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# In Vivo Characterization of G Protein-Coupled Estrogen Receptor in Mammary Tumorigenesis

Nicole Marjon

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**IN VIVO CHARACTERIZATION OF G PROTEIN-COUPLED  
ESTROGEN RECEPTOR IN MAMMARY TUMORIGENESIS**

by

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B.S. Biochemistry and Molecular Biology  
University of New Mexico, 2005

**DISSERTATION**

Submitted in Partial Fulfillment of the  
Requirements for the Degree of

**Doctor of Philosophy  
Biomedical Sciences**

The University of New Mexico  
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**May, 2015**

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## **DEDICATION**

To Kristopher, Lilli, and Kylee Marjon

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# **In Vivo Characterization of G Protein-Coupled Estrogen Receptor in Mammary Tumorigenesis**

**Nicole A. Marjon, MS, PhD, Biomedical Sciences, University of New Mexico, 2015**

## **ABSTRACT**

The steroid hormone, estrogen (17 $\beta$ -estradiol or E2), is involved in numerous and varied physiological processes. Until recently, all E2-dependent effects were thought to be propagated exclusively through the classical estrogen receptors (ERs), ER $\alpha$  and ER $\beta$ . However, in double ER $\alpha$ / $\beta$  knockout mice, select E2-dependent effects remain, suggesting the existence of additional E2 receptors, such as the G protein-coupled estrogen receptor (GPER) 1.

E2 plays a central role in the progression of breast cancer, and inhibiting E2 signaling in women with breast cancer increases long-term survival. The role of GPER in breast cancer is largely unknown. E2 stimulation of GPER activates the mitogen activated protein kinase (MAPK) cascade as well as phosphoinositide 3-kinase (PI3K), suggesting a role for GPER in proliferation and cell survival. Additionally, GPER is responsible for E2-dependent proliferation in select breast cancer cells *in vitro*. Clinically, GPER expression is correlated with increased size of the primary tumor and occurrence of distant metastasis. Although these data implicate GPER in breast carcinogenesis, its role *in vivo*, where tumor cells exist in a complex microenvironment, remains unclear. Therefore, this study focuses on the *in vivo* effects of GPER on mammary tumorigenesis.

GPER KO mice were bred with MMTV-PyMT mice, a model of mammary carcinogenesis, to determine the effects of GPER expression on tumor development and

progression. Tumor latency and extent of hyperplasia was unaffected, suggesting GPER does not play a role in early tumor development. However, in late stage tumorigenesis, GPER KO mice displayed smaller tumors and decreased metastases, demonstrating a role for GPER in tumor growth and progression. To distinguish the effects of GPER in the tumor parenchyma and microenvironment, GPER expressing PyMT tumor epithelial cells (WT/PyMT) or GPER KO PyMT cells (KO/PyMT) were each orthotopically transplanted into GPER WT and GPER KO recipient mice, and analyzed for tumor growth and metastasis. WT/PyMT tumor size was unaffected by the microenvironment, whereas KO/PyMT tumors were larger in KO recipient mice compared to WT recipient mice. With respect to metastasis, WT/PyMT mice metastasized more frequently in WT compared with KO mice, while KO/PyMT cell metastasis was unaffected by the microenvironment. These data suggest GPER expression in the tumor microenvironment and epithelium differentially regulates tumor growth and metastasis. Finally, because GPER expression regulates tumor progression, the effects of administering a GPER-selective agonist or antagonist in the PyMT model were determined. While the GPER-selective agonist G-1 did not affect tumor size or metastasis, the GPER-selective antagonist G36 decreased E2-mediated metastasis.

Together, these data are the first *in vivo* demonstration of GPER augmenting tumor growth and progression. Further, pharmacologically inhibiting GPER decreases metastasis, suggesting GPER could be a viable candidate for targeted therapy in breast cancer.

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## ABBREVIATIONS

[<sup>3</sup>H]E2 – tritiated 17 $\beta$ -estradiol

AF1 – activation function 1 domain

AF2 – activation function 2 domain

AI – aromatase inhibitor

AKT – protein kinase B

ATCC – American Type Culture Collection

BCL2 – B-cell lymphoma 2

BMM – bone marrow derived macrophage

BSA – bovine serum albumin

cAMP – cyclic adenosine monophosphate

CTGF – connective tissue growth factor

DAB – 3,3' diaminobenzidine tetrahydrochloride

DAPI – 4',6-diamidino-2-phenylindole

DCIS – ductal carcinoma *in situ*

DMEM – Dulbecco's modified eagle medium

E1 – estrone

E2 – 17 $\beta$ -estradiol

E3 – estriol

EAE – experimental autoimmune encephalomyelitis

ECM – extracellular matrix



EGF – epidermal growth factor

EGFR – epidermal growth factor receptor

ER – estrogen receptor

ERE – estrogen response element

ERK – extracellular signal-regulated kinase

ER $\alpha$  – estrogen receptor  $\alpha$

ER $\beta$  – estrogen receptor  $\beta$

FISH – fluorescence *in situ* hybridization

FSH – follicle-stimulating hormone

FSHR – follicle-stimulating hormone receptor

G protein – heterotrimeric G protein

G-1 – GPR30-selective compound 1 (agonist)

G15 – GPR30-selective compound 15 (antagonist)

G36 – GPR30-selective compound 36 (antagonist)

GFP – green fluorescent protein

GnRH – gonadotropin releasing hormone

GPCR – G protein-coupled receptor

GPOR – G protein-coupled estrogen receptor

GRK – G protein-coupled receptor kinase

H&E – hematoxylin and eosin

HB-EGF – heparin-bound epidermal growth factor

HER2 – human epidermal growth factor receptor 2

HET – heterozygous

HRP – horseradish peroxidase

HSP – heat shock protein

IFN $\gamma$  – interferon  $\gamma$

IHC – immunohistochemistry

IL-10 – interleukin 10

IL-4 – interleukin 4

K<sub>d</sub> – dissociation constant

K<sub>i</sub> – inhibition constant

KO – knockout

LCIS – luminal carcinoma *in situ*

LH – luteinizing hormone

LHR – luteinizing hormone receptor

LT – large T antigen from polyoma virus

MAPK – mitogen-activated protein kinase

mER $\alpha$  – membrane-associated estrogen receptor  $\alpha$

MMP – matrix metalloproteinase

MMTV – mouse mammary tumor virus

mRFP – monomeric red fluorescent protein

NGS – normal goat serum

NO – nitric oxide

P-histone H3 – phosphorylated histone H3

PBS-T – phosphate-buffered saline

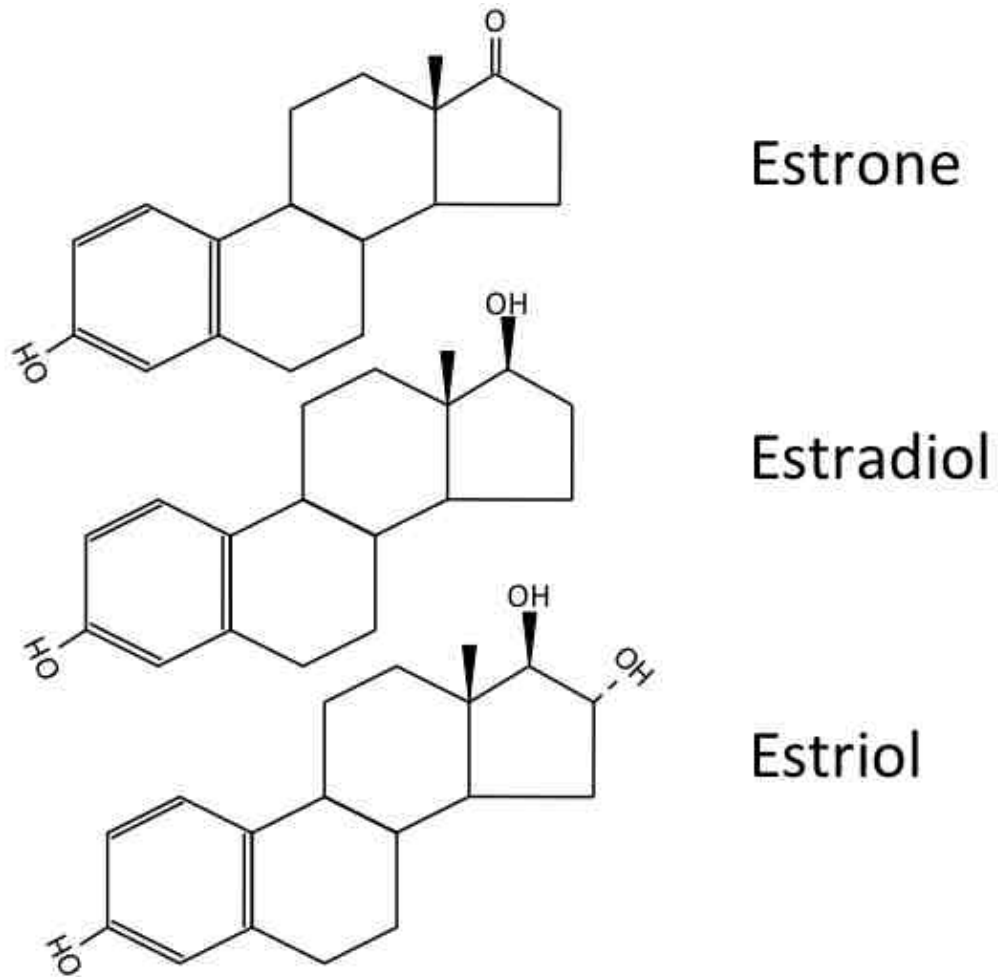
PFA – paraformaldehyde

PH – pleckstrin homology domain  
PI3K – phosphoinositide 3-kinase  
PLC – phospholipase C  
PP2A – protein phosphatase 2  
PR – progesterone receptor  
PyMT (MT) – polyoma middle T antigen  
qPCR – relative-quantitative PCR  
RBA – relative binding affinity  
SERM – selective estrogen-receptor modulator  
ST – small T antigen from polyoma virus  
TAM – tumor associated macrophage  
TF – transcription factor  
TNF – tumor necrosis factor  
WT – wild type

**CHAPTER 1**  
**INTRODUCTION**

## **1.1 ESTROGEN**

Estrogen is the primary female sex hormone and is in the family of steroid hormones, which includes glucocorticoids, mineralocorticoids, androgens, and progestins. There are three naturally occurring estrogens produced by humans including estrone (E1), estradiol (E2), and estriol (E3), and are named based on the number of hydroxyl groups they contain. E1 has one hydroxyl group, E2 has two hydroxyl groups, and E3 contains three hydroxyl groups (Figure 1.1). E1 is the predominant estrogen produced in post-menopausal women and is produced primarily by adipocytes (1), while E3 is produced by the placenta and is the predominant circulating estrogen during pregnancy (3). E2 is the principal circulating estrogen in premenopausal women and is well characterized in the regulation of the female reproductive system (4). There are two E2 isoforms,  $17\alpha$ -E2 and  $17\beta$ -E2; however,  $17\beta$ -E2 is the more physiologically active E2 and will be abbreviated as E2 throughout this dissertation (5).



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

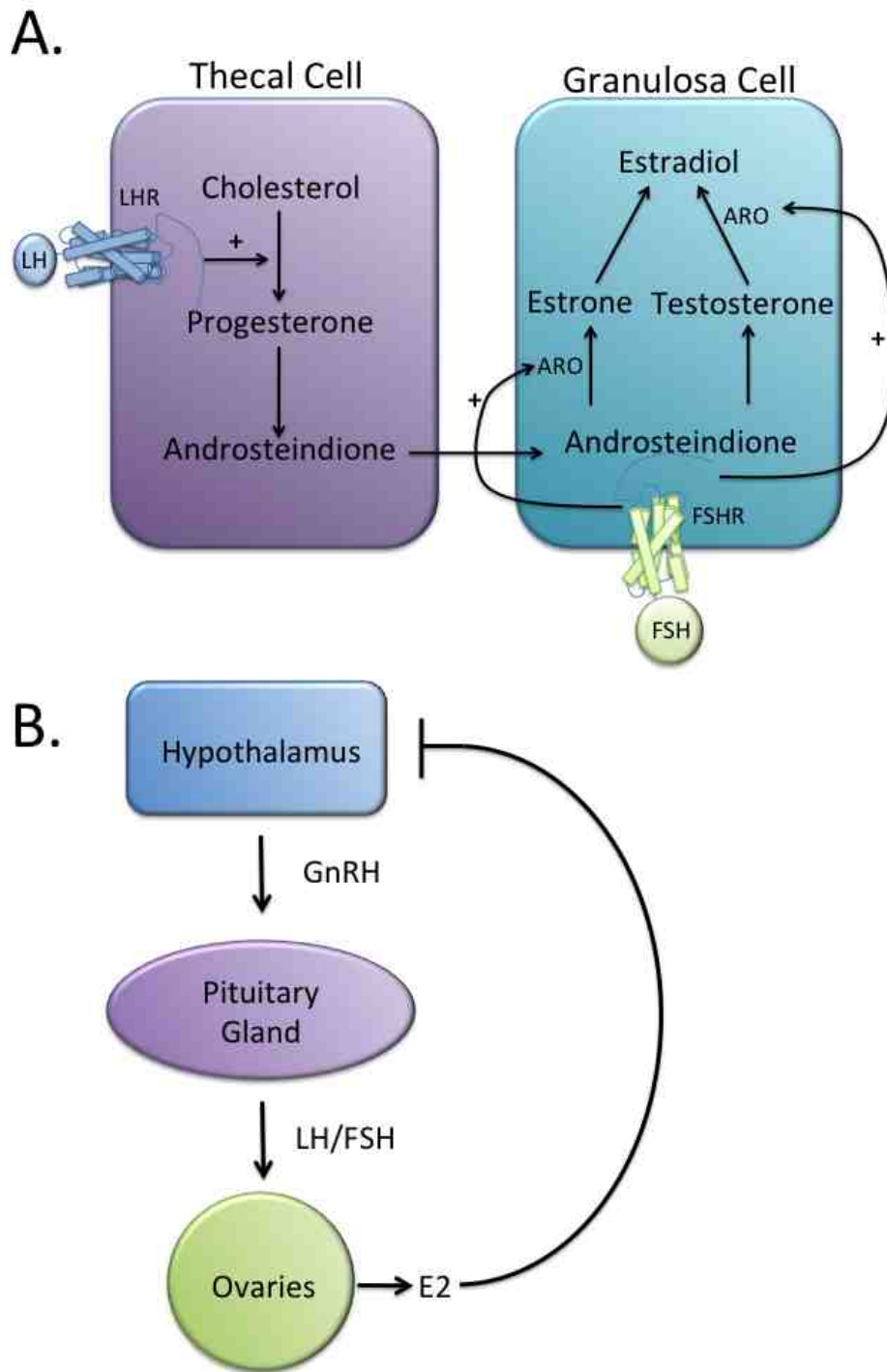
### 1.1.1 Estrogen Synthesis and Regulation

In females, the primary site of E2 synthesis is the ovarian follicle, although the liver, adrenal gland, adipocytes, bone, vascular endothelium, and regions of the brain also produce comparatively low amounts of E2 (4, 6, 7). These extra-ovarian sites of estrogen production, especially adipocytes, are important for the generation of E2 in postmenopausal women. In the ovarian follicle, there is cooperation between thecal and granulosa cells to convert cholesterol to E2 (8, 9). Through a multistep process in the thecal cell, cholesterol is converted to androstenedione or testosterone (8). Because the thecal cell does not express aromatase, the p450 enzyme required to convert androgens to estrogens, androstenedione and testosterone are transported to the granulosa cell, where androstenedione and testosterone are aromatized producing E1 and E2, respectively (10, 11). The majority of E1 is subsequently converted to E2 in the granulosa cell or peripheral tissues (Figure 1.2A).

The biosynthesis of E2 in the ovary is tightly regulated by the hypothalamus and pituitary gland. The hypothalamus releases gonadotropin releasing hormone (GnRH), which signals to the pituitary gland to release luteinizing hormone (LH) and follicle stimulating hormone (FSH). LH receptors (LHRs) are present at a high concentration on thecal cells, and stimulation of LHR induces the conversion of cholesterol to androstenedione (9). Simultaneously, FSH binds to FSH receptors (FSHRs) found on granulosa cells and activates aromatase, converting androstenedione to E1 or testosterone to E2 (12). E2 at high levels feeds back to the hypothalamus to inhibit the production of GnRH (Figure 1.2B) (8, 9). This negative feedback loop results in cyclic production of E2 in females of reproductive age and controls the menstrual cycle. The ovarian

production of E2 ceases when women enter menopause, which typically occurs in the fourth or fifth decade of life.





**Figure 1.2 Ovarian E2 production.** A. Premenopausal production of estradiol occurs in the ovarian follicle thecal and granulosa cells. In the thecal cell, LH stimulates the conversion of cholesterol to androstenedione, which is transported to the granulosa cell and converted to estrone or testosterone and ultimately estradiol. Aromatase (ARO) is responsible for the conversion of androgens to estrogens and is positively regulated by FSH. B. The hypothalamus secretes gonadotropin releasing hormone (GnRH), which stimulates the pituitary gland to secrete LH and FSH, which act on the ovarian follicle to stimulate E2 production. Secreted E2 subsequently inhibits hypothalamic GnRH release.

## **1.2 Classical Estrogen Receptors**

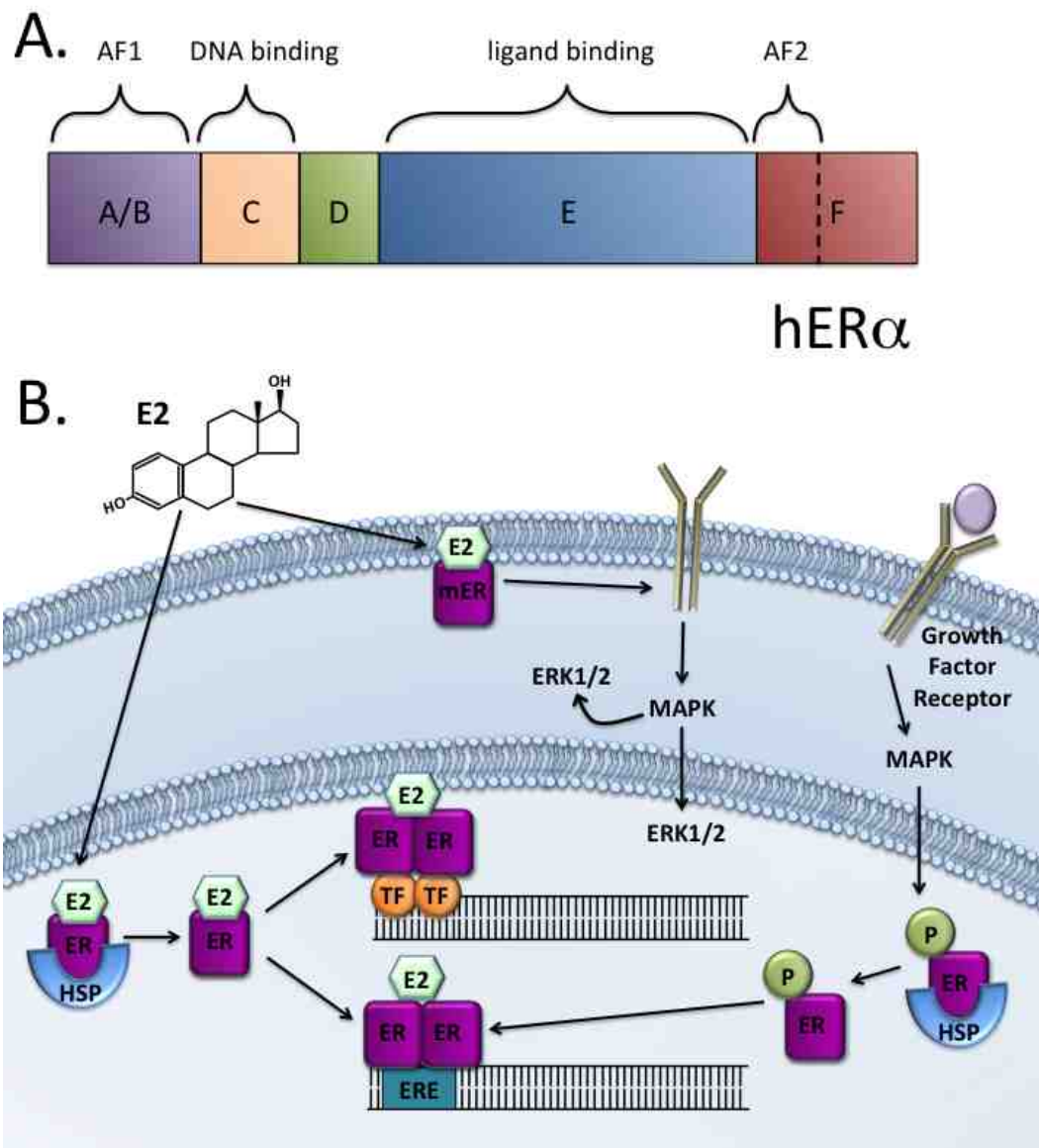
In 1930, E2 was identified as the ovarian-derived hormone when it was purified in crystallized form in the laboratory of Allen and Doisy (13). Early reports described a role for E2 in sexual maturity due to E2-dependent actions, including increased uterine wet weight, modified vaginal tissue, and vaginal and uterine proliferation (13). It was believed that E2 regulated metabolism in the absence of a specific E2 receptor until the development of tritiated E2 ( $[^3\text{H}]E2$ ) (14). In the Jensen laboratory,  $[^3\text{H}]E2$  was administered to female rats and was exclusively retained by estrogen responsive tissues, including the uterus and vagina where it caused proliferation. The retention of E2 and E2-dependent proliferation were blocked by the antiestrogen nafoxidine in a dose dependent manner, providing evidence for the existence of an E2 receptor (14). In 1973, the first estrogen receptor (ER) was extracted from rat uterus and was renamed ER $\alpha$  subsequent to the discovery of the second ER, ER $\beta$ , in 1996 (15, 16). ER $\alpha$  and ER $\beta$  are designated classical ERs. Classical ERs have a similar basic structure consisting of five domains designated A/B, C, D, E, and F (Figure 1.3A), with >60% overall sequence homology (17-19). Like other steroid receptors, ER $\alpha/\beta$  act as transcription factors after binding their ligand. The E domain is required for estrogen binding and the C domain is responsible for DNA binding (20). The E domain also contains activation function 2 (AF2) that is involved in ligand-dependent activation of transcription. In the amino terminal A/B domain, ER $\alpha$  also contains activation function 1 (AF1) responsible for ligand-independent activity (19).

### 1.2.1 Classical ER Signaling

ER $\alpha$  and ER $\beta$  are located primarily in the nucleus and stabilized in their inactive form by association with heat shock proteins (HSPs) (14). When activated by E2 binding, the classical ERs are released from HSPs, dimerize, and bind to estrogen response elements (EREs) in DNA where they recruit coactivators and corepressors to modulate target gene expression (21). Typically ER $\alpha$  binds to EREs to regulate transcription; however, using an ER $\alpha$  mutant that is unable to interact with DNA, Jakacka et al demonstrated ER $\alpha$  is also capable of interacting with transcription factors, coregulators, and corepressors to modulate gene expression independent of direct DNA binding (22). In another non-classical pathway, ligand-independent activation of the classical ERs occurs through MAPK-dependent phosphorylation of ER $\alpha$ , regulating transcription of ER $\alpha$  target genes (23, 24). These three pathways of ER $\alpha$ / $\beta$  activity culminate in the modulation of transcription and are therefore classified as long-term genomic responses. This is in contrast to activation of rapid, non-genomic signaling typically occurring downstream of membrane-associated receptors (Figure 1.3B).

Rapid, non-genomic signaling is defined by the activation of second messengers such as adenylyl cyclase or phospholipase C (PLC), and generally occurs on the order of seconds to minutes. Although ER $\alpha$  acts predominantly as a transcription factor, it has been reported to associate with the membrane through palmitoylation of its E domain and in that location to activate second messengers. Membrane-associated ER $\alpha$  (mER $\alpha$ ) associates with caveolin 1. When bound by E2, mER $\alpha$  dimerizes and associates with heterotrimeric guanine nucleotide-binding (G) proteins to produce cAMP, initiate Ca<sup>++</sup> mobilization, and stimulate kinase cascades (25). One of the best-described roles for

mER $\alpha$  is the production of NO in the vasculature leading to vasodilation, which has been verified *in vivo* (26). Another widely investigated role for mER $\alpha$  is cellular proliferation in the absence of nuclear ER $\alpha$ -mediated transcription. In breast cancer cell lines, mER $\alpha$  transactivates epidermal growth factor receptor (EGFR) and insulin-like growth factor 1 receptor, thereby phosphorylating ERK and increasing cell proliferation (25). While this is an intriguing role for mER $\alpha$ , the prevalence of mER $\alpha$  in human breast cancer samples is debated (Figure 1.3B) (27).



**Figure 1.3 Classical ER structure and signaling.** A. ER $\alpha$  has five major domains. DNA binding occurs in the C domain and the E domain is responsible for ligand binding. These domains are separated by the “hinge” region D domain. The N terminal activation function 1 (AF1) domain is required for ligand-independent transcription, while the activation function 2 (AF2) domain located in the C terminus and is responsible for ligand-dependent transcription. ER $\beta$  has a similar structure to ER $\alpha$ . B. Classically, E2 binds ER $\alpha/\beta$  in the nucleus releasing the receptor from heat shock proteins (HSP). The receptors dimerize and bind to estrogen response elements (EREs) in the DNA to modulate transcription. Additionally, once activated ER $\alpha/\beta$  can bind transcription factors to regulate transcription of genes not traditionally defined as estrogen-responsive genes. Ligand-independent ER $\alpha$  activation also occurs through kinase signaling downstream of growth factor receptors through phosphorylation of ER $\alpha$ . Lastly, ER $\alpha$  associates with the cell membrane (mER) and activates rapid, non-genomic signaling. In cultured breast cancer cells, mER transactivates growth factor receptors, enhancing proliferation.

### **1.3 G protein-Coupled Estrogen Receptor 1**

A double ER $\alpha$ / $\beta$  knockout mouse was generated to enhance the understanding of E2 and its receptors in normal physiology. The majority of E2-dependent effects were abolished in the double knockout mice; however, some rapid E2-dependent actions remained. Further, in cell lines, rapid E2 signaling remained in the presence of the ER $\alpha$  antagonist ICI-182,780, or were induced by this compound (28). These observations suggested the existence of a novel E2 receptor later determined to be the G protein-coupled receptor (GPCR) GPR30, which has been renamed G protein-coupled estrogen receptor 1 (GPER).

#### **1.3.1 GPCR Biology**

GPCRs are defined by their structure and interaction with G proteins. While many receptor types can interact with heterotrimeric G proteins, including receptor tyrosine kinases and classical steroid receptors, the term GPCR typically refers to a family of seven-transmembrane domain receptors. GPCRs are classically present on the cell membrane. When activated by their ligands, GPCRs recruit G proteins and initiate rapid downstream signaling via activation of adenylyl cyclase and PLC (29). Following activation, GPCRs are rapidly phosphorylated by a G protein-coupled receptor kinase (GRK), which recruits arrestins to bind the C-terminal tail of GPCRs, which in turn sterically block interactions with G proteins, inhibiting further signaling. Arrestins then initiate their own signaling cascade to facilitate trafficking of GPCRs targeted for recycling or degradation (30, 31). Arrestins have also been implicated in GPCR-dependent transactivation of tyrosine kinase receptors (32).

GPCRs are involved in a wide variety of physiologic activities, and 40% of currently prescribed pharmaceutical drugs target this receptor superfamily. It has been predicted through whole genome-sequence analysis that more than 800 GPCRs exist in the human genome. Roughly 300 GPCRs are believed to be olfactory receptors, and of the remaining receptors, over 100 have no known ligand and are classified as orphan receptors (33).

### **1.3.2 Discovery and Characterization of GPER**

In the 1990's, many research groups cloned orphan GPCRs with the goal of identifying their natural ligands and physiologic functions. In the late 1990's, GPR30 was cloned and reported to be expressed at higher levels in ER $\alpha$ <sup>+</sup> (MCF7) breast cancer cells as compared to ER $\alpha$ <sup>-</sup> (MDA-MB-231) breast cancer cells, leading Filardo and colleagues to hypothesize GPR30 was involved in rapid E2 signaling (34, 35). To test this hypothesis, ERK1/2 signaling was evaluated in MCF7 (ER $\alpha$ / $\beta$ <sup>+</sup>, GPR30<sup>+</sup>), SKBR3 (ER $\alpha$ / $\beta$ <sup>-</sup>, GPR30<sup>+</sup>), and MDA-MB-231 (ER $\alpha$ <sup>-</sup>, ER $\beta$ <sup>+</sup>, GPR30<sup>-</sup>) cell lines. E2-dependent ERK1/2 phosphorylation (P-ERK1/2) was stimulated in MCF7 and SKBR3 cells, but it was absent in MDA-MB-231 cells. Further, P-ERK1/2 could be induced in MDA-MB-231 cells transfected with a GPR30 plasmid in response to E2, tamoxifen, and ICI-182,780, but not in response to 17 $\alpha$ -estradiol or progesterone (35). These data suggested GPR30 expression was required to activate rapid E2-dependent signaling; however, there was no evidence that E2 interacted with the receptor.

In 2005, two groups independently demonstrated E2 binding to GPR30-expressing cells. In one report, Thomas *et al.* demonstrated that [<sup>3</sup>H]E2 bound to membranes of SKBR3 cells that are GPR30<sup>+</sup> and ER $\alpha$ / $\beta$ <sup>-</sup> with high affinity ( $K_d = 3.3$

nM), while testosterone, progesterone, and cortisol were unable to bind. Unlike the classical ERs, E1 and E3 displayed an extremely low binding affinity with a relative binding affinity (RBA) of <1% when compared to E2. As previously proposed, tamoxifen and ICI-182,780 were able to bind to GPR30 with a 10% RBA as compared to E2. To confirm these results, [<sup>3</sup>H]E2 binding was examined in the presence and absence of GPR30 transfection in HEK293 cells, which do not express the classic E2 receptors or GPR30. E2 binding was observed in GPR30-transfected HEK293 cells, but not in untransfected cells (36). In the second report, Ravenkar *et al.* used fluorescently labeled E2 to examine the binding affinity as well as location of estrogen binding in GPR30-transfected COS7 cells, which lack both classical ERs and GPR30. In addition, a fusion protein of GPR30 and green fluorescent protein (GFP) was exogenously expressed in COS7 to determine cellular location. GPR30 was located intracellularly on the endoplasmic reticulum and Golgi apparatus, in contrast to the classical plasma membrane localization of most GPCRs. These results were verified using an antibody directed against GPR30 in MCF7, SKBR3, MDA-MD-231, JEG, and HEC50 cells endogenously expressing GPR30. Using E2-Alexa633 as the tracer, E2 displayed a  $K_i$  of 6.6nM in GPR30-transfected COS cells, similar to the  $K_d$  determined for [<sup>3</sup>H]E2 by Thomas *et al.* Additionally, E2-Alexa546 colocalized with intracellular pools of GPR30-GFP in permeabilized cells, but not in unpermeabilized cells, when imaged using confocal fluorescence microscopy. These results suggest GPR30 is present on the endoplasmic reticulum and Golgi apparatus, where it is able to bind E2 (37). These two reports provided the first evidence of E2 binding to GPR30, and GPR30 was subsequently



renamed G protein-coupled estrogen receptor 1 (GPER) by the International Union of Basic and Clinical Pharmacology.

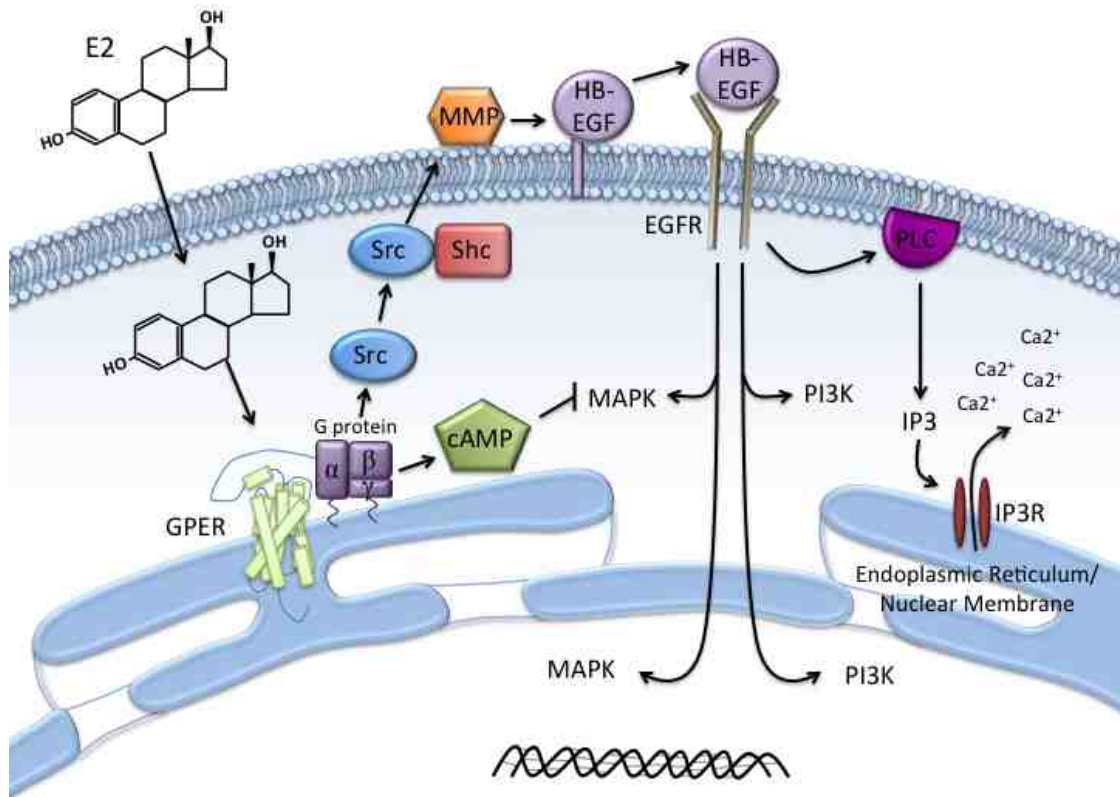
### 1.3.3 GPER Signaling

GPER activates rapid non-genomic signaling resulting in epithelial cell proliferation and motility. Filardo and colleagues demonstrated GPER-dependent P-ERK1/2 was blocked by the EGFR inhibitor tyrphostin AG 1478 (AG 1478), suggesting GPER signals via EGFR transactivation. P-ERK1/2 was also inhibited by Src family tyrosine kinase inhibitors, PP2, and heparin-bound-EGF (HB-EGF)-neutralizing antibodies. Furthermore, pertussis toxin and a G $\beta\gamma$ -sequestering peptide also inhibited GPER-dependent ERK1/2 activation (35). Therefore, it was concluded that GPER signaled through G $\beta\gamma$ -dependent Src activation ultimately resulting in transactivation of the EGFR following cleavage of proHB-EGF by matrix metalloproteinases (MMPs). Integrin  $\alpha 5\beta 1$  activation and Shc recruitment were subsequently determined to be required for EGFR transactivation (38). Although GPER induces P-ERK1/2 downstream of EGFR, it also inhibits P-ERK1/2 via cAMP production resulting from G $\alpha_s$  activation. This dual action of GPER tightly regulates ERK1/2 activation, which ensures the potent actions of ERK1/2 are not sustained (39).

Further investigation of the signaling downstream of GPER-dependent EGFR transactivation revealed E2-stimulated intracellular Ca<sup>++</sup> release as well as activation of phosphatidylinositol 3-kinase (PI3K). COS7 cells exogenously expressing GPR30 were loaded with the calcium sensitive dye Indo-1AM and stimulated with E2 in the presence and absence of pertussis toxin and AG 1478. E2-stimulated intracellular Ca<sup>++</sup> mobilization was sensitive to pertussis toxin and AG 1478, demonstrating Ca<sup>++</sup>

mobilization occurs downstream of GPER-dependent EGFR transactivation. To investigate PI3K activation, GPER-GFP and the PH domain of AKT fused to monomeric red fluorescent protein (PH-mRFP) were expressed in COS7 cells. Cells were stimulated with tamoxifen, E2, and E2 in the presence of AG 1478, pertussis toxin, and the PI3K inhibitor, LY294002. The localization of PH-mRFP was analyzed in GPER-expressing cells by confocal microscopy. In unstimulated cells, PH-mRFP was dispersed throughout the cell. However, upon stimulation with E2 or tamoxifen, PH-mRFP translocated to the nucleus. AG 1478, pertussis toxin, and LY294002 inhibited the nuclear translocation of PH-mRFP (37). Therefore, E2 stimulated the activation of nuclear PI3K through GPER-dependent transactivation of EGFR (Figure 1.4).

Downstream of GPER-initiated rapid signaling, transcription of genes such as Bcl-2, cyclin D, and *c-fos* is regulated, enhancing cell proliferation and survival (40-42). Many of the genes indirectly regulated by GPER are involved in proliferation and cell survival, as would be expected downstream of PI3K and MAPK signaling. Therefore, while E2-dependent effects are often separated into rapid signaling and long-term genomic actions, the lines can become blurred as rapid signaling modulates gene transcription.



**Figure 1.4 GPER-mediated signaling.** GPER stimulated by E2 activates heterotrimeric G proteins leading to Src activation. Src recruits Shc and activates matrix metalloproteinases (MMPs). MMPs cleave cell surface-bound heparin-bound EGF causing the autocrine and paracrine activation of EGFR. EGFR activates MAPK and PI3K and enhances calcium mobilization. MAPK and PI3K mediate rapid non-genomic effects in the cytoplasm and translocate to the nucleus to modulate transcription. GPER stimulation also activates adenylyl cyclase to produce cyclic AMP (cAMP), which inhibits EGFR-mediated MAPK activation, thereby regulating MAPK signaling.

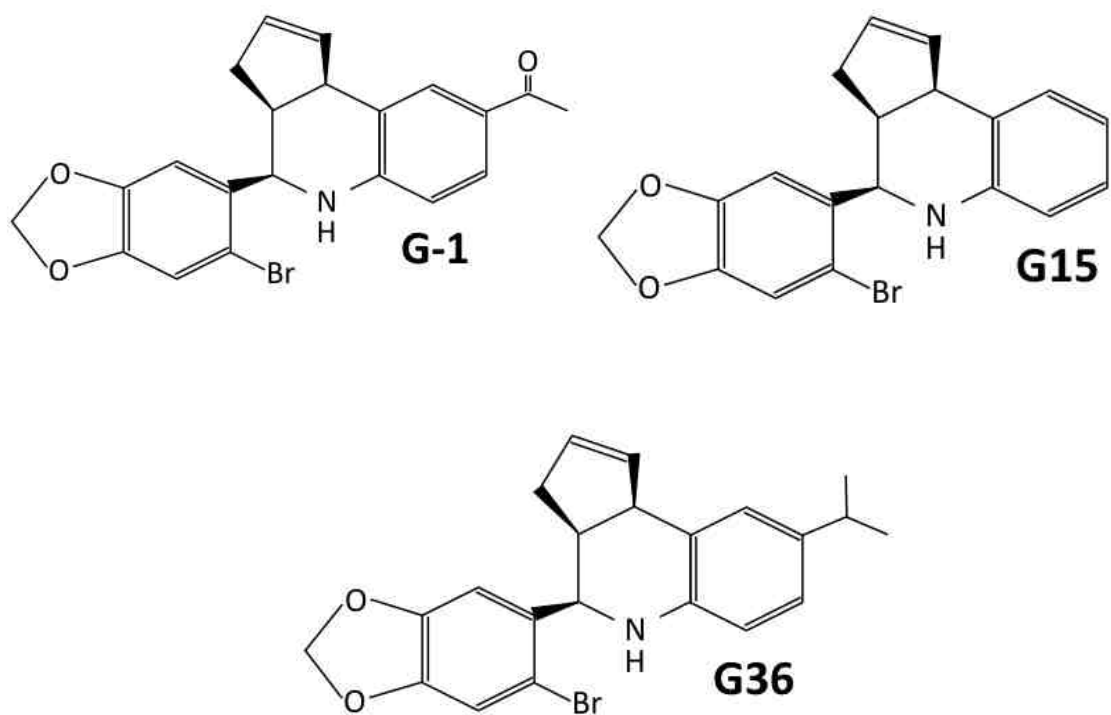
### 1.3.4 GPER Ligands

Using cell systems that either express GPER but lack ER $\alpha$ / $\beta$  or by exogenously expressing GPER in cells that lack all three estrogen receptors, GPER has been characterized as an E2 receptor. However, because the classical ERs, specifically ER $\alpha$ , are also able to activate rapid E2 signaling, it is essential to determine GPER-specific physiologic outcomes (43). Since GPER and ER $\alpha$  share many of the same ligands, including E2, ICI 182,780, and tamoxifen, it is difficult to study the effects of GPER in isolation. Therefore, virtual screening of a 10,000 chemical compound library was followed by flow cytometric binding assays to discover GPER-selective ligands (44). COS7 cells were transiently transfected with exogenous estrogen receptors and binding of E2-Alexa633 was measured by flow cytometry. The cells were preincubated with the prospective compound followed by addition of E2-Alexa633 and assayed for inhibition of E2-Alexa633 binding. One compound, a substituted dihydroquinoline, was determined to selectively inhibit E2-Alexa633 binding in COS7 cells expressing GPER and has been named G-1 (GPR30-specific compound 1) (Figure 1.5). The  $K_i$  of G-1 for GPER was determined to be 11 nM. To determine the activity of G-1, intracellular Ca<sup>++</sup> mobilization and PI3K activation were analyzed. In COS7 cells transiently transfected with ER $\alpha$ , ER $\beta$ , or GPER, G-1 was able to initiate intracellular calcium release only in cells expressing GPER, whereas E2 initiated Ca<sup>++</sup> release in cells expressing any of the three known E2 receptors. Similarly, while E2 induced PI3K activation in COS7 cells expressing ER $\alpha$ ,

ER $\beta$ , or GPER, G-1 was only able to induce PI3K activation in cells expressing GPER (44). Therefore, G-1 is classified as a highly selective-GPER agonist and has been widely used to examine the contribution of GPER to E2 signaling and physiology.

Following the discovery of G-1, it became clear a GPER-selective antagonist would enhance the understanding of GPER-dependent E2 signaling, especially in situations where all three ERs were present. Virtual screening and synthetic chemistry were used to identify G-1-like compounds, particularly those lacking potential hydrogen-bonding moieties. Candidate compounds were assessed to determine if they inhibited E2 mediated Ca<sup>++</sup> mobilization in GPER-expressing cells. G15, a compound similar in structure to G-1, except that it lacks the ethanone moiety, was determined to have antagonistic properties and competitively bound GPER with a K<sub>i</sub> of about 20nM (Figure 1.5). G15 displayed very weak affinity for ER $\alpha$  and ER $\beta$  with a K<sub>i</sub> >10 $\mu$ M. Further testing revealed that G15 inhibited GPER-mediated Ca<sup>++</sup> mobilization, PI3K activation, and *in vivo* uterine proliferation. Although 1  $\mu$ M G15 was unable to activate ER $\alpha$ -mediated PI3K activation, it bound weakly to ER $\alpha$  and ER $\beta$  at 10  $\mu$ M and at that concentration was able to activate transcription of an ERE reporter, at about 20% the maximal efficacy of E2 (45). Because G-1 lacked this activity, even at 10 $\mu$ M concentrations, it was hypothesized that the additional steric hindrance due to the ethanone moiety of G-1 prevented access and/or binding to the ligand-binding pocket of ER $\alpha$  and ER $\beta$ . Therefore, to mimic the steric bulk of G-1, but to prevent possible hydrogen bonding of the keto group to GPER, resulting in activation, a G-1 analog was synthesized in which the keto group was converted to a methyl group. The compound synthesized was named G36 (Figure 1.5) (46). G36 did not bind the classical ERs or

activate ERE-dependent transcription at 10 $\mu$ M. However, like G15, it was able to antagonize GPER-mediated Ca<sup>++</sup> mobilization and PI3K activation, demonstrating G36 is a more selective GPER antagonist than G15 (46). Thus, there are now three GPER-selective ligands that together have greatly enhanced our understanding of GPER biology.



**Figure 1.5 GPER-selective ligands.** Chemical structures of GPER-selective agonist (G-1) and antagonists (G15 and G36).

## **1.4 Estrogen Biology**

E2 is the main female sex steroid and plays a role in regulating female reproduction and the development of secondary sex characteristics. Serum E2 levels are low in females until the onset of puberty, at which time E2 levels rise and initiate breast development, widening of the pelvis, and increased fat deposition (12). During reproductive years, spikes in E2 trigger ovulation, and coordinated changes in E2 and progesterone levels stimulate endometrial proliferation and thickening to prepare the uterus for possible implantation of a fertilized egg. If implantation does not occur, E2 and progesterone levels return to baseline and the endometrium is shed producing menses (12). Successful implantation preserves high E2 levels, which are sustained throughout pregnancy to maintain the endometrium and cause vasodilation to enhance blood flow to the fetus, among other actions (47, 48). Although the role of E2 is best described in female reproduction, it affects a wide variety of physiologic systems in the male and female. E2 is neuroprotective (49), maintains bone density (50, 51), reduces the incidence of cardiovascular disease (52), and modulates the immune system (53). It is also involved in male reproduction by promoting testicular development, spermatogenesis, and sperm maturation (54-56). These diverse effects of E2 can be attributed to the classical ERs and GPER.

### **1.4.1 E2 in Breast Development**

The mammary gland is composed of branching ductal structures embedded in the mammary fat pad. The ducts are made up of a single layer of luminal epithelial cells surrounded by a layer of myoepithelial cells. In mammals, mammary gland development begins during embryogenesis, and at birth, males



and females have developed similar rudimentary ductal structures in the mammary fat pad (57). The development of the rudimentary mammary gland is unaffected in ER $\alpha$ , ER $\beta$ , ER $\alpha/\beta$ , and GPER knockout mice, suggesting that the initial development of the mammary gland is E2-independent (58). In females, with the onset of puberty, the ducts grow and branch in response to E2 until they reach the distal end of the mammary fat pad (59). However, the mammary gland is not fully mature until the third stage of growth during pregnancy and lactation when branching again increases and the luminal epithelial cells produce and secrete milk. Following cessation of milk production, involution occurs, demarcated by apoptosis of the epithelial cells, which are replaced by adipocytes (60).

About 15-25% of normal breast epithelial cells express ER $\alpha$ , while the majority of luminal epithelial and myoepithelial cells express ER $\beta$ . The mammary gland of ER $\alpha$  knockout mice does not develop beyond the rudimentary ducts formed during embryogenesis (61). Using an orthotopic transplant model, it was determined that ER $\alpha$  is required in both the luminal epithelial cells and the stroma. However, proliferating mammary cells are ER $\alpha$  negative, indicating E2 stimulates proliferation through a paracrine mechanism (62). In contrast ER $\beta$  and GPER knockout mice have no defects in peripubertal mammary gland development (58).

## **1.5 Breast Cancer**

Breast cancer is the most common type of cancer among women, excluding nonmelanoma skin cancer. Data gathered from 2006-2008 demonstrates women have a 1:8 lifetime risk of developing breast cancer, with probability dramatically increasing

after the age of 40. Although early detection and improved treatment options have decreased the mortality rate, breast cancer remains the second most common cause of cancer-related death in women as about 40,000 women die of breast cancer each year in the United States (63). Breast cancer is an exceptionally heterogeneous disease, and thus tumors are stratified into groups to predict prognosis and response to treatment.

### **1.5.1 Breast Cancer Classifications**

Many parameters have been used to classify breast cancer and patients with breast cancer including clinical, histologic, immunopathologic, molecular, and genomic markers. More recently, researchers have become interested in generating clinically assessable micro- and macroenvironmental markers. Currently, clinical parameters (patient age, tumor size, and lymph node status) are used in conjunction with histologic and immunopathologic information to determine patient prognosis and predict response to treatment (64, 65). Histologic subtypes of breast cancer are based on cytologic and architectural features. *In situ* carcinoma describes a tumor that has not invaded through the basement membrane. These tumors are further divided into ductal and lobular carcinoma *in situ* (DCIS and LCIS, respectively) with a higher prevalence of DCIS than LCIS. Histologically, LCIS is highly uniform whereas DCIS is more heterogeneous and further sub-classified as comedo, cribriform, micropapillary, papillary, or solid (65). Invasive or infiltrating carcinoma describes tumors that have invaded into the surrounding tissue through the basement membrane. These tumors are sub-classified as tubular, ductal lobular, invasive lobular, infiltrating ductal, mucinous (colloid), medullary, or papillary carcinoma (66). Infiltrating ductal carcinoma accounts for about 75% of invasive breast carcinoma cases and invasive lobular carcinomas account for

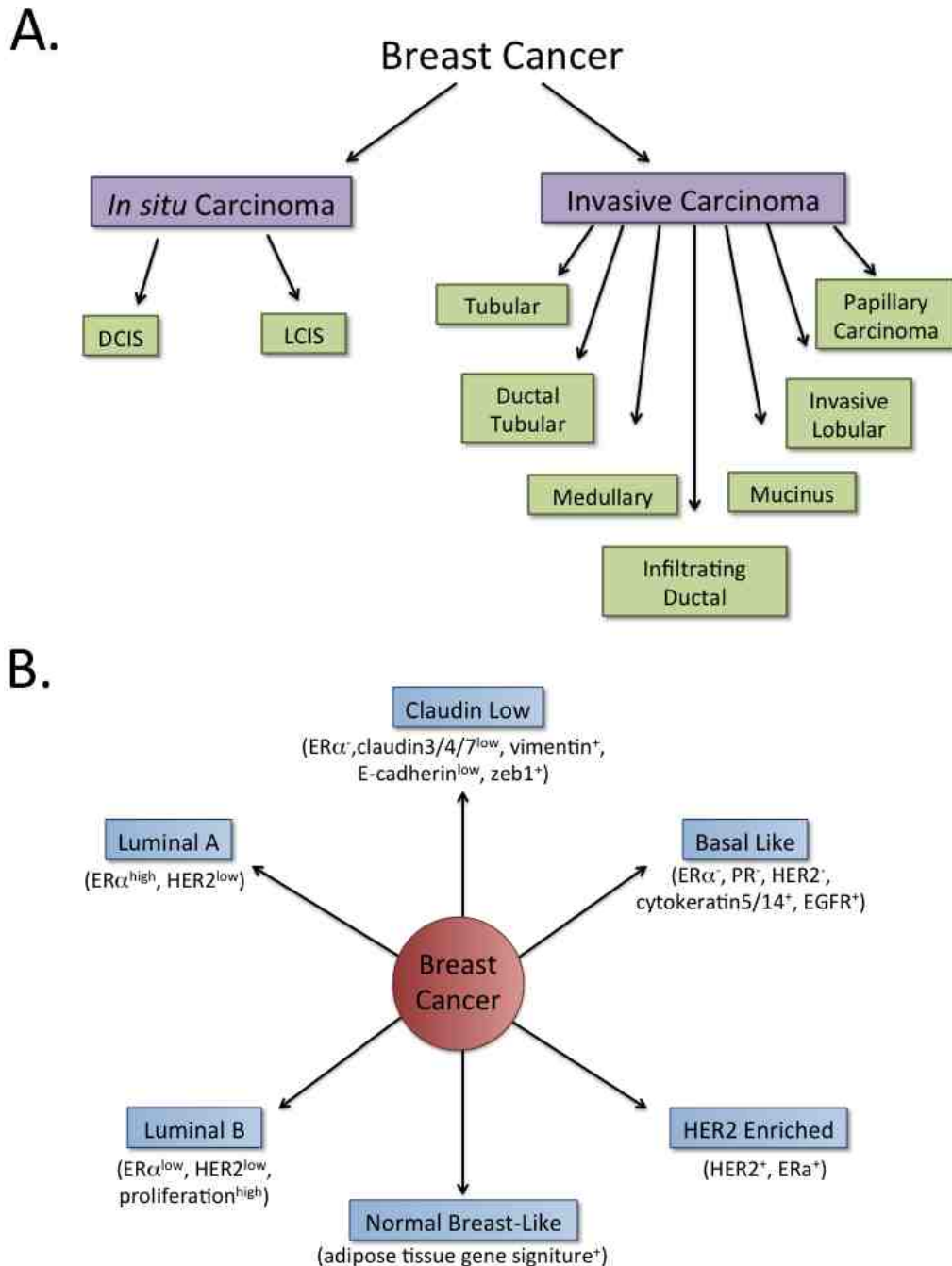
another 10% of cases, making up 85% of invasive tumors (67). Infiltrating ductal carcinoma is further classified into well differentiated, moderately differentiated, and poorly differentiated groups based on nuclear pleomorphism, glandular or tubule formation, and mitotic index (Figure 1.6A) (65).

While histological classification is useful to determine prognosis and how well a tumor may respond to chemotherapy, it does not aid in determining how well a patient will respond to molecularly targeted therapies. Therefore, in combination with histological classification, tumors are further classified based on immunopathologic markers. Presently, the most common markers include ER $\alpha$ , progesterone receptor (PR) and HER2, detected by immunohistochemistry (IHC) (64). Ambiguous IHC results for HER2 are subjected to fluorescence in situ hybridization (FISH) to evaluate the tumor for HER2 gene amplification (68). PR and ER $\alpha$  are typically correlated as ER $\alpha$  drives the expression of PR. ER $\alpha$ <sup>+</sup> tumors are correlated with a better outcome and can be targeted by endocrine therapies including ER $\alpha$  inhibitors and aromatase inhibitors (64). Additionally, patients with PR<sup>+</sup>/ER $\alpha$ <sup>-</sup> tumors are often treated with endocrine therapy as PR expression serves as evidence for ER $\alpha$  activity (69). HER2<sup>+</sup> tumors make up 15-20% of breast cancer cases and previously predicted poor patient outcome because HER2 drives tumor proliferation and aggressiveness (69). However, upon the discovery of HER2-targeted therapies, mortality decreased by 30-35% so now HER2<sup>+</sup> tumor predicts a treatable patient population (70). Currently, triple negative tumors (PR<sup>-</sup>/ER $\alpha$ <sup>-</sup>/HER2<sup>-</sup>) are associated with poor outcome, and there are no successful targeted treatment strategies for triple negative disease (71). These three markers also exemplify the

intratumoral heterogeneity that exists in breast cancer because only a small percentage of cells may express these receptors.

More recently, molecular classifications of breast cancer have been identified based on microarray data obtained from patient samples and are designated as the intrinsic molecular subtypes of breast cancer (72, 73). The molecular subtypes include claudin low ( $ER\alpha^-$ , claudin3/4/7<sup>low</sup>, vimentin<sup>+</sup>, E-cadherin<sup>low</sup>, zeb1<sup>+</sup>), basal-like ( $ER\alpha^-$ , PR<sup>-</sup>, HER2<sup>-</sup>, cytokeratin 5/14<sup>+</sup>, EGFR<sup>+</sup>), HER2-enriched (HER2<sup>+</sup>,  $ER\alpha^-$ ), normal breast-like (adipose tissue gene signature<sup>+</sup>), luminal A ( $ER\alpha^{high}$ , HER2<sup>low</sup>), and luminal B ( $ER\alpha^{low}$ , HER2<sup>low</sup>, proliferation<sup>high</sup>) (Figure 1.6B) (74). The widely used immunopathologic markers group cancers based on the expression of a few proteins, whereas the intrinsic molecular subtypes are able to further stratify cancer subtypes based on the expression of hundreds of mRNAs. However, this strategy is too expensive to be routinely implemented clinically. Therefore, researchers have narrowed down the number of genes required to make molecular profiling of patient samples clinically feasible. PAM50, a panel of 50 genes detected by RT-PCR, is a cost effective substitute for microarray analysis. PAM50 in combination with clinical parameters has a 94% sensitivity with a 97% negative predictive value when used to predict pathological complete response to treatment (75). Although PAM50 and similar gene expression signatures have been implemented in the clinic, many physicians still rely on immunohistochemical analysis of  $ER\alpha$ ,  $ER\beta$ , and HER2 to determine prognosis and treatment (76). The more information gained about individual breast cancers, the more difficult it is to group breast cancers into subtypes, demonstrating the need for personalized medicine. Many scientists have suggested using the intrinsic molecular

subtypes as tangible points on a breast cancer spectrum to help stratify and successfully treat patients.



**Figure 1.6 Breast cancer classifications.** A. Histopathological classification of breast cancer, defined by tissue morphology. B. Intrinsic molecular subtypes of breast cancer, determined by gene microarray.

### **1.5.2 Breast Cancer Microenvironment**

Many studies analyzing breast carcinogenesis focus on the epithelial tumor cells and ignore the stroma or tumor microenvironment. However, in a study by Barcellos-Hoff, cleared mammary fat pads were irradiated before transplantation of non-transformed epithelial cells. The irradiated stroma caused transformation of the epithelial cells resulting in tumor development, exemplifying the importance of the microenvironment in inhibiting tumor development (77). More physiologically relevant is the effect of chronic inflammation on tumor formation, which causes infiltration of immune cells, production of reactive oxygen species, and fibroblast activation leading to epithelial dysregulation and tumor formation (78-81). While stromal alterations are able to initiate the development of breast cancer, precancerous and cancerous cells also modify the microenvironment, further enhancing tumor survival, growth, and the ability to metastasize. Stromal alteration can enhance the release of growth factors, increase blood supply to the tumor, or change the make-up of the ECM to aid in migration and metastasis (82-84). However, these alterations can also cause genetic and epigenetic changes in the epithelial cells making the tumor more aggressive (82). In this manner, the tumor cells and microenvironment form a feedback loop to augment tumor progression.

Infiltrating immune cells, specifically macrophages, have been demonstrated to enhance tumorigenesis (85). Macrophages are extremely plastic cells that respond to multiple stimuli produced during injury and infection. Three populations of activated macrophages have been described, namely classically activated, regulatory, and wound

healing. Macrophages are often activated along a spectrum, rarely fitting neatly into any one category (86). Typically, macrophages become classically activated during infection when pro-inflammatory cytokines such as interferon- $\gamma$  (INF $\gamma$ ) and tumor necrosis factor (TNF) are produced. When classically activated, macrophages are capable of clearing invading pathogens and inhibiting tumorigenesis. Wound healing macrophages are generated after injury in response to IL-4 and produce ECM components such as collagen, while secreting very low levels of pro-inflammatory cytokines. Regulatory macrophages develop in response to glucocorticoids or IL-10 and secrete IL-10, which dampens proinflammatory immune responses (86). Tumor associated macrophages (TAMs) are polarized by factors in the tumor microenvironment and fall on the spectrum between wound healing and regulatory macrophages (86). TAMs secrete IL-10 and inhibit the production of inflammatory cytokines, thus protecting tumor cells from attack by the immune system (87). TAMs also aid in angiogenesis, remodel the ECM, and produce growth factors (88, 89). Collectively, the actions of TAMs enhance tumor growth and metastasis.

E2, which is present in high concentration in breast tumors, is able to polarize macrophages. Although the actions of E2 on macrophages can be ambiguous, many reports demonstrate a decrease in proinflammatory cytokine release by macrophages in the presence of E2 through activation of ER $\alpha$  and GPER (90, 91). Therefore, E2 in the tumor microenvironment may play a role in reeducating macrophages and decreasing their anti-tumoral response.



### 1.5.3 ER $\alpha$ in Breast Cancer

Prolonged exposure to estrogen, defined as early menarche and late menopause, or high serum E2 levels increases the risk of developing breast cancer. A receptor-independent mechanism has been described where estrogen metabolites, specifically catechol-estrogens, can form DNA adducts leading to mutagenesis and the development of cancer (92). A non-transformed, ER $\alpha^-$  breast epithelial cell line, MCF10F, was malignantly transformed when exposed to physiologic concentrations of E2 due to exposure to estrogen metabolites (93). However, an ER $\alpha$ -dependent mechanism has also been proposed whereby ER $\alpha$  stimulation by E2 causes increased breast cell proliferation providing increased opportunity for development DNA mutations. The mutations are unable to be effectively repaired because DNA repair machinery is less efficient in rapidly dividing cells (94). Crossing ER $\alpha$  knockout mice with mice that overexpress the oncogene Wnt-1 provided *in vivo* evidence for ER $\alpha$  initiating breast cancer. In ER $\alpha^-$ /Wnt-1 mice, 50% of tumors occurred by 11 months compared with 5 months for the ER $\alpha^+$ /Wnt-1 mice, suggesting ER $\alpha$  contributes to breast cancer development (95). In addition, inhibiting ER $\alpha$  with tamoxifen or raloxifene is a successful breast cancer prevention strategy in pre-menopausal women (96-98).

E2 also promotes the growth and distant metastasis of existing breast tumors through stimulation of ER $\alpha$ . E2-dependent tumor growth was first clinically observed in 1896 when oophorectomy dramatically decreased the size of breast tumors. In more recent years, the use of partial or full ER $\alpha$  antagonists such as tamoxifen, raloxifene and ICI-182,780 have been successful therapies in ER $\alpha^+$  breast cancer, decreasing recurrence rates and increasing disease free survival (96-98). Approximately 70% of human breast

cancers are ER $\alpha$ <sup>+</sup> and the majority responds to endocrine therapy that inhibits either ER $\alpha$  or the production of E2 by aromatase (59, 99). ER $\alpha$ -dependent growth is not only observed in premenopausal women, but also in postmenopausal women despite low levels of circulating E2. E2 in postmenopausal women is produced from extra-ovarian sites, particularly adipose tissue, making increased body fat a risk factor for breast cancer (100). Additionally, the intratumoral concentration of E2 in postmenopausal women with breast cancer are 20-fold higher than circulating E2, due in part to the production of E2 by tumor cells expressing high levels of aromatase as well as increased binding to ER $\alpha$  (101, 102). Intratumoral E2 targets ER $\alpha$  in the nucleus, regulating genes to increase proliferation and decrease apoptosis. As opposed to normal breast tissue, in tumors, stimulation of ER $\alpha$  drives proliferation of the cells expressing ER $\alpha$ , demonstrating a deviation from normal E2-induced paracrine signaling (103). An extranuclear ER $\alpha$ -dependent pathway has been reported that activates kinase cascades, such as the MAPK cascade, thereby increasing cell proliferation and decreasing cell death (104). The extranuclear signaling has also been implicated in cytoskeletal reorganization resembling alterations involved in metastasis (105). Therefore, it is well established that ER $\alpha$  enhances breast tumor growth as well as progression to metastasis.

#### **1.5.4 Anti-Estrogen Treatment of Breast Cancer**

Many cases of breast cancer are detected while they are still contained in the breast, meaning there is no detectable spread of disease to the draining lymph nodes or distant sites. Therefore, initial treatment primarily involves surgical removal of the tumor. However, after removal of the tumor, 50% of women die from dormant micrometastases undetectable at the time of diagnosis (106). Consequently, adjuvant therapy is initiated

after the removal of the primary tumor to eradicate these micrometastases (107). The type of adjuvant therapy depends on the histological grade of the tumors as well as the expression of ER $\alpha$  and HER2. HER2<sup>+</sup> tumors are treated with one year of anti-HER2 therapy subsequent to or in combination with standard chemotherapy (108). If the tumor is ER $\alpha$ <sup>+</sup>, endocrine therapy is initiated (109). As of 2010, the American Society of Clinical Oncology suggests premenopausal women with ER $\alpha$ <sup>+</sup> cancer receive tamoxifen for 5 years as aromatase inhibitors (AIs) are contraindicated in premenopausal women. If the patient is postmenopausal after 5 years of treatment with tamoxifen, then 5 years of additional treatment with AIs is recommended (110, 111). In postmenopausal women, the use of combination treatment with tamoxifen and AIs is recommended, but the exact order and length of treatment is debated. AIs can be used initially as an adjuvant therapy for 5 years or AI treatment can be initiated following treatment with tamoxifen. The length of tamoxifen treatment before the initiation of AIs is also debated. The guidelines state AIs can be initiated after 2-3 years of tamoxifen or after 5 years of tamoxifen treatment (109). There are many examples of treatment paradigms, but all guidelines recommend the use of AIs alone or in combination with tamoxifen for treatment of breast cancer in postmenopausal women.

Tamoxifen is a selective estrogen receptor modulator (SERM) that inhibits the AF2 domain of ER $\alpha$ , but not the AF1 domain. In the breast, where ER $\alpha$  activity is primarily due to AF2 domain activity, tamoxifen acts as an antagonist (112). However, in the bone, cardiovascular system, and endometrium tamoxifen acts as an agonist, due to the recruitment of different coregulators (113). Consequently, tamoxifen is beneficial in the bone, reducing the occurrence of osteoporosis, and can also be cardioprotective

(114). Although tamoxifen treatment can cause proliferation and even carcinogenesis in the endometrium in postmenopausal women, the benefits for breast cancer treatment outweigh the risks (115). A meta-analysis of clinical trials comparing adjuvant tamoxifen treatment for 5 years to no adjuvant tamoxifen treatment determined treatment of patients with ER $\alpha$ <sup>+</sup> cancer reduced the rate of recurrence by 39%. This effect was maintained at least ten years after cessation of tamoxifen treatment in both pre- and postmenopausal women (116).

While tamoxifen targets ER $\alpha$ , AIs inhibit the enzyme that produces E2. The comparison of five years of AI or tamoxifen treatment in postmenopausal women determined AIs decrease the risk of recurrence by an additional 5% as compared with tamoxifen, but the overall survival is not different (117). However, switching to an AI after 2-3 years of tamoxifen for a total of 5 years of treatment decreased the risk of recurrence and increased overall survival compared to tamoxifen alone for 5 years. Therefore, there are many treatment combinations for postmenopausal women with ER $\alpha$ <sup>+</sup> breast cancer that continue to be evaluated by randomized clinical trials (109).

### **1.5.5 GPER in Breast Cancer**

The role of E2 in breast cancer has been classically ascribed to ER $\alpha$ . However, accumulating evidence suggests that GPER may also affect the growth and progression of breast cancer. GPER transactivates EGFR leading to activation of MAPK and PI3K cascades (35). Both of these signaling cascades have been implicated in carcinogenesis due to the ability to increase cell proliferation and survival. GPER stimulation in MCF7 (ER $\alpha$ <sup>+</sup>, GPER<sup>+</sup>) and SKBR3 (ER $\alpha$ <sup>-</sup>, GPER<sup>+</sup>) increases cell proliferation through transactivation of EGFR (35). GPER stimulation in these cell lines also upregulates the

mRNA and protein expression of the proto-oncogene c-fos (41). Additionally, a study using SKBR3 cells demonstrated E2 stimulation of GPER increases the expression of connective tissue growth factor (CTGF), required for the migration of SKBR3 cells (118). These studies suggest that, similar to ER $\alpha$ , GPER stimulation results in cell proliferation and metastasis that could play a role in breast cancer *in vivo*. In 2006, a large cohort of breast cancer samples was examined for the expression of GPER. Approximately 60% of the samples expressed GPER, with roughly 50% of ER $\alpha$ <sup>-</sup> tumors expressing GPER, indicating an intact E2 signaling pathway in many ER $\alpha$ <sup>-</sup> tumors. This study also demonstrated increased tumor size and presence of distant metastases correlated with increased GPER expression (119).

Approximately 50% of patients develop resistance to tamoxifen and relapse with a more aggressive cancer phenotype. Many mechanisms of resistance have been described, but a relatively new proposed mechanism is that activation of GPER by tamoxifen enhances tumor proliferation thereby negating its actions on ER $\alpha$  (120). MCF7 cells were cultured in tamoxifen to induce tamoxifen resistance. It was demonstrated that GPER-dependent AKT activation partially mediated the observed tamoxifen resistance (120). Additionally, a study examining breast cancer samples comparing patients treated with tamoxifen to those who were not administered tamoxifen revealed that GPER expression was negatively correlated with relapse-free survival and independently predicts a poor relapse-free survival in patients who received tamoxifen as a monotherapy in an adjuvant setting (121). These studies suggest GPER may be a good therapeutic target in patients with ER $\alpha$ <sup>-</sup> cancer as well as in patients treated with tamoxifen.

## **1.6 PyMT Model of Breast Cancer**

Multiple transgenic mouse models of breast cancer are available to study the *in vivo* effects of a specific treatment or a protein of interest (60, 122). Many murine models of breast cancer express oncogenes under the control of the mouse mammary tumor virus (MMTV) promoter, which is primarily activated in mammary tissue. Common models of breast cancer include HER2/neu overexpression, P53 knockout, and polyoma middle T antigen (PyMT) expression (60).

PyMT is derived from the murine polyoma virus that initiates tumor formation in multiple tissues in infected mice. Three early viral proteins are present in tumor cells and are named large, middle, and small tumor antigen (LT, MT, and ST respectively). All three proteins are essential in the replication of the viral genome and have been implicated in the transformation of epithelial cells (123). However, the MT antigen (PyMT) is necessary and, in many cases, sufficient for cell transformation. PyMT does not have intrinsic catalytic activity. Instead, it associates with the cell membrane through a hydrophobic region in the C-terminus, where it acts as a scaffold for cellular signaling proteins including Src family kinases, protein phosphatase 2A (PP2A), Shc, PI3K, and PLC- $\gamma$  (124). Through binding to these cellular proteins, their normal spatio-temporal regulation is disrupted, leading to cell transformation and tumor development.

The MMTV promoter restricts PyMT expression primarily to the female mammary tissue although low levels are detected in the salivary glands, ovaries, epididymis, and seminal vesicles. However, tumor formation is generally restricted to the female mammary gland. Multifocal lesions first develop near the teat and extend into the mammary gland (123). There are four stages of tumor development in MMTV-PyMT

mice that histologically resemble human tumors of the same grade. First, the mice develop hyperplasia (4 weeks of age) followed by adenoma/mammary intraepithelial neoplasia (6-8 weeks of age), early carcinoma (8-12 weeks of age), and late carcinoma (10-14 weeks of age). As the tumors develop, ER $\alpha$  and PR expression are lost and ErbB2 (HER2/neu) expression increases, suggesting more aggressive disease (125). During late stage carcinoma, 94% of mice develop lung metastases, which is dependent on the recruitment of macrophages to the tumor (126). Because this model mimics human tumors and metastasizes to the lung, it is an excellent model to use to examine the role of proteins, such as GPER, in the development and progression of breast cancer.

### **1.7 Project Rationale**

E2 drives normal and neoplastic breast cell proliferation and survival. These E2 effects have traditionally been ascribed to the classical ER, ER $\alpha$  (6, 59). However, new evidence suggests that the non-classical E2 receptor, GPER increases proliferation of breast cancer cells *in vitro* (118).

ER $\alpha$  is a nuclear steroid receptor that typically modulates gene expression of target genes, occurring over several hours. However, E2 also mediates rapid non-genomic cellular responses including activation of ERK1/2, PI3K, and Ca<sup>++</sup> mobilization. Some of these rapid responses can be attributed to E2 stimulation of mER $\alpha$  to associate with G proteins initiating downstream signaling cascades (25). In cells lacking ER $\alpha$  and the other classical ER, ER $\beta$ , rapid E2 signaling still occurs (28). This ER $\alpha$ -independent rapid signaling has now been attributed, at least in part, to GPER. Growing evidence *in vitro* demonstrates a role for GPER in E2-mediated cell proliferation, survival, and migration, all hallmarks of tumor progression and aggressiveness. GPER expression in

patient samples has been correlated with increased tumor size, distant metastasis, and recurrence of breast cancer (119). Further, patients treated with tamoxifen have an increased risk of recurrence if their tumors express GPER (121). In breast tissue, tamoxifen inhibits ER $\alpha$ ; however, tamoxifen activates GPER, which could contribute to tamoxifen resistance (35, 36). Furthermore, approximately 50% of ER $\alpha$  tumors maintain GPER expression, suggesting a partially intact E2 signaling pathway that could drive proliferation (119). Collectively, this data suggests GPER may play a role in enhancing breast cancer aggressiveness, although strong *in vivo* evidence does not exist. Therefore, it is important to establish GPER as a contributor to breast cancer initiation and progression to determine if it could represent a novel therapeutic target.

## **1.8 Hypothesis and Specific Aims**

Although GPER has been associated with aggressive breast cancer and enhances breast cancer cell proliferation, *in vivo* evidence for GPER enhancing breast cancer is lacking. We hypothesize GPER augments breast cancer growth and metastasis in the MMTV-PyMT *in vivo* model of breast tumorigenesis.

### **1.8.1 Specific Aims**

**Aim 1. Determine the significance of GPER in mammary tumor growth and progression in the MMTV-PyMT murine model of mammary carcinogenesis**

**1.1** Elucidate tumor size, grade and receptor status from GPER WT and KO MMTV-PyMT mice

**1.2** Assess lungs from GPER WT and KO MMTV-PyMT mice for the presence of metastases



**Aim 2. Analyze the disparate effects of GPER expression in the tumor parenchyma and microenvironment in the MMTV-PyMT model**

- 2.1 Determine if GPER expression in the tumor microenvironment affects growth and metastasis of orthotopically transplanted GPER WT tumors
- 2.2 Evaluate the effects of GPER expression in the tumor microenvironment on the growth and metastasis of orthotopically transplanted GPER KO tumors
- 2.3 Assess the role of GPER expression in macrophages in tumor growth and metastasis in orthotopically transplanted GPER WT tumors

**Aim 3. Assess the consequences of GPER-targeted compounds on the progression of tumors in the MMTV-PyMT model**

- 1.1 Analyze the size and grade of tumors from MMTV-PyMT mice treated with GPER-selective compounds in the presence and absence of E2
- 1.2 Assess lungs from treated MMTV-PyMT mice for the presence of metastases

## **CHAPTER 2**

### **GPER REGULATES MAMMARY TUMORIGENESIS**

## **2.1 Abstract**

17 $\beta$ -estradiol (E2) is known to enhance breast cancer development and tumor growth through the activation of ER $\alpha$ . The discovery that ER $\alpha$  stimulates proliferation in breast tumor cells led to major advances in breast cancer treatment, drastically improving the prognosis for women with ER $\alpha$ <sup>+</sup> breast cancer. However, patients whose tumors do not express ER $\alpha$  or become resistant to treatment have fewer options and rely on chemotherapy, which has many side-effects. This suggests targeting ER $\alpha$  alone is not sufficient to eliminate breast cancer. The discovery of G protein-coupled estrogen receptor (GPER) suggested an additional mechanism through which E2 could exert its effects in breast cancer. Although there have been studies demonstrating a correlation of GPER expression with larger tumors, increased incidence of distant metastasis and recurrence *in vivo* as well as a proliferative role for GPER *in vitro*, *in vivo* evidence is lacking. To determine the role of GPER *in vivo*, we intercrossed MMTV-PyMT (PyMT) mice, a model of mammary tumorigenesis, with GPER knockout (KO) mice to determine if GPER expression affects tumor size and progression. Tumor latency in PyMT mice lacking GPER was not different than control mice. However, by 12-13 weeks of age, GPER KO PyMT mice displayed smaller tumors with decreased proliferation and fewer lung metastases. Therefore, we have provided the first *in vivo* evidence that GPER plays a critical role in breast tumor growth and distant metastasis.

## **2.2 Introduction**

The steroid hormone, 17 $\beta$ -estradiol (estrogen, E2), is the primary female sex hormone necessary for the development of secondary sexual characteristics in women (12). Specifically, E2 mediates the development of breast tissue during puberty and pregnancy by enhancing the proliferation of ductal epithelial cells (60). Similar to normal development, E2 also promotes breast cancer by promoting the proliferation, migration, and invasion of breast tumor cells both *in vitro* and *in vivo* (6). By inhibiting the activity of the classical estrogen receptor ER $\alpha$ , many cancer-promoting effects of E2 in cultured cells and mice are reduced; therefore, the actions of E2 in breast cancer have been attributed almost exclusively to ER $\alpha$  (127-129). Clinically, drugs administered to women with breast cancer to block the production of estrogen or inhibit ER $\alpha$ , thereby inhibiting E2 signaling, increase long-term survival (116). One of the most commonly prescribed adjuvant treatments for breast cancer is tamoxifen, a selective estrogen receptor modulator (SERM) that acts as an antagonist for ER $\alpha$  in the breast, inhibiting tumor growth. Treatment with tamoxifen for 5 years after surgery decreases mortality by 31% (116). However, only patients with ER $\alpha$ <sup>+</sup> tumors are eligible for treatment, and many ER $\alpha$ <sup>+</sup> tumors do not respond to tamoxifen or become resistant during treatment or upon recurrence (103). While tamoxifen is a successful therapy, breast cancer is still the second most common cause of cancer-related death in women in the US (63). Additionally, aromatase inhibitors, which inhibit the production of E2, are more efficacious for the prevention and treatment of breast cancer (117). These data suggest inhibition of E2 signaling solely through ER $\alpha$  is insufficient and other E2 receptors may be involved in the initiation and/or progression of breast cancer.

G protein-coupled estrogen receptor 1 (GPER) is a novel estrogen receptor whose role in breast carcinogenesis has yet to be determined. E2 stimulation of GPER activates the MAPK cascade as well as PI3K, among other pathways, and increases proliferation of breast cancer cells, suggesting it may play a role in one or more events of breast carcinogenesis (35, 37, 41, 118, 130). A small number of retrospective studies have examined the correlation between GPER expression in breast tumor samples and clinical outcomes. A study of 361 breast cancer patients found GPER expression correlated with increased size of the primary tumor and prevalence of distant metastasis (119). Another study demonstrated an increased recurrence rate in GPER<sup>+</sup> invasive ductal breast cancers (131). Most recently, a study of invasive breast cancer samples demonstrated GPER expression is an independent prognostic factor for decreased disease free survival in patients treated with tamoxifen (121). While these studies suggest a role for GPER in breast cancer, there is no direct experimental evidence that GPER plays a role in the initiation and/or progression of breast cancer.

In this study, the MMTV-PyMT mouse was crossed with GPER KO mice to investigate the contribution of GPER to breast carcinogenesis. MMTV-PyMT mice express the polyoma middle T antigen (PyMT) primarily in mammary tissue, under control of the mammary tumor virus (MMTV) promoter, resulting in the spontaneous development of mammary tumors (123). PyMT mice develop hyperplastic lesions in their mammary glands at 4 weeks of age that progress through the stages of adenoma, early carcinoma and late carcinoma over the subsequent 8-10 weeks(125). Histologically, PyMT tumors at each stage of development closely resemble similar stage human tumors. Furthermore, changes in biomarkers during PyMT mammary tumor

development are similar to human breast cancer as tumors progress from hyperplasia to carcinoma, including loss of ER $\alpha$ , progesterone receptor and integrin  $\beta$  expression, and an increase in the expression of Neu and cyclin D1 (125). Finally, analogous to human breast cancer, early tumor growth in PyMT mice is E2-dependent, demonstrating the PyMT model of mammary carcinogenesis to be a clinically relevant model of human breast cancer (132).

In the present study, we demonstrate the presence of GPER promotes growth and metastasis of late stage mammary tumors in PyMT mice, although we observed no difference in tumor latency between GPER WT and GPER KO mice. As the tumors progress, GPER KO mice displayed smaller tumors that exhibited a lower grade when compared to GPER WT and GPER heterozygote (HET) mice. Consistent with decreased size, GPER KO tumors displayed reduced proliferation compared to GPER WT mice. Most importantly, GPER KO mice exhibited fewer lung metastases compared to GPER WT mice. Taken together, these data demonstrate that silencing GPER decreases tumor size and the number of distant metastases, suggesting that pharmacological inhibition of GPER may represent a novel approach to reduce morbidity from breast cancer.

## **2.3 Methods**

**2.3.1 Mice.** FVB/N-Tg(MMTV-PyVT)634Mul/J (MMTV-PyMT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). GPER KO mice were provided by Jan Rosenbaum (Proctor & Gamble, Cincinnati, OH) and described previously (133). GPER KO mice were backcrossed 10 generations onto FVB/NJ mice. GPER KO mice were intercrossed with MMTV-PyMT mice to produce MMTV-PyMT mice that were wild type (WT), heterozygous, or knock out (KO) with respect to GPER. Animals were

housed at the animal research facility at the University of New Mexico Health Sciences Center and maintained under a controlled temperature of 22–23 °C with a 12-h light, 12-h dark cycle and fed a normal chow *ad libitum*. Tumors were allowed to grow until mice were 7 weeks or 12-13 weeks old. Tumors were weighed as a measurement of tumor size. Tumors and lungs were then resected, fixed in 4% PFA, and paraffin embedded. All procedures were approved by and carried out in accordance with the institutional protocols.

**2.3.2 Ovariectomy.** PyMT mice were anesthetized with isoflurane and placed in a nose cone while lying on a 37 °C heating pad. Buprenorphine (0.1 mg/kg) was injected prior to surgery. A small incision in the skin was made on the midline of the dorsal side of the mouse just cranial to the hipbones. The skin was gently separated from the underlying muscle to the left and right of the initial incision. An incision through the muscle was made slightly lateral to the spine. The ovary was gently pulled through the incision in the muscle with blunt forceps. While grasping the oviduct with forceps, a cut was made just below the ovary to remove the ovary. The incision in the muscle was closed using polydioxanone synthetic absorbable sutures (PDS\* Plus, Ethicon) and the skin incision was closed with 9 mm stainless steel tissue clips (ez CLIPS, Stoelting). The mice were then allowed to recover in a 37 °C recovery chamber.

**2.3.3 Tamoxifen Treatment.** When PyMT mice were 4-weeks-old, a 60-day release pellet containing tamoxifen (5 mg/pellet) (Innovative Research of America) was subcutaneously implanted on the left dorsal side of the mouse just below the rib cage. Tumors were resected at 12 weeks of age and weighed as a measurement of tumor size.

**2.3.4 Relative Quantitation of PyMT gene expression.** Mammary tumors from 10-

week-old WT/PyMT and KO/PyMT mice were removed and stored in Trizol (Sigma-Aldrich). Tumors were homogenized using a Polytron tissue homogenizer. RNA isolation was performed in Trizol using phenol-chloroform extraction according to the manufacturers instructions. cDNA was created via reverse transcription of 1 µg RNA with the iScript cDNA synthesis kit (BioRad) using the GeneAmp PCR system 9700 (Applied Biosystems, Inc.) according to manufacturers directions. Quantification of PyMT mRNA relative to cytokeratin 18 mRNA was performed using Fast SYBR Green (Molecular Probes) with the 7500 Fast Real-Time PCR System (Applied Biosystems, Inc). A relative standard curve for each primer was created from a mixture of the sample RNA extracted from the tumors with dilution values of 1.0, 0.25, 0.0625, and 0.0156. The relative concentrations were expressed as arbitrary units and plotted against the logarithm (base 20) of the dilution values. Linear regression was used to create a standard curve. The standard curves were used to determine the relative amount of PyMT and cytokeratin 18 in each sample. The relative concentration of PyMT was then normalized to the relative concentration of cytokeratin 18, which served as the endogenous control. The primer sequences were: PyMT\_Forward: 5'- CGG CGG AGC GAG GAA CTG AGG AGA G -3' Cytokeratin 18\_Forward: 5'- CAA GTC TGC CGA AAT CAG GGA C -3'.

**2.3.5 Tumor Histology.** The largest tumors from 12-13 week old mice were sectioned (5 µm) and stained with hematoxylin (Sigma-Aldrich) and Eosin (RICCA Chemical Company). Tumor grade was determined by a veterinary pathologist, Donna Kusewitt, DVM, PhD (The University of Texas MD Anderson Cancer Center). Grading PyMT tumors has been previously described (125). Briefly, tumor grade is determined by tissue architecture, degree of cellular atypia, and invasion into the surrounding stroma. Based



on these parameters, tumors were assigned grades as follows: (1) Hyperplasia: Densely packed acini filled or bridged by epithelial cells that have little to no cellular atypia. There is no invasion into the surrounding stroma. (2) Adenoma/mammary intraepithelial neoplasia (MIN): Increased proliferation of epithelial cells with acini mostly filled with cells. There is minimal cellular atypia and no invasion is present. (3) Early carcinoma: Florid proliferation with loss of acinar definition. There is moderate cellular atypia and early stromal invasion. (4) Late carcinoma: Solid sheets of cells with very few or no acini present. There is a high mitotic index consistent with increased proliferation. Marked cellular atypia and pronounced stromal invasion are present. Sections were imaged with a Nikon DS-Fi1 camera mounted on a Nikon Eclipse E400 microscope running NIS-Elements software.

The extent of tumor necrosis was determined and categorized by the number and size of necrotic areas. The score of necrosis is as follows: 1 = few small areas; 2 = few larger areas or moderate number of smaller areas; 3 = extensive areas.

**2.3.6 Immunostaining analysis.** For immunostaining, 5  $\mu\text{m}$  sections were deparaffinized, rehydrated, permeabilized in PBS containing 0.01% Triton X-100, and blocked in 3% normal goat serum (NGS) diluted in PBS plus 0.1% Tween-20 (PBS-T).

To evaluate the proliferation rate, microwave antigen retrieval was performed in 0.1 M sodium citrate (pH 6). Sections were stained overnight in a 1:100 dilution of the anti-phospho-histone H3 (P-histone H3, phospho Ser10, EMD Millipore) followed by detection with an anti-rabbit IgG antibody conjugated to Alexa 488 (Molecular Probes). Coverslips were mounted with Vectashield mounting medium with DAPI (Vector Laboratories). One random field from 3 sections was imaged with a Zeiss 200M Axiovert

microscope using MetaMorph software. The number of P-histone H3 positive cells was determined and normalized to the total number of cells per field. Apoptosis was evaluated in the same manner, except the primary antibody was directed against cleaved caspase-3 (Asp 175) (Cell Signaling).

To detect ER $\alpha$ , microwave antigen retrieval was performed in TET buffer (10mM Tris, 1mM EDTA, 0.05% Tween-20; pH 9) and endogenous peroxide activity was quenched in 3% H<sub>2</sub>O<sub>2</sub> before permeabilization. Sections were incubated overnight in a 1:100 dilution of anti-ER $\alpha$  antibody (MC-20) (Santa Cruz Biotechnology). The sections were washed in PBS-T and incubated with anti-rabbit IgG antibody conjugated to horseradish peroxidase (HRP) (Molecular Probes). 3,3'-Diaminobenzidine (DAB) (Sigma-Aldrich) was used as the substrate to detect the presence of the HRP-conjugated antibody. Coverslips were mounted with Permount mounting medium (Fisher Scientific). Three random fields per section were imaged with a Nikon DS-Fi1 camera mounted on a Nikon Eclipse E400 microscope running NIS-Elements software. The number of ER $\alpha$ -positive cells was determined and normalized to the total number of cells per field.

**2.3.7 Lung Metastasis.** Three 5  $\mu$ m sections separated by 100  $\mu$ m were prepared from the lungs of 12-13 week old animals. Sections were deparaffinized, rehydrated, and stained with H&E. Metastatic tumor foci present in the lung parenchyma are defined as a tightly clustered group of 10 or more hematoxylin-positive cells that excludes eosin-stained stroma. Total metastatic foci were counted in the three lung sections to determine the extent of metastases.

**2.3.8 Whole Mount.** Number 4 mammary glands from 7 week old PyMT mice were fixed in 4% PFA on stretched skin overnight at room temperature. The glands were

removed from the skin, incubated overnight in 100% acetone to remove the fat, washed in deionized water, and stained with carmine overnight. To generate carmine stain, carmine alum lake (1g, Sigma-Aldrich) and aluminum potassium sulfate (2.5g, Sigma-Aldrich) were dissolved in 500 mL of distilled water by boiling for 20 min and then filtered. After staining, the glands were washed in deionized water and dehydrated in ethanol before storing them in methyl salicylate (Sigma Aldrich). Glands were imaged with MoticCam 2300 running Motic software on an Olympus SZH dissection microscope.

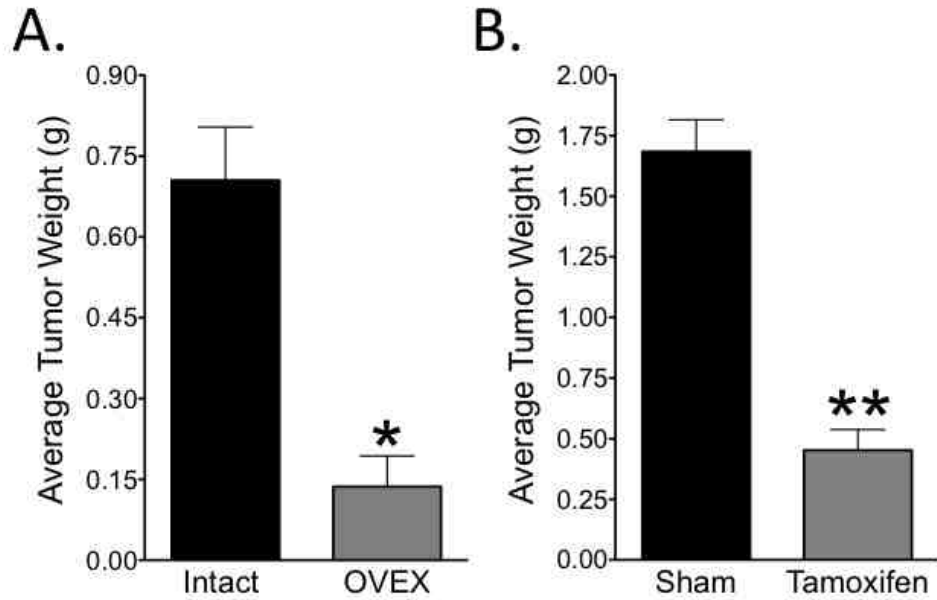
**2.3.9 Statistical Analysis.** Tumor size in ovariectomized and tamoxifen treated mice, hyperplasia in 7 week glands, P-histone H3 positive cells, relative concentration of PyMT RNA, ER $\alpha$  expression, and extent of lung metastasis were evaluated using two-tailed student's t-test with a p-value threshold of .05. Tumor size of WT/PyMT, HET/PyMT and KO/PyMT mice was assessed using one-way ANOVA with Bonferroni correction for multiple comparisons as a post-hoc test. Tumor grade and extent of necrosis were analyzed using a two-tailed Fisher's exact test with a p-value threshold of .05. Correlation between ER $\alpha$  and tumor grade was assessed using Pearson's correlation analysis with a p-value threshold of .05.

## **2.4 Results**

### **2.4.1 Ovariectomy and tamoxifen treatment reduce mammary tumor size.**

Although GPER has been correlated with human breast tumor growth and metastasis, *in vivo* studies examining the effects of GPER on breast cancer initiation, growth, and progression have not been performed. To study the role of GPER in breast cancer, we used the MMTV-PyMT (PyMT) model of mammary carcinogenesis, which has been

reported to be an E2-responsive tumor model (132). To verify the E2-responsiveness of tumor growth, PyMT mice were ovariectomized to remove the majority of circulating estrogen. The size of tumors from ovariectomized mice was compared to tumors from non-ovariectomized mice at 12 weeks of age. Mammary glands were palpated two times per week to detect the presence of tumors. Both cohorts of mice developed tumors between 7 and 8 weeks of age, suggesting there was no apparent effect of ovariectomy on tumor latency. However, by 12 weeks of age, the tumors in the non-ovariectomized mice were 5-fold larger than those in the ovariectomized mice (Figure 2.1A), indicating tumor growth in PyMT mice is highly E2-responsive. Steroid hormones such as glucocorticoids, progestins, androgens, and estrogens stimulate the MMTV promoter and increase the expression of PyMT, whereas E2 is a weak activator of the MMTV promoter. Therefore, to determine receptor-dependent E2 activity on tumor size, tamoxifen pellets were subcutaneously implanted in 4-week-old PyMT mice. When mice were 12-weeks of age, tumors were resected and weighed to determine tumor size. Tumors from mice treated with tamoxifen were about 3.5-fold smaller than tumors from sham treated mice, demonstrating the actions of E2 are receptor-dependent and not secondary to MMTV promotion (Figure 2.1B). Additionally, ovariectomy was slightly more effective than tamoxifen at decreasing tumor size indicating that ER $\alpha$  may not be the only estrogen receptor regulating tumor growth. Because of its E2 responsiveness, this model is appropriate for examining the role of GPER in mammary tumorigenesis.

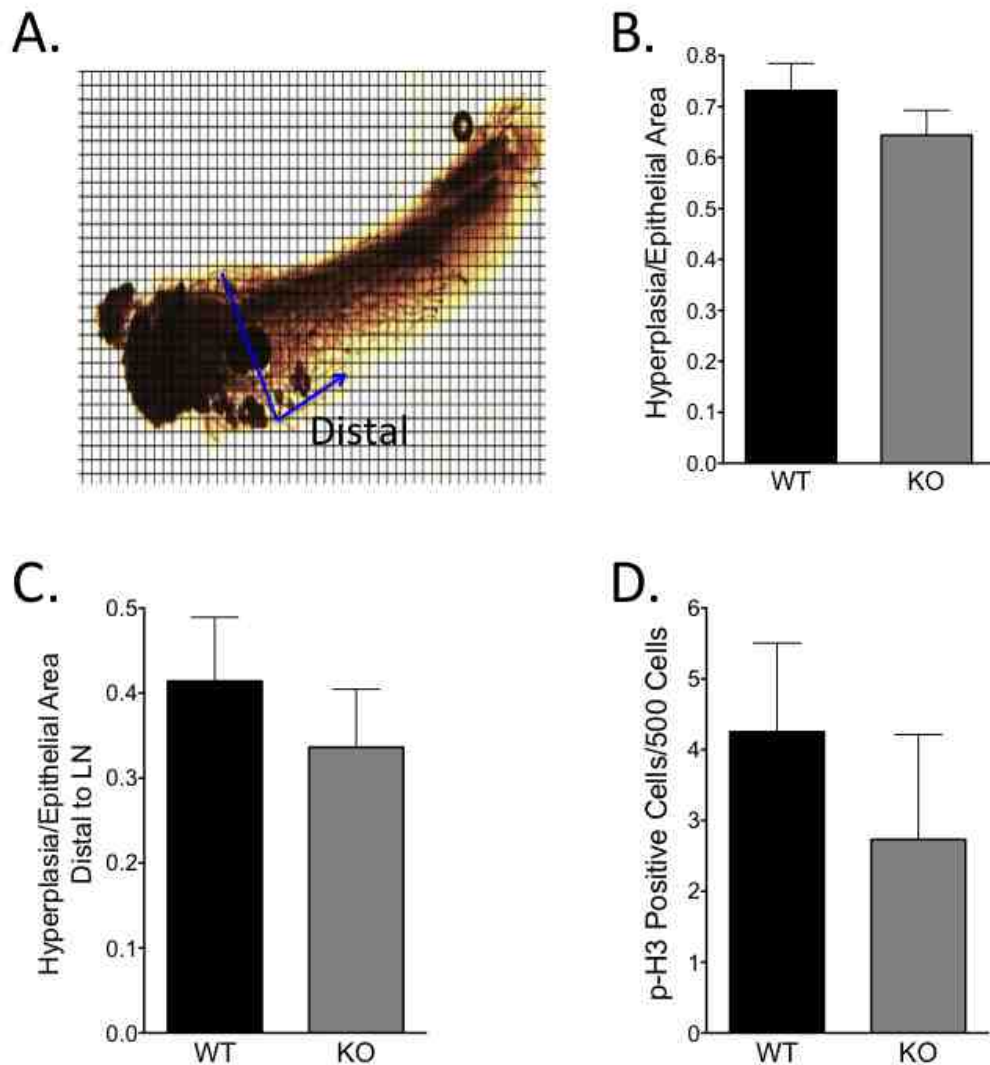


**Figure 2.1 Estrogen enhances mammary tumorigenesis.** A) PyMT mice were ovariectomized at 3 weeks of age to remove the majority of circulating E2. Tumors were resected and weighed as a measure of tumor size when mice were 12-weeks-old. B) 60-day release tamoxifen pellets were subcutaneously implanted into 4-week-old PyMT mice. Tumors were resected and weighed as a measure of tumor size when mice were 12-weeks-old. Two-tailed Student's t-test with a p-value threshold of .05 was used for statistical analysis.

\* p < .05  
 \*\* p < .01

**2.4.2 GPER deficiency does not affect the initiation of tumor formation or early tumor growth.** Male PyMT mice were intercrossed with female FVB mice lacking GPER (KO) to generate male and female PyMT GPER heterozygous (HET) mice (HET/PyMT). The male HET/PyMT and female HET/FVB mice were bred to produce PyMT GPER WT, HET, and KO offspring (WT/PyMT, HET/PyMT, KO/PyMT, respectively). In both WT/PyMT and KO/PyMT mice, tumors were palpable between 7 and 8 weeks of age, suggesting there is no difference in tumor latency. To assess differences in early tumor development, whole mounts of the number 4 mammary glands from 7-week-old WT/PyMT and KO/PyMT mice were stained with carmine to determine the extent of hyperplasia. In Image J, a grid was overlaid on the whole mount images and each box was evaluated for the presence of hyperplasia and epithelium (Figure 2.2A). Total hyperplasia relative to total epithelium was  $0.73 \pm .05$  for WT/PyMT and  $0.64 \pm .05$  for KO/PyMT, demonstrating no statistical difference ( $p > .05$ ) (Figure 2.2B). To evaluate if the lesions in WT/PyMT mice had extended farther into the mammary gland than in KO/PyMT mice, indicating more advanced disease, hyperplasia distal to the lymph node relative to total epithelium distal to the lymph node was determined. There was no difference in the extent of hyperplasia distal to the lymph node between WT/PyMT ( $0.41 \pm .08$ ) and KO/PyMT ( $0.34 \pm .07$ ) mice ( $p > .05$ ) (Figure 2.2C). Next we addressed proliferation in early tumors by staining number 2/3 mammary glands from 7-week-old mice with an anti-phospho-histone H3 (P-histone H3) antibody, a marker of the M phase of the cell cycle. We found no difference in the number of proliferating cells between tumors from WT/PyMT (4/500 cells) and KO/PyMT (3/500 cells) mice ( $p > .05$ ) (Figure 2.2D). Collectively, these data indicate GPER does not affect the development or early

growth of mammary tumors.

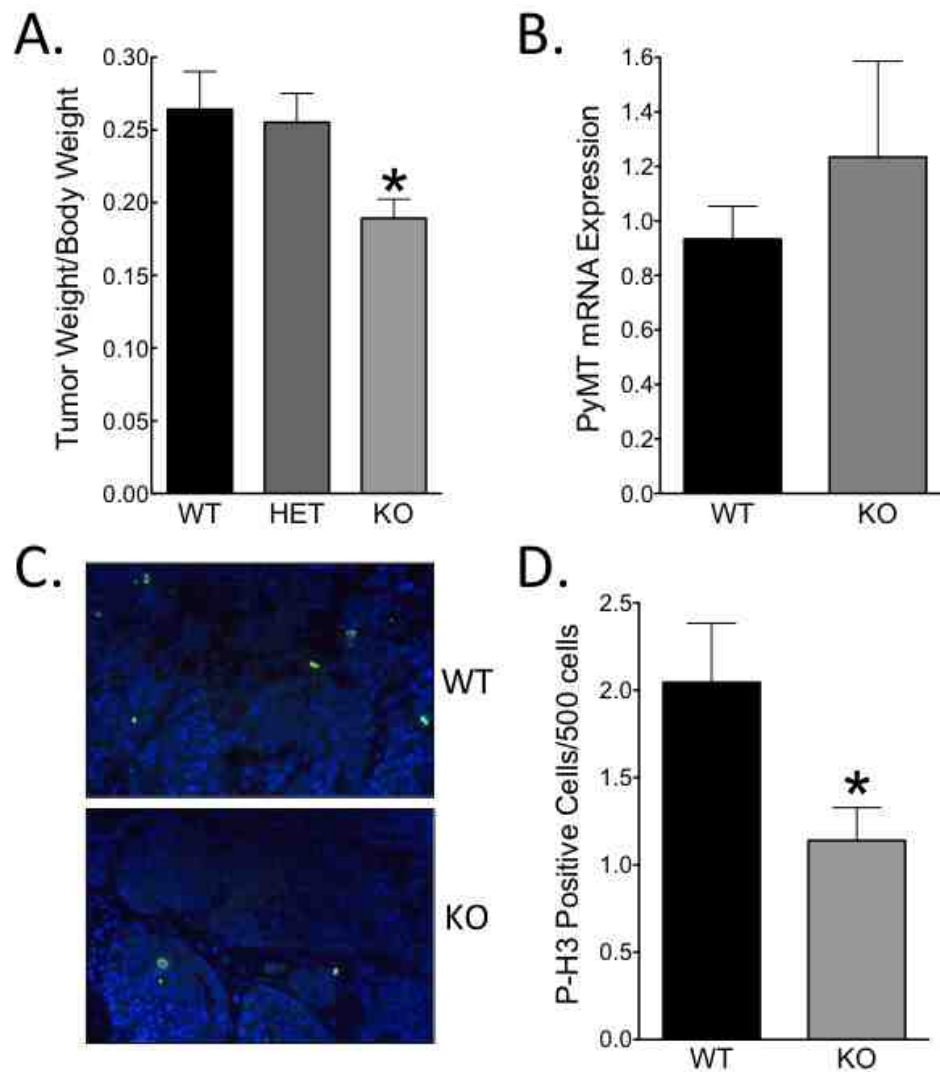


**Figure 2.2 Absence of GPER does not affect early tumor development.** A) Whole mounts of number 4 mammary glands from 7-week-old WT/PyMT and KO/PyMT mice were stained with carmine to visualize hyperplasia. Using Image J software, a grid was overlaid on the image, and each box was analyzed for the presence of hyperplasia and normal epithelium. Hyperplasia was analyzed in the total gland and distal to the lymph node. B) Quantification of total gland hyperplasia. The number of boxes containing hyperplasia was normalized to the number of boxes containing total epithelium, which is defined as hyperplastic and normal epithelium. C) Quantification of hyperplasia distal to the lymph node performed in the same manner as in B. D) Number 2/3 mammary glands from 7-week-old WT/PyMT and KO/PyMT mice were stained with anti-phospho histone-H3 (p-H3) antibody to determine proliferation rate. Statistical analysis was done using two-tailed Student's t-test with a p-value threshold of .05.



**2.4.3 Mammary tumor growth is reduced in the absence of GPER.** Although tumor development is unaffected by lack of GPER, tumors from older mice were evaluated to determine whether GPER affects tumor progression. When mice were 12-13-weeks-old, tumors were removed and weighed, as a measure of tumor size. There was no difference between WT/PyMT and HET/PyMT tumor size; however, tumors from KO/PyMT mice were 28% smaller than WT/PyMT mice ( $p < .05$ ) (Figure 2.3A). To verify the difference in tumor size was not indirectly caused by decreased expression of PyMT mRNA in KO/PyMT mice, real time PCR was used to determine the level of PyMT expression relative to cytokeratin 18, a marker of epithelial cells, in tumors from WT/PyMT and KO/PyMT mice. No difference in the relative expression of PyMT RNA between WT/PyMT and KO/PyMT mice was detected ( $p > .05$ ), indicating differences in tumor size were not indirectly caused by a decrease in PyMT expression (Figure 2.3B).

Because tumors in KO/PyMT mice were smaller than WT/PyMT tumors, proliferation and apoptosis of the tumor cells were analyzed to determine the relative contribution of each of these factors to overall tumor size. To evaluate the proliferation rate, the number of cells positive for p-histone H3, which stains cells in the M phase of the cell cycle, was determined and normalized to the total number of cells. Tumors from KO/PyMT mice exhibited a 44% lower proliferation rate than tumors from WT/PyMT mice ( $p < .05$ ) (Figure 2.3C). Apoptosis was investigated using an antibody directed against cleaved caspase-3 and similarly analyzed by microscopy. Although the positive control exhibited cleaved caspase-3 expressing cells, the tumors did not display cleaved caspase-3-positive staining regardless of the genotype. Therefore, WT tumors are larger, in part, due to increase cell proliferation with no appreciable apoptosis.



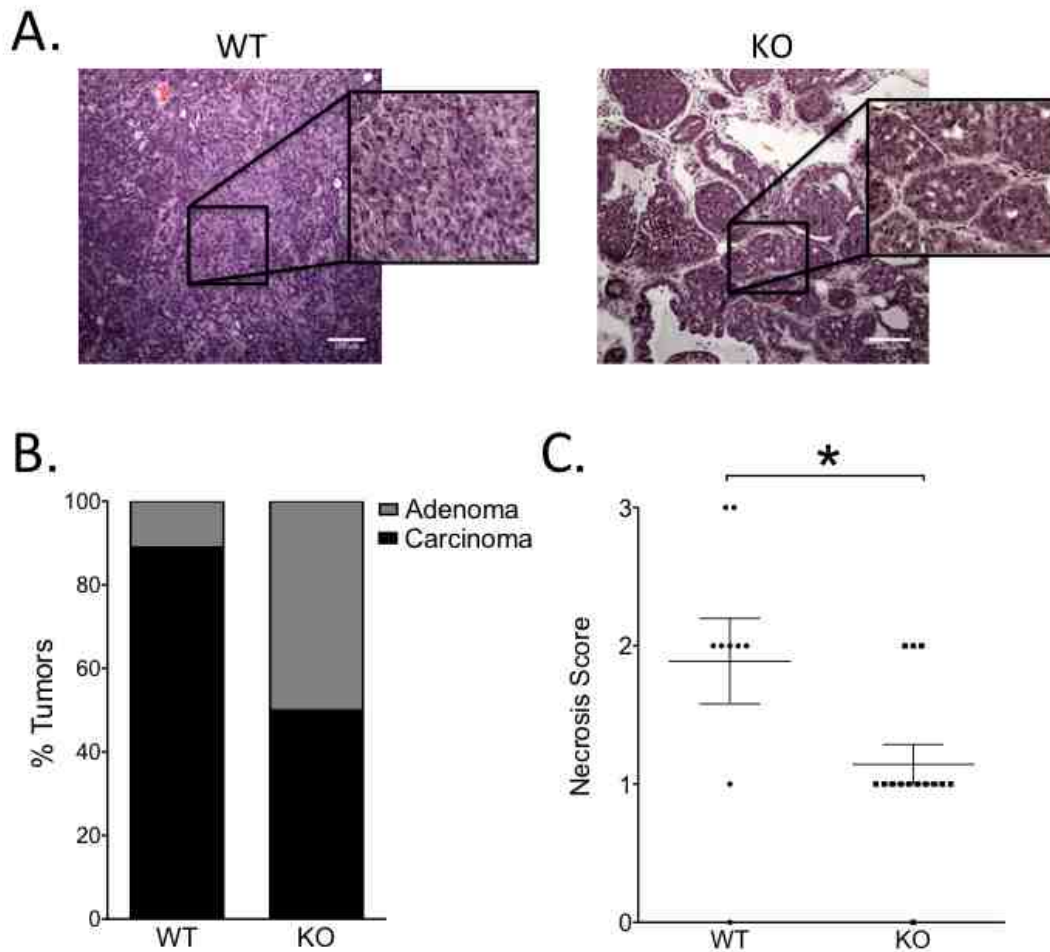
**Figure 2.3 KO/PyMT mice have smaller tumors with decreased proliferation compared to WT mice.** A) Tumors from WT/PyMT and KO/PyMT mice were resected and weighed at 12-13-weeks-old as a measure of tumor size. B) Quantification of PyMT gene expression relative to cytokeratin 18 from 10-week-old WT/PyMT and KO/PyMT mice. C) Images of P-histone-H3 (P-H3) staining on tumors from 12-13-week-old mice. Blue represents DAPI staining and green represents P-H3-positive cells. D) Quantification of P-H3-positive cells in tumors from WT/PyMT and KO/PyMT mice. Statistical analysis was done using two-tailed Student's t-test with a p-value threshold of .05.

\*  $p < .05$

**2.4.4 GPER is associated with predictors of poor prognosis.** To evaluate tumor aggressiveness, sections of the largest tumor from WT/PyMT and KO/PyMT mice were stained with H&E, analyzed to determine grade, and classified as either low-grade (hyperplasia and adenoma) or high-grade (early and late carcinoma) (Figure 2.4A). The majority of tumors from WT/PyMT mice lost acinar definition appearing as solid sheets of cells, and had invaded through the basement membrane into the surrounding stroma, indicative of carcinoma. In contrast, many tumors from KO/PyMT mice appeared to maintain acinar structure, although the acini were filled with cells; in addition, fewer tumors invaded through the basement membrane in the KO/PyMT mice. Based on histological parameters, tumors were graded and it was determined that 90% of tumors from WT/PyMT mice were carcinomas versus 50% of tumors from KO/PyMT mice, demonstrating a strong trend for KO/PyMT tumors to exhibit a lower tumor grade than WT/PyMT tumors ( $p = .056$ ) (Figure 2.4B).

During tumor resection, it appeared that tumors from KO/PyMT mice contained a smaller necrotic center than WT/PyMT mice. Because necrosis is an independent predictor for poor prognosis, a smaller necrotic center suggests less aggressive tumors (134). Necrosis was analyzed by evaluating the number and size of necrotic areas categorized using a scale from 0-3 with 0 signifying a lack of necrosis and 3 representing large and/or many areas of necrosis. The majority (78%) of tumors from WT/PyMT mice were given a score of 2 or greater compared with 21% of tumors from KO/PyMT mice receiving a score of 2 and no tumors from KO/PyMT mice receiving the highest score of 3 (Figure 2.4C). Thus, tumors lacking GPER exhibit fewer and smaller areas of necrosis compared to tumors that express GPER. Taken together, these data suggest KO/PyMT

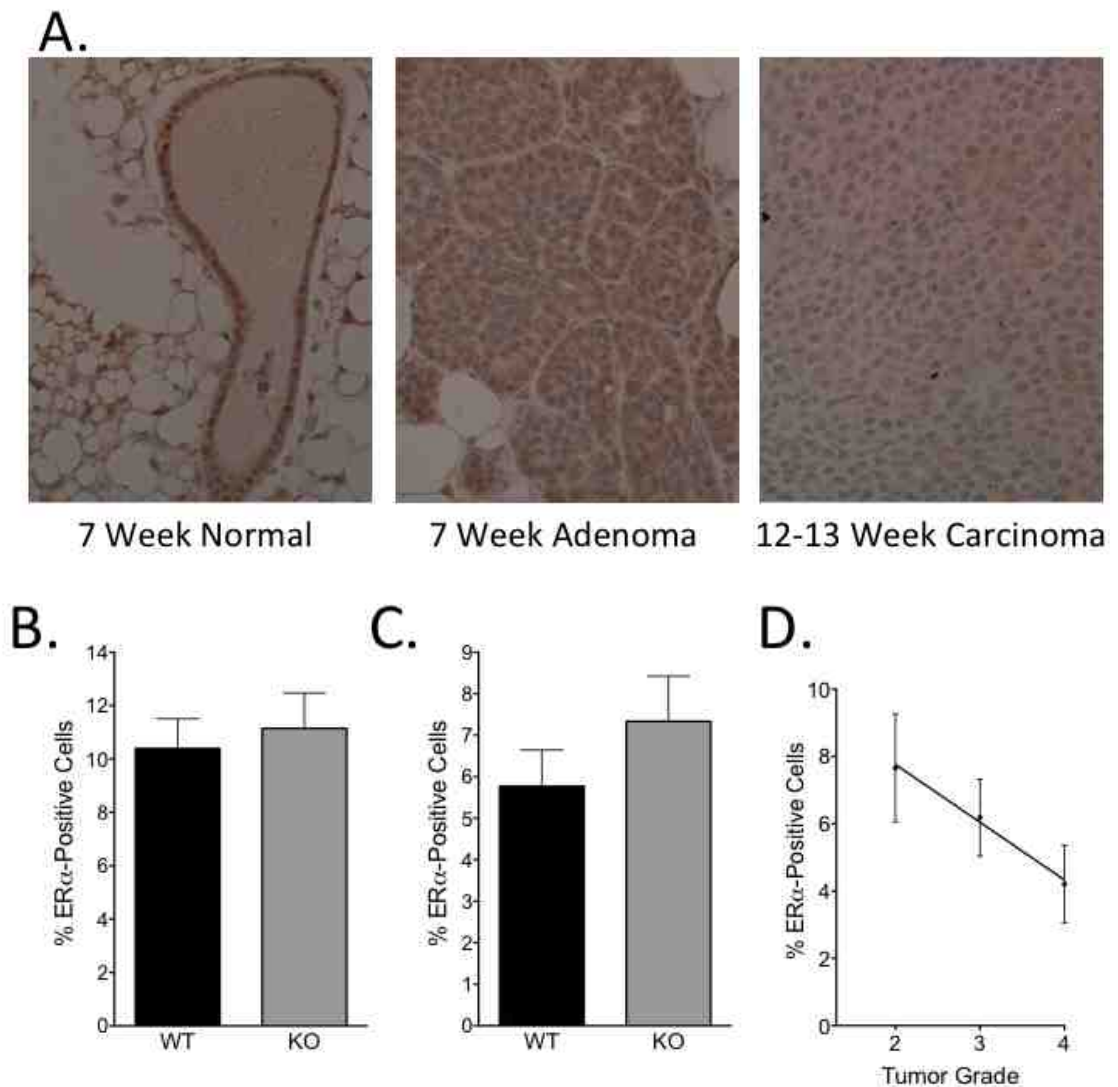
tumors are less aggressive than WT/PyMT tumors.



**Figure 2.4** There is a trend for tumors from WT/PyMT mice to correlate with predictors of poor prognosis compared with KO/PyMT mice. A) Representative images of tumors from WT/PyMT and KO/PyMT mice stained with H&E. WT/PyMT mice had little to no acinar definition, whereas acinar structures were present in many tumors from KO/PyMT mice. B) Quantification of tumor grades from WT/PyMT mice and KO/PyMT mice. C) Quantification of extent of necrosis for WT/PyMT and KO/PyMT mice. Two-tailed Pearson's chi-square test with a p-value threshold of .05 was used for statistical analysis.

\*  $p < .05$

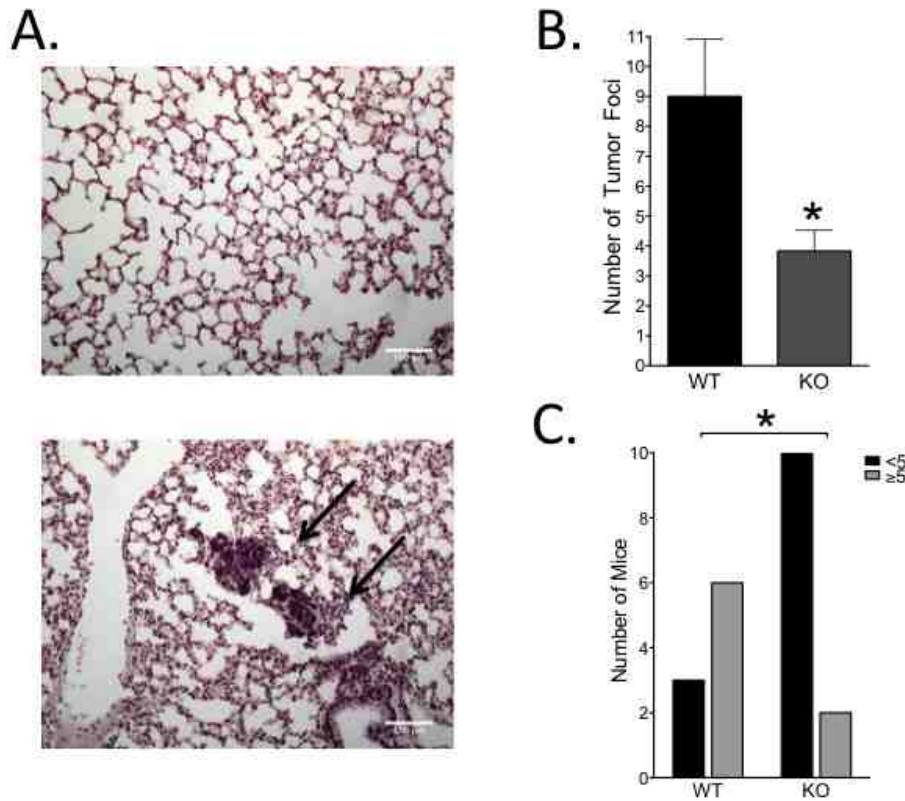
**2.4.5 ER $\alpha$  expression is unaffected by GPER deficiency.** As ER $\alpha$  drives proliferation in approximately 70% of human breast tumors and tamoxifen successfully inhibited PyMT tumor growth, we considered that eliminating GPER could affect the expression of ER $\alpha$  in the tumors of PyMT mice, thus indirectly affecting tumorigenesis (59). To examine ER $\alpha$  expression, tumor sections from 7-week-old and 12-13-week-old mice were stained and analyzed for the number of ER $\alpha$ -positive cells as well as staining intensity. Tumors from 7-week-old mice displayed faint ER $\alpha$  staining in the hyperplastic/adenomatous regions, and intense staining in the adjacent normal tissue, suggesting that as tumors form ER $\alpha$  expression is lost (Figure 2.5A). The number of ER $\alpha$ -positive cells in three random fields was determined and was not different between tumor from 7-week-old WT/PyMT and KO/PyMT mice (Figure 2.5B). Tumors from 12-13-week-old mice displayed faint ER $\alpha$  staining that was less intense than that of 7-week hyperplastic tissue (Figure 2.5A). Consistent with 7-week staining, the number of ER $\alpha$ -expressing cells was not different between the two genotypes (Figure 2.5C). Thus, manipulating the expression of GPER does not affect the expression of ER $\alpha$ . Because patients with ER $\alpha$ -positive tumors have a better prognosis than those with ER $\alpha$ -negative tumors, the number of ER $\alpha$ -expressing cells in tumors from 12-13-week-old PyMT tumors were analyzed with respect to tumor grade. Consistent with patient data, ER $\alpha$  expression is correlated with a lower tumor grade ( $p = .05$ ) (Figure 2.5D).



**Figure 2.5 GPER expression does not affect the percent of ER $\alpha$ -positive cells.** A) Sections (5  $\mu$ m) of tumors from 7-week-old and 12-week-old PyMT mice were stained with an anti-ER $\alpha$  antibody. B) Quantification of ER $\alpha$ -positive cells relative to total epithelial cells in 3 random fields in tumors from 7-week-old WT/PyMT and KO/PyMT mice. C) Quantification of ER $\alpha$ -positive cells relative to total epithelial cells in 3 random fields in tumors from 12-13-week-old WT/PyMT and KO/PyMT mice. D) Correlation analysis between tumor grade and percent of ER $\alpha$ -positive cells from 12-13-week-old mice. Differences in ER $\alpha$  expression were statistically analyzed by two-tailed Student's t-test with a p-value threshold of .05. Correlation between ER $\alpha$  expression and tumor grade was analyzed using simple linear regression with a p-value threshold of .05.

**2.4.6 GPER deficiency yields fewer metastases to the lung.** While tumor size, grade, proliferation rate, and estrogen receptor status are predictive of aggressiveness in breast tumors, the most reliable predictor of survival in patients is the presence of distant metastases (64, 67). The most common metastatic sites for human breast cancer are the lungs, liver, and bone (106). PyMT tumors predominantly metastasize to the lung, making it an appropriate model to use to evaluate metastasis (60). To assess the extent of metastasis, the lungs of 12-13-week-old KO/PyMT and WT/PyMT mice were stained with H&E and the number of tumor foci, designated as a group of 10 or more densely packed cells, was determined (Figure 2.6A). Lungs from WT/PyMT mice contained  $9.0 \pm 1.9$  metastatic foci per lung while lungs from KO/PyMT mice contained  $3.8 \pm 0.69$  metastatic foci, demonstrating an 58% reduction in metastases in KO/PyMT mice ( $p < .05$ ) (Figure 2.6B). While the majority of mice had metastasis by this age, 83% of KO/PyMT mice had fewer than 5 metastasis compared to 33% of WT/PyMT mice. These data demonstrate that PyMT mice lacking GPER have a decreased incidence of metastasis.





**Figure 2.6 Lack of GPER reduces metastatic burden in PyMT mice.** A) Lungs of WT/PyMT and KO/PyMT mice were removed when mice were 12-13-weeks-old and stained with H&E to determine the extent of metastasis. Top image represents normal mouse lung tissue. Bottom image represents mouse lung tissue containing tumor foci (arrows). B) Quantification of the number of tumor foci in WT/PyMT and KO/PyMT mice analyzed by two-tailed Student's t-test with a p-value threshold of .05. C) The number of mice exhibiting <5 or  $\geq 5$  metastatic foci per lung was analyzed by two-tailed Fisher's exact test with a p-value threshold of .05.  
\*  $p < .05$

## **2.5 Discussion**

Several reports have demonstrated cancer cell lines proliferate in response to the GPER-selective agonist G-1 and that E2-dependent proliferation is reduced upon silencing of GPER (28, 41, 118, 135). While these data suggest GPER may promote breast tumor growth, its importance in breast cancer initiation, growth and progression remains unclear. This is the first report describing a role for GPER in an *in vivo* model of breast cancer.

Initially, PyMT mice intercrossed with GPER KO mice were used to assess the contribution of GPER to early mammary tumor development using 7-week-old mice. Both WT/PyMT and KO/PyMT mice developed palpable tumors by 7 weeks of age, consistent with there being no difference in tumor latency. These tumors were analyzed for differences in proliferation and the extent of hyperplasia throughout the gland. At this early time point, there was no difference in the proliferation rate or hyperplasia, implying GPER does not play a role in tumor development or early tumor cell proliferation.

The role of GPER in late stage tumor growth and progression was evaluated by measuring tumor size and distant metastasis. Tumors from KO/PyMT mice were smaller than tumors from WT/PyMT mice, likely due to decreased tumor cell proliferation. Further, there was a trend for KO/PyMT mice to exhibit lower grade tumors than WT/PyMT mice, suggesting tumors from KO/PyMT mice are less aggressive than tumors from WT/PyMT mice. While tumor size, grade and proliferation rate are used clinically to predict patient outcomes, the best parameter to determine prognosis is the presence of distant metastases. In breast cancer, the primary sites of metastasis are bone, liver, and

lung. Although PyMT mice do not develop bone or liver lesions, metastatic lung foci are routinely observed by 12 weeks of age. In this regard, there were significantly fewer metastatic foci in the lungs of KO/PyMT mice compared to WT/PyMT mice. These data establish a role for GPER in growth and metastasis of mammary tumors in a reliable *in vivo* model of breast cancer (125). Evaluating data from early and late stages of tumorigenesis, we propose GPER increases the aggressiveness of established tumors, but has minimal effect on tumor development.

The classical E2 receptor ER $\alpha$  is known to increase proliferation rate and tumor growth in breast tumors (6). Therefore, the tumor expression of ER $\alpha$  was evaluated at 7 and 12-13 weeks of age showing no difference between GPER KO and GPER WT tumors. Staining intensity in hyperplastic regions of tumors from 7-week-old mice is faint compared to adjacent normal tissue. Staining intensity decreases further in 12-13-week-old mice compared to hyperplasia from 7-week-old mice. Therefore, ER $\alpha$  may play a dominant role in early mammary tumor development through its robust proliferative effects, as evidenced by reduced tumor size in ovariectomized and tamoxifen treated mice (Figure 2.1), rendering GPER KO less consequential at early times, with the growth- and metastasis-promoting effects of GPER becoming determinant later in tumor development as tumors lose ER $\alpha$  expression. While tamoxifen is an effective treatment for ER $\alpha$ <sup>+</sup> breast cancer, 30% of breast tumors do not express ER $\alpha$ . Further, 30% of ER $\alpha$ <sup>+</sup> tumors that initially responded to tamoxifen become resistant to the ER $\alpha$ -targeted therapy (136). Given the possible role of GPER in tamoxifen-resistant and ER $\alpha$ <sup>-</sup> tumors, GPER could be targeted to reduce tumor growth and metastasis. Additionally, while tamoxifen is a selective estrogen receptor modulator (SERM) that inhibits ER $\alpha$  in the breast, it

activates GPER, possibly contributing to tamoxifen resistance and the development of tamoxifen associated uterine cancer (35). Administering a GPER-selective antagonist following or in combination with tamoxifen could represent an approach to inhibit resistance and improve the efficacy of tamoxifen and other SERMs.

This is the first *in vivo* study demonstrating a role for GPER in the progression of breast cancer, which reveals a novel target in hormone therapy for breast cancer. For decades, tamoxifen has been successful in treating patients with breast cancer, although many tumors are initially resistant or develop resistance, and when breast tumors recur, many are resistant to tamoxifen (137). GPER is expressed in 60% of ER $\alpha$ <sup>-</sup> tumors, and GPER expression is associated with increased recurrence after adjuvant treatment with tamoxifen as a monotherapy (119, 121). Therefore, it is possible that targeting GPER would increase patient survival in women with the more aggressive ER $\alpha$ <sup>-</sup> tumors and could be an effective combination treatment with tamoxifen. Additionally, GPER-selective small molecule inhibitors (G15 and G36) have been developed, making GPER an attractive clinical target.

**CHAPTER 3**

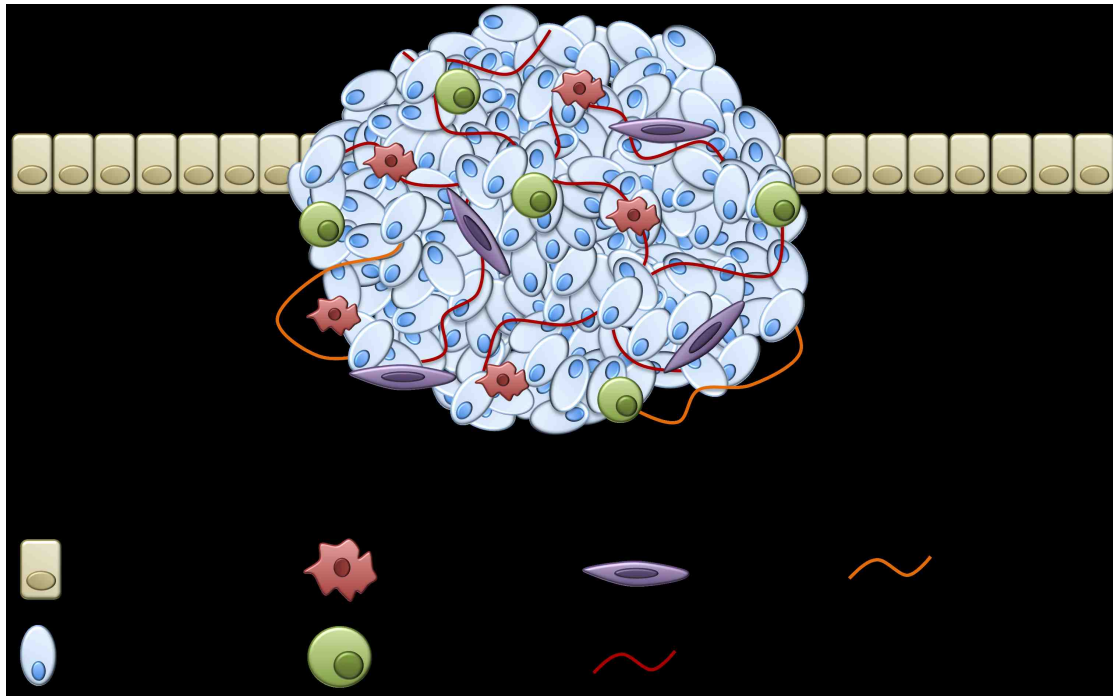
**IDENTIFYING THE ROLE OF GPER IN THE  
MICROENVIRONMENT OF MAMMARY CARCINOMA**

### **3.1 Abstract**

Traditionally, mutations or signaling pathways that drive breast cancer are analyzed in tumor epithelial cells; however, breast cancer growth and its ability to metastasis is also regulated by the tumor microenvironment. The microenvironment is comprised of fibroblasts, extracellular matrix components, immune cells, blood vessels, and lymphatic vessels, which can each be modulated to affect tumor growth and aggressiveness. In addition to its effects in cultured tumor cells, G protein-coupled estrogen receptor (GPER) increases the proliferation and migration of fibroblasts, one of the central members of the tumor microenvironment, suggesting GPER stimulation in the tumor microenvironment could enhance tumorigenesis. Additionally, knocking out GPER in the MMTV-PyMT model of breast carcinogenesis decreases tumor size and metastasis, although the distinct roles of GPER in the tumor parenchyma versus the microenvironment are unclear (Chapter 2). To distinguish the effects of GPER in the tumor parenchyma and microenvironment, GPER expressing PyMT tumor epithelial cells (WT/PyMT) were orthotopically transplanted into GPER WT and GPER KO recipient mice and analyzed for tumor growth and metastasis. The reciprocal experiment (KO/PyMT cells into WT and KO mice) was also performed. WT/PyMT tumor size was unaffected by the microenvironment, whereas KO/PyMT tumors were larger in KO recipient mice compared to WT recipient mice. With respect to metastasis, WT/PyMT mice metastasized more frequently in WT compared with KO mice, while KO/PyMT cell metastasis was unaffected by the microenvironment. These data suggest GPER expression in the tumor microenvironment regulates tumor growth and metastasis.

## **3.2 Introduction**

Breast cancer is classically defined as dysregulation of epithelial cells leading to uncontrolled proliferation, increased cell survival, and the ability to metastasize to distant sites. Although breast cancer manifests in epithelial cells, changes in the microenvironment are critical to formation and progression of breast tumors (138). The mammary gland is a secretory organ formed by a branching duct structure embedded in the mammary fat pad. The ducts are comprised of an inner layer of luminal epithelial cells responsible for milk secretion surrounded by an outer layer of myoepithelial cells. The luminal epithelial cells make up the parenchyma of the breast and are supported by their microenvironment, or stroma, which includes myoepithelial cells, blood vessels, lymphatic vessels, immune cells, fibroblasts, and extracellular matrix (ECM) (12). The stroma communicates with the epithelium to maintain normal breast homeostasis through many mechanisms including direct cell-cell contact, release of paracrine factors, and changes in the structure and components of the ECM. Consequently, alterations in the normal microenvironment lead to disruption of normal gland function and, conceivably, cancer development (77, 82, 139). Additionally, tumor epithelial cells release signals to modulate the microenvironment. Once the microenvironment has been modified, it enhances tumor growth and proliferation (Figure 3.1).



**Figure 3.1 Mammary tumor microenvironment.** Mammary tumors are surrounded by a complex microenvironment that includes fibroblasts that secrete ECM, blood vessels, lymphatic vessels, and multiple types of immune cells (2).



Macrophages are part of the innate immune system that perform a versatile role in the microenvironmental regulation of carcinogenesis. Tissue remodeling that occurs during tumor formation releases pro-inflammatory cytokines, which recruit and activate macrophages. Once activated, macrophages release tumoricidal products, such as tumor necrosis factor- $\alpha$  (TNF $\alpha$ ) and nitric oxide (140). Inflammatory macrophages are also capable of recognizing tumor cell neo-antigens, phagocytosing tumor cells, and presenting antigens to the adaptive immune system, thereby activating the adaptive immune system. Tumoricidal capabilities of macrophages have been observed *in vitro* in co-culture experiments as well as *in vivo* in bone marrow and liver metastases (89, 141). Historically, it was believed successful tumors simply excluded macrophages to evade their tumoricidal actions. However, it was later recognized that low-level chronic inflammation occurring in disease states, such as ulcerative colitis, correlates with tumor formation (142). Using mouse models of inflammatory disease, it was determined “smoldering inflammation” enhances tumor development through production of reactive oxygen species (ROS) causing DNA damage and mutations in epithelial cells. Additionally, in over 80% of tumors, including thyroid, lung, hepatocellular, and breast carcinoma, the presence of infiltrating macrophages is correlated with poor prognosis. In breast cancer, macrophages are recruited to benign tumors as the tumors shift to malignancy, indicating macrophages may enhance cancer progression (89). These clinical observations resulted in further investigation into the role of tumor-associated macrophages (TAMs). In mouse models of carcinogenesis, depleting macrophages by administering chlodronate-containing liposomes reduces angiogenesis, tumor growth, and metastasis (85, 141, 143). Additionally, tumors in MMTV-PyMT mice crossed with

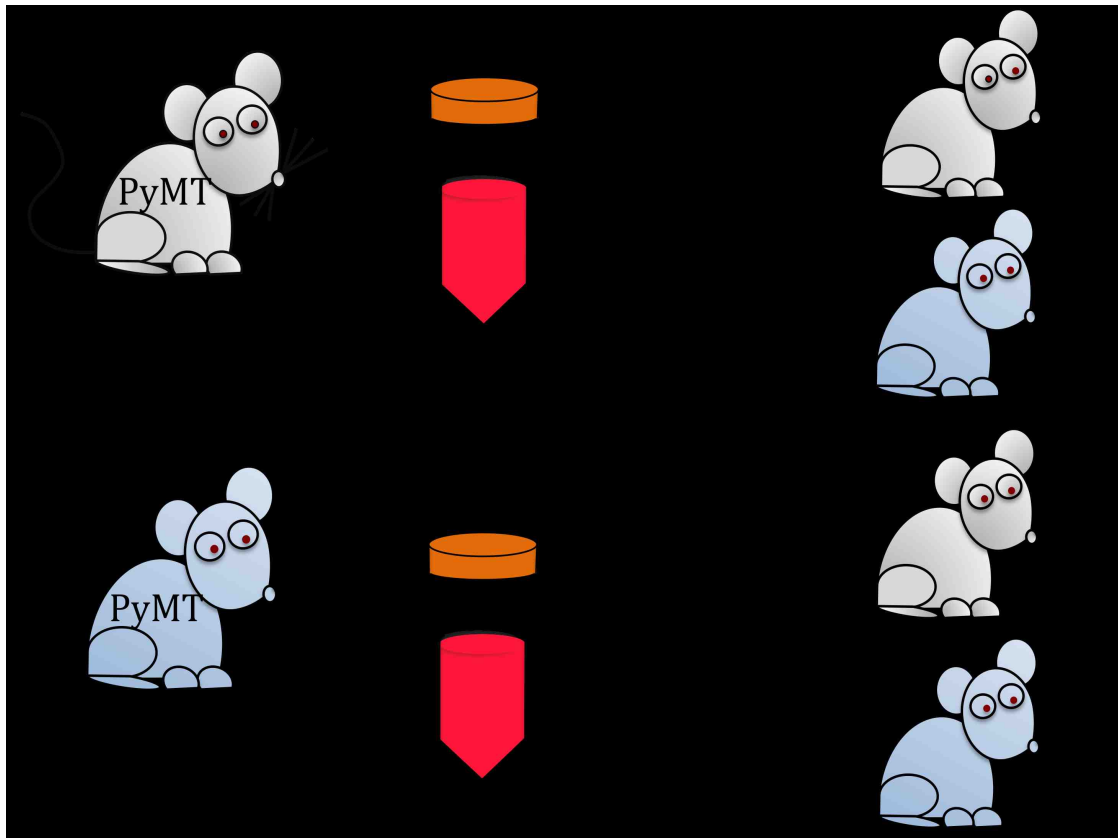
*Csf1<sup>op/op</sup>* transgenic mice contain significantly fewer TAMs, do not progress to malignancy as rapidly as WT mice and are unable to metastasize (126). It is now well accepted that, although macrophages are able recognize and kill precancerous cells, they also initiate epithelial cell transformation during chronic inflammation. , and in existing tumors, macrophages increase tumor growth and enhance the ability of tumors to metastasize.

Macrophages respond to multiple cues in their environment including cytokines and steroid hormones, such as 17 $\beta$ -estradiol (E2). Although the consequences of E2 in macrophages are controversial, in many circumstances, E2 decreases the inflammatory response of macrophages (91). Premenopausal women have lower circulating levels of TNF- $\alpha$ , a proinflammatory cytokine, compared with postmenopausal women and men under normal conditions. Premenopausal women also display an attenuated TNF- $\alpha$  response during septic shock as compared with men, providing *in vivo* evidence E2 may affect macrophage polarization. In cultured human primary macrophages, E2 inhibits the translocation of NF- $\kappa$ B, decreasing the expression of TNF- $\alpha$  and presumably other proinflammatory cytokines (144). Additionally, in the autoimmune disease multiple sclerosis (MS), E2 is anti-inflammatory and neuroprotective. Until recently, the actions of E2 in macrophages have been largely attributed to ER $\alpha$  and to a lesser extent ER $\beta$ . In 2009, Blasko et al., describe a role for the novel E2 receptor G protein-coupled estrogen receptor (GPER) in decreasing secretion of TNF- $\alpha$  and IL-6 from macrophages, thereby inhibiting disease progression in the experimental allergic encephalomyelitis (EAE), a mouse model of MS (90). Because E2 is present in high levels in the breast cancer

microenvironment, it may decrease macrophage secretion of pro-inflammatory cytokines, consequently inhibiting the anti-tumor immune response via ER $\alpha$  or GPER (101).

In chapter 2 of this dissertation, *in vivo* evidence is provided for GPER augmenting late stage breast cancer growth and metastasis in the MMTV-PyMT model of mammary tumorigenesis. In this model, lack of GPER reduces tumor cell proliferation and metastasis; however, because a global GPER KO mouse was used, it is unclear if the effects of GPER are due to its actions in the tumor parenchyma or microenvironment. Therefore, we employed an orthotopic transplant model to examine the discrete effects of GPER in the tumor cells and microenvironment (Figure 3.2). Epithelial cells from PyMT mice expressing GPER (WT/PyMT) were orthotopically transplanted into GPER WT or KO recipient mice to evaluate the effect of the microenvironment on tumor growth and metastasis. No difference in tumor size was observed when WT/PyMT cells were transplanted into either WT or KO recipient mice. Evaluation of lung metastasis revealed WT/PyMT cells metastasize more frequently in WT recipient mice than in KO mice. The reciprocal experiment was also performed in which epithelial cells from PyMT mice lacking GPER (KO/PyMT) mice were orthotopically transplanted into WT and KO recipient mice. KO/PyMT cells generated larger tumors in KO recipient mice compared to WT recipient mice, although there was no difference in the extent of metastasis. Because macrophages are required for metastasis in the MMTV-PyMT model and E2 affects macrophage polarization through GPER stimulation, WT/PyMT cells were orthotopically transplanted in combination with GPER WT or KO bone marrow derived macrophages (WT/BMM or KO/BMM, respectively) to investigate the role of macrophages in tumor size and metastasis. Larger tumors formed in the presence of

WT/BMM compared to KO/BMM, although the number of metastasis between mice receiving WT/BMM or KO/BMM was not different. Therefore, it can be concluded GPER expression in the microenvironment affects tumor growth and metastasis.



**Figure 3.2 Orthotopic transplant model.** Tumors were removed from 7-week-old PyMT mice, minced, and digested. Organoids were purified via differential centrifugation and stored in liquid nitrogen. The number 4 mammary fat pads of WT and KO recipient mice were cleared when mice were 3-weeks-old. When recipient mice were 5-weeks-old, cells were orthotopically transplanted into the cleared number 4 mammary fat pads.

### **3.3 Methods**

**3.3.1 Mice.** FVB/N-Tg(MMTV-PyVT)634Mul/J (MMTV-PyMT) and FVB/NJ mice were purchased from The Jackson Laboratory (Bar Harbor, ME). GPER KO mice were provided by Jan Rosenbaum (Proctor & Gamble, Cincinnati, OH) and described previously (133). GPER KO mice were backcrossed 10 generations onto FVB/NJ mice. Animals were housed in the animal research facility at the University of New Mexico Health Sciences Center and maintained under a controlled temperature of 22–23°C with a 12-h light, 12-h dark cycle and fed a normal chow *ad libitum*. All procedures were approved by and carried out in accordance with institutional protocols.

**3.3.2 Primary mammary tumor epithelial cell isolation.** Tumors were removed from 7-week-old GPER WT or KO PyMT mice. Tumors were minced in epithelial medium containing DMEM-F12 (cellgro, mediatech) + 5% FBS (JRH Biosciences) 1% penicillin-streptomycin-glutamine (GIBCO) + 10 µg/ml insulin (Cell Applications) + 0.5U/ml hydrocortisone (Sigma-Aldrich) + 10 ng/ml EGF (Molecular Probes). Minced tumors were dissociated in epithelial medium containing 3 mg/ml collagenase A (Roche) and 250 U/ml Hyaluronidase (Sigma-Aldrich) for 3 hours at 37°C with gentle rocking. Dissociated cells were strained through a 500 µM sterile mesh nylon filter. Filtered cells were centrifuged at 48 g for 3 min. The resulting pellet was washed three times with HBSS + 5% FBS and centrifuged at 200 g for 2 min. The resulting organoids were resuspended in epithelial cell media + 10% DMSO and stored in liquid nitrogen.

**3.3.3 Fat pad clearing and tumor cell transplantation.** When WT or KO FVB/NJ mice were 3-weeks-old, the number 4 mammary fat pads were cleared to remove the epithelium, while leaving part of the mammary fat pad. An inverse Y incision was made

on the ventral side of the mouse, and the skin was gently separated from the underlying muscle to expose each number 4 mammary gland. The mammary tissue proximal to the fat pad lymph node was removed. Saline was instilled into the subcutaneous space to prevent fibrotic adhesions, and the skin incision was closed using steel tissue clips (ez CLIPS, Stoelting). Two weeks after the fat pad was cleared, the inverse Y incision was reopened, and 10,000 WT/PyMT or KO/PyMT organoids resuspended in 30  $\mu$ l of phenol red-free DMEM-F12 were injected directly into each cleared number 4 mammary fat pad. The skin incision was closed using steel tissue clips.

### **3.3.4 Isolation and differentiation of bone marrow derived macrophages**

**(BMM).** Bone marrow from GPER WT or KO FVB/NJ mice was collected from 6-8-week-old mice as previously described. Briefly, the femur and tibia were cleaned and rinsed in 70% ethanol. The epiphyses was removed from both ends of the bone and the marrow was flushed out with ice cold DMEM + 10% FBS using a 27 G needle. The bone marrow was passed through a 40  $\mu$ m cell strainer (BD Biosciences) and centrifuged at 100 g for 5 min to isolate bone marrow cells. The cells were resuspended in DMEM + 10% FBS + 20% L929 conditioned media (differentiation media) and seeded in a 150 mm untreated petri dish. The media was changed on days 4 and 6, and the cells were used on day 7. To generate L929 conditioned medium, L929 cells were grown to confluency. Once confluent, the media was changed and left for 7 days, at which time the media was collected and filtered through a 0.22  $\mu$ m polyethersulfone membrane bottle top vacuum filter (Corning, Sigma-Aldrich).

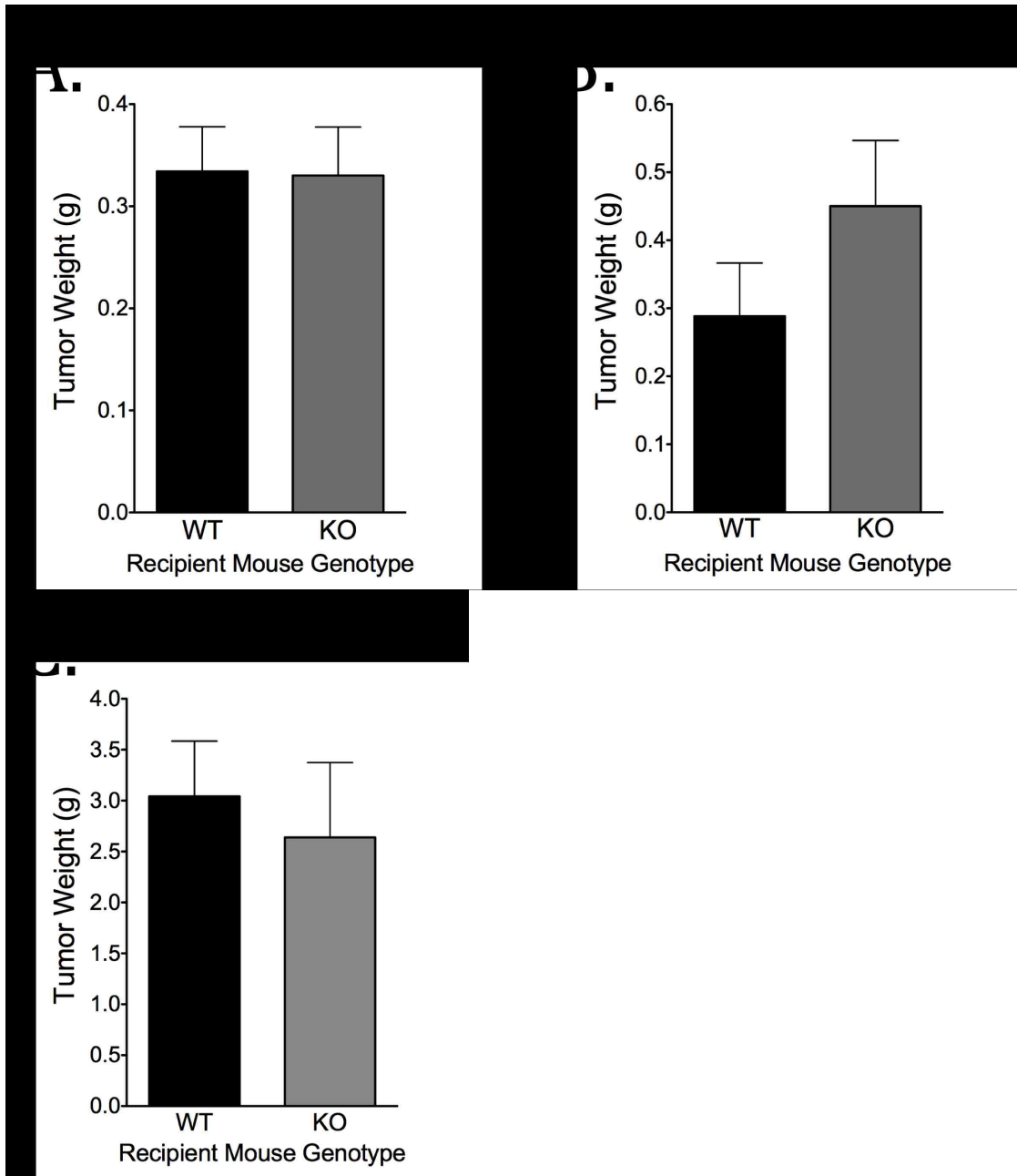
**3.3.5 Co-injection of WT/PyMT cells with WT/BMM and KO/BMM.** A clonal cell line previously generated from a PyMT mouse was used in this experiment and

cultured in epithelial cell medium. WT/PyMT cells mixed with WT/BMM or KO/BMM at a 1:9 or 1:3 ratio were resuspended at a concentration of  $1 \times 10^6$  cells per 100  $\mu$ l in phenol red-free DMEM-F12. Cells were orthotopically injected into the un-cleared number 4 mammary glands of a FVB/NJ mouse.

### **3.4 Results**

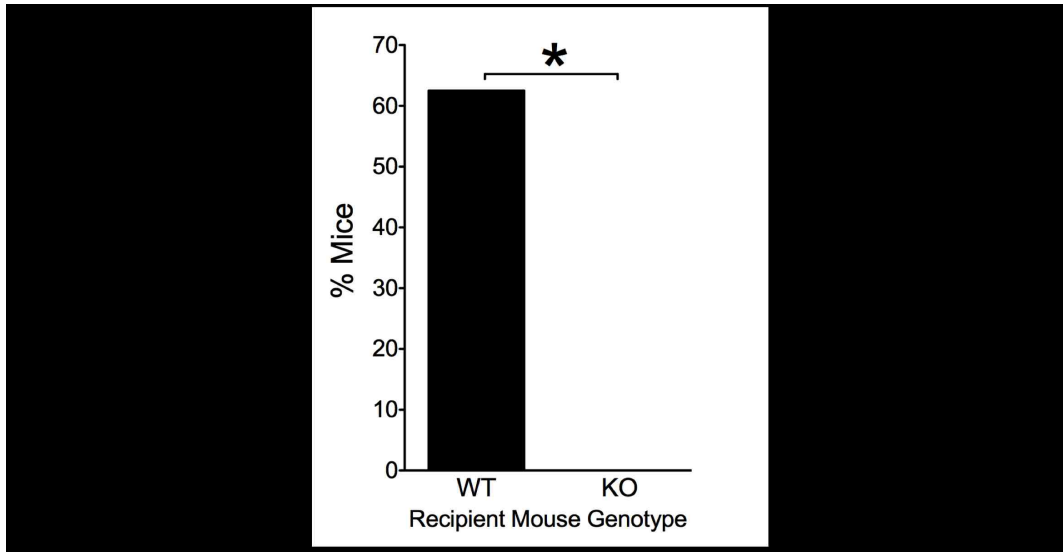
**3.4.1 GPER expression in the microenvironment of GPER<sup>+</sup> tumors does not affect tumor size, but increases the incidence of metastasis.** E2 is known to enhance proliferation and metastasis of tumors via activation of ER $\alpha$ , and we provide evidence in chapter 2 that GPER expression contributes to E2-mediated advancement of breast cancer. To further elucidate the role of GPER in the tumor parenchyma and microenvironment, an orthotopic transplant model was used (Figure 3.2). WT/PyMT cells were orthotopically injected into the cleared number 4 mammary fat pads of WT and KO recipient mice. Tumors were removed and weighed as a measure of tumor size at various time points after injection. Five weeks post-injection, no difference in tumor size was observed between WT ( $0.32 \pm 0.07$  g) and KO ( $0.23 \pm 0.03$  g) recipient mice ( $p = .25$ ) (Figure 3.3A). Tumor weights appeared to deviate between WT ( $0.29 \pm 0.08$  g) and KO ( $0.45 \pm 0.1$  g) at 8 weeks after cell injection, although there was no statistical difference between the two groups ( $p = .23$ ) (Figure 3.3B). A final time point of 12 weeks post-injection was examined, and again, there was no difference between tumor size in WT ( $3.042 \pm 0.54$ ) and KO ( $2.638 \pm 0.74$ ) recipient mice ( $p = .67$ ), suggesting the microenvironment does not affect the size of WT/PyMT tumors (Figure 3.3C).





**Figure 3.3 GPER expression in the microenvironment does not affect tumor size of WT/PyMT tumors.** WT/PyMT cells were orthotopically transplanted into WT and KO recipient mice. Tumors were removed and weighed at different time points after tumor injection including: A) 5 weeks B) 8 weeks or C) 12 weeks. Results were analyzed by Student's t-test.

In addition to influencing tumor size, the microenvironment regulates the ability of tumor cells to metastasize by modifying the ECM, increasing vascular permeability, and increasing cell migration and invasion into the surrounding stroma (2, 82). Previously, it was determined the lack of GPER inhibits metastasis in the MMTV-PyMT model (chapter 2); however, it is unclear if this effect is due to the absence of GPER in the tumor parenchyma or microenvironment. Accordingly, the lungs from WT and KO mice with WT/PyMT tumors were sectioned, stained with H&E, and analyzed for the presence of distant metastases (Figure 3.4A). No metastatic foci were observed in the lungs of KO mice, whereas lungs from 63% of the lungs from mice contained metastases ( $p=.03$ ) (Figure 3.5B). These data demonstrate GPER expression in the tumor microenvironment augments metastasis of WT/PyMT tumors, while not affecting tumor size.



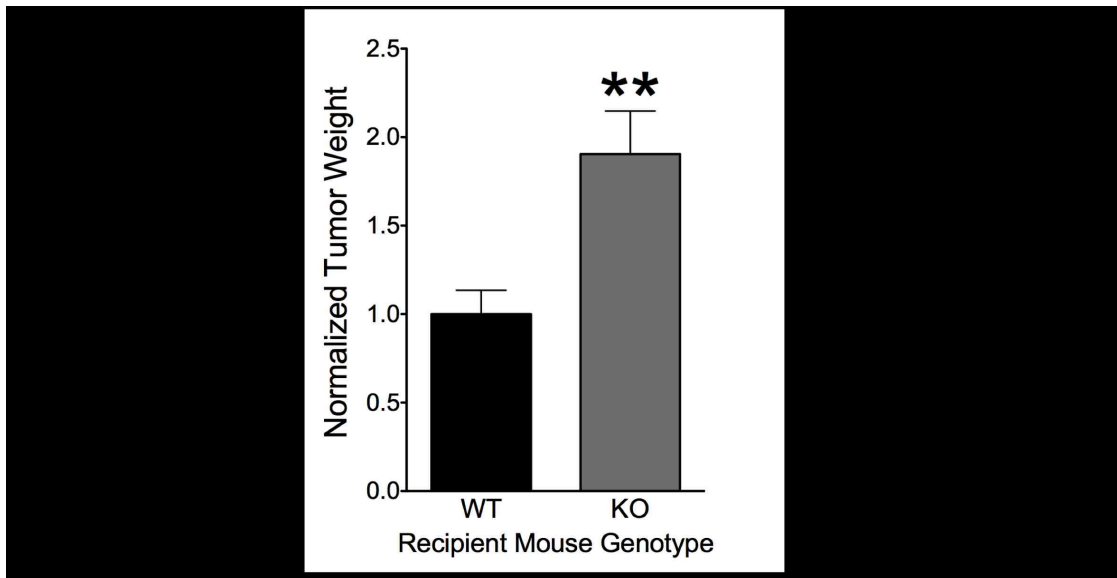
**Figure 3.4 WT/PyMT cells metastasize more frequently in WT mice compared with KO mice.** Lungs of WT and KO mice bearing WT/PyMT tumors were analyzed for the presence of metastatic foci. Data are displayed as the percentage of mice with metastases. Statistical analysis was performed using Fisher's exact test.

\*  $p < .05$

### **3.4.2 GPER<sup>-</sup> tumors are larger in a microenvironment lacking GPER, but the extent of metastasis is unaffected by microenvironmental GPER expression.**

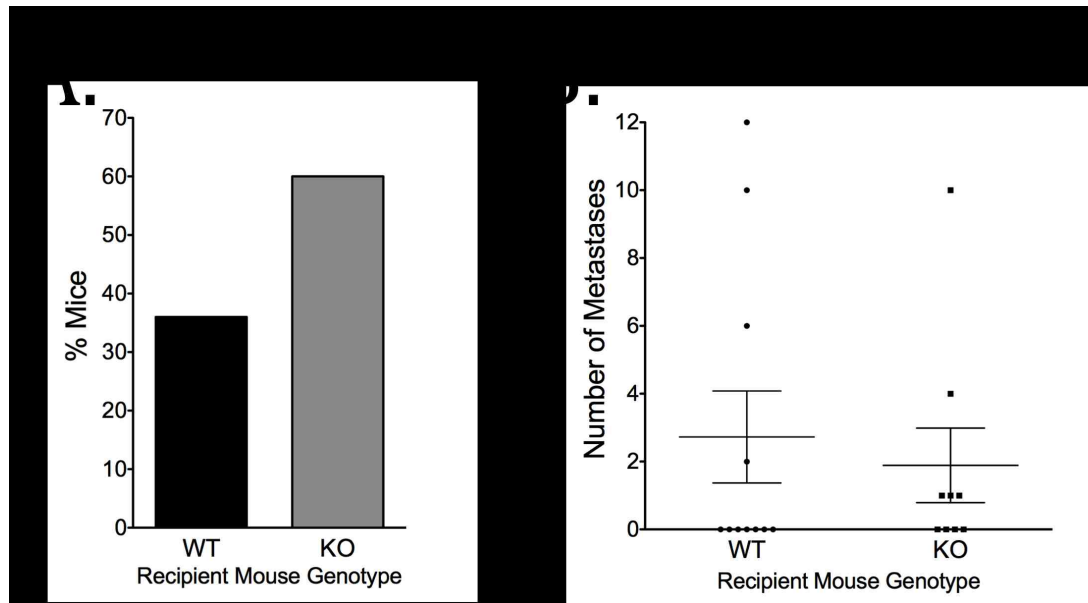
Although clinically the majority (62%) of invasive breast tumors express GPER in the tumor epithelium, a large proportion of breast tumors do not (119). Therefore, to determine how GPER expression in the microenvironment of a GPER<sup>-</sup> tumor affects tumor growth and progression, KO/PyMT cells were orthotopically transplanted into WT and KO recipient mice. Tumors were resected at 12 weeks after transplantation and weighed to determine tumor size. Tumors in KO recipient mice were 2-fold larger than those in WT recipient mice ( $p = .003$ ) (Figure 3.5), demonstrating a growth advantage for KO/PyMT tumors in a GPER<sup>-</sup> microenvironment.

The lungs of WT and KO mice bearing tumors from KO/PyMT cells were assessed in the same manner described above. No difference was observed between the percent of mice displaying metastasis between WT (31%) and KO (45%) mice ( $p = .50$ ) (Figure 3.6A). While the incidence of metastasis was not different, it is possible the metastatic burden, defined as the number of metastases per lung, could be different between WT and KO mice. Therefore, the number of metastatic foci in each lung was assessed. WT mice had  $2.7 \pm 1.4$  metastatic foci per lung compared to  $1.9 \pm 1.0$  foci in KO mice ( $p = .70$ ) (Figure 3.6B). These data suggest that although the absence of GPER in the microenvironment results in larger KO/PyMT tumors, the microenvironmental expression of GPER does not affect the ability of GPER<sup>-</sup> tumors to metastasize.



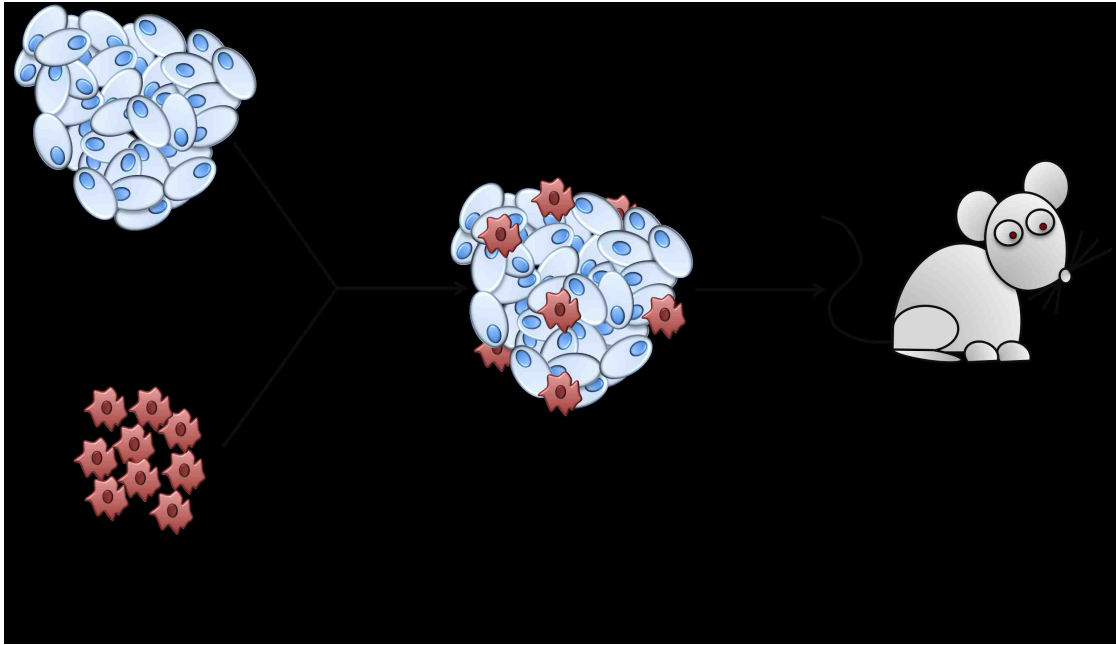
**Figure 3.5 KO/PyMT tumors are larger in a KO vs. WT microenvironment A)** KO/PyMT cells were orthotopically transplanted into WT and KO recipient mice. Tumors were removed and weighed 12 weeks after transplantation. Results were analyzed by Student's t-test.

\*\* p < .01



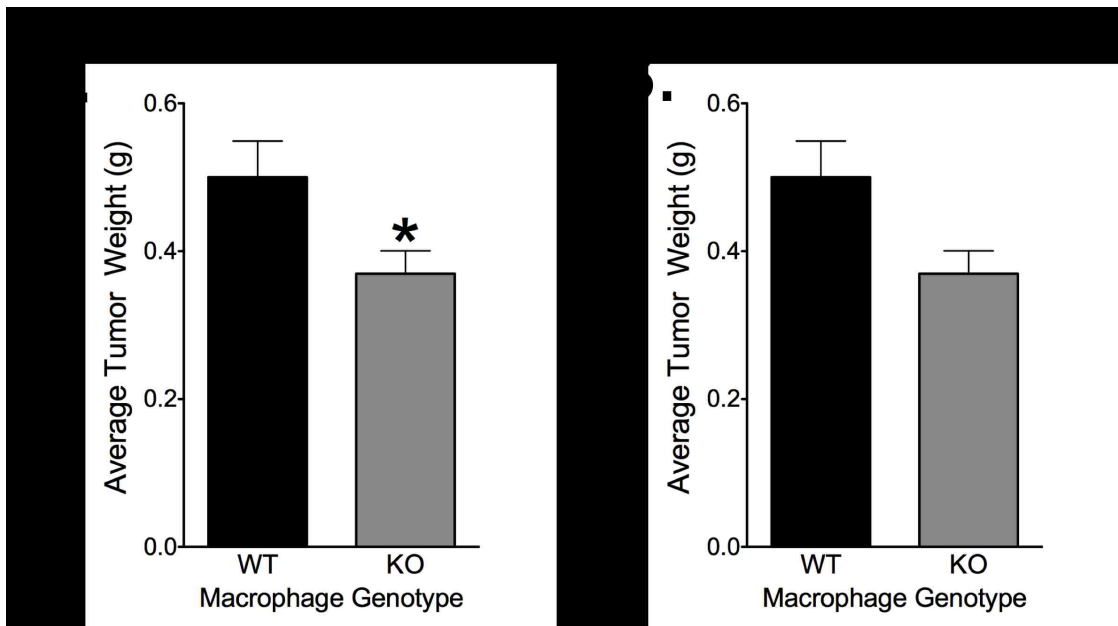
**Figure 3.6 GPER expression in the microenvironment of KO/PyMT tumors does not affect metastasis.** A) Lungs from WT and KO mice bearing KO/PyMT tumors were analyzed for the presence of metastases. Statistical analysis was performed using Fisher's exact test. B) The number of metastatic foci/lung was determined. Statistical analysis was done with Student's t-test with a p-value threshold of .05.

**3.4.3 Lacking GPER in TAMs decreases tumor size.** Altering microenvironmental GPER expression influenced tumor size and incidence of metastasis; however, the cell types responsible for these differences remain unknown. TAMs enhance tumor growth and metastasis by releasing growth factors, such as EGF, and by dampening the anti-tumor immune response (89). To examine the effect of GPER expression in macrophages on tumor size, a mixture of 90% WT/PyMT cells and 10% WT/BMM or KO/BMM was orthotopically transplanted into WT mice (Figure 3.7). Tumors co-injected with KO/BMM demonstrated a 30% reduction in tumor size ( $0.37 \pm 0.03$  g) compared to tumors with WT/BMM ( $0.5 \pm 0.05$  g) ( $p < .05$ ) (Figure 3.8A). To determine if the effects of altering GPER expression in macrophages could be enhanced, the macrophage population was increased to 25% of the transplanted cells. In agreement with data obtained from 10% macrophages, there was a trend for tumors to be 20% smaller when co-injected with KO/BMM ( $5.1 \pm 0.34$ ) compared with WT/BMM ( $4.2 \pm 0.15$ ) ( $p = .08$ ) (Figure 3.8B). These data demonstrate that lacking GPER expression in TAMs decreases tumor growth, suggesting GPER stimulation in macrophages enhances mammary tumor growth.



**Figure 3.7 Orthotopic transplant of macrophages and WT/PyMT cells.** WT/PyMT cells were mixed with WT/BMM or KO/BMM in culture and orthotopically transplanted into a WT mouse. The ratio of BMM:PyMT cells varied between 1:9 and 1:3.



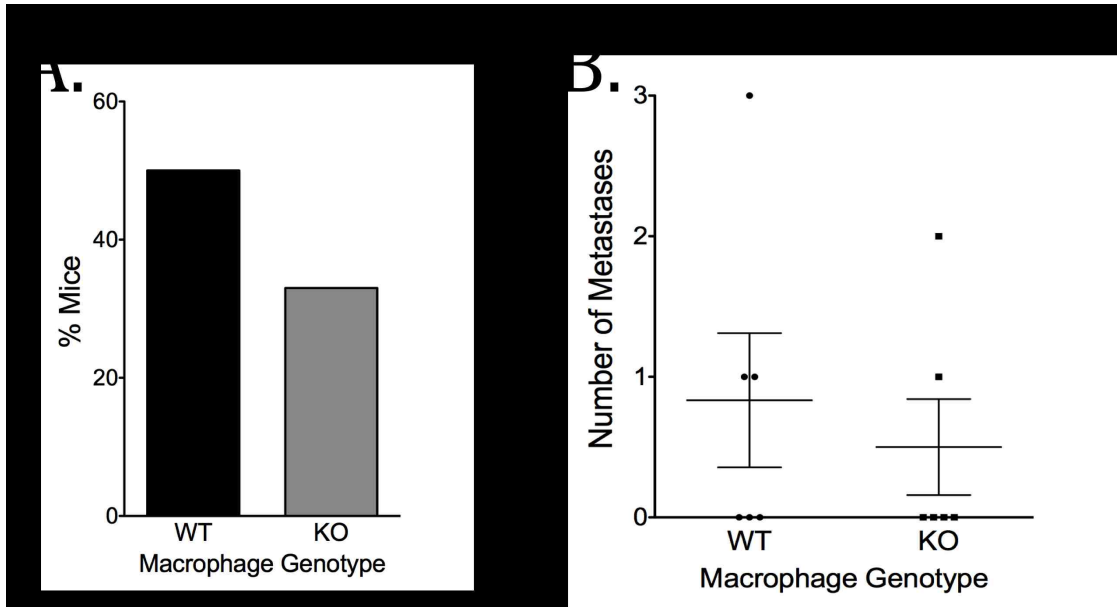


**Figure 3.8 Tumors containing KO/BMM are smaller than tumors containing WT/BMM.** WT/PyMT cells co-injected with 10% (A) or 25% (B) WT/BMM or KO/BMM were weighed as a measure of tumor size. Results were analyzed using Student's t-test.

\*  $p < .05$

#### **3.4.4 GPER expression in macrophages does not affect extent of metastasis.**

Previously published data demonstrate TAMs are necessary for tumors to metastasize in the PyMT model (126). Further, in breast cancer models, a CSF-1-EGF feedback loop has been described, in which the tumor cells release CSF-1 to recruit macrophages causing the macrophages to release EGF initiating epithelial cell migration toward the vasculature (145). This feedback loop enhances breast cancer metastasis. To examine the role of GPER expression in macrophages on metastasis, the lungs of mice co-injected with WT/PyMT cells and either WT/BMM or KO/BMM were stained with H&E and analyzed for the presence of metastasis. No significant difference was observed in the percent of mice with metastases from tumors containing WT/BMM (50%) or KO/BMM (33%) (Figure 3.9A). There was also no difference in metastatic burden, defined as the number of metastases per lung, between tumors with WT/BMM ( $0.83 \pm 0.48$ ) or KO/BMM ( $0.50 \pm 0.34$ ) (Figure 3.9B). Therefore, GPER expression in macrophages does not affect the ability of tumors to metastasize.



**Figure 3.9 The co-injection of WT/BMM or KO/BMM with PyMT cells does not affect the extent of metastasis.** A) The lungs of mice co-injected with PyMT cells and WT/BMM or KO/BMM were analyzed for the presence of distant metastases. Results were statistically analyzed using Fisher's exact test. B) The number of metastases per lung were counted. Results were analyzed using Student's t-test.

### **3.5 Discussion**

Although it has been demonstrated that GPER plays a role in breast cancer progression, its precise role in tumor growth and metastasis remains to be clarified. Previously, we established that lack of GPER in the PyMT model of mammary carcinogenesis decreases tumor size and extent of metastasis. Because the GPER KO PyMT mice were generated using a global KO mouse, it was not possible to determine if the observed differences in tumor progression were due to absence of GPER in the tumor parenchyma or microenvironment. Accordingly, WT/PyMT and KO/PyMT cells were each orthotopically transplanted into WT and KO recipient mice. The size of WT/PyMT tumors was not different regardless of GPER expression in the microenvironment. These data suggest proliferation and survival effects of GPER expression in the epithelium are dominant to the effects of GPER in the microenvironment. In contrast, KO/PyMT tumors were larger in KO mice than in WT mice. There are two general explanations for these data. One is the presence of GPER in the microenvironment inhibits tumor growth. Therefore, when GPER is absent in the microenvironment, tumor cells are released from negative regulation by microenvironmental GPER and grow more rapidly. Another explanation is that lacking GPER in the microenvironment directly stimulates tumor growth. Resolving the mechanism of microenvironmental GPER regulation of tumor growth is not trivial, and requires analysis of individual components in the microenvironment. Additionally, because tumor cells are transplanted into a global KO mouse, it is important to investigate the effects of GPER expression on the macroenvironment, including release of hormones such as insulin, estrogen, progesterone, and glucocorticoids, which could affect tumor size.

Just as the microenvironment is involved in tumor growth, it also regulates metastasis. Therefore, lungs of WT and KO recipient mice bearing WT/PyMT tumors were analyzed for the presence of metastatic foci. WT/PyMT cells metastasized frequently in WT mice, although they did not metastasize in KO mice, demonstrating the WT microenvironment positively regulates the ability of GPER<sup>+</sup> tumor cells to metastasize. The observed difference in metastasis may be due to changes in the microenvironment of the primary tumor, the microenvironment of the metastatic site, changes in vasculature, or global changes that modulate cell survival as they metastasize. Conversely, no differences in metastasis were observed when KO/PyMT cells were transplanted into WT or KO mice, which may be due to an indirect effect of tumor size. Among other variables, increased tumor size is a risk factor for metastasis (146). Consequently, because KO/PyMT tumors are larger in KO mice they may metastasize more frequently than smaller tumors in WT mice, masking the positive effects of microenvironmental GPER on metastasis. Therefore, lungs of KO and WT mice should be analyzed for metastatic foci when KO/PyMT tumors are the same size rather than being analyzed at the same time point.

Multiple components of the microenvironment are involved in regulating mammary tumor growth and metastasis (2, 82). In mouse models of mammary carcinogenesis, macrophages enhance tumor progression through the secretion of growth factors and anti-inflammatory cytokines after being polarized by soluble factors from tumor microenvironment (89). GPER decreases the LPS-induced secretion of inflammatory cytokines, but in the context of a mammary tumor, the effects of GPER on macrophage polarization are unknown (90). To determine if GPER expression in

macrophages affects tumor growth and metastasis, WT/BMM and KO/BMM were orthotopically co-injected with WT/PyMT cells into a WT recipient mouse. Co-injection of WT/PyMT cells and WT/BMM resulted in larger tumors than when KO/BMM were co-injected; however, there was no effect on the ability of the tumors to metastasize. These data suggest that in the context of mammary carcinoma, GPER expression in TAMs may augment tumor growth. There are many possibilities for why the absence of GPER expression in macrophages does not affect metastasis in this model. One possibility is GPER expression in macrophages may increase the secretion of anti-inflammatory cytokines, dampening the immune response to the tumor, resulting in increased tumor cell survival. Additionally, the expression of GPER in macrophages may increase the release of growth factors, thereby augmenting tumor growth. However, increasing tumor growth and survival does not necessarily correlate with metastatic capability (147). Furthermore, intrinsic experimental complications exist. In the experiment, tumor cells and BMM are transplanted into GPER WT immunocompetent mice. Consequently, the transplanted tumors recruit endogenous GPER WT macrophages to the tumor site, diluting the possible effects of the injected KO/BMM. Additionally, the number of injected BMM retained at the tumor site is unknown. Therefore, although 10-25% of the injected cells were KO/BMM, many of these macrophages may have migrated away from the tumor. The experimental design would be improved by using a conditional macrophage depletion model, such as the MAFI mouse, as the recipient mice, thereby inhibiting endogenous macrophage recruitment to the tumor site (148). Further, co-injecting labeled macrophages would help estimate the number of macrophages retained by the primary tumor. These experimental changes would ensure the effects

mediated by GPER KO macrophages would not be obscured by endogenously recruited macrophages, and the number of macrophages could be optimized for possible migration away from the tumor.

Collectively, these data demonstrate a role for GPER expression in the tumor microenvironment on both tumor growth and progression, although the effects seem to be contradictory in some instances. For example, although the absence of GPER in the microenvironment does not affect tumor size in WT/PyMT derived tumors, selectively lacking GPER in macrophages decreases tumor size formed by WT/PyMT cells. This suggests the role of GPER in the tumor microenvironment is multifaceted, and while GPER expression in TAMs enhances tumor growth, GPER expression in another component of the microenvironment may inhibit tumor growth. Consequently, a more thorough analysis of the activity of GPER in individual components of the microenvironment would help in the development of GPER-targeted therapies for breast cancer.

**CHAPTER 4**

***IN VIVO* ADMINISTRATION OF GPER-SELECTIVE  
ANTAGONIST G36 REDUCES MAMMARY TUMOR METASTASIS**



## **4.1 Abstract**

Treatment for breast cancer is shifting away from chemotherapy and toward therapeutics aimed at targeting specific proteins or mutations present in an individual patient's tumor. One successful target is the classical estrogen receptor, ER $\alpha$ , which is inhibited in breast cancer by tamoxifen thereby decreasing breast cancer-related mortality. Tamoxifen and other targeted drugs not only improve patient outcomes, but also decrease toxicity associated with traditional chemotherapeutics. Unfortunately, there are limited clinically available targeted therapies for breast cancer, and resistance to therapy is a recurring obstacle. To enhance the successful use of tailored treatment in patients with breast cancer, novel therapeutic targets need to be discovered. The G protein coupled estrogen receptor (GPER) has been implicated in breast cancer progression both *in vitro* and *in vivo*. However, the effect of GPER-selective compounds on breast cancer growth and metastasis remains unknown. Using the PyMT mouse model of tumorigenesis we demonstrate that pharmacologically inhibiting GPER decreases E2-mediated lung metastasis, although it has no effect on tumor size or grade. Therefore, GPER may be a novel therapeutic target to decrease metastasis.

## **4.2 Introduction**

Breast cancer accounts for 30% of new cancer cases among women and approximately 40,000 women die of breast cancer in the United States each year (63). Because of early detection and advances in treatment, breast cancer associated mortality has significantly decreased. One major advance in breast cancer treatment was recognition that the classical estrogen receptor, ER $\alpha$ , drives proliferation of breast cancer

cells (6). A higher proliferation rate leads to an increased mutation rate, more advanced tumor grade, and increased frequency of metastasis (94). Therefore, drugs designed to inhibit ER $\alpha$  or the release of estrogen are now the standard of care for patients with ER $\alpha$  expressing tumors (111). Additionally, it was determined that 25% of breast tumors overexpress the epidermal growth factor family member ErbB2/HER2/neu, which is also implicated in driving tumor progression. This discovery led to the development of the small molecule inhibitor lapatinib and anti-HER2 monoclonal antibody trastuzumab. ER $\alpha$  and HER2 targeted therapies are extremely successful due to inhibition of receptors known to drive the progression of breast cancer (69). Furthermore, they are selectively used in patient populations whose tumors express ER $\alpha$  or overexpress HER2, increasing their efficacy and decreasing side effects in patients who would not benefit from treatment (149). Unfortunately, many tumors eventually become resistant to targeted therapies. The intrinsic heterogeneity of tumors is a common cause of drug resistance. For example, a breast tumor is classified as ER $\alpha^+$  if 1% or more of its cells express the receptor. However, subsequent to ER $\alpha$  inhibition, ER $\alpha^-$  cells evolve to drive tumor growth and progression (150, 151). Therefore, there is an impetus to discover novel targeted therapies to combat resistance. There are multiple means to detect novel tumor drivers including high-throughput screening, cancer genome searches to identify mutations or duplication, and hypothesis driven research on individual proteins (152). Using hypothesis driven research, the non-classical estrogen receptor, G protein-coupled estrogen receptor 1 (GPER) has been implicated in the progression of breast cancer and as a possible therapeutic target.

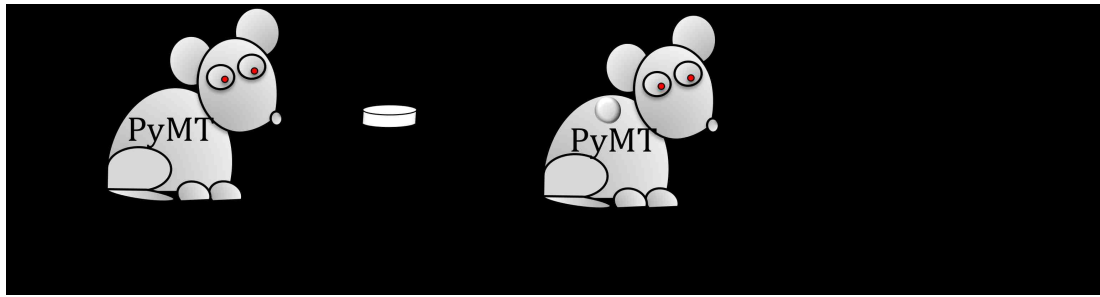
GPER was initially found to mediate rapid E2-dependent signaling in MCF7 (ER $\alpha$ <sup>+</sup>, GPER<sup>+</sup>) and SKBR3 (ER $\alpha$ <sup>-</sup>, GPER<sup>+</sup>) cells via transactivation of EGFR causing the activation of MAPK and PI3K, known tumor promoters (35, 37). Further, activation of GPER *in vitro* stimulates proliferation and migration of breast cancer cells that are inhibited by silencing GPER expression (41, 118). Although GPER appears to enhance tumorigenic features of breast cancer cells *in vitro*, its clinical value was questioned. Therefore, multiple groups analyzed GPER expression in patient samples with respect to clinical parameters of poor prognosis and found GPER expression in primary tumors correlated with increased tumor size, distant metastasis, and increased recurrence (119, 121, 131). While these data strongly implicate GPER in the progression of breast cancer, direct *in vivo* evidence was still lacking. Accordingly, we used the MMTV-PyMT (PyMT) model of mouse mammary carcinogenesis. PyMT mice were intercrossed with GPER knockout mice to analyze tumor progression with respect to GPER expression. We demonstrated mice lacking GPER have smaller tumors, decreased tumor grade, and reduced number of metastases compared with WT PyMT mice (chapter 2). These *in vivo* data demonstrate that the lack of GPER hinders mammary tumor progression, suggesting GPER enhances tumorigenesis. Therefore, *in vitro*, *in vivo*, and clinical data collectively indicate GPER enhances tumor growth and metastasis.

One of the most widely used adjuvant therapies in breast cancer is the selective estrogen receptor modulator (SERM) tamoxifen, which inhibits ER $\alpha$  in breast tissue. It is estimated 30% of breast tumors are initially resistant to tamoxifen because they lack ER $\alpha$ , and 50% of ER $\alpha$  expressing tumors develop resistance (136). There are many explanations for tamoxifen resistance including non-classical activation of ER $\alpha$  via the

MAPK cascade, increased E2 sensitivity in the tumor cells, or E2-independent growth through manipulation of another signaling pathway such as HER2 (103). However, a new hypothesis for tamoxifen resistance was made upon discovery of GPER. It was suggested that while tamoxifen inhibits ER $\alpha$ -mediated E2 signaling, GPER-mediated signaling is still intact, leading to decreased efficacy of tamoxifen. Furthermore, tamoxifen is a GPER agonist that augments cell proliferation via transactivation of EGFR. Therefore, not only is GPER-mediated E2 signaling not inhibited by tamoxifen, but tamoxifen also activates GPER-dependent cell proliferation (120). In patient samples analyzed for GPER expression, it was discovered that 40% of ER $\alpha$ <sup>+</sup> tumors express GPER (119). Further, GPER expression correlated with increased recurrence after adjuvant monotherapy with tamoxifen. Thus, it is possible that inhibiting GPER while administering tamoxifen would reduce resistance and recurrence. While some patients develop resistance to tamoxifen, other patients do not express ER $\alpha$  and thus, cannot be treated with tamoxifen. 50% of ER $\alpha$ <sup>-</sup> tumors express GPER, suggesting there may be an intact, E2-dependent pathway driving proliferation and GPER could be a therapeutic target in ER $\alpha$ <sup>-</sup> tumors (119). Therefore, pharmacologically inhibiting GPER in breast cancer could decrease tamoxifen resistance as well as treat patients whose tumors do not express ER $\alpha$ .

A GPER-selective agonist (G-1) and antagonist (G36) were developed using computer modeling, flow cytometric binding assays, and synthetic chemistry (44, 46). G-1 stimulates cell proliferation and migration in a GPER-dependent manner in cultured breast cancer cells through transactivation of EGFR, resulting in MAPK and PI3K activation (44). Therefore, G-1 stimulation of GPER activates similar, if not identical,

pathways as the endogenous ligand E2, and has been widely used *in vitro* and *in vivo* to dissect the physiologic roles of GPER (44, 90, 135). Conversely, G36 has been demonstrated to inhibit GPER-dependent Ca<sup>++</sup> mobilization, PI3K activation, and MAPK activation following E2 or G-1 stimulation. Neither of these compounds demonstrates binding to ER $\alpha$  or ER $\beta$  or activation of transcription downstream of the estrogen response element (ERE), validating their selectivity for GPER at concentrations up to 10  $\mu$ M (46). These compounds can be successfully used *in vivo*, in the presence of all three estrogen receptors to isolate the effects of GPER and determine the outcome of GPER-targeted therapy. Therefore, to delineate the consequences of GPER-targeted therapy in breast cancer, MMTV-PyMT mice were ovariectomized and treated with 90-day release pellets containing no compound (sham), G-1, E2, G36, and E2 in combination with G36 (E2+G36) (Figure 4.1). While tumor size increased compared to sham with the addition of E2, G-1 had no effect, and G36 was unable to inhibit the E2-mediated tumor growth. The extent of metastasis was increased with the addition of E2 but was unaffected by G-1 when compared with sham pellet. However, G36 reduced E2-mediated metastasis, and mice receiving G36 in the absence of E2 exhibited no metastases. These data suggest that although targeting GPER does not affect tumor size, inhibiting GPER reduces the ability of the tumor to metastasize, making GPER a potential therapeutic target in patients with breast cancer.



**Figure 4.1 Experimental protocol for treatment with GPER-selective compounds.** PyMT mice were ovariectomized when mice were 3-weeks-old to remove the majority of circulating estrogen. When mice were 4-weeks-old, 90-day release pellets were subcutaneously implanted on the dorsal side of the mice. Pellets contained E2 (0.36 mg/pellet), G-1 (3 mg/pellet), G36 (2mg/pellet), and E2 + G36, in which an E2 and a G36 pellet were both implanted. Tumors were resected when mice were 12-weeks-old and weighed as a measurement of tumor size. Lungs were also removed to determine extent of metastasis.

## **4.3 Methods**

**4.3.1 Mice.** FVB/N-Tg(MMTV-PyVT)643Mul/J (MMTV-PyMT) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Animals were housed in the animal research facility at the University of New Mexico. They were maintained on a 12-h light and 12-h dark cycle with a controlled temperature of 22-23°C and fed normal chow *ad libitum*. Mice were ovariectomized at 3 weeks of age to decrease endogenous E2 production as described previously (chapter 2). At 4 weeks of age, 90-day release pellets were subcutaneously implanted on the dorsal midsection of the mouse. Pellets were purchased from Innovative Research of America and contained E2 (0.36 mg/pellet), G-1 (3 mg/pellet), and G36 (2 mg/pellet). Mice in the E2+G36 group has 2 pellets implanted, one with each compound. Tumors and lungs were resected when mice were 12 weeks of age. Tumors and the left lung were fixed in 4% PFA at room temperature overnight and embedded in paraffin. The right lung was stored in Trizol (Sigma-Aldrich) at -80°C to preserve RNA. All procedures were approved by and carried out in accordance with the institutional protocols.

**4.3.2 Histologic analysis of tumor sections.** Three 5 µm sections of the largest tumor from each mouse were stained with H&E. The sections were analyzed for tumor grade, mitotic index, and extent of necrosis by veterinary pathologist Donna Kusewitt, DVM, PhD (The University of Texas MD Anderson Cancer Center). Grading PyMT tumors was performed as previously described (125). Briefly, grade is based on tissue architecture, degree of cytologic atypia, and invasion into the surrounding stroma. The grades are as follows: (1) Hyperplasia: Densely packed acini filled or bridged by epithelial cells that have little to no cytologic atypia. There is no invasion into the surrounding stroma. (2)

Adenoma/mammary intraepithelial neoplasia (MIN): Increased proliferation of epithelial cells with acini mostly filled with cells. There is minimal cellular atypia and no invasion is present. (3) Early carcinoma: Florid proliferation with loss of acinar definition. There is moderate cellular atypia and early stromal invasion. (4) Late carcinoma: Solid sheets of cells containing very few or no acini with a high degree of proliferation (Figure 4.2). Marked cellular atypia and pronounced stromal invasion are present. Mitotic Index is a measure of the proliferation rate and is defined as the number of mitotic figures per high-powered field. Lastly, necrosis is categorized as the number and size of necrotic areas. The score of necrosis is as follows: 1 = few small areas; 2 = few larger areas or moderate number of smaller areas; 3 = extensive areas.

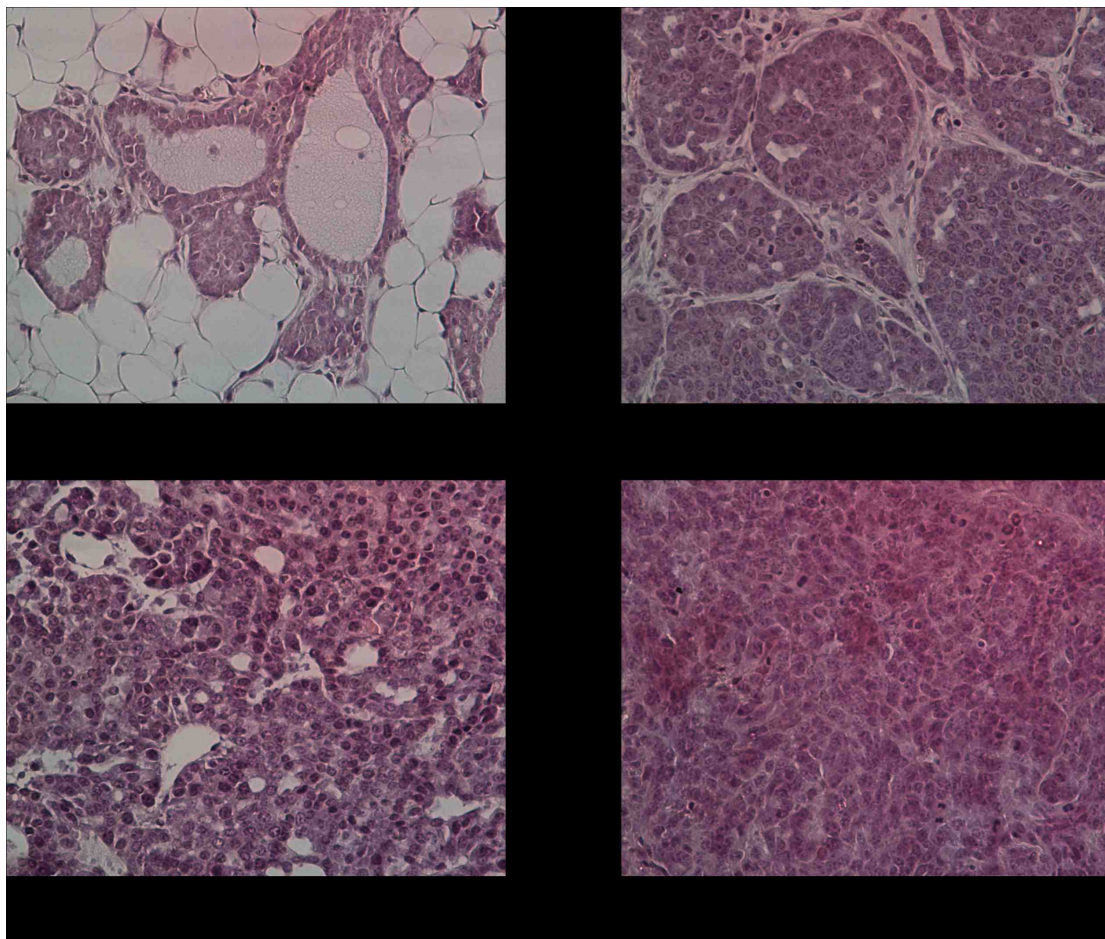
**4.3.3 Analysis of Lung Metastasis.** Because PyMT RNA is not expressed in the lung unless tumor cells are present as a result of metastasis, the right lung of each mouse was analyzed for the presence of PyMT RNA by relative-quantitative PCR (qPCR). RNA was extracted using Trizol-chloroform (Sigma-Aldrich), following the manufacturer's instructions. cDNA was created via reverse transcription of 1  $\mu$ g RNA with the iScript cDNA synthesis kit (BioRad) using the GeneAmp PCR system 9700 (Applied Biosystems, Inc.) according to manufacturers directions. Quantification of PyMT mRNA relative to 36B4 mRNA was performed using Fast SYBR Green (Molecular Probes) with the 7500 Fast Real-Time PCR System (Applied Biosystems, Inc), using relative standard curves, as previously described (chapter 2) Briefly, a standard curve using a mixture of sample cDNA was created for each primer set. The Ct values each lung sample were compared first to the standard curve to determine the relative amount of PyMT or 36B4



cDNA in each sample. The relative amount of PyMT cDNA was then normalized to the relative amount of 36B4 cDNA.

Three 5  $\mu\text{m}$  sections separated by at least 100  $\mu\text{m}$  were created from the left lung of each mouse. The sections were stained with H&E and analyzed for the number of metastatic foci. A metastatic focus was defined as a group of 10 or more cells stained darkly with hematoxylin, excluding eosin positive stroma.

**4.3.4 Statistical Analysis.** Caliper measurements of tumors were analyzed by two-way ANOVA with Bonferroni's correction for multiple comparisons as a post-hoc test. Tumor size, mitotic index, qPCR evaluation of lung metastasis, and number of lung metastases were compared using one-way ANOVA with Bonferroni's correction for multiple comparison test as a post-hoc test, when applicable. Tumor grade, amount of necrosis, and presence of metastases were compared using chi-squared analysis followed by Fisher's exact test with corrected p-value when applicable.

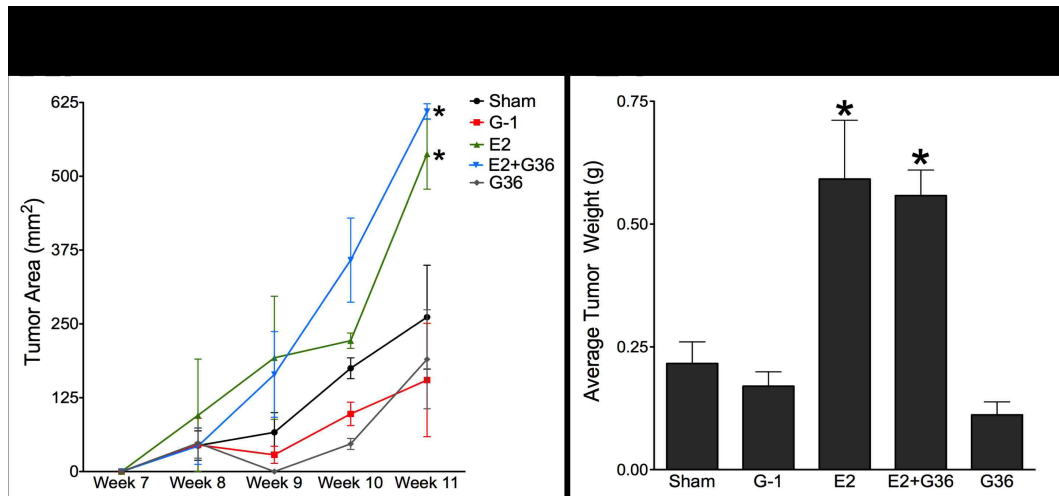


**Figure 4.2 Representative images of each tumor grade.** Tumor sections (5  $\mu\text{m}$ ) were stained with H&E. Tumors progress from well differentiated (grade 1) to poorly differentiated (grade 2) as illustrated above.

## **4.4 Results**

**4.4.1 GPER-selective compounds do not affect tumor size.** We previously determined knocking out GPER in the MMTV-PyMT model of mammary carcinogenesis reduces tumor size and metastasis (chapter 2). While these data demonstrate decreasing the expression of GPER in patients with breast cancer may improve patient outcome, they do not necessarily predict the outcome of pharmacologic inhibition of GPER. Examining the effects of GPER-selective compounds on *in vivo* cancer progression will aid in determining if GPER is a good candidate for targeted therapy in breast cancer. Therefore, PYMT mice were ovariectomized at 3 weeks of age to reduce the endogenous E2. Pellets containing GPER-targeted compounds, including G-1, E2, E2+G36, and G36, were subcutaneously implanted one week after ovariectomy. Mice were palpated two times per week to determine if GPER-selective compounds affected tumor latency. All mice displayed palpable tumors between 7 and 8 weeks of age, suggesting tumor latency is not affected by targeting the classical estrogen receptors or GPER. Once large enough, tumors were measured with calipers two times per week to monitor tumor growth. Differences in tumor size between groups were first observed at 9 weeks of age. However, no significant differences were detected until 11 weeks of age, at which time tumors in mice treated with E2 or E2+G36 were 2-fold larger than sham treated mice. However, G-1 and G36 alone had no effect of tumor size compared to sham (Figure 4.3A). Tumors were removed when mice were 12 weeks of age, and, similar to caliper measurements, the average weight of tumors from mice treated with E2 ( $0.59 \pm 0.11$  g) was about 2.5-fold greater than sham ( $0.22 \pm 0.04$  g), and G36 had no effect on E2-mediated tumor growth. Further, tumors from mice treated with G-1 or G36 were not

different in size from sham (Figure 4.3B). Collectively, these data suggest targeting GPER with a selective agonist or antagonist does not affect the size of mammary tumors.

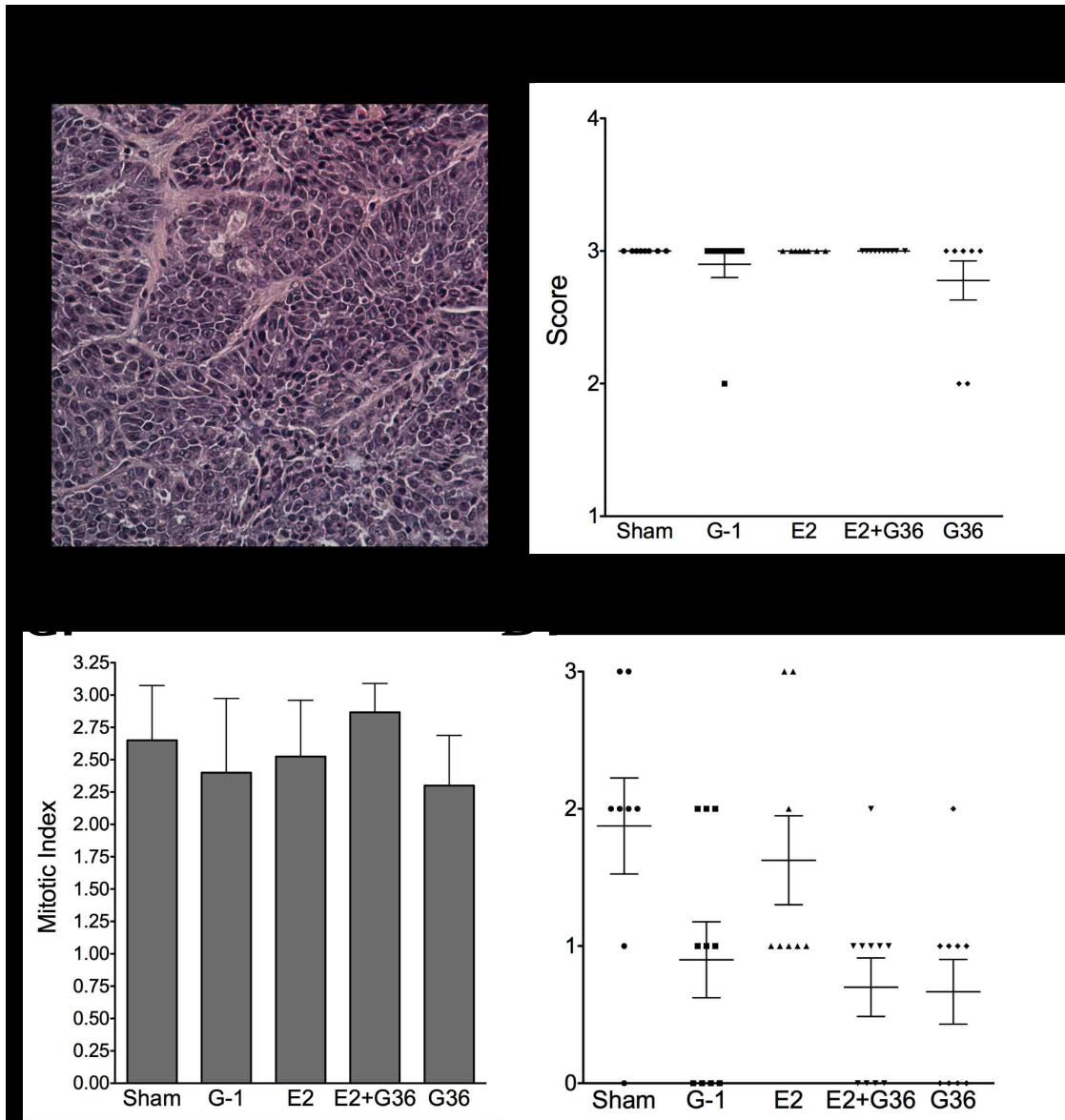


**Figure 4.3 The GPER-selective agonist or antagonist has no effect on tumor size.** A) PyMT mice were ovariectomized at 3 weeks of age and treatment pellets were implanted at 4 weeks of age. Tumor measurements were obtained using calipers beginning at 7 weeks of age through 11 weeks of age. Data was statistically analyzed by two-way ANOVA with Bonferroni correction for multiple comparisons as a post-hoc test. B) Tumors were resected when mice were 12-weeks-old and weighed as a measurement of tumor size. One-way ANOVA with Bonferroni correction for multiple comparisons as a post-hoc was used to statistically analyze tumor weights.

\*  $p < .05$ , compared to sham

**4.4.2 Treatment of PyMT mice with GPER-selective compounds does not affect tumor grade, proliferation rate, or extent of necrosis.** Although GPER-selective compounds did not alter tumor size, it is possible parameters indicative of tumor aggressiveness were affected. Therefore, sections from the largest tumor from each mouse were stained with H&E and analyzed for tumor grade, mitotic index, and extent of necrosis as markers of aggressive disease. Tumors from all treatment groups appeared as sheets of cells with a loss of acinar definition, and where acini could be visualized, they were completely filled with cells (Figure 4.4A). Cytologic atypia and marked areas of necrosis were also apparent, and many tumors exhibited early stage invasion into the surrounding stroma. Based on these histological observations, each tumor was graded. Sham, E2 and E2+G36 all had an average tumor grade of  $3 \pm 0$ . Mice treated with G-1 had an average grade of  $2.9 \pm 0.1$  and G36 treated mice had an average of  $2.77 \pm 0.15$ , and were not statistically different than sham treated mice (Figure 4.4B). These data demonstrate there is no difference in tumor grade between groups. Next, the number of mitotic figures per high power field was determined as a measure of the proliferation rate, which is an independent marker of poor prognosis. In all treatment groups, the mitotic index was between 2 and 3 with no significant differences as measured by one-way ANOVA [ $F(4, 38) = .26, p = .90$ ] (Figure 4.4C). Finally, the extent of necrosis was assessed because it is correlated with poor prognosis and aids in metastasis by promoting cell detachment (134). There was a trend for tumors from sham and E2 treated mice to contain increased necrosis compared with G-1, E2+G36 or G36 alone ( $p = .06$ ) (Figure 4.4D). These data suggest GPER ligands do not affect parameters of poor prognosis

typically analyzed in the primary tumor including tumor grade, cell proliferation, and necrosis.

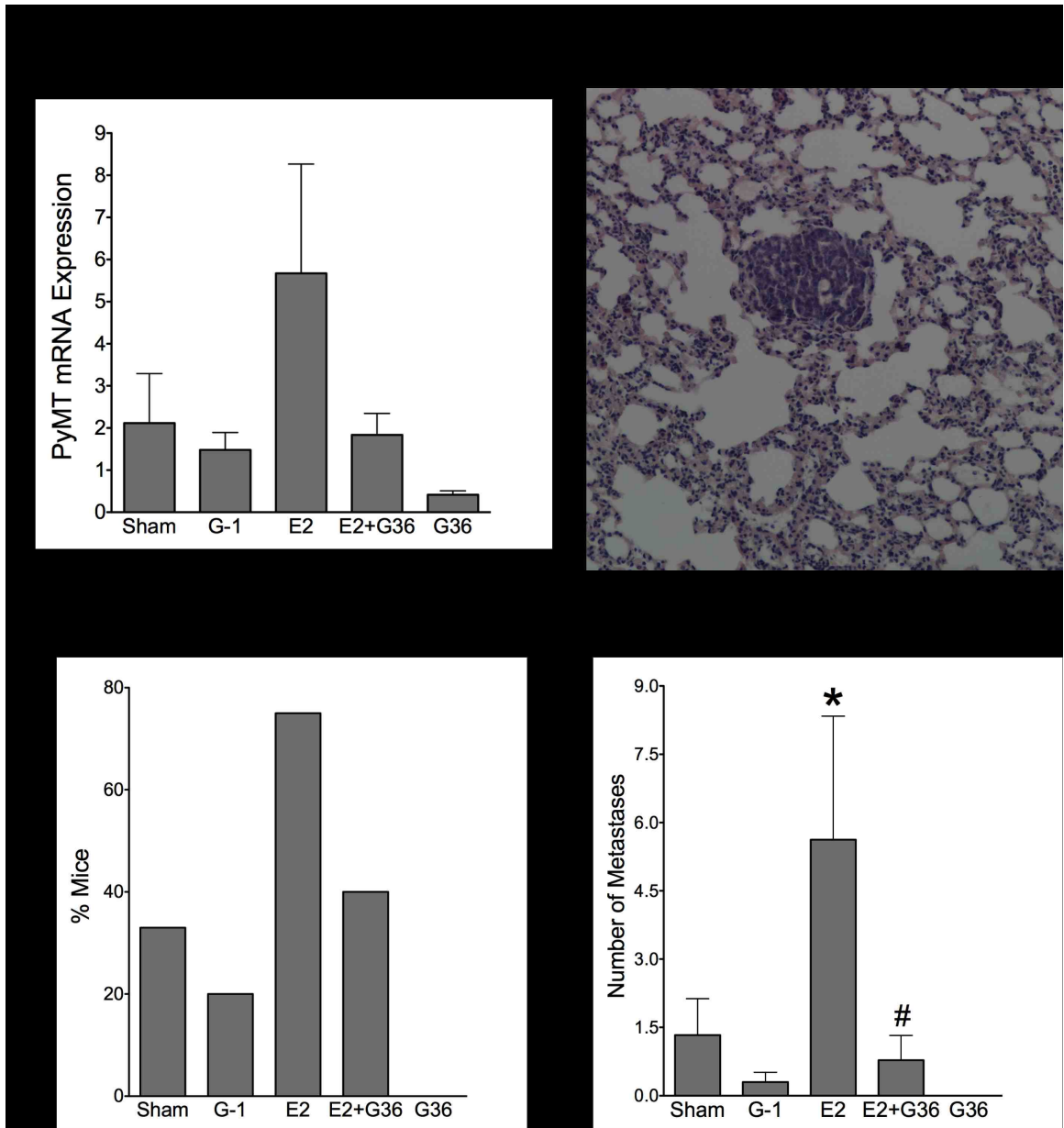


**Figure 4.4 GPER-selective compounds do not affect histological parameters of poor prognosis in PyMT mice.** A) Representative image of 5 $\mu$ m tumor sections stained with H&E. B) Tumor sections stained with H&E were analyzed for tumor grade. Statistical analysis was performed by chi-squared analysis. C) Mitotic index, defined as the number of mitotic figures per high-powered field was determine in sections stained with H&E. One-way ANOVA was performed to analyze results. D) Extent of necrosis was determined by the number and size of necrotic areas present in tumor sections stained with H&E and analyzed by chi-squared analysis.



**4.4.3 Pharmacologic inhibition of GPER impedes mammary tumor metastasis.** Tumor size, grade, and proliferation rate are used clinically to predict the presence of metastasis and patient outcome (64). However, because metastasis is a complex process occurring in multiple organs, it is possible to affect the frequency of metastasis without affecting the aforementioned aspects of the primary tumor (106). Accordingly, RNA was extracted from the right lung of each mouse to assess the presence of PyMT RNA, which would not be present in lungs unless PyMT expressing mammary tumor cells had metastasized. The lungs of sham, G-1, and G36 treated mice expressed very low levels of PyMT RNA, suggesting minimal metastases. However, there was a trend for G36 to inhibit the E2-mediated increase in PyMT RNA (Figure 4.5A). To further assess the extent of metastasis, the left lung of each mouse was paraffin embedded, sectioned, and stained with H&E (Figure 4.5B), and the presence of metastatic tumor foci was assessed. Although not statistically significant, it is important to note that 33% of sham treated mice had metastatic foci as compared to 75% of E2 treated mice. Additionally, there was a trend for G36 treatment in combination with E2 to reduce the incidence of E2-dependent metastasis by 60%. Lastly, lungs from mice treated with G36 displayed no metastases, but again this was not significant compared to sham (Figure 4.5C). To further assess the extent of metastasis, the number of metastases was determined. The number of metastases was not different between sham ( $1.3 \pm 0.8$ ) and G-1 ( $0.3 \pm 0.2$ ) treated mice and although G36 treated mice had no metastases, it was not significant compared with sham. E2 treated mice had an average of  $5.6 \pm 2.7$  metastases per lung, which is 4 times the number of metastases compared to sham treated mice. G36 reduced the number of E2-mediated metastases to  $0.77 \pm 0.55$ , bringing metastasis in

mice treated with E2+G36 back to the level of sham treated mice (Figure 4.5D). Therefore, although GPER-selective compounds do not affect the size or grade of mammary tumors, pharmacological inhibition of GPER decreases the extent of metastasis.



**Figure 4.5 Administration of GPER-selective antagonist, G36, inhibits metastasis in PyMT mice.** A) PyMT mRNA expression in lungs from PyMT mice was analyzed by relative quantitative PCR to determine extent of lung metastasis. Statistical analysis was performed using one-way ANOVA. B) Representative image of a metastatic focus in lung tissue. C) The incidence of metastasis was determined by the number of mice in each treatment group displaying metastases in their lungs. Chi-squared test was used for statistical analysis. D) The number of metastases in the lung of each mouse was determined to assess the extent of metastasis. One-way ANOVA with Bonferroni correction for multiple comparisons as a post-hoc was used for statistical analysis.

\*  $p < .05$ , compared to sham

#  $p < .05$ , compared to E2

## **4.5 Discussion**

Breast cancer treatment is evolving towards tailored therapeutics for each patient, depending on the expression profile of her tumor. This shift toward personalized medicine has increased 5-year survival rates and decreased treatment side effects. Unfortunately, only a handful of targeted therapies exist, and many patients are not eligible for, or become resistant to these therapies, indicating the need to develop novel treatment modalities (149). One obstacle in developing novel therapies is discovering proteins or mutations that not only drive progression of breast cancer but can also be targeted in a therapeutically meaningful manner (152). Using hypothesis driven research, we have identified GPER as a possible therapeutic target. When absent, GPER hinders tumor growth, decreases tumor grade, and impedes metastasis (chapter 2). However, in chapter 3 we demonstrated that lack of GPER in the microenvironment enhances tumor growth of GPER<sup>+</sup> cells. These seemingly contradictory results suggest that global inhibition of GPER might not provide straightforward results. Therefore, the effects of a GPER-selective agonist and antagonist were analyzed in the PyMT mouse model of mammary tumorigenesis.

Ovariectomized, PyMT mice were treated with the GPER-selective agonist, G-1, and GPER-selective antagonist, G36, in the presence and absence of E2, the endogenous GPER ligand. Based on previously published data demonstrating G-1 increases proliferation and migration in cultured cells and our own data that lacking GPER inhibits tumor progression, we hypothesized G-1 would increase tumor size and metastasis (135). However, treatment of PyMT mice with G-1 had no apparent affect on tumor size,

proliferation rate, tumor grade, or incidence of metastasis. The apparent disagreement between previously obtained data and the effect of G-1 in PyMT mice may point to a role for opposing effects of GPER in the tumor microenvironment and parenchyma. For example, targeting GPER in the stroma may inhibit tumor growth and metastasis, thereby negating the positive effect GPER has on proliferation and metastasis when stimulated in the tumor epithelial cells. Another possibility is that using pellets as the drug delivery system may have affected the intratumoral concentration of G-1 over time. Analysis of E2 release from pellets revealed a large bolus of E2 is released in the first few days after implantation and then levels off to a much lower level (153). The dynamics of G-1 release from the pellet are unknown. However, if a large amount of G-1 is released in the first few days after implantation, the concentration of compound subsequently released may not be high enough to affect tumor growth and metastasis. Therefore, an in-depth study of G-1 release from pellets needs to be performed in addition to analyzing the effects of G-1 on the tumor microenvironment.

As expected, E2 increased tumor size. However, G36 did not reverse the E2-dependent increase in tumor size, suggesting dominance of the growth-promoting effects of ER $\alpha$ . Somewhat surprisingly, E2 had no effect on tumor grade or proliferation rate. A more specific marker of proliferation such as Ki67 or phospho-histone H3 should be analyzed as the mitotic index is a rough estimation of proliferation rate. Furthermore, a low dose of E2 was used that may have decreased its effectiveness, especially when the dynamics of E2 release from pellets is considered (153). However, this low-dose E2 significantly increased the incidence of metastasis, which was blocked by combination therapy with G36, suggesting GPER stimulation is required for E2-dependent metastasis

in the PyMT model of mammary tumorigenesis. Additionally, no metastases were observed in mice treated with G36 alone, although this was not statistically different from sham. Although inhibiting GPER did not affect tumor size, it did significantly decrease extent of metastasis. Since most breast cancer mortality does not result from the primary tumor, but rather, from metastasis to distant sites, GPER is a viable therapeutic target.

Targeting E2-mediated pathways is not a novel therapeutic strategy as tamoxifen is the most widely used targeted therapy in breast cancer (116). Premenopausal women with breast cancer are post-surgically treated with tamoxifen for 5 years, and postmenopausal women are treated with a combination of aromatase inhibitors and tamoxifen (111). However, 50% of breast tumors develop resistance to tamoxifen (136). One possible reason for resistance is activation of GPER during tamoxifen treatment. Therefore, inhibiting GPER in combination with tamoxifen may improve efficacy and inhibit resistance. Further, tumors that do not express ER $\alpha$  may respond to G36, providing a new option in treatment for women with ER $\alpha$ <sup>-</sup> tumors. Therefore, pharmacologically inhibiting GPER in combination with current therapies is an intriguing treatment modality to inhibit E2-mediated metastasis in breast cancer that could increase disease free survival.

**CHAPTER 5**  
**CONCLUSIONS AND FUTURE DIRECTIONS**

## **5.1 Summary and Future Directions**

We aimed to determine the effects of the non-classical estrogen receptor, GPER, on breast cancer development, growth and progression. Often, E2 drives breast carcinogenesis, and these E2-dependent effects have historically been attributed to the classical E2 receptor, ER $\alpha$ , due to its ability to modulate gene transcription and increase tumor cell proliferation and survival (6). Furthermore, antagonizing ER $\alpha$  with drugs, such as tamoxifen is a successful treatment strategy in patients with ER $\alpha$ <sup>+</sup> breast cancer (6). GPER also increases epithelial cell proliferation in an E2-dependent manner; however, unlike ER $\alpha$ , which classically behaves as a transcription factor, GPER is responsible for rapid non-genomic E2-dependent signaling (28, 118, 135). Because GPER is an estrogen receptor that increases cell proliferation, it was reasonable to believe it plays a role in hormone responsive female cancers, including breast cancer. *In vitro* evidence in breast cancer cell lines demonstrates GPER enhances cell proliferation and migration through transactivation of the EGFR (118). Additionally, evaluation of clinical samples detected GPER expression in 62% of invasive breast cancers, which correlated with increased tumor size and presence of distant metastasis, and GPER was associated with increased recurrence after tamoxifen treatment (119, 121). While these *in vitro* and clinical data strongly implicated GPER in breast cancer progression, direct *in vivo* evidence was still lacking. Accordingly, we investigated the *in vivo* impact of GPER expression on breast cancer development, growth, and progression using the MMTV-PyMT murine model of mouse carcinogenesis.

GPER KO mice were crossed with PyMT mice to generate PyMT GPER WT, HET, and KO mice (WT/PyMT, HET/PyMT, KO/PyMT). Using this model, it was



determined GPER does not affect early tumor development evidenced by no difference in proliferation rate or extent of hyperplasia at 7 weeks of age. However, by 12 weeks of age, tumors in KO/PyMT mice were smaller than tumors in WT/PyMT mice. Evaluation of P-histone-H3 expression demonstrated the proliferation rate in tumors from KO/PyMT was reduced compared with tumors from WT/PyMT mice, suggesting the difference in tumor size is due to the effect of GPER on proliferation. Furthermore, KO/PyMT mice contained fewer metastases compared to WT/PyMT mice, implicating GPER in breast cancer metastasis and consistent with *in vitro* reports of GPER stimulating epithelial cell migration (118). Collectively, these results establish a role for GPER in mammary tumor growth and progression and are the first direct *in vivo* evidence for a role of GPER in breast cancer. While these results are exciting, it is important to acknowledge the KO/PyMT mouse is a global knockout, making it difficult to differentiate the mechanism by which GPER regulates tumor growth. Therefore, an orthotopic transplant model was used to differentiate the contribution of GPER expression in the tumor cell versus the microenvironment.

WT/PyMT cells were orthotopically transplanted into WT and KO FVB mice to assess the microenvironmental role of GPER on tumor size and distant metastasis. Tumor size did not differ between WT and KO mice, suggesting the proliferative and survival effects of GPER in the tumor parenchyma are dominant over any effect GPER may be having in the microenvironment. However, there is no direct evidence in this model demonstrating GPER expression in tumor cells increases proliferation or survival. To determine the possible growth promoting effects of GPER in tumor epithelium, primary WT/PyMT and KO/PyMT cells will be cultured and assessed for differences in

proliferation rate as well as cell survival. While this simple experiment analyzes a direct effect of GPER on epithelial cell proliferation, it does not account for epithelial-derived secreted factors that may modulate the microenvironment. For example, GPER stimulation causes the cleavage of HB-EGF, which could ultimately result in VEGF production and increased angiogenesis, thus indirectly regulating tumor size (154). To delineate these indirect tumor-promoting effects, an in-depth analysis of tumors derived from WT/PyMT and KO/PyMT cells needs to be performed to assess differences in vasculature and stromal cells.

Metastasis to the lungs of WT and KO mice bearing WT/PyMT tumors was also assessed. While metastasis occurred in WT mice, no metastases were present in the lungs of KO mice. These data suggest GPER in the microenvironment augments the ability of tumors to metastasize, although the mechanism has not been elucidated. Multiple modifications in the tumor microenvironment are responsible for increased metastasis, including alterations to the ECM, increased blood vessel permeability, and signaling from multiple cell types (139). Investigation into the mechanism of metastasis should initially focus on gross differences between primary tumors in WT and KO mice. These differences include modifications in the type of stromal collagen, changes in density or distribution of vasculature, and the relative quantity of microenvironmental cell types, such as fibroblasts and immune cells (139). Next, cultured tumor chunks will be analyzed for secreted factors including growth factors and cytokines to determine if GPER affects the production of factors regulating metastasis. Finally, the effects of GPER in individual stromal cell types will be assessed. For example, an EGF-CSF-1 signaling axis occurs between macrophages and mammary tumor cells, which enhances

metastasis (145). Thus, if GPER mediates the secretion of EGF from macrophages, it may enhance metastasis through this signaling axis. Once individual cell effects are determined, co-culture invasion assays with these individual cell types will be used in combination with signaling inhibitors to determine the mechanism of microenvironmental GPER-mediated metastasis. Furthermore, the ability for tumors to survive and grow in the distant site can be affected by the microenvironment of the metastatic organ (106). Therefore, an experimental metastasis model will be used in which WT/PyMT cells are intravenously injected into WT and KO mice. This method bypasses the invasion and intravasation steps required for metastasis and focuses on the ability of the tumor cells to seed and survive at a distant site (155).

The reciprocal experiment was also performed in which KO/PyMT cells were orthotopically transplanted into WT and KO mice. Tumors transplanted into KO mice were significantly larger than those in WT mice, suggesting GPER in the microenvironment hinders tumor growth. In a similar manner as described above, it will be necessary to determine differences in vascular density and distribution and the relative quantity of stromal cells to begin to understand how lacking GPER in the microenvironment augments tumor growth. Further, investigation into the role of GPER in individual stromal cell types will aid in understanding microenvironmental regulation of KO/PyMT tumor size.

In contrast to tumor size, no difference was observed in the ability of KO/PyMT tumors to metastasize in WT or KO mice. These data appear contradictory to the data obtained from WT/PyMT tumors, which metastasize more frequently in WT mice than KO mice. However, it is important to recognize that KO/PyMT tumors are larger in KO

compared to WT mice, and tumor size is an independent risk factor for metastasis (146). Therefore, to better assess the effects of microenvironmental GPER on metastasis of GPER<sup>-</sup> tumors, KO/PyMT cells will be orthotopically transplanted into WT and KO mice and the lungs will be assessed for metastasis when tumors are the same size as determined by caliper measurements. The results from this experiment will clarify if GPER in the microenvironment affects the ability of KO/PyMT tumors to metastasize or if the metastasis-promoting effects of GPER in the microenvironment of KO/PyMT tumors are being masked by the difference in tumor size.

Macrophages in the microenvironment have been implicated in tumor growth and progression (89). Because GPER modulates the polarization of macrophages and decreases the release of pro-inflammatory cytokines, bone marrow derived macrophages (BMM) from WT and KO mice (WT/BMM and KO/BMM, respectively) were co-transplanted with WT/PyMT cells into a WT mouse (90). This experiment demonstrated GPER expression in macrophages enhances tumor growth, but has no effect on the ability of the tumor to metastasize. However, a confounding factor in this experiment was the endogenous expression of GPER<sup>+</sup> macrophages in the recipient mouse. Therefore, once the tumor begins to form, endogenous GPER<sup>+</sup> macrophages will be recruited to the tumor site, potentially diluting the effects of KO/BMM. Furthermore, because the transplanted macrophages were unlabeled, it is unclear how many were retained by the tumor. Therefore, in the future this experiment will be conducted in transgenic mice allowing conditional macrophage ablation such as MAFIA mice to deplete endogenous macrophages (148). Tumors will be orthotopically transplanted into macrophage-depleted mice receiving adoptively transferred WT/BMM or KO/BMM. Differences in

tumor size and metastasis with respect to GPER expression in macrophages will then be assessed. Additionally, GPER-dependent macrophage polarization will be assessed *in vitro* by analyzing secreted cytokines in WT/BMM and KO/BMM. Furthermore, co-culture experiments with PyMT cells and WT/BMM and KO/BMM will be performed to examine differences in tumor cell proliferation, survival, and invasion.

Ultimately, defining the role of GPER in breast carcinogenesis will determine if it is a good candidate for targeted therapy. Inhibiting E2-dependent signaling is a successful treatment paradigm in patients with ER $\alpha$ <sup>+</sup> tumors (99, 103, 116). One of the most common adjuvant therapies for breast cancer is tamoxifen, which antagonizes ER $\alpha$ -dependent signaling in breast cancer. Although 70% of tumors express ER $\alpha$  and are treated with tamoxifen, many become resistant to treatment (59). Furthermore, the 30% of tumors that do not express ER $\alpha$  are ineligible for treatment with tamoxifen, often leading to treatment with toxic chemotherapeutics (136). The discovery of GPER revealed a novel, targetable E2-dependent signaling pathway that could help explain tamoxifen resistance, as tamoxifen is a GPER agonist (120). Therefore, PyMT mice were ovariectomized to remove the majority of circulating E2 and treated with E2, the GPER-selective agonist G-1, and the GPER-selective antagonist G36 in the presence or absence of E2. As expected, E2 increased tumor size and the extent of metastasis. G36 had no effect on E2-dependent tumor size, suggesting the proliferative effect of ER $\alpha$  is dominant to that of GPER, making GPER inhibition inconsequential in tumor size. However, G36 inhibited E2-mediated metastasis, demonstrating a role for GPER in mammary tumor metastasis. Although G-1 enhances the proliferation of cultured breast cancer cells *in vitro*, it did not affect the size of *in vivo* tumors. It is possible the effect of G-1 on the

microenvironment inhibits tumor growth, negating its proliferative effect in the epithelium. Additionally, G-1 did not affect the extent of metastasis, although G36 inhibited E2-mediated metastasis. These apparently contradictory results indicate ER $\alpha$  and GPER may cooperate to enhance breast cancer metastasis. *In vitro* examination of ER $\alpha$ - and GPER-dependent migration and invasion will help to clarify the role of each receptor in metastasis and how they may interact to enhance metastasis. Further, analyzing the effects of G36 on the tumor microenvironment will aid in understanding how GPER may indirectly cooperate with E2 to increase the incidence of metastasis. Finally, ER $\alpha$  expression in patient samples has been negatively correlated with lymph node involvement and distant metastasis; however, ER $\alpha$ -positive samples will be analyzed with respect to GPER to determine if GPER enhances metastasis in ER $\alpha$ <sup>+</sup> tumors (64). The reciprocal analysis will also be done in GPER<sup>+</sup> samples to reveal a possible cooperation between GPER and ER $\alpha$  in metastasis. These data demonstrate pharmacologically inhibiting GPER *in vivo* inhibits breast cancer metastasis. Therefore, G36 may be a viable treatment to decrease tamoxifen resistance or to treat patients with ER $\alpha$ <sup>-</sup> tumors.

## **5.2 Overall Conclusions**

This seminal work establishes an *in vivo* role for GPER in breast cancer growth and metastasis. Prior to the discovery of GPER, the understanding of E2-dependent effects in breast cancer was incomplete. Since that time, many studies have indicated a possible role for GPER in breast cancer; however, this is the first *in vivo* demonstration that GPER expression in both the tumor parenchyma and microenvironment enhances tumorigenesis. Furthermore, pharmacologic inhibition of GPER was demonstrated to decrease distant metastasis, the foremost cause of breast cancer-related mortality, suggesting GPER is a good candidate for targeted therapy.

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