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G-Protein Coupled Estrogen Receptor Regulation of Migration and Metastasis in the Breast

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G PROTEIN-COUPLED ESTROGEN RECEPTOR REGULATION
OF MIGRATION AND METASTASIS
IN THE BREAST

by

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B.S. Biochemistry
Oklahoma Christian University, 2007

DISSERTATION

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DEDICATION

To those who believed in me when I no longer did

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These seven years in graduate school have been the most difficult years of my life, challenging me in ways about which no one had warned me. I learned being smart is not always enough. I also learned how powerful the support of my family and friends truly is.

To my mom and dad: you have put up with my endless questions of "why" and "how" since I learned to talk. You listened as I excitedly explained what I learned but put restrictions on what could be discussed while eating. Even into grad school, when you had no idea what I was talking about, you still cared enough to listen and ask questions. You supported me when I failed and taped my data to the fridge when it was finally statistically significant. You encouraged me to pursue my passion for science, even if it meant hard lab stools instead of the fancy, comfortable chairs in the business school.

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G PROTEIN-COUPLED ESTROGEN RECEPTOR REGULATION OF MIGRATION AND METASTASIS IN THE BREAST

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ABSTRACT

Proliferation and migration are critical steps within normal mammary development and breast cancer progression. While 17β-estradiol (E2) stimulates proliferation in normal and breast cancer cells through estrogen receptor-α (ERα) and G Protein-Coupled Estrogen Receptor-1 (GPER) [1-3], GPER regulation of E2-dependent migration has not been fully examined. GPER upregulates pathways necessary for increased migration, including vimentin, matrix metalloproteinase-9, and mitogen activated protein kinase (MAPK) signaling [1, 4-6]. GPER may also increase phospho-focal adhesion kinase (p-FAK) through Src activation [7, 8]. As a tumor develops, it produces transforming growth factor-β (TGF-β) to activate normal fibroblasts in the stroma, transforming them into cancer-associated fibroblasts (CAFs) and resulting in increased metastasis. As metastasis is the primary cause of cancer-related death, it is critical to examine how CAF activation is regulated [9-11]. GPER upregulates connective tissue growth factor, an enhancer of TGF-β-induced fibroblast activation, and is

correlated with increased breast cancer metastasis [12-14], suggesting a role for GPER in fibroblast activation and metastasis.

In this study, GPER activation increased MCF10A breast epithelial cell migration. Concomitant with GPER-dependent migration, increased expression of proteins supporting collective migration was observed, including vimentin, p-FAK, E-cadherin, and β-catenin, without increased proliferation. GPER inhibited PyMT breast cancer cell individual migration. Additionally, GPER increased normal fibroblast activation and proliferation in an EGFR-ERK-dependent manner but inhibited migration *in vitro*. *In vivo*, the absence of GPER expression in fibroblasts increased tumor metastasis and metastatic lesions size but did not affect collagen production. This is the first study to demonstrate a role for GPER in migration of normal breast epithelial cells, activation of normal fibroblasts, and the inhibition of tumor metastasis.

Separately, the role of GPER in the classical uterine responses to estrogen activity, proliferation and water imbibition, traditionally attributed to ERα activation, was examined. G-1, a GPER-selective ligand, stimulated lumenal epithelial cell proliferation but not imbibition. AB-1, an ERα- and ERβ-selective ligand that activates genomic responses but not rapid signaling, stimulated proliferation and imbibition. However, rapid signaling is required for complete imbibition as AB-1 only induced ~60% of the E2-induced imbibition.

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ABBREVIATIONS

17β-HSD	17β-hydroxysteroid dehydrogenase
7TM	seven-transmembrane
ADAM-17	a disintegrin and metalloprotease-17
AF-1	activation function-1
AF-1	activation function-2
AI	aromatase inhibitor
ANOVA	Analysis of Variance
AQP	aquaporin
ATCC	American Type Culture Collection
Bcl-2	B-cell lymphoma 2
CAF	cancer-associated fibroblast
cAMP	cyclic adenosine monophosphate
CTGF	connective tissue growth factor
DAPI	4',6'-diamidino-2-phenylindole
DBD	DNA binding domain
DCIS	ductal carcinoma *in situ*
DMEM	Dulbecco's modified eagle medium
DMSO	dimethylsulfoxide
E1	estrone
E2	17β-estradiol
E3	estriol
ECIS	electric cell-substrate impedance sensing

ECM	extracellular matrix
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
Egr1	early growth response 1
EMT	epithelial-mesenchymal transition
eNOS	endothelial nitric oxide synthase
ERα	estrogen receptor-α
ERβ	estrogen receptor-β
ERE	estrogen response element
ERK1/2	extracellular signal-regulated kinase-1/2
FAK	focal adhesion kinase
FBS	fetal bovine serum
FSH	follicle-stimulating hormone
FSHR	follicle-stimulating hormone receptor
GDP	guanosine diphosphate
GTP	guanosine triphosphate
GnRH	gonadotropin releasing hormone
GPCR	G protein-coupled receptor
GPER	G Protein-Coupled Estrogen Receptor 1
HB-EGF	heparin-bound epidermal growth factor
HER-2	human epidermal growth factor receptor 2
H&E	hematoxylin and eosin
HIF-1α	hypoxia-inducible factor-1α

HRE	hormone response element
HSP90	heat shock protein 90
IGF-1R	insulin-like growth factor-1 receptor
KO	knockout
LBD	ligand-binding domain
LH	luteinizing hormone
LHR	luteinizing hormone receptor
MAPK	mitogen-activated protein kinase
MEC	mammary epithelial cells
mERα	membrane-associated estrogen receptor-α
MMP	matrix metalloproteinase
MMTV	murine mammary tumor virus
NFκβ	nuclear factor-κ B
NGS	normal goat serum
NO	nitric oxide
P4	progesterone
PBS	phosphate-buffered saline
PBS-T	phosphate-buffered saline + 0.1% Tween 20
PFA	paraformaldehyde
PI3K	phosphotidylinositol 3-kinase
PKA	protein kinase A
PR	progesterone receptor
PRMT1	protein arginine *N*-methyltransferase 1

P/S	penicillin/streptomycin
PyMT	polyoma middle T antigen
SEM	standard error of the mean
SERM	selective estrogen receptor modulator
SMA	α-smooth muscle actin
SRF	serum response factor
TAM	tamoxifen
TEB	terminal end bud
TF	transcription factor
TGF-β	transforming growth factor-β
TNBC	triple negative breast cancer
VEGF	vascular endothelial growth factor
WT	wild type

CHAPTER 1

INTRODUCTION

1.1 Estrogen

Estrogen, the primary female sex hormone, is the best characterized member of the steroid hormone family that includes progestins, androgens, glucocorticoids, and mineralocorticoids. There are three primary estrogens produced by the human body, estrone (E1), estradiol (E2), and estriol (E3) (Figure 1.1). E1 is produced principally outside of the ovaries by adipocytes and is responsible for most of the estrogenic effects in postmenopausal women [15]. Throughout pregnancy, E3 produced by the placenta becomes the dominant estrogen [16]. While both the 17α- and 17β-estradiol isoforms of E2 are produced, 17β-estradiol, designated E2 throughout this study, is more physiologically active. In the adult, non-pregnant, premenopausal female, E2 is the most potent estrogen [17] with a wide range of physiologic effects, including maturation of the reproductive and mammary tissues, maintenance of bone density [18], and immune system modulation [19], as well as neuro- and cardioprotection [20, 21]. E2 is also important in male fertility with effects on testicular and sperm development [22-24].

While E1 and E3 are predominantly produced by adipocytes and the placenta, respectively, E2 is predominately produced in the ovarian follicle, although local synthesis does occur in low amounts in adipocytes, liver, adrenal gland, bone, vascular endothelium, and brain tissue [25-27]. Within the ovarian follicle, theca interna and granulosa cells are regulated by the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH) to initiate the multi-step process of converting cholesterol into E2. Regulating the synthesis of gonadotropins, the hypothalamus releases nocturnal pulses of gonadotropin releasing hormone (GnRH) that bind to the GnRH receptors on gonadotrope cells within the anterior pituitary gland to stimulate production and secretion

of LH and FSH [28] (Figure 1.2 A). LH then binds to the LH receptors present on the theca interna cells in the ovary, triggering the conversion of cholesterol into progesterone (P4) and, subsequently, to the androgens androstenedione or testosterone. FSH can also stimulate the production of P4 within the granulosa cells; however, granulosa cells are incapable of converting P4 into androgen due to the lack of 17β-hydroxysteroid dehydrogenase. Any P4 produced within the granulosa cells must be transported to the theca interna cells to be converted to androgen. Because theca interna cells do not express aromatase, the p450 enzyme required to convert androgen into E2, the androgen must be transported to the granulosa cells where the final aromatization can occur [29] (Figure 1.2 B). The E2 is then released into the serum and disseminated throughout the body. High E2 serum levels inhibit hypothalamic secretion of GnRH, thereby forming a negative feedback loop and the basis for the oscillatory nature of estrogen production and menstrual cycle [28].

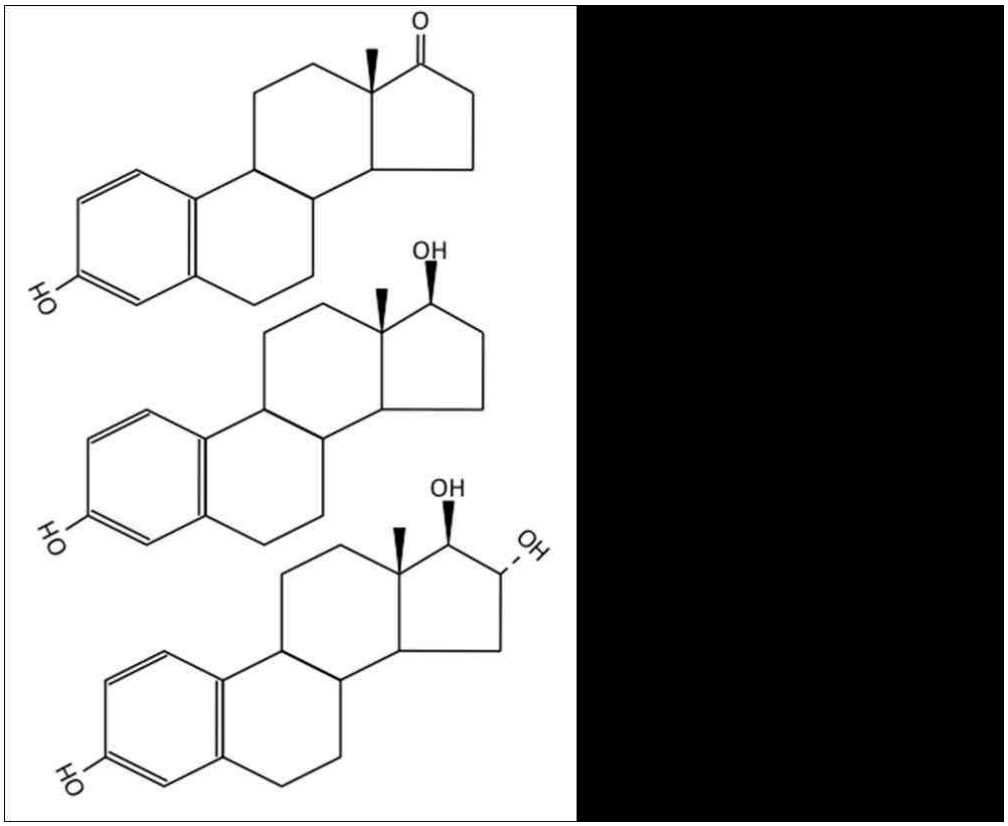
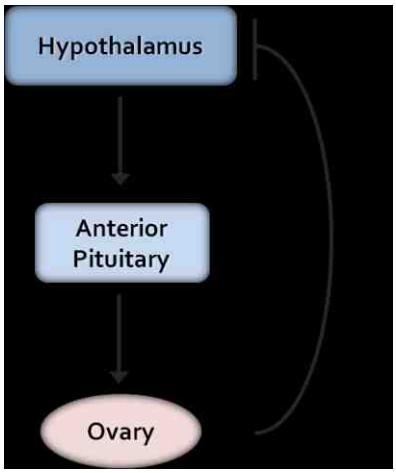


Figure 1.1 Physiologic estrogens. Chemical structures of the three naturally occurring estrogens.

A



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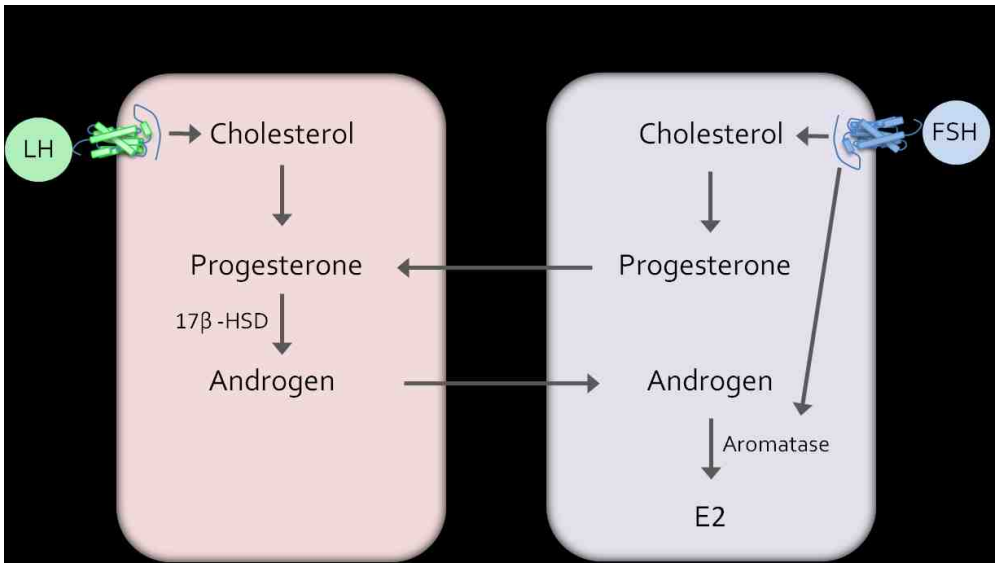


Figure 1.2 E2 synthesis. A) The hypothalamus releases gonadotropin releasing hormone (GnRH) inducing the anterior pituitary gland to produce luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH and FSH stimulate the ovarian production of E2. High E2 levels inhibit GnRH. **B)** LH binds LH receptors on ovarian theca interna cells, converting cholesterol into progesterone into androgens androstenedione or testosterone through 17β-hydroxysteroid dehydrogenase (17β-HSD). FSH binds FSH receptors on ovarian granulosa cells, producing progesterone. Progesterone produced in the granulosa cells must be transported to theca interna cells and converted to androgen. Androgen must be transported to granulosa cells where FSH increases aromatase conversion of androgen into E2.

1.2 Classical Estrogen Receptors

While E2 was first isolated in 1930 [30], it was several decades before estrogen receptor-α (ERα) was characterized in 1973 as a receptor responsible for estrogen-dependent signaling [31]. Following the development and analysis of a mice with a targeted deletion of the ERα gene in 1993 [32], it became apparent ERα was not solely responsible for all estrogenic effects, as some E2-dependent effects persisted, and a second estrogen receptor, ERβ, was discovered shortly thereafter in 1996 [33].

ERα (*ESR1*) and ERβ (*ESR2*) are members of the highly conserved nuclear receptor family of transcription factors. ERα and ERβ both contain six structural domains: A/B, C, D, E, and F. The central C domain is comprised of two zinc fingers responsible for DNA binding and is the most highly conserved domain with 95% sequence homology between ERα and ERβ. This allows for both receptors to bind to the same *cis*-acting hormone response elements (HREs), termed estrogen response elements (EREs), located within the promoter regions of target genes [34]. While the C domains of ERα and ERβ are highly conserved, variability within the NH_2-terminal, A domain allows for the recruitment of diverse co-regulatory proteins and transcription factors. This leads to distinct, non-redundant roles for the two receptors, particularly within the immune, skeletal, cardiovascular and central nervous systems [35-37]. The A/B domain also contains the constitutively active activation function-1 (AF-1). A second activation function (AF-2) and the ligand-binding domain (LBD) are located within the E domain. Sixty percent sequence homology between the two receptors within the E domain results in similar binding affinities for E2 (K_d = 0.05 – 0.1nM) [34, 38] (Figure 1.3 A).

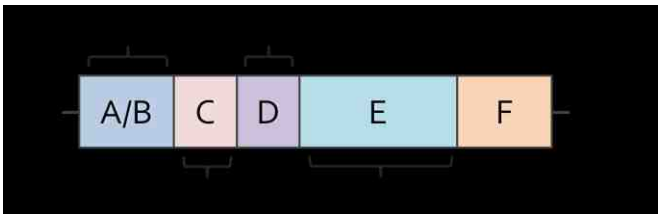
1.2.1 Classical Estrogen Receptor Signaling

Due to its small, lipophilic structure, estrogen passively crosses the cellular and nuclear membranes to bind to the predominantly nuclear localized estrogen receptors. E2 binding to the LBD induces a conformational change, leading to the release of inhibitory heat shock protein 90 (HSP90) chaperones from the D domain and the dimerization of the receptor along the LBD. ERα and ERβ are transcriptionally active in both homo- and α/β heterodimerized conformations due to homology within the DNA binding domain (DBD) and LBD of the ERs [39]. The dimerized receptor then binds to the ERE present within the promoter sequence of the target gene and regulates transcription, either positively or negatively depending upon the recruited cofactors. As ERα and ERβ are members of the nuclear receptor family of transcription factors, the ERE contains two half-sites with the sequence RGGTCA, separated by three base pairs [39]. The ER dimer may also alter transcription of non-ERE containing genes through association with the AP-1 or SP-1 transcription factors and their associated binding sites [40, 41]. Additionally, phosphorylation by MAPK of Ser^{118} within the AF-1 region of ERα leads to ligand-independent transcriptional activation of estrogen-responsive genes [42] (Figure 1.3 B).

Apart from the long-term genomic responses induced by transcriptional activation, ERα and ERβ also effect rapid signaling, non-genomic cellular responses, as quickly as a few seconds after ligand binding. While the genomic effects are enacted through nuclear localized ER functioning as a transcription factor, many of the non-genomic effects occur through palmitoylated, and thus membrane associated, ERα (mERα) [43]. These rapid signaling effects include the production of phospholipase C and cAMP leading to the influx of Ca^{2+} and associated downstream signaling, the release

of nitric oxide within the vascular endothelial cells resulting in vasodilation, and the activation of Src and thereby the PI3K and MAPK pathways [43, 44] (Figure 1.3 B).

A



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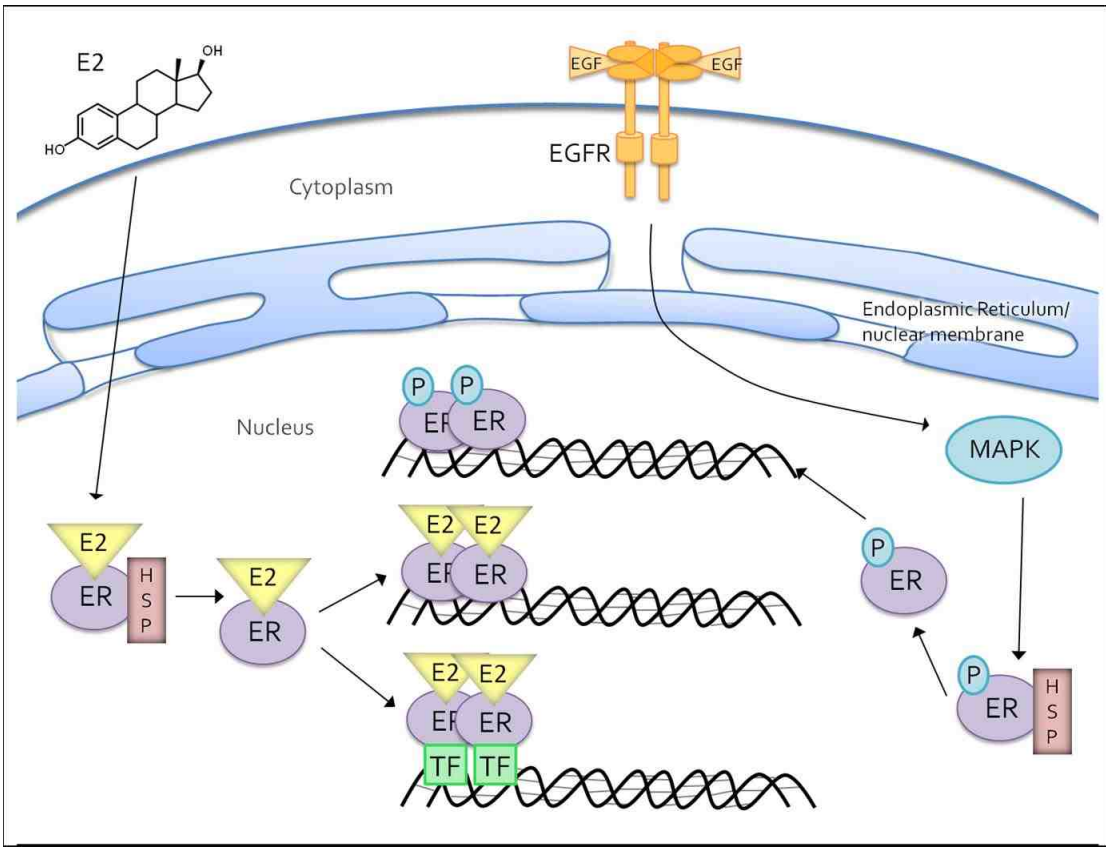


Figure 1.3 ER structure and signaling. A) ERα and ERβ contain six domains. The A/B domain contains activation function-1 (AF-1) and recruits cofactors. The C domain contains the DNA binding domain (DBD). AF-2 and the ligand-binding domain (LBD) are in the E domain. **B)** E2 crosses the membranes to bind ER inducing a conformational change and releasing of the inhibitory heat shock protein 90 (HSP90) chaperones. ER dimerizes along the LBD and binds to the estrogen response element (ERE) or recruits transcription factors (TF) to regulate transcription. MAPK phosphorylates ERα, inducing ligand-independent transcription.

1.3 G Protein-Coupled Estrogen Receptor 1

In addition to the classical estrogen receptors, ERα and ERβ, a third estrogen receptor, the G Protein-Coupled Estrogen Receptor 1 (GPER), has been identified. Unlike the classical receptors, GPER is a member of the G protein-coupled receptor (GPCR) superfamily and, as such, does not act directly as a transcription factor. GPER was first cloned in the late 1990's and named GPR30, designating it as an orphan seven-transmembrane (7TM) GPCR [45-48]. Previously demonstrated to play a role in estrogen signaling [1, 49], GPR30 was characterized as an independent estrogen receptor in 2005 and renamed GPER [50, 51].

1.3.1 GPER Signaling

GPER is the only known GPCR with a steroid ligand identified in vertebrates [52]. Unlike other known GPCRs, GPER was determined to localize primarily on the endoplasmic reticulum with possible rapid cycling to the plasma membrane [53, 54]. GPER binds E2 with a slightly weaker binding affinity (K_d = 3 - 6.6nM) than the classical ERs (K_d = 0.05 - 0.1nM); however, it remains within the physiological range [55].

As a GPCR, GPER associates with the heterotrimeric G proteins, $G_{\alpha s}$ and Gβγ [50, 51]. E2 activation of GPER induces a conformational change in the G_α subunit allowing for the exchange of the inactive bound GDP for GTP [56]. Following dissociation from the Gβγ subunit and GPER, $G_{\alpha s}$ then activates adenylyl cyclase and cAMP, leading to calcium-dependent signaling [49, 51]. Meanwhile, the Gβγ subunit activates Src, leading to the activation of matrix metalloproteinases (MMPs) to cleave heparin-bound epidermal growth factor (HB-EGF) and subsequent transactivation of the

epidermal growth factor receptor (EGFR) and the mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling pathways [1]. Activation of the MAPK cascade and transcription factors, such as Elk-1 and serum response factor (SRF), induce proliferation through the upregulation of c-fos, Bcl-2, cyclin D1, connective tissue growth factor (CTGF), and early growth response 1 (Egr1), among others [13] (Figure 1.4). GPER signaling through Src also leads to the development of SHC-integrin α5β1 complexes, structures critical for fibronectin matrix assembly and cellular movement and migration [57]. Additionally, GPER may induce cellular effects independent of G proteins. Following ligand binding, GPER is phosphorylated by GPCR kinase, leading to the recruitment of β-arrestin2 [58]. In most other GPCRs, β-arrestins, once recruited to a ligand-bound receptor, act as an adaptor protein, scaffolding large complexes of intracellular signaling molecules, such as Src, ERK1/2, PI3K, and NFκB [59, 60]. Thus, it may be possible for GPER, through β-arrestin2, to directly activate the MAPK and other kinase cascades to induce proliferation rather than relying on G protein signaling alone.

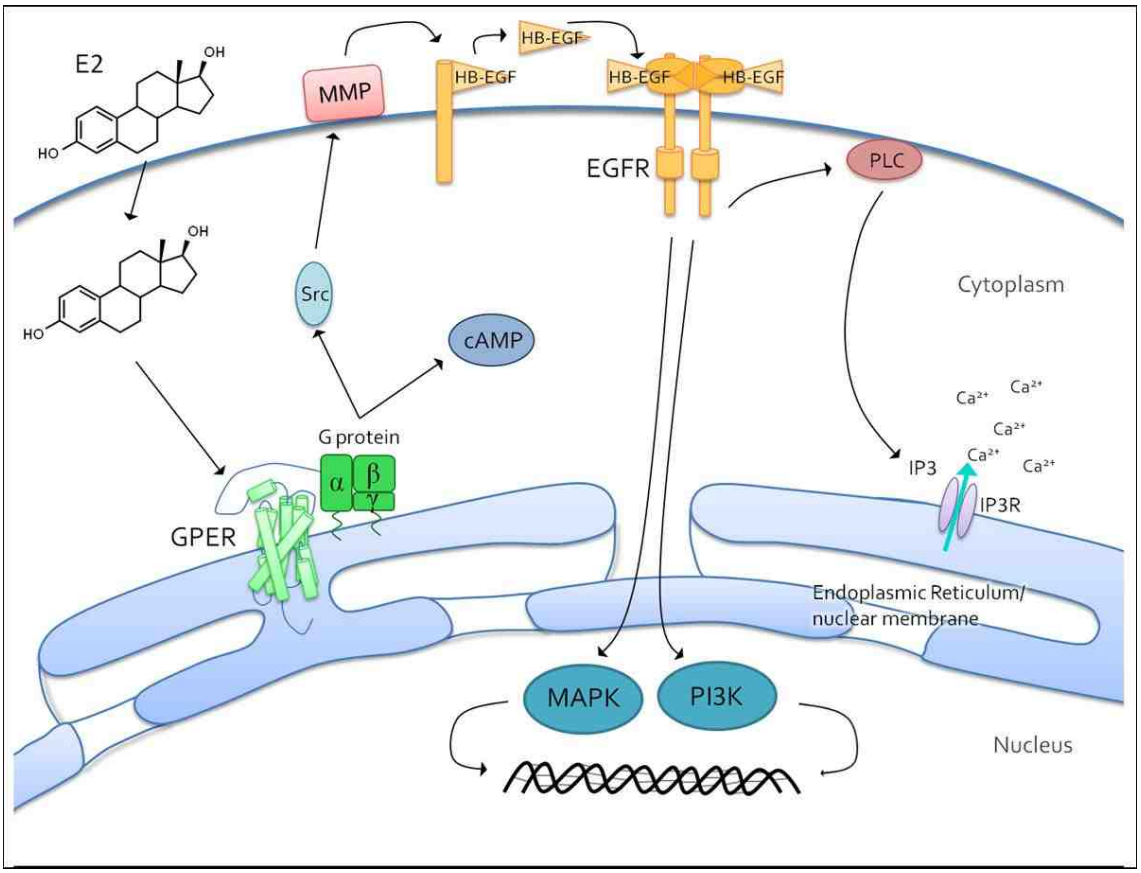


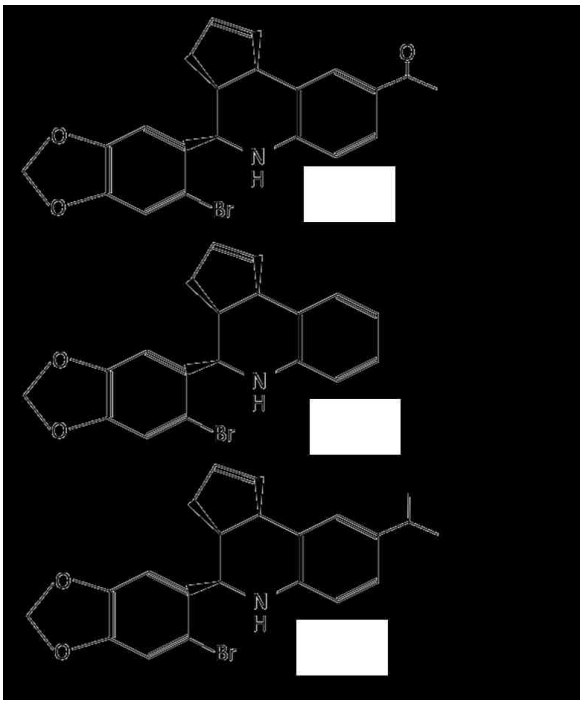
Figure 1.4 GPER signaling. E2 binds GPER and releases $G_{\alpha s}$ which activates adenylyl cyclase and cAMP resulting in calcium flux. The Gβγ subunit activates Src. Src activates matrix metalloproteinases (MMPs) to cleave heparin-bound epidermal growth factor (HB-EGF) and transactivates epidermal growth factor receptor (EGFR). EGFR stimulates the mitogen activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K) signaling and transcription.

1.3.2 Selective Ligands

Because ERα, ERβ, and GPER are often co-expressed, are activated by the same endogenous ligand E2, and share many signaling pathways, it is critical, but challenging, to ascertain the precise role of each individual receptor. This can be accomplished by manipulating the receptor expression levels, either by deleting a receptor or by examining each receptor in a system or cell type expressing only that receptor. However, it is also important to examine the activation or inhibition of each receptor in the presence of the other two to determine possible interactions. This can be achieved through the development of synthetic, selective ligands. A synthetic, GPER-selective agonist, G-1, was identified from a library of 10,000 molecules and well characterized (Figure 1.5 A). A substituted dihydroquinoline, G-1 selectively inhibits the binding of E2 to GPER while showing no inhibition of E2 binding to ERα or ERβ. G-1 activates intracellular calcium signaling and PI3K pathway in a GPER-dependent manner. No ERα- or ERβ-dependent effects, either genomic or rapid signaling, were apparent in response to G-1 [53]. Additionally, two GPER-selective antagonists, G15 and G36, have been developed based upon the GPER-selective G-1 scaffold (Figure 1.5 A). Because G15, the first antagonist identified, weakly inhibits ERα at concentrations greater than 10μM, the highly selective G36 was developed, exhibiting no inhibition of ERα or ERβ signaling [55, 61]. In the initial screens, an agonist selective for ERα and ERβ, AB-1, was also identified (Figure 1.5 B). AB-1, a phenol-substituted oxabicyclo[3.3.1]nonene, is able to selectively bind and activate ERα- and ERβ-dependent genomic effects, while exhibiting no activity toward GPER [62, 63]. These selective ligands, together with targeted gene deletion,

allow the individual roles of ERα, ERβ, and GPER to be unraveled both *in vitro* and *in vivo*.

A



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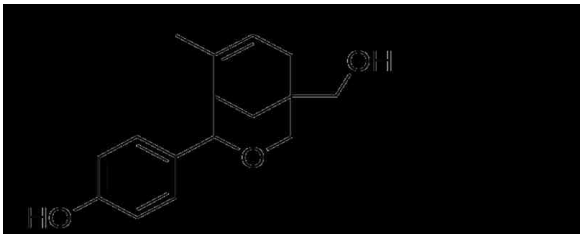


Figure 1.5 Selective ligands. A) Chemical structures of GPER-selective agonist G-1 and antagonists G15 and G36. **B)** Chemical structure of ERα- and ERβ-selective agonist AB-1.

1.4 Estrogen in Uterine Physiology

The uterus is comprised of three primary layers, the outer muscular myometrium, the basal layer of the endometrium, and the functional layer of the endometrium lining the lumen of the uterus. In the adult, non-pregnant, pre-menopausal female, the menstrual cycle induced by cyclic ovarian E2 production results in a biphasic uterine physiology. The follicular, or proliferative, phase is characterized by proliferation of epithelial cells in the functional layer of the endometrium as E2 levels increase to a maximal level at ovulation. Following ovulation, E2 levels decrease precipitously due to the negative GnRH feedback loop. During the luteal, or secretory, phase, progesterone (P4) levels increase, inducing the differentiation and proliferation of stromal cells to transform the endometrium into a receptive environment for implantation. In the absence of implantation, the functional layer is shed as P4 levels decrease [64].

1.4.1 Estrogen Receptor Expression and Function in Normal Uterus

ERα, ERβ, and GPER are all expressed in each of the three uterine layers; however, the expression levels of each receptor vary throughout the menstrual cycle. Within the human endometrium, ERα protein levels greatly increases during the proliferative phase in both epithelial and stromal cells, then decreases during the secretory phase [65, 66]. GPER mRNA expression also increases during the proliferative phase, particularly within the epithelial cells. However, while the mRNA levels decrease during the secretory phase, the GPER protein levels remain constant [67]. ERβ protein levels in the endometrium remain constant throughout the cycle [66]. Within the human myometrium, ERα mRNA is expressed to a much higher level than ERβ mRNA, although ERβ mRNA expression becomes dominant during pregnancy and post-

menopause [68, 69]. Expression of estrogen receptors is also dynamic in the postmenopausal human endometrium, as ERα protein levels decrease throughout the endometrium and GPER localization shifts from the epithelium to primarily within the stromal cells [70, 71]. Aromatase is also produced within the endometrial stromal cells, allowing for increased levels of local E2 aromatization [72].

E2 has long been known to induce proliferation and edema, or water imbibition, within the endometrial epithelial cells [73, 74]. These responses have become classical estrogen responses within the uterus [74]. Through the use of mice lacking ERα and ERβ through genetic deletion (knockout (KO)), the role of each of these receptors has been clarified. Although both receptors are required for normal male and female fertility, as ERαKO mice are sterile and ERβKO mice are either infertile or subfertile, their cellular effects are quite distinct [32, 75]. ERαKO uteri are hypoplastic and exhibit half of the normal wet weight, a measure of water imbibition [32]. While there is no change in wet weight compared to wild type mice, ERβKO mice develop endometrial hyperplasia and a decreased level of epithelial cell differentiation [75]. Taken together, it becomes clear E2 stimulation of ERα increases both epithelial cell proliferation and uterine wet weight. ERβ appears to play a regulatory role, dampening ERα-induced proliferation, consistent with anti-proliferative findings *in vitro* [76]. Although proliferation occurs in the lumenal epithelial cells of the endometrium, E2 activates ERα receptors localized within adjacent stromal cells, triggering epithelial proliferation through a paracrine response independent of ERα epithelial expression [77, 78]. The role of GPER in these classical estrogenic uterine responses has yet to be determined.

1.4.2 Estrogen Receptor and Function in Endometrial Cancer

Endometrial cancer is the most common gynecologic malignancy with 49,560 estimated new cases and 8,190 deaths in the US in 2013 [79]. The primary risk factor for development of endometrial cancer is prolonged exposure to estrogen, particularly estrogen unopposed by P4 [80]. Additionally, women receiving the selective estrogen receptor modulator (SERM) tamoxifen (TAM) for treatment of breast cancer are at an increased risk of developing endometrial cancer [81]. Because TAM is a SERM, it acts as an ER agonist in some tissues and an antagonist in other. *In vitro*, TAM induces proliferation in numerous endometrial cancer cell lines [82, 83]. TAM targets the AF-2 domain of ERα and ERβ, sterically hindering the binding of E2 within the LBD, and preventing the ensuing conformational change within the receptor. In tissues such as the breast, where ligand-dependent AF-2 activity is dominant, TAM serves as an ERα antagonist and is one of the recommended therapeutic for ERα positive breast cancer [84, 85]. In endometrial tissue, however, ligand-independent AF-1 activity and recruitment of coregulatory proteins is more prevalent, and TAM serves as an ERα agonist [86]. TAM has also been shown *in vitro* to act as a GPER agonist in both endometrial and breast cells [50, 87].

Estrogen receptor expression has been correlated with endometrial cancer stage and prognosis. In stage I/II tumors, indicating the tumor has not yet invaded beyond the body of the uterus and cervix, high ERα expression is indicative of a good prognosis. Decreased ERα expression levels are correlated with markers of poor outcome, such as aggressive histological subtypes, adnexal spread, and recurrence, regardless of tumor stage [88]. ERβ expression was not correlative with outcome in early stage tumors [89].

Expression of both ERα and ERβ, however, is decreased in higher grade and advanced stage endometrial cancer associated with myometrial invasion and de-differentiation [90]. Contrary to ERα, high GPER expression levels correlate with markers of poor prognosis, such as high grade, advanced stage, cervical involvement, and aggressive histology, as well as an overall lower survival rate. This correlation becomes more pronounced with high-grade III/IV tumors, those that have spread beyond the uterus and cervix. In addition, the expression of GPER inversely correlated with ERα expression levels [70]. In a positive feedback loop possibly leading to the progression of the tumor, TAM stimulation can also increase GPER expression levels *in vitro* [91]. Despite this *in vitro* and correlative data, very little is known concerning the role of GPER in both normal and malignant endometria.

1.5 Estrogen in Breast Physiology

The mammary gland is unique as the majority of the organ development and maturation occurs after birth during puberty. Within both male and female embryos, thickening of the ventral skin develops by birth into a rudimentary ductal structure supported by the surrounding mammary fat pad. The gland continues to grow in proportion with the body until puberty [92]. At puberty, the ovaries begin their cyclic production of E2, and additional development occurs within the female mammary gland. The repetitive cycles of E2 signaling induces proliferation within the terminal end buds (TEBs) resulting in the elongation and branching of the ductal structure throughout the fat pad. During pregnancy, the final step for full maturation occurs as branching and alveologenesis reach peak levels. In a fully developed, lactating mammary gland, the

ducts are lined by a single layer of milk producing epithelial cells surrounded by contractile myoepithelial cells to assist in milk ejection. Upon the cessation of lactation, the ductal structure undergoes apoptotic remodeling known as involution [73].

1.5.1 Estrogen Receptors in Normal Breast Physiology

Once more, the roles of ERα and ERβ in mammary development have been elucidated through the use of KO mice. While ERα, ERβ, and GPER are all expressed, prepubertal development is independent of estrogen signaling as no defects are observed in ERαKO, ERβKO, or GPERKO mice [35, 93]. However, ERαKO mice never develop beyond the rudimentary structure present at birth [94]. The mechanism of this developmental defect is complicated by multiple roles for ERα within the epithelial and stromal compartments. One method of determining the role of these receptors in individual components of the mammary gland is through the orthotopic transplant model. Prior to the pubertal development, the epithelial duct structure is restricted to the proximal end of the mammary fat pad and can be surgically removed. New epithelium is then orthotopically transplanted, allowing it to grow in a physiologically relevant environment [95, 96]. ERα is expressed in both the epithelial and stromal cells of the developing mammary gland; however, ERα is heterogeneous, expressed in only 10-15% of lumenal epithelial cells [2]. When ERαKO and ERα wild type (WT) mammary epithelial cells (MEC) are mixed *in vitro* and transplanted into an ERαWT fat pad, only the ERαKO cells proliferate, although they are unable to do so without the ERαWT MEC co-transplantation [97]. Thus, consistent with previous findings, E2 activates ERα to stimulate the proliferation of adjacent ERα negative cells [2]. This paracrine signaling is believed to occur through ERα-induced cleavage of membrane bound amphiregulin by a

disintegrin and metalloprotease (ADAM)-17. Amphiregulin then activates EGFR signaling and proliferation on adjacent ERα negative cells [98]. In support of this, amphiregulinKO and ADAM-17KO mice both show impaired ductal development [99].

In addition to the effects seen within the ERαKO epithelial cells, the ductal defects may also be attributed to stromal cell impairment. To determine these stromal effects, an ERαKO fat pad denuded of epithelia was orthotopically transplanted into the fat pad of an ERαWT mouse. As the endogenous WT ducts grew, they avoided the KO fat pad [97]. Without the pro-proliferative ERα stromal signals, the anti-proliferative signals from the highly expressed stromal ERβ likely became dominant, inhibiting elongation within the fat pad [100]. ERβKO mice exhibit delayed, but normal, branching and ductal development [101]. The delay is likely caused by the irregularity of the estrous cycle due to ovarian perturbation [102]. ERβKO mice have difficulty lactating due to a lack of terminal epithelial cell differentiation. With the removal of the ERβ inhibitory signal, the mammary gland becomes filled with cysts as the mice age, due to excessive epithelial proliferation and minimal differentiation [101]. This data further demonstrates the need to examine the cross-talk and interaction not only between the three estrogen receptors, but also between the epithelial and stromal compartments of the breast.

1.6 Estrogen in Breast Cancer

Breast cancer is the most frequent form of cancer and second leading cause of cancer-related deaths in women with an estimated 232,340 new cases diagnosed and 39,620 deaths in the US in 2013. It is estimated one in eight women will develop breast cancer over the course of their lifetime [79]. Prolonged lifetime exposure to estrogen,

through early menarche, late menopause, or hormone replacement therapy, increases the risks of breast cancer, whereas the lack of cycling estrogen levels and induction of epithelial differentiation during pregnancy and lactation is protective [103-106]. This carcinogenic effect of estrogen exposure is primarily due to two mechanisms. First, E2 exposure has been shown to result in DNA adducts and mutations [107]. Second, DNA repair systems are unable to clear these adducts and other damage incurred as E2 increases the proliferation rate of the epithelial cells, resulting in increased accumulation of DNA mutations [108]. Evidence for the role of estrogen in breast cancer progression first became apparent in the 1880's. It was discovered that removal of the patient's ovaries resulted in the regression of the tumor [109]. However, not all tumors respond similarly to estrogen. As a result, a complex breast cancer classification system has arisen.

Upon a diagnosis of breast cancer, tumor samples are taken and tested for various factors to determine stage, grade, and subtype. These factors combine to provide a recommended treatment plan and prognosis. Breast cancer is staged according to size, lymph node involvement, and distant metastasis [110]. The tumor is also graded based on differentiation, defined as tubule formation or nuclear pleomorphism, and proliferative activity, defined by the mitotic index [111]. While stage and grade of a tumor are good indicators of the current level of tumor progression, they reveal very little about the genetic profile of the tumor and thus about the response to treatment. For this reason, additional histological markers are examined, the most important of which are the expression levels of ER, progesterone receptor (PR), and human epidermal growth factor receptor 2 (HER2), a member of the EGFR family with no known ligand [112]. Of all

breast cancers, 75-80% are initially ER and PR positive and hormone responsive, allowing for the use of anti-estrogen and aromatase inhibitor (AI) treatment. HER2 overexpression occurs in 10-15% of breast cancer increasing the likelihood of either homodimerization or dimerization with another member of the EGFR family and results in the activation of the MAPK and PI3K signaling cascades. HER2 overexpressing tumors are good candidates for the dimerization blocking anti-HER2 antibody trastuzumab [113]. However, the remaining 10-15% does not express these three receptors. Classified as triple negative breast cancer (TNBC), patients presenting with these tumors have fewer treatment options [114]. Additional information is now available to assist in characterizing tumors further through the increased implementation of a new test, PAM50, which examines expression levels of 50 additional genes [115]. Taken together, this diagnostic information has resulted in improved prognosis and outcome for breast cancer patients [116].

1.6.1 Classical Estrogen Receptors in Breast Cancer

ERα is initially expressed in 75-80% of all breast cancer. Expression levels often increase as low-grade ductal carcinoma *in situ* (DCIS) progresses to high-grade DCIS. Once the tumor begins to invade the surrounding tissue, ERα expression decreases in most tumors [117]. While it is thought E2 can induce tumorigenesis without ERα through the induction of DNA adducts, long-term exposure to E2 fails to induce tumorigenesis in ERαKO mice [118]. ERα proliferation signaling is likely a main cause for this pro-tumorigenic role. Unlike the paracrine signaling mechanism found in normal mammary epithelium, ERα positive breast cancer cells stimulate their own proliferation in a loosely regulated manner [119]. ERα increases the expression of both myc and cyclin D1 to

promote cell cycle progression. While this transcriptional regulation is also present in normal mammary epithelial cells, ERα is able to induce these effects in breast cancer cells more frequently and in a ligand-independent manner through increased activation from overexpressed HER2. The interaction between HER2 and ERα also activates the MAPK and PI3K cascades to stimulate additional proliferation [120]. ERβ continues to regulate ERα-induced proliferation by inhibiting the transcription of cyclin D1, myc, p53, p21, FOXO1, and other cell cycle regulators, thereby inducing G1 cell cycle arrest [121]. ERβ exhibits anti-tumorigenic effects by decreasing the levels of de-differentiation within breast cancer cells, a hallmark of tumor progression. However, ERβ expression levels often begin to decrease immediately in DCIS, preventing ERβ from inhibiting proliferation and de-differentiation [122].

In addition to increasing proliferation, E2 also increases the metastatic abilities of breast cancer cells. Metastasis is a multi-step process in which tumor cells develop a motile phenotype, leave the primary tumor site by entering the blood or lymphatic system, and circulate throughout the body to establish a secondary tumor site. Breast cancer most often develops metastatic lesions within the lung, brain, and bone. E2 increases motility in breast, endometrial, and ovarian cancer cells *in vitro* [5, 123, 124]. One method by which ERα may increase migration is through E2 activation of protein arginine *N*-methyltransferase 1 (PRMT1). PRMT1 transiently methylates ERα which allows ERα to form a cytoplasmic complex with PI3K, SRC, and focal adhesion kinase (FAK). The increased focal adhesion turnover results in increased migration [125].

Because of the pro-tumorigenic role of ERα, several antagonists were developed as breast cancer therapies, the most effective of which was TAM. Unlike in the

endometrium, TAM functions as an effective ERα antagonist in breast cancer cells. In premenopausal women with ERα positive breast cancer, administration of TAM for five years is the recommended first line of treatment [85]. However, 30-50% of ERα positive patients with advanced disease do not respond to TAM. Of those patients that do respond, TAM is not effective after 5 years of treatment due to acquired resistance [126]. Regardless of ERα status, TAM functions as a GPER agonist, and this agonism is thought to be one way breast cancer cells acquire TAM resistance [127].

1.6.2 GPER in Breast Cancer

While the role of GPER in normal mammary biology is relatively understudied, GPER has been repeatedly demonstrated to play a significant role in breast cancer. In 321 breast cancer cases, 60% expressed GPER at or above levels found in normal breast tissue. 40% of the tumors co-expressed GPER and ERα, and, interestingly, of the 122 ERα negative tumors, half expressed GPER. This high GPER expression correlated with increased tumor size, HER2 overexpression, and increased metastasis [14]. This contrasts with the inverse correlation of ERα expression levels with each of these variables [128]. However, in a separate study, loss of GPER expression correlated with increased breast cancer metastasis [129]. It is important to note that in all of these studies, the expression of GPER was assessed in the tumor cells only, not in the surrounding microenvironment. Additionally, GPER positive patients treated with TAM had higher GPER expression levels and a lower relapse free survival rate than those either not treated with TAM or treated with AI [130].

Similar to ERα, GPER can exert pro-tumorigenic effects through stimulation of both proliferation and migration. First, GPER increases proliferation in both ERα positive

and negative breast cancer cells *in vitro* [1, 3]. While much of this proliferation is induced through the signaling mechanism previously described, alternative mechanisms may also exist within the deregulated environment of the cancer cell. GPER expression levels are increased by EGFR activation, particularly in ERα negative breast cancer cells, allowing the tumors to remain estrogen-responsive in the absence of ERα [131]. This overexpression correlates with EGFR and HER2 overexpression in 70% of breast tumors, significantly increasing the level of EGFR transactivation and proliferative signaling within the cell [130]. Second, several possible pathways for induction of migration have recently been identified. Interaction between the insulin-like growth factor-1 receptor (IGF-1R) and other GPCR family members, through their G proteins, has been shown to result in migration of breast cancer cells *in vitro* [132]. This transactivation of the IGF-1R has now also been verified to occur through GPER, resulting in the stimulation of both proliferation and migration of ERα positive breast cancer cells [4]. Possibly the most significant effect of GPER on tumor cell migration is through the upregulation of CTGF. In a microarray study of GPER-regulated gene transcription, CTGF, a known migration stimulant, had the highest induction [13]. GPER-dependent transcription of CTGF is increased even further under the hypoxic conditions commonly found within a tumor [133]. As a tumor grows, the existing vasculature becomes insufficient to fully support the tumor, resulting in poorly oxygenated, or hypoxic, regions furthest from the vasculature and the transcription of hypoxia-inducible factor (HIF)-1α. Among a vast array of pro-tumorigenic effects, HIF-1α increases GPER expression levels, thereby increasing CTGF levels [134]. This increased CTGF results in increased proliferation and migration of breast cancer cells *in vitro* [13, 135]. However, within a tumor, the majority

of CTGF is not produced by the tumor cells themselves, but by the nearby stromal fibroblasts, suggesting an interesting role for GPER in the tumor-stroma signaling interplay.

1.6.3 Breast Cancer Microenvironment

While extensive effort and research has been put forth to understand and target tumor cells, focusing on the tumor alone misses the important contributions of the microenvironment in promoting tumorigenesis. Although most tumor cells develop from an epithelial origin, they receive significant regulation, either pro- or anti-tumorigenic, from the surrounding stroma. The influence can be so strong that a debate has developed as to whether the first carcinogenic signal originates in the epithelium, thereby activating the stroma, or in the stroma, inducing transformation of the epithelium [136-138]. Thus it is important to examine both the efferent signaling pathway, from the tumor cells to the stroma, and the afferent pathway, from the stroma to the tumor cells. The stroma of the mammary gland is distributed throughout the adipocytes of the fat pad and is quite heterogeneous, containing fibroblasts, inflammatory and immune cells, and blood vessels with pericytes, endothelial, and smooth muscle cells. The stroma plays a significant role in both carcinogenesis and tumor progression. In the orthotopic transplant model previously discussed, irradiation of the denuded mammary fat pad prior to transplantation of new normal epithelium resulted in tumor development [139]. Once breast tumors have developed, the presence of altered, or desmoplastic, extracellular matrix (ECM) is indicative of a more aggressive tumor and worse prognosis [140]. Although the ECM is composed of more than 300 different proteins, collagen, fibronectin, and tenascin-C are

the most common ECM components in breast cancer and are produced primarily by cancer-associated fibroblasts in the stroma [141].

Fibroblasts are the most common cell type within the stroma; however, most of these fibroblasts are quiescent. When the surrounding tissue is wounded, these fibroblasts become activated, transforming into α-smooth muscle actin (SMA) expressing myofibroblasts that assist in the wound repair and tissue remodeling [142]. Cancer is a chronic, highly inflammatory malignancy, resulting in many of the same fibroblast activating signals found in wounds. As such, it is often referred to as "the wound that never heals" [143]. These signals recruit surrounding fibroblasts and begin to activate them. In the initial stages of tumor development, fibroblasts are anti-tumorigenic, inhibiting the growth of the tumor via gap junctions [144]. As the tumor progresses, the fibroblasts become pro-tumorigenic and are classified as cancer-associated fibroblasts (CAFs) [145]. The presence of an elevated number of CAFs recruited to the tumor is a marker of increased tumor size, metastasis, and poor prognosis [9, 10]. One mechanism of CAF activation is through the transforming growth factor (TGF)-β signaling pathway. Within the ECM, TGF-β forms a complex with CTGF through which CTGF increases the affinity of TGF-β for its receptor and enhances the activation of the Smad signaling cascade to induce activation. A positive feedback loop is established as TGF-β then upregulates CTGF gene transcription [12]. *In vitro* evidence indicates increased expression of CTGF may be sufficient to induce fibroblast activation [146], and since GPER upregulates CTGF, these findings suggest GPER may stimulate fibroblast activation.

Once activated, CAFs begin remodeling and stiffening the tumor ECM by decreasing production and degradation of the collagen IV found in normal mammary microenvironment and increasing the secretion of collagens I and III, fibronectin, and tenascin-C [141]. CAFs also produce lysyl oxidase that increases the covalent bonds within collagen fibrils, further stiffening the ECM [147]. Several pro-tumorigenic effects result from this compressed, desmoplastic ECM. The compressed ECM increases the concentration of local soluble growth factors, thereby increasing tumor proliferation [148]. It also leads to higher interstitial pressure within the tumor, preventing drug diffusion, thereby minimizing therapeutic efficacy. In a mouse model of pancreatic adenocarcinoma, pharmaceutical inhibition of CAFs resulted in lower interstitial pressure and increased chemotherapeutic efficiency [149]. The desmoplastic ECM also provides a better substrate for tumor cell migration. In a recent study, tumor cell migration was shown to be dependent upon the desmoplastic ECM. It also demonstrated CAFs generate force- and protease-mediated tracks in the ECM, allowing the metastasizing tumor cells to follow behind [150]. These tracks have also been shown *in vivo* in the margins of human breast cancer samples [151]. Once in the vasculature, CAFs protect the tumor cells from cell death as a result of anoikis and immune surveillance. By metastasizing with their own primary site CAFs, the tumor cells are more likely to successfully establish a secondary, metastatic lesion. Presumably, the metastasizing CAFs begin remodeling the secondary site ECM and creating a pro-tumorigenic environment more quickly than newly recruited and activated secondary site fibroblasts [152]. GPER has been shown, through increased CAF production of CTGF, to increase the proliferation and migration of both CAFs and breast cancer cells *in vitro* [13, 153]. The ability of

GPER to increase CTGF, a known fibroblast activator, combined with the pro-metastatic effect of CAFs, suggests a role for GPER in the transformation of normal mammary fibroblasts into CAFs, resulting in the increased tumor cell migration and metastasis associated with high GPER expression.

1.7 Project Rationale

The impact of E2 on breast development and maturation, as well as in the development and progression of breast cancer, has been well characterized. E2 increases proliferation of normal breast epithelial and tumor cells. It also increases the motility of tumor cells and fibroblasts. An important challenge is to elucidate the role of specific estrogen receptors in these responses. ERα increases proliferation through the rapid activation of MAPK and PI3K signaling cascades and the genomic upregulation of myc and cyclin D1 transcription [39, 43]. More recently, it has become evident that GPER-mediated E2 signaling likely plays an important but poorly characterized role in breast cancer etiology. Unraveling the specific role of GPER will enhance our understanding of breast cancer etiology, and will be particularly relevant for the study of tamoxifen-resistant breast cancer and tamoxifen-induced endometrial cancer [91, 127, 130]. GPER increases proliferation within tumor cells through the activation of the MAPK and PI3K cascades [1, 51]. It also increases CTGF production by CAFs *in vitro*, a key signaling molecule in the migration and metastasis of tumor cells [13]. This suggests an important role for GPER in microenvironmental regulation of tumor progression, the understanding of which is critical to improving treatment and survival of breast cancer patients.

1.8 Hypothesis and Specific Aims

While the majority of estrogenic effects in the breast have been attributed to ERα, recent studies provide strong evidence for a significant contribution of GPER to estrogen-dependent responses in both normal and tumor tissue. However, the molecular mechanisms by which GPER mediates tumor progression, and the specific tissue targets of GPER activity, remain unclear. The studies described herein were designed to address some of these gaps.

Thus, we hypothesize activation of GPER induces breast epithelial cell migration and fibroblast activation, thus increasing breast cancer metastasis.

SPECIFIC AIM 1: Determine the role of GPER activation on breast epithelial cell and tumor cell migration *in vitro*.

1.1: Assess the effect of GPER activation on epithelial cell migration.

1.2: Determine the effect of GPER activation on breast tumor cell migration.

SPECIFIC AIM 2: Determine the role of GPER activation in fibroblast-dependent breast cancer metastasis.

2.1: Assess the effect of GPER in fibroblast activation and function *in vitro.*

2.2: Assess the effect of GPER in fibroblast-dependent metastasis *in vivo.*

SPECIFIC AIM 3: Determine the role of GPER, ERα, and ERβ in normal endometrium estrogenic responses.

2.1: Examine the effects of ERα and ERβ in normal endometrium *in vivo* using selective compounds.

2.2: Examine the effect of GPER in normal endometrium *in vivo* using selective compounds.

CHAPTER 2

GPER REGULATES BREAST EPITHELIAL AND CANCER CELL MIGRATION

2.1 Abstract

Migration is a critical process in the development and progression of both normal mammary ductal structures and breast cancer. While E2 regulation of cell motility has been demonstrated repeatedly, the role of GPER in mediating E2-dependent migration in both normal and cancer cells has not been fully examined. GPER is known to upregulate pro-migratory MAPK and PI3K signaling pathways, increase vimentin, and increase the production and activation of MMP-9, all necessary actions for increased migration [1, 4-6]. It has also been theorized GPER may increase p-FAK through activation of Src [7, 8]. However, GPER may also inhibit breast cancer cell migration [53]. In the present study, GPER activation was demonstrated to increase collective migration within the non-tumorigenic MCF10A breast epithelial cell line. Concomitant with GPER-dependent migration, increased expression and activation of proteins supporting collective migration was observed, including vimentin, p-FAK, E-cadherin, and β-catenin, without increased proliferation. Conversely, GPER inhibited the individual migration of tumorigenic PyMT breast cancer cells derived from a mouse breast cancer model.

2.2 Introduction

The development and maturation of the mammary gland beyond a rudimentary structure begins at puberty and is dependent upon ovarian 17β-estradiol (E2) production. The repetitive cycles of E2 production induces proliferation and migration within the epithelial cells of the terminal end buds (TEBs) resulting in the elongation and branching of the mammary ducts throughout the fat pad [73]. E2 induces proliferation in normal and breast cancer cells through both the classical estrogen receptor ERα and the GPCR GPER

[1-3] and inhibits proliferation through ERβ [76]. E2 also regulates the migration necessary for elongation and branching. In collective migration, as exemplified in branching morphogenesis, leading cells found in the TEB form actin- and vimentin-dependent membrane protrusions enriched with integrins and matrix metalloproteinases (MMPs) [6, 154, 155]. Integrins are heterodimeric extracellular matrix (ECM) receptors comprised of an α and β subunit. Depending upon the specific composition of the ECM, any of twenty-four known integrin heterodimers may be expressed [156]. The primary integrin found within migrating mammary epithelial cells is α5β1 due to the high fibronectin content within the ECM of normal mammary gland [157]. Intracellularly, integrin-ECM binding initiates the assembly of focal adhesions at the attachment site, inducing the autophosphorylation of focal adhesion kinase (FAK). This phosphorylation allows Src to bind, further phosphorylating and activating FAK. Activated FAK then serves as a scaffold for pro-migratory downstream signaling cascades, recruiting Src, MAPK, and PI3K [158]. Cell migration is accomplished through the formation of focal adhesions along the leading edge of a cell and dissolution of adhesions along the trailing edge. Without FAK and the downstream Src recruitment, focal adhesions would remain adhered to the ECM, preventing migration [8]. In addition to migration, leading cells must also proteolytically degrade the ECM through the production and activation of MMPs to generate a migratory path through the basement membrane surrounding the epithelial cells [159]. Because cell-cell contacts such as adherens and tight junctions remain intact during this process, cells attached to the leading cells are passively pulled along, resulting in the elongation of the mammary duct [154].

E2 regulates this migration at multiple points throughout the process. Through ERα, E2 activates Src thereby regulating the activation of FAK, the downstream pro-migratory MAPK and PI3K signaling, and the turnover of focal adhesions [7, 158]. As a result, ERαKO mice exhibit no elongation or branching of ducts upon stimulation with E2, never developing beyond the rudimentary structure present at birth [94].

The pro-migratory MAPK and PI3K pathways can also be initiated through GPER transactivation of the EGFR and interaction with the insulin-like growth factor-1 receptor (IGF-1R) [1, 4]. GPER also increases migration through the upregulation of vimentin within the leading cells and increased MMP-9 activity [5, 6]. Additionally, Src can be activated by E2 in an ERα-independent, G protein-dependent manner and is significantly elevated in tamoxifen-resistant breast cancer cells [7, 8]. Since Src is a critical activator of FAK, this suggests a role for GPER in the regulation of migration through Src and FAK.

The migration observed in ductal morphogenesis is mechanistically similar to the process by which breast cancer cells metastasize. Metastasis is a five step process by which cells 1) invade through the basal lamina into the surrounding tissue, 2) intravasate into the blood or lymphatic vessels within the primary tumor site, 3) survive apoptosis and immune surveillance while circulating through the vascular system, 4) extravasate into the tissue of a secondary tumor site, and 5) establish a metastatic lesion. Breast cancer most often develops metastatic lesions within the lung, brain, and bone, and the presence of distant metastases elevates a breast cancer case to the most advanced stage and worst predicted outcome [110].

Metastasis of cancer cells has been observed to occur through two primary methods: individual cell migration or collective migration. While both migration methods are often detected, leukemia, lymphoma, and most solid stromal tumors seem to preferentially metastasize through individual migration, and most epithelial tumors appear to metastasize through collective migration [160, 161]. The mechanism for both collective and individual tumor cell migration is nearly identical to branching morphogenesis with integrin, MMP, and FAK involvement, although much more loosely regulated than in normal breast development [161]. However, individual migration requires the dissolution of cell-cell contacts, most notably the downregulation of E-cadherin. E-cadherin is a transmembrane protein that dimerizes with E-cadherin on adjacent epithelial cells forming adherens junctions. E-cadherin is linked to the actin cytoskeleton through β-catenin and assists in epithelial cell apical-basal polarization [162]. Loss of E-cadherin and adherens junctions results in a loss of apical-basal cell polarity and de-differentiation of epithelial tumor cells into a more motile, mesenchymal phenotype, permitting individual tumor cells to migrate away from the main tumor mass. Thus, decreased E-cadherin expression is a primary hallmark of tumor cells undergoing the epithelial-to-mesenchymal transition (EMT) [160]. The loss of E-cadherin alone is sufficient to induce migration and invasion, increasing metastasis [163]. High E-cadherin expression levels are frequently found in ERα positive breast cancer since ERα decreases expression levels of snail and slug, the primary transcriptional repressors of E-cadherin [164, 165]. However, as ERα decreases with advanced tumor progression, expression of E-cadherin is concomitantly reduced, increasing the probability of tumor metastasis [163]. The loss of ERβ also leads to a decrease in E-cadherin expression, consistent with

the role of ERβ in promoting differentiation [101, 166]. The loss of ERα and expression of normal to high levels of GPER are both independently correlated with increased breast cancer metastasis [14, 128], again suggesting a strong role for GPER in migration. However, GPER may also inhibit breast cancer cell migration [53]. Indeed, in a separate study, loss of GPER expression correlated with increased breast cancer metastasis [129]. It is not currently known under what conditions GPER may promote or inhibit migration.

In the present study, GPER activation is demonstrated to increase collective migration within breast epithelial cells. This is confirmed by the upregulation of the collective migration markers vimentin, p-FAK, E-cadherin, and β-catenin, as well as the lack of increased proliferation. Conversely, GPER inhibits the individual migration of breast cancer cells.

2.3 Methods

2.3.1 Cell lines and reagents. Immortalized, non-transformed, non-tumorigenic MCF10A human breast epithelial cells (ATCC, Manassas, VA; catalog number CRL-10317) were cultured in MCF10A complete media (DMEM/F-12 supplemented with 5% horse serum, 10μg/mL insulin, 100ng/mL cholera toxin, 0.5μg/mL hydrocortisone, 20ng/mL recombinant epidermal growth factor (EGF) and 1% penicillin/streptomycin (P/S)). PyMT breast cancer cells, a clonal immortalized, tumorigenic cell line, were previously generated in the lab from the mammary tumors of a 7wk FVB/N-Tg(MMTV-PyVT)634Mul/J (MMTV-PyMT) transgenic mouse, that overexpresses the polyoma middle T antigen (PyMT) under the control of the mouse mammary tumor virus (MMTV) promoter [167]. PyMT cells were maintained in epithelial cell medium

(DMEM/F-12 supplemented with 5% fetal bovine serum (FBS), 10µg/mL insulin, 0.5µg/mL hydrocortisone, 10ng/mL EGF and 1% P/S). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37°C. Dulbecco's Modified Eagle Medium (DMEM)/F12, phenol red-free DMEM/F12, E2, FBS, NGS, horse serum, insulin, cholera toxin, hydrocortisone were from Sigma. EGF and penicillin/streptomycin (P/S) were from Invitrogen. G-1 was synthesized as described [53] and provided by Dr. Jeffrey Arterburn (New Mexico State University).

2.3.2 Wound healing assay. Wound healing "scratch" assays were performed as previously described [168]. Briefly, cells were passaged either into 24-well cell culture plates or onto 12mm glass coverslips (Electron Microscopy Sciences, Hatfield, PA) for immunostaining. Confluent cells were cultured in phenol red-free MCF10A media with all supplements listed above for 24 hr, then washed and placed in starvation media (phenol red-free DMEM/F-12 supplemented with 2% dextran-charcoal-stripped FBS, 10µg/mL insulin, 100ng/mL cholera toxin, 0.5µg/mL hydrocortisone, and 1% P/S) for 24hr to induce cell synchronization as previously described [169]. Following synchronization, cells were pretreated for 24 hours with dimethylsulfoxide (DMSO) vehicle control, E2 (10nM and 100nM), G-1 (10nM and 100nM), or EGF (10nM). Following pretreatment, a 0.5cm wound was scratched into the center of the confluent cells with a 2µL pipet tip with an average width of 200µm. The treatment media was replaced to remove cell debris. The wound was imaged at four marked locations with a Zeiss 35 Axiovert microscope using MetaMorph® software at 0, 12, 15, 18, 21, and 24hr post-wound. Migration was analyzed by measuring the total area for each wound using

MetaMorph® software. The area of the wound at each time point was expressed as the percentage of the 0hr wound area remaining.

2.3.3 Electric cell-substrate impedance sensing (ECIS) wound healing assay. ECIS migration assays were performed in the ECIS model Zθ (Applied Biophysics) as previously described [170]. Briefly, PyMT cells were seeded into ECIS electrode arrays (8W10E) (Applied Biophysics) in complete PyMT epithelial media. Confluent cells were washed, placed in starvation media (phenol red-free DMEM/F-12 supplemented with 10µg/mL insulin, 0.5µg/mL hydrocortisone, and 1% P/S) for 24h, and pretreated with DMSO vehicle control, E2 (100nM), G-1 (100nM), or EGF (10nM) in starvation media for 48h. Treatment media was changed, and baseline impedance was measured in ECIS normal mode (1µA at 4 kHz) at 5min intervals for 1hr. The monolayer was electrically wounded with an elevated field pulse of 5mA at 60 kHz for 2min, resulting in a rapid decrease in impedance. The increasing impedance indicative of migration was measured in ECIS normal mode for 24hr. Impedance for each time point was normalized to the first impedance measurement after wounding.

2.3.4 Transwell® migration assay. MCF10A cells were placed in starvation media described above for 24hr and pretreated for 48 hours in starvation media with DMSO vehicle control, E2 (10nM and 100nM), G-1 (10nM and 100nM), or EGF (10nM). Cells were resuspended and seeded into 8µm pore size Transwell® inserts (Costar) in a 24-well plate at 1 x 10^5 cells/well. Starvation media containing treatment was added to both the upper and lower chamber and 10% c.s. FBS was added to the lower chamber as a chemoattractant. After 48h of incubation at 37^{o}C, non-migrated cells were removed from the upper side of the membrane with cotton swabs, and the cells on the lower surface of

the membrane were fixed in cold methanol on ice for 10min. Membranes were mounted with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Five random fields from each membrane were imaged with a Zeiss 200M Axiovert microscope using MetaMorph® software. The total number of cells for each membrane were counted and normalized to the vehicle control.

PyMT migration assays were performed similarly. Cells were placed in starvation media for 24hr. Cells were seeded into 8μm pore size Transwell® inserts (Costar) in a 24-well plate at 2 x 10^4 cells/well. Starvation media containing DMSO vehicle control, E2 (100nM), G-1 (100nM), or EGF (10nM) was added to both the upper and lower chamber and 10% c.s. FBS was added to the lower chamber as a chemoattractant. Cells were allowed to migrate for 24hr.

2.3.5 Immunostaining analysis. For immunostaining, cells were cultured on 12mm acid-washed, lysine coated, glass coverslips and fixed with 4% paraformaldehyde for 20 min at 20^{o}C. The cells were permeabilized with cold methanol for 10 min on ice and blocked with 5% normal goat serum (NGS) diluted in phosphate-buffered saline (PBS) for 15 min at 20^{o}C. Cells were stained with primary antibody diluted in 5% NGS + PBS 1hr at 20^{o}C in a humidified chamber followed by detection with secondary antibody for 1hr at 20^{o}C. Coverslips were mounted on slides with Vectashield mounting medium with DAPI (Vector Laboratories). Three random fields from each coverslip were imaged with a Zeiss 200M Axiovert microscope using MetaMorph® software. Primary antibodies used were rabbit anti-Ki-67 (Thermo Fisher), mouse anti-phospho-FAK (Y397) (BD Biosciences), mouse anti-vimentin (3B4) (Millipore), mouse anti-E-cadherin (BD Biosciences), and mouse anti-β-catenin (BD Biosciences). Secondary antibodies were anti-rabbit and anti-

mouse IgG antibodies conjugated to Alexa 488 (Molecular Probes). Preliminary studies examining vimentin, p-FAK, β-catenin, and E-cadherin are all n=1.

2.3.6 Statistics. Statistical analysis was performed using GraphPad Prism® version 4.03. Comparisons of results between different treatment groups were determined using a one-way Analysis of Variance (ANOVA) followed by a Bonferroni correction for multiple comparisons as a post-hoc test. Data represents the mean ± SEM of three or more separate experiments. P-values less than 0.05 were considered to be significant.

2.4 Results

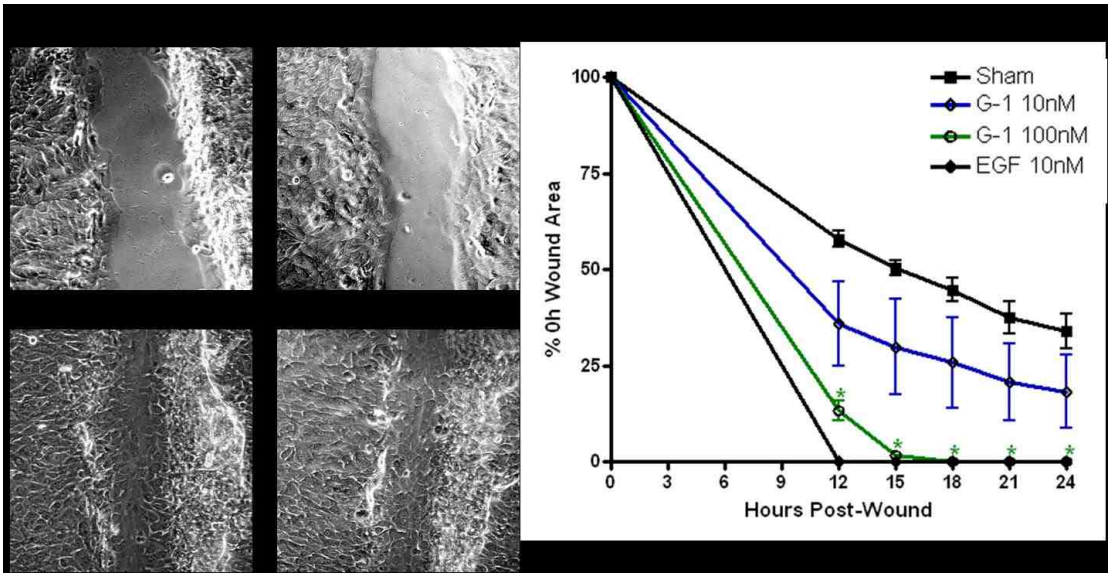
2.4.1 GPER increases breast epithelial cell migration

Although E2 activation of GPER stimulates many pathways involved in migration, such as increased MAPK and PI3K signaling, vimentin, and MMP production, no studies have been performed to determine the effect of GPER on normal breast epithelial cell migration required for proper breast development. In order to study such effects, MCF10A cells were selected as an ideal *in vitro* model system. MCF10A cells are immortalized, non-transformed human breast epithelial cells isolated from a reduction mammoplasty and have many features of normal epithelial cells. Genetically, they exhibit a near-diploid karyotype with minimal rearrangement. *In vitro*, these cells demonstrate three-dimensional growth in collagen controlled by hormones and growth factors and an inability to grow in anchorage-independent conditions. Finally, *in vivo* MCF10A cells are non-tumorigenic and as such, when injected into immune compromised mice, no tumor will develop [171]. In addition, MCF10A cells express only GPER, containing insignificant levels of ERα or ERβ, allowing specific examination of the role of GPER

activation by both the endogenous ligand, E2, and the synthetic, GPER-selective ligand, G-1 [172].

Using MCF10A cells, a wound healing "scratch" assay was performed to determine the role of GPER in normal breast epithelial migration. MCF10A cells were seeded to confluency, starved of growth factors and E2, and pretreated for 48hr with E2 or G-1. The cells were wounded and migration was observed. G-1 and E2 both stimulated significantly increased migration in a dose-dependent manner, as compared to the sham control (Figure 2.1). At 100nM, G-1 activation of GPER induced a significant increase in migration beginning at 12hr after the initial wounding with full wound closure by 18hr (Figure 2.1 A). Likewise, 100nM E2 also significantly increased migration, though at a slower rate than G-1, and did not achieve full wound closure by 24hr post-wound (Figure 2.1 B). As GPER is the only known estrogen receptor expressed within MCF10A cells, this may indicate a differential GPER response to the two ligands. This functional selectivity between two ligands binding the same GPCR and eliciting qualitatively different outcomes has been previously reported [173]. Importantly, both ligands significantly increased epithelial cell migration. Visual inspection indicated the cells did not break off and migrate individually, instead migrating as a sheet to close the wound, indicative of the collective migration seen in ductal morphogenesis.

A



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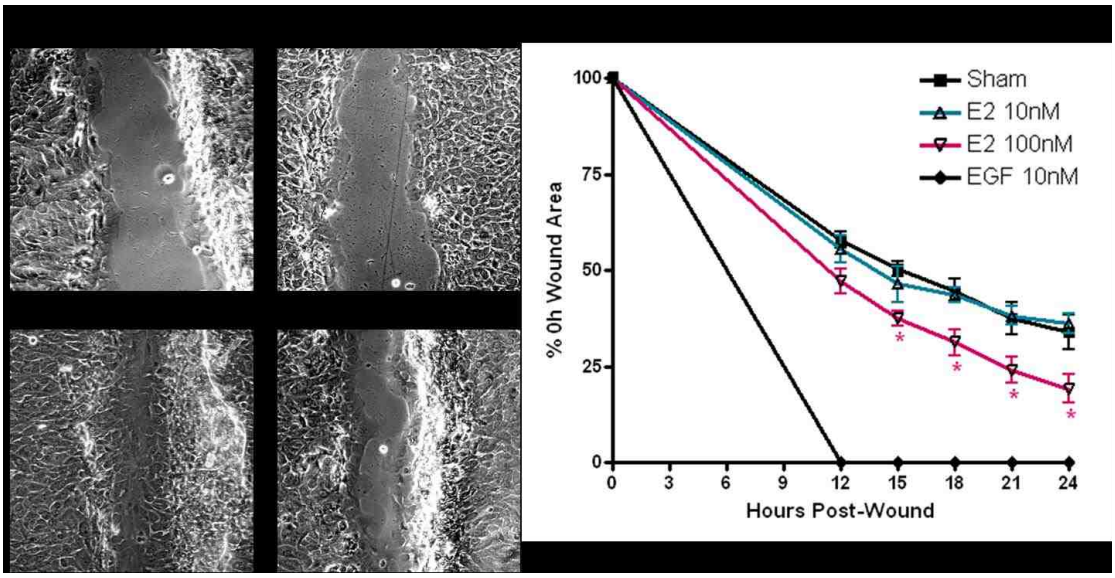
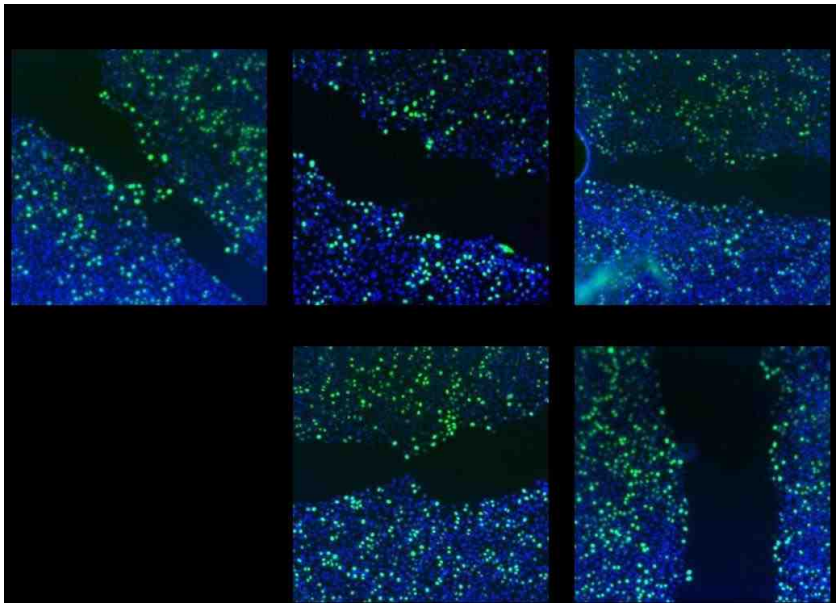


Figure 2.1 GPER increases breast epithelial cell migration. Wound healing scratch assays were performed on MCF10A cells pretreated for 48hr with **A)** G-1 or **B)** E2. The wound area was photographed at 0, 12, 15, 18, 21, and 24hr post-wound. Images shown are 24hr post-wound. Migration was quantified as described in Methods.
*p < .05 compared to sham.

2.4.2 GPER has no effect on proliferation in migrating epithelial cells

While the sheet-like movement induced by GPER activation is highly indicative of collective migration, it is possible the cells were not migrating, but proliferating into the wound, effectively closing the wound by increasing cell numbers. MCF10A cells have previously been demonstrated to migrate collectively, independent of proliferation [6]. However, because GPER is known to increase proliferation, it is important to distinguish between the two responses as a cause of the wound closure. To do so, MCF10A cells were grown on coverslips, and a wound healing assay was performed as described above. Proliferation was analyzed through the immunodetection of Ki-67, a nuclear protein present in all cells within any stage of the cell cycle except G_0. Although proliferating cells were detected, the proliferation was uniform throughout the monolayer, and there was no difference in proliferation following treatment with E2 or G-1 as compared to the sham control (Figure 2.2). This confirms the increased wound closure induced by GPER activation is a result of increased cell migration.

A



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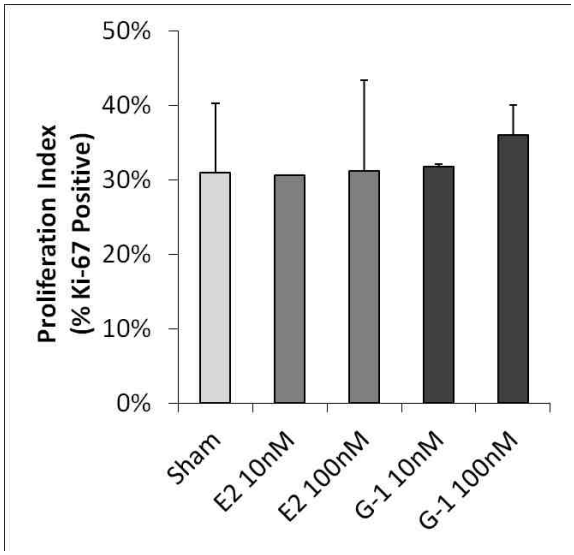


Figure 2.2 GPER has no effect on proliferation in migrating epithelial cells. A) MCF10A cells treated with E2 or G-1 were immunostained to detect the proliferation marker Ki-67 (green) and counterstained with DAPI nuclear stain (blue) **B)** Quantitation of proliferation. Data is expressed as a percentage of total cells positive for Ki-67 at the wound edge.

2.4.3 GPER increases expression of proteins required for collective migration

Visual inspection of MCF10A cell migration in wound assays indicates a collective migration phenotype (Fig. 2.1). To confirm this, preliminary studies were performed measuring the expression of proteins induced during and required for collective migration. Migratory MCF10A cells are known to increase vimentin expression in a polarized manner along the leading edge of the cells, assisting in the formation of membrane ruffles and protrusions necessary for migration. Vimentin expression is not seen in cells away from the wound edge [6]. MCF10A cells treated with E2 or G-1 increased vimentin along the wound edge, but not away from the wound, as compared to the sham control (Figure 2.3). Vimentin was polarized to the leading edge of the cell, in front of the nucleus, indicative of a migrating cell. Additionally, collective migration necessitates an increase in cell-cell contacts, specifically adherens junctions. GPER activation by both E2 and G-1 increases the level of E-cadherin within the migrating cells along the wound edge as compared to the control (Figure 2.3). There was no corresponding E-cadherin increase in the non-migratory cells distant from the wound. To test the localization of this additional E-cadherin to the adherens junctions, β-catenin protein levels and localization at the cell membrane was examined as a marker of fully formed adherens junctions. The increase in E-cadherin and adherens junctions in GPER-activated migrating cells was confirmed by a concomitant increase in membrane localized β-catenin (Figure 2.3). Finally, the expression level of phosphorylated FAK (p-FAK) was examined. P-FAK is indicative of the formation and dissolution of focal adhesions, a macromolecular complex necessary for migration linking the ECM with intracellular signaling machinery through integrins [8]. GPER activation by both E2 and

G-1 increased p-FAK expression, as compared to the sham control, in a pattern consistent with localization to focal adhesions (Figure 2.3). Cumulatively, this data indicates GPER activation does significantly increase collective migration of breast epithelial cells.

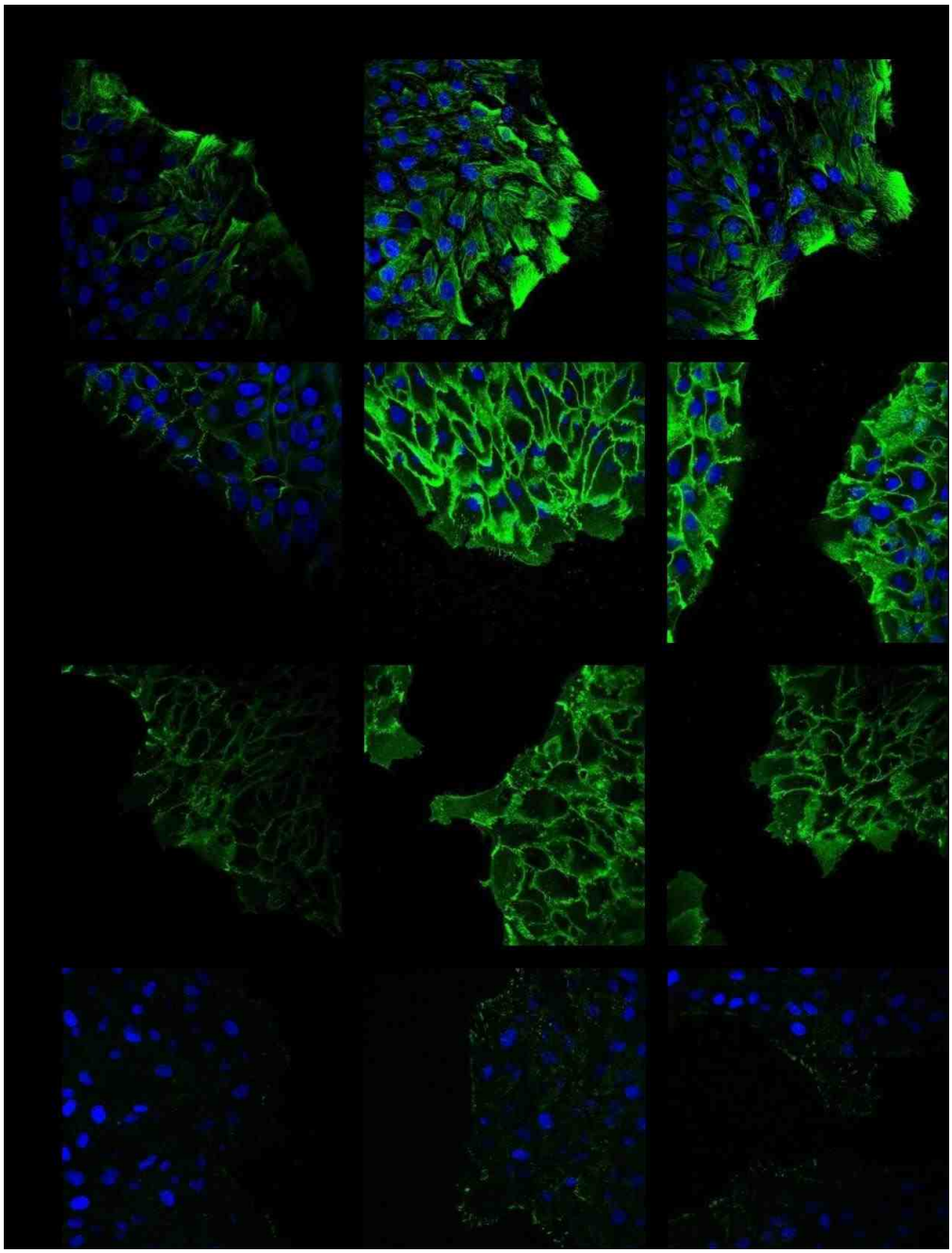


Figure 2.3 GPER increases expression of proteins required for collective migration. MCF10A cells were treated with E2 or G-1 and wounded. Cells were stained for collective migration markers vimentin, E-cadherin, β-catenin, or p-FAK, as indicated (green) and counterstained with DAPI nuclear stain (blue).

2.4.4 GPER has no effect on individual migration in epithelial cells

To test the role of GPER activation on individual migration of breast epithelial cells, MCF10A cells were again E2 and growth factor starved, pretreated for 48hr, and seeded into the upper chamber of a Boyden chamber Transwell® migration assay. A chemoattractant, 10% c.s. FBS, was placed in the bottom chamber, and cells were allowed to migrate for 48hr. In this assay, migrating cells must detach from surrounding cells to migrate individually through the pores in the membrane [168]. Compared to the sham control, no increased migration was detected following GPER activation by either G-1 or E2 at any dose (Figure 2.4). Thus, GPER does not induce individual migration in breast epithelial cells, consistent with the GPER-dependent increase in adherens junctions previously observed.

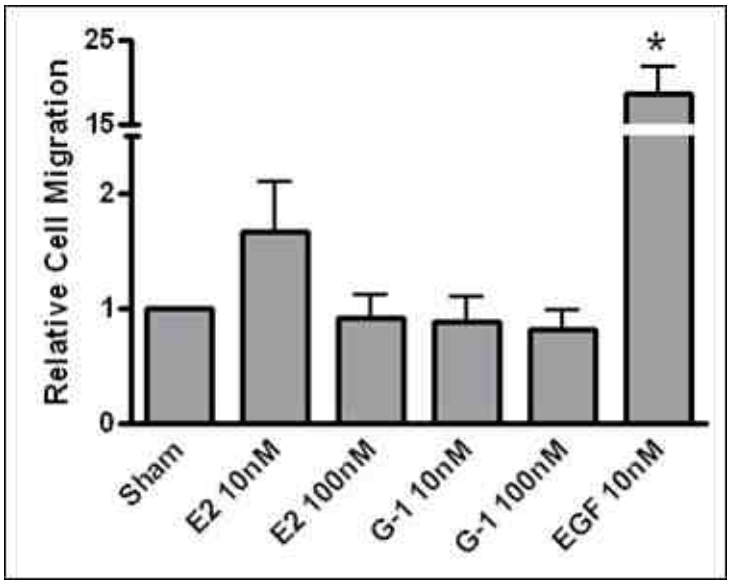


Figure 2.4 GPER has no effect on individual migration in epithelial cells. Transwell® migration assays were performed on MCF10A cells pretreated with E2 or G-1 for 48hr. Cells were allowed to migrate for 24hr. Cells that migrated through the membrane were counted and normalized to sham-treated controls. *p < .05 compared to sham.

2.4.5 GPER has no effect on combined individual and collective migration in breast cancer cells

While GPER has now been shown to induce collective migration in breast epithelial cells, the role of GPER in cancer cell migration is still unclear. GPER has been shown to both increase and inhibit breast cancer cell migration [13, 53]. To further examine the role of GPER in migration, a PyMT breast cancer cell line previously isolated and cloned from the mammary tumors of an MMTV-PyMT mouse was selected. Unlike MCF10A cells, PyMT cells express all three estrogen receptors, thus allowing any interactions affecting migration to be examined. Initially, migration of PyMT cells was assessed in a "scratch" wounding assay; however, in all treatment samples, a combination of sheet-like migration and individual cell migration was observed (data not shown). Because of the individually migrating cells, quantitation of migration was not possible. Thus, the test was repeated using an electric cell-substrate impedance sensing (ECIS) wound healing assay. This method allows for migration measurement based upon the inherent electrical current impedance properties of cell membranes. PyMT cells were E2 and growth factor starved, pretreated for 48hr, and seeded into specially developed chamber slides, containing 10 electrodes/chamber. In normal sensing mode, the ECIS machine measures impedance by applying a low electrical current that does not impact the cells [170]. A damaging current is then applied, disrupting cells directly over the electrode, thereby creating a "wound" similar to that in the scratch assay. Immediately following the wounding, there is a precipitous drop in impedance. Cells not directly over the electrode are unharmed. The normal sensing mode then measures impedance levels over time to determine the migration of cells over the electrode to cover the wound [170].

GPER activation by G-1 and E2 did not increase PyMT breast cancer cell migration, as compared to the sham control (Figure 2.5).

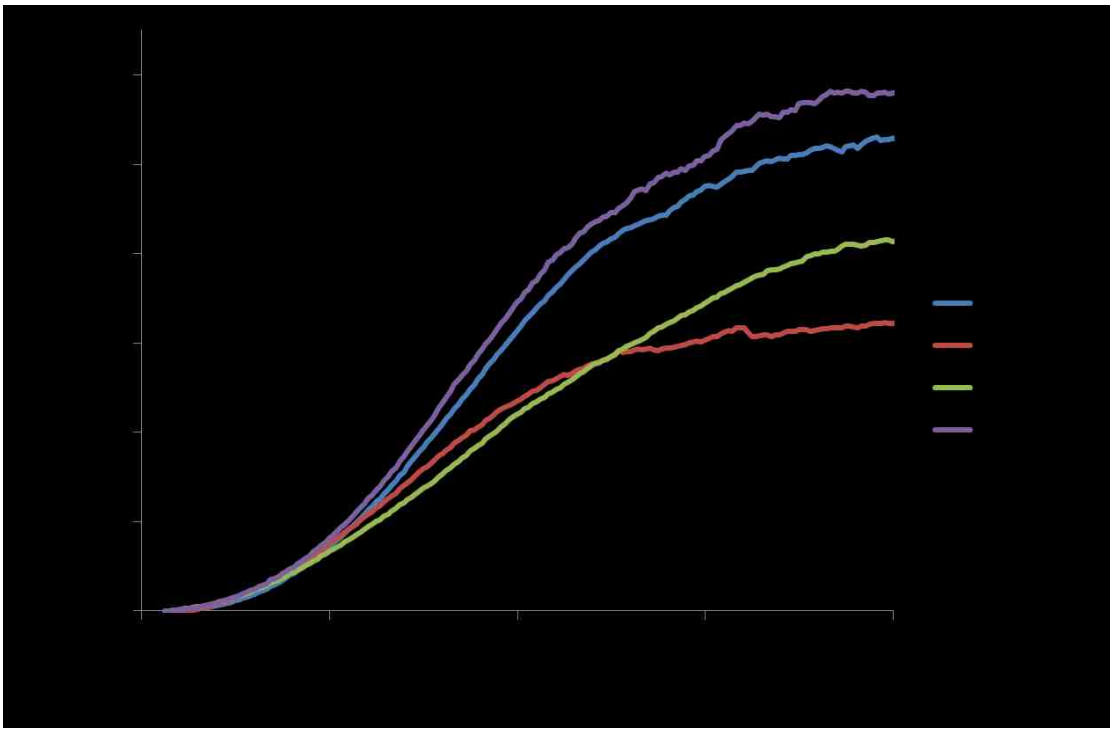


Figure 2.5 GPER has no effect on combined individual and collective migration in breast cancer cells. ECIS migration assays were performed on PyMT cells pretreated with E2 or G-1 for 48hr. Impedance was measured for 16hr and normalized to the first impedance measurement after wounding.

2.4.6 GPER inhibits individual migration in breast cancer cells

Because the ECIS wound healing assay measures both collective and individual migration indiscriminately and PyMT cell individual migration had been observed, a Transwell® migration assay was also performed to measure individual migration alone. PyMT cells were E2 and growth factor starved and seeded into the upper chamber of a Boyden chamber Transwell® migration assay. A chemoattractant, 10% c.s. FBS, was placed in the bottom chamber, and cells were allowed to migrate for 24hr. GPER activation by both E2 and G-1 significantly inhibited the individual migration of PyMT breast cancer cells as compared to the sham control (Figure 2.6). This result indicates GPER activation does inhibit individual cell migration in this model.

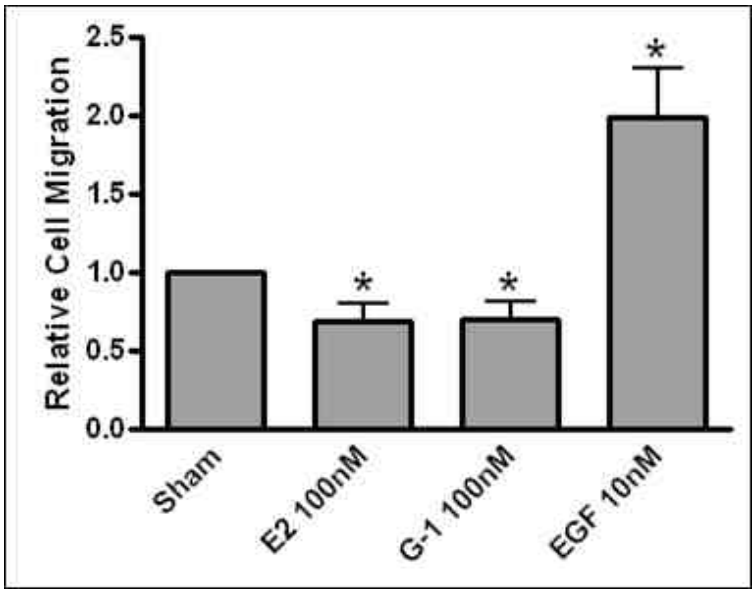


Figure 2.6 GPER inhibits individual migration in breast cancer cells. Transwell® migration assays were performed on PyMT cells treated with E2 or G-1. Cells were allowed to migrate for 24hr. Cells that migrated through the membrane were counted and normalized to sham-treated controls. *p < .05 compared to sham.

2.5 Discussion

Migration is a critical process in the development and progression of both normal mammary ductal structures and breast cancer. While E2 regulation of motility has been demonstrated repeatedly, the role of GPER in mediating E2-dependent migration has not been fully examined. GPER is known to upregulate pro-migratory MAPK and PI3K signaling pathways, increase vimentin, and increase the production and activation of MMP-9, all necessary actions for migration [1, 4-6]. It has also been theorized GPER may increase p-FAK through activation of Src [7, 8]. However, GPER may also inhibit breast cancer cell migration [53]. This is the first report of GPER inducing collective normal breast epithelial cell migration while inhibiting individual breast cancer cell migration.

GPER activation by both the endogenous ligand, E2, and the synthetic, selective ligand, G-1, results in increased migration of the non-tumorigenic MCF10A breast epithelial cell line (Figure 2.1). Because it was possible the cells were simply proliferating to fill wound, rather than migrating into it, a proliferation assay was performed. There was no difference in proliferation between GPER-activated and control-treated cells (Figure 2.2). This result would seem to contradict previous reports of GPER-induced breast epithelial cell proliferation [1]. However, GPER likely stimulates both proliferation and migration primarily through activation of the MAPK pathway. Previous reports demonstrate the MAPK cascade will not simultaneously induce both proliferation and migration within the same cell as these two signals inhibit one another through opposing regulation of cyclin D1 and thus the cell cycle [174, 175]. Within these studies, additional signaling along the wound edge resulted in a migratory signal, while

away from the wound edge proliferation occurred [175]. The lack of increased proliferation within the monolayer distant from the wound in the current study is likely an artifact of the experimental process, as the wound healing scratch assay requires a confluent monolayer. Upon reaching confluency, MCF10A cells exhibit contact inhibition and suppress proliferation [171]. Unlike in the confluent epithelial cells lining the mammary ducts *in vivo*, the MCF10A cells in this experiment were grown directly on tissue-culture treated plastic or glass and were not in contact with significant levels of ECM. Confluent epithelial cells grown on ECM similar to that found *in vivo* exhibit a lower threshold for growth factor concentration required to overcome the contact inhibition [176]. The threshold may be lower due to integrin-ECM binding stimulating additional activation of the MAPK pathway to induce proliferation [177]. This threshold is lowered still further with a stiffer ECM composition [176]. This is consistent with increased levels of the much stiffer collagen I surrounding the highly proliferative terminal end buds in the developing mammary gland *in vivo* [178].

To further confirm migration, as well as determine the method of migration, preliminary studies of several proteins required for collective migration were performed (Figure 2.3). The first step in migration is the formation of membrane ruffles and protrusions. These require coordinated remodeling of the actin and filamentous cytoskeleton. Treatment of MCF10A cells with E2 or G-1 both resulted in increased vimentin, polarized to the leading edge of the cell, indicative of migration. These membrane protrusions are enriched with integrins to bind the ECM and form focal adhesions. FAK is recruited to focal adhesions where it is autophosphorylated resulting in the binding of Src, further activation, and focal adhesion turnover [158]**.** The coordinated

formation of adhesions along the leading edge and dissolution along the trailing edge is essential for cell migration. Concomitant with increased migration, GPER activation results in increased p-FAK localized to the focal adhesions.

Characteristic of collective migration, GPER activation induced sheet-like migration of the epithelial cells. In collective migration, migrating cells increase cell-cell contacts, specifically adherens junctions, allowing the leading cells to pull the remainder of the cells along. This migration is necessary for the ductal morphogenesis and elongation occurring during mammary gland maturation [154]. Indeed, GPER activation results in increased adherens junctions as evidenced by increased E-cadherin and β-catenin, confirming GPER stimulation of collective migration. Consistent with increased cell-cell contacts, GPER activation does not result in increased individual migration within epithelial cells (Figure 2.4).

GPER exhibits seemingly contradictory migratory effects in the PyMT breast cancer cell line to those demonstrated in epithelial cells. Rather than stimulating migration, GPER activation inhibits PyMT individual migration (Figure 2.6), while having no effect on migration measured by ECIS (Figure 2.5). This inhibition may be due to the presence of ERβ in PyMT cells. While MCF10A cells only express GPER, PyMT cells express all three known estrogen receptors. In addition to its role in inhibiting proliferation, ERβ has recently been shown to inhibit migration and invasion of breast cancer cells as well [179, 180]. This inhibition can occur in a ligand-independent manner through the phosphorylation of ERβ S105 by ERK1/2 [179]. Since GPER activation increases ERK1/2 activation [1], it is possible GPER activation is inducing the phosphorylation of ERβ S105, thereby inhibiting PyMT breast cancer cell migration.

The role of ERβ S105 phosphorylation may also explain the lack of migration inhibition observed by ECIS measurement. Because PyMT breast cancer cells do not exhibit the contact inhibition present in epithelial cells and phosphorylated ERβ is unable to inhibit proliferation in a ligand-independent manner [179], the PyMT cells may have filled the wound over the electrode through GPER-induced proliferation. This would negate the GPER migration inhibition demonstrated in the individual transwell migration assay. This suggests a possible novel interaction between GPER and ERβ in the regulation of breast cancer cell migration.

CHAPTER 3

GPER REGULATION OF FIBROBLAST ACTIVATION AND FUNCTION

3.1 Abstract

As a breast tumor develops, the normal mammary fibroblasts in the surrounding stroma are recruited and activated by the increased inflammatory cytokines, primarily TGF-β, produced by the tumor, transforming into cancer-associated fibroblasts (CAFs). The pro-tumorigenic role of activated CAFs in tumor growth and metastasis has been recently characterized. The increased presence of CAFs within the tumor is correlated with increased tumor size, metastasis, and poor prognosis [9, 10]. Since metastasis is the primary cause of cancer-related death [11], it is critical to understand the mechanisms by which normal fibroblasts become activated and transformed into CAFs. Stimulation of GPER increases the production of connective tissue growth factor (CTGF), a significant enhancer of TGF-β-induced fibroblast activation [12, 13]. Additionally, GPER expression is correlated with increased breast cancer metastasis [14], suggesting a role for GPER in fibroblast activation and subsequent tumor metastasis. This study demonstrates GPER activation leads to increased normal mammary fibroblast activation and proliferation in an EGFR-ERK-dependent manner. However, GPER fibroblast activation did not result in increased migration as anticipated, but inhibited fibroblast migration *in vitro*. This inhibition was consistent *in vivo* as the loss of GPER fibroblast expression leads to increased tumor metastasis and larger metastatic lesions without increased primary tumor size. This increased metastasis was not a result of altered ECM, as GPER did not affect collagen production either *in vitro* or *in vivo*. This is the first study to demonstrate a role for GPER in both the activation of normal fibroblasts and the inhibition of tumor metastasis.

3.2 Introduction

Breast cancer is the most common cancer among women with 1 in 8 women developing it and is the second most common cause of cancer deaths for women [79]. The stage of cancer progression present at the time of diagnosis is a critical determining factor for both the prognosis of the patient and the course of treatment. There is a five-year survival rate of 89% for breast cancer as a whole; however, the survival rate for patients with advanced stage cancer upon diagnosis decreases precipitously [79]. Breast cancer staging is based upon the size of the primary tumor, lymph node involvement, and the presence of distant metastasis. The presence of distant metastasis, most commonly to the lung, brain, and bone for breast cancer patients, is indicative of stage IV progression and correlates with a poor prognosis [110]. The presence of distant metastases at diagnosis lowers the five-year survival rate to just 24% [79]. Metastasis and the growth of these secondary tumors is the most common cause of cancer-related mortality [11].

Metastasis occurs when tumor cells invade through the basement membrane and extracellular matrix (ECM) surrounding the tumor, intravasate into the nearby blood and lymphatic vascular systems, circulate throughout the body, extravasate into a secondary site, and establish a metastatic lesion. Tumor metastasis requires significant interaction and signaling between tumor cells and the surrounding stroma. The pro-tumorigenic stromal influence can be so significant that a debate has developed as to whether the first carcinogenic signal originates in the epithelium, thereby activating the stroma, or in the stroma, inducing transformation of the epithelium [136]. Thus it is important to examine both the signaling from the tumor cells to the stroma and signaling from the stroma to the tumor cells. The stroma of the mammary gland is distributed throughout the adipocytes of

the fat pad and is quite heterogeneous, containing blood vessels with pericytes, endothelial, and smooth muscle cells, inflammatory and immune cells, and fibroblasts.

While most of the fibroblasts present in the normal mammary gland are quiescent, cytokines and growth factors present in the tumor microenvironment recruit and activate the nearby fibroblasts. Although normal fibroblasts can be transformed into cancer-associated fibroblasts (CAFs) through the actions of platelet derived growth factor-α/β, basic fibroblast growth factor, and interleukin-6, by far the most common method of activation is through the transforming growth factor (TGF)-β signaling pathway [181, 182]. TGF-β is secreted by both tumor cells and fibroblasts as an inactive homodimer and bound through chaperone proteins to the ECM. Upon integrin-mediated deformation or matrix metalloproteinase (MMP) degradation of the ECM, TGF-β is released [183]. The affinity of TGF-β for its receptors, TGF-β receptor I/II, is greatly enhanced through interaction with connective tissue growth factor (CTGF) [12]. Upon binding, TGF-β activates the Smad signaling cascade and transcription of Smad-responsive genes, including CTGF. In the initial stages of tumor development, activated fibroblasts are anti-tumorigenic, inhibiting the growth of the tumor via gap junctions connecting the fibroblasts to each other [144]. As the tumor grows, TGF-β increases reactive oxygen species production within the fibroblasts, which is thought to be responsible for the inhibition of the gap junctions [184]. With the loss of the gap junctions, the fibroblasts become pro-tumorigenic and are classified as CAFs [145]. The activation of CAFs is not reversible, and, unlike fibroblasts activated during non-pathological, wound-healing processes, CAFs do not respond to apoptotic signals, resulting in a stable population of constitutively activated fibroblasts creating a highly fibrotic, desmoplastic tumor

microenvironment [182, 185]. This increased presence of CAFs within the tumor is correlated with increased tumor size, metastasis, and poor prognosis [9, 10].

Once activated, CAFs begin remodeling and stiffening the tumor ECM by decreasing production and degradation of the collagen IV found in normal mammary basal lamina and increasing the secretion of collagens I and III, fibronectin, and tenascin-C [141]. CAFs also produce lysyl oxidase which increases the covalent bonds within collagen, further stiffening the ECM [147]. This desmoplastic ECM provides a better substrate for tumor cell migration [150]. However, CAFs exert a direct influence on tumor cell metastasis as well. Increased CAF production of TGF-β and CTGF independently increases tumor cell migration [153, 186]. Tumor cells, in turn, stimulate increased CAF production of MMPs and collagen [187]. Increased MMP production and contractility allow CAFs to generate protease- and force- mediated tracks in the ECM, allowing the invading tumor cells to follow behind [150, 151]. Once in the vasculature, CAFs protect the tumor cells from cell death. By metastasizing with their own primary site CAFs, the tumor cells are more likely to successfully establish a secondary, metastatic lesion [152]. It is thought the metastasizing CAFs begin remodeling the secondary site ECM and creating a pro-tumorigenic environment more quickly than newly recruited and activated secondary site fibroblasts. Indeed, breast cancer cells co-injected with CAFs exhibit increased tumor growth, angiogenesis, and metastasis as compared to co-injection with normal fibroblasts [10]. In a separate study, co-injection with normal fibroblasts overexpressing CTGF also increased tumor growth and metastasis [188].

In CAFs, activation of GPER increases production of CTGF, thereby increasing the proliferation and migration of both CAFs and breast cancer cells *in vitro* [13, 153]. This role in fibroblasts may account for the high GPER expression correlated with increased breast cancer metastasis [14, 189], since GPER activation inhibits breast cancer cell migration, an important step in the metastatic process (Figure 2.6). However, in a separate study, loss of GPER expression correlated with increased breast cancer metastasis [129]. It is important to note that in all of these studies, the expression of GPER was assessed in the tumor cells, not in the surrounding stroma. Thus it is possible that differences in GPER stromal expression may account for the variances in these studies. The ability of GPER to increase CTGF, a known fibroblast activator, combined with the pro-metastatic effect of CAFs, suggests a role for GPER in the transformation of normal mammary fibroblasts into CAFs, resulting in the increased tumor cell migration and metastasis associated with high GPER expression.

This study demonstrates GPER activation leads to increased normal mammary fibroblast activation and proliferation *in vitro* in an EGFR-ERK-dependent manner. However, GPER fibroblast activation did not result in increased migration as anticipated, but inhibited fibroblast migration *in vitro*. This inhibition was consistent *in vivo* as the loss of GPER expression in fibroblasts leads to increased tumor metastasis and larger metastatic lesions without increased primary tumor size. This increased metastasis was not a result of altered ECM, as GPER did not affect collagen production either *in vitro* or *in vivo.* Thus, GPER stimulation likely leads to the activation of normal fibroblasts while inhibiting full transformation into CAFs, resulting in anti-metastatic activity similar to that of activated normal fibroblasts present in the early stages of tumor growth [144,

145]. This is the first study to demonstrate a role for GPER in both the activation of normal fibroblasts and the inhibition of tumor metastasis.

3.3 Methods

3.3.1 Cell lines and reagents. PyMT breast cancer cells, a clonal immortalized, tumorigenic cell line, were previously generated in the lab from the mammary tumors of a 7wk FVB/N-Tg(MMTV-PyVT)634Mul/J (MMTV-PyMT) transgenic mouse, that overexpresses the polyoma middle T antigen (PyMT) under the control of the mouse mammary tumor virus (MMTV) promoter [167]. PyMT cells were maintained in epithelial cell medium (DMEM/F-12 supplemented with 5% fetal bovine serum (FBS), 10μg/mL insulin, 0.5μg/mL hydrocortisone, 10ng/mL EGF and 1% penicillin/streptomyocin (P/S)). Cells were cultured in a humidified atmosphere containing 5% CO_2 at 37^{o}C. Dulbecco's Modified Eagle Medium (DMEM)/F12, phenol red-free DMEM/F12, E2, FBS, NGS, insulin, and hydrocortisone were from Sigma. EGF and P/S were from Invitrogen. G-1 and G36 were synthesized as described [53] and provided by Dr. Jeffrey Arterburn (New Mexico State University). EGFR inhibitor Tyrphostin AG1478, Src inhibitor PP2, and MEK inhibitor U0126 were from Calbiochem.

3.3.2 Mice. FVB/N-Tg(MMTV-PyVT)634Mul/J (MMTV-PyMT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Previously described GPER KO mice were provided by Jan Rosenbaum (Proctor & Gamble, Cincinnati, OH) [190]. GPER KO mice were backcrossed 10 generations onto FVB/NJ mice. FVB GPER KO mice were intercrossed with MMTV-PyMT mice to produce GPER wild type (WT) or knock out

(KO) MMTV-PyMT mice. Animals were housed at the animal research facility at the University of New Mexico Health Sciences Center. They were maintained under a controlled temperature of 22–23°C with a 12hr light, 12hr dark cycle and fed a normal chow *ad libitum*. All procedures were approved and carried out in accordance with institutional protocols.

3.3.3 Primary mammary fibroblast isolation and activation. To isolate normal primary mammary fibroblasts, mammary glands from 10 week-old FVB GPER WT or KO female mice were resected. Tissue was minced and dissociated in DMEM/F12 medium containing 3mg/ml collagenase A (Roche) and 250U/ml hyaluronidase (Sigma) for 3hr at 37°C with gentle rocking. Dissociated cells were strained through a 500μm sterile nitex mesh filter. Filtered cells were centrifuged at 48g for 3min at 4°C to remove the large epithelial cell clusters and debris. The supernatant was transferred to a clean tube and centrifuged at 200g for 2min at 4°C to remove the remaining epithelial cells and hematopoietic cells. The supernatant was again transferred to a clean tube and centrifuged at 500g for 5min at 4°C. The supernatant was discarded, and the fibroblast pellet was washed in DMEM/F12 media and centrifuged at 500g for 5min at 4°C twice. Isolated fibroblasts were then either plated in full fibroblast media (DMEM/F12, 10% FBS, and 1% P/S) or stored in liquid nitrogen in 90% FBS + 10% dimethylsulfoxide (DMSO). Cells were confirmed as fibroblasts by expression of fibroblast marker vimentin and absence epithelial marker cytokeratin-18.

For optimal fibroblast activation, FVB GPER WT or KO fibroblasts were seeded at low confluency (3 x 10^3 cells/well) in 24-well plate and placed in fibroblast starvation media (phenol red-free DMEM/F12, 0.5% dextran-charcoal-stripped FBS (c.s. FBS), 1%

P/S) for 24hr. Cells were then treated in starvation media with DMSO vehicle control, E2 (100nM), G-1 (100nM), or TGF-β (10ng/mL) for 72hr. For inhibitor studies, G36 (500nM), U0126 (250nM), AG1478 (10μM), or PP2 (10nM) were added simultaneously with treatment. Primary fibroblasts were discarded after 10 passages.

3.3.4 Immunostaining analysis. For cell-based immunostaining, primary FVB GPER WT or KO normal fibroblasts were cultured on 12mm acid-washed, lysine coated, glass coverslips and fixed with 4% paraformaldehyde for 20min at 20°C. The cells were permeabilized with cold methanol for 10min on ice and blocked with 5% normal goat serum (NGS) diluted in phosphate-buffered saline (PBS) for 15min at 20°C. Cells were stained with primary antibody diluted in 5% NGS + PBS 1hr at 20°C in a humidified chamber followed by detection with secondary antibody for 1hr at 20°C. Coverslips were mounted on slides with Vectashield mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Three random fields from each coverslip were imaged with a Zeiss 200M Axiovert microscope using MetaMorph® software. Fluorescence intensity was quantitated using ImageJ software (National Institute of Health). Briefly, the total fluorescence intensity for each image was measured. For background subtraction, the fluorescence intensity for three random background selections was divided by the pixel area of the background selections and multiplied by the total pixel area of the image to determine the image background intensity. The image background intensity was subtracted from the total fluorescence intensity to determine the adjusted intensity. This was then divided by the number of cells in the image (as analyzed by nuclear staining) to determine the intensity per cell. Each sample was then normalized to sham for a final relative intensity per cell.

For tissue-based immunostaining, 5μm tissue sections were deparaffinized, rehydrated, permeabilized in PBS + 0.1% Triton X-100, and blocked in 3% NGS diluted in PBS + 0.1% Tween-20 (PBS-T). Microwave antigen retrieval was performed in 0.1 M sodium citrate (pH 6). The tissue was stained with primary antibody diluted in 3% NGS + PBS-T overnight at 4^{o}C in a humidified chamber followed by detection with secondary antibody for 1hr at 20^{o}C. Coverslips were mounted on slides with Vectashield mounting medium with DAPI (Vector Laboratories). At least three random fields from each sample were imaged with a Zeiss 200M Axiovert microscope using MetaMorph® software.

Primary antibodies used were rabbit anti-Ki-67 (Thermo Fisher), mouse anti-smooth muscle actin (Abcam), rabbit anti-phospho-p44/42 MAPK (ERK1/2) (Thr202/Tyr204) and rabbit anti-p44/42 MAPK (ERK1/2) (Cell Signal), rabbit anti-CTGF and rabbit anti-collagen I (Novus Biologicals), and rabbit anti-collagen IV (N-terminal) (Sigma). Secondary antibodies were anti-rabbit and anti-mouse IgG antibodies conjugated to Alexa 488 or Alexa 555 (Molecular Probes).

3.3.5 Transwell® migration assay. Primary GPER WT or KO normal mammary fibroblasts were placed in starvation media described above for 24hr and pretreated for 72 hours in starvation media with DMSO vehicle control, E2 (100nM), G-1 (100nM), or TGF-β (10ng/mL). Cells were resuspended and seeded into 8μm pore size Transwell® inserts (Costar) in a 24-well plate at 1.5 x 10^4 cells/well. Starvation media containing treatment was added to both the upper and lower chamber and 10% c.s. FBS was added to the lower chamber as a chemoattractant. After 24h of incubation at 37^{o}C, non-migrated cells were removed from the upper side of the membrane with cotton swabs, and the cells on the lower surface of the membrane were fixed in cold methanol on ice for 10min.

Membranes were mounted with Vectashield mounting medium with DAPI (Vector Laboratories). Five random fields from each membrane were imaged with a Zeiss 200M Axiovert microscope using MetaMorph® software. The total number of cells for each membrane were counted and normalized to the vehicle control.

For the co-culture migration assays, GPER WT or KO fibroblasts were seeded into 24-well plates, placed in fibroblast starvation media for 24hr, and pretreated with DMSO vehicle control, E2 (100nM), G-1 (100nM), or TGF-β (10ng/mL) for 72hr. The cells were then thoroughly washed in PBS to remove all treatment. PyMT cells previously placed in PyMT starvation media (phenol red-free DMEM/F-12 supplemented with 10µg/mL insulin, 0.5µg/mL hydrocortisone, and 1% P/S) for 24hr were resuspended in fibroblast starvation media and seeded into 8µm pore size Transwell® inserts (Costar) at 2 x 10^4 cells/well. The lower chamber of the wells contained the 72hr treated GPER WT or KO fibroblasts in plain fibroblast starvation media (Figure 3.7 A). PyMT cells were allowed to migrate for 24hr.

3.3.6 Mammary fat pad clearance and tumor transplantation. The inguinal (#4) mammary fat pads of 3 week-old FVB GPER WT female mice were cleared of endogenous epithelium as previously described [95, 96]. On the ventral side of the mouse, an inverse Y incision was made, and the skin was gently separate from the peritoneum. The mammary tissue proximal to the fat pad lymph node was removed. Saline was added to the cavity to prevent fibrotic adhesions, and the skin incision was closed using steel tissue clips (EZ CLIPS, Stoelting). The mice were given one week to heal, and the incision was reopened. For mice receiving 25% fibroblasts, 225,000 PYMT GPER WT tumor cells and 75,000 fibroblasts of indicated genotype were resuspended in

30µl of DMEM/F12 media and injected into each cleared mammary fat pad. For mice receiving 50% fibroblasts, 150,000 PYMT GPER WT tumor cells and 150,000 fibroblasts of indicated genotype were injected. Tumor controls of 225,000 (25%) or 150,000 (50%) PYMT GPER WT tumor cells without exogenous fibroblasts were injected. The skin incision was closed using steel tissue clips. The tumors were allowed to grow for 6 weeks before resection. Lungs were insufflated with 4% PFA to preserve architecture. All tissue was fixed overnight in 4% PFA and paraffin embedded.

3.3.7 Heidenhain's AZAN Trichrome staining. Trichrome stain was performed as previously described [191]. Five µm tissue sections were deparaffinized, rehydrated, and placed in 60^{o}C azocarmine solution in water bath for 1hr. Slides were washed, placed in aniline-alcohol and acetic-alcohol for 1min each, followed by 5% phosphotungstic acid for 30min. After 1hr in Heidenhain's blue-orange, slides were rinsed in acidified water, dehydrated, and mounted using Permount mounting medium (Thermo Fisher).

3.3.8 Histological analysis of lung metastasis. Three, 5µm lung sections separated by at least 100µm were deparaffinized, rehydrated, and stained with hematoxylin and eosin (H&E). A metastatic tumor focus present in the lung parenchyma is defined as a tightly clustered group of 10 or more hematoxylin-positive cells with minimal eosin-stained stroma. Total metastatic foci were counted in the three lung sections. The area of each metastatic lesion was determined using ImageJ software (National Institute of Health).

3.3.9 Statistics. Statistical analysis was performed using GraphPad Prism® version 4.03. Comparisons of results between two treatment groups were determined using two-tailed student's t-test. Comparisons of results between multiple treatment groups were determined using a one-way Analysis of Variance (ANOVA) followed by a Bonferroni

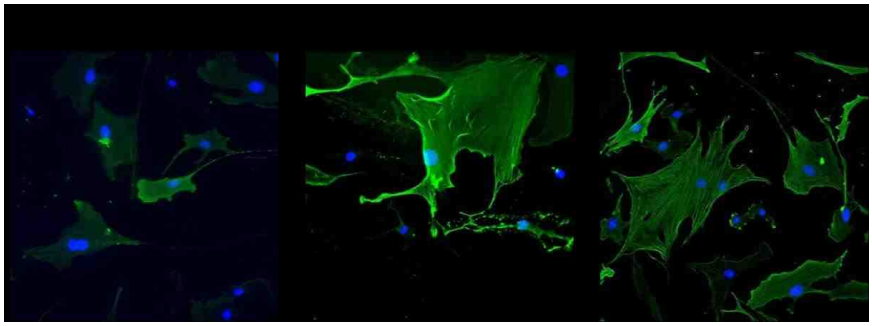
correction for multiple comparisons as a post-hoc test. For both methods of analysis, data represents the mean ± SEM of three or more separate experiments. P-values less than 0.05 were considered to be significant.

3.4 Results

3.4.1 GPER activates normal mammary fibroblasts

Characterization of the *in vitro* effects of GPER activation within isolated human mammary CAFs has recently begun [153]. However, the role of GPER in promoting the activation of fibroblasts into CAFs remains unexamined. Therefore, primary normal mammary fibroblasts were isolated from FVB/NJ GPER wild-type (FVB WT) and FVB/NJ GPER knockout (FVB KO) mice. Following E2 and growth factor starvation for 24hr, the fibroblasts were treated for 72hr with the GPER-selective agonist, G-1, and expression of smooth muscle actin (SMA) was measured. Among the initial stages of fibroblast activation is the upregulation of SMA, as the fibroblasts begin to exhibit a myofibroblast phenotype. Accordingly, the expression of SMA is the most commonly accepted marker of fibroblast activation [192]. Activation of GPER in FVB WT fibroblasts resulted in a two-fold increase of SMA expression as compared to sham (Figure 3.1 A, B). This activation was completely inhibited by the addition of the GPER-selective antagonist, G36 (Figure 3.1 C), indicating GPER contributes to the activation of normal fibroblasts. Additionally, neither E2 nor G-1 treatment induced SMA expression, and thus fibroblast activation, in FVB KO fibroblasts (Figure 3.1 D).

A



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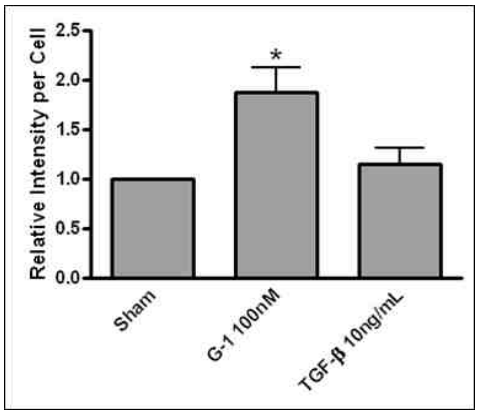

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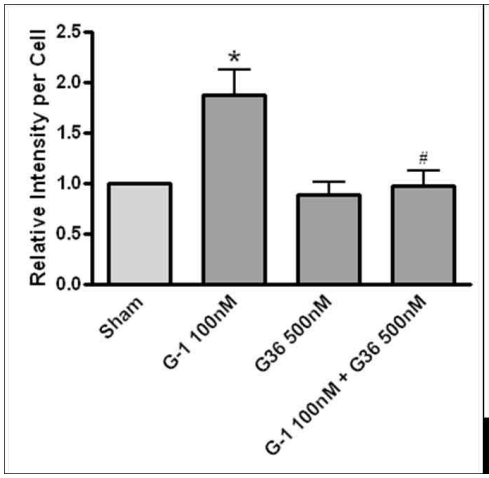

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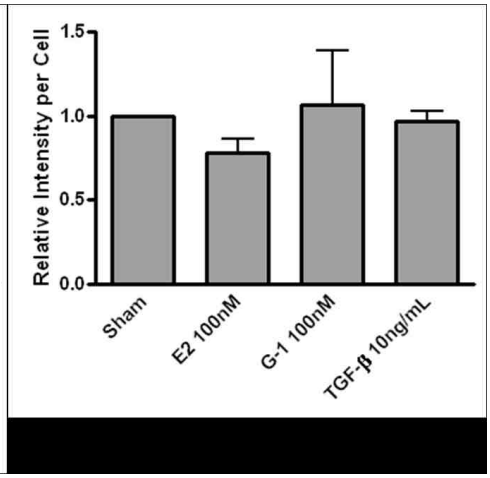


Figure 3.1 GPER activation promotes normal mammary fibroblasts activation. **A)** FVB WT fibroblasts were treated for 72hr with G-1, immunostained for SMA (green) and counterstained with DAPI nuclear stain (blue). **B)** FVB WT SMA expression was quantitated as described in methods. **C)** FVB WT fibroblasts treated 72hr with G-1, G36, or combined. SMA was quantitated as described. **D)** FVB KO fibroblasts treated 72hr with E2 or G-1. SMA was quantitated as described. *p < .05 compared to sham.

3.4.2 GPER increases fibroblast proliferation

Because GPER is known to increase CAF proliferation [153], a proliferation assay was performed on FVB WT and KO normal fibroblasts. As in the activation assay, the fibroblasts were starved for 24hr and treated with E2 or G-1 for 72hr. The cells were immunostained for Ki-67 and proliferating cells were quantitated. G-1, but not E2, resulted in significantly increased fibroblast proliferation as compared to sham (Figure 3.2 A). This proliferation was abrogated by the addition of G36 (Figure 3.2 B) and absent in FVB KO fibroblasts (Figure 3.2 D). The lack of increased proliferation following treatment with E2 could be due to an inhibitory effect by either ERα or ERβ as E2 activates all three estrogen receptors present in the fibroblasts. A balance between ERα-induced proliferation and ERβ-dependent inhibition would also account for the absence of any change in proliferation in FVB WT fibroblasts treated with a combination of E2 and GPER-selective antagonist, G36 (Figure 3.2 C). This is consistent with the role of ERβ as an inhibitor of ERα-induced proliferation [76].

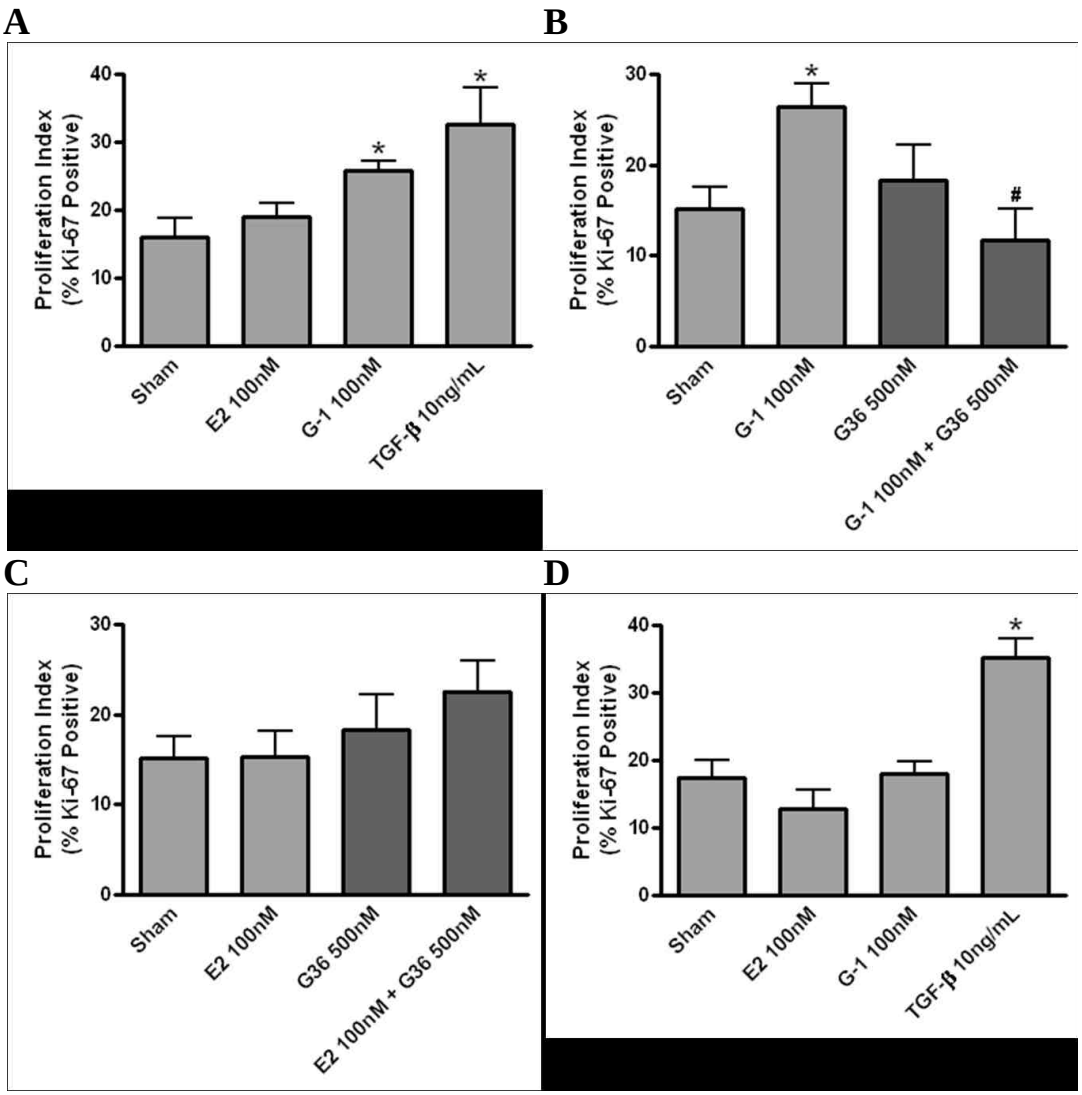
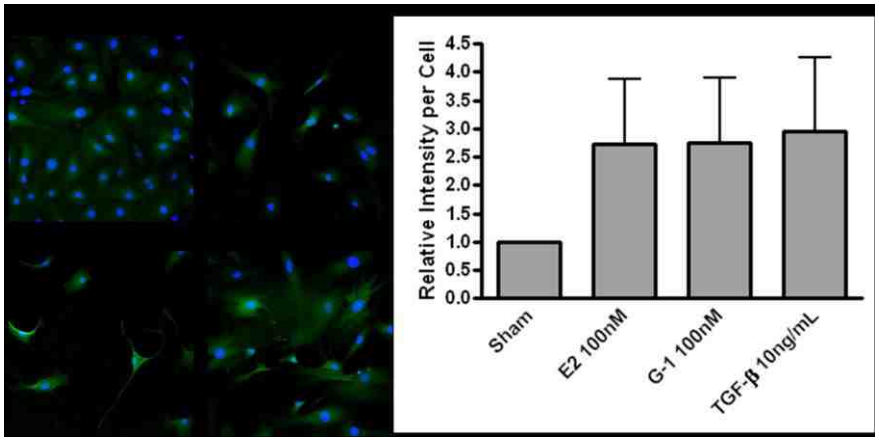


Figure 3.2 GPER activation increases fibroblast proliferation. Fibroblasts were treated for 72hr and immunostained to detect the proliferation marker Ki-67. Data is expressed as the percentage of total fibroblasts positive for Ki-67. **A)** FVB WT fibroblasts treated with E2 or G-1. **B)** FVB WT fibroblasts treated with G-1, G36, or combined. **C)** FVB WT fibroblasts treated with E2, G36, or combined. **D)** FVB KO fibroblasts treated with E2 or G-1. *p < .05 compared to sham. #p < .05 compared to G-1.

3.4.3 GPER activates fibroblasts through MAPK

The pathway by which GPER induced fibroblast activation was next examined. GPER signaling most commonly occurs through MMP cleavage of heparin-bound EGF (HB-EGF) resulting in the transactivation of EGFR and the MAPK cascade, specifically mediated through ERK-1/2-dependent signaling (Figure 1.4). Activation of EGFR and MAPK is known to result in fibroblast activation. To determine if GPER-dependent activation occurs through this pathway, FVB WT fibroblasts were activated by E2 and G-1 as described above, and phospho-ERK (p-ERK) was examined by immunodetection. While not statistically significant, GPER activation by both G-1 and E2 trends towards increased p-ERK within the fibroblasts (Figure 3.3 A). Furthermore, treatment with the MEK, and thus ERK, inhibitor U0126 abolishes GPER-dependent fibroblast activation, as determined by SMA expression (Figure 3.3 B). Additionally, EGFR inhibition by AG1478 also abolishes fibroblast activation (Figure 3.3 C), indicating the transactivation of EGFR and ERK are required for GPER-induced fibroblast activation.

A



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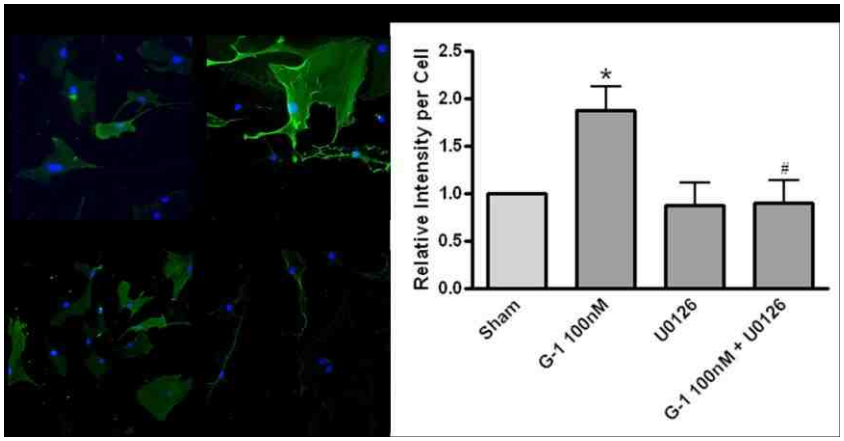

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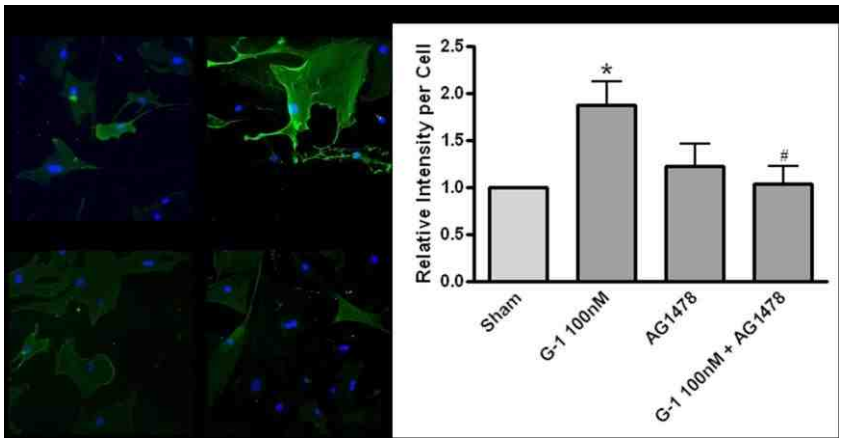

Figure 3.3 GPER activation promotes fibroblasts activation through MAPK. A) FVB WT fibroblasts treated 72hr with E2 or G-1 and immunostained for p-ERK (green) and counterstained with DAPI nuclear stain (blue). **B, C)** FVB WT fibroblasts treated 72hr with G-1 and **B)** U0126, an ERK inhibitor, or **C)** AG1478, an EGFR inhibitor, and immunostained for SMA (green) and DAPI (blue). All images quantitated as described in Methods. *p < .05 compared to sham. #p < .05 compared to G-1.

3.4.4 GPER activation of fibroblasts is Src-independent

The ability of GPER to signal through Src within fibroblasts not only leads to possible MMP-independent activation of EGFR and ERK, it may also lead to increased fibroblast migration through the phosphorylation of FAK and turnover of focal adhesions. To determine if Src is involved in GPER-induced signaling in normal fibroblasts, FVB WT fibroblasts were treated with G-1 and PP2, a Src inhibitor, for 72hr. Inhibition of Src did not significantly decrease fibroblast activation, as determined by SMA expression (Figure 3.4), indicating Src is not necessary for GPER-dependent fibroblast activation.

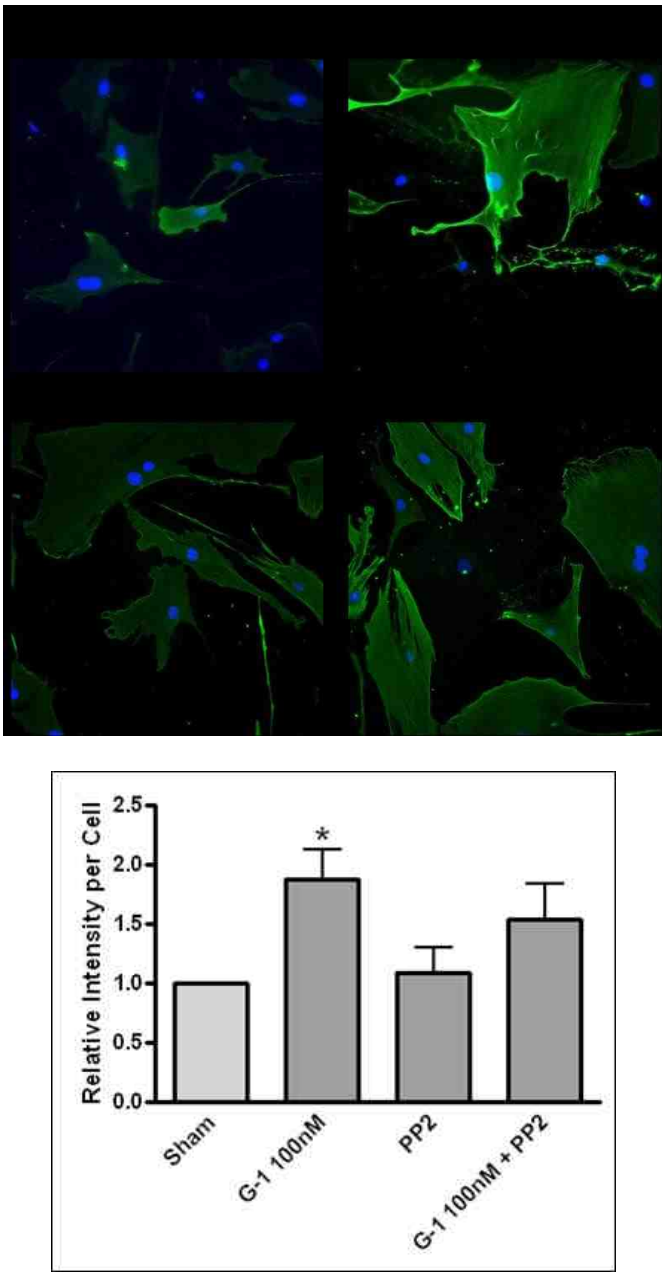

Figure 3.4 GPER activation induced fibroblast activation is Src-independent. FVB WT fibroblasts treated 72hr with G-1 and PP2, a Src inhibitor, and immunostained for SMA (green) and counterstained with DAPI nuclear stain (blue). All images quantitated as described in Methods. *p < .05 compared to sham.

3.4.5 GPER does not stimulate CTGF production in normal fibroblasts

CTGF has a prominent role in the activation of normal fibroblasts and migration of CAFs [188]. Production of CTGF within CAFs is significantly increased by the activation of GPER [153]. To determine the effect of GPER activation on CTGF production in normal mammary fibroblasts, FVB WT fibroblasts were treated with E2 or G-1 for 72hr, and CTGF was measured by immunostaining. GPER activation did not alter CTGF expression, as compared to sham, in FVB WT fibroblasts (Figure 3.5 A). Combination treatment with G36 in FVB WT fibroblasts (Figure 3.5 B) or treatment with E2 or G-1 in FVB KO fibroblasts also exhibited no effect on CTGF production (Figure 3.5 C). Although GPER reportedly increases CTGF production in both tumor cells and CAFs thereby inducing proliferation and migration [13, 153], it does not increase CTGF production in normal mammary fibroblasts.

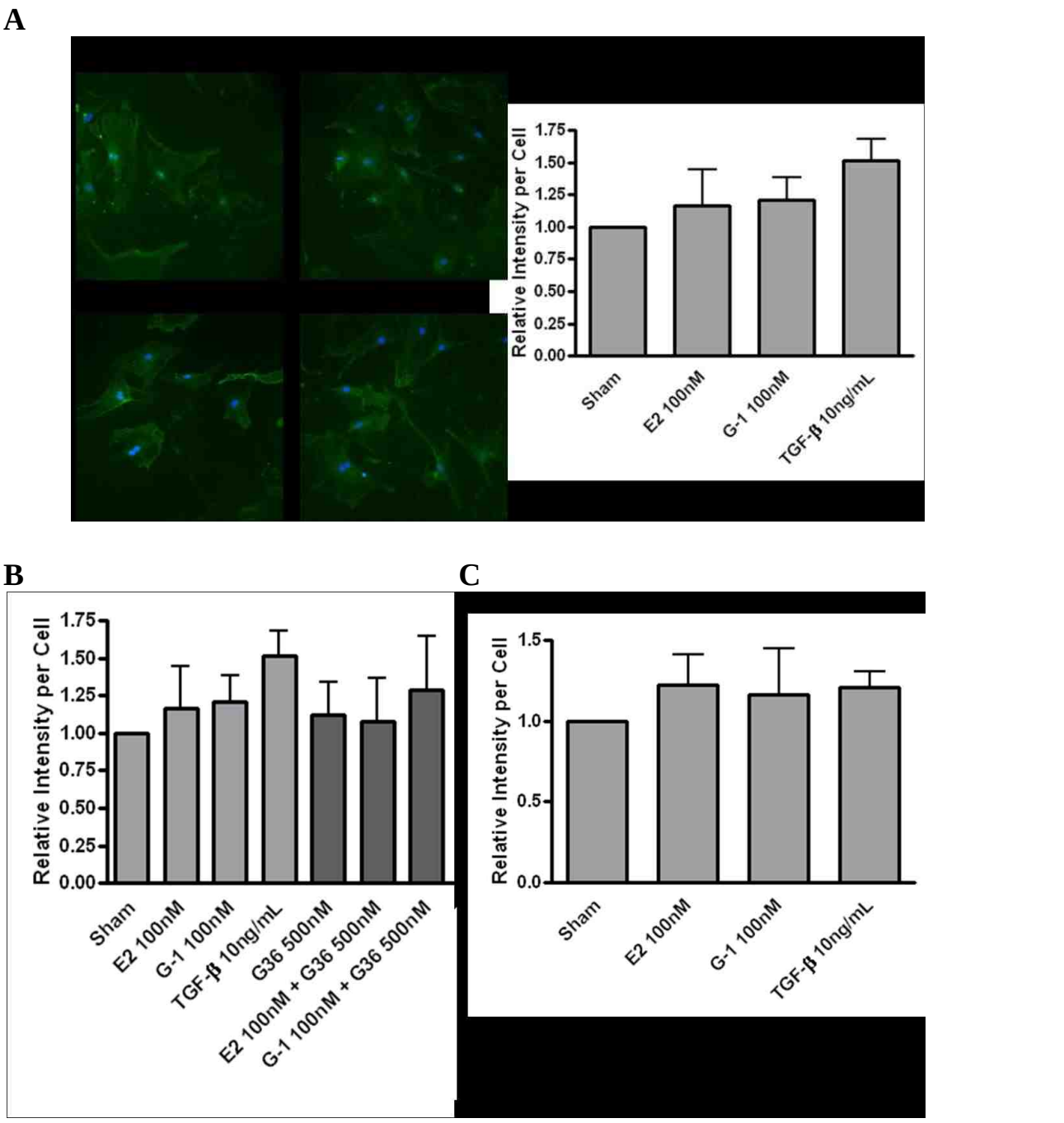

Figure 3.5 GPER activation does not stimulate CTGF production in normal fibroblasts. Fibroblasts were treated 72hr, immunostained for CTGF (green), and counterstained with DAPI nuclear stain (blue). **A)** FVB WT fibroblasts treated with E2 or G-1. **B)** FVB WT fibroblasts treated with E2, G-1, G36, or combined. **C)** FVB KO fibroblasts treated with E2 or G-1. All images quantitated as described in Methods.

3.4.6 GPER inhibits normal fibroblast migration

While GPER increases SMA expression and proliferation within normal mammary fibroblasts, full transformation into CAFs also requires an increase in motility. To test migration in GPER-activated fibroblasts, a Transwell® migration assay was performed. FVB WT fibroblasts were starved for 24hr and pretreated with G-1 for 72hr for full activation prior to being seeded into Transwell® chambers and allowed to migrate for 24hr. Activation with G-1 resulted in significant inhibition of normal fibroblast migration as compared to sham (Figure 3.6 A). FVB WT fibroblasts treated with G-1 and G36 together (Figure 3.6 B) or FVB KO fibroblasts treated with G-1 exhibited no migration inhibition (Figure 3.6 C). Instead, FVB WT fibroblasts treated with either G36 alone or in combination with G-1 increased migration, as compared to sham, indicating GPER expression results in inhibition of a basal level of migration.

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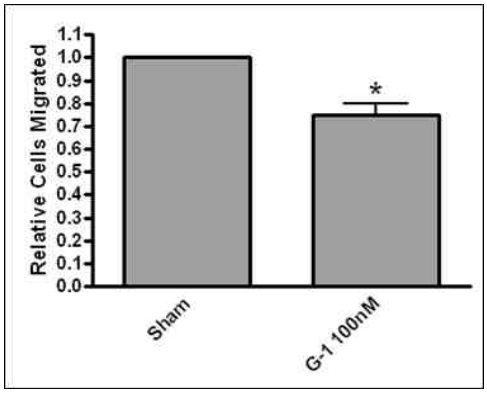

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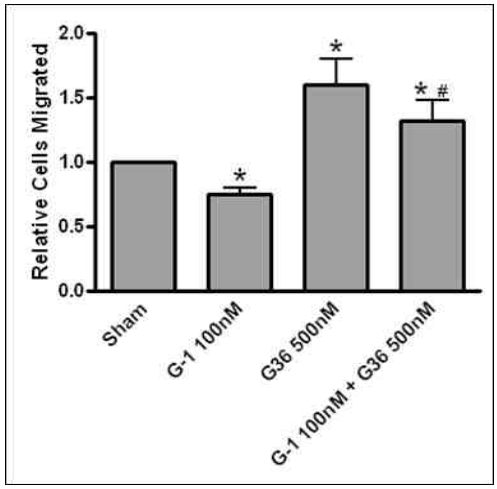

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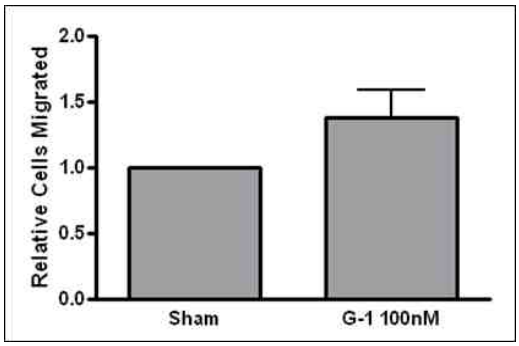
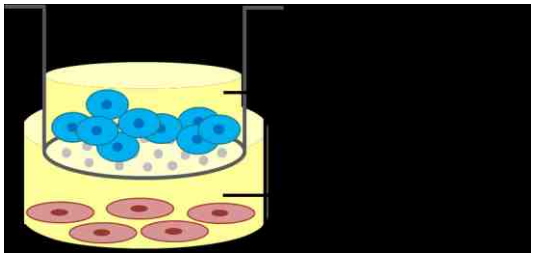


Figure 3.6 GPER activation inhibits normal fibroblast migration. Transwell® migration assays were performed on fibroblasts pretreated for 72h and allowed to migrate for 24hr. Cells migrating through the membrane were counted and normalized to sham-treated controls. **A)** FVB WT fibroblasts treated with G-1. **B)** FVB WT fibroblasts treated with G-1, G36, or combined. **C)** FVB KO fibroblasts treated with G-1. *p < .05 compared to sham.

3.4.7 GPER mediates fibroblast-induced tumor cell migration

Tumor cell activation of CAFs results in CAF-induced tumor cell migration and the development of a positive feedback loop [153, 186, 187]. To determine if GPER-activated normal fibroblasts have any effect on breast cancer cell migration, a co-culture Transwell® migration assay was performed. FVB WT fibroblasts were treated with E2 or G-1 for 72hr. After removal of treatment, untreated PyMT GPER WT (PyMT WT) breast cancer cells were placed in Transwell® inserts in the fibroblast-containing wells and allowed to migrate for 24hr (Figure 3.7 A). While not statistically significant, there is a pronounced trend toward increased breast cancer cell migration in response to GPER-activated fibroblasts (Figure 3.7 B). Additionally, the G-1 treated FVB WT fibroblasts exhibit significantly increased proliferation, as compared to sham (Figure 3.7 C), and a nearly two-fold increase over proliferation without exposure to breast cancer cells (Figure 3.2 A). This data suggests once more that the signaling between tumor cells and fibroblasts in the microenvironment increases the pro-metastatic abilities of both cell types and activation of GPER appears to enhance this signaling.

A



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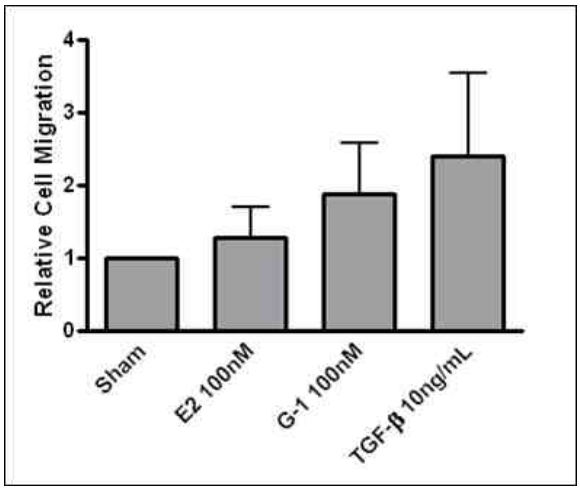

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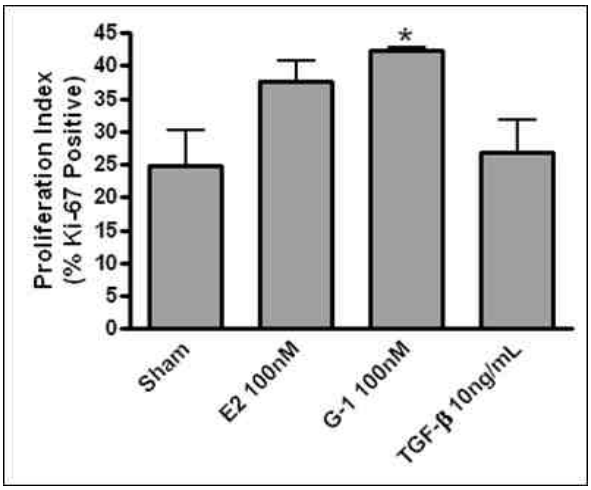
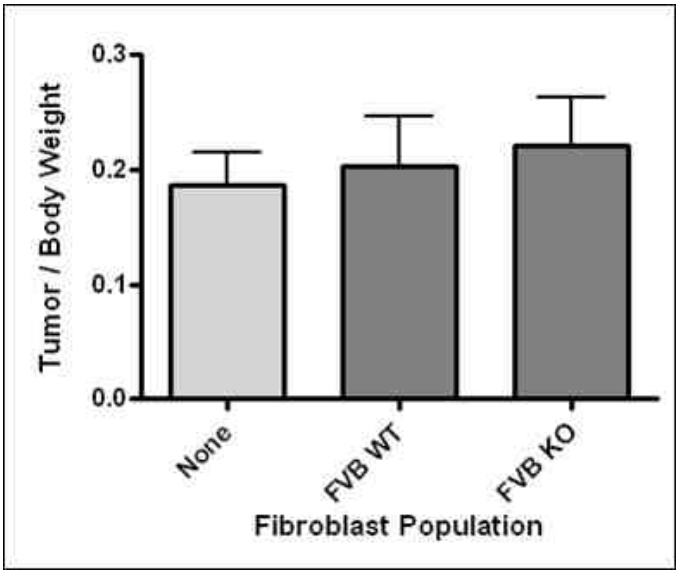


Figure 3.7 GPER activation mediates tumor cell-induced fibroblast proliferation. **A)** Transwell® co-culture migration assays setup. GPER WT PyMT cells were seeded in top chamber with 72hr pretreated fibroblasts on bottom. Cells migrating through the membrane after 24hr were counted and normalized to sham-treated controls. **B)** Migration of PyMT cells exposed to E2 or G-1 pretreated FVB WT fibroblasts. **C)** E2 or G-1 pretreated FVB WT fibroblasts exposed to migrating PyMT cells were immunostained to detect the proliferation marker Ki-67. Data is expressed as the percentage of total fibroblasts positive for Ki-67. *$p < .05$ compared to sham.

3.4.8 GPER fibroblast expression does not affect tumor size

To determine the effects of GPER expression in fibroblasts on tumor growth *in vivo*, the orthotopic transplant mouse model was employed. Prior to pubertal development, the epithelial ductal structure is restricted to the proximal end of the mammary fat pad and can be surgically removed. Tumor cells can then be orthotopically transplanted into the cleared fat pad, allowing growth in a physiologically relevant environment free of any endogenous epithelial growth [95, 96]. In this study, PyMT WT breast cancer cells were mixed with either FVB WT or KO normal fibroblasts and co-injected into the cleared inguinal fat pad of FVB WT mice to determine the effect of GPER fibroblast expression on tumor growth after 6 weeks. The tumor cells and fibroblasts were mixed at a low, 25% fibroblast ratio and a higher, 50% fibroblast ratio. By having two fibroblast concentrations, it can be determined if the concentration of fibroblasts present within the tumor impacts tumor growth and metastasis in a dose-dependent manner. Additionally, at the 50% fibroblast ratio, any effects of endogenous FVB WT fibroblasts present in the recipient fat pad will be overwhelmed. There were no significant differences in tumor weight in either 25% or 50% FVB WT or KO fibroblast-co-injected tumors, as compared to control tumors injected with no exogenous fibroblasts (Figure 3.8 A, B), indicating GPER expression in fibroblasts does not impact tumor growth and size.

A



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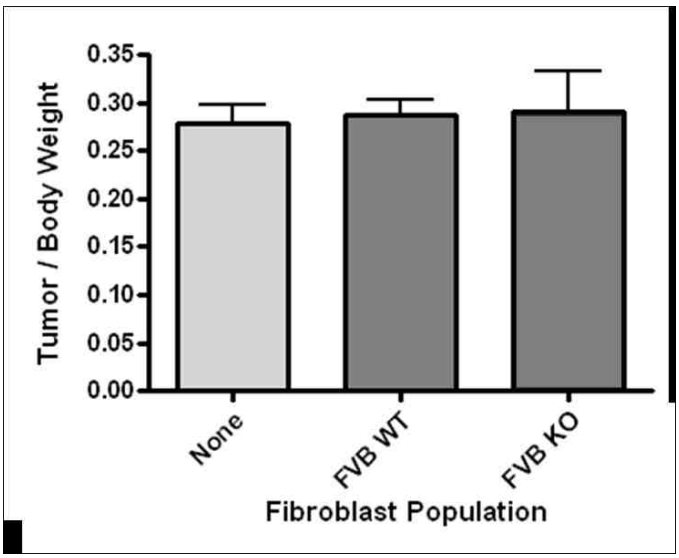


Figure 3.8 GPER expression in fibroblasts does not affect tumor size. PyMT WT breast cancer cells were mixed with either FVB WT or FVB KO normal fibroblasts at **A)** 25% or **B)** 50% fibroblast ratio and co-injected into the cleared inguinal fat pad of FVB WT mice. Control tumors (None) did not contain any exogenous fibroblasts. After 6 weeks, tumors were weighed and normalized to the body weight of the mouse.

3.4.9 Loss of GPER expression in the mammary fat pad increases ECM production

One of the primary, pro-metastatic functions of CAFs is the conversion of the normal mammary ECM into the much stiffer, desmoplastic tumor ECM, primarily through increased production and crosslinking of collagen I and degradation of collagen IV [141, 147, 182]. To determine if GPER expression within the fat pad alters tumor desmoplasia, PyMT WT breast cancer cells were orthotopically transplanted without exogenous fibroblasts into the cleared inguinal fat pad of FVB WT or KO mice. Injecting tumors into an FVB GPER KO mouse allows for analysis of the role of GPER in all of the cells present in the cleared mammary fat pad, including blood vessels, immune cells, and fibroblasts, all of which may influence ECM production. The tumors were sectioned, and a Heidenhain's AZAN trichrome stain (trichrome) was performed, staining the ECM components collagen and laminin, as well as cartilage and mucus, within the tumors blue [191]. PyMT WT tumors grown in FVB KO fat pads display an increased amount of ECM as compared to tumors grown in an FVB WT microenvironment (Figure 3.9), indicating GPER expression in the tumor microenvironment inhibits ECM production.

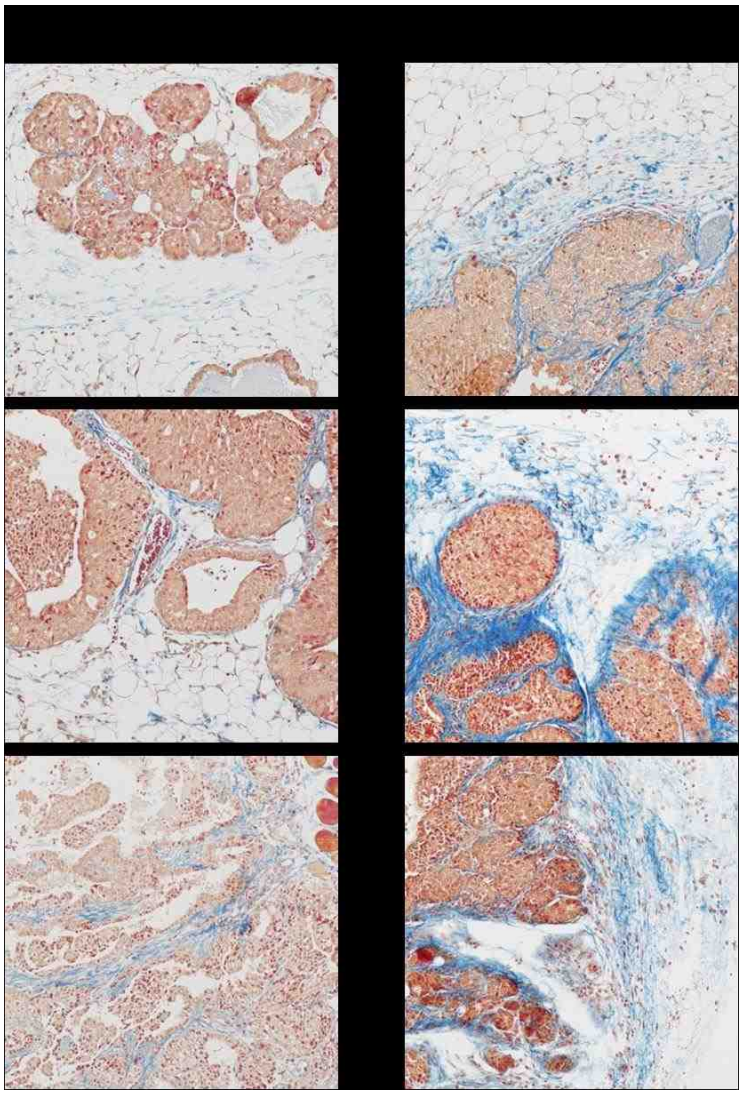
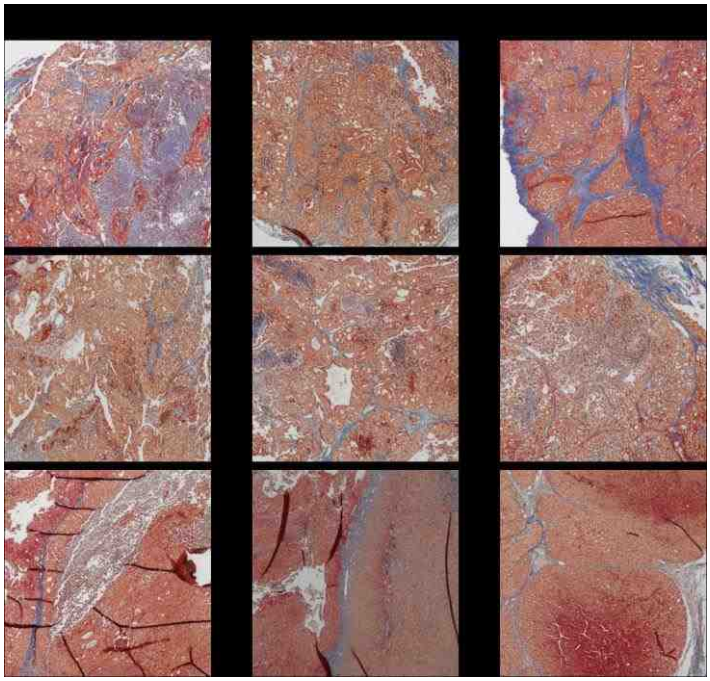


Figure 3.9 Loss of GPER expression in the mammary fat pad increases ECM production. PyMT WT cells were orthotopically transplanted into the cleared inguinal fat pad of FVB WT or KO mice. Tumors were sectioned and a Heidenhain's AZAN trichrome stain was performed, staining the ECM within the tumors blue. Three images from three different mice shown for both FVB WT and KO mice.

3.4.10 GPER does not affect fibroblast collagen production

Since fibroblasts are the primary stromal cell responsible for ECM production, it was determined if this increased ECM production was a result of GPER loss in fibroblasts specifically, rather than a loss of GPER in all of the cells localized within the stroma as examined above. PyMT WT tumor cells and either FVB WT or KO normal fibroblasts were orthotopically transplanted into FVB WT mice, and the tumors were trichrome-stained to determine ECM content. While the amount of ECM was quite varied within each tumor, as well as between tumors, there were no apparent differences in ECM production between tumors containing FVB WT or KO fibroblasts (Figure 3.10 A). Because trichrome stains all types of collagen blue, the specific effect of GPER fibroblast expression on the production of collagen I, the predominant ECM component produced by CAFs, was determined by immunostaining. While the levels of collagen I within the tumors was quite heterogeneous overall, there appeared to be no difference in collagen I levels comparing tumors co-injected with FVB WT or KO fibroblasts (Figure 3.10 B). While CAFs are the predominant source of collagen I within a tumor, collagen produced by endothelial cells was also detected within blood vessel walls [193]. To quantitate the collagen I produced specifically by GPER-activated fibroblasts in the absence of tumor cells, Western blot analysis was performed for collagen I and IV production by FVB WT (Figure 3.11 A) or FVB KO fibroblasts (Figure 3.11 B) treated *in vitro* with either E2 or G-1 for 72hr. Consistent with the *in vivo* results, there was no significant difference in collagen I or IV secretion by GPER-activated FVB WT or KO fibroblasts *in vitro*.

A



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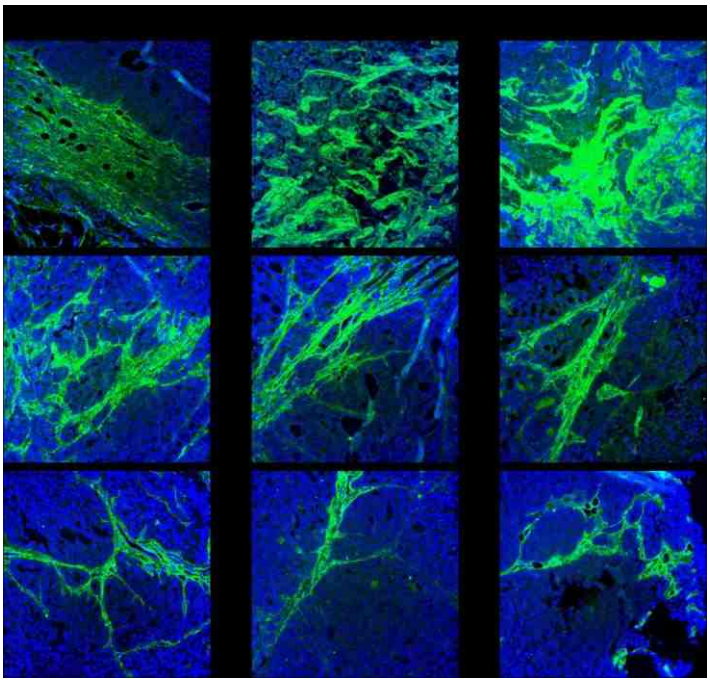


Figure 3.10 GPER expression does not affect fibroblast collagen production *in vivo.* PyMT WT cells were mixed with FVB WT or KO fibroblasts at a 50% ratio and co-injected into FVB WT mice. After 6 weeks, tumors were **A)** trichrome stained and **B)** immunostained for collagen I (green) and counterstained with DAPI (blue).

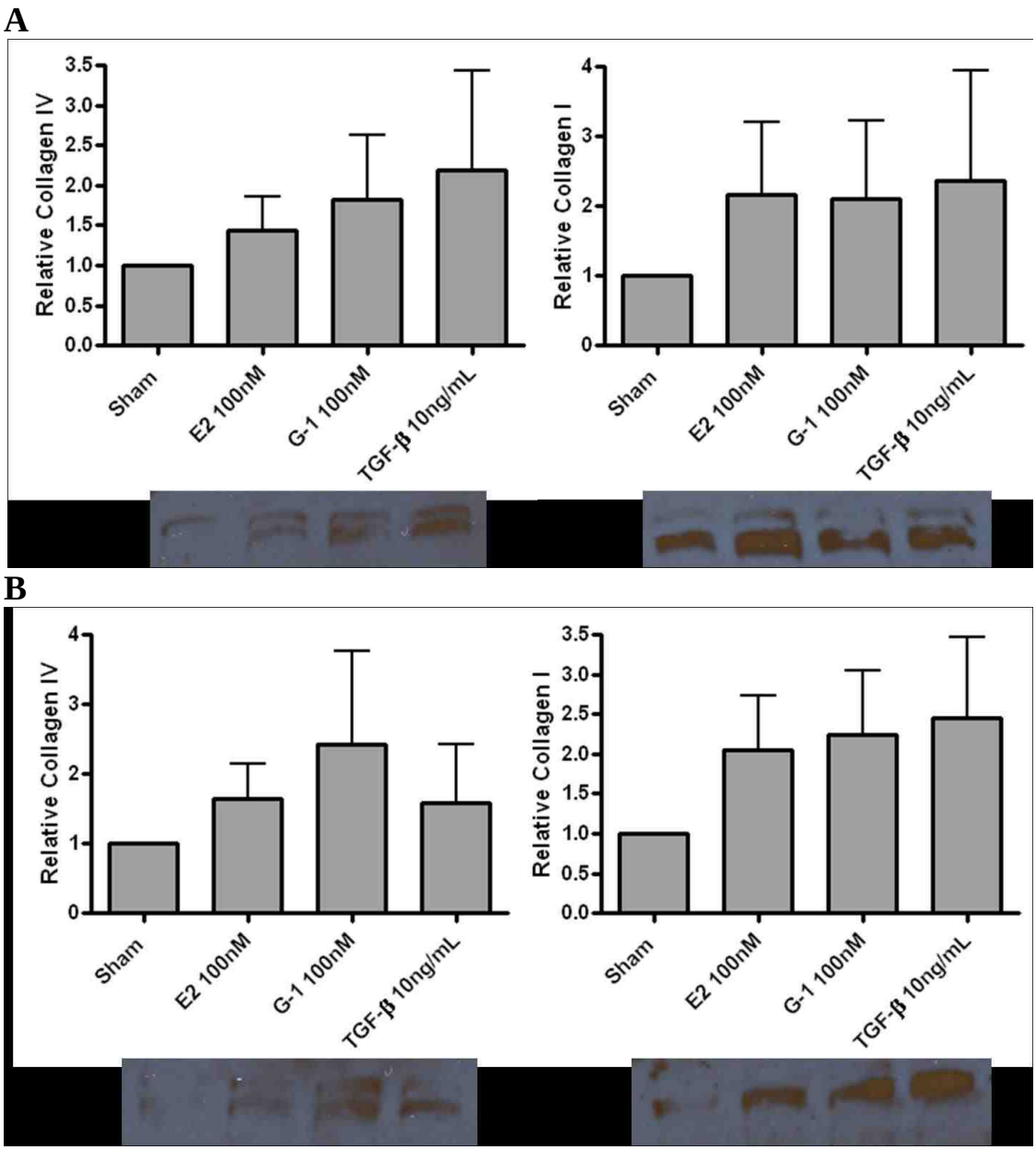


Figure 3.11 GPER activation does not affect fibroblast collagen production *in vitro.* A) FVB WT or **B)** FVB KO fibroblasts treated for 72hr with E2 or G-1. Collagen I and IV secreted into the media were analyzed by Western blot. Data was normalized to sham.

3.4.11 Loss of GPER increases lung metastasis

Although GPER activates normal mammary fibroblasts (Figure 3.1), this activation resulted in decreased fibroblast migration *in vitro* (Figure 3.6). Since migratory and co-metastasizing CAFs promote breast cancer metastasis [150, 152], GPER-induced migration inhibition would suggest a possible role for GPER in the inhibition of tumor metastasis. However, these experiments in the present study were done *in vitro* with fibroblasts in isolation, without any exposure to tumor cells. As previously discussed, the cross-talk between fibroblasts and tumor cells is critical. To determine the functional effect of GPER expression in normal mammary fibroblasts in a physiologically relevant environment on tumor metastasis, the lungs of the FVB WT or KO fibroblast and PyMT WT tumor bearing mice were removed and examined for metastatic lesions by H&E staining. Orthotopically transplanted PyMT tumor cells consistently metastasize to the lungs [194]. Tumors transplanted with 50% FVB WT or KO fibroblasts did not exhibit any significant differences in the number of metastatic lesions present in the lungs, as compared to tumors transplanted without exogenous fibroblasts (Figure 3.12 A). However, PyMT WT tumors containing 50% FVB KO fibroblasts developed significantly more metastatic lesions within the lungs than either control tumors or FVB WT fibroblast tumors (Figure 3.12 B). To further determine the extent of metastasis, each metastatic lesion was measured. Both 25% and 50% FVB KO fibroblasts significantly increased the area of each metastatic lesion (Figure 3.12 C), as well as increased total metastatic area within the lung tissue (Figure 3.12 D). This indicates the loss of GPER within fibroblasts fosters a pro-metastatic environment in which metastatic tumors exhibit increased growth.

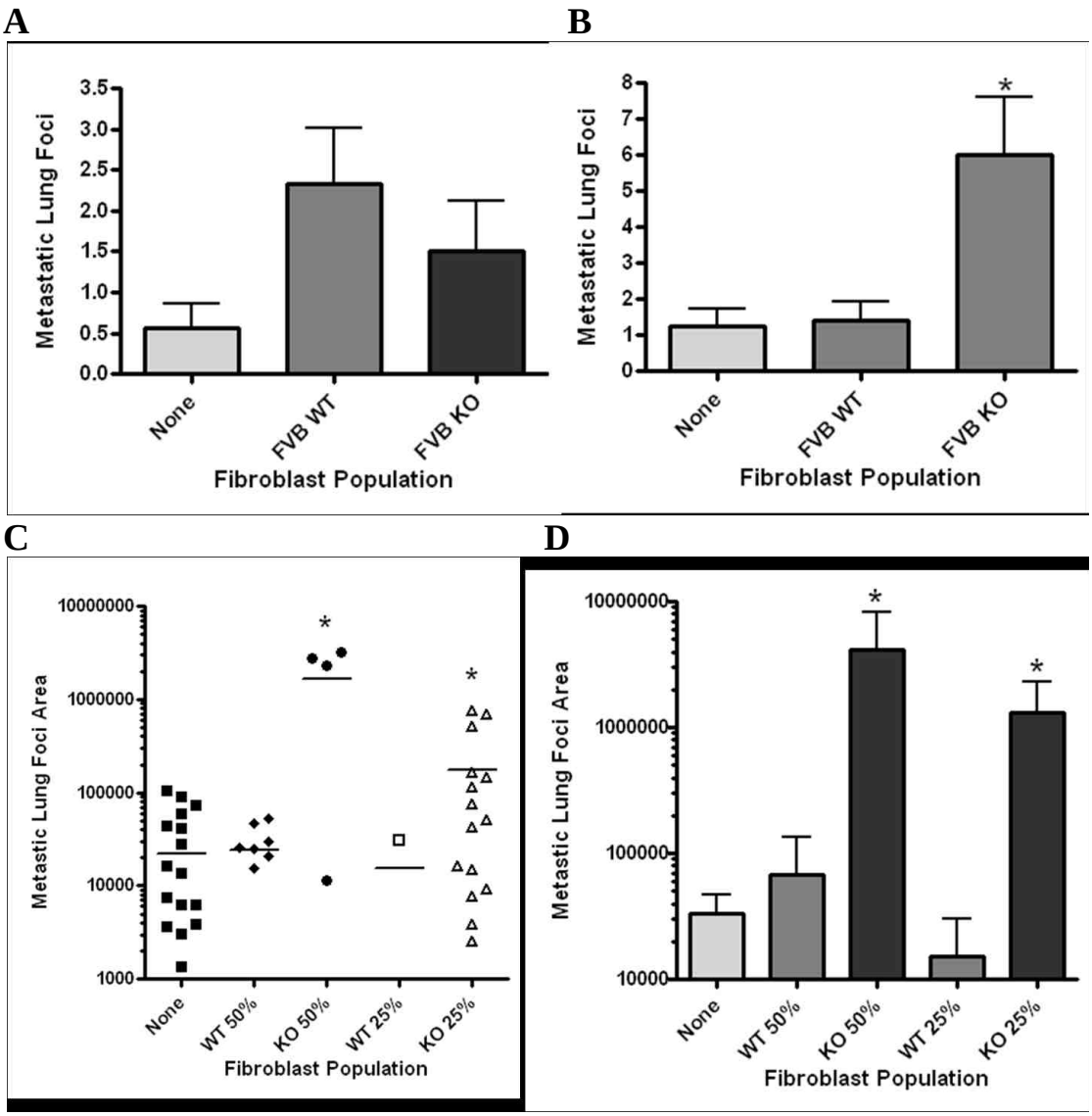


Figure 3.12 Loss of GPER increases lung metastasis. PyMT WT breast cancer cells were mixed with either FVB WT or FVB KO normal fibroblasts at **A)** 25% or **B)** 50% fibroblast ratio and co-injected into the cleared inguinal fat pad of FVB WT mice. Control tumors (none) did not contain any exogenous fibroblasts. After 6 weeks, lungs were H&E stained and metastatic foci were counted. **C)** Area of each metastatic lesion was measured. **D)** Total metastatic area was measured for each mouse. Area is measured in relative units. *$p < .05$ compared to none.

3.5 Discussion

Important research has been conducted recently characterizing the pro-tumorigenic role of activated cancer-associated fibroblasts in tumor growth and metastasis. The increased presence of CAFs within the tumor is correlated with increased tumor size, metastasis, and poor prognosis [9, 10]. Since metastasis is the primary cause of cancer-related death, it is critical to understand the mechanisms by which normal fibroblasts become activated and transformed into CAFs. Because this activation primarily occurs through CTGF-enhanced TGF-β signaling and GPER significantly increases CTGF production in CAFs and breast cancer cells [13, 153], the role of GPER in the activation of normal mammary fibroblasts was examined.

Upon activation, fibroblasts begin producing SMA to increase motility and contractility and adopt a myofibroblast phenotype. Activation of GPER in primary, normal, mammary fibroblasts by the synthetic ligand, G-1, results in the *de novo* expression of SMA indicative of an activated fibroblast (Figure 3.1 A, B). This fibroblast activation was accompanied by increased GPER-dependent proliferation (Figure 3.2 A) and required the activation of EGFR and ERK signaling (Figure 3.3). However, this activation did not require Src activity (Figure 3.4). In the classical GPER signaling pathway, E2 binding to GPER results in Gβγ subunit activation of Src. Src then either directly transactivates the EGFR or activates MMPs to cleave HB-EGF, thus activating the EGFR (Figure 1.4). While no known studies have demonstrated Src-independent GPER activation of EGFR, recent studies have shown direct interaction of GPCRs with both EGFR and IGF-1R through the Gβγ subunit [132, 195]. Additionally, IGF-1R-dependent proliferation in both MCF-7 breast cancer cells and Ishikawa endometrial

cancer cells requires GPER [4]. Thus, GPER may transactivate EGFR either directly or through interaction with IGF-1R, rather than in a Src-dependent manner, thereby increasing fibroblast proliferation.

In CAFs and breast cancer cells, GPER stimulation of ERK and its downstream AP-1 element results in increased production of CTGF [13, 153]. However, in normal fibroblasts activated by GPER, as defined by SMA production, CTGF was not upregulated (Figure 3.6). GPER-dependent normal fibroblast activation also decreased migration (Figure 3.4 A), and inhibition of GPER increased migration 50% compared to control (Figure 3.4 B). Though unexpected, this migration inhibition is consistent with a recent study in breast cancer cells demonstrating GPER-dependent ERK phosphorylation of Smad inhibits the TGF-β migration signal [196]. This study is supported by increasing evidence of MAPK inhibition of TGF-β signaling [197-199]. The regulation of the interaction between these two fundamental cellular pathways is complex and still poorly understood. When fibroblasts are exposed to breast cancer cells, there is increased TGF-β produced by both cell types [186]. Thus, when GPER-activated fibroblasts and PyMT breast cancer cells are co-cultured, GPER-dependent fibroblast proliferation is increased (Figure 3.7 C), and there is a trend toward increased stimulation of breast cancer cell migration (Figure 3.7 B). This suggests a role for GPER in mediating the fibroblast-tumor signaling.

To further examine the effects of GPER expression in fibroblasts on the paracrine signaling between tumor cells and fibroblasts, tumors were grown orthotopically in vivo in the presence of fibroblasts either expressing or lacking GPER. The absence of GPER expression in fibroblasts resulted in an increased number and size of metastatic lesions

(Figure 3.12). While GPER expression in the stroma as a whole, including blood vessels, immune cells, and fibroblasts, decreased ECM production (Figure 3.9), the loss of GPER in fibroblasts alone did not increase ECM levels, particularly collagen (Figure 3.10, 11). Although the fibroblasts were normal when transplanted, TGF-β produced by the nearby tumor cells should have transformed them into CAFs [181, 182, 200]. However, this *in vivo* activation could not be assessed within this study. Consistent with the *in vitro* studies, it is possible GPER-mediated MAPK signaling within the fibroblasts dampens the activating TGF-β stimulus, thus inhibiting the ability of the fibroblasts to support metastasis. Additionally, TGF-β signaling increases reactive oxygen species production, inhibiting the gap junctions present in activated normal fibroblasts [184]. Fibroblasts maintaining gap junction communication exhibit an anti-metastatic phenotype [144]. When GPER expression in fibroblasts is eliminated, TGF-β signaling would no longer be suppressed by the GPER-mediated MAPK signaling, thereby completing the positive feedback loop and inducing the further transformation of activated fibroblasts into CAFs. This transformation result in increased metastasis of both the CAFs and tumor cells. Thus, GPER stimulation likely leads to the activation of normal fibroblasts while inhibiting full transformation into CAFs, resulting in anti-metastatic activity similar to that of activated normal fibroblasts present in the early stages of tumor growth [144, 145].

Tumor cells metastasizing with primary site fibroblasts exhibit less cell death, from both apoptosis and immune surveillance, within the blood and are able to establish a greater number of metastatic lesions [152]. In addition, these lesions grow larger than those depleted of primary site CAFs [152], similar to the effect seen in tumors containing

fibroblasts lacking GPER. This is the first study to demonstrate a role for GPER in both the activation of normal fibroblasts *in vitro* and the inhibition of tumor metastasis *in vivo*, possibly through the inhibition of TGF-β activation.

CHAPTER 4

ESTROGEN RECEPTOR EFFECTS IN THE MOUSE ENDOMETRIUM

4.1 Abstract

The classical responses to estrogen activity within the uterus, proliferation and water imbibition, are traditionally attributed to ERα activation resulting in both increased gene transcription and rapid signaling. However, with the discovery of GPER, a new estrogen receptor capable of inducing proliferation, this assumption required re-examination. This study began examining the individual E2-dependent responses of ERα and GPER, as well as the independent roles of ERα transcriptional regulation and rapid signaling through the use of selective ligands. GPER-dependent proliferation within the lumenal epithelial cells was demonstrated through the GPER-selective agonist, G-1. This proliferation was abolished by the GPER-selective antagonist, G15. However, GPER is not involved in imbibition as G-1 was unable to induce a response and G15 did not inhibit the E2-induced imbibition. To determine the mechanism by which ERα induces imbibition and proliferation, AB-1, an ERα and ERβ selective ligand that activates genomic responses but not rapid signaling, was used. Because ERβ does not increase endometrial proliferation or imbibition [201], AB-1-induced proliferation or imbibition can be reasonably assigned to ERα. Both proliferation and imbibition can occur independently of ERα rapid signaling. However, rapid signaling appears to be required for complete imbibition as AB-1-stimulated gene transcription only induced ~60% of the E2-induced imbibition.

4.2 Introduction

In the adult, non-pregnant, pre-menopausal female, the menstrual cycle induced by cyclic ovarian 17β-estradiol (E2) production results in a biphasic uterine physiology.

The follicular, or proliferative, phase is characterized by proliferation of epithelial cells in the innermost functional layer of the endometrium as E2 levels increase to a maximal level at ovulation. During the secretory phase, progesterone (P4) levels increase, inducing the differentiation and proliferation of stromal cells to transform the endometrium into a receptive environment for implantation. In the absence of implantation, the functional layer is shed as P4 levels decrease [64]. The E2-dependent proliferation and edema observed during the proliferative phase are well described and accepted as classical responses within the uterus [73, 74]. However, the mechanisms by which they occur are still being characterized.

E2 activates ERα and induces proliferation within the lumenal epithelial cells of the endometrium [32]. This increased proliferation likely occurs through both the rapid signaling and genomic responses of ERα. Upon estrogen binding, ERα dimerizes and binds either directly to an estrogen response element (ERE) within the promoter region of a target gene or to the binding site of an associated cofactor, such as the AP-1 or SP-1 transcription factors (Figure 1.3) [40, 41]. ERα is able to either promote or repress transcription of the target gene, depending on the cofactors recruited. Several proliferation genes are upregulated, including myc and cyclin D1, primary regulators of the cell cycle [202]. While this increase has been attributed solely to the transcriptional abilities of ERα, evidence in breast cancer cells indicates this transcription may also be dependent upon ERα stimulating rapid signaling through cAMP and protein kinase A (PKA) [202]. ERα rapid signaling responses may also increase epithelial cell proliferation through activation of the MAPK and PI3K signaling cascades [43].

E2 is also responsible for the increased fluid accumulation within the endometrium during the proliferative phase of the menstrual cycle [203]. ERα increases uterine blood vessel permeability through increased production of vascular endothelial growth factor (VEGF) and nitric oxide (NO). The compromised endothelial barrier is unable to restrict protein movement, resulting in increased serum proteins entering the uterine stroma [204-206]. This protein influx creates an osmotic imbalance, leading to increased water within the interstitial spaces in the stroma. The water is then passively and actively transported across the lumenal epithelial cells and into the lumenal space, where it will be excreted as uterine lumenal fluid. E2 increases the expression of the water channel aquaporins (AQP) through the activation of ERα, thereby increasing the lumenal fluid [207]. The process of E2-induced water imbibition can be inhibited by actinomyocin D, an inhibitor of protein translation and transcription, indicating transcriptional regulation and *de novo* protein synthesis is critical for ERα activity [204]. However, as in ERα-induced proliferation, it is unknown if ERα rapid signaling is required for this gene transcription.

The discovery of the estrogen receptor GPER has required reanalysis of estrogenic responses previously attributed to ERα. E2 stimulated proliferation in both ERα positive Ishikawa and ERα negative HEC1A endometrial cancer cell lines was abolished following GPER knockdown [87]. This proliferation was induced through the activation of the MAPK cascade, consistent with the known GPER signaling pathway (Figure 1.4). Additionally, although tamoxifen (TAM) acted as an ERα antagonist within Ishikawa cells, treatment with TAM resulted in increased cell growth, consistent with TAM serving as GPER agonist [50, 87]. Proliferation stimulated by TAM activation of

GPER may explain why women treated with TAM are at an increased risk of developing endometrial cancer [81]. However, the role of GPER in proliferation of normal endometrium *in vivo* has yet to be examined.

In this study, selective ligands for GPER, ERα, and ERβ were used to determine the role of each receptor in the classical uterine estrogen responses, proliferation and water imbibition. The GPER-selective agonist, G-1, demonstrated GPER-dependent proliferation within the lumenal epithelial cells. This proliferation was abolished by the GPER-selective antagonist, G15. However, GPER is not involved in imbibition. To determine the mechanism by which ERα induces imbibition and proliferation, AB-1, an ERα and ERβ selective ligand that activates genomic responses but not rapid signaling, was used. Because ERβ does not increase endometrial proliferation or imbibition [201], AB-1-induced proliferation or imbibition can be reasonably assigned to ERα. Both proliferation and imbibition can occur independently of ERα rapid signaling. However, rapid signaling appears to be required for complete imbibition as AB-1-stimulated gene transcription only induced ~60% of the E2-induced imbibition.

4.3 Methods

4.3.1 Mice. Ovariectomized, 10 week-old C57Bl/6J female mice were purchased from Harlan Laboratories. Animals were housed at the animal research facility at the University of New Mexico Health Sciences Center. They were maintained under a controlled temperature of 22–23°C with a 12hr light, 12hr dark cycle and fed normal chow *ad libitum*. All procedures were approved and carried out in accordance with institutional protocols.

4.3.2 AB-1 uterine analysis. C57Bl/6 female mice were ovariectomized at 10 weeks of age and allowed to rest and acclimate to their environment for 10-14 days. This time also allows any residual ovarian E2 circulating throughout the body to be eliminated. Mice were subcutaneously injected once daily for three days with 100µL of sham control, E2 (2ng or 10ng) (Sigma) or AB-1 (2µg, 10µg, or 90.9µg) (ChemDiv). All compounds were dissolved in ethanol. Injections were 10% ethanol containing treatment and 90% aqueous vehicle (0.9% (w/v) NaCl with 0.1% (w/v) albumin and 0.1% (v/v) Tween-20). Ethanol alone (10µL) was added to 90µL aqueous vehicle as control. 24hr after final injection, each mouse was weighed, and the uterus was removed, weighed, fixed in 4% PFA, and embedded in paraffin.

4.3.3 GPER uterine analysis. C57Bl/6 female mice were ovariectomized at 10 weeks of age and allowed to rest and acclimate to their environment for 10-14 days. This time also allows any residual ovarian E2 circulating throughout the body to be eliminated. Mice were subcutaneously injected with 100µL of sham control, E2 (200ng), G-1 (40ng, 200ng, or 1000ng) (Dr. Jeffrey Arterburn, New Mexico State University), G15 (272ng, 900ng, or 2725ng) (Dr. Jeffrey Arterburn, New Mexico State University), or G15 (272ng, 900ng, or 2725ng) combined with E2 (200ng) or G-1 (200ng). All compounds were dissolved in ethanol. Injections were 10% ethanol containing treatment and 90% aqueous vehicle as described above. 18hr after injection, each mouse was weighed, and the uterus was removed, weighed, fixed in 4% PFA, and embedded in paraffin.

4.3.4 Immunostaining analysis. For proliferation analysis, 5µm uterine tissue sections were deparaffinized, rehydrated, permeabilized in PBS + 0.1% Triton X-100, and blocked in 3% normal goat serum (NGS) diluted in PBS + 0.1% Tween-20 (PBS-T).

Microwave antigen retrieval was performed in 0.1 M sodium citrate (pH 6). The tissue was stained with rabbit anti-Ki-67 (Thermo Fisher) primary antibody diluted in 3% NGS + PBS-T overnight at 4^{o}C in a humidified chamber followed by detection with anti-rabbit secondary antibody conjugated to Alexa 488 (Molecular Probes) for 1hr at 20^{o}C. Coverslips were mounted on slides with Vectashield mounting medium containing 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Three random fields from each sample were imaged with a Zeiss 200M Axiovert microscope using MetaMorph® software.

4.3.5 Statistics. Statistical analysis was performed using GraphPad Prism® version 4.03. Comparisons of results were determined using two-tailed student's t-test. Data represents the mean ± SEM of three or more separate experiments. P-values less than 0.05 were considered to be significant.

4.4 Results

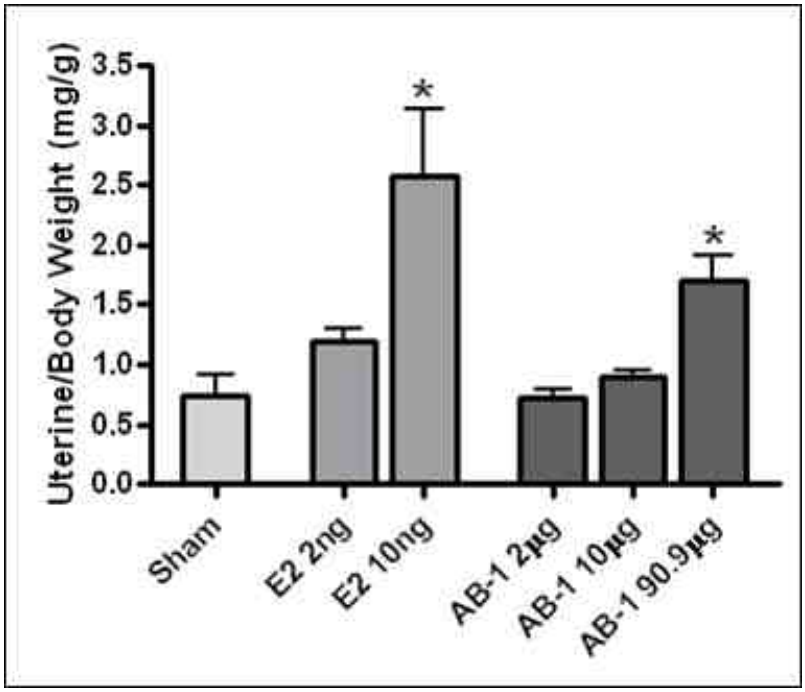
4.4.1 ERα transcriptionally regulates water imbibition and epithelial proliferation

ERα activity occurs through both genomic and rapid signaling. Often the individual roles of these two mechanisms are blurred, as it is difficult to separate genomic- and rapid signaling-induced activity *in vivo*. AB-1 is a selective agonist for both ERα and ERβ [62, 63, 208], demonstrating negligible ability to bind GPER (Dr. Eric Prossnitz, unpublished observations). AB-1 binding to ERα and ERβ increases gene transcription [63, 208] with an EC_{50} of 15nM, compared to 0.1nM for E2. However, AB-1 antagonizes ERα-induced calcium mobilization and PI3K activation rapid signaling (Dr. Eric Prossnitz, unpublished observations). Thus the use of AB-1 allows for the

separation of ER transcriptional activity from rapid signaling. While ERβ is expressed in the uterus, ERα expression and activity are predominant in the mature, premenopausal, non-pregnant uterus [68, 69]. Additionally, ERβ activation does not result in increased proliferation [76, 201]. Therefore, the effects of AB-1 on water imbibition and proliferation can be reasonably assigned to ERα. To examine the ability of ERα transcriptional activity to induce water imbibition in the mouse uterus, sexually mature female mice were ovariectomized to remove the predominant source of premenopausal E2. The mice were injected once daily for three days with either E2 or AB-1. The uteri were removed and weighed, as increased uterine weight relative to body weight is a measure of imbibition. Both E2 and AB-1 significantly increased uterine wet weight, and thus imbibition, in a dose dependent manner, as compared to sham control (Figure 4.1 A). However, AB-1 was not able to achieve the same level of water uptake as E2, inducing ~60% of the E2-induced imbibition. This diminished response is not attributable to the affinities of AB-1 (EC_{50} = 15nM) and E2 (EC_{50} = 0.1nM) for ERα, as the highest AB-1 dose (90.9μg) was nearly 10,000-fold higher than the necessary E2 dose (10ng), far exceeding the differences in affinities. This suggests a role for ERα-dependent rapid signaling in the enhancement of transcription responsible for water uptake.

To determine the role of ERα-mediated transcription on lumenal epithelial cell proliferation, Ki-67 positive lumenal epithelial cells were quantitated. Both AB-1 and E2 significantly increased lumenal epithelial cell proliferation by five-fold as compared to the sham control (Figure 4.1 B). In contrast to uterine water imbibition, AB-1 and E2 induced proliferation equally, indicating ERα-mediated transcriptional activity is sufficient for ERα to fully stimulate proliferation.

A



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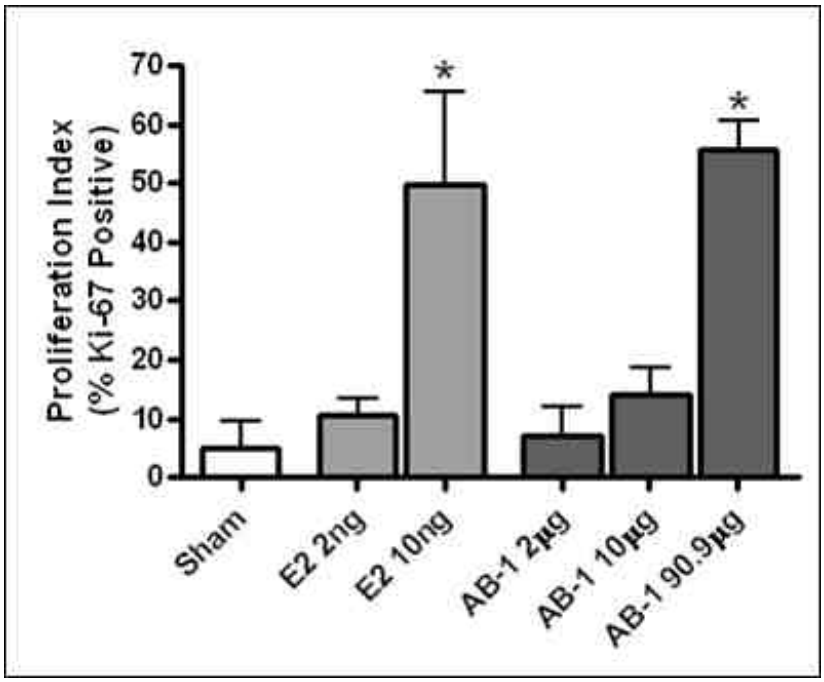
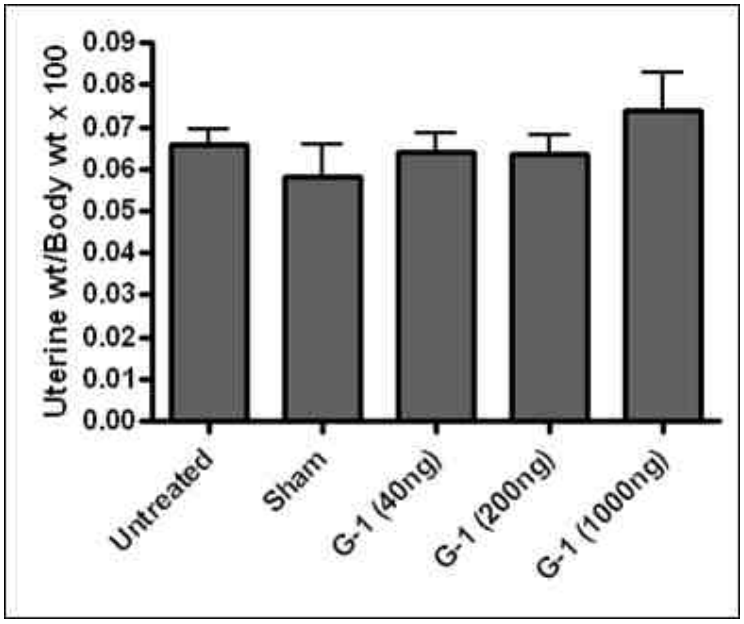


Figure 4.1 ERα transcriptionally regulates water imbibition and epithelial cell proliferation. Mice were injected once daily for three days with E2 or AB-1. **A)** The uterus was weighed and normalized to body weight. **B)** Uteri were immunostained to detect the proliferation marker Ki-67. Data is expressed as the percentage of total lumenal epithelial cells positive for Ki-67. *p < .05 compared to sham.

4.4.2 GPER does not regulate water imbibition

To determine the role of GPER in E2-stimulated water imbibition within the uterus, sexually mature female mice were ovariectomized and injected with G-1, a GPER selective agonist. After 18hr, the uteri were removed and weighed. Activation of GPER by G-1 did not result in water imbibition at any dose (Figure 4.2 A). To confirm GPER activation by the endogenous ligand, E2, did not impact imbibition, E2 or E2 in combination with G15 was injected, and wet weight was measured after 18hr. As a GPER-selective antagonist, G15 should block any E2 activation of GPER. Thus any E2-induced effect exhibited following treatment with G15 can be solely attributed to ERα or ERβ. Treatment with E2 resulted in significantly increased weight, and thus water uptake, and treatment with G15 was unable to inhibit this effect (Figure 4.2 B), confirming GPER is not necessary for E2-induced water imbibition.

A



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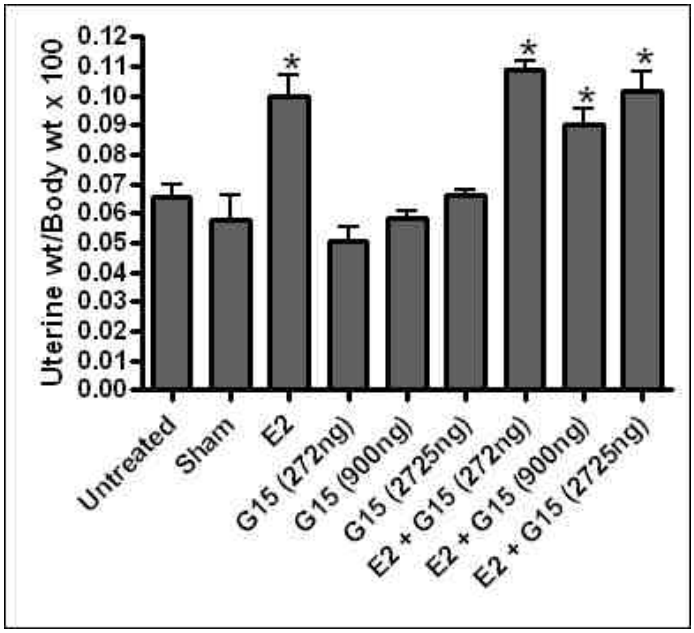
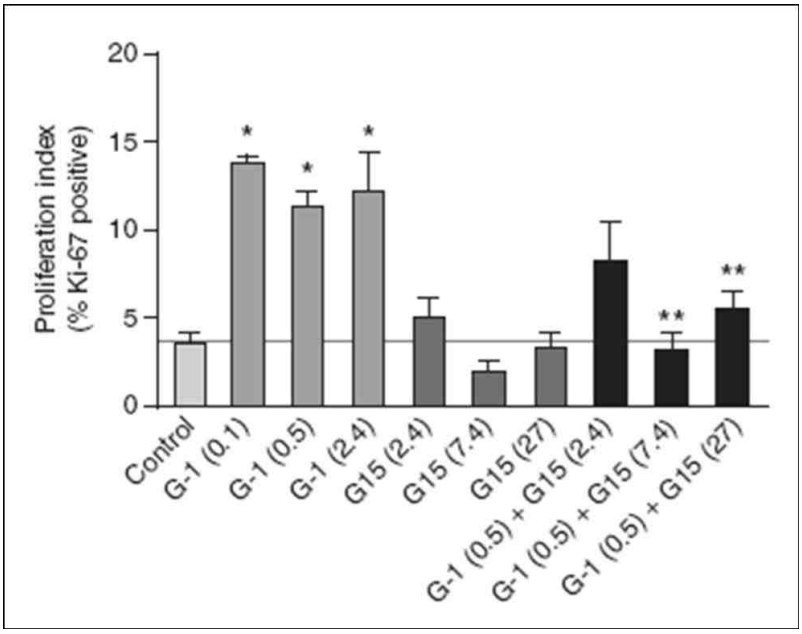


Figure 4.2 GPER does not regulate water imbibition. Mice were injected once with **A)** G-1 or **B)** E2, G15, or E2 and G15 combined. After 18hr, the uterus of each mouse was weighed and normalized to body weight. *p < .05 compared to sham.

4.4.3 GPER increases uterine epithelial cell proliferation

Since GPER increases proliferation of endometrial cancer cells *in vitro* [50, 87, 209], sexually mature ovariectomized mice were treated with either G-1 for 18hr, and proliferation was quantitated. At each dose, G-1 significantly increased lumenal epithelial cell proliferation compared to sham control, and treatment with G15 abrogated the G-1-induced response (Figure 4.3 A). To confirm the role of GPER in E2-stimulated proliferation, mice were treated with E2 or E2 and G15. E2 significantly increased proliferation as compared to the control, and G15 partially inhibited the E2-induced proliferation (Figure 4.3 B), indicating E2 stimulates uterine proliferation through both ERα and GPER.

A



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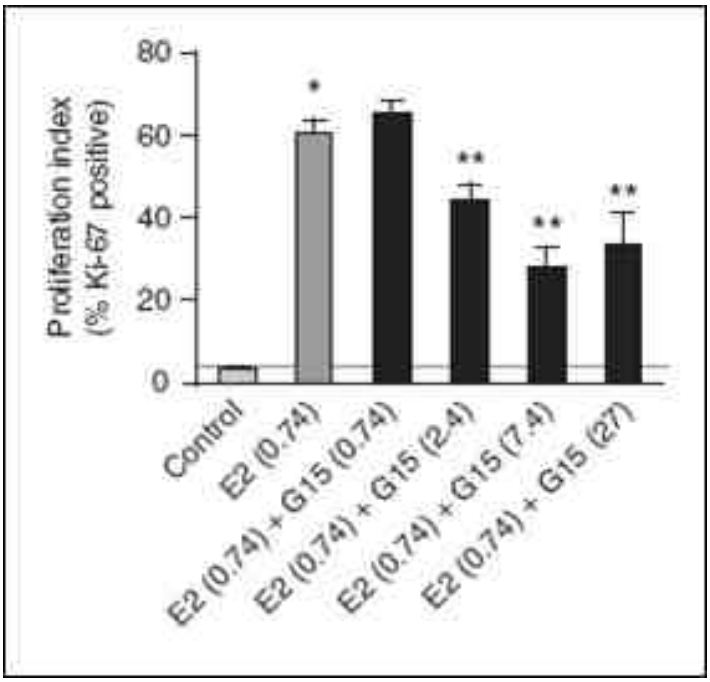

Figure 4.3 GPER increases uterine epithelial cell proliferation. Mice were injected once with **A)** G-1, G15, or G-1 and G15 combined or **B)** E2 or E2 and G15 combined. After 18hr, the uteri were immunostained to detect the proliferation marker Ki-67. Data is expressed as the percentage of total lumenal epithelial cells positive for Ki-67. *p < .05 compared to sham. **p < .05 compared to G-1 or E2.

4.5 Discussion

The classical markers of estrogen activity within the uterus, proliferation and water imbibition, are traditionally attributed to ERα signaling. However, with the discovery of GPER, a new estrogen receptor capable of inducing proliferation, this assumption required re-examination. This study began assessing the individual E2-dependent responses of ERα and GPER, as well as the roles of ERα transcription regulation and rapid signaling through the use of selective ligands.

E2 activation of ERα induces cellular effects through both the regulation of transcription and the activation of rapid signaling pathways. Because they often occur concomitantly, it has been difficult to specifically assign ERα-dependent effects to either of these two mechanisms *in vivo*. The development of the ERα- and ERβ-selective ligand, AB-1, and the demonstration of its ability to activate transcriptional activity while antagonizing rapid signaling allows for mechanistic distinction of these two responses. While ERβ is expressed in the uterus, ERα expression and activity are predominant in the mature, premenopausal, non-pregnant uterus [68, 69]. Additionally, ERβ activation does not result in increased proliferation [76, 201]. Therefore, the effects of AB-1 on water imbibition and proliferation can be reasonably assigned to ERα. Through AB-1, it was determined ERα transcriptional activation is responsible for both increased water uptake and proliferation within the endometrium (Figure 4.1 A). However, maximal water uptake appears to require rapid signaling as well, since excess AB-1 was unable to induce a response of the same amplitude as E2-induced imbibition. This rapid signaling is most likely production of NO through ERα-dependent MAPK phosphorylation and activation of endothelial nitric oxide synthase (eNOS). ERα-induced NO production and

vasodilation is an important step in the imbibition process and is independent of *de novo* protein synthesis [210]. The increased ERα-dependent transcription and *de novo* synthesis of VEGF and the water channel AQP stimulate water uptake, though this response is incomplete in the absence of NO production. Although E2 does not increase water imbibition through GPER alone (Figure 4.2), GPER may be able to stimulate rapid signaling necessary to elicit a full E2 water imbibition response. This could be determined by treating mice with AB-1 to induce ERα-dependent transcription combined with G-1 to stimulate GPER-dependent rapid signaling.

While the water imbibition response does not require GPER activity, E2-induced proliferation does require GPER activation to mediate a complete response and can be partially inhibited through treatment with a GPER antagonist (Figure 4.3). In a recent study, E2-stimulated xenograft endometrial tumor growth was almost completely inhibited following treatment with G36, a second-generation GPER-selective antagonist [209]. One of the most intriguing implications of GPER-induced endometrial proliferation lies in the ability of tamoxifen (TAM) to serve as a GPER agonist. Women treated with TAM for breast cancer are at an increased risk of developing endometrial cancer [81]. While ERα is agonized by TAM in the uterus, ERα expression is inversely correlated with markers of poor outcome in endometrial cancer, such as aggressive histological subtypes, adnexal spread, myometrial invasion, de-differentiation and recurrence [88, 90]. High GPER expression levels are inversely correlated with ERα expression, and positively correlate with markers of poor prognosis, such as high grade, advanced stage, cervical involvement, and aggressive histology, as well as an overall lower survival rate. This positive correlation becomes more pronounced with high-grade

III/IV tumors [70]. Likewise, high endometrial GPER expression has been significantly correlated with the development of TAM-induced endometrial pathology as well as with increased progression rate of endometrial polyps or hyperplasia [127].

Aside from these correlative studies, direct evidence demonstrates TAM stimulated proliferation and invasion of endometrial cancer cells occurs in a GPER-dependent manner [91]. TAM activation of GPER and downstream MAPK results in increased production of pro-migratory MMP-2 and -9 and IL-6, a cytokine associated with aggressive endometrial cancer and a shorter survival period [211]. Blocking GPER signaling abrogates the pro-tumorigenic effects of TAM [91], presenting intriguing treatment possibilities. To further explore this role in tumor growth and metastasis, mice with endometrial tumors could be treated with TAM to inhibit ERα in combination with G15 or G36 to inhibit GPER, thus inhibiting all of the known E2 receptors. Additionally, adjuvant treatment with the GPER inhibitors G15 or G36 may minimize the malignant endometrial effects of TAM treatment of breast cancer, thereby increasing patient survival.

CHAPTER 5

CONCLUSIONS AND FUTURE DIRECTIONS

5.1 Summary and Future Directions

This study provides evidence for the contribution of GPER to the migration of both normal breast epithelial and breast tumor cells and to the activation and function of fibroblasts in the breast tumor microenvironment. Proliferation and migration are two of the most critical steps within both normal mammary development and breast cancer progression. The role of E2 in increasing proliferation in normal and tumor cells through both ERα and GPER is well characterized [1-3]. ERα is critical for the coordinated proliferation and migration necessary for proper ductal elongation and morphogenesis within the breast during puberty [94]. However, ERα seems to have an inhibitory effect on breast cancer metastasis, as expression has been inversely correlated with metastasis [128].

While the role of GPER in the migration of normal breast epithelial cells remains largely unexamined, GPER activation increases breast cancer cell migration *in vitro*, primarily through inducing the fibroblast and breast cancer cell production of CTGF [13]. However, GPER may also inhibit breast cancer cell migration [53]. It is not currently known under what conditions GPER promotes or inhibits migration. In a retrospective study, GPER expression in breast cancer samples directly correlated with increased tumor size and metastasis [14]. However, in a separate study, loss of GPER expression correlated with increased breast cancer metastasis [129]. It is important to note that in all of these studies, the expression of GPER was assessed in the tumor cells, not in the surrounding stroma.

In addition to clinical correlation studies, the role of GPER in breast cancer metastasis has been directly examined using the mouse mammary tumor virus-polyoma

middle T antigen (PyMT) mouse model of breast cancer, which spontaneously develops mammary tumors in a manner similar to the development of human tumors [167, 212]. The hormonal dependence of PyMT tumors has been demonstrated as tumors allowed to develop in the absence of ovarian E2 or treated with tamoxifen (TAM) exhibit a much smaller tumor size. Treatment with the GPER-selective antagonist G36 does not affect tumor size or grade; however, G36 does inhibit E2-induced lung metastasis (Nicole Marjon, unpublished observations). Similarly, PyMT GPER knockout (PyMT KO) mice exhibit significantly fewer metastatic lesions than control PyMT GPER WT (PyMT WT) mice. PyMT WT tumor cells orthotopically transplanted into the non-tumorigenic mammary fat pad of FVB KO recipient mice also develop significantly fewer metastatic lesions as compared to FVB WT recipient mice (Nicole Marjon, unpublished observations). Taken together, these data suggest GPER within the tumor microenvironment is a critical component of E2-induced breast cancer metastasis.

Metastasis is responsible for the vast majority of cancer-related deaths and is a complex process involving not only the tumor cells, but also a wide variety of cell types present within the tumor microenvironment [11]. These include the vascular system, with both established and newly formed blood vessels, and the immune system, with both adaptive T cells and innate macrophages and natural killer cells. The loss of GPER expression affects both of these systems through increased vasoconstriction and a pro-inflammatory environment [213-216]. The microenvironment may also impact tumor growth and metastasis through metabolic regulation [217]. GPER KO mice exhibit metabolic dysregulation resulting in increased obesity compared to GPER WT mice [216]. Although the effect of GPER within these systems has been individually studied,

the role of GPER in these systems has not yet been examined in the context of the tumor microenvironment.

In addition to the vascular and immune cells present within the microenvironment, a principal cell type aiding tumor metastasis through both direct and indirect interaction are cancer-associated fibroblasts (CAFs). Normal mammary fibroblasts are recruited by the tumor cells and transformed into CAFs primarily through the actions of TGF-β produced by the tumor cells [145, 182]. The increased presence of CAFs within the tumor microenvironment is correlated with increased tumor size and metastasis [9, 10]. Once activated, CAFs begin remodeling and stiffening the tumor ECM by decreasing production of collagen IV found in normal mammary basal lamina and increasing the secretion of MMPs, collagens I and III, fibronectin, and tenascin-C [141]. This stiffer, fibrotic ECM, referred to as desmoplastic ECM, provides a better substrate for tumor cell migration [150]. CAFs also directly aid in tumor cell metastasis by leading tumor cells out of the primary tumor site, protecting them from apoptosis and immune surveillance within the blood stream, and increasing their ability to successfully establish a secondary tumor site [10, 150-152]. GPER-dependent production of CTGF within CAFs increases both fibroblast and breast cancer cell proliferation and migration [13, 153]. Since CTGF is also a potent fibroblast activator, this study examined the role of GPER in the activation and function of normal mammary fibroblasts.

Because cancer development, growth, and metastasis are often the result of deregulated physiological processes, the role of GPER in normal breast epithelial cell migration was first examined. Using methods to analyze both collective and individual cell migration, GPER was demonstrated to increase collective migration without

impacting individual migration. In breast cancer cells, however, a different role for GPER was observed as individual migration was inhibited. It is possible the differences observed between the MCF10A and PyMT cell lines may be due to the fundamental differences between human, non-tumorigenic, breast epithelial cells and mouse breast cancer cells. To determine which of these differences, if any, impact the ability of GPER to affect cell migration, collective and individual migration assays should be performed on human breast cancer cells and normal mouse mammary epithelial cells.

GPER-dependent inhibition of PyMT breast cancer cell migration may also be due to the presence of ERβ. While MCF10A cells only express GPER, PyMT cells express all three known estrogen receptors. In addition to its role in inhibiting proliferation, ERβ has recently been shown to inhibit migration of breast cancer cells as well [179, 180]. This inhibition can occur in a ligand-independent manner through the phosphorylation of ERβ S105 by ERK1/2. Of note, phosphorylated ERβ is unable to inhibit proliferation in a ligand-independent manner [179]. Thus it is possible GPER-selective activation by G-1 in PyMT cells may transactivate ERβ through ERK1/2 resulting in migration inhibition. If ERβ is inhibiting GPER-stimulated migration, knocking down ERβ in PyMT tumor cells should result in increased migration. Additionally, transfecting ERβ into MCF10A cells may inhibit GPER-induced migration, thus demonstrating a role for ERβ in regulating GPER-dependent migration in normal breast epithelial cells as well.

The role of ERβ S105 phosphorylation inhibiting migration may also explain the lack of migration inhibition observed by ECIS measurement. Because PyMT breast cancer cells do not exhibit the contact inhibition present in epithelial cells and

phosphorylated ERβ is unable to inhibit proliferation in a ligand-independent manner [179], the PyMT cells may have filled the wound over the electrode through GPER-induced proliferation. Thus it is important to analyze PyMT breast cancer cell proliferation in response to GPER activation. Additionally, to confirm the potential masking effect of GPER-dependent proliferation on migration inhibition, migration of G-1 treated PyMT cells in the presence of a proliferation inhibitor should be measured. By inhibiting proliferation, GPER-induced PyMT migration should increase due to the absence of opposing MAPK signals.

Although GPER activation in PyMT cells inhibited migration, when exposed to GPER activated normal fibroblasts, PyMT migration trended toward an increase, though this increase was not statistically significant. In this experiment, the PyMT cells and fibroblasts shared media and thus were able to stimulate one another in a paracrine manner through secreted factors. However, evidence indicates physical interaction between fibroblasts and cancer cells has a much greater effect on the activation of both cell types through juxtacrine signaling [150, 186, 218]. It is necessary to determine the impact of GPER fibroblast activation on the effects of fibroblast-tumor cell contact. The interaction may stimulate the production of CTGF reported in CAFs treated with G-1. CTGF production was not stimulated in GPER-activated normal fibroblasts, suggesting an additional signal, perhaps TGF-β, from the tumor cells is necessary for complete transformation of GPER-activated normal fibroblasts into CAFs.

It is possible this fibroblast-tumor cell contact may also amplify the statistically insignificant effects on tumor cell migration observed in co-culture experiments performed in this study. However, GPER-activated fibroblasts may inhibit the tumor cell

migration when in direct contact, similar to the *in vivo* metastasis model in which co-injection with GPER KO fibroblasts in direct contact with the tumor cells resulted in more metastatic lesions than GPER WT fibroblasts. To examine this interaction, GPER-activated fibroblasts and PyMT breast cancer cells could be co-cultured on an artificial basement membrane in an organotypic system as previously described [150]. In this system, fibroblast-tumor interaction results in measurable invasion into the basement membrane. From this experiment, it can be determined if direct interaction between GPER-activated fibroblasts and PyMT breast cancer cells alters the migratory and invasive abilities of both cell types. If direct interaction with GPER KO fibroblasts resulted in increased PyMT cancer cell invasiveness, this would provide clues into possible mechanisms by which GPER in fibroblasts inhibit tumor metastasis *in vivo*. This organotypic *in vitro* experiment also removes the confounding factor of endogenous GPER WT fibroblast effects present in the GPER KO fibroblast tumors transplanted into GPER WT mice.

The organotypic model of fibroblast and cancer cell interaction also allows for better measurement of ECM components produced by the fibroblasts along the invasion track [150]. While GPER activation of normal fibroblasts alone *in vitro* did not affect collagen production, perhaps GPER-activated fibroblast interaction with PyMT breast cancer cells is necessary for increased collagen I and fibronectin production. Fibroblast-tumor cell contact has been demonstrated to significantly increase fibroblast production of collagen and fibronectin [219]. It is also possible for fibroblasts to alter the ECM composition through increased production of MMPs. Fibroblasts use MMPs to degrade the anti-metastatic collagen IV and create tracks for the tumor cells to follow [150, 151,

187]. GPER increases the proteolytic activity of MMP-2 and -9 as part of the transactivation of EGFR [49, 220], and a recent *in vitro* study in ovarian cancer cells demonstrated GPER activation increased invasiveness through the increased production of MMP-9 [5]. GPER-dependent fibroblast MMP production may be increased through direct fibroblast-tumor cell contact [187]. MMP production by GPER-activated fibroblasts alone or in the presence of PyMT breast cancer cells can be assessed through gel zymography, the standard method for assessing MMP production [221].

In addition to increasing tumor cell migration and invasion, co-metastasizing CAFs protect the tumor cells within the blood stream resulting in a greater number of metastatic lesions [152]. These lesions grow larger than those depleted of primary site CAFs [152], similar to the effect seen in tumors containing GPER KO fibroblasts *in vivo*. A difference in GPER WT and KO fibroblast metastatic ability would be consistent with the *in vitro* data demonstrating GPER WT fibroblast activation by G-1 inhibited fibroblast migration. To determine if GPER KO fibroblasts are metastasizing with the tumor cells more than GPER WT fibroblasts, GPER KO mice can be crossed with GFP transgenic mice to produce fluorescently labeled primary mammary fibroblasts with or without GPER expression. PyMT breast cancer cells and either GFP+ GPER WT or GFP+ GPER KO fibroblasts can be transplanted into non-GFP expressing FVB recipient mice and allowed to grow. The metastatic lesions in the lungs can be examined for the presence of GFP+ cells. Because neither the recipient mouse nor the transplanted tumor cells express GFP, any GFP+ cell present in the metastatic lung lesion must be a primary site fibroblast. By quantitating the number of primary site fibroblasts, it can be determined if GPER expression in fibroblasts inhibits fibroblast metastasis resulting in

fewer and smaller lesions as observed in this study. If there is determined to be a difference in the number of GPER WT and KO fibroblasts in the metastatic lesions, it may be due to inhibition of either migration and invasion necessary for intravasation or extravasation and establishment of the secondary site [152].

To distinguish between the two metastatic processes of extravasation and intravasation, mixed PyMT tumor cells and either GFP+ GPER WT or KO fibroblasts can be inoculated directly into the bloodstream, bypassing intravasation. This approach allows for selective examination of the role GPER in fibroblasts during extravasation and the establishment of the secondary tumor site. If GPER inhibits fibroblast-dependent ECM remodeling, the fibroblasts would be unable to provide the beneficial effects of metastasizing CAFs within the bloodstream and in establishing a pro-tumorigenic microenvironment in the secondary tumor site. This would result in fewer, smaller lesions than those produced by GPER KO fibroblasts as observed in this study. If GPER inhibits fibroblast-dependent invasion there would be minimal difference in the number and size of lesions from GPER WT and KO fibroblasts, since this step will be bypassed. It is more likely, however, that GPER has effects on both invasion and ECM production, and direct vascular injection would result in a partial increase in metastatic lesion size and number, as compared to the orthotopic transplant model performed in this study.

5.2 Overall Conclusions

This study demonstrated alternate roles for GPER in normal and tumor breast cells through the promotion of normal epithelial cell migration and the inhibition of both breast cancer cell and normal fibroblast migration *in vitro*. GPER expression in fibroblasts also resulted in the partial inhibition of tumor metastasis and secondary tumor site growth *in vivo*. The anti-metastatic role of GPER in fibroblasts examined within this study suggests intriguing therapeutic possibilities. It also presents a possible cause for the discrepancies in the clinical correlation studies reporting that high GPER tumor expression is correlated with both increased and decreased metastasis as these reports only measured GPER expression in the tumor cells [14, 129]. If, as the data presented in this study suggests, GPER expression in fibroblasts is a key determinant in the metastatic potential of the tumor, it is critical to begin determining the expression levels of GPER not just within the tumor cells, but also within the stroma and surrounding fibroblasts for a more accurate prognosis. This data suggests that a high GPER expression level within the fibroblasts would indicate a lower likelihood of metastasis, and thus a more favorable prognosis. By therapeutically targeting the anti-metastatic ability of GPER-expressing fibroblasts with GPER-selective agonists like G-1, it may be possible to confine a tumor to the primary location. By reducing distant metastasis, better treatment options, minimizing the use of systemic chemotherapy and its harmful side effects, would be available resulting in improved patient survival. However, complications from treatment with a GPER agonist could arise if the tumor cells express high levels of GPER. Within tumor cells, GPER increases proliferation and may lead to increased migration [1, 3, 13]. As such, treatment with a GPER agonist would only be advisable when the fibroblasts

express high levels of GPER and the tumor cells express low levels of GPER. A more detailed understanding of the interconnected, complex effects GPER has on breast cancer metastasis through tumor cells, fibroblasts, and many other stromal cells will allow for better GPER-targeted treatment options to be developed to prevent metastasis and improve patient survival.

6.1 References

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