DECIPHERING THE MOLECULAR MECHANISMS OF ESTROGEN SIGNALING IN GASTROINTESTINAL TUMORS

A Dissertation Presented to

the Faculty of the Department of Biology and Biochemistry

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

By

Fahmi Mesmar

May 2017

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ABSTRACT

Colorectal and pancreatic cancers are predominant gastrointestinal (GI) tumors with estimated 90,970 deaths in the United Sates in 2016, representing ~ 60% of the total GI tumors related-mortalities. Several investigations, including meta-analysis, preclinical, and *in vitro* studies have established the protective role of estrogens and related receptors against GI tumors.

The main estrogen nuclear receptor in the colon is estrogen receptor beta (ERβ/ESR2). During colon cancer progression, ERβ expression is considerably reduced.

Re-expressing ERβ in colon cancer cell line (SW480) induces significant changes in miRnome. miR-205 is among the upregulated genes which directly targets the oncogene PROX1. *In vivo* studies demonstrated that both ERβ and miR-205 exert anti-metastatic effects.

SW620, a highly metastasized human colorectal cancer cell line was used for further analysis. This cell line dose not express any ERα or ERβ, and ERβ was introduced using lentiviral.

Here we show that 17β-estradiol (E2) has an ERβ-dependent as well as ERβ-independent effects suggesting possible role of alternative receptors in estrogen signaling in colonic epithelium such as G-protein coupled estrogen receptor 1 (GPER1).

In response to E2 or GPER1-selective agonist G1 treatments, several oncogenic long non-coding RNAs (lncRNAs) show downregulation, including MALAT1, NEAT1, ZEB1-

AS1 and HOTAIR. Several of these lncRNAs are involved in epithelial-to-mesenchymal transition (EMT) and tumor metastasis. Collectively, our data demonstrate that estrogen singling in the colon has anti-metastatic effects by modulating the expression of the cancer-related noncoding RNAs.

EMT transition is frequently linked to a chemoresistance phenotype, which is a common phenomenon in pancreatic cancer. Genistein, a phytoestrogen, has a chemoenhancing effect when it combined with gemcitabine in pancreatic cancer cell lines (PANC1 and MiaPaCa2). Using RNA-seq, genistein induces expression of genes that are related to apoptosis, calcium signaling, and endoplasmic reticulum stress, which can all be linked to enhanced GPER1 activity. Genistein treatment also reduces the expression of several inflammation related genes including MUC1. We demonstrate that G1 treatment similarly reduces proliferation, but dose not fully mimic the apoptotic features of genistein. Collectively, these data indicate that using an estrogenic compound, such as genistein, may enhance the anti-proliferative and apoptotic effects of chemotherapeutic agents such as gemcitabine.

List of Manuscripts

Included in this thesis

1. Estrogen receptor beta reduces colon cancer metastasis through a novel miR-205 - PROX1 mechanism.
   Nguyen-Vu T, Wang J, **Mesmar F**, Mukhopadhyay S, Saxena A, McCollum CW, Gustafsson JÅ, Bondesson M, Williams C.
   Oncotarget. 2016 Jul 5;7(27):42159-42171.
   PMID: 27283988

2. Estrogen receptor beta and G protein-coupled estrogen receptor 1 mediate convergent functions and gene expression in colon cancer cells.
   Fahmi Mesmar, Linnea Pettersson, Maria Bondesson, Cecilia Williams
   Manuscript in preparation

3. Activation of GPER1 by genistein or G1 inhibits the growth of pancreatic cancer.
   Fahmi Mesmar, Sebastian DiLorenzo, Chin-Yo Lin, Ander Berkenstam, Cecilia Williams.
   Manuscript in preparation

Other publications generated during graduate studies

4. Single-Molecule Sequencing Reveals Estrogen-Regulated Clinically Relevant lncRNAs in Breast Cancer. Jonsson P, Coarfa C, **Mesmar F**, Raz T, Rajapakshe K, Thompson JF, Gunaratne PH, Williams C.
   Mol Endocrinol. 2015 Nov;29(11):1634-45.
   PMID: 26426411

5. Vitamin D Induces Global Gene Transcription in Human Corneal Epithelial Cells: Implications for Corneal Inflammation. Reins RY, **Mesmar F**, Williams C, McDermott AM.
   Invest Ophthalmol Vis Sci. 2016 May 1;57(6):2689-98.
   PMID: 27196318

6. Genome-wide effects of MELK-inhibitor in triple-negative breast cancer cells indicate context-dependent response with p53 as a key determinant.
   Simon M, **Mesmar F**, Helguero L, Williams C.
   PLoS One. 2017 Feb 24;12(2).
   PMID: 28235006

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1. Introduction

1.1. Gastrointestinal Cancer

Gastrointestinal cancer (GI-cancer) is defined as a tumor originated from the gastrointestinal tract, or from the accessory organs of the digestive system. Worldwide, GI tumors are the leading causes of cancers-related mortalities in both genders. The colon and rectum (colorectal) as well pancreas form most common GI tract malignancies. These tumors collectively account for 185,000 estimated new cases and approximately 90,970 deaths a year in United States only [1]. Glandular epithelium forms the most common histological type of colorectal and pancreatic tumors which called adenocarcinoma (http://www.cancerresearchuk.org). Tobacco, obesity and alcohol are common risk factors to develop GI adenocarcinomas [2].

1.2. Colon cancer

1.2.1. Colorectal cancer (CRC): incidence, risk factors and prevention

Although the survival rate of CRC has been enhanced during the past three decades, colorectal cancer still remains in the third place of cancer deaths after prostate and lung in men, and breast and lung in women. In 2016, approximately 95,270 were diagnosed with a colon cancer and 39,220 cases

with rectal cancer in both sexes. An estimated 49,190 cases form both colon and rectal cancers were expected in 2016 [2]. The risks to develop CRC are increased with age, familial history, inflammatory bowel diseases (such as ulcerative colitis and Chron's disease) and eating habits like high consumption of red and processed meat [1]. Using non-steroidal anti-inflammatory drugs (NSAID), and supplements with different effects such as vitamin D, calcium, and phytoestrogens, have been reported to reduce the incidence of CRC [3]. Despite of the availability of early diagnostic tools and preventive medicine we are in urgent need to find and develop a preventive regime as well as new therapeutics to reduce the incidence and enhance the survival of CRC patients.

1.2.2. Molecular pathogenesis of colorectal cancer

CRC develops via deactivation of tumor suppressor genes or gain-of-function in oncogenes. Genetic mutations may be sporadic, inherited or familial. 70% of CRC are due to sporadic mutations. The disease progression starts with adenoma formations, that subsequently accumulate mutations and thus over the time, form at the end carcinoma. Adenomas are not cancerous lesions. Adenoma evolves to carcinoma, the cancerous lesion, due to consecutive mutations (commonly are point mutations) in genes, which include adenomatous polyposis coli (*APC*), KRAS proto-oncogene, GTPase (*KRAS)*, and finally tumor protein p53 (*TP53)*.

In addition to point mutations, microsatellite instability (MIS), chromosomal instability (CIN), and methylation of CpG islands also paly a significant role in affecting critical genes which maintain the colonic epithelium.

MIS occurs due to mutations or loss of expressions in DNA mismatch repair genes that include: *MLH1*, *MSH2*, *MSH6*, *PMS1*, *PMS2* or *EPCAM*. Recent development in high-throughput technologies revealed the involvement of many other pathways that contribute to CRC. These include: alterations in WNT, MAPK, PI3K and TGF-β/SMAD signaling pathways, which are crucial for CRC development and metastasis [4, 5]. In CRC, WNT signaling pathway is frequently altered at the beginning of tumor formation, followed by dysregulation in RAS-MAPK and in some cases in RAF-MAPK signaling pathways. PI3K signaling pathway can be involved via mutation in *PIK3CA* (*PI3K*) gene or via loss of function in *PTEN* a tumor suppressor gene. TGF-β signaling pathway can be activated when the cells exhibit loss of the q arm of chromosome 18, which encodes *SMAD2* and *SMAD4* genes. The final step in this tumorigenic process involves losing of *TP53* function (**Fig.1.1**) [4].

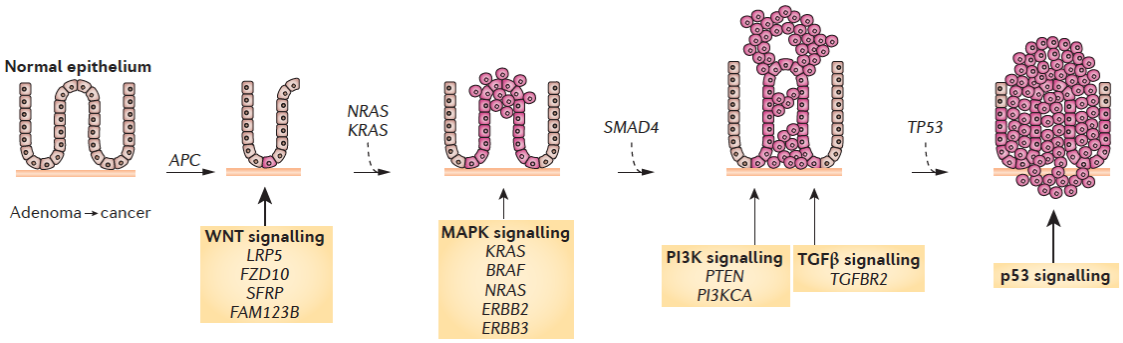

Figure 1.1. Adenoma to carcinoma classical model [4]

1.2.3. Protective effects of estrogen against colon tumorigenesis (epidemiological and preclinical data)

Colorectal cancer statistics data show that the incidence of CRC in men is higher than in women [6]. In addition, hormonal replacement therapies (HRT) have been shown to exert protective roles against CRC development. Post-menopausal women who have been treated with HRT exhibit a 30% decrease in CRC incidence [7, 8]. It has been established that HRT reduces the risk to develop other gastrointestinal tumors such as esophageal and gastric cancers [9]. Also, a meta-analysis reports show that the consumption of soy rich food reduces the incidence of CRC in women [10]. *In vitro* studies report an inhibition of colon cancer proliferation and induction in apoptosis during phytoestrogen treatments [11, 12]. Furthermore, animal studies support the estrogen protective effects. In mice, adding estrogens or phytoestrogens to the diet protect ovariectomized mice from azoxymethane-induced colon cancer [13]. Lastly, the same protective functions by estrogen have been also observed in

ovariectomized murine with dimethylhydrazine-induced colon tumor [14], as well as in $Apc^{Min/+}$ colon tumorigenesis model [15, 16].

1.2.4. Estrogen receptors (ERs), their functions and ligands

Estrogens mediate their intracellular effects mainly by binding and activating two nuclear receptors; estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). The ERα encoding gene, *ESR1* is located on human chromosome 6q25.1-q25.2, while ERβ is encoded by *ESR2,* and the gene is located on chromosome 14q23.2-q23.3 [17].

Estrogen receptors are composed of three functional domains: the NH_2-terminal domain (NTD) contain ligand-independent activation function domain (AF-1), DNA binding domain (DBD) which mainly recognizes and binds to the estrogen response element (ERE), and the COOH-terminal ligand-binding domain (LBD) which encompasses the ligand-dependent activation function domain (AF-2) [17]. The DBD is consisted of two zinc finger domains which recognize and bind DNA major groove [18].

A short helical region at AF-2 domain (LBD) called helix 12 (H12) determines the effects of the ligands. Changes in the orientation of H12 helix define the functions of the ligands as an agonist or antagonist [19].

Agonist treatment results in the dissociation of nuclear receptors from their scaffolding heat shock proteins (Hsp70 and Hsp90), enabling the proteins to dimerize and translocate into nucleus where they exert their genomic functions.

In the nucleus the activated ERs modulate gene transcription either by direct binding to ERE or indirectly by interacting with other transcription factors such as Fos/Jun (AP-1), SP-1, or NFκB [3, 19]. Rapid non-genomic signaling of ligand-activated receptors has been also reported, through modulating the cellular activities of the kinases and phosphatases [19]. In addition to the estrogen nuclear receptors, estrogens can also activate a G protein-coupled estrogen receptor 1 (GPER1), which is encoded by a gene on chromosome 7p22.3.

Activation of GPER1 can result in both transcriptional regulation and rapid non-genomic signaling [20]. The roles of the GPER1 in cancer are heterogeneous and context-dependent. GPER1 activation may promote the proliferation of ovarian and some breast cancers, on the other hand, it can play a tumor suppressive function in prostate for instance [21]. In the colon, GPER1 functions are not fully elucidated.

The role of estrogen nuclear receptors has been extensively studied in different cancers. While ERα promotes cancer progressions, ERβ (full isoform) exerts anti-tumorigenic functions in prostate, breast, ovarian, and colon cancers [17]. ERβ-agonist interaction induces conformational changes promoting ERβ translocation to the nucleus. There are various available ERβ agonists which can fully activate the receptor such as 17β estradiol (E2), or partially activate the receptor such as genistein [19]. Antagonists either work by interfering with the active confirmation of the receptor or by inducing its degradation. For instance, ICI 164,384 is an

ERβ antagonist. ICI 164,384 fully blocks the interaction between the LBD and the activation domain rendering the ERβ inactive [22]. Estrogens are the main endogenous agonist for ERs. Estrogens are steroidal hormones derived from androgenic precursor by aromatization, primarily in the ovary, but can also have effects in other tissues including adipose, muscle and nervous tissues [23]. The most abundant and potent form of estrogen is 17β estradiol (E2). E2 oxidation leads to formation of less active forms which include estrone (E1) and estriol (E3) [23]. In colonic epithelium, E2 is converted to E1 by 17β-hydroxysteroid dehydrogenase (17β-HSD2 and 4). E1 has been reported to have an anti-proliferative effect in the colon [13, 24, 25].

1.2.5. ERβ functions in colorectal cancer

In addition to the aforementioned epidemiological and preclinical data regarding estrogen's protective functions in the colon, there is a plethora of supporting evidence that illustrates ERβ involvement in colon tumorigenesis.

Primarily, in colonic epithelium the predominant estrogen nuclear receptor is ERβ, while ERα exhibits no or very limited expression [26, 27].

These data establish a general agreement that estrogen signaling in the colon is via ERβ. Likewise, during the development of CRC, the expression of ERβ is significantly reduced [28-30]. Loss of ERβ expression has been linked to poor CRC prognosis [27]. DNA methylation study demonstrates that 90% of CRC tissue develop a methylation at the *ESR2* promoter [31]. Moreover, single

nucleotide polymorphisms in the promoter sequence of *ESR2* is associated with an improved survival among CRC patients [32]. Several *in vitro* studies have confirmed previous findings. Re-expressing ERβ in SW480, a human colon cancer line results in proliferation inhibition and 60% decrease in tumor weight when xenografted in a fat pad of immunodeficient mice treated with E2 [33]. Apparently, ERβ decreases the proliferation of SW480 by reducing *c-MYC* expression.

Over and above, *in vivo* experiments demonstrate the important roles of ERβ in maintaining colonic epithelium. Although ERβ absence in ($ER\beta^{-/-}$) knockout mice is insufficient stimulus to develop a colon cancer, their colonic epithelium have showed a disruption in tight-junction, hyper-proliferation and decrease in apoptosis as well as in differentiation [34]. These data raise a question to study ERβ functions in different intestinal tumorigenesis models. For example, ($ER\beta^{-/-}$)($Apc^{Min/+}$) mice have showed an increase in the numbers and the sizes of formed adenoma when they compared with $Apc^{Min/+}$ controls [35]. Furthermore, azoxymethane (AOM)/dextran sodium sulfate (DSS) colitis-associated colon cancer model provides additional evidence of ERβ's protective role during the disease progression. Colonic epithelium of (AOM/DSS – $ER\beta^{-/-}$) mice have showed a higher dysplasia coupled with increase in inflammation in comparison with (AOM/DSS – WT) [36].

1.2.6. Estrogen and ERβ signaling in the colon

Despite the established data regarding the protective role of estrogens and ERβ against the development of colon cancer, the exact molecular mechanisms remain unclear. The anti-tumorigenic effects of estrogens can occur through different mechanisms such as activation of ERβ, the main estrogen nuclear receptor in the colon, or through alternative receptors such as GPER1, or even through its metabolites such as E1.

The available molecular data suggest that ERβ can protect against colon cancer by targeting various signaling pathways such as proliferation, inflammatory and apoptosis.

Re-expressing ERβ in human colon cancer cell lines (SW480, HT29 and HCT116) downregulates IL6 inflammatory network as well as reduces the expression of several oncogenes such as MYC, MYB and PROX1 [33, 37]. The impairment in IL6 inflammatory pathway may be attributed to ERβ interference with NFκB signaling [38]. In another human colon cancer cell line HCT8, restoring ERβ expression inhibits cell proliferation by modulating the expression of crucial cell cycle regulators such as reducing cyclin E expression and enhancing p21 expression cyclin-dependent kinase inhibitor [39]. In LoVo colon cancer cell line, ERβ induces an apoptosis by increasing p53 signaling [40]. Another aspect of ERβ genomic function is regulating microRNAs (miRNAs) expression. Studies have demonstrated that ERβ reduces the expression of oncogenic miR-17 cluster

as well as upregulates miR-205 a tumor suppressor miRNA [41]. miR-205 ectopic expression inhibits the migration and metastasis of human colon cancer cell lines by targeting PROX1 a key oncogene in colon cancer [42]. Collectively, these data suggest that ERβ exhibits a tumor suppressive role in the colonic epithelial.

1.2.7. Epithelial mesenchymal transition in colon cancer metastasis

Epithelial mesenchymal transition (EMT) is a developmental process in which the epithelial cells acquire mesenchymal spindle-shaped fibroblast-like morphology. During the EMT process, cells become more motile and less adhesive. Because of this, EMT can play an important role during cancer cell metastasis [43]. Because EMT is tightly regulated during the developmental stages there are several factors can be involved including transcription factors (TFs), miRNAs, lincRNAs, and EMT-associated signaling pathways.

EMT process is accompanied by a decrease in E-cadherin (*CDH1*), claudin (*CLDN1*) and occludin (*OCLN*) expressions as well as an increase in vimentin (VIM) expression, which results in alterations in cell polarity and adhesion [44]. Various TFs can induce EMT transition in a direct and/or an indirect fashion. Among these, ZEB1, ZEB2, SNAIL/SNAI1 and SLUG/SNAI2 transcription factors can induce EMT and cancer metastasis [43, 44]. Role of various noncoding RNAs in EMT will be discussed in next section.

1.2.8. Role of Non-coding RNAs in colon cancer

Several non-coding RNAs (ncRNAs) including microRNA (miRNAs) and long non-coding RNAs (lncRNAs) can be involved in CRC progression. miRNAs are short RNAs, their mature form is 20–24 nucleotides. miRNAs generally recognize a specific sequence at 3'UTR region of their target mRNA result in transcription repression [5].

lncRNAs are another class of ncRNAs. lncRNAs are 200 nucleotides or more in length. They are mainly transcribed by RNA polymerase II and their expression is tightly regulated similar to mRNAs. The majority of lncRNAs are 5'-methylguanosine capped and poly-adenylated at their 3' end. The major feature of lncRNAs is the tendency to fold into thermodynamically higher-order structures which allow them to interact with DNA, RNA and proteins [45].

lncRNAs can modulate gene expression by various mechanisms at different levels. Transcriptional processes can be stimulated or repressed when lncRNAs interact with transcriptional machinery proteins or with chromatin remodeling enzymes. At the post-transcriptional level, lncRNAs can affect the mRNA stability, the translation and splicing of their targets (**Fig. 1.2**). In addition to foregoing, lncRNAs are also able to interact with miRNA. The proposed models of miRNA-lncRNA interactions include: miRNA-triggered lncRNA degradation, miRNA-sequestration by lncRNA, lncRNA competing miRNA for the mRNA, or lncRNAs generating miRNA [46].

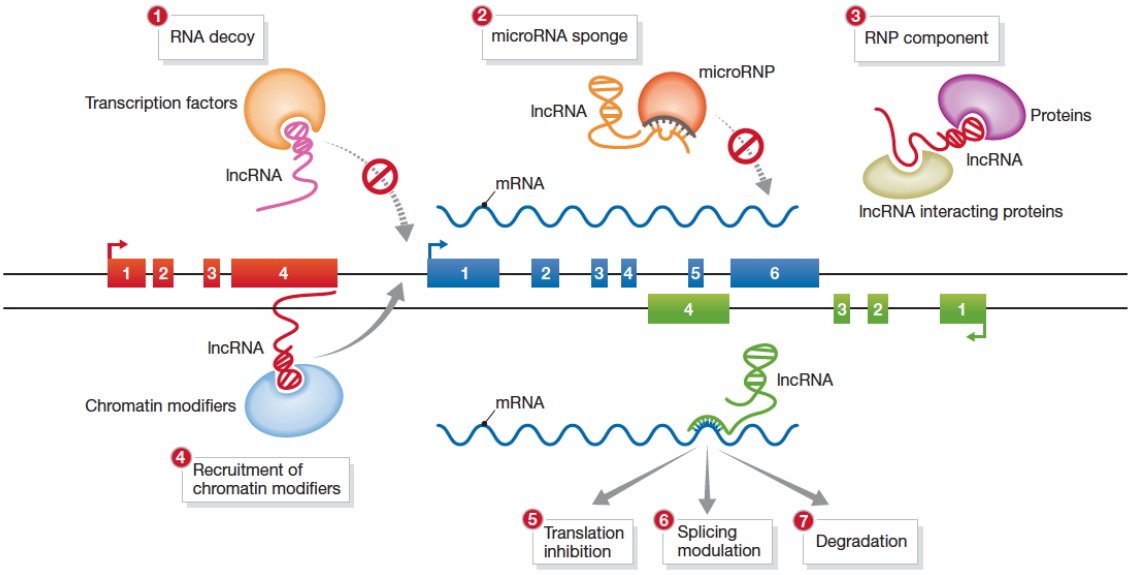

Figure 1.2. Mechanisms of lncRNA functional interactions [47]

During the development of colon cancer, ncRNAs often exhibit deviations from normal cellular activity. The expression of ncRNAs can be linked to CRC initiation, progression and/or metastasis (**Fig. 1.3**) [5]. Like many other cancers, the metastatic CRC is the major cause of death. Several ncRNAs promote the carcinoma – metastasis transition. ncRNAs may play a role to facilitate the early steps of tumor metastasis such as migration, invasion and epithelial-mesenchymal transition (EMT), or the late steps including extravasation, angiogenesis and colonization processes [5, 48, 49].

During the EMT, the immotile polarized epithelial cells acquire the motile mesenchymal phenotypes [48]. Since EMT encompasses changes in gene expression, it has been reported that many ncRNAs can be involved during this

process. It has been reported that miR-200 family are significantly repressed during EMT [48]. Reduction in miR-200 expression promotes stemness of the CRC which is correlated with a more aggressive phenotype [50].

Dysregulation in HOX transcript antisense RNA (HOTAIR), a lncRNA, has also been implicated in EMT. HOTAIR promotes CRC metastasis through EMT process presumably by modulating cancer epigenome [51].

Another important event during cancer metastasis is invasion. Several miRNA and lncRNA have been reported to repress or induce cancer invasion. We have reported that miR-205 is able to reduce migration and invasion of colon cancer cell lines (SW480 and HT29) by targeting PROX1, a crucial oncogene for CRC [42].

Metastasis associated lung adenocarcinoma transcript 1 (MALAT1) is a lncRNA which promotes cancer metastasis by multiple mechanisms. MALAT1 is able to activate ERK/MAPK and Wnt/β-catenin oncogenic pathways. MALAT1 is also able to interact with tumor suppressors miRNAs such as miR-101 and miR-217 and affects their functions [49]. Figure 1.3 illustrates various examples of ncRNAs that involved in the tumorigenesis of the colonic epithelium (**Fig. 1.3**).

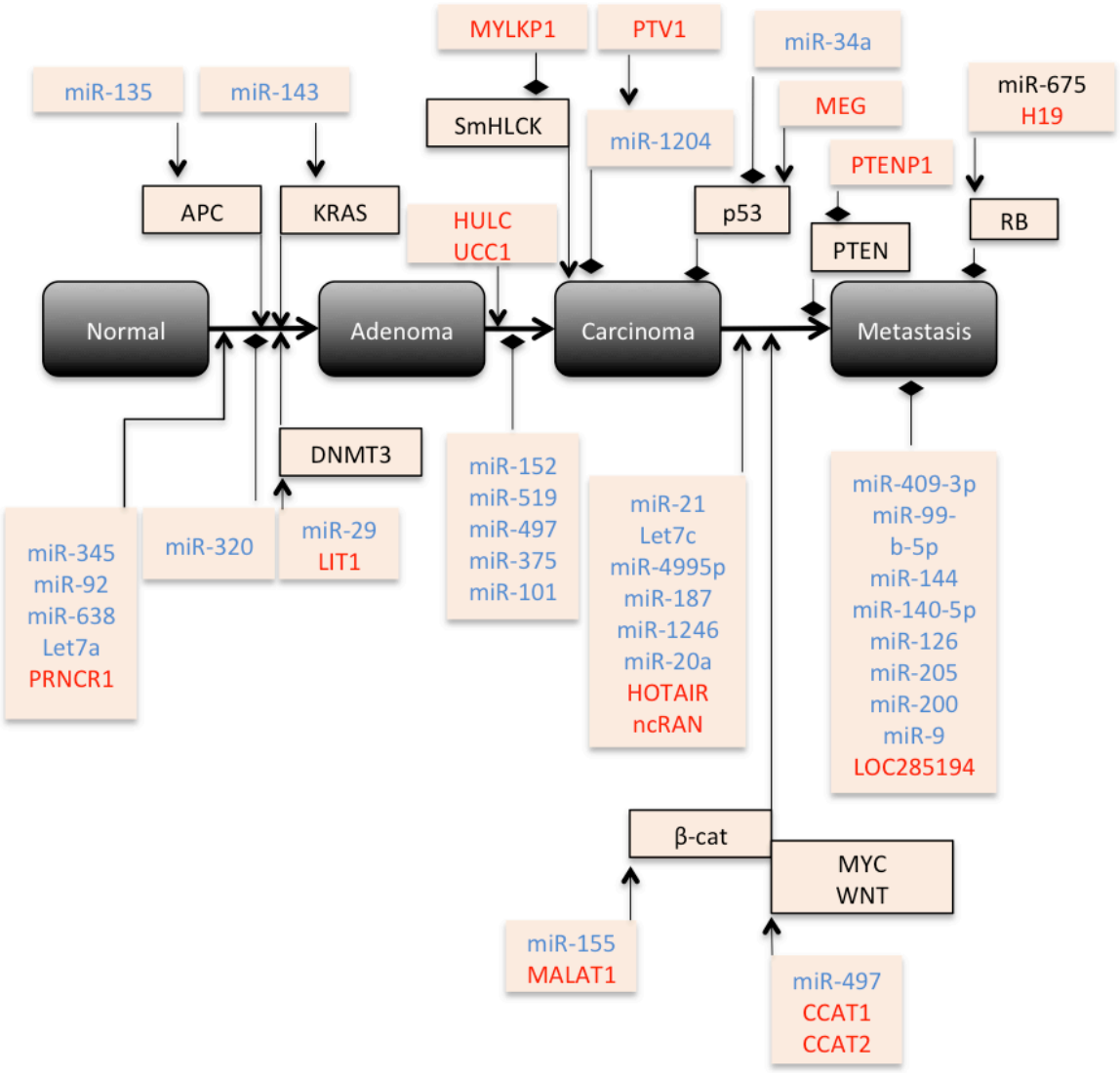

Figure 1.3. Contribution of various miRNA (blue) and lncRNA (red) during CRC. Arrow-headed lines indicate activation while diamond-headed lines indicate inhibition [5].

1.3. Pancreatic cancer

1.3.1. Pancreatic cancer: incidence, risk factors, and prevention

Pancreatic cancer is a highly aggressive malignancy which is characterized by rapid progression, metastasis, and chemoresistance [52]. In 2016, the number of the estimated new cases was 53,070 in United Sates only, and the estimated deaths from pancreatic tumor was 41,780, placing it as the fourth leading cause of cancer related deaths in United Sates [2]. The overall 5-year survival rate for pancreatic cancer patients is less than 5% and only 15% - 20% for whom undergo tumor resection [52, 53]. The risks to develop pancreatic cancer are increased with age, family history, diabetes and with hereditary pancreatitis [54, 55].

Pancreatic tissue is composed of two functional units: exocrine and endocrine. Exocrine pancreas consists of duct and acinar cells, which are involved in digestion process, whereas, endocrine pancreas consists of alpha, beta, delta and PP cells that are involved in glucose metabolism. The majority (85%) of pancreatic cancer is believed to arise from duct cells forming **pancreatic ductal adenocarcinoma (PDCA)** [52, 54, 55]. As in colon cancer, use of non-steroidal anti-inflammatory drugs (NSAID) has been reported to reduce the incidence of pancreatic cancer [56].

1.3.2. Molecular pathogenesis of pancreatic ductal adenocarcinoma (PDAC)

Genomic and molecular pathology analysis of the PDAC proposed an evolving and multistep tumor progression model. As in adenoma–carcinoma colon cancer model there are sequential steps to develop PDAC. During PDAC the normal duct starts to acquire mutations to develop abnormal premalignant lesions referred to pancreatic intraepithelial neoplasia (**PanIN**). PanIN accumulates genetic alterations, which can be graded in types 1, 2, and 3 [52, 55] (**Fig.1.4**). Figure 1.4. illustrates PanIN-PDAC cancer model. The majority (90%) of PanIN consist of *KRAS* mutations including early PanIN. In addition, KRAS signaling pathway can be activated by upstream epidermal growth factor receptor (EGFR). Overexpression of EGFR has been reported in more than 90% of pancreatic cancers [57]. After the activation of KRAS singling pathway, the inactivation mutations in tumor suppressor genes (*INK4A/CDKN2A*), *TP53*, *SMAD4* and *BRCA2* are crucial to develop the final malignant lesion (**Fig. 1.4**) [52, 55]. These genes are involved in the regulation of several pathways including: cell cycle, apoptosis, TGF–β signaling and DNA damage response which provide multiple molecular candidates for the available therapeutic agents.

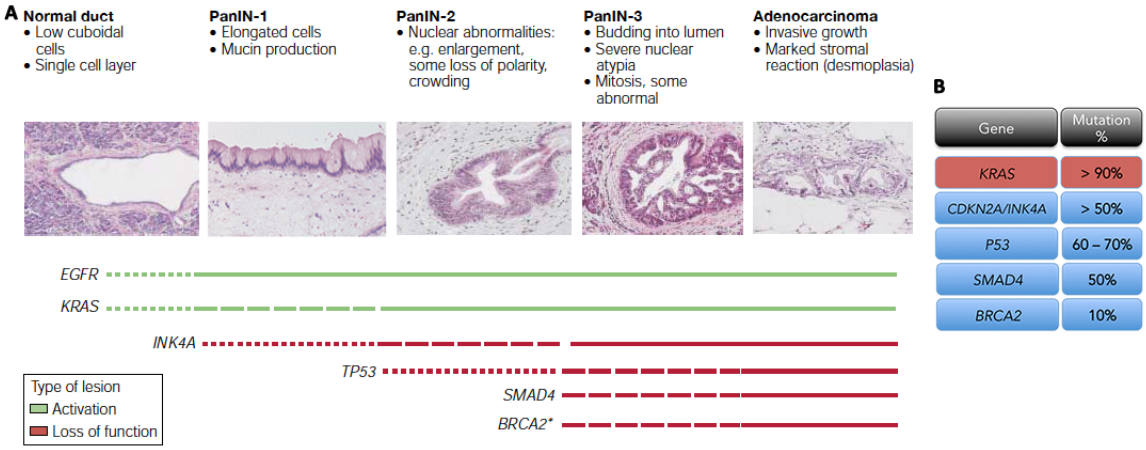

Figure 1.4. Pancreatic intraepithelial neoplasia - pancreatic ductal adenocarcinoma (PanIN – PDAC) cancer model. (A) Low power view of PanIN – PDAC cancer model. **(B)** Common gene mutations. Red colored boxes represent the percentage of activation mutations in KRAS. Blue colored boxes represent the percentage of deactivation mutations in tumor suppressor genes [52, 58].

1.3.3. PDAC chemoresistance and treatment

Intrinsic or acquired chemoresistance is a crucial feature of PDAC. Several pathways have been implicated in PDAC chemoresistance including the acquisition of mesenchymal phenotype as well as activation of redundant pathways. Activation of EMT transition induces chemoresistance in cancer cells. Mesenchymal phenotype is associated with cell dedifferentiation and stem cell-like features. Reversing EMT to MET sensitizes tumor cells to several chemotherapies such as gemcitabine [59]. In addition to the EMT process, activation of the NFκB pathway and overexpression of multidrug resistance (MDR) genes also induce chemoresistance [60].

Gemcitabine is the first-line drug for PDAC. Gemcitabine is a deoxycytidine analog. To exert it's anti-proliferative effects, gemcitabine is transported and metabolized inside the cells and converted to it's active triphosphate form, gemcitabine (dFdCTP). This reaction is catalyzed by three different kinases where deoxycytidine kinase (dCK) regulates the rate-limiting step. Gemcitabine exerts its anti-tumorigenic effects by interfering with DNA replication and deoxynucleotide synthesis as well as via activation of apoptosis [61].

The underlying mechanisms of gemcitabine chemoresistance are not fully understood. Alteration in gene expression that are related to gemcitabine metabolism cascade is a potential chemoresistance mechanism. For instance, downregulation in gemcitabine transporter proteins such as (hENT) reduces gemcitabine cellular uptake and thus induces chemoresistance. Likewise, a reduction in gemcitabine activating kinases such as (dCK) renders the gemcitabine inefficient. Upregulation in phosphatases and/or deaminases deactivate gemcitabine which leads to drug resistance [61].

Alterations in pathways that are unrelated to gemcitabine transportation and metabolism can be also implicated in chemoresistance. For example, active NFκB pathway contributes in gemcitabine resistance. In pancreatic cancer cell lines (BxPc-3, Capan-1, T3M4 and PancTu-1) gemcitabine elucidates a dose-dependent induction of NFκB [62].

Activation of NFκB pathway can be due to high levels of MUC4 [63], increase in CXCR4 receptor [64] or via NFκB–HIF1α-CXCR4 positive feedback loop [65].

Although gemcitabine is the standard drug to treat PDAC the mechanisms of resistance still not well understood and further studies shall be conducted to elucidate the mechanisms of gemcitabine chemoresistance.

1.3.4. Genistein is a phytoestrogen and a typical chemosensitizing agents

Genistein is a phytoestrogen, which has a structure similar to the 17β-estradiol (E2) (**Fig. 1.5**) [66], with weak estrogenic activity [67].

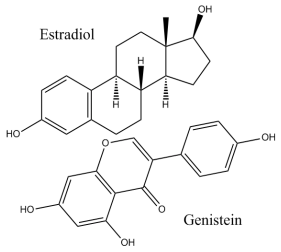

Figure 1.5. Chemical structures of 17β-estradiol and genistein [66]

Genistein can be a typical chemosensitizing agent since it is non-toxic on normal tissue and possesses anti-tumor activities [66]. In addition, genistein can also interfere with several oncogenic pathways; cell cycles, apoptosis, angiogenesis and metastasis. Studies have shown that, genistein inhibits protein-tyrosine kinase (PTK), Akt and NFκB signaling pathways. Furthermore, genistein is able to

induce apoptosis either by up regulating pro-apoptotic effectors such as Bax, Bad and Bak or by downregulating anti-apoptotic molecules such as Bcl-2 and Bcl-xL. Moreover, studies have published that genistein is able to induce cell cycle arrest in different cancer cell lines [66, 67]. In addition to that, genistein shows anticancer effects in various *in vivo* cancer models like tumor xenografts and chemical-induced cancers. In these models genistein acts as a cancer preventive agent which is able to reduce tumor growth, tumor angiogenesis and metastasis [67]. Thus it is reasonable to use genistein as a cancer chemopreventive agent and/or chemosensitizing agents. Several studies demonstrated a synergistic or additive effect of genistein when combined with chemotherapeutic agents such as gemcitabine [68], erlotinib [69], 5-fluorouracil (5-FU) [70] and docetaxel or cisplatin [71]. Genistein additive effects have been shown in different type of cancers including prostate, breast, lung [67, 72, 73], and pancreas [68-71]. Despite the promising *in vitro* and *in vivo* anti-tumor efficacy and chemosensitization of genistein, clinical phase I/II did not show an enhancement in patient survival when genistein was combined with gemcitabine [74, 75] or erlotinib [74]. Further studies are needed to understand the exact molecular mechanism of action of genistein, which cancer patients are ideal for the use of genistein, and finally which chemical modifications of genistein are needed to enhance solubility and bioavailability.

1.4. Emerging roles of GPER1 in cancers

The G protein-coupled estrogen receptor (GPER1/GPR30) is a 7-transmembrane receptor involved in both rapid non-genomic estrogen signaling and in transcriptional regulation [76]. The cellular localization of GPER1 remains controversial. GPER1 is mainly localized on the intracellular membranes such as endoplasmic reticulum [77]. In addition to that, nuclear localization has been also observed [78]. Based on mRNA studies, GPER1 is widely expressed in various human tissues. This includes female reproductive system such as ovaries, uterus, mammary glands and in GI system such as liver, pancreas and colon [79].

GPER1 can be activated by several ligands including: natural estrogens, phytoestrogens, synthetic selective agonists, selective estrogen receptor modulators (SERMs) and selective estrogen receptor downregulators (SERDs) (**Fig. 1.6**). In comparison with ERα and ERβ, GPER1 has lower binding affinities to E2. 0.1 nM of E2 are enough to activate ERα and ERβ, while GPER1 requires 4-6 nM of E2 to be activated [77, 80]. In addition to E2, highly selective GPER1 ligands have been identified including selective agonist G1 [81], and two selective antagonists G15 [82] and G36 [83]. It has been reported that G1 at concentrations up to 10 μM has no binding activities to ERα, ERβ, or to 25 other G protein-coupled receptors [81].

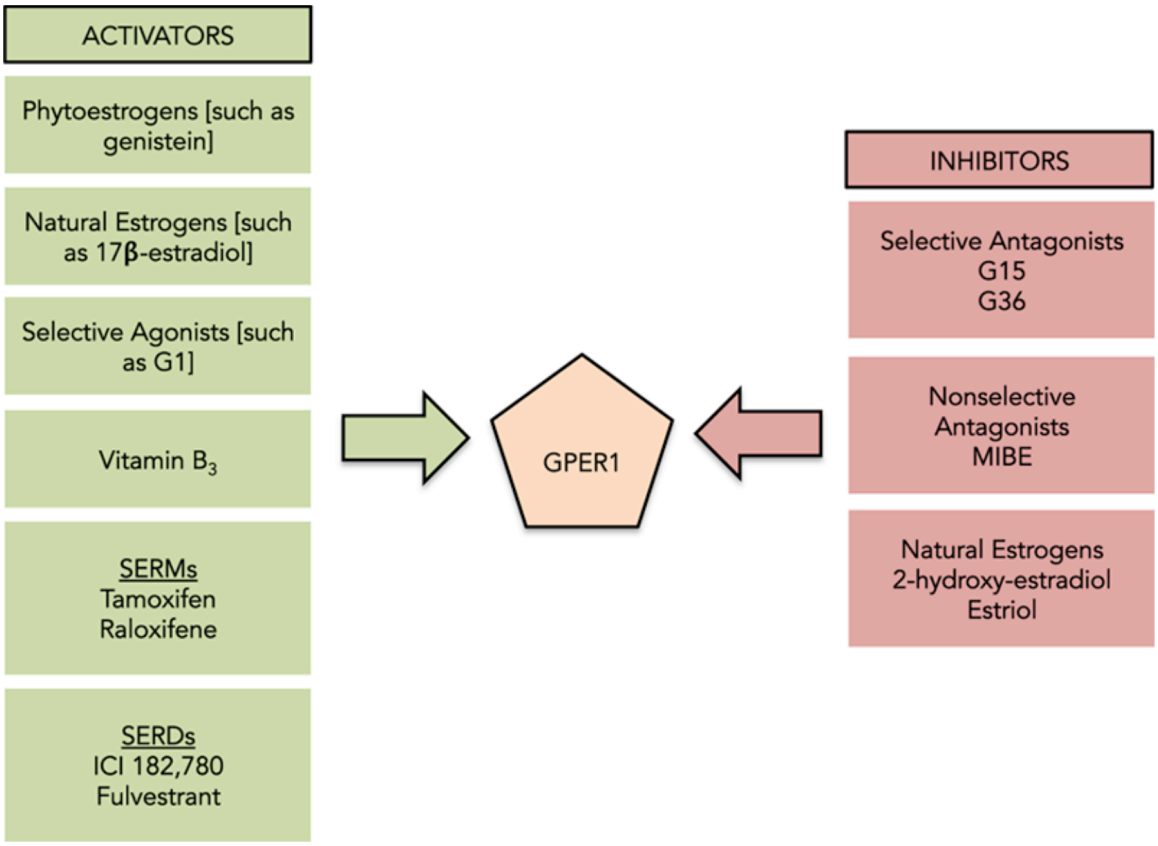

Figure 1.6. GPER1 ligands. Green highlighted boxes are activators. Red highlighted boxes are inhibitors. SERMs: selective estrogen receptor modulators. SERDs: selective estrogen receptor downregulators. MIBE: ethyl 3-[5-(2-ethoxycarbonyl-1-methylvinyloxy)-1-methyl-1H-indol-3-yl]but-2-enoate [76].

GPER1 regulates several signaling pathways that are involved in cell cycle, apoptosis, autophagy, inflammation, and angiogenesis. All of these pathways are implicated in cancer cell proliferation and metastasis. The downstream targets of GPER1 are involved in MAPK/ERK1/2, EGF-EGFR-PI3K-AKT, calcium mobilization and cAMP singling pathways [84-86]. The net effect of GPER1 activation on cellular phenotypes is extremely context dependent. It have been shown that GPER1 acts as tumor suppressor in ovarian [87], breast [88, 89], and endometrial

cancers [90]. Furthermore, clinical studies have demonstrated that using SERMs can enhance the efficiency of chemotherapies in prostate cancer [91] and in non-reproductive cancers such as pancreatic cancer [92] possibly by activating GPER1.

2. Material and methods

2.1. Cell culture and maintenance

All Cell lines were obtained from American Type Culture Collection (ATCC) (Manassas, VA). MiaPaCa2, PANC1, SW480, SW620 and HEK293 cell lines were cultured in high glucose with L-glutamine DMEM media (Sigma-Aldrich, St. Louis, MO) supplemented with 10% fetal bovine serum (FBS), and 1% penicillin and streptomycin (Sigma-Aldrich, St. Louis, MO). HT29 was cultured in RPMI (Sigma-Aldrich, St. Louis, MO) media supplemented with 10% fetal bovine serum (FBS), and 1% penicillin and streptomycin. All cell lines were cultured at 37°C with 5% CO_2 under humidity condition.

2.2. RNA extraction

RNA was extracted by using 1:5 TRIzol (Invitrogen Carlsbad, CA): Chloroform (Sigma-Aldrich, St. Louis, MO) method and purified by miRNeasy or RNeasy spin column (Qiagen, Chatsworth, CA). On column DNase treatment was performed to remove genomic DNA (Qiagen, Chatsworth, CA). After that, RNA concentration was measured using NanoDrop 1000 spectrophotometer (Thermo-Fisher-Scientific Waltham, US-MA). RNA purity was determined by measuring 260/280 and 260/230 ratios. Samples with approximately 2 (260/280) ratio and between 2.0 – 2.2 for 260/230 ratio were used for further analysis. RNA quality was measured using Agilent 2100 BioAnalyzer (Agilent technologies,

Palo Alto, CA). Samples with RNA integrity (RIN) > 9.5 were used for sequencing.

2.3. Complementary DNA (cDNA) synthesis and quantitative PCR (qPCR)

Biorad iScript kit (Bio-Rad, Hercules, CA) was used for cDNA synthesis. 1 µg of RNA was used as starting material. iScript master mix buffer contains reverse transcriptase, dNTPs, and mixture of oligo(dT) and random hexamer primers. The reaction setup was done according to manufacturers recommendations. Primer 3 software (http://bioinfo.ut.ee/primer3-0.4.0/primer3/) was used to design the forward and reverse primers for qPCR. The designed primers usually flank the exon-exon junction and were ordered either from IDT (Coralvile, NA) or Invitrogen and the amplification product did not exceed 150 bp. For mRNA expression level analysis, qPCR iTaq universal SYBR green supermix (Bio-Rad) was used. The qPCR reaction was performed either on 7900HT Fast Real-Time PCR System (Applied Biosystems) or CFX96™ Touch System (Bio-Rad). 18S, GAPDH or ARHGDIA were used as reference genes for mRNA expression level measurements. Melting curves were detected and analyzed for all measured genes. ΔΔCt-method was used to measure relative expression and fold change ($2^{-\Delta\Delta Ct}$) was calculated. Unpaired two-tailed t-test was used for statistical testing, and results were considered significant if the p value < 0.05.

2.4. Library preparation, RNA sequencing, and data analysis

RNA-seq was performed in two biological replicates at Sweden's National Genomics Infrastructure (NGI). The libraries were prepared from polyA-tailed RNA, and sequenced by using Illumina HiSeq rapid mode approach. At least 15 million single-reads (SR) with 50bp in length were generated for each sample. Reads were mapped against human genome (GRCh37) using Tophat/2.0.4. Reads with multiple alignments were removed using picard-tools/1.29, htseq/0.6.1 was used to count reads for each transcript and cufflinks/2.1.1 was used to normalize the reads count to the length of each transcript. The total number of reads for each sample (Fragments Per Kilobase per Million, FPKM) were calculated. Limma-Voom [93] or DEseq2 [94] methods were used to calculate the differential gene expression (DGE) and corresponding fold changes, p-values, and adjusted p-values; (False discovery rate (FDR). Genes were denoted significantly differentially expressed when FDR < 0.05, $\log_2$FC ≥ |0.4|, and FPKM (treated) > 1. Enriched sub-networks and gene ontologies/biological functions were identified using Pathway Studio's Expression regulatory sub-network enrichment, Elsevier's Pathway Studio (11.2.5.9) (https://www.elsevier.com/solutions/pathway-studio-biological-research) or DAVID bioinformatics website [95].

2.5. Immunoblotting

Protein crude extract was prepared using RIPA buffer (Invitrogen) supplemented with proteinase inhibitors (Sigma-Aldrich). Protein concentration was measured by using Pierce 660 protein assay kit (Thermo-Fisher-Scientific). Approximately 30 – 60 µg of protein were boiled and reduced with β mercaptoethanol and then loaded onto 10% polyacrylamide gel. Sodium dodecyl sulfate (SDS) electrophoresis was performed and proteins were transferred onto polyvinylidene difluoride (PVDF) membrane using Trans-Blot Turbo Transfer System (Bio-Rad). The membrane was blocked with 5% non-fat milk (Bio-Rad) in Tris-buffered saline 0.1%-Tween-20 (TBST) for 1 hour at room temperature (RT). The membrane was incubated overnight at 4°C with the primary antibody in 1% milk TBST. The primary antibody was detected with species specific secondary antibody conjugated with horseradish peroxidase for 2 hours at RT. The blot was processed using ECL kit Clarity Western ECL Substrate Kit (Bio-Rad) and the reaction was detected by using ChemiDoc Imaging Systems (Bio-Rad).

2.6. Lentivirus particles and stable cell line generation

Lentivirus particles were generated in HEK293 cells. Fully confluent HEK293 cells were transfected with four plasmids (three packing plasmids were used pLP1, pLP2, and pLP/VSVG) combined with either pLenti_ERβ (isoform 1) or an empty vector (plasmids were gifts from Dr. Anders Ström University of Houston). Plasmids were mixed with FugeneHD (Promega, San Obispo, CA) and Opti-MEM (Life Technologies, Grand Island, NY). HEK293 cells were transfected for 5 hours at 37°C and 5% CO_2 then the media was changed with a regular one. 48 hours after the transfection the virus containing media was filtered, collected in cryotubes and stored at -80°C. Stable SW620_pLentiERβ (SW620_ERβ) and SW620_pLentiEmpty (SW620_Ctrl) cells were generated via virus transduction. Cells were incubated with virus particles mixed with complete media supplied with polybrene (Sigma-Aldrich). Finally transduced cells were selected with 10 µg/ml of blasticidin (Invitrogen).

2.7. Cell proliferation assay

3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, PES: phenazine ethosulfate) MTS reagent (Promega, Madison, WI,) was used to measure cell proliferation. For each used cell lines, approximately 3000 cells were plated in quintuple fashions in 96-well plates. Cell proliferation was measured by adding 20 µl of tetrazolium (MTS) reagent to 100 µl of media followed by incubation for 2 hours at 37°C, 5% CO2. The absorbance was

measured at 490 nm using Multiskan GO Microplate spectrophotometer (Thermo-Fisher-Scientific). Significance was determined by using unpaired two-tailed t-test and results were considered significant if the p value < 0.05.

2.8. Cell preparation, labeling, and microinjection in zebrafish larvae

Cell were plated in 6-well plate at least 24 hours before harvesting. Cell viability and cell count were determined by using trypan blue staining followed by counting using countess devise (Invitrogen). CellTracker **CM-DiI** fluorescent dye (Thermo-Fisher-Scientific) was used for cell labeling. Approximately $3x10^6$ cells were incubated with 2µM CM-DiI for 5 min at 37°C then for 15 min at 4°C. To discard the unbound materials, cells were washed twice with RPMI. Finally, the labeled cells were prepared in 30 µl of RMPI and kept on ice until injection. Approximately 5nL corresponding to 500 cells were injected in the yolk of zebrafish (*Danio rerio*) larvae at 48 hours post-fertilization (hpf) followed by incubation at 32°C. We used transgenic zebrafish larvae with green fluorescent protein (GFP)-tagged vascular system (Tg(kdrl:EGFP) mitfab^{692}). All of the injected larvae were examined under fluorescent microscope 2-3 hours after the injection. Poorly injected embryos, i.e., direct injection into blood stream were excluded from the study. Tumor cell dissemination in the fish body (mainly in the tail) was detected using fluorescence microscopy at 24 and 48 hours post

injection. Fisher's exact test was used for statistical testing. Results were considered significant if the p value < 0.05

3. Chapter 1: Estrogen receptor beta reduces colon cancer metastasis through a novel miR-205 - PROX1 mechanism[1]

3.1. Introduction

Prospero homeobox 1 (PROX1) is a transcription factor which is implicated in various type of cancers including pancreatic, brain and colon tumors [96]. PROX1 expression may be crucial during the progression of colorectal cancer by promoting adenoma to carcinoma transition [97] as well as tumor metastasis [98, 99]. Re-expressing ERβ in colon cancer cell lines reduces PROX1 expression levels [37] and simultaneously induces the expression of miR205 [41]. PROX1-3'UTR is a putative miR-205 target sequence. Using TargetScan (http://www.targetscan.org/vert_71/) miR-205 contains four possible binding sites on PROX1 3'UTR. In current study my colleague (Trang Vu) demonstrated the miR-205 – PROX1 interactions experimentally. Furthermore, we have shown that ectopic expression of miR-205 reduces PROX1 RNA and protein levels. Here we provide a novel mechanism of ERβ antitumor effects through miR-205 – PROX1 axis.

[1]This chapter has been published in Oncotarget. 2016 Jul 5;7(27):42159-42171 entitled with **Estrogen receptor beta reduces colon cancer metastasis through a novel miR-205 - PROX1 mechanism.** Nguyen-Vu T, Wang J, **Mesmar F**, Mukhopadhyay S, Saxena A, McCollum CW, Gustafsson JÅ, Bondesson M and Williams C.

3.2. Supplemental material and methods

3.2.1. Transfection of human miR-205 mimic

To study the effect of miR-205 on cell metastasis we transfected HT29 and SW480 cell lines with miRNA mimic. miR-205 mimic and scramble controls were ordered from Dharmacon (GE Healthcare, Lafayette, CO). Cells were plated in 6-well plate until they reached 90% confluency. Cells were transfected either with 50nM of miR-205 mimic or scramble control for 24 hours. DharmaFECT (0.1%) in antibiotic free media was used as transfection reagent. Media was changed to complete media 24 hours after the transfection. Then the cells were prepared for subsequent experiment.

3.3. Results and Discussion

3.3.1. ERβ and PROX1 are negatively correlated in colon epithelium

We collected the RNA expression data for colorectal adenocarcinoma TCGA-COADREAD cohort from The Cancer Genome Atlas (TCGA). The cohort contains 382 tumor and 51 normal samples. The data show a significant decrease in a (*ESR2*) ERβ expression in tumor when compared with normal tissues (**Fig.3.1A**) accompanied with a significant increase in *PROX1* expression (**Fig.3.1B**). ERβ and PROX1 expressions show a negative correlation (p value < 0.005) in both tumor and normal tissues (r = – 0.24) (**Fig.3.1C**). We validated the clinical data through the use of colon cancer cell line. PROX1 RNA and protein levels are significantly decreased when we re-expressed ERβ through plasmid [37]. The mechanism is presumably indirect through upregulation of miR-205 in ERβ expressing cells [41], then subsequently allowing the miR-205 to regulate *PROX1* expression. Several studies showed that miR-205 may paly an anti-tumorigenic role in various type of tumors including colorectal [100]. In agreement with others our studies illustrate that miR-205 expression is significantly decreased during tumor progression [current study]. Collectively, during the development of CRC the ERβ and miR-205 expression are significantly decreased, while PROX1 expression is increased and promoting CRC progression.

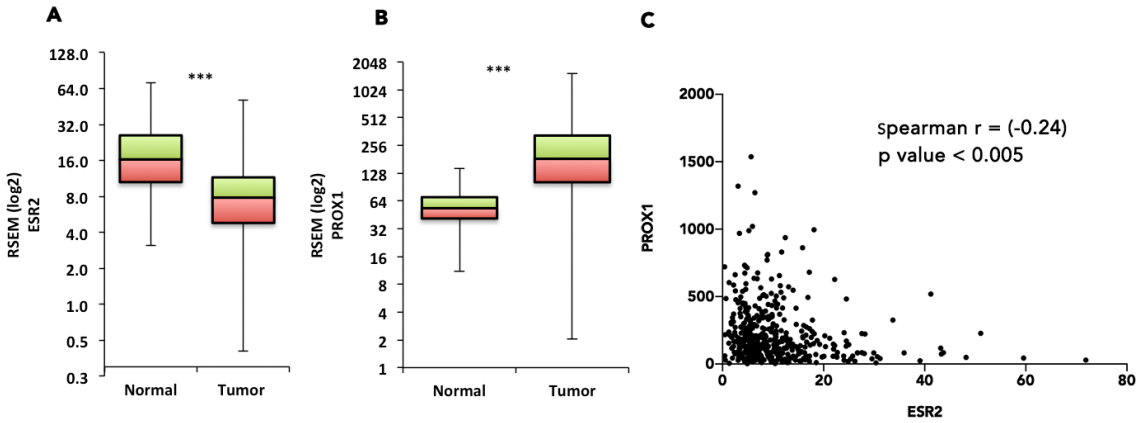

Figure 3.1. Expression of *ESR2* and *PROX1* in clinical samples. (A) The expression of ERβ (*ESR2*) is significantly decreased in colorectal cancer (p value < 0.005). **(B)** *PROX1* expression is significantly increased in colorectal cancer (p value < 0.005). In figures (A and B) y axis represents $\log_2$ of RNA-Seq by Expectation Maximization (RSEM) values and x axis represents 51 normal and 382 tumor samples. Parametric unpaired t-test with Welch's correction was used to calculate p values. **(C)** Spearman coefficient shows a negative correlation between *ESR2* and *PROX1* (r = –0.24) with p value < 0.005 in colorectal tissue. The data were collected from The Cancer Genome Atlas (TCGA).

3.3.2. ERβ and miR-205 reduces cell metastasis *in vivo*

We previously demonstrated an increase in miR-205 expression levels in the presence of ERβ expression [41]. Ectopic expression of miR-205 in breast cancer cell lines (MCF7 and MDA-MB-231) suppresses cell growth and invasion [101], probably by reversing EMT [100]. Likewise, the miR205 expression is significantly reduced during colon cancer metastasis [102]. We used zebrafish larvae to study tumor metastasis. We compared two colon cancer cell lines SW480_ERβ and HT29_ERβ with their corresponding controls. We did the experiment in triplicates. ERβ expressing cells SW480 and HT29 show a significant reduction in tumor invasion and metastasis (**Table 3.1**) (**Fig.3.2**).

Table 3.1					
Cell line	% metastatic cells	p value	Cell line	% metastatic cells	p value
SW480			**HT29**		
ERβ	20.8	0.02	**ERβ**	11.0	0.008
Ctrl	36.2		**Ctrl**	25.9	
Fisher's exact test used to calculate p values					

To assess the effect of miR-205 on cell invasion and metastasis we conducted *in vivo* cell metastasis experiment. We compared the micro-metastasis in zebrafish tail of SW480 and HT29 cells transfected with miR-205 mimic or scramble control. Ectopic expression of miR-205 suppresses tumor metastasis in both cell lines (**Table 3.2**) (**Fig.3.2**).

Table 3.2					
Cell line	% metastatic cells	p value	Cell line	% metastatic cells	p value
SW480			**HT29**		
miR-205 mimic	9.1	0.006	**miR-205 mimic**	5.8	0.001
Ctrl	21.0		**Ctrl**	17.8	
Fisher's exact test used to calculate p values					

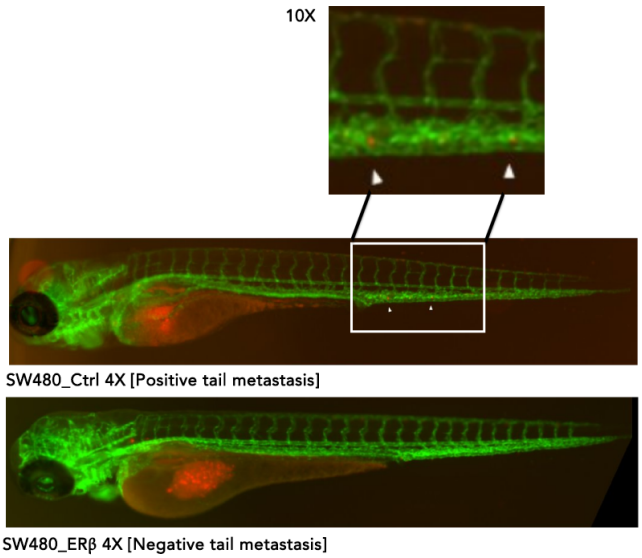

Figure 3.2. Zebrafish micro-metastasis assay. miR-205 and ERβ inhibit tumor metastasis of SW480 and HT29 cell lines (we compared SW480_ERβ with SW480_Ctrl)

3.4. Conclusion

Although there is a plethora of evidence of the protective functions of estrogen and ERβ against colon cancer the molecular mechanisms are not well understood. Estrogens are involved in complex cellular regulatory networks basically through modulation of gene expressions. In response to estrogen, ERβ modulates the expression of various classes of coding and noncoding genes. We provide an example of such complex network in colon epithelium. We demonstrate that ERβ has the ability to reduce tumor metastasis via miR-205 – PROX1 pathway. Further studies are required to characterize ERβ protective functions against the colon cancer as well as its role in advance metastatic disease.

4. Chapter 2: Estrogen receptor beta and G protein-coupled estrogen receptor 1 mediate convergent functions and gene expression in colon cancer cells

4.1. Introduction

Despite the availability of early diagnostic tools like colonoscopy and regular screening for tumor markers such as carcinoembryonic antigen (CEA), colorectal cancer (CRC) still remains the third cause of cancer-related deaths in both men and women [2]. Genetics and environmental factors play a pivotal role in the etiology of CRC. Low physical activity, smoking, poor dietary habits, obesity and inflammatory bowel disease (IBD) contribute to the development of CRC [1].

In addition, genetic alterations in mismatch repair genes, in WNT signaling genes, and many other genes are also risk factors to develop CRC [103]. From a preventive perspective, using of non-steroidal anti-inflammatory drugs (NSAID), estrogens (in women), and phytoestrogens reduce the incidence of CRC [3, 7, 8].

The protective effect of estrogens has also been proven experimentally in various CRC animal models, including inflammation-induced and APC^{Min}-induced colon cancer [13-16]. Multiple data indicate that estrogens exert their protective effects in colon by modulating ERβ activity [26, 27].

Clinically, ERβ expression is decreased during colon cancer progression [28-30], and has been linked to poor CRC prognosis [27]. Furthermore, 90% of CRC tissue

show methylation at the *ESR2* gene [31]. Single nucleotide polymorphisms in *ESR2* promoter is associated with an improved survival among CRC patients [32].

Preclinical studies confirmed the deleterious phenotypes in colonic epithelium in absence of ERβ. Colonic epithelium of ($ER\beta^{-/-}$) mouse model shows morphological alterations as well as increase in proliferation and decrease in apoptosis [34]. ERβ deficiency promotes cancer progression in various colon cancer-induced models [35, 36]. Xenograft and *in vitro* studies confirmed ERβ tumor suppressive functions [33].

ERβ is a nuclear receptors (NRs). NRs are able to orchestrate arrays of cellular functions such as growth, differentiation and metabolism through a complex signaling networks via activating/repressing transcriptional machinery or crosstalk with other signaling pathways [104]. Involvement of ncRNAs such as miRNA and lncRNA in signaling introduces a new layer of complexity in NRs networks and raises new questions on the mechanism of NRs and how they interact with regulate ncRNAs.

In a previous study, we demonstrated an example of ERβ – microRNA regulatory network. We showed that ERβ reduces colon cancer metastasis by downregulating PROX1 through enhancing miR-205 expression. Here, we tested another layer of transcriptional regulation by examining the effect of ERβ on lncRNAs expression in CRC.

lncRNAs are implicated in many cellular processes such as transcription, chromatin remodeling, post-transcriptional processing and trafficking that change cellular phenotypes [105]. Thus, lncRNAs can have a role in health and diseases including cancer. lncRNAs can be implicated in cancer initiation, progression and metastasis [5]. For instance, increase in prostate cancer associated non-coding RNA-1 (PRNCR1) lncRNA expression is associated with CRC initiation [106]. Upregulation in HOTAIR and MALAT1 lncRNAs is implicated in CRC invasion and metastasis [107, 108]. We hypothesized that estrogens and ERβ are able to modulate the expression of critical lncRNAs which are important for CRC development and progression.

4.2. Supplemental material and methods

4.2.1. Quantification of ERβ using radioactive ligand-binding assay

Radioactive competitive binding assay was used to quantify ERs in stably transduced SW620. Approximately $3x10^5$ cells were seeded in 6-well plates and incubated at 37°C with 5% CO_2 for 24 hours. Cells were then treated with 0.1 nM [3H]-E2 (41.3 Ci/mmol specific activity) in absence or presence of 1 μM unlabeled E2 in 10% DCC-fetal bovine serum for 5 hours at 37°C. After incubation, the unbound materials were aspirated and the adherent cells were washed three times with cold PBS. Cells were lysed with a lysis buffer and the lysate were mixed with LSC-cocktail (Emulsifier-Safe; Packard BioScience, Waltham, MA). Radioactivity was measured using liquid scintillation counter (LS-6000-SC; Beckman-Coulter, Brea, CA). Bound radioactivity values were expressed in disintegrations per minute (DPM). The experiment was performed six times for both SW620_ERβ and SW620_Mock. Finally the mean of DPM readings were normalized to the protein concentration and cell number.

4.2.2. Ligands treatments

Cells were cultured for 24 hours prior the treatments. Cells were treated either with 10nM E2 or 1 μM G1 in complete growth media for 24 hours at 37°C with 5% CO_2.

4.2.3.qPCR confirmation

Primer pairs have been used in qPCR

Gene Name	Primer Name	Primer Sequence 5′ – 3′
ESR2/ERβ	hESR2_F	ACTTGCTGAACGCCGTGACC
	hESR2_R	CAGATGTTCCATGCCCTTGTT
MALAT1	hMALAT1_F	GACGGAGGTTGAGATGAAGC
	hMALAT1_R	ATTCGGGGCTCTGTAGTCCT
NEAT1	hNEAT_F	TCGGGTATGCTGTTGTGAAA
	hNEAT_R	TGACGTAACAGAATTAGTTCTTACCA
ZEB1-AS1	hZEB2-AS1_F	GTAGGGACGCGGTCAGAAAG
	hZEB2-AS1_R	GCAAGCGGAACTTCTAGCCT
HOTAIR	hHOTAIR_F	GGCGGATGCAAGTTAATAAAAC
	hHOTAIR_R	TACGCCTGAGTGTTCACGAG
LINC00657/ NORAD	hLINC00657_F	AGCGAAGTCCCGAACGACGA
	hLINC00657_R	TGGGCATTTCCAACGGGCCAA
ZEB1	hZEB1_F	GCTCACACATAAGCAGTAAGA
	hZEB1_R	AAAGAGACGGTGAAGAACAC
SLUG/SNAI2	hSLUG_F	TTTCTGGGCTGGCCAAACATAAGC
	hSLUG_R	CCGCAGATCTTGCAAACACAAGGT
CDH1	hCDH1_F	GGATGTGCTGGATGTGAAT
	hCDH1_R	GGCAGTGTAGGATGTGATTT
VIM	hVIM_F	CCAGCTAACCAACGACAAA
	hVIM_R	TCCTCTCTCTGAAGCATCTC
18S_rRNA	18S_rRNA_F	GCTTAATTTGACTCAACACGG
	18S_rRNA_R	AGCTATCAATCTGTCAATCCT
ARHGDIA	hARHGDIA_F	CAGGAAAGGCGTCAAGATTG
	hARHGDIA_R	GTCAGGAACTCGTACTCCTC

4.2.4.Immunoblotting

ERβ (GeneTex, San Antonio, TX), Slug, Snail, ZEB1 (cell signaling), E-cadherin and vimentin (Santa cruz) were detected by incubating the membrane overnight at 4°C with 1:1000 of primary antibody. GAPDH was used as loading control; 1:5000 of primary antibody was used (Thermo Fisher Scientific).

4.3. Results and Discussion

4.3.1. ERβ is re-expressed and functional in SW620 cell line

SW620 cells were either transduced with ERβ lentivirus (SW620_ERβ) or empty vector (SW620_Ctrl). Receptor quantification was performed by using radioactive competitive ligand-binding assay. ERβ-expressing cells show significant binding of labeled E2 when compared with E2 saturated one. Control cells do not show signal higher than the background (**Fig. 4.1A**).

The calculated number of ERβ receptors are approximately 3,211±216 receptors per cell (5.4 fmol/10^6cell). Under regular culture conditions MCF7 a human breast cancer cell line expresses approximately 59 fmol/10^6cell of ERα [109]. The number of ERβ in SW620 is 10 times less than ERα in MCF7, we conclude that ERβ expression level falls within physiological ranges. ERβ expression was confirmed by using qPCR (**Fig. 4.1B**) and western blot (**Fig. 4.1C**). Cell morphology analysis shows that ERβ expressing SW620 cells exhibit more round-shaped epithelial-like morphology when compared with the control cells which are more spindle-shaped fibroblast-like morphology (**Fig. 4.1D**). This morphology may be related to known ERβ functions of inhibiting EMT and maintaining epithelial differentiation [110, 111].

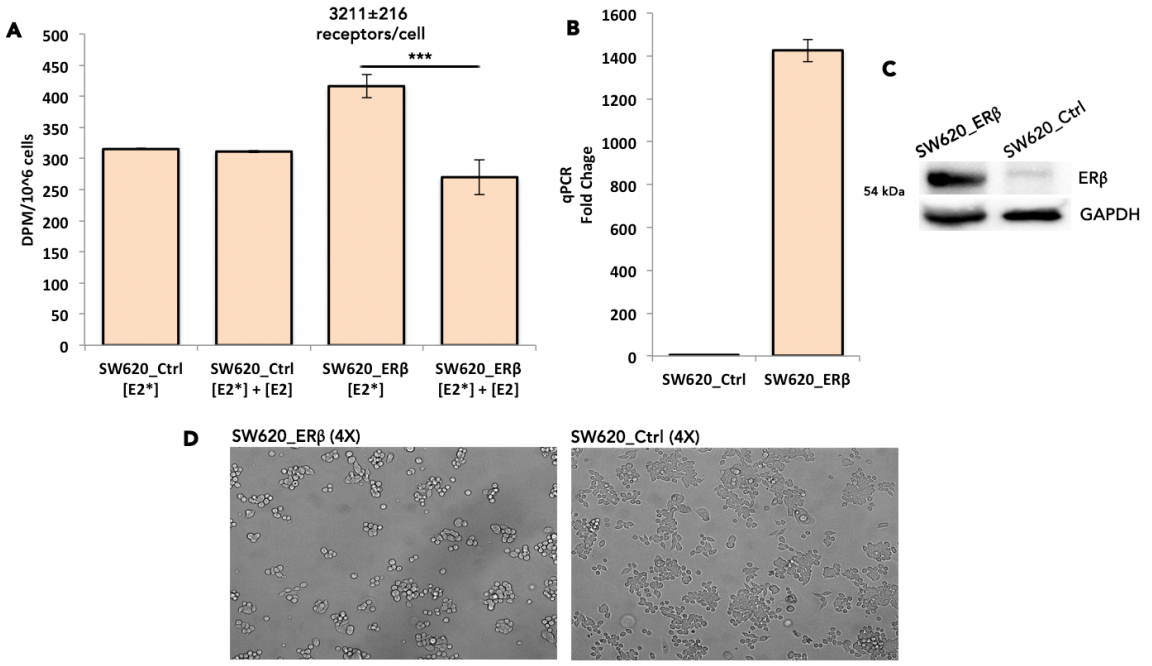

Figure 4.1. Re-expressing ERβ in SW620 using lentivirus. (A) ERβ quantification using radioactive competitive ligand-binding assay. Approximately 3,211 receptors are expressed per cell in SW620_ERβ cell line. Y-axis represents disintegration per minute (DPM) per million cells. Samples include control (empty plasmid) and ERβ transduced treated with radiolabel E2* alone or combined with unlabeled E2 in excess. Error bars represent standard error of the mean (SEM) between the trials. Unpaired two-tailed t-test was used to calculate p value (*** $p < 0.005$). **(B)** Fold change of ERβ transcript was determined by qPCR. **(C)** Western blot confirms ERβ expression **(D)** ERβ expressing cells recapture round-shaped phenotype whereas control cells have more spindle-shaped morphology.

4.3.2. ERβ reduces SW620 proliferation and metastasis

To study ERβ effects on cell growth we used MTS cell proliferation assay. ERβ-expressing SW620 cells show a significant decrease in cell proliferation ($p < 0.005$) even in absence of ligand (**Fig. 4.2A**). This confirms previous reports which showed that ERβ expression reduces cell proliferation independently of E2 in colon [33], and breast [112] cancer cells. ERβ expression reduces cell proliferation probably by suppressing the expression of considerable oncogenes

such as MYC and EGFR [33, 41]. Further, we have previously demonstrated that ERβ is able to reduce cell migration and invasion of various colon cancer cell lines such as SW480 and HT29 [41, 42]. These cell lines are characterized by a lower metastatic capabilities compared to SW620 [113].

To study the metastasis of SW620 ± ERβ we injected the labeled tumor cells into the yolk sac of zebrafish larvae. Tumor metastasis into the circulatory system was observed 24 and 48 hours after the injection. The advantages of this assay, include the ability to study the tumor metastasis *in vivo* in relatively short period of time. Moreover, using a transparent zebrafish line allows for live imaging.

A key feature of the larval stage embryos is that they have not yet developed an adaptive immune system, which enables xenograft studies [114-116]. Using this assay, ERβ-expressing SW620 exhibits a significant reduction in tumor metastasis ($p < 0.05$) (**Fig. 4.2B & C**). Although we demonstrated before that ERβ can inhibit tumor metastasis via miR-205-PROX1 pathway, further studies are required to comprehensively understand the underlying mechanisms of ERβ anti-metastasis functions in the colon cancer.

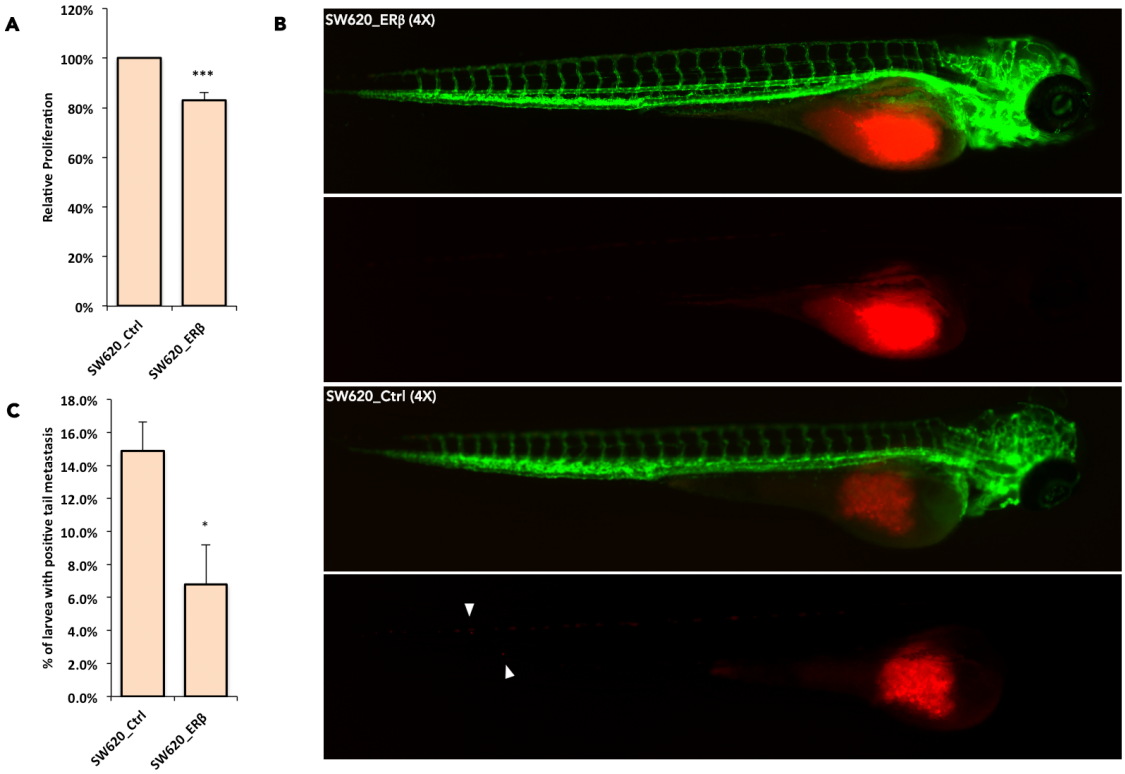

Figure 4.2. ERβ reduces cell proliferation and metastasis. (A) MTS assay shows a significant reduction in cell proliferation among ERβ expressing SW620 cells. Y-axis represents relative proliferation and error bars represent the standard deviation (SD) of two independent trials. Unpaired two-tailed t-test was used to calculate p value (*** $p < 0.005$). **(B)** Zebrafish metastasis assay shows a signifcant reduction in metastasis when ERβ is expressed. **(C)** Percentage number of larval embryos that developed tail metastasis are indicated. Error bars represent standard error of the mean (SEM) of three independent trials. Fisher's exact test was used to calculate the significance difference between two groups (* $p < 0.05$).

4.3.3. Gene expression profiling shows downregulation of cell adhesion genes in SW620.

We have completed the gene expression profiling for two isogenic colon cancer cell lines: the primary cell line SW480 and the lymph node metastatic SW620 cell line. SW480 cells have an round-shaped morphology while SW620 is characterized by spindle-shaped morphology.

RNA-seq differential expression analysis shows that the majority of selected genes with applied cutoffs are highly expressed in SW480 (**Fig. 4.3A**), indicating downregulation during metastasis transformation. 1656 genes are upregulated in SW480 cells (**Fig. 4.3B**) while 692 genes are upregulated in SW620 cells (**Fig. 4.3C**).

Gene Ontology analysis of SW620 downregulated genes shows an enrichment in cell adhesion process (**Fig. 4.3D**). Loss of cell adhesion molecules is consistent with progression and transformation to the metastatic stage. Balance interactions between epithelial cells and extracellular matrix is important to maintain cell proliferation, differentiation, survival and apoptosis [117].

SW620 cell line shows downregulation of genes which are important to communicate with surrounding environment and organization of extracellular matrix. Other group used quantitative proteomics profiling to compare SW480 with SW620. Downregulation in cytoskeleton-related proteins and cellular adhesion molecules in SW620 cell line has been observed [118]. Defects in cells–interactions with the extracellular matrix are crucial to initiate the metastasis [117]. Consequently, It has been shown that well-differentiated colon tumors express higher level of adhesion molecules such as intracellular adhesion molecules (ICAMs) when compared with undifferentiated tumors [119]. Likewise, primary colon cancers express higher levels of adhesion molecules than

metastatic lesions [120]. We concluded that SW620 cell line is a suitable *in vitro* model to study tumor metastasis.

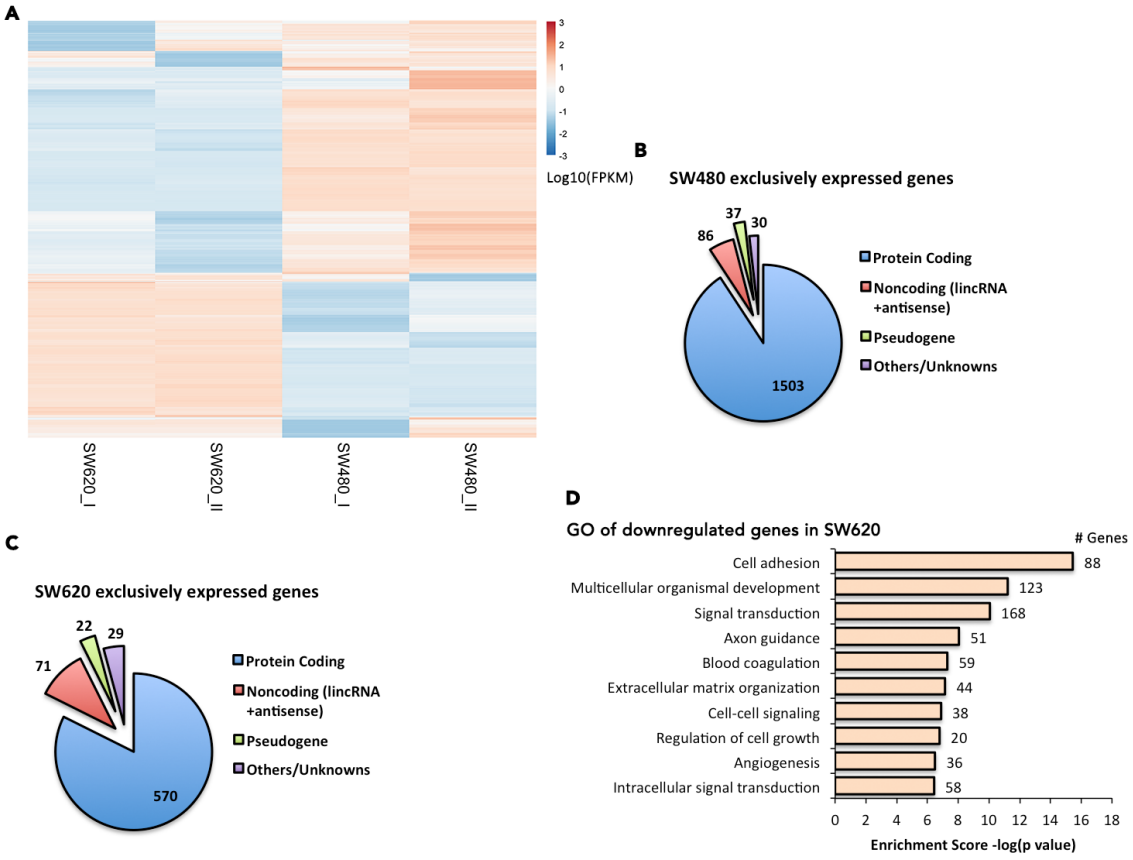

Figure 4.3. Gene expression profiling of SW480 and SW620 cell lines. (A) Heatmap is representing $\log_{10}$FPKM values for the top 1,200 differentially expressed genes (FDR < 0.05, $\log_2$FC ≥ 3 and $\log_2$FC ≤ -2). **(B)** Pie chart shows transcript classes of the 1,656 genes that are upregulated in SW480 cell line. **(C)** Pie chart shows gene classes of the 692 genes that are upregulated in SW620 cell line. **(D)** Gene Ontology (GO) pathway analysis of protein coding genes. Top ten GO terms of biological processes for downregulated genes in SW620. X-axis represents enrichment score ($-\log_{10}$(p value)). Pathway studio software was used to produce gene ontologies and p values. Numbers on the side of each bar represent the genes which are involved in corresponding pathway.

4.3.4.SW480 cell line expresses high levels of known oncogenic lncRNAs

Another set of differentially expressed genes between SW620 and SW480 cells are lncRNAs including lincRNA and antisense RNA (**Fig. 4.3A & B**). 157 lncRNAs genes are differentially expressed between the SW480 and SW620 cell lines (**Fig. 4.4A**). SW480 expresses higher level of several cancer-related lncRNAs. Among these oncogenic lncRNAs are colon cancer associated transcript 1 (CCAT1), nuclear paraspeckle assembly transcript 1 (NEAT1), Pvt1 oncogene (PVT1), and non-coding RNA activated by DNA damage (LINC00657/NORAD), as well as less characterized lncRNAs such as cancer susceptibility 8 (CASC8/LINC00860) (**Fig. 4.4B**).

Upregulation in oncogenic lncRNAs may be important to enforce transition from malignant carcinoma to metastatic lesion. For instance, CCAT2 may play a role during CRC helping the cells to acquire the metastasis phenotype [121]. We have noticed that several of SW480 upregulated lncRNAs are located on chromosome 8q24 such as CCAT1, PVT1 and CASC8. This region also encompasses MYC gene, suggesting a potential common regulatory mechanism. Some studies showed that SW480 cell line has higher MYC RNA and protein levels in comparison with SW620 cell line [122], which suggest higher expression level of 8q24 genes in SW480. Other studies demonstrated regulatory interactions between MYC and CCAT1 [121, 123], and PVT1 in

colorectal cancer [124, 125]. These functional interactions can play pivotal roles during disease progression.

Further studies are required to characterize the mechanisms of regulation of lncRNAs in CRC and how they are important for tumor metastasis. Elucidating the functions of uncharacterized lncRNAs which are differentially expressed in SW480 and SW620 cell lines are also required.

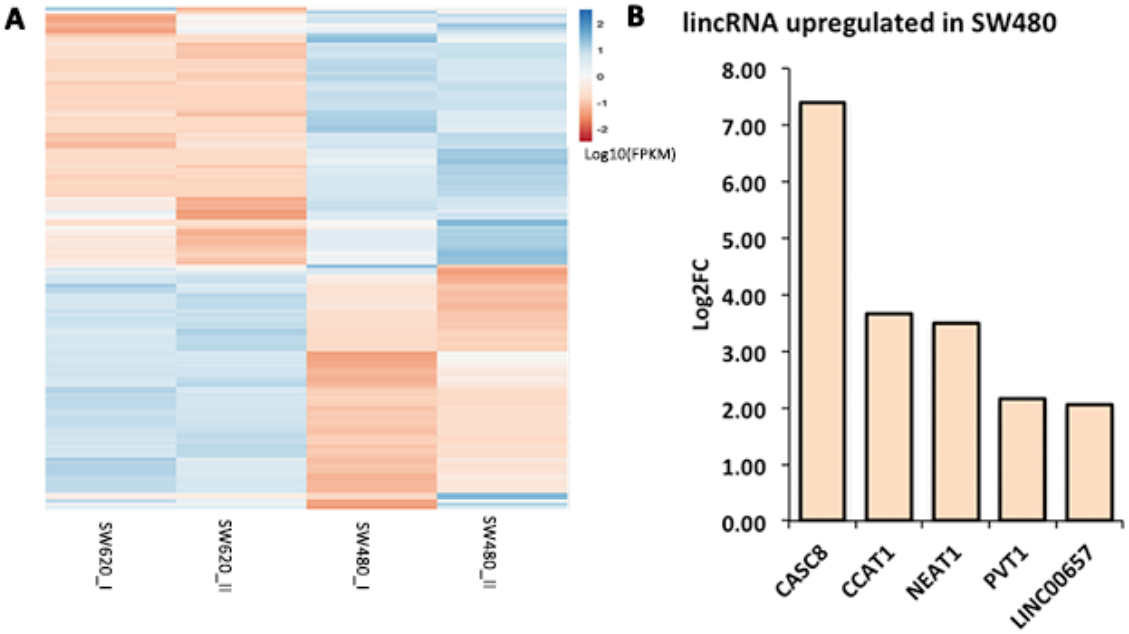

Figure 4.4. Differential expression analysis of lncRNAs in SW480 and SW620 cell lines. (A) Heatmap represents log_{10}FPKM values for lncRNA genes (FDR < 0.05 and log2FC ≥ |2|) that are differentially expressed in SW480 and SW620 **(B)** Examples of SW480 upregulated lncRNAs. Y-axis represents log_2 of fold change (FC).

4.3.5. Gene expression in response to ERβ expression and E2 treatment in SW620 cell line

We compared gene expression profiles of SW620_ERβ cells with SW620_Ctrl cells in absence of ligand treatment. We used DEseq2 software to measure the differential gene expression [94]. ERβ per se has no influence on gene expression in SW620 cells in absence of the ligand (**Fig. 4.5A**). Our previous functional assays have shown a reduction in SW620 proliferation and metastasis when cells express ERβ, suggesting a ligand-independent mechanism.

Next we compared SW620_ERβ E2-treated cells with SW620_ERβ vehicle-treated. SW620_ERβ cells were treated either with 10nM of E2 or with DMSO for 24 hours. E2-treated samples, in duplicates, demonstrate a clear separation in term of global gene expression when compared with DMSO-treated samples (**Fig. 4.5B**). 8,325 genes show differential expression (FDR < 0.05 and $\log_2$FC ≥ |0.4|); of which 4,039 genes are upregulated and 4,249 genes are downregulated in response to E2 in ERβ-expressing cells (**Fig. 4.5C**). Applying a cut-off of FPKM value ≥ 1 (in treated samples), results in 6,863 protein-coding genes and 113 noncoding RNA genes defined as differentially expressed (**Fig. 4.5D**). Gene Ontology of the downregulated genes shows an enrichment in cell cycle, cell division and mitosis biological processes (**Fig. 4.5E**). These results support our functional data of reduced proliferation upon ERβ expression. Our experiment indicates that the transcriptional effect by ERβ is significantly

enhanced in presence of ligand. However, our setup dose not exclude ERβ-independent estradiol-mediated effects, through activating other estrogen receptors such as GPER1.

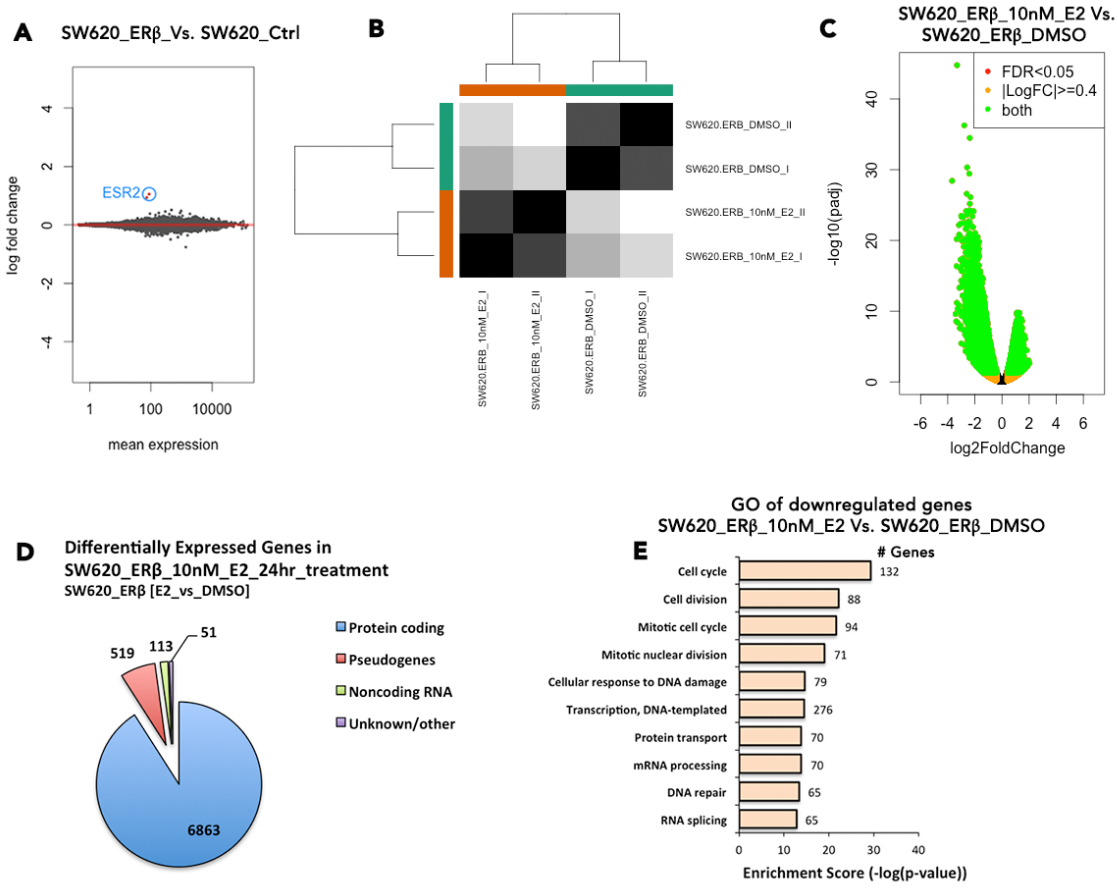

Figure 4.5. Major transcriptional effects in response to E2 in the presence of ERβ in SW620 colon cancer cell line. (A) MA plot compares SW620_ERβ with SW620_Ctrl in absence of E2 treatment. Data show no significant difference between two groups. **(B)** Euclidean distance was used to study the relationship between sample replicates. E2-treated samples are clustered together. **(C)** Volcano plot reports $-\log_{10}$ FDR on the Y-axis and $\log_2$ fold change of differentially expressed genes on the X-axis. Here we compare SW620_ERβ treated with 10nM of E2 for 24 hours with SW620_ERβ treated with DMSO. **(D)** Pie chart shows gene types of 7,546 differentially expressed genes upon E2 treatment. **(E)** Gene Ontology (GO) of downregulated protein-coding genes. X-axis represents the enrichment score ($-\log_{10}$ p value). Pathway studio software was used to produce gene ontologies and p values. Numbers on the side of each bar represent the genes which are involved in corresponding pathway.

4.3.6. E2 treatment reduces the expression of several oncogenic lncRNAs

Focusing on non-coding transcripts, 113 noncoding lncRNAs, including both lincRNAs and antisense RNA genes, demonstrate differential expression in SW620_ERβ cells in response to 24-hour of 10nM E2 treatment compared to vehicle treatment (**Fig. 4.5D**). Among them are several oncogenic lncRNAs such as MALAT1, NEAT1, HOTAIR and ZEB1-AS1 are significantly reduced (**Fig. 4.6A & B**).

MALAT1 is a single exon 8.7 Kb RNA which mainly localized at nuclear speckles [126]. Upregulated MALAT1 is implicated in different kind of cancers such as colon [127], and pancreas [128]. High level of MALAT1 expression predicts poor prognosis in CRC [127]. In addition, MALAT1 has also been reported to play an important role in CRC proliferation, invasion and metastasis [108, 129].

Our experiment demonstrate a 0.15 (FC) reduction in MALAT1 expression after E2 treatment, which is consistent with what has been observed in breast cancer. Zhao Z *et al* observed a decrease in MALAT1 expression in breast cancer cell lines (MCF7 and MDA-MB-231) in response to E2 in a dose-dependent manner [130].

Another significantly downregulated lncRNA in our experiment is HOTAIR. Upregulation in HOTAIR gene is associated with poor CRC prognosis, low survival [131, 132], and tumor metastasis through EMT pathway [133]. Likewise,

ZEB1-AS1 has been reported to promote the metastasis of osteosarcoma by interfering with miR-200 tumor suppressive functions [134]. High level of NEAT1 expression predicts poor prognosis and metastasis in both colon [135] and endometrial cancers [136]. Our data illustrate that estrogen treatment of the metastasized colon cancer cell line SW620 represses the expression of several oncogenic lncRNAs, including important EMT inducers. We propose that downregulation of these lncRNAs can be a potential novel mechanism of cancer prevention and inhibition of tumor metastasis by estrogen and ERβ.

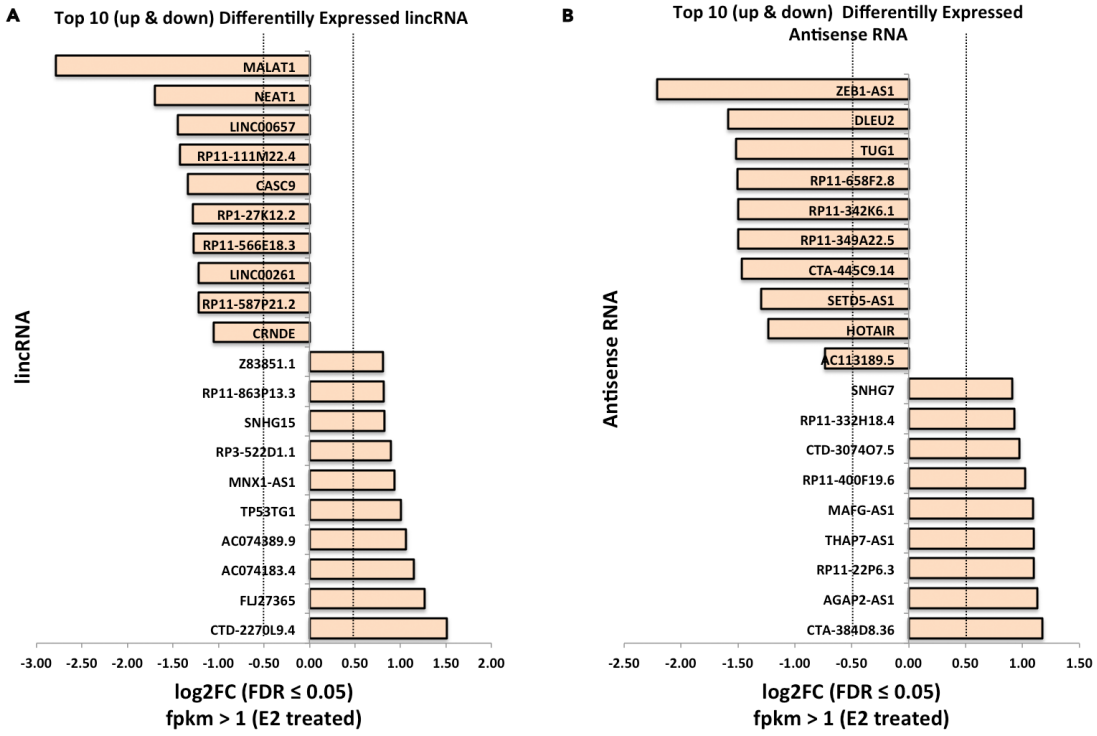

Figure 4.6. Expression profile of lncRNAs in SW620_ERβ cells after 24 hours of 10nM E2 treatment. (A) Differential expression of lincRNAs in SW620_ERβ ± E2. Several cancer-related lincRNAs are downregulated upon E2 treatments such as MALAT1, NEAT1 and LINC00657. **(B)** Differential expression of antisense lncRNAs in SW620_ERβ ± E2. Oncogenic antisense lncRNAs are downregulated upon E2 treatment, such as ZEB-AS1 and HOTAIR. X-axis represents $\log_2$ fold changes in both A and B.

4.3.7.qPCR verifies RNA-seq data indicating ERβ ligand-independent and ligand-dependent effects.

We used qPCR to validate RNA-seq data. Cancer-related lncRNA genes and EMT markers were selected for the analysis. We studied gene expressions in SW620_ERβ ± E2 and SW620_Ctrl ± E2. We are able to confirm all regulators indicated by RNA-seq. Interestingly, most of the selected genes have shown downregulation upon E2 treatment even in absence of ERβ (**Fig. 4.7**) proposing an E2-ERβ-independent effects.

Supporting a potential non-ERβ mediated mechanism, another study has shown that treating human colon cancer cell line (LoVo, which dose not express ERα or ERβ) with E2 reduces cell proliferation and migration by reducing the cell cycle-regulating proteins [40]. We propose such changes may be mediated by a third estrogen receptor: GPER1.

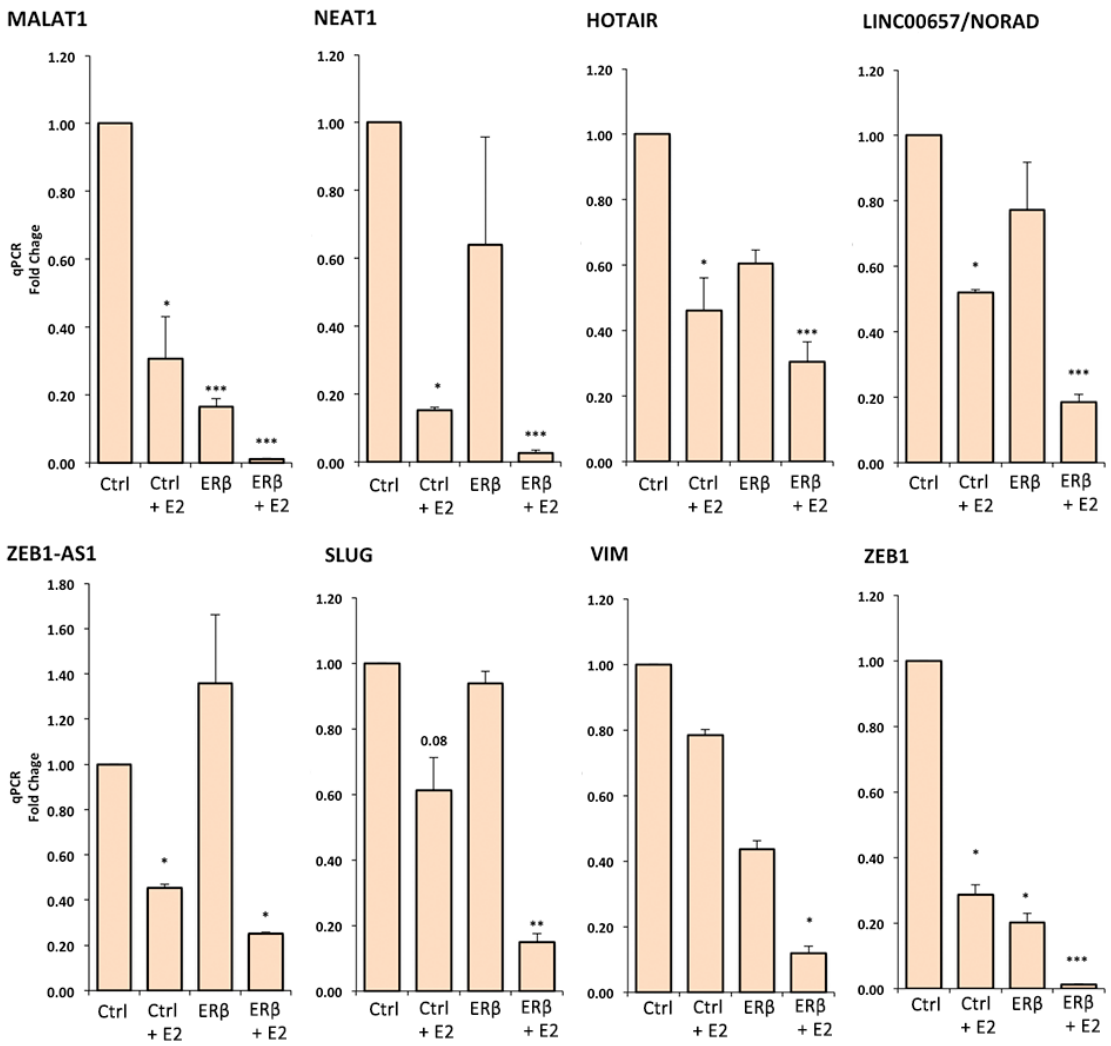

Figure 4.7. qPCR confirms RNA-seq results of the selected genes in SW620 cell line. Y-axis represents fold change ($2^{-\Delta\Delta Ct}$) for each tested gene. X-axis indicates samples. Control (Ctrl) and ERβ-expressing samples were treated with DMSO or with 10nM of E2 for 24 hours. Data represent three independent biological (culture) replicates. Unpaired two-tailed t-test was used to calculate p values (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$). Error bars represent SEM of the fold change values.

4.3.8. GPER1 has a potential role on gene regulation in CRC

Quantitative-PCR data proposed the involvement of other estrogen receptors in SW620 cell line in response to E2 treatment. We elected GPER1 as potential receptor.

GPER1 has high affinity for E2 [80], and the activation of GPER1 leads to rapid non-genomic signaling as well as transcriptional regulation [20]. The physiological and pathological role of GPER1 in the colon epithelium has not been explored. TCGA-COADREAD cohort shows that *GPER1* is expressed in both normal and tumor tissues, and its expression is significantly decreased in cancerous tissue (**Fig.4.8A**). *GPER1* expression is confirmed in two colon cancer cell lines, SW480 and SW620, at both RNA (**Fig.4.8B**) and protein levels (**Fig.4.8C**).

To test whether GPER1 has a potential role in transcriptional regulation in colon cancer cell line we treated SW620 with 1 μM of G1 a GPER1 agonist for 24 hours. We selected this dose to activate GPER1 receptor since this concentration was previously used by other investigators [137, 138]. We further focused on genes that are differentially expressed in response to E2 treatment in SW620 cells in absence of ERβ (**Fig 4.7**). lncRNAs MALAT1, ZEB1-AS1 and LINC00657 are significantly downregulated upon G1 treatment (**Fig.4.8D**). Since MALAT1 and ZEB1-AS1 are contributed to EMT, we tested some EMT markers after G1 treatment. *SLUG*, *ZEB1*, and *VIM* demonstrate significant

downregulation while E-cadherin transcript (*CDH1*) shows upregulation (**Fig.4.8D**).

Despite the role of GPER1 in tumorigenesis is not yet well established. Several reports have shown that the activation of GPER1 by its selective G1 agonist exerts anti-tumorigenic effects. *In vitro* and *in vivo* studies have demonstrated that G1 treatment has anti-proliferative function in various types of cancers, such as castration-resistance prostate cancer [139], both ER-negative and ER-positive breast cancers [138, 140], as well as in ovarian cancers [141].

G1 – GPER1 anti-proliferative effects are generally mediated by induction of Ca^{+2} mobilization [140], which by itself is able to activate the apoptotic process [142]. In addition, G1 is also able to upregulate p21, a p53-regulated cyclin kinase inhibitor, in c-Jun/c-Fos–dependent fashion [143, 144]. Recent reports demonstrated that G1-treated triple-negative breast cancers exhibit less proliferation, motility, invasion and angiogenesis [137, 145]. The authors observed that G1 anti-tumorigenic effects are mediated by inhibiting NFκB signaling and subsequent downstream targets and pathways such as IL6 and EMT [137, 145]. Inhibition of cell migration via G1–GPER1–NFκB pathway has also been established in non-small cell lung cancer [146]. Here we demonstrate that activation of GPER1 by G1 downregulates several EMT-related genes in colon cancer cells.

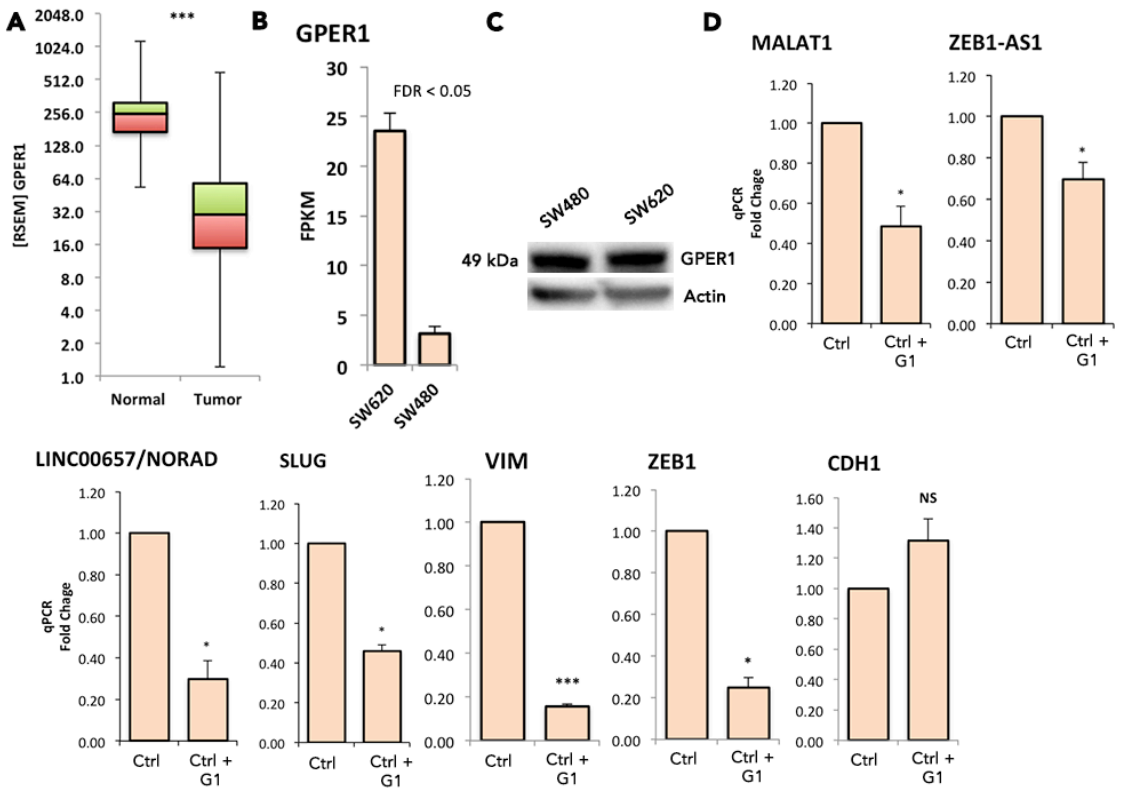

Figure 4.8. Potential role of GPER1 on gene regulation in CRC. (A) *GPER1* expression is significantly decreased in CRC tissues (p value < 0.005). Y-axis represents $\log_2$ of RNA-Seq by Expectation Maximization (RSEM) values and X-axis represents 51 normal and 382 tumor samples. Parametric unpaired t-test with Welch's correction was used to calculate p value. Data were collected from The Cancer Genome Atlas (TCGA). **(B)** *GPER1* expression in SW620 and SW480 cell lines is represented by FPKM value. *GPER1* expression is significantly higher in SW620 (FDR < 0.05) **(C)** Western blot shows GPER1 protein in SW620 and SW480 cell lines. **(D)** Gene expression analysis of MALAT1, ZEB-AS1, LINC00657/NORAD, *SLUG*, *VIM* and *ZEB1* upon treatment with 1μM G1 ligand a GPER1 agonist for 24 hours. *CDH1* shows insignificant upregulation. Data represent two independent biological replicates. Unpaired two-tailed t-test was used to calculate p values (* p<0.05, ** p<0.005, *** p<0.0005). Error bars represent ±SEM of fold change values.

4.3.9. Comprehensive analysis of E2 and G1 transcriptional activities in SW620 cell line

For further understanding of the transcriptional activity of E2 in colonic epithelium without ERβ, we treated SW620 cell line with 10nM of E2 for 24 hours, and conducted RNA sequencing. 1,700 genes are identified as transcriptionally regulated in response to E2 in absence of ERβ (**Fig.4.9A**). These include MALAT1 and NEAT1, there expressions are reduced by 0.37 and 0.34 FC respectively. The majority of these genes are also differentially expressed in E2-treated ERβ-expressing SW620 cells (**Fig.4.9B**). We can conclude that considerable number of genes are regulated in absence of ERβ, possibly by other estrogen receptor(s).

Gene Ontology analysis of downregulated genes due to E2 treatment reveals set of genes that are involved in transcriptional regulation, cilium assembly and microtubules anchoring (**Fig. 4.9C**). Primary cilia are sensory specialized microtubules that are present in most human cell types. Signaling in cilia is largely mediated by G-protein-coupled receptors (GPCR) [147]. Targeting microtubule dynamics is an efficient way to reduce cancer growth and metastasis [148]. As a GPCR, GPER1 is expressed in the colon [149] and a potential estrogen receptor in SW620 cell line. To study GPER1 genome-wide effect we treated SW620 cell line with 1μM of G1 for 24 hours and performed RNA sequencing. 319 genes are differentially expressed in response to G1 treatment

(**Fig.4.9D**). Of these, 168 genes are also regulated by E2 treatment in absence of ERβ (**Fig.4.9E**). Our interpretation of these data is that, GPER1 indeed regulates a high number of genes in response to E2 or G1 in SW620 cell line. Gene Ontology analysis of E2/G1 commonly regulated genes (n=168) reveals an enrichment in cilium assembly, indicating that active GPER1 interferes with microtubules assembly (**Fig.4.9E and F**). By analyzing enrichment among the 319 G1-GPER1 regulated genes, we find an enrichment in several biological processes that are related to known GPER1 physiological functions such as calcium, cholesterol and lipid metabolisms (**Fig.4.9E and F**) [76]. In addition, GPER1 regulates the expression of several EMT genes. Many EMT drivers are downregulated, such as MALAT1 (**Fig.4.9G**) accompanied with downregulation in its downstream targets (**Fig.4.9H**). Moreover, G1-treated cells demonstrate a reduction in the expression of several mesenchymal markers, including Vimentin and Fibronectin, and upregulation of the epithelial marker such as E-cadherin (**Fig.4.9G**). ZEB1, E-cadherin, and Vimentin exhibit changes at the protein level after 48 hours of 1 μM G1 treatments (**Fig.4.9I**). Although Slug and Snail show changes at the RNA level (by qPCR and RNA-seq), we do not observe corresponding changes at the protein level.

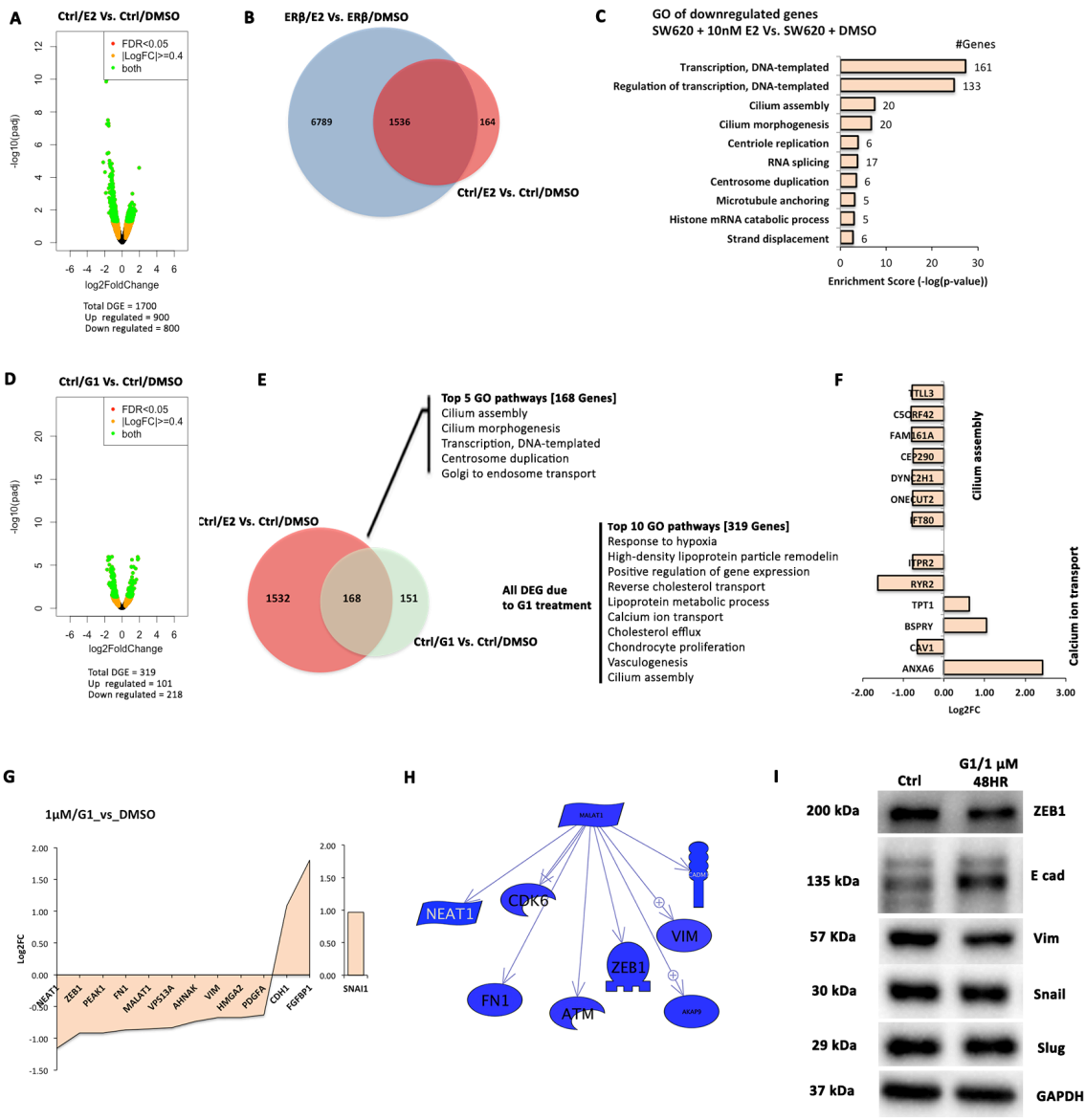

Figure 4.9. Comprehensive analysis of E2 and G1 transcriptional activities in the SW620 cell line. (A) Volcano plot represents differentially expressed genes in SW620 cells treated with 10nM of E2 for 24 hours. Y-axis demonstrates $-\log_{10}$FDR, X-axis demonstrates $\log_2$ fold change of differentially expressed genes**. (B)** Venn diagram indicates differentially expressed genes in SW620_Ctr ± E2 overlapped with SW620_ERβ ± E2. Selected cutoff values are FDR < 0.05, log2FC ≥ |0.4|, FPKM (treated) > 1. **(C)** Gene Ontology (GO) of downregulated protein-coding genes. X-axis represents the enrichment score ($-\log_{10}$ p value). Pathway studio software was used to produce gene ontologies and p values. Numbers on the side of each bar represent the genes which are involved in corresponding pathway. **(D)** Volcano plot represents differentially expressed genes in SW620 cells treated with 1μM of G1 for 24 hours. Y-

axis demonstrates $-\log_{10}$FDR, X-axis demonstrates $\log_2$ fold change of differentially expressed genes. **(E)** Venn diagram indicates differentially expressed genes in SW620_Ctrl ± E2 overlapped with SW620_ERβ ± G1. Selected cutoff values are FDR < 0.05, log2FC ≥ |0.4|, FPKM (treated) > 1. Gene Ontologies of the overlapped genes and all differentially expressed genes upon G1 treatment are indicated in the figure. p value is < 0.05 for all GO. **(F)** Log_2FC of genes belong to two GPER1-related biological processes i.e. calcium ion transport (p value 0.008) and cilium assembly (p value 0.004). **(G)** Gene expression of EMT-related genes. Y-axis represents $\log_2$FC. **(H)** Subnetwork analysis of G1 regulated genes shows MALAT1 and its targets (p value p-value enrichment 2.35×10^{-6}). **(I)** Immunoblot of several EMT–related proteins.

4.3.10. Metastasis associated lung adenocarcinoma transcript 1 (MALAT1) is significantly elevated in CRC clinical samples and it is a potential target for estrogen signaling

One of the most studied lncRNAs is MALAT1 because of its significant role during cancer development and progression [126]. In CRC, MALAT1 is implicated in various tumorigenic processes including proliferation, invasion and metastasis [108, 129].

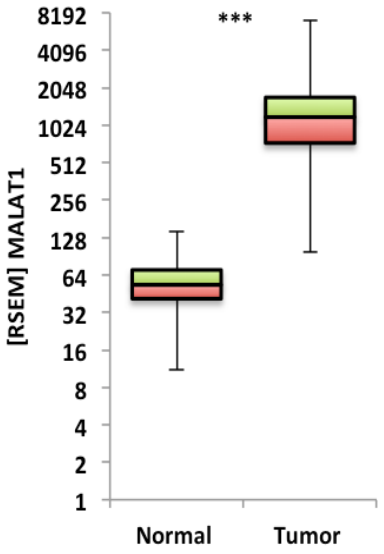

Figure 4.10. MALAT1 expression is significantly increased in CRC tissues. Y-axis represents log_2 of RNA-Seq by Expectation Maximization (RSEM) values and X-axis represents 51 normal and 382 tumor samples. Parametric unpaired t-test with Welch's correction was used to calculate p value. Data were collected from The Cancer Genome Atlas (TCGA). *** $p < 0.005$.

Analyzing TCGA data we confirm that, MALAT1 expression is significantly elevated in CRC tumor samples (**Fig. 4.10**). Here we show that the activation of estrogen signaling pathways by E2 or G1 represses MALAT1 expression in SW620 cell line (**Fig. 4.7** and **Fig. 4.8D**). The effect may be mediated by GPER1 and enhanced by ERβ expression.

Negative regulation of MALAT1 by E2 has also been shown in breast cancer [130, 150]. Interestingly, the decrease in MALAT1 expression is in ERα-

independent way [130]. We demonstrate that GPER1 mediates the repression of MALAT1 expression in response to its selective ligand and by E2 in the SW620 colon cancer cell line.

MALAT1 is known to mediate gene regulation through multiple mechanisms including transcriptional and post-transcriptional regulations. MALAT1 enhances several cancer-related cellular processes such as cell cycle and EMT [126]. It have been reported that MALAT1 can promote EMT in lung [151], bladder [152], oral squamous cell carcinoma [153], and cervical tumors [154]. MALAT1 mediates EMT transition by repressing the expression of E-cadherin (*CDH1*) [151], and enhances the expressions and/or the activities of several mesenchymal-related transcription factors such as Slug, ZEB1 and ZEB2 [152], Snail [154], as well as NFκB [153]. We show several aspects of MALAT1 regulatory scheme and functions in (**Fig. 4.11**) and possible transcriptional regulation through ERβ and GPER1 receptors, the precise epistatic relationships in this pathway remain to be resolved. Further mechanistic studies are required to understand how steroidal and non-steroidal ligands suppress the expression of MALAT1 and other EMT-related lncRNAs such as ZEB1-AS1 and HOTAIR, and how this impacts of estrogenic functions in the colon and CRC.

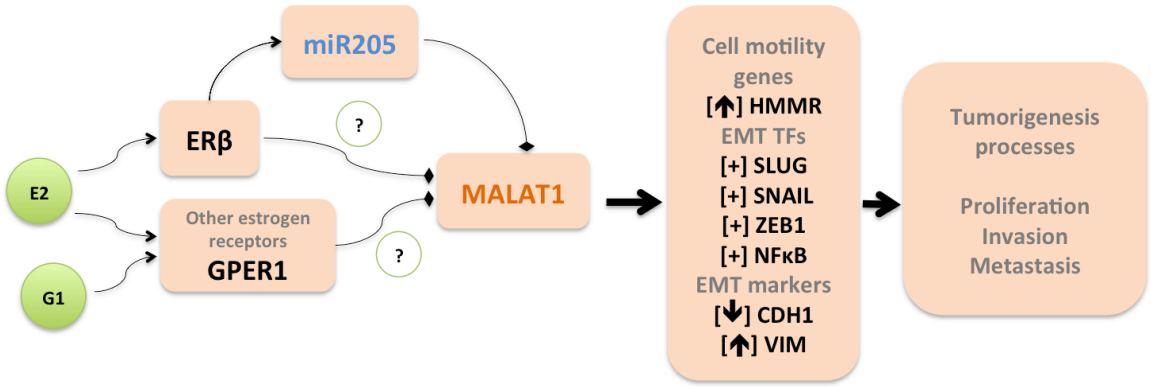

Figure 4.11. Proposed regulatory mechanism of MALAT1 by estrogen signaling and downstream effects in CRC. We observed before that ERβ upregulates miR205. It has been shown that MALAT1 is a miR205 target gene in bladder cancer [155]. MALAT1 expression is implicated in tumor metastasis. For instance, MALAT1 enhances the expression of cell motility-related genes such as HMMR [156]. HMMR shows downregulation by 0.09 FC in E2 treated SW620_ERβ. MALAT1 has also been linked to EMT signaling pathway by enhancing the expression and/or the activity of several EMT-TFs such as Slug, Snail, ZEB1 [152-154], and NFκB [137, 145, 146]. We assumed that GPER1 or ERβ attenuate the EMT singling by reducing MALAT1 expression.

4.4. Conclusion

Estrogens and related compounds have a protective effects against the development of colon cancer. It has been widely accepted that estrogens in the colon exert their effects by modulating the activity of ERβ, the main estrogen nuclear receptor in colonic epithelium. Many studies have shown that ERβ expression is lost during the progression of colon cancer.

In our current study, we demonstrate that re-expression of ERβ in SW620 human colon cancer cell line reduces cell metastasis. Further, our results show that lncRNAs are potential transcriptional targets of the activated ERβ. Various oncogenic lncRNAs are significantly downregulated in E2/ERβ treated SW620.

Among these, EMT-promoting lncRNAs such as MALAT1, HOTAIR and ZEB1-AS1. Interestingly, some lncRNAs and other coding-RNAs are responded to E2 treatment in absence of ERβ. We hypothesized that GPER1 may mediate this effect. In current study we demonstrate that several lncRNAs are regulated by the GPER1-selective G1 agonist and GPER1 has anti-tumorigenic effects in CRC, aligning with what others have shown in several other types of cancers. Our data provide new insights into the role of estrogens, ERβ, and GPER1 in colon cancer cells. We propose that both ERβ and GPER1 has potential protective effects against colon cancer. Further mechanistic studies are required to understand both ERβ and GPER1 functions in the colon epithelial cells and whether there is a potential crosstalk between two receptors. Since the activation of GPER1 modulates gene expression, which seems enhanced by ERβ, further studies are required to elucidate which transcription factors, what expression levels, and how these transcription factors activate GPER1-genomic cascade. In addition, further studies are needed to uncover the ERβ – MALAT1 regulation. One potential mechanism is via ERβ binding sites at MALAT1 promoter or may be by indirect regulation via miR-205. Because GPER1 is expressed in tumors, and appears to interfere with tumor metastasis, further studies using CRC models are needed to understand potential therapeutic opportunities.

5. Chapter 3: Activation of GPER1 by genistein or G1 inhibits the growth of pancreatic cancer

5.1. Introduction

Pancreatic ductal adenocarcinoma (PDAC) is an aggressive deadly disease with limited treatment options. PDAC constitutes 90% of all pancreatic tumors [157]. In 2016; 53,070 new PDAC cases were diagnosed and 41,780 deaths occurred in US only, making PDAC the 4th leading cause of cancer-related death in the United States [2]. Although the five-year survival rate has been improved for most cancers, PDAC shows a modest improvement from 5% to 8% during the last four decades, with a median survival of six months [2]. Low survival is attributed to lack of effective chemotherapies and late diagnosis of PDAC [158]. Currently, gemcitabine, a deoxycytidine analogue is the first-line of therapy for PDAC. Unfortunately, most tumors develop chemoresistance [61]. Combination therapies such as gemcitabine with other chemotherapeutic agents (including EGFR, mTOR, and Akt inhibitors) have been widely tested, but prolonged survival for only a few months accompanied by a significant toxicity have been observed [159]. The need for safe, affordable and less toxic drugs in order to augment the efficacy of chemotherapies is indispensable.

Genistein is a phytoestrogen enriched in soy diets. It has been proposed that genistein acts as a chemotherapeutic-enhancing agent against multiple types of cancers, including prostate [160], and breast [161]. Preclinical studies using different animal models have reported an antitumor function of genistein [66]. Genistein treatment results in inhibition of tumor growth, angiogenesis and metastasis as well as induction of apoptosis [66, 162]. Multiple reports show that genistein is able to augment the antineoplastic effects of many chemotherapeutic drugs such as gemcitabine [68], erlotinib [69], 5-fluorouracil (5-FU) [70], and docetaxel or cisplatin in different types of cancers [71]. For instance, in pancreatic cancer, adding genistein to 5-FU enhances the antitumor effect of 5-FU and reducing tumor growth *in vitro* and *in vivo* [70]. However, Genistein concentrations used to demonstrate the *in vitro* effects have in many cases been high (50-200 μM) and the mechanism of action is not fully understood. Genistein affects multiple cancer-related pathways, it directly binds and modulates the activities of estrogen receptors (ERs and GPER1), as well as, modulates NFκB, MAPK/ERK, and PI3K/Akt/mTOR pathways. However, its mechanism of action in PDAC has not been demonstrated. In order to provide a comprehensive understanding of molecular mechanisms of genistein in PDAC, along with the mechanistic background for its reported chemoenhancing properties, we have conducted series of experiments. In current study we used two PDAC cell lines MiaPaCa2 and PANC1. We did multiple cell proliferation studies using different

concentrations of genistein only, combined with gemcitabine, or gemcitabine only. 40nM of gemcitabine and 50 μM of genistein were used for subsequent experiments. Both cell lines were treated with 40nM of gemcitabine ± 50 μM of genistein for 24 hours, followed by RNA sequencing, pathway analysis, quantitative real-time PCR (qPCR) and demonstration of the proposed molecular mechanism.

5.2. Supplemental material and methods

5.2.1. Genistein, gemcitabine, G1, and G15 treatments

MiaPaCa2 and PANC1 were cultured as described before. Cells were treated with indicated concentrations of genistein (Sigma-Aldrich), G1 (Tocris Bioscience, Avonmouth, Bristol), or G15 (Tocris Bioscience) which all of them were prepared in DMSO. DMSO was used as a vehicle control. The final concentration of DMSO did not exceed 0.1%. Gemcitabine (Sigma-Aldrich) was used in 20 or 40 nM and prepared in phosphate buffered saline.

5.2.2. Data mining and analysis

Cell lines were treated with 50 μM of genistein, 40nM gemcitabine or with both of them combined for 24 hours. RNA was isolated, measured and sent for sequencing. Row read counts were used to measure the differential gene expression using limma-voom software [93].

5.2.3. Apoptosis assays (flow cytometry and immunoblotting)

Cell apoptosis was assessed by studying PARP and caspase 3 cleavage. PARP and cleaved PARP were detected by using 1:1000 of anti-PARP antibody (Cell Signaling Technology Inc. Beverly, MA). Caspase 3 and the cleaved version were detected by using 1:1000 of two different primary antibodies (Cell Signaling). For PANC1 Annexin V and DNA staining were used to confirm cell apoptosis during G1 treatment. Annexin V was detected by FITC (BD bioscience, Bedford, MA) and DNA by using propidium iodide (PI) solution (BD Bioscience) according

to manufacturer's instructions. BD LSRFortessa analyzer was used to detect the fluorescence. Flowjo was used for data analysis.

5.2.4.qPCR confirmation

Primer pairs have been used in qPCR

Gene Name	Primer Name	Primer Sequence 5′ – 3′
MUC1	hMUC1_F	TGCCGCCGAAAGAACTACG
	hMUC1_R	TGGGGTACTCGCTCATAGGAT
PLCG2	hPLCG2_F	ACTCCAAAGATTTCGAGCGAG
	hPLCG2_R	GAGTGCCATATAGGATGGTGAAG
DDIT3	hDDIT3_F	GGAAACAGAGTGGTCATTCCC
	hDDIT3_R	CTGCTTGAGCCGTTCATTCTC
ITPR1	hITPR1_F	GCGGAGGGATCGACAAATGG
	hITPR1_R	TGGGACATAGCTTAAAGAGGCA
VDR	hVDR_F	TCTCCAATCTGGATCTGAGTGAA
	hVDR_R	GGATGCTGTAACTGACCAGGT
CHAC1	hCHAC1_F	GAACCCTGGTTACCTGGGC
	hCHAC1_R	CGCAGCAAGTATTCAAGGTTGT
18S_rRNA	18S_rRNA_F	GCTTAATTTGACTCAACACGG
	18S_rRNA_R	AGCTATCAATCTGTCAATCCT
GAPDH	hGAPDH_F	GACCACAGTCCATGCCATCA
	hGAPDH_R	CATCACGCCACAGTTTCCC

5.3. Results and Discussion

5.3.1. Genistein inhibits the growth of pancreatic cancer cell lines and enhances gemcitabine efficacy

Previous studies have reported that genistein reduces the proliferation of several pancreatic cancer cell lines [68-70]. Further, genistein is also able to enhance the anti-proliferative effect of 5-FU on MiaPaCa2 xenografts [70]. In order to test the reproducibility of these reports and explore the chemoenhancing effects, we determined the effective inhibitory concentration of genistein on cell proliferation using two pancreatic cancer cell lines, MiaPaCa2 and PANC1.

Both cell lines are belonged to the quasimesenchymal subtype (according to Collisson *et al*. [163]). At 50 μM of genistein both cell lines show a significant reduction in cell proliferation (**Fig. 5.1A**), supporting previous reports of anti-proliferative effects starting from 25 μM up to 100 μM of genistein depending on the used cell lines [68-70]. To test whether genistein is able to potentiate the anti-growth function of gemcitabine, we treated both cell lines with different concentrations of genistein combined with 40 nM of gemcitabine for 96 hours. We confirmed that, PANC1 is more resistance to gemcitabine, as it has been previously reported [164]. However, when we included genistein, an additive effect is observed from 25 μM of genistein in PANC1 cell line, whereas MiaPaCa2 cell line, which shows a stronger response to gemcitabine alone, needs up to 100 μM of genistein for significant additive effects (**Fig. 5.1B**).

Thus, genistein has anti-proliferative and chemoenhancing effects in both pancreatic cancer cell lines, with prominent outcome in PANC1 the more chemoresistant cell line.

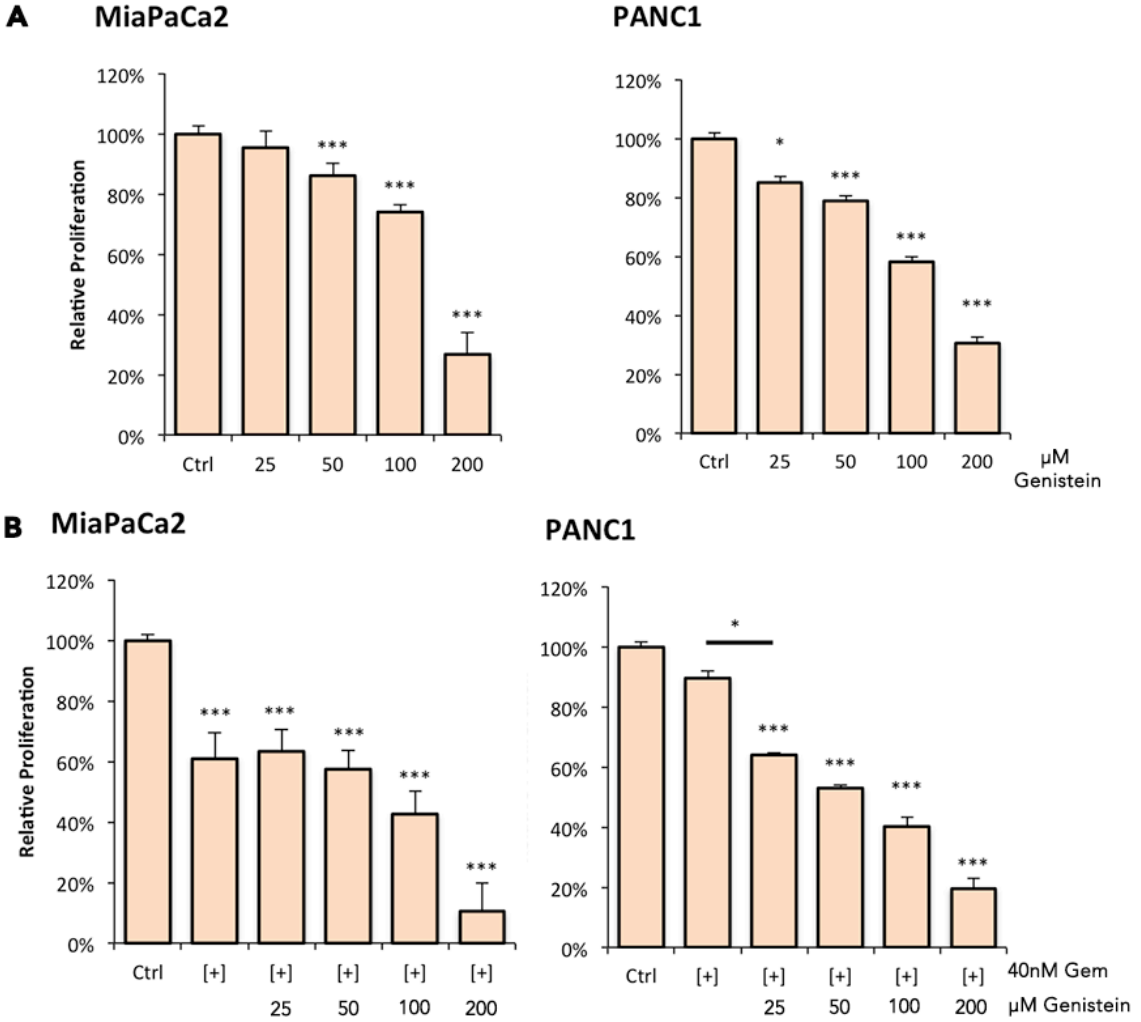

Figure 5.1. The effect of genistein, gemcitabine or combination treatments on the proliferation of MiaPaCa2 and PANC1 cell lines using MTS assay. (A) Cells were treated with increasing concentrations of genistein and then incubated for 96 hours. Controls were treated with DMSO. **(B)** Cells were treated with either 40 nM of gemcitabine only or in combination with increasing concentrations of genistein for 96 hours. In both A and B, Y-axis represents relative proliferation. Error bars represent ±SEM of five replicates. Unpaired two-tailed t-test was used to calculate p values (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$).

5.3.2. RNA-seq specifies gemcitabine patterns that induce apoptosis and activate pro-inflammatory pathways

To identify the effect of gemcitabine at gene expression level, we treated both MiaPaCa2 and PANC1 with 40nM of gemcitabine for 24 hours and conducted RNA-seq of the polyA-tailed transcriptome. Gemcitabine treatment induces differential expression of 3471 and 2641 genes in MiaPaCa2 and PANC1, respectively, of which 834 genes are regulated in both cell lines (**Fig. 5.2A**). We explored which regulatory sub-networks are enriched in this data set (834 commonly regulated genes), and we find that these genes are primarily regulated by MAPK1, PI3K/Akt pathway, TGFB1, and NFκB activator TNFα (**Fig. 5.2B**). Figure 5.2C, illustrates all genes regulated by gemcitabine that are also known to be modulated by TNFα (**Fig. 5.2C**). These include key members of the NFκB signaling pathways such as the NFκB subunits RELB and NFKB2, and the cytokine IL-8, which all are significantly upregulated upon gemcitabine treatment in both cell lines (**Fig. 5.2D**). Such changes have been correlated with cell proliferation, migration and angiogenesis, and associated with poor prognosis in pancreatic and other cancers [165, 166]. Using gene ontology classifications for the differentially expressed genes, we find that those with functions within cell cycle, DNA damage, DNA repair, and apoptosis are significantly enriched (**Fig. 5.2E**). Gemcitabine is a nucleoside analogue, which interferes with DNA synthesis, thereby induces DNA damage, reduces cell

mitosis, and eventually induces cell apoptosis [61]. These effects are clearly visible at gene expression level in our data. Activation of apoptosis can be caspase-mediated since we observed an upregulation of caspases in both cell lines for example (CASP2) or via alternative pathways such as activation of p38 mitogen – activated protein kinase (MAPK) in response to cellular stress [167], which is also suggested by our data (**Fig. 5.2B**).

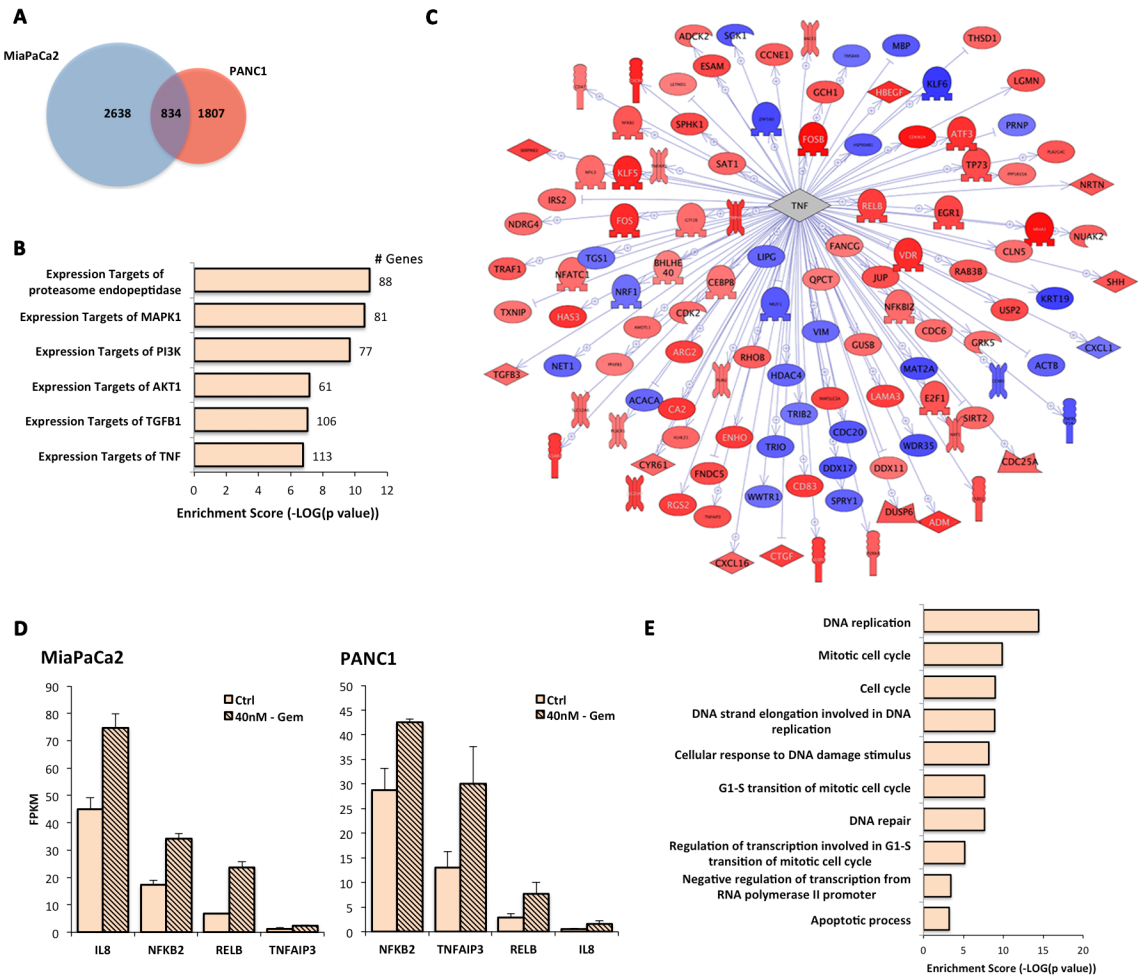

Figure 5.2. Transcriptomic effects of gemcitabine treatment in MiaPaCa2 and PANC1 cell lines. (A) Venn diagram demonstrates differentially expressed genes upon 24 hours of gemcitabine treatment, as identified by RNA-seq. **(B)** Subnetwork analysis of the 834 overlapped genes shows a number of common regulators. **(C)** 113 differentially expressed genes are known to be regulated by TNF signaling (p value enrichment 1.7×10^{-7}). **(D)** FPKM values of gemcitabine-upregulated NFκB genes **(E)** Enriched biological functions among 834 commonly differentially expressed genes.

5.3.3. RNA-seq reveals genisteins effect on the intrinsic apoptotic pathway in response to endoplasmic reticulum stress

To understand the molecular mechanisms of genistein anti-cancer functions, we conducted RNA-seq for MiaPaCa2 and PANC1 cells treated with 50 μM of genistein for 24 hours. Genistein treatment changes the expression of 552 genes in MiaPaCa2 (350 upregulated and 202 downregulated) and 1,040 genes in PANC1 (567 upregulated and 473 downregulated), as depicted by volcano plots (**Fig. 5.3A**). 146 genes are differentially expressed in both cell lines (**Fig. 5.3B**). Functional annotation of the 146 genes reveals an enrichment among the biological processes of calcium ions transport, post-transcriptional modifications (PTM), ATP response, apoptosis signaling in response to endoplasmic reticulum stress, and estradiol signaling pathways (**Fig. 5.3C**). Two of these pathways are particularly relevant to genistein functions: releasing of sequestered Ca^{2+} to the cytosol and the activation of intrinsic apoptotic pathway in response to endoplasmic reticulum stress [162].

Upon genistein treatment we find the expression of DNA damage inducible transcript 3 (DDIT3) being significantly upregulated in both cell lines (**Fig. 5.3D**). DDIT3 is marker of endoplasmic reticulum stress and it has also been shown to be upregulated in breast cancer in response to genistein [168]. Endoplasmic reticulum stress can activate cell apoptosis. We also identify that mucin 1 (MUC1) is downregulated in genistein-treated cells (**Fig. 5.3D**).

Pancreatic cancer cells which express high levels of MUC1 are more resistant to chemotherapeutic agents such as gemcitabine [169]. Furthermore, our RNA-seq data show downregulation in kinesin family member 20B (KIF20B) (**Fig. 5.3D**). In gastric cancer, genistein has been reported to induce cell cycle arrest by decreasing the expression of KIF20A [170]. Collectively, these data indicate that genistein can potentiate the anti-tumor functions of chemotherapeutic agent by multiple mechanisms.

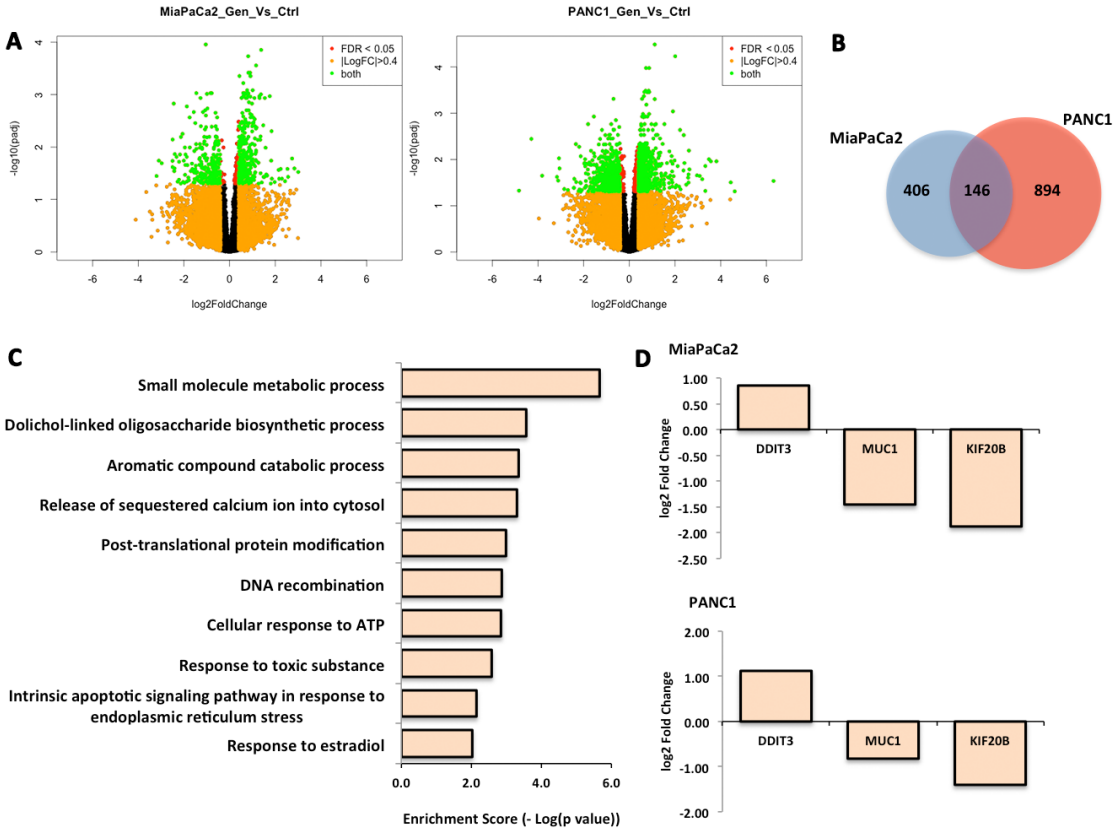

Figure 5.3. Effects of genistein treatment on gene expression in MiaPaCa2 and PANC1 cell lines. (A) Volcano plots depict genistein-induced gene expression in two pancreatic cancer cell lines MiaPaCa2 and PANC1. **(B)** Venn diagram represents differentially expressed genes in both cell lines MiaPaCa2 and PANC1 after applying selected cutoffs (FDR < 0.05, FPKM (treated) >1 and $\log_2$FC ≥ |0.4|). 146 genes are differentially expressed in both cell lines. **(C)** Functional annotation of the differentially expressed genes in both cell lines reveals the enriched pathways. Y-axis shows the enrichment score [-$\log_{10}$(p value)]. **(D)** $\log_2$FC for three significantly changed genes which are potential genistein targets (FDR < 0.05).

5.3.4. The chemoenhancing pathways of genistein treatment are revealed by RNA-seq

Our analysis of gemcitabine-affected genes has identified several regulators which are linked to chemoresistance, including P13/AKT1 [62, 171, 172], TGFB1 [173], and TNFα [62, 174] as figure 5.2B shows. Targeting key signaling pathways is important for efficacious gemcitabine treatment.

Furthermore, our analysis of genistein-mediated transcriptomic effects indicate potential mechanisms for its proposed chemoenhancing effects. This includes activation of apoptosis through endoplasmic reticulum stress. This apoptotic mechanism is less affected by chemoresistance [175] and can be utilized to augment the effect of chemotherapeutic agents.

Also genistein mediates downregulation of MUC1, which can also be beneficial, as overexpression of mucins is implicated in chemoresistance in multiple tumors, including pancreatic cancers [169, 176]. In order to detail the particular effects of the combination treatment, we performed RNA-seq comparing a 24 hours combinatory treatment of 40 nM gemcitabine and 50 μM genistein with 40 nM gemcitabine only. We compared gene expression in MiaPaCa2 and PANC1 cell lines. 154 genes are significantly changed in both cell lines (**Fig. 5.4A**). We detailed exactly which biological functions, sub-networks and genes that potentially mediate the chemoenhancing effects. Apoptosis in response to endoplasmic reticulum stress, influx of intracellular Ca^{2+} (**Fig. 5.4B**), p-38 MAPK

signaling pathway (p value $1.0x10^{-7}$) are significantly enriched (**Fig. 5.4C**). Moreover, our data show significant increase in vitamin D receptor (VDR), calpain 5 (CAPN5), inositol 1,4,5-trisphosphate receptor type 1 (ITPR1), phospholipase C gamma 2, (PLCG2), ChaC glutathione specific gamma-glutamylcyclotransferase 1 (CHAC1), and caspase 9 (CASP9) during genistein gemcitabine combination treatment compared with gemcitabine only (**Fig. 5.4D, 5.4E**). The effect of genistein (alone or combined with gemcitabine) on calcium influx accompanied with apoptosis due to endoplasmic reticulum stress as well as enrichment in estradiol signaling ($p>0.01$) lead us to propose that, in MiaPaCa2 and PANC1 cell lines genistein mediates its effects via the G protein-coupled estrogen receptor (GPER1).

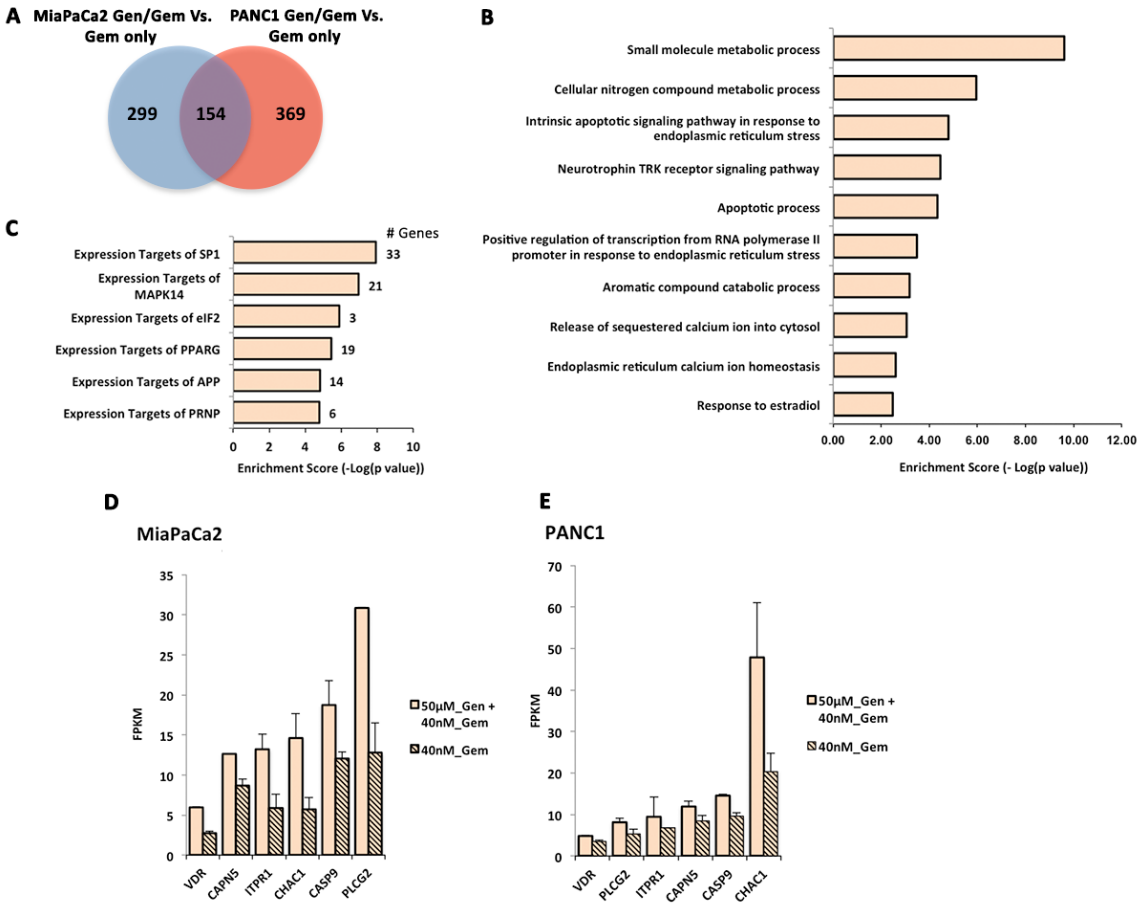

Figure 5.4. The effect of genistein and gemcitabine combination on gene expression in MiaPaCa2 and PANC1 cell lines. (A) Venn diagram shows differentially expressed genes in both cell lines comparing genistein and gemcitabine combination with gemcitabine only after applying cutoffs (FDR < 0.05, FPKM (treated) >1 and logFC ≥ |0.4|). 154 genes are differentially expressed in both cell lines. **(B)** Enriched biological functions among the 154 commonly differentially expressed genes. **(C)** Subnetwork analysis of the 154 overlapped genes shows a number of common regulators such as SP1 (p value $1.0x10^{-8}$) and MAPK14 (p value $1.0x10^{-7}$). **(D)** FPKM values of the proposed genistein-targets when we added genistein to gemcitabine in MiaPaCa2 and **(E)** PANC1, demonstrating a significant increase (FDR < 0.05).

5.3.5. GPER1 is a potential genistein target gene in pancreatic cancer cell lines

We set up the hypothesis that GPER1 can mediate the genistein mechanisms in pancreatic cells. To test this hypothesis, we first excluded the involvement of the nuclear estrogen receptors by confirming their non-expressed status [FPKM<0.1 in both cell lines] (data not shown). Then we confirmed the expression of GPER1 at transcript and protein levels. We noted a higher level of its transcript in PANC1 using both RNA-seq and qPCR (**Fig. 5.5A**), accompanied by a slightly higher protein level in in PANC1 (**Fig. 5.5B**). Secondly, we explored the specific regulation of several proposed direct and indirect GPER1 target genes using our RNA-seq data. For instance, VDR, CAPN5, ITPR1, PLCG2, CHAC1, and CASP9 show significant upregulation upon genistein treatment in both cell lines and they are GPER1 potential targets (**Fig. 5.4D, 4E**). Furthermore, the enriched pathways accompanied with functional annotations of differentially expressed genes propose GPER1 activation. We used these data to build a model to show genistein mechanism of action in pancreatic cancer which is presumably by GPER1 signaling (**Fig. 5.5C**).

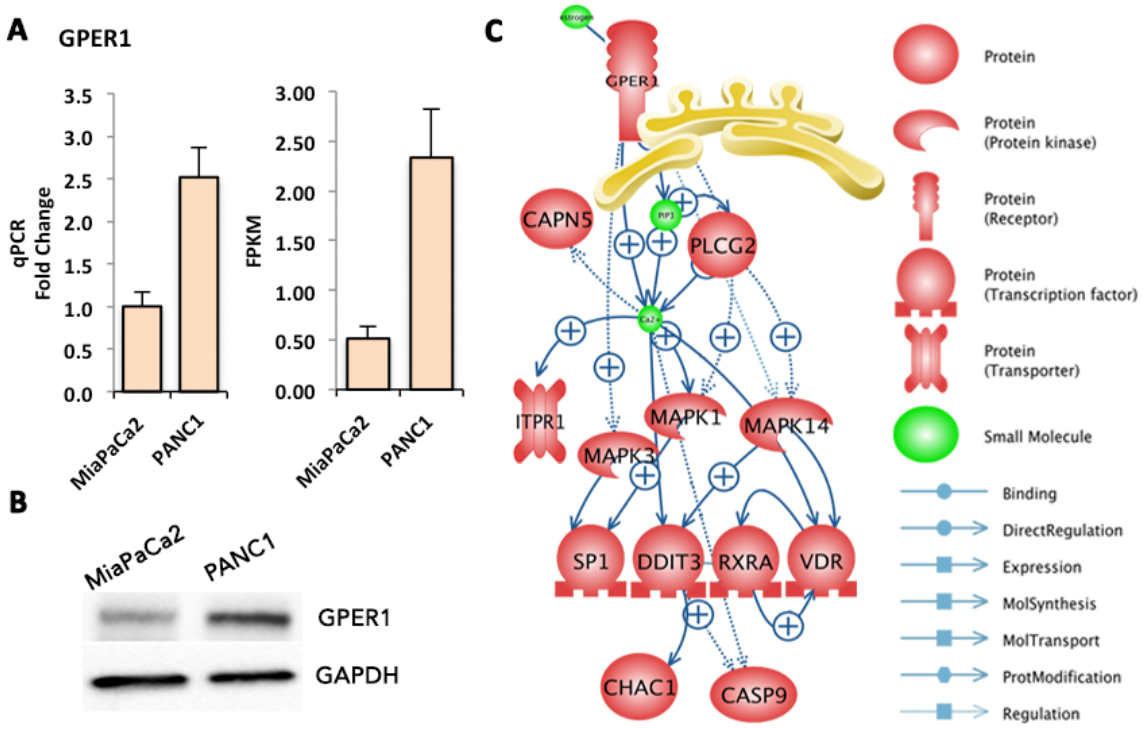

Figure 5.5. Proposed molecular mechanism of anti-proliferative effects of genistein on pancreatic cancer cell lines. (A) GPER1 expression in MiaPaCa2 and PANC1 cell lines using qPCR, RNA-seq, and **(B)** Western blot. GPER1 protein is indicated at 42 kDa, and GAPDH was used as loading control. **(C)** Proposed molecular mechanism of the additive anti-proliferative effect of genistein through the activation of GPER1 receptor.

5.3.6. Selective GPER1 agonist (G1) reduces pancreatic cancer cell proliferation

To test whether the anti-proliferative and chemoenhancing effects of genistein is truly mediated by GPER1, we treated MiaPaCa2 and PANC1 cell lines with G1 a GPER1 selective agonist then we measured cell proliferation. It has been demonstrated that G1 is highly selective agonist. G1 at concentrations up to 10 μM has no binding activities to both estrogen nuclear receptors (ERα and ERβ) or to 25 other G protein-coupled receptors [81]. G1 selectivity has also been tested in *Gper* knockout mice. *Gper*$^{-/-}$ mice do not respond to G1 treatment [177, 178]. We treated both MiaPaCa2 and PANC1 with different concentrations of G1 for 48 and 72 hours followed by proliferation assay. 2 μM of G1 are enough to produce significant reduction in cell proliferation 48 hours after the treatment (**Fig. 5.6A**). After 72 hours a concentration-dependent reduction of proliferation is noted from 0.5 μM in MiaPaCa2 and from 1 μM in PANC1 (**Fig. 5.6B**). To validate that anti-proliferative effect of G1 is through GPER1, we measured proliferation for cells treated with G1 combined with G15 (a GPER1 antagonist) in 1:5 ratio. G15 interferes with G1 and partially rescues cell proliferation (**Fig. 5.6C**). The binding efficacy is 10 times less for G15, which can explain why no full rescue is achieved in 1:5 ratio concentrations. However, a 10-fold excess of G15 would result in levels so high that it may generate unspecific or toxic side effects.

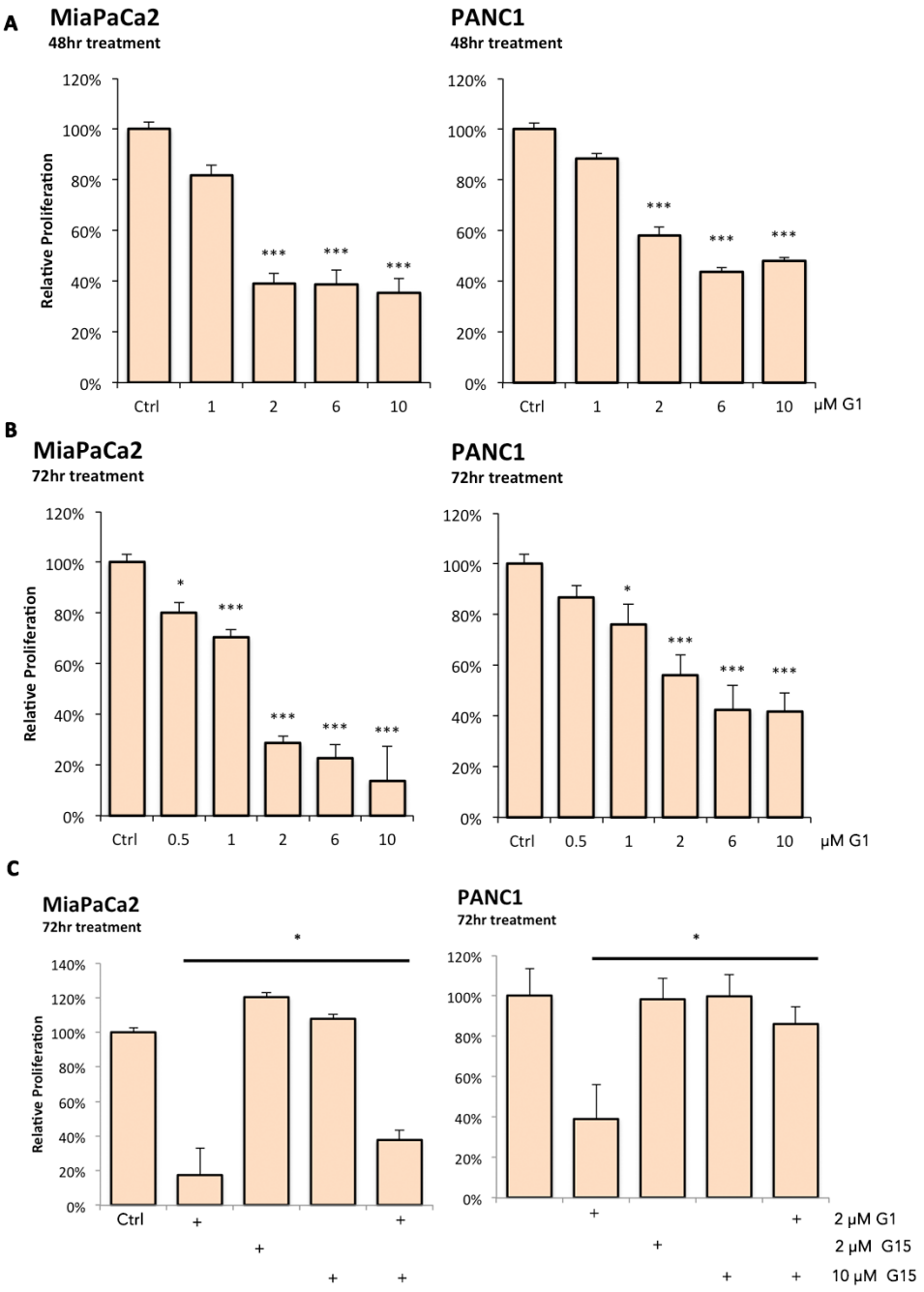

Figure 5.6. GPER1 mediates anti-proliferative effects in pancreatic cancer cell lines. (A) Relative proliferation after 48 hours of G1 treatment, 2 μM of G1 are enough to reduce cell proliferation by at least 40% in both cell lines. **(B)** Relative proliferation after 72 hours of G1 treatment. **(C)** G15, a GPER1 antagonist interferes with G1 anti-proliferative effect in both cell lines. Error bars represent ± SEM. Unpaired two-tailed was used to calculate the significance. *p<0.05, **p<0.005, ***p<0.0005 statistical significances.

5.3.7. G1 induces cell apoptosis by activating caspase 3 and confirmed for PANC1 by flow cytometry

We tested whether GPER1 activation by G1 induces cell apoptosis. MiaPaCa2 and PANC1 were treated with 2 μM of G1 for 48 hours. G1 treated cells show an increase in PARP and caspase 3 cleavage (**Fig. 5.7A**) indicating that GPER1 is pro-apoptotic receptor in pancreatic cancer cell lines. Although several studies showed G1 selectivity, however GPER1-independent effect has also been reported. It have been shown that, G1 exerts anti-proliferative effect although GPER1 expression was reduced by siRNA [179]. May be using siRNA knockdown GPER1 is insufficient to abolish the entire receptor activity. Another report demonstrated that G1 exerts an effect on microtubule structures of *Gper*$^{-/-}$ endothelial cells [180]. In this study, although G1 alters the microtubules structures at low concentration (200 nM), the effect on DNA synthesis has been observed at relatively high concentration (3 μM) of the ligand.

Broselid *et al*, confirmed G1 selectivity using GPER1-shRNA. G1-induced apoptotic singling was clearly abolished in GPER1-shRNA cells [181]. Collectively, we assume that G1 acts via GPER1 signaling but we need to validate our data by knocking out *GPER1* using shRNA. To confirm the apoptosis upon G1 treatment, we used propidium iodide - Annexin V staining. PANC1 shows significant increase in PI-Annexin double staining upon G1 treatment (**Fig. 5.7B**).

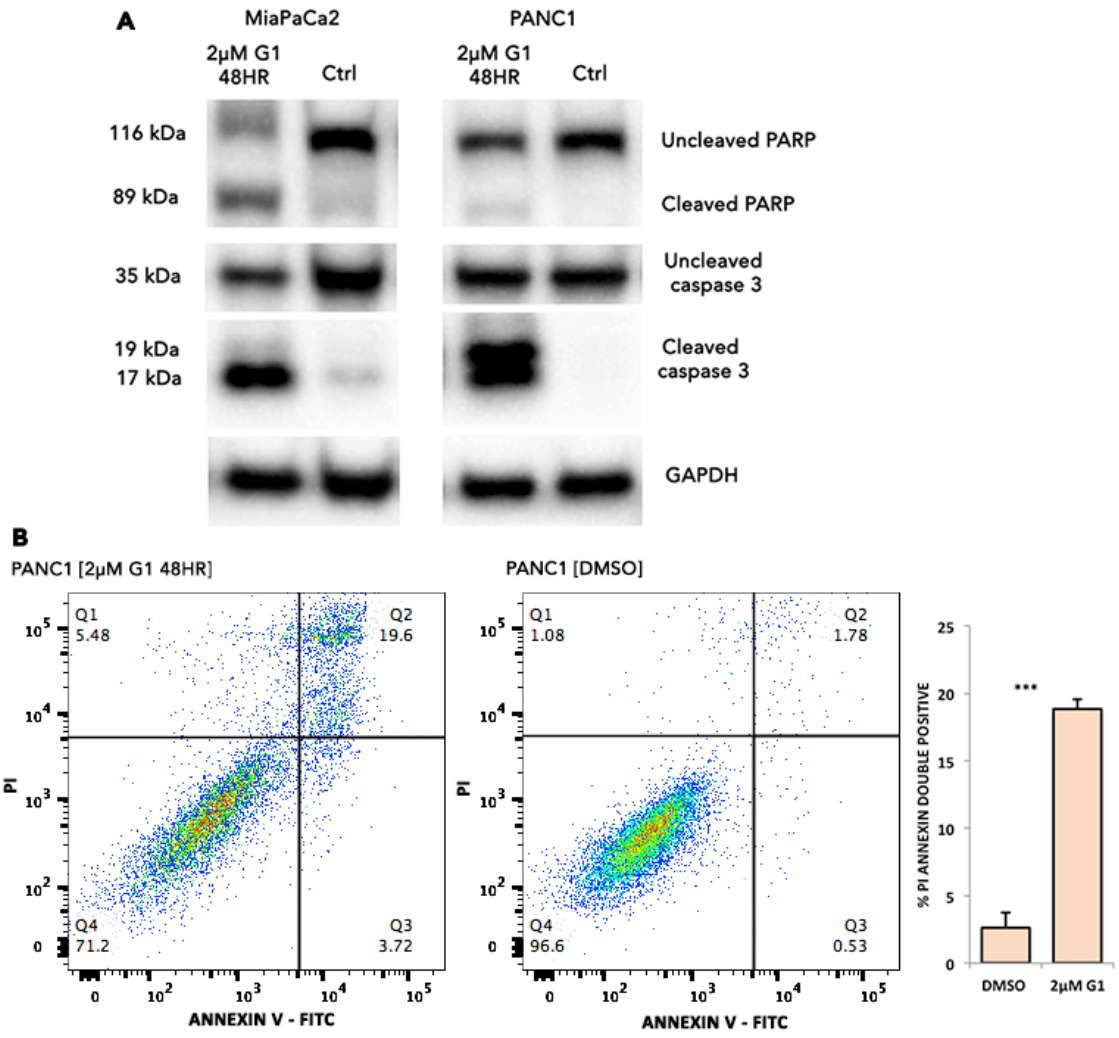

Figure 5.7. G1 treatment induces apoptosis in MiaPaCa2 and PANC1 cell lines. (A) G1-treated cells show PARP cleavage and caspase 3 activation. MiaPaCa2 are more susceptible to G1 ligand. **(B)** Propidium iodide - Annexin V staining confirms cell apoptosis. Approximately 20% of PANC1 show double staining after 48 hours of 2 μM G1 treatment. The experiment was performed in triplicate. Significant difference between DMSO and G1 treated samples is observed. Error bar represents ± SEM. Unpaired two-tailed t-test was used to calculate p value (*** $p<0.005$).

5.3.8.qPCR confirms RNA-seq data

We selected genes that are involved in apoptosis in response to endoplasmic reticulum stress and can be regulated by cytosolic Ca^{+2}. We confirmed that *DIDTI3*, *ITRP1* and *VDR* are significantly upregulated in both cell lines. CAPN5 shows significant upregulation only in MiaPaCa2 while PLCG2 and CHAC1 are only unregulated in PANC1 (**Fig. 5.8**). Our RNA-seq experiment shows downregulation in MUC1 expression level, which we confirm it by qPCR. All qPCR tested genes are consistent with RNA-seq data and show same direction of fold change as well.

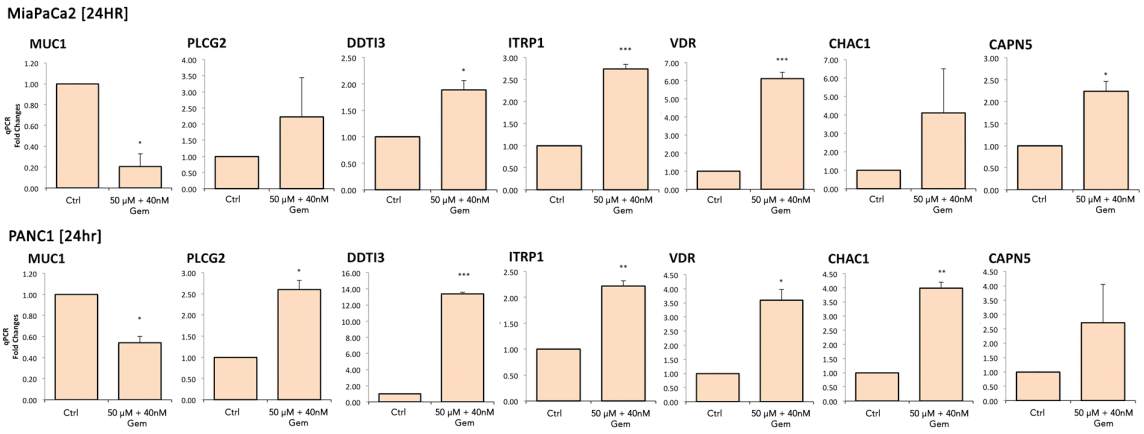

Figure 5.8. qPCR confirmation of selected genes. Y-axis represents fold change. Error bars represent ±SEM of two biological replicates. Unpaired two-tailed t-test was used to calculate p values (* $p<0.05$, ** $p<0.005$, *** $p<0.0005$).

5.4. Conclusion

Acquired resistance to chemotherapy in pancreatic cancer is a common phenomenon among treated patients. Gemcitabine is the first-line chemotherapeutic agent against pancreatic cancer. Due to reduction in gemcitabine transporters, overexpression of deactivating enzymes or activation of chemoresistance-related pathways like NFκB, patients develop drug-resistance. Phytoestrogens like genistein have been widely tested as chemoenhancing agents. When gemcitabine is combined with genistein, the cells show an additional reduction in cell proliferation, especially in gemcitabine less-responsive pancreatic cancer cell line such as PANC1. Using RNA-seq we are able to propose a mechanism of action for genistein effects on pancreatic cancer. We find that genistein reduces NFκB related signaling molecules such as MUC1. Furthermore, in genistein only or when combined with gemcitabine, cells undergo apoptosis presumably due to endoplasmic reticulum stress and an increase in cytoplasmic calcium. These two enriched pathways lead us to propose that GPER1 is genistein target gene in pancreatic cancers. GPER1 activation by G1 reduces cell proliferation and induces apoptosis in MiaPaCa2 and PANC1 pancreatic cancer cell lines. We conclude that the use of genistein is a feasibly and useful way to enhance the efficacy of chemotherapeutic especially in resistance tumors. Finally, further works and experiments are still required to enhance genistein solubility and bioavailability to be use in the clinic.

6. Concluding remarks and future directions

Gastrointestinal (GI) cancers are the leading causes of cancer-related deaths for both men and women. The requirements for new diagnostics markers, drug targets, and preventive medicines are crucial to enhance disease survival. Estrogens and phytoestrogens are significantly associated with a lower-risk to develop gastrointestinal tumors. Large cohort studies showed that estrogens have a protective effect against various gastrointestinal tumors like colorectal, esophageal and gastric cancers [7-9, 182], as well as pancreatic cancers [communicating data]. The underlying mechanisms behind protective function of estrogens are still under investigation.

Once the cancerous cells are being developed and evade the preventive anti-tumor mechanisms, cancer cells acquired additional characteristics, which turn the disease into aggressive and life-threatening illness. The leading causes of poor prognosis and low survival are the acquisition of metastasis and chemoresistance phenotypes, which remain the major challenges in the field.

In current work, we studied estrogen signaling in two GI tumors: colon and pancreatic cancers. In colon, ERβ is the main nuclear estrogen receptor. In addition to ERβ, the cytoplasmic estrogen G protein-coupled receptor which GPER1 is also expressed. During cancer progression, colon epithelium loses completely ERβ expression while GPER1 is significantly reduced.

Since we were initially interested in studying cancer preventive role of the E2/ERβ axis in the colon, we re-expressed ERβ in human colon cancer cell lines. ERβ expression results in anti-oncogenic effects, which agreed with clinical, pre-clinical and epidemiological data. Colleagues and I conducted further experiment to dissect ERβ mechanisms in the colon epithelium. We showed that ERβ is able to enhance the expression of miR-205 a tumor suppressor microRNA. miR-205 suppresses tumor metastasis by targeting EMT process. These data triggered us to study ERβ effects on more advanced stages during the tumorigenesis such as metastasis. To study metastasis we used an elegant *in vivo* zebrafish model. We confirmed that ERβ has anti-metastasis functions in colon cancer. Follow up experiments were conducted by studying ERβ functions in SW620, a highly metastasized colon cancer cell line.

High-throughput RNA-seq analysis of E2 treated SW620_ERβ cell line shows significant downregulation in cell cycle related genes and several oncogenic lncRNAs, confirming ERβ tumor suppressive functions. Interestingly, E2 has genomic effect in SW620 cells in absence of ERβ. Several differentially expressed genes upon E2 treatment show same changes when GPER1 was activated by its selective ligand G1. MALAT1 a lncRNAs is significantly reduced upon E2 or G1 treatments. High expression of MALAT1 has been linked to more aggressive, poor prognostic and metastatic disease. Moreover, activation of GPER1 reduces EMT transition. G1 treatment induces the expression of *CDH1* an epithelial marker gene,

simultaneously reduces the expression mesenchymal related genes such *VIM* and *ZEB1*. Collectively, we conclude that the activation of estrogen signaling in the colon has protective and anti-tumorigenic functions through ERβ and/or GPER1 singling. Although GPER1 expression is reduced during cancer progression, apparently its level is slightly elevated in metastatic lesions, which makes it a potential drug target [139].

Apparently, estrogen acts as ligand for both NR and GPCR in colonic epithelium and regulates numerous protein coding and noncoding RNAs generating a complex regulatory networks. Modeling such regulatory networks is crucial to understand the net effects of estrogen on colonic epithelium, as well as on the surrounding environment.

We showed that estrogen treatment represses the expression of cell cycle and mitosis genes. This data lead us to conclude that estrogens singling may paly an important role for the maintenance and the hemostasis of epithelial cells. Probably studying estrogen functions in healthy intestinal tissue is also important to understand the mechanism of action. It is also critical to study in detail the negative impacts of estrogen signaling on EMT and whether estrogen counteracts EMT-activating ligands such as TGFβ. Since GPER1 roles in the colon are not well established more studies are required to elucidate the mechanism of action. *In vivo Gper* knockout studies are crucial to understand GPER1 functions in the intestine. Moreover, evaluating the effect of G1/GPER1 during the progression of colon

cancer such in AOM/DSS model will help us to understand its role during the tumorigenesis.

We showed that estrogen singling interferes with metastasis by modulating EMT signaling which is frequently linked to chemoresistance phenotype. Since the acquisition of chemoresistance is a common phenomenon in pancreatic cancer, we studied the chemoenhancing properties of genistein in two pancreatic cancer cell lines MiaPaCa2 and PANC1. Genistein is a naturally occurring non-toxic phytoestrogen [67]. We showed that genistein is able to augment the anti-proliferation outcome of gemcitabine. Moreover, our data suggested that genistein is able to activate apoptotic pathways due to endoplasmic reticulum stress. This pathway is less affected by chemoresistance [175]. Although genistein is a multi-targeted agent our sub-network and gene ontology analysis demonstrated that most of genistein related transcriptional changes are linked to GPER1 singling. We demonstrated that, the activated GPER1 reduces the proliferation and induces the apoptosis of pancreatic cancer cell lines. Collectively, our data suggested that adding estrogenic compounds such as genistein to the chemotherapy is an efficient way to enhance the effect of therapeutic regimen. Additional *in vivo* works are needed to characterize the genistein or G1 anti-tumor functions at higher level. Models such as patient-derived xenograft (PDX) can be suitable for such studies which can be utilized for patient selection for particular therapeutic regimen. Furthermore, more researches are required to study drug toxicity, safety and

efficacy of G1 or any other GPER1 ligands. Finally, further studies are need to modify genistein physical and chemical properties to enhance its solubility and the efficacy.

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