logFC < 0$) are represented in shades of blue. The row dendrogram in each expression heatmap showed that significant DE genes were separated into two clusters of upregulated and downregulated genes. The column dendrogram in each expression heat map showed that the samples were separated into two groups: control (vehicle-treated) and 1 μM G1-treated. Interestingly, none of the genes was differentially expressed in the 100 nM G1-treated group. The top 50 significantly induced and top 50 significantly repressed genes upon 1 μM G1 treatment were provided in supplementary table 6.1 and supplementary table 6.2, respectively.

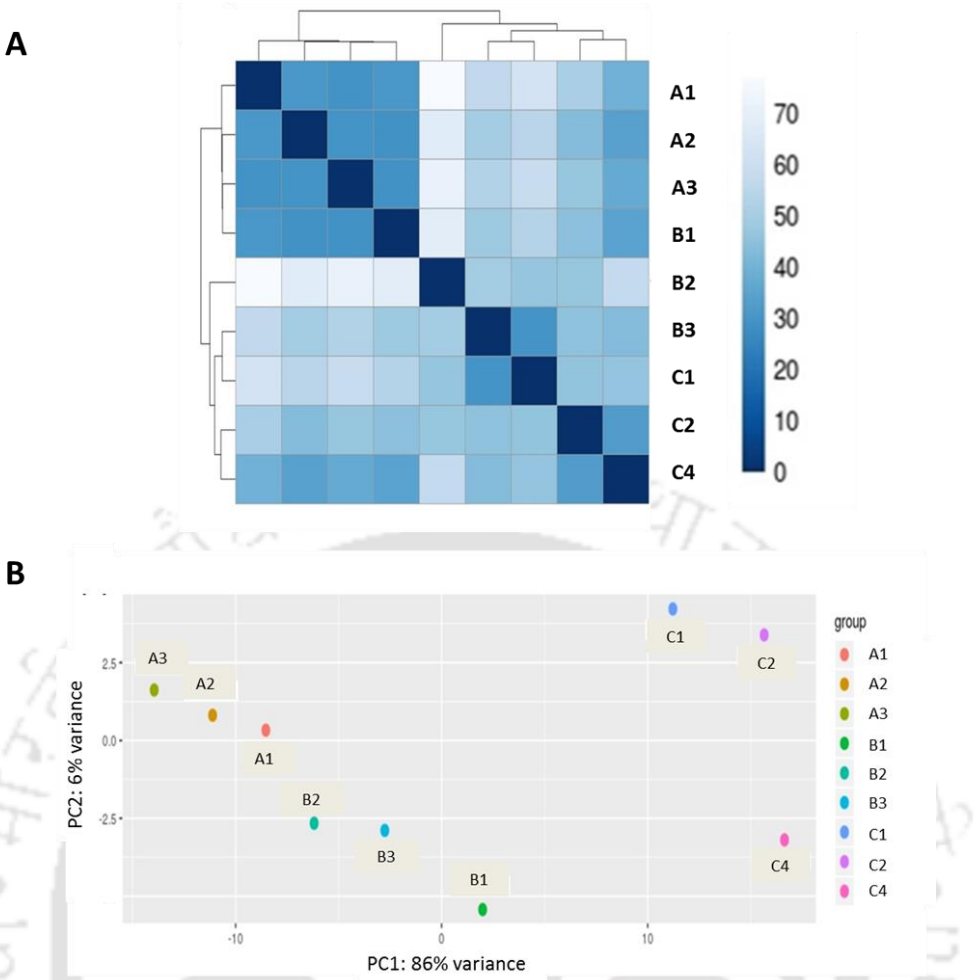

Figure 6.3: RNA-seq data visualization: (A) Heatmap of sample-to-sample distances**:** Sample to sample heatmap of three samples with their respective biological replicates. In this figure sample A is represented as control, 100 nM G1 is represented as B and 1 µM G1 is represented as C. The heatmap was generated with DeSeq2 software package in R. (B) Principal component analysis (PCA) plot**:** Principal component analysis between control (ethanol-treated), 100 nM and 1 µM G1 treatment groups with their replicates. Each point represents a treatment sample. Samples with similar gene expression profiles are clustered together. Sample groups are indicated by using different colours, as indicated in the legend provided. Percentages on each axis represent the percentages of variation explained by the principal components. In this figure sample A is represented as control, 100 nM G1 is represented as B and 1 µM G1 is represented as C. The PCA plot was generated with DeSeq2 software package in R.

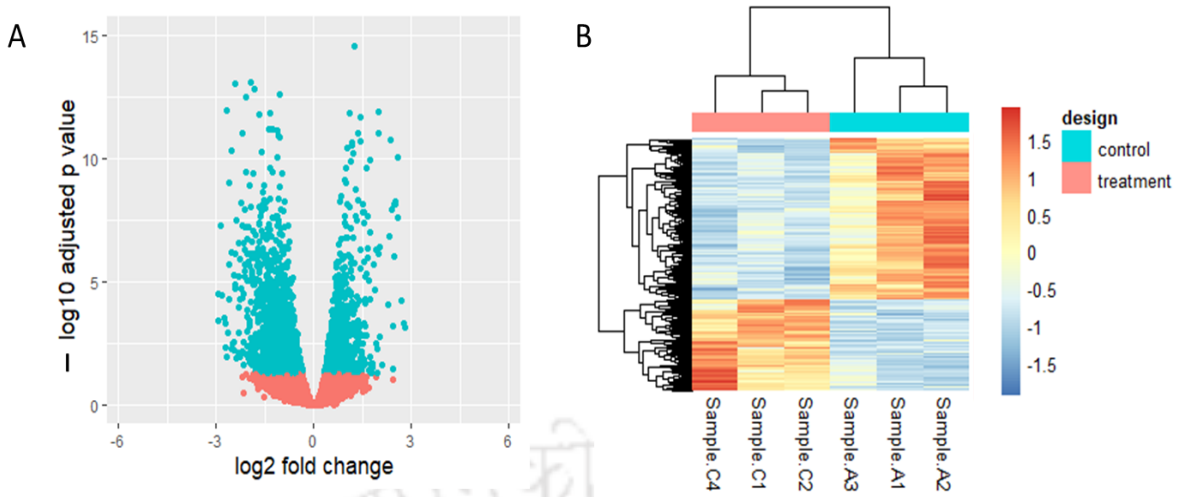

Figure 6.4. Summary of RNA-seq data: (A) Volcano plot. Each point represents a gene, and is plotted based on its –log10Padj and log_2FC values. The blue dots indicate genes (n = 2676) that are significantly modulated by 1 µM G1. The red dots represent non-significant genes. (B) Expression heatmap of 2676 significantly modulated genes. A1, A2, and A3 are control samples and C1, C2, and C4 are 1 µM G1-treated samples. On the heatmap, the colour for each gene represents its log-normalized count.

6.2.6. Gene set enrichment analysis

The genes regulated by 1 µM G1 treatment motivated the mining of enriched gene sets using the fGSEA package in R. Gene set enrichment analysis was used to determine whether a priori defined set of genes shows statistically significant, concordant differences between the vehicle control and 1 µM G1-treated group. 1 µM G1 treatment enriched the G2/M checkpoint, apoptosis, hypoxia, P53, and other gene sets (Figure 6.5). Notably, the G2/M checkpoint gene set was negatively enriched, whereas the apoptosis gene set was positively enriched. Taken together, the pathway analysis and cell-based assay results indicate that selective activation of GPER in MCF-7 cells inhibits proliferation by G2/M-phase arrest and induces apoptosis.

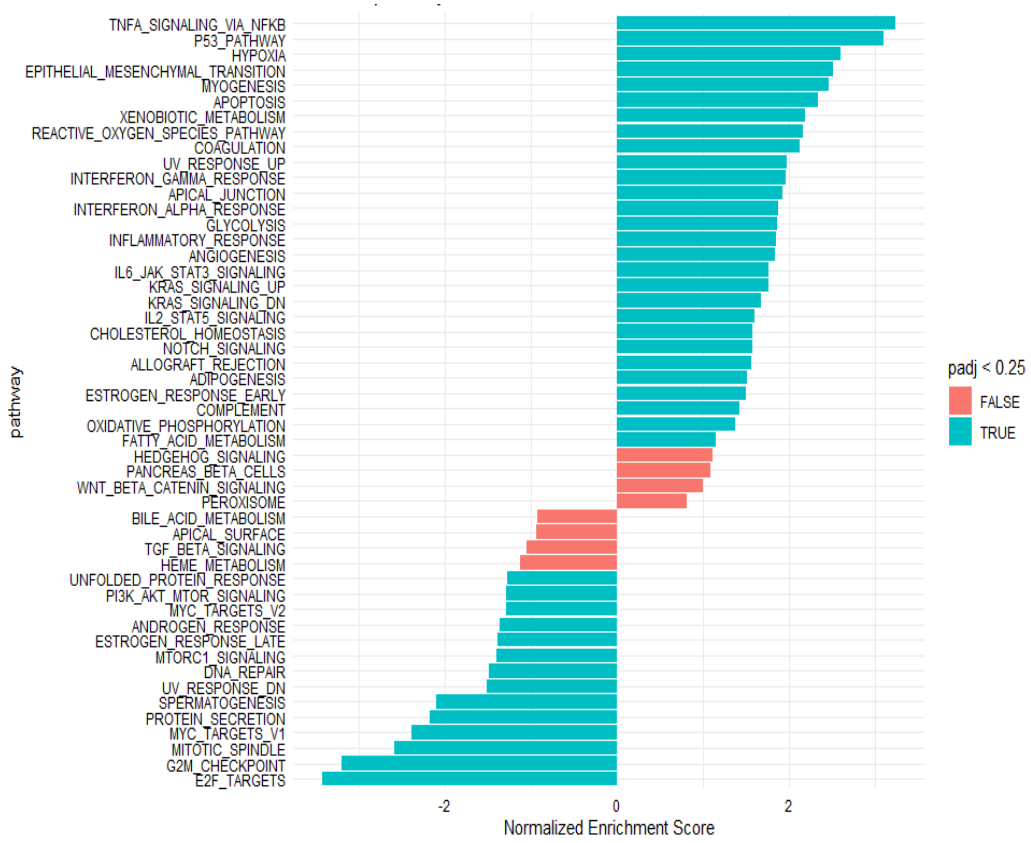

Figure 6.5. Gene set enrichment analysis. Bar chart showing the fGSEA results. The fGSEA package in R was used to analyze the RNA-seq data to identify the enriched hallmark gene sets in MCF-7 cells following 1µM G1 treatment. Bars represent NES (Normalized Enrichment Score). Blue bars correspond to the significantly enriched gene sets, and red bars represent significantly negative enriched gene sets based on a 25% cut-off for FDR.

6.2.7. Estrogen stimulation rendered the MCF-7 or T47D cells more responsive to G1

Based on the pathway analysis and cell-based assays, we concluded that GPER activation led to apoptosis in breast cancer cells. To investigate the effect of G1 in the presence or absence of 10 nM E2 or 100 nM PPT, we designed MTT assays to determine the IC_{50} values of G1 in MCF-7 or T47D cells following 120 h of treatment, in the presence or absence of E2 (Figure 6.6, A,B) or PPT (Figure 6.6, C,D). The IC_{50} values of G1 in MCF-7 and T47D cells without E2 or PPT were 3364 nM and 6888 nM, respectively. E2 treatment reduced the IC_{50} in MCF-7 cells to 943 nM and T47D cells to 2508 nM. Likewise, PPT treatment reduced the IC_{50} to 1335 nM in MCF-7 cells and 3615 nM in T47D cells. The presence of E2 or PPT increased the responsiveness of the cells to G1 stimulation. The MCF-7 cells were more responsive to G1 in the presence or absence of E2 or PPT, compared to the T47D cells.

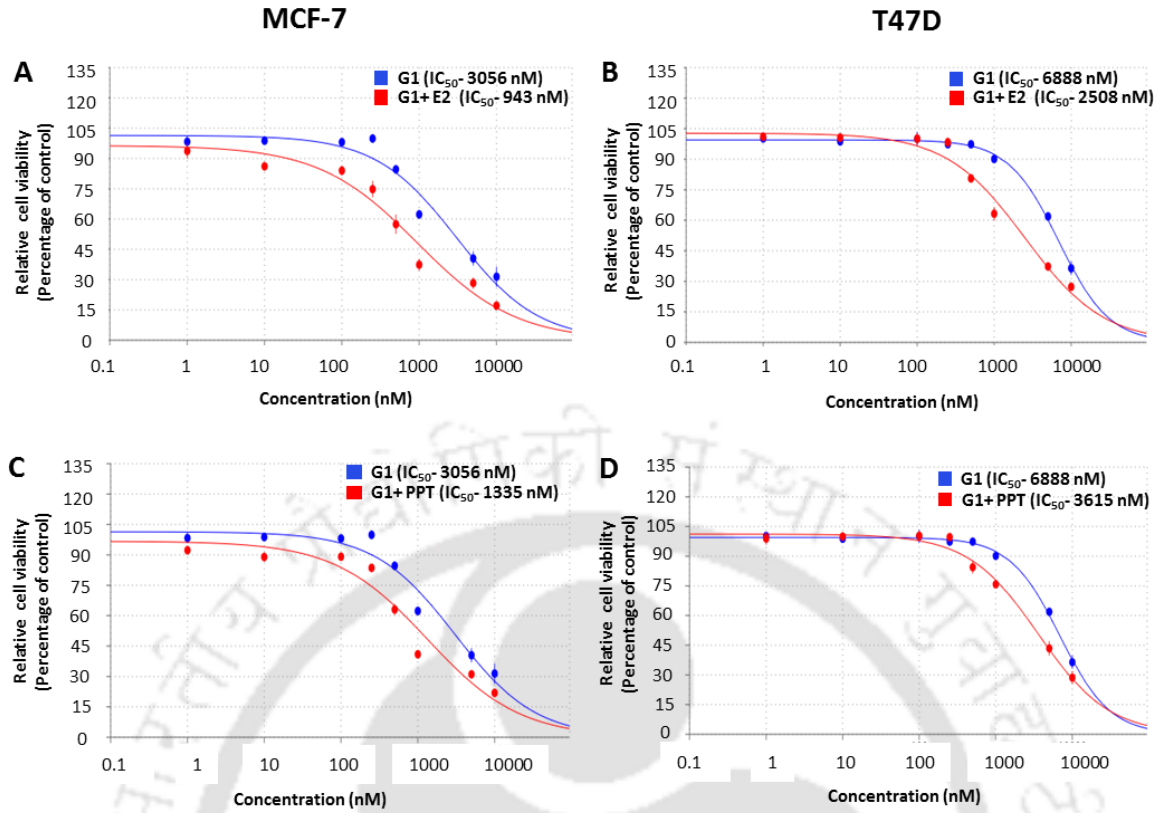

Figure 6.6: Estrogen stimulation renders MCF-7 or T47D cells more responsive to GPER agonist G1: MCF-7 (A,C) or T47D (B,D) cells were seeded into 96 well plates and allowed to acclimatize and grow for 48 h. Cells were treated with indicated concentrations of G1, in the presence or absence of 10 nM E2 or 100 nM PPT in M2-medium. The treatment was continued for 120 h, replenishing the treatment medium every 48 h. The treatment was terminated by removing the spent medium, followed by a DPBS wash. The cells were incubated with MTT reagent for 3 h. The excess reagent was removed, and the formazan crystals were dissolved in DMSO. Absorbance at 570 nm and 690 nm was taken, and the difference in the absorbance (A_{570}-A_{690}) was considered as a measure of cell viability. The viability of the ethanol-treated cells was assigned the value of 100, and treated cells were expressed relative to the control. IC_{50} of the groups treated with indicated concentrations of G1, in the presence or absence of 10 nM E2 or 100 nM PPT, were calculated using AAT bioquest IC_{50} calculator.

6.3. Discussion

GPER is a putative tumor suppressor; its expression is down-regulated in breast cancers, and high expression is associated with better patient survival[151]. GPER signaling cross-talks with other signaling pathways[83,144,152]. G1, a non-steroidal synthetic ligand, was used in our investigation to activate GPER selectively. G1 is often employed to examine GPER-specific effects because of its selective affinity for GPER over ERα or ERβ. G1 has enabled investigations into mechanisms of GPER signal transduction, which conforms to the typical GPCR signaling paradigm. It involves events downstream of Gα, and Gβγ subunits. The effect of GPER activation by G1 in breast cancer is still not clearly understood. Most studies have shown that G1 inhibits cell growth in GPER-expressing cancer cells. Wei et al. (2014) demonstrated that G1 suppresses proliferation by inducing G2/M cell cycle arrest and

apoptosis, by downregulating the G2-checkpoint regulator cyclin B, and induction of mitochondrial-related apoptosis[148]. G1 inhibits cell proliferation and induces intracellular calcium (Ca^{2+}) levels, as reported by Vo et al. (2019)[149]. However, on the contrary, Wang et al. (2012) found that G1-mediated activation of GPER led to proliferation and cell cycle progression, which were abolished with GPER-specific antagonist G15[105].

Despite extensive research on GPER (G-protein-coupled estrogen receptor) signaling, the target transcriptome influenced by GPER signaling remains poorly understood. Initial insights into GPER targets were gained through transcriptome analysis of SkBr-3 cells treated with estrogen or tamoxifen for 1 h, which revealed the induction of diverse transcription factors in response to these stimuli[103]. Subsequently, Toprak et al. (2020) demonstrated that treatment of ovarian cancer cell lines with 1 μM G1, a specific GPER agonist, for 72 h resulted in the downregulation of the mitosis pathway and the upregulation of the p53 pathway. In our study, we observed that prolonged activation of GPER led to the enrichment of apoptosis-related gene sets. Our findings were consistent with the experimental observations made by Wei et al. (2014), who showed that G1 stimulation induced G2/M cell cycle arrest in breast cancer cells[148].

In the previous chapters, we discussed that GPER and ERα expression are positively correlated in breast tumors and estrogenic stimulation upregulates GPER expression in breast cancer cells. Based on the following observation, we wanted to investigate whether the upregulation of GPER expression upon estrogen treatment will affect the responsiveness of the cells towards G1. We reported that estrogen-mediated induction of GPER makes cells more responsive to G1. The IC_{50} of G1 in the E2- or PPT-treated groups was lower than in the vehicle-treated group. We believe that in response to estrogenic stimulation, such a mechanism would prevent the mammary epithelium from proliferating excessively, hence avoiding tumor development.

GPER and ERα play crucial roles in breast cancer. They have contrasting effects on breast cancer cells. Based on our observations, we believe, the former induces cell cycle arrest and apoptosis, while the latter induces proliferation. When GPER and ERα positively co-express in breast tumors, their contrasting downstream signaling effects appear to balance each other. Despite these improvements, a few recent investigations suspected G1-mediated cellular effects to be off-target, especially at higher G1 concentrations[41,105,106]. Further studies

are needed to point out how GPER and its agonist, such as G1, can be considered a potentially new pharmacological tool to reduce the growth of breast tumors.

CHAPTER 7

Conclusions and future prospects

7.1. Conclusions

GPER is a seven-transmembrane G-protein-coupled estrogen receptor that mediates rapid estrogen actions. GPER has gained traction in the field of breast cancer due to the following reasons: it is a non-canonical membrane estrogen receptor[16–18], it explains the EGF-like effects of estrogen[16], and it contributes to tamoxifen resistance[35,42,91]. The acknowledgement of its clinical relevance is reflected in the plethora of publications addressing GPER expression in breast cancer, and its association with standard clinico- or histo-pathological markers[29,30], patient survival[27,32], endocrine resistance[35], or metastasis[29].

The significance of GPER-ERα co-expression, has not been adequately addressed, although it is appealing on several counts. GPER-ERα co-expression entails favourable prognosis and cross-talk between GPER-ERα is a proof of the functional interaction that is made possible by their co-expression. However, there are discrepancies in the nature of their association; obfuscating the nature of their relationship, its significance, and the underlying mechanistic basis. In this aspect, our contribution is noteworthy. GPER mRNA, and protein expression were analyzed in ERα-positive or -negative breast tumors from two independent cohorts using an assortment of methods, such as analysis of tumors from the TCGA-BRCA cohort, western blotting, and RT-qPCR. According to our findings, GPER expression is positively associated with ERα expression in breast cancer.

Positive GPER-ERα co-expression in breast tumors has clinical significance. The Kaplan-Meier Plotter was employed to gauge the effect of GPER expression on the survival of patients with ERα-positive or -negative tumors. Analysis of survival data revealed that high GPER expression is associated with significantly longer overall survival of patients with ERα-positive breast tumors. In contrast, patients with ERα-negative tumors have poor overall survival with higher GPER expression. The contrasting impact of GPER expression on survival depending on ERα status is intriguing. Taken together, the present study shows the utility of GPER as a prognostic marker in breast cancer.

The molecular basis behind the low GPER expression in ERα-negative tumors is still not clearly understood. Manjegowda et al. (2017) recently reported the loss of GPER expression in breast cancer cells and attributed it to DNA methylation-dependent silencing. They highlighted the significance of the methylation in the terminal 8 CpGs of the upstream CpG island (upCpGi) in the GPER locus. In this thesis, using targeted bisulfite sequencing,

we demonstrated the likely association of DNA methylation in the suppression of GPER expression in ERα-negative breast tumors. The methylation status at the terminal 8 CpGs of the upstream CpG island can serve as a prognostic marker to assess the clinical behaviour of breast tumors. Given the role of GPER in patient survival, epigenetic restoration of GPER expression in the ERα-positive/GPER-negative tumors using pharmacological agents can help in better prognosis. However, the role of histone deacetylation in regulating GPER expression in breast cancer still needs to be explored.

Our study provides strong evidences in favour of the role of liganded-ERα in inducing GPER expression, thereby providing basis the for their co-expression in breast cancer. This study has provided molecular insights into the regulation of GPER by estrogen. Estrogen-mediated induction of GPER has cell biological implications. GPER signaling activates the EGFR-MAPK pathway, which in turn activates the unliganded ERα, due to the phosphorylation of serine 118. This cross-talk between GPER and ERα is a proof of the functional interaction that is made possible by their co-expression. However, the cross-talk described so far has been unidirectional. Estrogen-mediated induction of GPER via ERα means that the cross-talk is bidirectional; each impacting the expression or function of the other.

GPER has greater cell biological implications in the context of breast cancer development and progression. Since its discovery, GPER has been considered a therapeutic target in breast cancer. The data on divergent effects of GPER activation include both promotion[108,153] and inhibition[149] of cell proliferation. Analysis of our RNA-sequencing results revealed that G1 stimulation enriched the apoptosis-related genes and depleted the G2/M cell cycle checkpoint genes. Selective activation of GPER inhibited the growth of MCF-7 cells. The cells were arrested in the G2/M phase of the cell cycle and destined for apoptosis. The response of the MCF-7 cells to G1 stimulation was then examined in the presence of E2 or PPT. E2 or PPT treatment increased the responsiveness of the breast cancer cells to G1 stimulation. In view of the negative impact of GPER activation on cell proliferation, estrogen induction of GPER may produce a balancing effect to counteract the pro-proliferative effects of activated ERα. Such a mechanism would prevent excessive proliferation of the mammary epithelium in the face of estrogenic stimulation, thereby preventing tumorigenesis. On the other hand, higher expression of GPER in tamoxifen resistant cells, estrogen induction of GPER may render ERα dispensable for growth and

proliferation of the mammary epithelium. Such a mechanism would subserve the emergence of endocrine therapy resistant breast tumors[35,42].

Our study clearly suggested a positive association between GPER and ERα expression in breast cancer. This research provides novel insights into the estrogen-regulation of GPER expression via ERα in breast cancer. In our opinion, the clinical and *in vitro* findings reported in this thesis will significantly contribute to the area of GPER biology and eventually to breast cancer patients.

7.2. Future prospects

The present investigation sheds new light on the involvement of GPER in breast cancer. Our contributions are noteworthy in elucidating the estrogen regulation of GPER expression in breast cancer cells. Estrogen regulation of GPER via ERα has clinical relevance. PR, a downstream transcriptional target of the estrogen-ERα signaling pathway, has been used to assess the estrogen responsiveness of breast tumors as GPER is also a downstream transcriptional target of the estrogen-ERα signaling pathway. We anticipate that even in the absence of PR, GPER may serve as an independent or an additional marker for identifying estrogen-responsive breast tumors.

Besides short-term non-genomic effects, GPER also mediates the long-term genomic effects of estrogen. The genomic effects of GPER activation are not completely understood. The RNA-seq results will contribute to a better understanding of the effects of long-term GPER activation in breast cancer cells. Investigators interested in GPER biology can use, and independently analyze, the RNA-seq data for insights into the genomic effects of GPER activation.

The biological functions of the GPER transcript variants are unclear. We have shown the expression of GPER-v2, -v3, and -v4 in breast tumors and breast cancer cell lines. As far as we are aware, no previous reports have been made on the expression profile of these GPER variants in breast tumor samples. While visualizing these transcripts in the UCSC Genome Browser (genome build hg19), we found another transcript (uc010ksd.1, GENCODE Transcript ID - ENST00000401670.1), which is annotated as GPER. This transcript has two exons and a long intron. It is similar to the known variants in terms of the open reading frame and 3'UTR, both contained in its second exon. We named the novel

GPER transcript as GPER-v5. Interestingly all the transcript variants of GPER differ in their 5'UTRs. 5'UTRs influence translability and stability of mRNAs and thus contribute to post-transcriptional regulation of gene expression. The influence of the different 5'UTRs on GPER protein expression has not been studied. It would be interesting to investigate whether alterations in GPER expression at the variant level impacts cell metabolism, and response to estrogen or endocrine disruptors, and are associated with pathological conditions.

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Appendix

Supplementary Table 3.1. List of materials, reagents, and kits used in this study

Items	Catalogue number	Company	Country
Phenol red- containing Dulbecco's modified Eagle's medium (DMEM)	AT-007	Himedia	Mumbai, India
Phenol red – containing Roswell Park Memorial Institute (RPMI) -1640	AT-028	Himedia	Mumbai, India
Phenol red- free Dulbecco's modified Eagle's medium (DMEM)	AT-190	Himedia	Mumbai, India
Pheol red – free Roswell Park Memorial Institute (RPMI)-1640	AT-120	Himedia	Mumbai, India
cs-FBS	RM10416	Himedia	Mumbai, India
Trypsin-EDTA	TCL-034	Himedia	Mumbai, India
Antibiotics	A018	Himedia	Mumbai, India
DPBS	TS-100	Himedia	Mumbai, India
FBS	10270106	Gibco	Grand Island, NY, USA
EmeraldAmp	RR320B	DSS Takara Bio Incorporation	New Delhi, India
G1	10008933	Cayman Chemical	MI, USA
Protein G plus-Agarose beads	IP04-1.5ML	Merck Millipore	Burlington, USA
Polyclonal histone H3 antibody	BB-AB0055	BioBharati LifeScience Pvt. Ltd	Kolkata, India
Normal rabbit IgG antibodies	AB0001	BioBharati LifeScience Pvt. Ltd	Kolkata, India
Estrogen Receptor α (D8H8) Rabbit mAb	8644S	Cell signaling technology	Danvers, Massachusetts, USA
Lipofectamine RNAimax	13778075	Thermo Scientific	PA, USA
PowerUp™ SYBR® Green PCR	A25743	Thermo Scientific	PA, USA
ERα-specific siRNA	4392420	Thermo Scientific	PA, USA

Scrambled siRNA	AM4611	Thermo Scientific	PA, USA
Clarity Western ECL Substrate	1705060	Bio-rad	Hercules, California, USA
Cell culture plasticwares		Eppendorf	Hamburg, Germany
All other reagents, salts, and buffers		Merck	Darmstadt, Germany
		Sisco Resarch Laboratories	Mumbai, India
		Sigma-Aldrich	MO, USA

Supplementary table 3.2: List of primers used for routine RT-PCR, real-time qRT-PCR, and ChIP.

Sl. No	Gene Name	Primer sequence (5'---->3')	Amplicon (base pair)	Annealing temperature (°C)	Remarks
1	**RPL35a**	Forward- CGGCCTCCAAGCT CT TAAG Reverse- CAGGTCCAGGG CTTGTACT	131	60	Used in qRT-PCR using cDNA synthesized from cell lines and breast tumors
2	**GPER-v2**	Forward- ATCTGGACGGCAGGT ACC Reverse- GAAGAACAGATGCTCCTCACAC	149	60	Used in qRT-PCR using cDNA synthesized from cell lines and breast tumors
3	**GPER-v3**	Forward- TGGACGGCAGCCCTGCTC Reverse- GCTGCTCACTCTCTGGGTAC	154	60	Used in qRT-PCR using cDNA synthesized from cell lines and breast tumors
4	**GPER-v4**	Forward- GCGGGTCTCT TCCTCTCTC Reverse- GCTGCTCACTCTCTGGGTAC	166	60	Used in qRT-PCR using cDNA synthesized from cell lines and breast tumors
5	**PR**	Forward- CGCGCTCTACCCTGCACTC Reverse- TGAATCCGGCCTCAGGTAGTT	121	60	Used in qRT-PCR using cDNA synthesized from cell lines
6	**Gapdh**	Forward- GGCCGGGGCCCACTTGAAG Reverse- TGGATGACCTTGGCCAGGGGG	174	68	Used in qRT-PCR using cDNA synthesized from mouse mammary gland
7	**GPER-ChIP**	Forward- ATCTGGACAGCCTCACGCAG Reverse- ACGGCCCATGAAGACTGTGC	348	58	Used in ChIP
8	**pS2**	Forward- CATTGCCTCCTCTCTGCTCC Reverse- ACTGTTGTCACGGCCAAGCC	423	58	Used in ChIP (+ve control)
9	**ESR1**	Forward- GCCCTACTACCTGGAGAA Reverse- CCCTTGTCATTGGTACTGG	132	60	Used in routine RT- PCR
10	**Cyclophilin A**	Forward- GGGCCGCGTCTCCTTTGAGC Reverse- GGCGTGTGAAGTCACCACCC	158	60	Used in routine RT-PCR
11	**GPER upCpGi**	Forward- ATTTAGAAGTAGGAGTGAGATT Reverse- ATCCCAAACATTCAAACCAA	465	53	Used to amplify bisulfite converted DNA

Supplementary table 3.3: Summary of the sequencing read data.

Treatment	Sample	Reads passed after filtering	Reads failed due to low quality	Reads failed due to too many N	Reads with adapter trimmed using fastP	Bases trimmed due to adapters using fastP	Overall alignment rate using HISAT2 (in %)
Vehicle (0.1% Ethanol)	A1	15151200	478998	5812	1606080	29796912	94.25
	A2	14885494	468992	5608	1329364	24873434	94.52
	A3	17318424	525390	6778	1711716	31229126	94.66
100nM G1	B1	16088528	477180	6442	1664584	32398762	94.55
	B2	16267278	494750	6160	1601466	29507242	94.62
	B3	16615052	520318	6262	1703590	31487992	94.48
1 µM G1	C1	15197230	497706	5742	1105054	20382242	94.27
	C2	16917668	502368	6484	1603392	29159112	94.15
	C4	15618252	499354	6054	1468392	27198184	93.82

Supplementary table 6.1. List of top 50 upregulated genes in 1 µM G1-treated group

Sl No.	Gene name	log_2FoldChange	pvalue	padj
1.	CYP1A1	4.405241671	7.02E-20	1.64E-16
2.	GDF15	2.854811957	6.08E-38	4.97E-34
3.	ENSG00000286116	2.812028748	1.91E-05	0.000464
4.	APOBEC3H	2.741271442	1.07E-05	0.000293
5.	RP11-398B16.2	2.68188368	9.23E-07	3.82E-05
6.	CCL22	2.573271951	7.81E-11	1.47E-08
7.	ACTA2	2.562125602	8.21E-14	3.95E-11
8.	UGT1A10	2.498712077	1.24E-11	3.55E-09
9.	UGT1A4	2.483322158	1.48E-11	4.11E-09
10.	UGT1A1	2.474649662	1.55E-11	4.11E-09
11.	UGT1A5	2.471746654	1.58E-11	4.11E-09
12.	UGT1A9	2.471746654	1.58E-11	4.11E-09
13.	UGT1A3	2.471746654	1.58E-11	4.11E-09
14.	UGT1A8	2.465100286	1.63E-11	4.11E-09
15.	ACTA2-AS1	2.45862102	5.45E-09	5.34E-07
16.	UPK3BP1	2.437428935	1.95E-05	0.000471
17.	UGT1A6	2.373049332	3.43E-11	7.39E-09
18.	RASD1	2.363372037	1.38E-14	8.7E-12
19.	UGT1A7	2.312463322	6.6E-10	8.85E-08
20.	CCDC33	2.269660488	1.47E-06	5.61E-05
21.	HMOX1	2.069547966	2.41E-28	1.31E-24
22.	CDKN1A	2.049963431	2.23E-42	3.65E-38
23.	LIF	2.038418124	1.9E-09	2.24E-07
24.	FOSL1	2.018855865	6.38E-05	0.001202
25.	ATF3	1.993634865	3.93E-09	4.2E-07

Sl no.	Gene name	log_2FoldChange	pvalue	Padj
26.	STRA6	1.993596348	8.26E-15	6.14E-12
27.	KRT81	1.971058809	2.19E-19	3.98E-16
28.	TNFRSF10C	1.96556167	7.54E-16	7.26E-13
29.	AHRR	1.92985369	0.000277	0.003834
30.	ENSG00000283710	1.914070201	0.000144	0.002306
31.	ANGPTL4	1.892230436	3.66E-09	3.96E-07
32.	ENSG00000284948	1.865336063	2.54E-07	1.34E-05
33.	EDA2R	1.832007905	1.51E-08	1.26E-06
34.	PLAU	1.768320508	0.001575	0.014695
35.	RP11-177H13.2	1.758863868	0.002059	0.017897
36.	TIMP3	1.748065122	2.1E-21	8.58E-18
37.	CYP24A1	1.736845771	4.19E-10	6.01E-08
38.	GADD45A	1.736573983	2.51E-13	1.05E-10
39.	LINC00475	1.732329902	7.83E-07	3.36E-05
40.	ENSG00000284386	1.720487315	0.000803	0.008776
41.	RND1	1.695986536	8.46E-08	5.43E-06
42.	LOXL2	1.647180685	9E-11	1.66E-08
43.	BTG2	1.639385373	3.42E-09	3.76E-07
44.	KRT83	1.638111808	6.86E-09	6.53E-07
45.	BBC3	1.620091561	5.91E-13	2.25E-10
46.	CREG2	1.619263443	5.14E-05	0.001008
47.	ALDH1A3	1.602770985	6.04E-05	0.00115
48.	HES2	1.59780641	4.37E-08	3.04E-06
49.	GPR87	1.591020628	5.48E-08	3.64E-06
50.	ENSG00000276966	1.577323866	1.72E-05	0.000425

Supplementary table 6.2: List of top 50 downregulated genes in 1 µM G1-treated group

Sl No.	Gene name	log_2FoldChange	pvalue	padj	Sl no.	Gene name	log_2FoldChange	pvalue	padj
1	CASC5	-2.95016	3.67E-07	1.8E-05	26	NCAPG	-2.21515	6.34E-07	2.78E-05
2	SMC2	-2.85164	1.84E-10	2.98E-08	27	CSE1L	-2.21245	1.21E-14	8.26E-12
3	BLM	-2.82016	4.81E-07	2.24E-05	28	CWC22	-2.19309	0.000365	0.004772
4	KIF20B	-2.76569	6.39E-06	0.000192	29	CLSPN	-2.19241	2.14E-10	3.34E-08
5	TOP2A	-2.69664	7.03E-16	7.19E-13	30	CDCA2	-2.16343	1.29E-09	1.59E-07
6	CEP135	-2.65556	3.7E-05	0.00078	31	IL6ST	-2.1625	4.01E-07	1.94E-05
7	C11orf82	-2.61612	7.19E-07	3.12E-05	32	CENPC	-2.15126	0.000123	0.002029
8	DTL	-2.61157	1.37E-12	4.67E-10	33	DNA2	-2.14186	1.69E-08	1.38E-06
9	TTK	-2.60893	1.38E-08	1.17E-06	34	RAD51AP1	-2.13983	2.66E-09	3.03E-07
10	EXO1	-2.5494	3.18E-09	3.57E-07	35	KIF23	-2.13561	2.92E-10	4.42E-08
11	ATAD2	-2.54426	5.71E-14	2.92E-11	36	AQR	-2.13192	3.43E-11	7.39E-09
12	NDC80	-2.46592	4.87E-08	3.32E-06	37	ESCO2	-2.11405	5.06E-08	3.42E-06
13	ANLN	-2.43909	3.14E-17	4.59E-14	38	NUP205	-2.11125	4.06E-08	2.9E-06
14	DLGAP5	-2.43106	1.65E-07	9.42E-06	39	GEN1	-2.10832	5.81E-08	3.83E-06
15	ERCC6L	-2.42114	2.11E-07	1.15E-05	40	ARHGAP11A	-2.0933	1.61E-16	1.88E-13
16	MCM10	-2.4192	4.63E-09	4.73E-07	41	ORC1	-2.07904	4.98E-13	1.99E-10
17	STIL	-2.37027	1.1E-10	1.95E-08	42	CENPI	-2.06855	8.28E-10	1.09E-07
18	DEPDC1	-2.30748	1.17E-09	1.45E-07	43	SLC4A10	-2.05756	3.36E-05	0.000722
19	CTD-2006C1.6	-2.30235	4E-05	0.00083	44	WDHD1	-2.04643	5.33E-06	0.000164
20	ENSG00000285948	-2.29889	1.79E-06	6.64E-05	45	NEK2	-2.04215	4.27E-09	4.45E-07
21	KIAA1524	-2.2988	5.26E-08	3.53E-06	46	BUB1B	-2.04105	1.06E-07	6.58E-06
22	RAD18	-2.28687	8.29E-09	7.66E-07	47	FANCD2OS	-2.04071	0.000198	0.00296
23	MRE11A	-2.27248	8.53E-07	3.58E-05	48	ZNF700	-2.03597	3.05E-06	0.000102
24	HELLS	-2.25414	1.81E-11	4.35E-09	49	ZNF430	-2.0235	0.000905	0.009668
25	PLK4	-2.23047	2.33E-07	1.25E-05	50	FREM2	-2.01541	1.14E-08	9.92E-07

List of abbreviations

AF1	Activation function1
ANOVA	Analysis of variance
AP1	Activator protein1
BAM	Binary Alignment Map
BBCI	Bhubaneswar Barooah Cancer Institute
BRCA 1	Breast cancer gene 1
BSA	Bovine serum albumin
CAF	Cancer associated fibroblast
cAMP	Cyclic adenosine monophosphate
cDNA	Complimentary deoxyribonucleic acid
ChIP	Chromatin immunoprecipitation
ChIP-Seq	Chromatin immunoprecipitation sequencing
CI	Confidence interval
CREBP	cAMP-response element-binding protein
csFBS	Charcoal stripped FBS
CycA	Cyclophilin A
Ct	Cycle threshold
DMFS	Distant metastasis-free survival
DDFS	Distant disease-free survival
DMEM	Dulbecco's Modified Eagle's Medium
DMSO	Dimethyl sulfoxide
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
DPBS	Dulbecco's phosphate-buffered saline
DRY	Aspartic acid- Arginine-Tyrosine
E1	Estrone
E2	17β-Estradiol
E3	Estriol
ECL	Enhanced chemiluminescence
EDTA	Ethylenediaminetetraacetic acid
EGF	Epidermal growth factor

EGFR	Epidermal growth factor receptor
EMT	Epithelial to mesenchymal transition
ER	Estrogen receptor
ERBB2	Erb-B2 Receptor Tyrosine Kinase 2
ERE	Estrogen response elements
ERK	Extracellular-signal-regulated kinase
EtBr	Ethidium bromide
EtOH	Ethanol
FBS	Fetal bovine serum
FDR	False discovery rate
GPER	G-protein coupled estrogen receptor
GPCR	G-protein coupled receptor
GSEA	Gene set enrichment analysis
gDNA	Genomic DNA
GLM	Generalised linear model
HB-EGF	Heparin-bound EGF-like growth factor
HCl	Hydrochloride
HEPES	4-(-2hydroxyethyl)-piperazineethanesulfonic acid
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia-inducible factor
HRE	Hypoxia-responsive element
HRP	Horseradish peroxidase
HR	Hazard ratio
Hsp	Heat shock protein
IDC	Invasive ductal carcinoma
HSD	Honestly significant difference
IC_{50}	Inhibitory concentration 50
IgG	Immunoglobulin G
IHC	Immunohistochemistry
IGF	Insulin-like growth factor
IP	Immunoprecipitation
IUPHAR	International Union of Pharmacology
Kb	Kilobase

kDa	Kilo Dalton
KM-plotter	Kaplan-meier plotter
LFC	Log fold change
LBD	Ligand binding domain
MMP's	Matrix metalloproteinases
MAPK	Mitogen-activated protein kinases
MCT	Monocarboxylate transporter
mER	Membrane associated ER
mL	Milliliter
mRNA	Messenger ribonucleic acid
MTT	Methylthiazolyldiphenyl-tetrazolium bromide
MCF-7	Michigan Cancer Foundation-7
NaCl	Sodium chloride
NCI	National Cancer Institute
nER	Nuclear estrogen receptors
NES	Normalized Enrichment Score
ns	Not significant
OS	Overall survival
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate-buffered saline
PBST	PBS containing 0.05% Tween 20
PCR	Polymerase chain reaction
PIP3	Phosphatidylinositol 3,4,5-trisphosphate
PKA	Protein kinase A
PPT	Propylpyrazoletriol
PR	Progesterone receptor
PKC	Protein kinase C
PM	Plasma membrane
pS2/TFF-1	Trefoil factor-1
qRT-PCR	Quantitative reverse transcription PCR
RFS	Relapse-free survival
RNA	Ribonucleic acid
RNase	Ribonuclease

RPKM	Reads per kilobases million
RNA-seq.	RNA Sequencing
RPMI-1640	Roswell Park Memorial Institute-1640 medium
RT-PCR	Reverse transcription-polymerase chain reaction
Scr	Scrambled
Sd	Standard deviation
SAM	Sequence Alignment Map
SDS	Sodium dodecyl sulfate
SDS-PAGE	SDS polyacrylamide gel electrophoresis
SERDs	Selective estrogen receptor down-regulators
SERMs	Selective estrogen receptor modulators
SHBG	Sex hormone-binding globulin
siRNA	Short interfering ribonucleic acid
SP1	Specificity protein 1
SRA	Sequence Read Archival
Ta	Annealing temperature
TAM	4-hydroxytamoxifen
TBST	Tris-buffered saline containing 0.05% Tween 20
TCGA	The Cancer Genome Atlas
Tm	Melting temperature
TGF	Transforming growth factor
TM	Transmembrane
TNBC	Triple-negative breast cancer
TF	Transcription factor
TSS	Transcription start site
UCSC	University of California, Santa Cruz
upCpGi	Upstream CpG island
UTR	Untranslated region
Var	Variants
nm	Nanomolar
µm	Micrometer
µM	Micromolar
µl	Microliter

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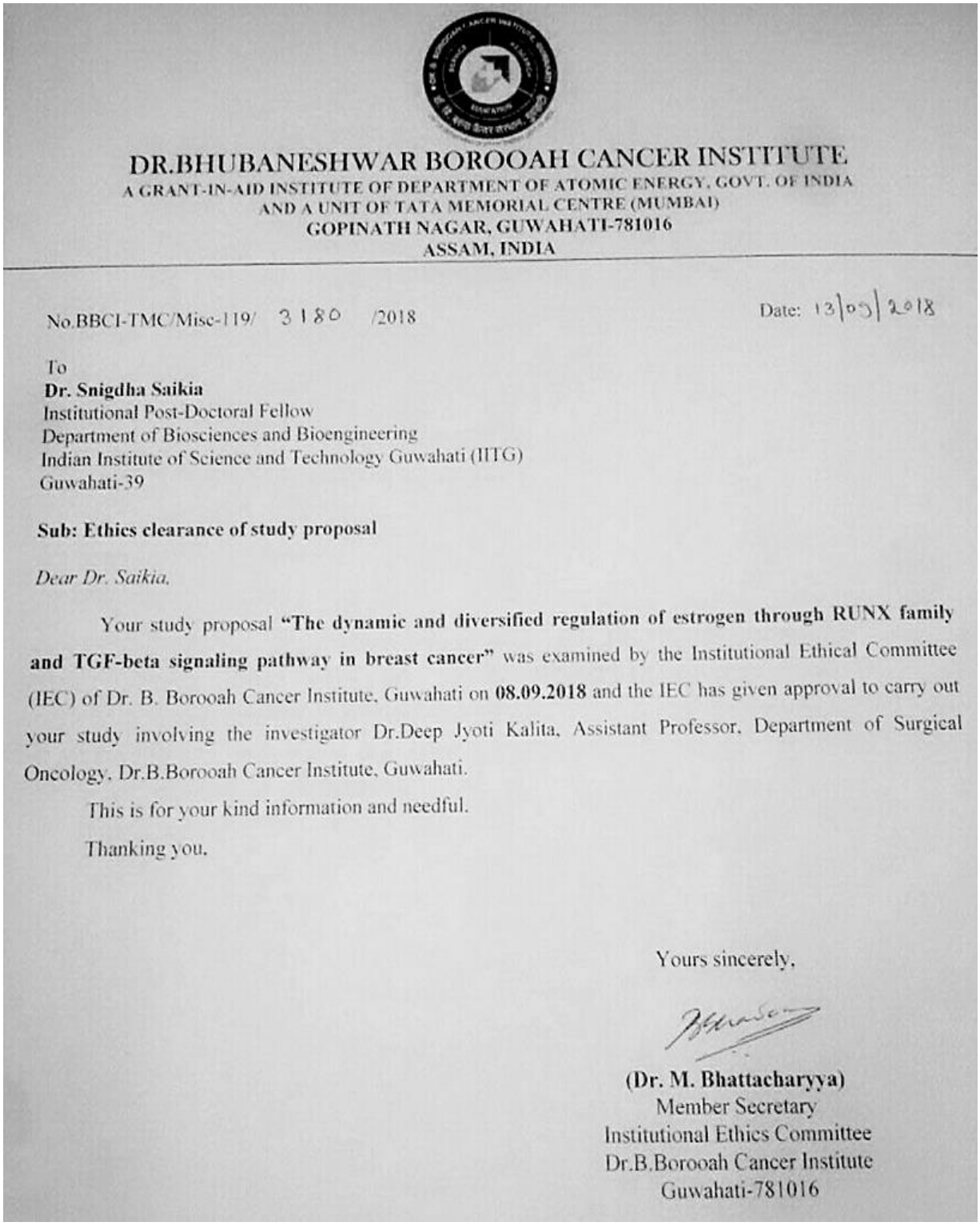
Chapter 4: Permission from the institute ethics committee to conduct mRNA and protein expression studies on breast tumor samples collected from BBCI, Guwahati.



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AND A UNIT OF TATA MEMORIAL CENTRE (MUMBAI)
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ASSAM, INDIA

No.BBCI-TMC/Misc-119/ 3180 /2018 Date: 13/09/2018

To
Dr. Snigdha Saikia
Institutional Post-Doctoral Fellow
Department of Biosciences and Bioengineering
Indian Institute of Science and Technology Guwahati (IITG)
Guwahati-39

Sub: Ethics clearance of study proposal

Dear Dr. Saikia,

Your study proposal **"The dynamic and diversified regulation of estrogen through RUNX family and TGF-beta signaling pathway in breast cancer"** was examined by the Institutional Ethical Committee (IEC) of Dr. B. Borooah Cancer Institute, Guwahati on **08.09.2018** and the IEC has given approval to carry out your study involving the investigator Dr.Deep Jyoti Kalita, Assistant Professor, Department of Surgical Oncology, Dr.B.Borooah Cancer Institute, Guwahati.

This is for your kind information and needful.

Thanking you.

Yours sincerely,

(Dr. M. Bhattacharyya)
Member Secretary
Institutional Ethics Committee
Dr.B.Borooah Cancer Institute
Guwahati-781016

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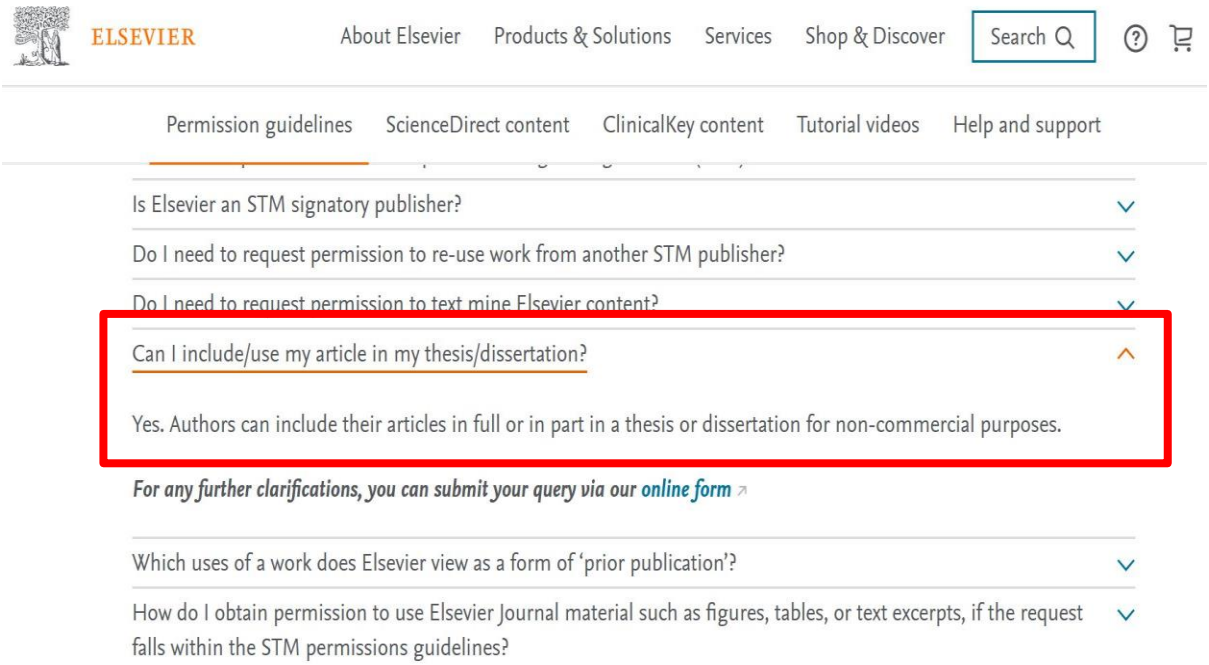

List of publications and presentations

Publications from thesis work:

1. **Pal, U.,** Manjegowda, M.C., Singh, N., Saikia, S., Philip, B.S., Kalita, D.J., Rai, A.K., Sarma, A., Raphael, V., Modi, D., Kataki., A.C. & Limaye A.M., The G-protein-coupled estrogen receptor, a gene co-expressed with ERα in breast tumors, is regulated by estrogen-ERα signalling in ERα positive breast cancer cells, BioRxiv (2022). https://doi.org/https://doi.org/10.1101/2022.06.14.496079.

2. **Pal, U.**, Sahu, A., Barah, P., & Limaye A.M., Transcriptomic data of MCF-7 breast cancer cells treated with G1, a G-protein coupled estrogen receptor (GPER) agonist, Data in Brief 41(2022) 107948.

Publications from outside thesis work:

1. **Pal, U.**, Manjegowda, M.C. & Limaye A.M., A novel transcript variant of human G-protein coupled estrogen receptor, Mol. Biol. Rep. 48 (2021) 2979–2983.

2. **Pal, U.**, Ghosh, S. & Limaye A.M., DNA methylation in the upstream CpG island of the GPER locus and its relationship with GPER expression in colon cancer cell lines, Mol. Biol. Rep. 47 (2020) 7547–7555.

3. Saikia, S., **Pal, U.**, Kalita, et al. RUNX1T1, a potential prognostic marker in breast cancer, is co-ordinately expressed with ERα, and regulated by estrogen receptor signalling in breast cancer cells, Mol. Biol. Rep. 48 (2021) 5399–5409.

4. A. Kumar, M.C. Manjegowda, D.J.S. John Mary, **U. Pal**, S. Kumar, A.M. Limaye, Estrogen receptor-α is a determinant of protocadherin-8 expression in breast cancer cells, Gene Reports. 14 (2019) 6–11. https://doi.org/10.1016/j.genrep.2018.10.014.

5. S. Katla, N. Mohan, S.S. Pavan, **U. Pal**, S. Sivaprakasam, Control of specific growth rate for the enhanced production of human interferon α2b in glycoengineered Pichia pastoris : process analytical technology guided approach, J. Chem. Technol. Biotechnol. 94 (2019) 3111–3123.

6. S. Katla, B. Karmakar, S.R.R. Tadi, N. Mohan, B. Anand, **U. Pal**, S. Sivaprakasam, High level extracellular production of recombinant human interferon alpha 2b in glycoengineered Pichia pastoris: culture medium optimization, high cell density cultivation and biological characterization, J. Appl. Microbiol. 126 (2019) 1438–1453.

Poster presentations:

- **Uttariya Pal**, Mohan C. Manjegowda, Mithilesh Kohale and Anil M. Limaye. Transcriptome profiling using next-generation sequencing of MCF-7 cells treated with GPER-specific agonist G1. Presented in "World cancer congress 2020", held in Jaipur, India (2020). **(Best poster award)**

- Mohan C. Manjegowda, **Uttariya Pal**, Paridhi Singhal Gupta, Ajay Kumar, Dixcy Jaba Sheeba J M, Gaurav Bhatt and Anil M. Limaye. Transcriptome profile of breast cancer cells treated with GPER1-specific agonist G1. Presented in "IACR", held in Kolkata, India (2018).

Workshops attended:

- "Genetic Variation Analysis Course", from 9th to 12th March, held in Online (University of Zurich) (2021).

- "Online workshop on Flow Cytometry Techniques and Applications", from 21st to 22nd December, held in NECBH, IIT Guwahati, India (2020).

- 20th INDO US FLOWCYTOMETRY SYMPOSIUM CUM WORKSHOP on " Applications of flowcytometry in biotechnology" from 13th to 16th March held in IIT, Guwahati, India (2019).