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Characterization of G protein-coupled estrogen receptor (GPER) in breast epithelial proliferation and morphogenesis

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Characterization of G Protein-Coupled Estrogen Receptor (GPER) in Breast Epithelial Proliferation and Morphogenesis

by

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DISSERTATION

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DEDICATION

To Robert and Beverly Scaling

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ABSTRACT

Estrogen (17β-estradiol, E2) plays an important role in regulating an array of functions in both male and female reproductive physiology. In the mammary gland, E2-induced proliferation, ductal outgrowth and subsequent branching morphogenesis is required for proper development of the breast. In males, E2 is required for proper testicular development, spermatogenesis, and sperm maturation in the epididymis, but can also negatively regulate these functions with inappropriate exposure.

The effects of E2 in these organs have long been attributed to classical estrogen receptors (ERα and ERβ), due to the observed effects in ER-/- mice; however, GPER is abundantly expressed in male and female reproductive organs, including the breast, testes and epididymis, and there is increasing evidence that GPER contributes to E2-induced functions in these tissues. For this study, we were interested in the contribution of GPER to E2-induced processes in 1) the breast; proliferation and morphogenesis, 2) the testes; regulation of spermatogenesis and morphology, and 3) the epididymis; specifically morphological regulation.

Since proliferation and morphogenesis in the mammary gland are under tight E2 control and GPER is able mediated E2-induced proliferation in breast cancer cells, we were interested to see if GPER mediates E2-induced proliferation and morphogenesis in breast epithelial cells (MCF10A cell line) and in human breast tissue. E2 and G-1 stimulation in human breast tissue led to significant increase in proliferation measured by Ki-67 staining, and led to distinct

morphological changes including an E2-induced increase in epithelial height in alveolar structures and a G-1-induced increase in luminal area after seven days in culture. E2-and G-1 also stimulated proliferation in MCF10A cells and this is dependent on epidermal growth factor receptor (EGFR) transactivation and mitogen activated protein kinase (MAPK) activation. Other observations in chapter 3, including a G-1-induced reduction in E-cadherin protein expression (breast tissue) and a G-1-induced increase in focal adhesion kinase (FAK) activation (MCF10A cells) suggest that regulation of GPER-mediated morphological changes involves regulation of cell-cell adhesion proteins.

To determine the contribution of GPER to E2-induced spermatogenesis and morphology in male reproductive organs, we subcutaneously implanted C57BL/6 male mice with 21-day release E2 and G-1 pellets, and then investigated morphological effects on the testes and epididymis. G-1 had no effect on spermatogenesis or testicular morphology (unlike estrogen which impairs proper testicular morphology and abolishes spermatogenesis); however, G-1 treatment significantly increased the luminal area of epithelial structures in the epididymis.

We have demonstrated in this study that while GPER doesn't mediate the entirety of estrogen's effect in female and male reproductive physiology, GPER-contributes to E2-induced proliferation in the mammary gland and to the regulation of morphogenesis of epithelial structures in the breast and the epididymis.

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ABBREVIATIONS

2-D—2 dimensional

3-D—3 dimensional

ADAM—a disentegrin and metalloprotease

ATCC—American type culture collection

BAX—BCL-associated X protein

BCL2—B-cell lymphoma 2

BM—basement membrane

BSA—bovine serum albumin

CHTN—cooperative human tissue network

DAPI—4',6-diamidino-2-phenylindole

DCIS—Ductal Carcinoma In Situ

DMEM—Dulbecco's modified eagle medium

DN—dominant negative

E2—17β-estradiol

ECM—extracellular matrix

EGF—epidermal growth factor

EGFR—epidermal growth factor receptor

ER—estrogen receptor

ERE—estrogen response element

ERK—extracellular signal-regulated kinases

ERα—estrogen receptor α

ERβ—estrogen receptor β

FAK—focal adhesion kinase

FBS—fetal bovine serum

FSH—follicle stimulating hormone

G1—Gap 1 phase of mitosis cell cycle

G-1—GPER-selecitve compound 1 (agonist)

G15—GPER-selective compound 15 (antagonist)

G36—GPER-selective compound 36 (antagonist)

GnRH—gonadatrophin releasing hormone

GPCR—G protein-coupled receptor

GPER—G protein-coupled estrogen receptor

GPR30—G protein-couple receptor 30

HB-EGF—heparin-bound epidermal growth factor

HER2—human epidermal growth factor receptor 2

hr—hour

HSP—heat shock protein

IBC—inflammatory breast cancer

IGFR—Insulin-like growth factor receptor

LH—luteinizing hormone

MAPK—mitogen-activated protein kinase

MEC—mammary epithelial cell

MEK—MAP kinase kinase

min—minute

MMP-matrix metalloprotease

NGS—normal goat serum

NIH—National Institutes of Health

NS—not significant

PBS—Phosphate buffered saline

PFA—paraformaldehyde

PH—pleckstrin homology

PH3—phospho-histone H3 antibody

PH-FP—pleckstrin homology conjugated fluorescent protein

PI3K—phosphoinositide 3-kinase

PIP3--phosphatidylinositol (3,4,5)-trisphosphate

PLC—phospholipase C

PKA—protein kinase A

PKC—protein kinase C

PM—plasma membrane

RT-PCR—reverse transcriptase polymerase chain reaction

SERM—selective estrogen receptor modulator

siRNA—small interfering ribonucleic acid

TBS—tris buffered saline

TBST—tris buffered saline with 1% Tween-20

1. INTRODUCTION

1.1 Estrogens

The estrogen family of steroid hormones regulates a wide variety of physiological processes within the human body. There are three estrogens that exert an effect at different times throughout female reproductive physiology; estriol, estrone and 17β-estradiol (estradiol, E2), the latter being the most biologically active estrogenic hormone in non-pregnant women between menarche and menopause (Anderson, 2002). Estrogens belong to the family of steroid hormones that include progesterone, testosterone, glucocorticoids and mineralcorticoids. Being a steroid hormone, estrogens are derived from cholesterol and ultimately synthesized by aromatization of testosterone. Biosynthesis of estrogens is regulated by the hypothalamic-pituitary-gonadal axis (**Fig 1.1**) (Bliss et al., 2010; McLachlan, 2000). Gonadotrophin releasing hormone (GnRH) is released from the hypothalamus which stimulates cells in the anterior pituitary to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH acts on the theca interna cells in the ovary, to stimulate the conversion of cholesterol to progesterone, and finally to androstenedione. Androstenedione then enters neighboring granulosa cells in the ovary, where FSH stimulates its conversion to testosterone (McLachlan, 2000). Testosterone is converted via aromatase into 17β-estradiol (E2) (**Fig 1.2**). E2 produced by the ovaries in turn acts on cells of the anterior pituitary, which regulate LH and FSH secretion, creating a negative feedback loop (McLachlan, 2000). In pre-menopausal women, E2 is produced primarily by granulosa cells in the

developing ovarian follicles, the corpus luteum and the placenta (Nelson and Bulun, 2001). E2 is also produced in smaller amounts by other tissues such as the adrenal glands, the liver, the breasts, and adipose tissue supported by the localization of aromatase expression in these tissues (Nelson and Bulun, 2001). E2 production in local tissues (other than the ovaries) is especially important in postmenopausal women, when ovarian function ceases (Nelson and Bulun, 2001).

1.1.1 Estrogen function in human physiology

E2 regulates a wide range of physiologic processes in many tissue types in both men and women. While E2 is traditionally thought of as a female sex hormone, there are many targeted effects of E2 throughout the body distinct from its role in development and maintenance of female reproductive organs. For example, E2 plays a role in cardioprotection (Guzzo, 2000), neuroprotection (Arnold and Beyer, 2009) inflammation (Dai et al., 2009) and maintenance of the skeletal system (Manolagas et al., 2002; Termine and Wong, 1998).

1.2 Classical estrogen receptor signaling

The biological effects of E2 have long been attributed to the two classical estrogen receptors (ERs), estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ). These receptors belong to the nuclear receptor superfamily, a family of ligand-dependent transcription factors. In the absence of ligand, ERs are localized largely in the nucleus (with a small percentage of cytoplasmic localization), where heat shock protein chaperones keep the receptors in an

inactive conformation. E2 binding induces conformational changes in these receptors, leading to the dissociation of inactivating proteins, dimerization and translocation of cytoplasmic receptors to the nucleus. Activated ERs bind to specific estrogen response elements (EREs) located within the promoter region of estrogen-regulated genes (**Fig 1.3**). Subsequently, ERs modulate transcription of estrogen-responsive genes, thus they mediate long-term or genomic estrogen signaling. Although products of different genes, ERα and ERβ share a high degree of sequence homology within their DNA binding domains (Couse and Korach, 1999; Korach, 1994); therefore is it not surprising that both receptors are able to bind EREs with similar specificity and affinity.

1.2.1 Non-genomic signaling through classical estrogen receptors

In addition to E2's ability to directly regulate gene transcription, E2 is known to induce rapid, non-genomic signaling in various cell types. These effects are insensitive to transcriptional and translational inhibition, and occur much faster than genomic signaling, on a scale of seconds to minutes. The rapid effects of E2 include activation of protein kinases (extracellular signal-related kinase (ERK, protein kinase A (PKA), protein kinase C (PKC) and phosphoinositide-3-kinase (PI3K)), production of second messengers such as cAMP and nitric oxide (NO) and changes in ion channel activity and intracellular calcium (Ca2+) levels (Levin, 2002). One of the earliest reports of rapid nongenomic E2-dependent activity was demonstrated in the 1970's, showing that 17β-estradiol induced a rapid stimulation of cAMP production and calcium flux in the rat endometrium (Pietras et al., 1975). Rapid, non-transcriptional E2 signaling

is mediated by classical ERs in their traditional locations (nucleus and cytoplasm), yet there is also evidence for the plasma membrane localization of classical ERs, which may also contribute to rapid signaling (Levin, 2002). ERα specifically has been shown to mediate E2-activation of MAPK (ERK-1 and ERK-2) in MCF-7 breast cancer cells as well as in Cos cells transfected with ERα cDNA (Levin, 2003; Pedram et al., 2006; Razandi et al., 2003).

1.2.2 Classical estrogen receptors in mammary gland biology

The mammary gland is a unique organ with regard to development, maintaining a very rudimentary structure from birth to the onset of puberty (Silberstein, 2001), wherein exposure to circulating E2 promotes continued development. In both humans and lower mammals, exposure to E2 causes the mammary gland to undergo proliferation, duct elongation and lateral ductal branching (Brisken and O'Malley, 2010). These effects are reported to be mediated by ERα, which is not surprising since ERα is abundantly expressed in mammary tissue (Brisken and O'Malley, 2010). ERβ is also expressed throughout the mammary gland and plays a role in mammary gland development. Although ERα and ERβ share similar mechanisms of action with regard to genomic E2 signaling, studies in mice with genetic deletion of either ERα or ERβ have revealed distinct phenotypes, suggesting these receptors regulate dissimilar cellular pathways in the breast. ERα-/- mice maintain a very rudimentary, neonatal mammary structure with no ductal elongation in response to E2, suggesting ERα is the key mediator of estrogenic effects on the mammary gland (Brisken and O'Malley, 2010). Grafting experiments with ERα-/- mice show

that epithelial ERα is required for ductal elongation in during puberty-associated development in the mammary gland (Couse and Korach, 1999). In contrast, mammary gland development occurs normally in ERβ-/- , which is responsible for terminal differentiation, and preparation of the mammary gland for lactation (Forster et al., 2002b).

In the human breast, estrogen receptor expression is similar to that seen in rodents. ERα is expressed in 15-30% of the luminal epithelial cells, whereas ERβ is present in most luminal epithelial and myoepithelial cells, as well as stromal cells. In humans, most breast epithelial proliferation occurs in ERα negative cells. Because ERα is rapidly degraded by the proteosome upon activation, it was proposed that ERα expression is down modulated in cells that have entered the cell cycle due to estrogen stimulation. To test this hypothesis ERα null murine mammary epithelial cells (MECs) were mixed with wild type MECs, and used to reconstitute a cleared mouse mammary fat pad. It was demonstrated that in the presence of wt MECs, ERα null cells were able to proliferate, indicating that estrogen promotes proliferation via a paracrine mechanism (Mallepell et al., 2006). This result is consistent with observations in human breast epithelia, demonstrating that the cells that proliferate do not express ERα (Brisken and O'Malley, 2010).

The estrogen-induced paracrine mechanism that promotes proliferation is likely mediated by amphiregulin, an EGF family member whose transcription is strongly induced by E2 (McBryan et al., 2008). Amphiregulin is a membrane-bound growth factor protein that is cleaved and released by a disentegrin and

metalloproteinase (ADAM) at the cell surface (McBryan et al., 2008; Willmarth and Ethier, 2006a). Cleaved amphiregulin binds to and activates the epidermal growth receptor (EGFR) on adjacent, ERα negative cells in the mammary gland, leading to EGFR signaling, and proliferation and subsequent ductal elongation (Brisken and O'Malley, 2010). In cells that co-express ERα and ERβ, ERβ acts to inhibit ERα-induced proliferation. This is thought to be due to the ability of ERβ to repress ERα-mediated cyclin D1 induction in response to estrogen, thus ERβ mediates an anti-proliferative role in the breast (Boonyaratanakornkit and Edwards, 2004; Liu et al., 2002). Directly coupled to its effect on epithelial proliferation in the developing mammary gland, E2 stimulates branching morphogenesis in the mammary gland, induced by ERα-mediated cell proliferation (Feng et al., 2007; Mallepell et al., 2006; Silberstein, 2001).

1.2.3 Estrogens in male physiology

Although androgen (testosterone) is the dominant male sex hormone, E2 does have an important role in the male reproductive system (Carreau et al., 2012). The effect of E2 on the male testes was demonstrated as early as the 1930's and 40's, when it was reported that high dose E2 exposure during development induced malformation of components of the male reproductive system (McLachlan, 2000). The role of E2 in male physiology became more relevant with the description and characterization of direct binding of E2 to a receptor (ERα) in mammalian testes and epididymis (Danzo et al., 1975; Danzo et al., 1977; Danzo et al., 1978; Danzo and Eller, 1979).

The role of E2 in spermatogenesis, and resultant fertility, was first demonstrated in studies with mice lacking ERα or aromatase. These mice exhibit impaired spermatogenesis and fertility (Korach, 1994; Lubahn et al., 1993), highlighting the effects of E2 on male reproductive physiology. Another indication of E2's importance in spermatogenesis is the correlation between exposure to environmental estrognens and decreased sperm counts in men over the past 60 years (Sharpe and Skakkebaek, 1993; Toppari et al., 1996).

E2's regulation of spermatogenesis stems from its ability to negatively regulate GnRH and FSH/LH secretion, similar to testosterone, as part of the hypothalamic-pituitary testicular axis (Handelsman et al., 2000). Studies in humans showed that administration of E2 could further enhance FSH/LH suppression that was induced by testosterone (Handelsman et al., 2000). The effects of E2 in the epididymis, where it is involved in sperm maturation (achievement of sperm motility) and the resorption of fluid through the efferent ductuals have also been demonstrated in ERα null mice (Hess et al., 1997).

The contribution of ERα to E2's actions in the male reproductive system were demonstrated in ERα null mice; however, in light of the discovery and of a novel estrogen receptor, G protein-coupled estrogen receptor (GPER), recent work has been devoted to the characterization of GPER in the male reproductive system, including expression patterns (Lazari et al., 2009; Rago et al., 2011) and signaling mechanisms (Chimento et al., 2011; Lucas et al., 2010). These will be reviewed in more detail in chapter four.

1.3 G protein-coupled estrogen receptor (GPER/GPR30)

In addition to E2 signaling mediated by classical ERs, ERα and ERβ, there is now a third estrogen receptor reported to mediate the effects of E2 in many different cells and tissues. The G protein-coupled estrogen receptor (GPER, originally named GPR30) is a seven transmembrane G protein-coupled receptor (GPCR) that binds and is activated by E2 (Carmeci et al., 1997). GPER was first cloned in the late 1990's by four different labs (Carmeci et al., 1997; O'Dowd et al., 1998; Owman et al., 1996; Takada et al., 1997). One lab cloned GPER from an ERα/β positive breast cancer cell line (MCF7) and they demonstrated that GPER is ubiquitously expressed in normal human tissues and primary breast carcinomas, and it's expression overlapped with that of classical ERα (Carmeci et al., 1997). The patterns of GPER expression indicated this receptor may be involved in physiologic responses specific to hormonally regulated tissue (Carmeci et al., 1997); however, at the time no natural ligand was known for this receptor, and it was given the orphan designation GPR30 (Carmeci et al., 1997).

In 2000, work performed by a group at Brown University, led by Edward Filardo, demonstrated E2-induced phosphorylation of ERK in breast cancer cells lacking classical ERs (Filardo et al., 2000). It was also observed that when GPER was expressed in ERα-, GPER-negative MDA-MB-231 breast cancer cells, they became increasingly E2-responsive in terms of E2-induced ERK phosphorylation, while this response was lacking in wild type MDA-MB-231 cells (Filardo et al., 2000). A similar effect of E2-responsiveness was observed in SKBr3 breast cancer cells, which express GPER but lack classical ERs (Filardo

et al., 2000). Inhibition of EGFR kinase function, neutralization of HB-EGF activity, and down modulation of HB-EGF at the cell surface demonstrated that E2-induced ERK activation was due to transactivation of the EGFR through cleavage of HB-EGF at the cell surface by (Filardo et al., 2000). Since GPER positive cells had been shown to be responsive to E2, the next step was to show specific binding of E2 to GPER.

The direct binding of E2 to GPER was shown in two studies in 2005. Revankar, et al. demonstrated direct E2 binding to GPER using fluorescently conjugated E2 in ER-negative Cos7 cell membranes transfected with GPER (Revankar et al., 2005). Revankar and colleagues also showed that GPER was localized to the endoplasmic reticulum (Revankar et al., 2005), unlike other 7TM GPCRs that are normally found at the plasma membrane. The intracellular localization for GPER is possible because the ligand, E2, is a steroid hormone that is able to pass freely through the membrane. At the same time, Thomas and colleagues at Brown University described the specific binding of tritiated E2 to ERα/β negative, GPER positive SKBr3 cell membranes, and human embryonic kidney cells transfected with GPER (Thomas et al., 2005).

1.3.1 GPER signaling

When GPER was initially characterized, transactivation of the epidermal growth factor receptor (EGFR) and subsequent activation of MAPK signaling in breast cancer cells were identified as key downstream events of GPER activation (Filardo et al., 2000); however, it had also been shown that these events could

be mediated by ERα (Razandi et al., 2003). Clearly it became important to determine the E2-induced signaling potentials and the mechanism by which GPER and ERα mediate E2 signaling independently. To address this question, Revankar et al, transfected Cos7 cells (lacking all known estrogen receptors) with either GPER or ERα, together with a fluorescently labeled reporter for EGFR-dependent signaling, the pleckstrin homology (PH) domain from Akt. This reporter protein serves as an indication of PI3K signaling and membrane localized phosphatidylinositol (3,4,5)-triphosphate (PIP3) accumulation. To demonstrate the fidelity of the reporter for EGFR activation, cells expressing the fluorescent reporter (PH-FP) were stimulated with epidermal growth factor (EGF). As expected, with EGF stimulation, PH-FP accumulated at the plasma membrane (Revankar et al., 2005). Upon E2 stimulation, cells expressing ERα or GPER showed specific accumulation of the PH-FP reporter; however, it was localized to the nucleus. Nuclear PH-FP accumulation was prevented by the PI3K inhibitor LY2940092. In order to determine if different ligands distinctively activate the two receptors, ERα or GPER transfected cells were treated with 4-hydroxytamoxifen (4-HT), a selective estrogen receptor modulator (SERM), shown to be a partial agonist against ERα and capable of activating ERK phosphorylation via GPER. Stimulation with 4-HT had no effect in cells transfected with ERα together with the PH-FP reporter (Revankar et al., 2005); however, 4-HT did activate nuclear PH-FP accumulation via PI3K in GPER expressing Cos7 cells, demonstrating that one ligand can produce different downstream signaling events via ERα versus GPER (Revankar et al., 2005).

When cells were pretreated with the EGFR inhibitor AG1478, GPER-induced but not ERα-induced PI3K activation was blocked, demonstrating again that E2 can activate PI3K via two distinct mechanisms depending on specific receptor expression (Revankar et al., 2005).

Taking these results together with Filardo's initial observations, the signaling pathway downstream of E2-dependent GPER activation is shown in **figure 1.4** (taken from Prossnitz et al., 2008). E2, being cell permeable, is able to pass through the membrane and bind intracellular GPER located on the endoplasmic reticulum. Upon E2 binding, GPER is activated along with its associated heterotrimeric G proteins, which in turn can activate Src. Activated Src can activate MMPs at the cell surface which cleave pro-HB-EGF from the cell membrane, allowing cleaved HB-EGF to bind to and activate the EGFR, leading to downstream activation of MAPK and PI3K activation and associated cellular process such as proliferative and pro-survival signaling (Filardo et al., 2000; Prossnitz et al., 2008). Quinn et al. recently demonstrated that the GPER-induced transactivation of the EGFR by HB-EGF was also dependent on α5β1 integrin activation and fibronectin matrix assembly SKBr3 breast cancer cells (Quinn et al., 2009).

1.3.2 GPER-selective compounds

Since E2 is able to bind and activate all three known estrogen receptors including ERα, ERβ and GPER, and both tamoxifen and ICI 182 780 (chemotherapies used as ERα down-modulators in breast cancer) have been

shown to agonize GPER, the identification of GPER-selective compounds was vital to facilitate further study of GPER specifically (**Fig 1.5**)

In an attempt to identify ligands specific to GPER, Bologa et al carried out a virtual screen of a library of approximately 10,000 GPCR-associated compounds to assess their structural similarity to E2. Based on the results, the top 100 compounds were tested for activity toward GPER using a competition binding assay. One compound, later named G-1, was subsequently identified as a selective GPER agonist (Bologa et al., 2006). G-1 competitively displaced binding of fluorescent E2 (E2-Alexa) in GPER-transfected cells, yielded an inhibition constant (Ki) for G-1 of 11nM, whereas the Ki for E2 binding to GPER is 5.7nM (Bologa et al., 2006). No significant affinity of G-1 for ERα or ERβ was observed. The specificity of G-1 as an agonist was confirmed by its ability to promote intracellular calcium immobilization in GPER-expressing Cos7 cells, but not cells expressing ERα/β (Bologa et al., 2006). Not long after the identification of G-1, a GPER-selective antagonist, G15 was identified. G-15 antagonizes E2-dependent GPER activation in vitro and in vivo, using a well-established assay of E2-induced murine uterine proliferation (Dennis et al., 2009). At concentrations of 10μM and above, G15 exhibits low-affinity cross reactivity to ERα, therefore an antagonist with enhanced selectivity to GPER, G36, was synthesized (Dennis et al., 2011). The identification of a GPER-selective agonist and antagonist provide us with the opportunity to selectively modulate GPER receptor function *in vitro* and *in vivo*.

1.4 Estrogen and breast cancer

In normal breast epithelia, 15-30% of epithelial cells express ERα, whereas the number of ERα-positive breast epithelia increases early in malignant progression to 60-70% (Brisken and O'Malley, 2010). As a result, in approximately two-thirds of women who have ER positive breast cancer, E2 stimulates proliferation and thus progression of tumorigenesis (Early Breast Cancer Trialists' Collaborative Group (EBCTCG), 2005). In these patients, Tamoxifen, a selective estrogen receptor modulator (SERM) is used to antagonize the binding of E2 to classical ERs, a successful strategy in attenuating the growth of ER positive breast cancer (Fisher et al., 2005). Tamoxifen and other SERMs have been shown to bind to and activate GPER (Ignatov et al., 2010; Vivacqua et al., 2006b), providing a possible mechanism for the progression of ER negative breast cancers.

In addition to GPER expression in breast cancer cell lines, GPER expression has also been observed and correlated with decreased survival in patients with hormone responsive cancers including breast (Arias-Pulido et al., 2010; Filardo et al., 2006; Kuo et al., 2007; Luo et al., 2011), endometrial (Smith et al., 2007) and ovarian (Smith et al., 2009). In a large study carried out in 2006 by Filardo et al. it was found that GPER expression in tumors was positively correlated with HER-2/neu growth factor receptor expression, tumor size and distant metastasis (Filardo et al., 2006), indicating GPER was an indicator of a more aggressive form of breast cancer. In this same study it was observed that approximately half of the ER negative breast tumors retained GPER expression

(Filardo et al., 2006), which may suggest that these tumors remain E2 responsive through GPER-associated signaling even in the absence of classical ERα signaling. This hypothesis highlights the possibility of the ability to antagonize GPER in conjunction with classical ERs in breast and other hormone responsive cancers to achieve a better treatment outcome.

1.5 Rationale for project

Although attempts to characterize GPER have increased exponentially since its initial discovery, the effects of GPER on breast proliferation and morphogenesis still need to be elucidated. It is well accepted that E2 is required for normal breast development and maintenance of proper mammary gland function (Couse and Korach, 1999; Forster et al., 2002b). E2 is a potent mitogen capable of promoting proliferation, both during development as well as in the maintenance of normal mammary gland physiology. E2's actions are mediated by modulation of gene transcription as well as activation of rapid signaling pathways (Kelly and Levin, 2001; Pedram et al., 2006). E2's capacity to promote proliferation is not limited to a phenotypically normal setting, as E2 is able to induce proliferation in breast tumors (Brisken and O'Malley, 2010), thereby promoting breast cancer progression. Because of E2's role in breast development and breast cancer, our ability to understand E2-induced physiologic processes mediated by all three estrogen (ERα, ERβ and GPER) receptors is vital. E2-induced processes in the body are generally attributed to the classical estrogen receptors, ERα and ERβ; however the identification of GPER as an estrogen receptor has complicated our understanding of E2 physiology. GPER

has been shown to produce confounding outcomes in response to SERMs such as tamoxifen and ICI 182, 780. These compounds function as ERα antagonists in the breast; however they are able to activate GPER, complicating the outcome when they are used therapeutically in patients with ER positive breast cancers that could presumably also express GPER (Pandey et al., 2009). It has also been shown that GPER-associated signaling pathways regulate cell proliferation, invasion, metastasis and other tumor-related cellular signaling. In addition to signaling, GPER may serve as a valuable predictor of cancer development and overall prognosis in E2-dependent cancers, such as breast, due to the fact that GPER expression is correlated with decreased survival, increased tumor size and distant metastasis (Filardo et al., 2006). Because of the correlation between GPER expression in breast tumors and tumor progression variables, and the fact that GPER is activated by two widely used ER antagonists, the ability to target this receptor in conjunction with classical ER therapy could prove to be very successful in patients with breast cancer. In addition, since ER negative breast cancers have a worse prognosis and fewer treatment options, treatment directed at GPER in these patients could be an effective therapeutic option. In order to someday target this receptor in cancer, the mechanisms by which GPER contributes to E2-induced processes, specifically E2-induced proliferation need to be determined.

1.6 Hypothesis

E2 is required for normal mammary gland development, function and proper morphology; however, it also is able to stimulate proliferation in breast tumors (Anderson, 2002). Studies performed in ER null mice have shown that ERα mediates E2-induced proliferation in the breast, and this directly correlates to ductal elongation and mammary gland branching morphogenesis (Brisken and O'Malley, 2010). The most recently identified estrogen receptor, GPER, has been shown to contribute to E2-induced signaling in breast cancer cells (Filardo et al., 2000; Quinn et al., 2009), and GPER expression in breast tumors has been correlated with poor prognosis, increased tumor size and distant metastasis and increased HER-2/neu expression in patients with breast cancer. Even though there is increasing evidence that GPER contributes to both normal estrogen biology in the mammary gland as well as in breast cancer, it is still unclear if GPER is directly involved in E2-induced proliferation and morphogenesis in the breast. Based on our current understanding of E2's actions in the breast, and GPER's role as an estrogen receptor, we hypothesize that GPER contributes to E2-induced proliferation and morphogenesis in the human breast.

1.6.1 Specific Aims

AIM 1 Determine if GPER activation contributes to estrogen-induced proliferation

1.1 Determine if GPER promotes proliferation in an immortalized, non-transformed human breast epithelial cell line, MCF10A

1.2 Elucidate the signaling pathway downstream of GPER activation leading to proliferation in MCF10A cells

1.3 Determine if GPER activation promotes proliferation in normal human breast tissue and breast tumor tissue

AIM 2 Determine if GPER contributes to estrogen-induced regulation of mammary gland morphogenesis

2.1 Determine if E2 and G-1 regulate alveolar morphology in human breast tissue

2.2 Determine if E2 and G-1 regulate mitotic spindle orientation in MCF10A cells *in vitro* in a 3-D model of breast epithelial morphogenesis

2.3 Determine if E2 and G-1 alter junctional E-cadherin expression in human breast tissue

AIM 3 Determine if GPER contributes to estrogen-induced regulation of spermatogenesis in murine testes

3.1 Determine the effects of E2 and G-1 on testes wet weight

3.2 Determine the effects of E2 and G-1 on morphological regulation and spermatogenesis within the epididymis of male mice

1.7 Figure legends

Figure 1.1 Regulation of 17β-estradiol production by the hypothalamic-pituitary-gonadal axis.

Gonadotrophin releasing hormone (GnRH) is released from the hypothalamus which stimulates cells in the anterior pituitary to release luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH act on cells in the ovary to stimulate the multistep conversion of cholesterol to E2. E2 produced by the ovaries inhibits the hypothalamus from releasing GnRH, creating a negative feedback loop.

Figure 1.2 Conversion of cholesterol to 17β-estradiol occurs in the ovary.

Luteinizing hormone (LH) released from the hypothalamus drives the conversion of cholesterol to progesterone to androstenedione in theca cells in the ovary. Androstenedione enters neighboring granulosa cells in the ovary, where follicle-stimulating hormone (FSH) released from the hypothalamus promotes its conversion into testosterone. Finally, aromatase converts testosterone into 17β-estradiol.

Figure 1.3 Estrogen activation of classical estrogen receptors ERα/β.

In the absence of ligand, classical ERs are localized primarily in the nucleus (some localization in cytoplasm), where heat shock proteins (HSP) and chaperones keep them in an inactive conformation. Binding of 17β-estradiol (E2) to ERs causes a conformation change which leads to the dissociation of HSPs,

dimerization of ERs and translocation of cytoplasmic receptors into the nucleus. Activated ER dimers bind to estrogen response elements (EREs) located within the promoter region of estrogen-regulated genes and modulate their transcription, thus mediating genomic estrogen signaling.

Figure 1.4 GPER-dependent signaling.

17β-estradiol (E2) is able to pass through the membrane and bind intracellular GPER (GPR30) located on the endoplasmic reticulum. Upon E2 binding, GPER is activated along with its associated heterotrimeric G proteins, which in turn can activate the non-receptor tyrosine kinase Src. Activated Src can activate matrix metalloproteases (MMPs) at the cell surface which leads to the cleavage of heparin bound EGF (HB-EGF) growth actors from the cell membrane, allowing pro-HB-EGF to bind to and activate the epidermal growth factor receptor (EGFR). Activation of the EGFR leads to downstream activation of the MAPK and PI3K pathways which promote proliferation and pro-survival signaling respectively.

Figure 1.5 GPER-selective Compounds.

Chemical structures of 17β-estradiol (E2; A), GPER-selective agonist G-1 (B), and GPER-selective antagonist G-36 (C).

1.8 Figures

Figure 1.1 Regulation of 17β-estradiol production by the hypothalamic-pituitary-gonadal axis

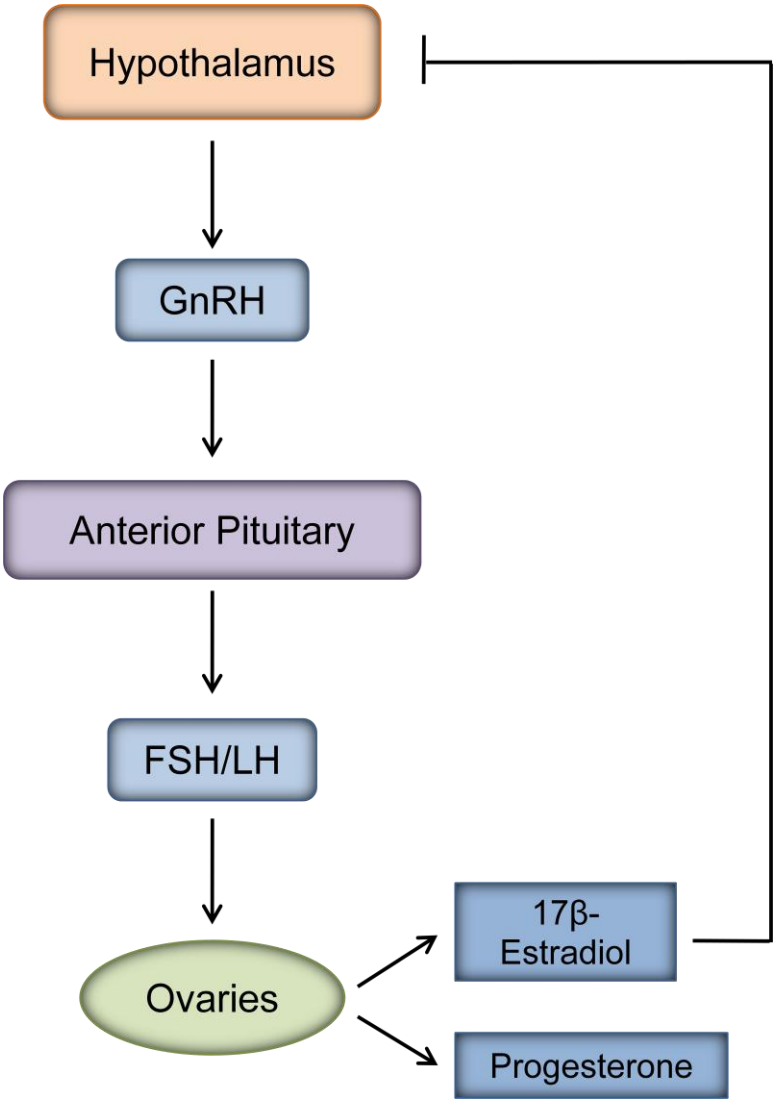


Figure 1.2 Conversion of cholesterol to 17β-estradiol occurs in the ovary

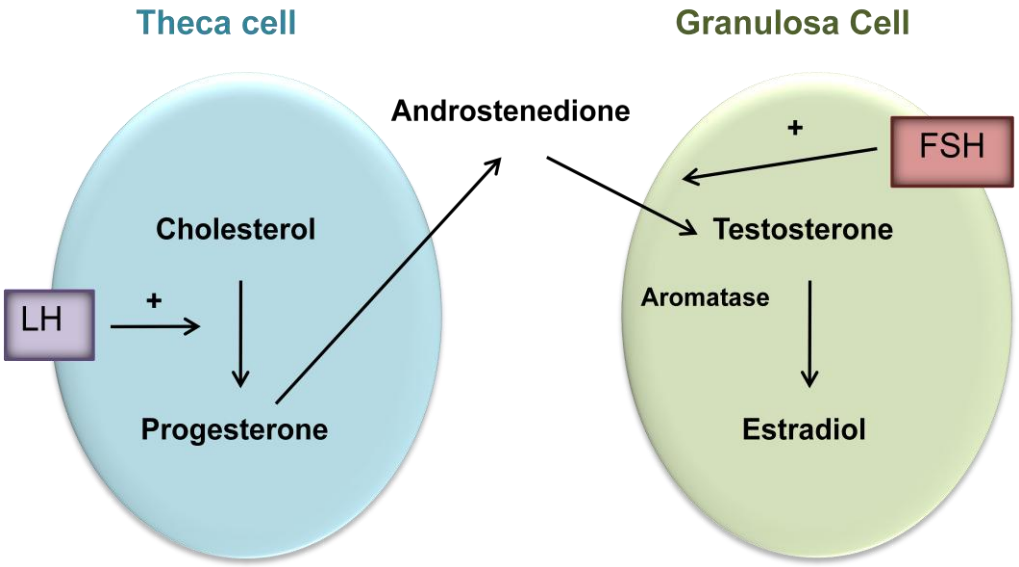


Figure 1.3 Estrogen activation of classical estrogen receptors ERα/β

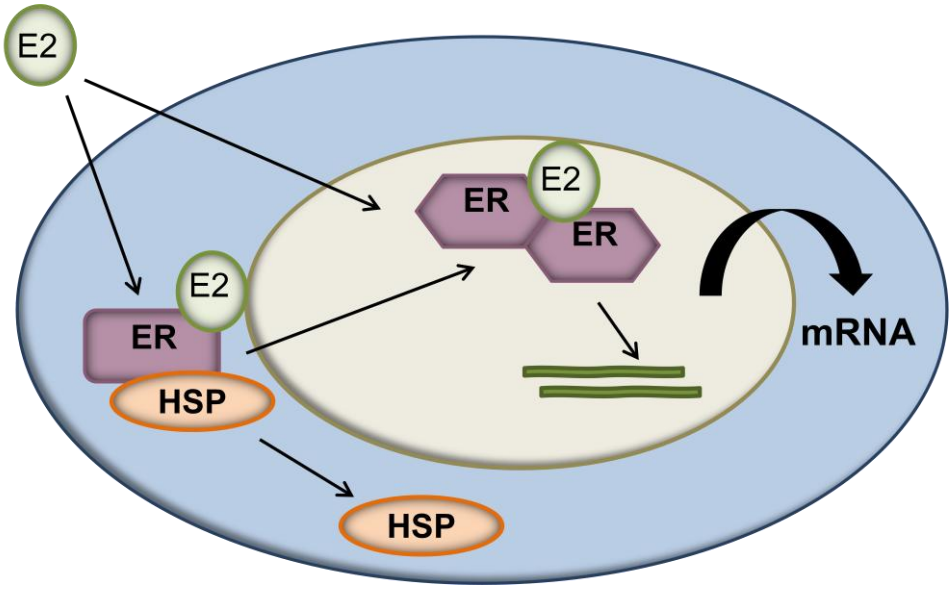


Figure 1.4 GPER-dependent signaling

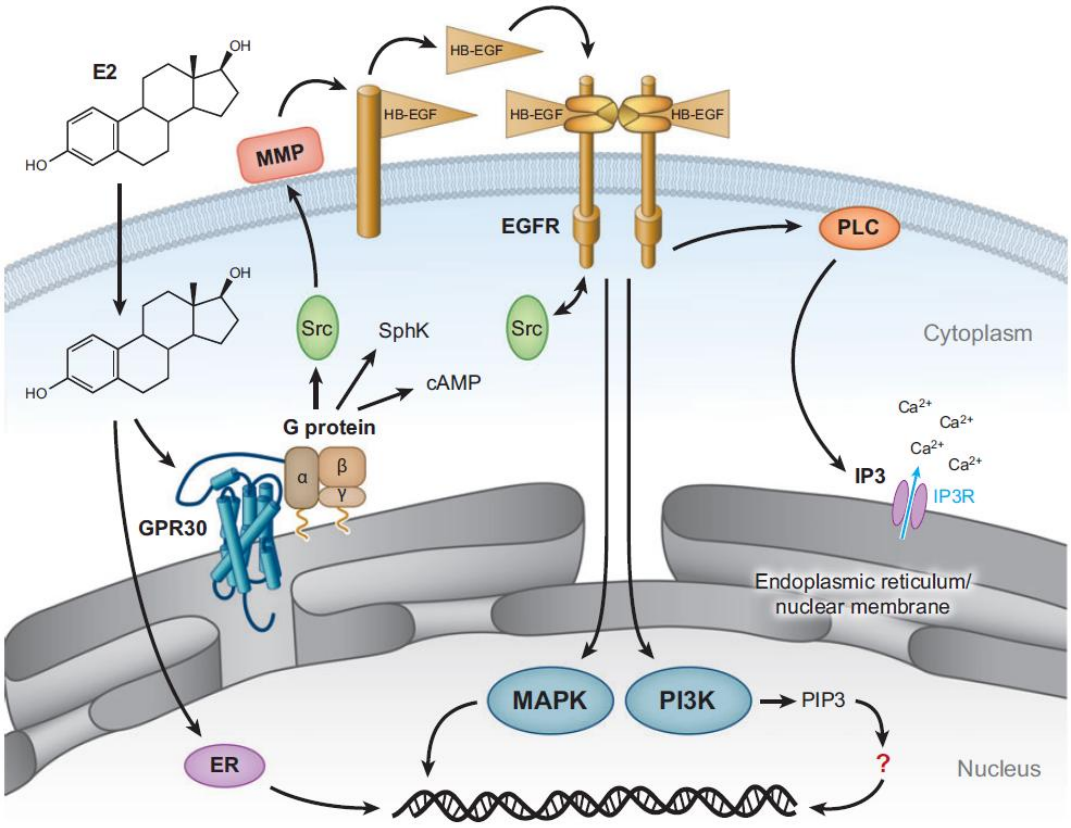
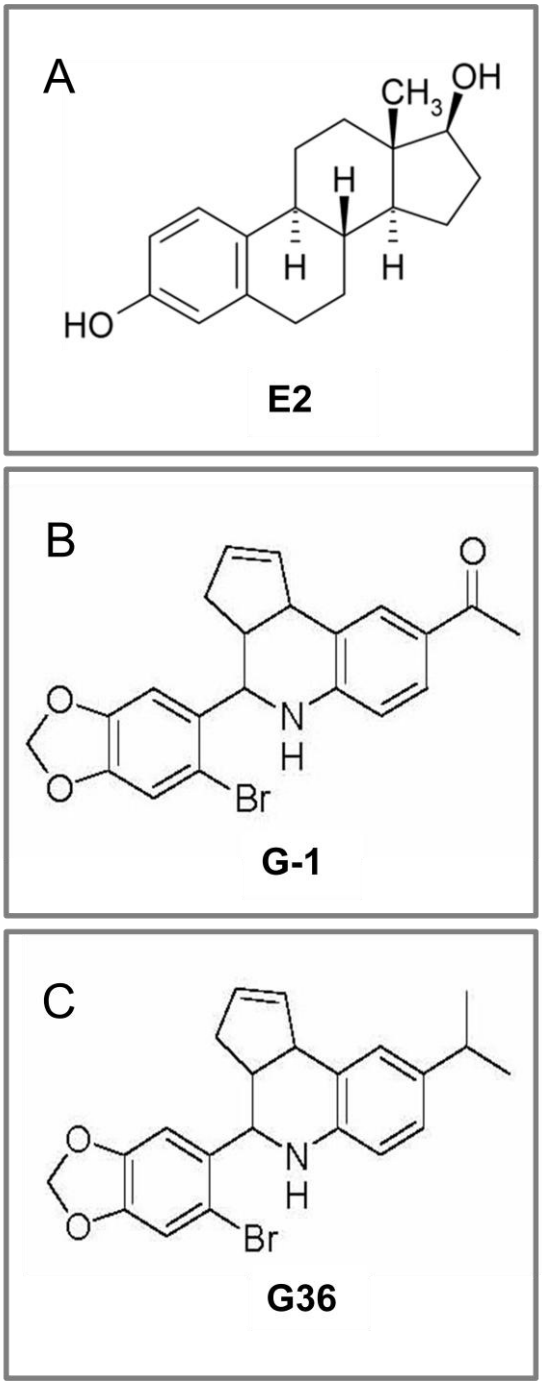


Figure 1.5 GPER Selective Compounds



2. Estrogen-induced activation of GPER and downstream proliferative signaling

2.1 Abstract

Estrogen (17β-estradiol, E2) signaling is required to promote continued development of the mammary gland at puberty. Importantly, E2 stimulates cell proliferation in the mammary gland, which leads to ductal elongation and regulation of breast morphology. In addition to a developmental setting, estrogen promotes proliferation in a tumorigenic setting, which is of the ways by which estrogen promotes breast cancer. The proliferative effects of E2 in the breast have long been attributed to the two classical estrogen receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ); however, the effects of a novel estrogen receptor, GPER, on breast epithelial proliferation remain unclear. We hypothesize that GPER contributes to E2-induced proliferation in the breast. In order to elucidate the effects of GPER activation on proliferation in the breast, we used a non-transformed breast epithelial cell line, MCF10A, as well as an organ culture model of human breast tissue. Activation of GPER by E2 and G-1 in human breast tissue led to a significant increase in proliferation measured by Ki-67 staining. GPER activation also led to a significant increase in proliferation in a breast epithelial cell line, MCF10A. We demonstrated, by western blot analysis and inhibition of signaling components, that E2- and G-1-induced proliferation in MCF10A cells is dependent on SRC, EGFR transactivation via activation by HB-EGF and ERK activation; however it is not dependent on MMP cleavage of HB-EGF at the cell surface. The specificity of GPER in E2-induced proliferation was confirmed by the ability of G36 to abrogate E2 and G-1-induced

proliferation in breast tissue and MCF10As as well as the ability of siRNA knockdown of GPER to prevent E2- and G-1-induced proliferation in MCF10A cells. The results of this study are the first demonstration of GPER-dependent proliferation in human tissue, and emphasize the contributing role of GPER in E2-induced breast proliferation.

2.2 Introduction

Normal growth and differentiation of the breast are under tight endocrine control. This is demonstrated by the fact that mammary gland development is not completed until the gland is exposed to circulating E2 at puberty, yet not in the absence of E2 (Couse and Korach, 1999). E2's actions in the breast are best characterized as occurring through genomic signaling by activation of ligand dependent transcription factors, including estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (Jensen and DeSombre, 1973; Kuiper et al., 1996). In the breast, E2 acts through ERα to promote proliferation of the epithelium in the developing gland at puberty, and consequently promotes ductal elongation and outgrowth (Brisken and O'Malley, 2010). ERβ appears dispensable for mammary gland growth and development in murine models (Korach et al., 1994), but is instead responsible for terminal differentiation of the mammary gland in preparation for lactation (Forster et al., 2002).

E2 induces breast epithelial proliferation through an autocrine mechanism, in which E2 activation of ERα causes these cells to release amphiregulin, which binds and activates its receptor on neighboring ER negative cells, leading to activation of proliferation pathways (Willmarth and Ethier, 2006). In this manner, cells that are ERα positive are distinct from those that are positive for proliferation markers such as Ki-67 (Nelson and Bulun, 2001). There is extensive evidence demonstrating E2's ability to promote proliferation *in vitro* in human mammary epithelial cells and breast cancer cells (Tan et al., 2009; Willmarth and Ethier, 2006a). E2 has also been shown to promote proliferation within the mammary

epithelium of human breast tissue cultured *ex vivo* (Eigeliene et al., 2006); exhibiting the proliferative role of E2 in a much more physiologically relevant model.

Although E2 is required for normal breast development, its ability to promote breast cancer is also well documented, making it a significant factor in assessing breast cancer risk. In a tumorigenic setting, the autocrine regulation of epithelial proliferation is lost, and proliferation positive cells are also positive for estrogen receptors (McBryan et al., 2008; Tan et al., 2009; Willmarth and Ethier, 2006). For these reasons it is critical to have a complete understanding of E2's mechanisms of action mediated through specific receptors in the breast.

More recently it has become evident that, in addition to genomic signaling, E2 can modulate rapid cellular signaling through the classical estrogen receptors (Razandi et al., 2003). There is evidence that ERs can initiate extra-nuclear signaling cascade complexes at the plasma membrane, often termed the signalsome (Levin, 2002). These signaling cascades recruit second messengers calcium and nitric oxide, receptor tyrosine kinases including the epidermal growth factor receptor (EGFR), insulin-like growth factor (IGF-1R), various G protein-coupled receptors (GPCRs) and protein kinases including phosphoinositide-3 kinase (PI3K), serine-threonine kinase Akt, mitogen-activated protein kinase (MAPK), nonreceptor tyrosine kinase Src, and protein kinases A and C (Levin, 2003).

Rapid E2-dependent signaling has also recently been demonstrated to occur through the novel G protein-coupled estrogen receptor, GPER (Filardo and

Thomas, 2005; Revankar et al., 2005), originally designated GPR30. E2 activation of GPER leads to transactivation of the EGFR and downstream activation of MAP kinase and PI3 kinase signaling cascades in breast cancer cells (Filardo et al., 2000). GPER expression has been observed in normal breast tissue and breast tumors and in a large study performed in 2006, GPER overexpression was correlated with an increased tumor size, distant metastasis and HER-2/*neu* expression (Filardo et al., 2006); suggesting GPER expression is a predictor of a more aggressive form of breast cancer. While Filardo and colleagues observed significant correlations between tumor size, HER-2/neu expression and GPER expression, confounding results have been presented since. Two independent studies carried out more recently weren't able to show a significant association between GPER expression and HER-2/neu status and although GPER positive tumors tended to be larger, the correlation was not significant (Kuo et al., 2007; Luo et al., 2011). There has also been a study assessing GPER expression and tumor progression variables in inflammatory breast cancer, where it was observed that co-expression of ERα and GPER was correlated with increased overall survival, and the absence of both ERα and GPER in inflammatory breast tumors was correlated with decreased overall survival (Arias-Pulido et al., 2010)

Observations of these studies complicate hypotheses of E2-induced regulation of breast cancer, highlighting the urgency in determining GPERs contribution to E2-induced processes in the breast. A contributing factor for the aggressive phenotype correlating with GPER expression may be epithelial

proliferation enhanced by GPER, given that this receptor has been previously shown to contribute to E2-induced proliferation *in vitro* in breast cancer cell lines,(Pandey et al., 2009; Vivacqua et al., 2006a; Vivacqua et al., 2006b) and *in vivo* in the murine endometrium (Dennis et al., 2009). The aim of the present study is to determine if GPER contributes to E2-induced epithelial proliferation in the phenotypically normal human breast. Based on the fact that 1) E2 promotes proliferation in hormone responsive tissue; 2) GPER promotes proliferation in breast and other cell lines and tissue, and 3) GPER expression correlates with breast cancer progression, we hypothesize GPER activation is responsible in part for E2-induced proliferation in the human breast.

As E2 is able to activate ERα, ERβ and GPER, in order to discriminate the roles of these individual receptors in proliferation, we have recently identified reagents with specificity toward GPER, including a GPER-selective agonist, G-1 (Bologa et al., 2006), and a GPER-selective antagonist, G36 (Dennis et al., 2009). In the present study we demonstrate that GPER is expressed in MCF10A cells, that express no ERα or ERβ (Debnath et al., 2003), and both E2 and GPER agonist G-1are able to stimulate proliferation in these cells. The E2-induced proliferation we observe in MCF10A cells is dependent on EGFR transactivation via soluble HB-EGF and subsequent activation of ERK; however, not dependent on activation of MMPs, a mechanism previously described (Filardo et al., 2000). Proliferation is also induced in human breast tissue explants in response to E2 and G-1, and we demonstrate that the proliferation is in part mediated by GPER, as the GPER-selective antagonist G36 partially

abrogates this effect. These results indicate that GPER contributes to E2-induced proliferation in the breast, and is the first demonstration of GPER-mediated proliferation in human tissue.

2.3 Results

2.3.1 Estrogen induces proliferation in MCF10A cells

MCF10A cells have been used extensively to study breast morphology *in vitro* because of their ability to recapitulate breast epithelial morphogenesis when cultured three dimensionally (3-D) on a reconstituted basement membrane (Debnath et al., 2003). Due to the fact that these cells are ERα and ERβ negative, they are not typically used in studies of E2- responsiveness, although we had seen GPER expression in normal breast tissue (unpublished) and wanted to investigate is this receptor was mediating E2 function in ER negative breast epithelial cells. To determine if MCF10A cells proliferate upon E2 stimulation, cells were cultured in the presence of either vehicle (sham) or E2, fixed and immunolabeled with an antibody that recognizes a mitosis-specific phosphorylated form of Histone H3 (ser10),(pH3), a component of the nucleosome. Following incubation of MCF10A cells for 24 hr with E2, we observed a dose dependent increase in proliferation compared to control treated cells; up to a 3-fold increase in cells treated with 100nM E2 (**Fig 2.1**).

2.3.2 MCF10A cells express GPER

Since we observed MCF10A cells to be E2 responsive, and they are reported to lack ERα and ERβ, we reasoned that they likely express GPER, as

the mediator of the E2-induced proliferative response. MCF10A cells demonstrated GPER expression as determined by both immunofluorescence staining and western blotting using a polyclonal antibody generated against a C-terminal peptide in the human GPER protein (**Fig 2.2**). Immunofluorescence staining revealed an intracellular pattern for GPER in MCF10A cells, consistent with an endoplasmic reticulum localization as described (Revankar et al., Science paper) (**Fig 2.2A**), that decreased considerably in intensity upon transfection with a GPER-specific siRNA (GPER siRNA), but not with transfection of non-specific, control siRNA (data not shown). Western immunoblotting using the anti-GPER antibody demonstrated the presence of a specific polypeptide in MCF10A cells (MW ~55kDa) (**Fig 2.2B**). This polypeptide was significantly diminished in cells transfected with GPER siRNA (**Figs 2.2B, 2.2C**). The polypeptide also decreased in intensity when the GPER specific antibody was pre-incubated with the antigenic peptide, but not after pre-incubation with a scrambled peptide (data not shown), confirming specificity of this antibody for GPER. An additional polypeptide of lower molecular weight (~45kDa) was also reduced by GPER siRNA (**Fig 2.2B**), suggesting the presence of GPER-specific degradation products or isoforms. The absence of ERα mRNA and ERα protein expression was confirmed in MCF10A cells by RT-PCR and immunofluorescence (data not shown).

2.3.3 Estrogen-induced proliferation is mediated by GPER in MCF10A cells

Based on the observations that GPER is expressed in MCF10A cells, and these cells exhibit increased proliferation in response to E2 stimulation, we

further evaluated the effect of GPER-selective agonist G-1 and GPER-selective antagonist G36 on proliferation in MCF10A cells. Cells stimulated with G-1 for 24 hours exhibited a dose-dependent increase in proliferation, up to a 3-fold increase at the highest dose (100nM) compared to control treated cells (**Fig 2.3A**). To establish that the increased proliferation was due to GPER activation, co-stimulation with agonists (E2 and G-1) and GPER-selective antagonist (G36) were carried out and pH3 immunodetection was used to quantitate proliferation. G36 significantly blocked both E2- and G-1-induced proliferation, but had no effect on EGF-induced proliferation (**Fig 2.3B**). To further demonstrate that the increased proliferation observed with E2 and G-1 treatment is due to activation of GPER, proliferation was assessed in GPER-specific siRNA-transfected cells (GPER siRNA) compared with non-specific siRNA-transfected cells. Cells in which GPER protein expression was knocked down by GPER siRNA exhibited significantly lower E2 and G-1 induced proliferation indexes as compared with control siRNA-transfected cells (**Fig 2.3C**). GPER siRNA transfection had no effect on EGF-induced proliferation in MCF10A cells (**Fig 2.3C**). Knockdown of GPER protein expression in MCF10A cells was confirmed by Western immunoblotting (**Fig 2.3D**).

2.3.4 E2 and G-1 induce ERK activation in MCF10A cells

As GPER is known to activate ERK (phosphorylation), and ERK activation is upstream of cellular proliferation (Zhang and Liu, 2002) we reasoned that GPER activation in MCF10A cells would result in ERK phosphorylation. Western immunoblotting of E2- and G-1- treated MCF10A cell lysates was performed with

an antibody against phosphorylated ERK (p-ERK). In preliminary experiments, we determined that E2 and G-1 resulted in a time-dependent increase in p-ERK as determined by band intensity relative to the loading control actin (data not shown) with peak activation occurring at 15 minutes. Therefore all subsequent stimulation experiments were terminated at 15 min. E2 and G-1 led to a significant increase in the phosphorylation of ERK when compared to control treated cells (**Fig 2.4A**), and treatment of cells with E2 or G-1 in combination with GPER-selective antagonist G36 reduced E2- and G-1-induced ERK phosphorylation significantly, while G36 alone had no effect. To more directly attribute the observed ERK activation to GPER, ERK activation following E2 or G-1 stimulation was assessed in cells that had been transfected with GPER siRNA or control siRNA. MCF10A cells transfected with GPER siRNA exhibited significantly lower E2- and G-1-induced ERK phosphorylation when compared to control siRNA transfected MCF10A cells (**Fig 2.4B**). GPER siRNA knockdown had no effect on EGF-induced ERK phosphorylation compared to control siRNA-transfected cells, demonstrating the specificity of the effects of GPER protein knockdown (**Fig 2.4B**).

2.3.5 Mechanism of E2- and G-1-induced ERK activation in MCF10A cells

The mechanism of E2- and G-1-induced GPER-dependent ERK phosphorylation was explored using pharmacologic signaling inhibitors. Since GPER is known to transactivate the EGFR in breast cancer cell lines (Filardo et al., 2000), we tested the ability of the EGFR-specific tyrosine kinase inhibitor, AG1478, to block E2- and G-1-induced ERK phosphorylation in MCF10A cells

(**Fig. 2.5A**). In addition, we also tested the MAPK Kinase (ERK) inhibitor, U0126 (**Fig 2.5B**) and the non-receptor tyrosine kinase SRC inhibitor, PP2 (**Fig 2.5C**) on their ability to block E2- and G-1-induced ERK phosphorylation in MCF10A cells. Pretreatment with AG1478 or U0126 prior to stimulation with GPER agonists significantly blocked E2- and G-1-induced ERK phosphorylation (**Figs 2.5A, 2.5B**), demonstrating that EGFR activation is a consequence of E2- and G-1-induced GPER activation in MCF10A cells. It has been shown previously that the non-receptor tyrosine kinase Src is activated downstream of GPCR activation in cancer cell lines (Frame, 2002), and there is evidence that Src can directly activate the intracellular domain of the EGFR (Migliaccio et al., 2006). Based on these observations, we sought to determine whether the Src inhibitor, PP2, could block E2- and G-1-induced ERK phosphorylation downstream of GPER activation in MCF10A cells. In cells pretreated with PP2, E2- and G-1-induced ERK phosphorylation was significantly blocked when compared with cells pretreated with Sham (**Fig 2.5C**); however, PP2 did not affect EGF-induced ERK phosphorylation in cells (**Fig 2.5C**).

These results demonstrate that Src is upstream of EGFR transactivation in MCF10A cells; however the direct mechanism by which Src activates EGFR in MCF10A cells is still unidentified. It has been reported in breast cancer cells, downstream of GPER, activated Src is able to activate MMPs at the cell surface to cleave pro-HB-EGF, allowing the soluble ligand to bind EGFR (Filardo et al., 2000) thus providing a distinct, extracellular mechanism for activation of EGFR by Src. In order to determine if this is occurring in MCF10A cells, or if Src is able

to activate the EGFR directly and intracellularly in MCF10A cells, we tested the ability a broad spectrum MMP inhibitor, GM6001 (also reported to inhibit ADAMs (Moss et al., 2007)), to block E2- and G-1-induced ERK phosphorylation. GM6001 had no effect on E2- and G-1-induced ERK phosphorylation in MCF10A cells when compared to cells pretreated with Sham (**Fig 2.5D**). The ability of GM6001 to inhibit MMPs was verified by gel zymography (data not shown). Our observations thus far indicate that Src can be activated in a GPER-dependent fashion and is required for EGFR transactivation downstream of GPER in MCF10A cells, although activation of MMPs are not required for E2- and G-1-induced ERK phosphorylation in MCF10A cells.

These results suggest that Src is activating EGFR directly; however, to determine the mechanism of EGFR transactivation downstream of Src activation, we utilized two reagents; a diphtheria toxin mutant, CRM-197 that sequesters or down-modulates surface-expressed pro-HB-EGF, inhibiting its mitogenic activity (Naglich et al., 1992), and an antibody specific for HB-EGF that neutralizes and blocks its ability to bind EGFR. Pro-HB-EGF is known to serve as the primary binding site for diphtheria toxin (Naglich et al., 1992), and the mutant form of diphtheria toxin, CRM-197, is able to bind pro-HB-EGF and sequester it, preventing its signaling ability. To test the hypothesis that GPER is transactivating the EGFR via HB-EGF, we measured ERK activation in MCF10A cells that had been pretreated with either CRM-197 or an HB-EGF neutralizing antibody prior to stimulation with E2 or G-1. Both CRM-197 and the HB-EGF neutralizing antibody were able to block E2- and G-1-induced ERK

phosphorylation compared to cells pretreated with vehicle, but had no effect on the ability of exogenous EGF to promote ERK phosphorylation (**Fig 2.5E, 2.5F**).

2.3.6 Mechanism of E2- and G-1-induced proliferation in MCF10A cells

In MCF10A cells, removal of exogenous EGF is sufficient to arrest cells in G1 of the cell cycle, yet does not promote apoptosis (Chou et al., 1999), therefore we sought to determine the effects of signaling inhibitors on E2- and G-1-induced proliferation in MCF10A cells and to determine if E2 and G-1 sufficient to promote proliferation in the absence of exogenous EGF. We first tested the EGFR inhibitor, AG1478, the MAPK Kinase (ERK) inhibitor, U0126, and the PI3 Kinase inhibitor, LY294002, for their ability to block E2- and G-1-induced proliferation in MCF10A cells. AG1478 completely blocked EGF-, E2- and G-1-induced proliferation in MCF10A cells (**Fig 26A**). MAPK Kinase inhibitor, U0126, also blocked E2- and G-1 induced ERK activation (**Fig 2.6A**); however, pretreatment of MCF10A cells with PI3Kinase inhibitor LY294002 had no effect on E2- and G-1-induced proliferation (**Fig 2.6A**), suggesting E2-and G-1-induced proliferation occurs independently of PI3Kinase activation. We also tested the ability of inhibitors of Src (PP2), MMP (GM6001), HB-EGF (CRM-197), and EGFR ligand binding (HB-EGF neutralizing antibody) to block E2- and G-1-induced proliferation. Pretreatment of MCF10A cells with PP2, CRM-197 or HB-EGF neutralizing antibody blocked E2- and G-1-induced proliferation compared to cells pretreated with vehicle (**Fig 2.6B**); however, none of these compounds inhibited exogenous EGF-dependent proliferation (**Fig 2.6B**). GM6001 had no effect on E2- and G-1-induced proliferation, similar to the non-effect of GM6001

on E2- and G-1-induced ERK phosphorylation (**Fig 2.6B**), suggesting that although Src is being activated downstream of GPER, MMP activation is not required for E2- and G-1-induced proliferation in MCF10A cells.

2.3.7 E2 and G-1 induce proliferation in a 3-D model of breast morphogenesis

Our observations thus far indicate that activation of GPER via E2 or G-1 leads to a significant increase in proliferation in MCF10A cells in monolayer culture (**Fig 2.1, 2.3A, 2.3B**), and this proliferation is dependent on the transactivation of the EGFR and subsequent phosphorylation of ERK (**Fig 2.6**). MCF10A cells are able to mimic breast epithelial morphogenesis when grown in Matrigel™ (Debnath et al., 2003). When seeded as single cells, MCF10A cells proliferate to form multicellular, hollow spheroids (**Fig 2.7)** similar to alveolar structures found in the human breast *in vivo*, making them a more physiologically relevant model to study proliferation. In the MCF10A 3-D model, cells are subject to proliferation regulation by the surrounding basement membrane, and follow a very ordered, reproducible timeline of events, making it a good model to study mitogenic stimulation of proliferation. We sought to determine if E2- and G-1 could induce proliferation in MCF10A cells in 3-D; in a setting that is governed by growth controls from the surrounding microenvironment, i.e. Matrigel™ (Debnath et al., 2003). Proliferation was detected in MCF10A cells stimulated in Matrigel™ for six days (treatments began on day four post-seeding and cells were fixed on day ten) by immunodetection of proliferation marker p-H3 (**Fig 2.8A**; green). Cells were co-labeled with an antibody raised against α-tubulin (**Fig 2.8A**; red), and nuclei are stained with Topro-3 (**Fig 2.8A**; blue). E2- and G-1 both induce a

significant increase in proliferation in MCF10A cells in 3-D after six days of treatment compared to control-treated cells (**Fig 2.8B**). E2- and G-1 treatment also led to an increase in cell number per spheroid (**Fig 2.8C**), indicating that E2 and G-1 stimulation lead to cell cycle completion in MCF10A cells.

2.3.8 GPER contributes to E2-induced proliferation in human breast tissue

Since GPER activation was able to mediate proliferation in non-tumorigenic MCF10A cells, we sought to determine if E2-dependent proliferation in human breast tissue, cultured as described (Eigeliene et al., 2006; **Fig 2.9**), was mediated in part by GPER. We confirmed expression of estrogen receptors in human breast tissue explants by immunohistochemical analysis (immunohistochemistry; IHC) using antibodies against GPER (**Fig 2.10C**) or ERα (**Fig 2.10D**). While ERα alpha expression and staining intensity was uniform throughout breast tissue samples, GPER staining intensity was more variable, although every sample in which proliferation was assessed was GPER positive, based on IHC.

Immunodetection of proliferation marker Ki-67 was used to elucidate the effect of GPER activation on proliferation in mammary explants after seven days in culture. Ki-67 proliferation marker was used to quantitate proliferation in breast tissue instead of pH3, since Ki-67 is a much broader proliferation marker, labeling all cells in the cell cycle, whereas pH3 only labels cells in mitosis. Due to the proliferation rates in breast tissue being much lower than that in MCF10A cells, we used Ki-67 immunostaining to capture a larger percentage of

proliferating cells. Treatment of breast tissue explants with E2 or G-1 significantly increased epithelial cell proliferation, compared to control (**Fig 2.10A**). Breast tissue explants treated with GPER antagonist G36 alone had no effect (**Fig 2.10B**); however, G36 significantly reduced E2- and G-1-dependent proliferation, suggesting that GPER contributes to E2-induced proliferation in primary human breast tissues.

2.3.9 E2 and G-1 promote proliferation in tumorigenic human breast tissue

In addition to investigating GPER's contribution to E2-induced proliferation in normal human breast tissue, we also were interested if E2 or G-1 were able to promote proliferation (measured by Ki-67 immunostaining) in human breast tumor tissue after seven days of stimulation. Treatment of breast tumor tissue explants with E2 or G-1 significantly increased epithelial cell proliferation, compared to control (**Fig. 2.11)**. Whereas explants treated with GPER antagonist G36 alone had no effect, G36 was able to significantly reduced E2- and G-1-dependent proliferation (**Fig. 2.11)**, suggesting that GPER activation contributes to E2-induced proliferation in primary breast tumor tissue, similar to the effect of E2 and G-1 on non-tumorigenic breast tissue.

2.4 Discussion

The proliferative effects of E2 in the breast are well established and have long been attributed to the classical estrogen receptors ERα and ERβ (Brisken and O'Malley, 2010). However, it is not known if the novel G-protein-coupled estrogen receptor, GPER, can promote E2-induced proliferation in the normal human breast. It was demonstrated that GPER can mediate proliferation in SKBr3 breast cancer cells (Pandey et al, 2009), endometrial cancer cells (Vivacqua et al., 2006b), and ovarian cancer cells (Albanito et al., 2007); however, there is also evidence that GPER inhibits proliferation of ER positive MCF7 breast cancer cells (Ariazi et al., 2010). Moreover, our group has shown that GPER promotes proliferation in vivo, in the murine endometrium (Dennis et al., 2009). Due to the ability of GPER to both promote and inhibit proliferation depending on cell type and estrogen receptor status, we were interested in the role of GPER in E2-induced proliferation in the normal human breast. We addressed this question by directly measuring GPER-dependent proliferation in a human breast epithelial cell line, MCF10A, and in human breast tissue. We showed that E2 and the GPER-selective agonist G-1 induce proliferation in MCF10A cells both in standard monolayer culture (**Fig 2.1**), and in a 3-D model of breast epithelial morphogenesis, where growth control cues are present (**Fig 2.8**). These cells express GPER but not ERα and ERβ (**Fig 2.2**), suggesting that E2-induced proliferation is dependent on GPER alone in MCF10A cells. To support the role of GPER in E2-induced proliferation, we used a GPER-selective antagonist, G36, as well as GPER-targeted siRNA in proliferation assays. Both treatments blocked E2- and G-1-induced proliferation (**Fig 2.3B, C)**.

Our results also demonstrate that E2 promotes proliferation in normal human breast tissue explants from reduction mammoplasty surgery (**Fig 2.10A)**, consistent with previous findings (Eigeliene et al., 2006). GPER-selective agonist G-1 also promoted proliferation in human breast tissue explant cultures compared to control treated tissue, albeit at a reduced level compared to E2 (**Fig 2.10B)**. Moreover, G36 completely blocked G-1-induced proliferation (**Fig 2.10B**). G36 also partially blocked E2-induced proliferation in human breast tissue explants, suggesting that E2–dependent proliferation in the human breast occurs through activation of multiple estrogen receptors, including GPER. Therefore this study is the first to demonstrate GPER-dependent proliferation in a normal human tissue.

Filardo and colleagues previously demonstrated that E2-dependent GPER activation leads to EGFR transactivation, with subsequent Erk-1 and Erk-2 activation in breast cancer cells (Filardo et al., 2000). GPER transactivation of the EGFR and subsequent activation of downstream signaling pathways is consistent with our prior observation that E2-dependent GPER activation stimulates the PI3K pathway, in an EGFR activation-dependent manner (Revankar et al., 2005). Therefore, in order to dissect the molecular pathway by which GPER promotes proliferation in a normal, non-tumorigenic setting, we targeted different components of the EGFR/MAPK signaling pathway (Filardo et al., 2000; Quinn et al., 2009). Our results reveal that E2- and G-1-induced GPER activation of leads to EGFR transactivation and subsequent ERK activation (**Fig 2.4**). Moreover, EGFR transactivation and subsequent ERK activation is required

for E2- and G-1-induced proliferation in MCF10A cells (**Fig 2.6**). PI3Kinase inhibitor LY294002 had no effect on E2- and G-1-induced proliferation, suggesting activation of the MAPK signaling pathway downstream of EGFR transactivation is independent of PI3Kinase activation. We also determined that in MCF10A cells, although activation of the non-receptor tyrosine kinase Src is required for GPER-dependent activation of ERK and proliferation, MMP activity is not required for EGFR transactivation (measured by ERK activation) (**Fig 2.5D**) or proliferation (**Fig 2.5B**). Nevertheless, we confirmed the requirement for HB-EGF to promote E2- and G-1-induced phosphorylation of ERK and proliferation downstream of GPER activation, because sequestering and down-modulating pro-HB-EGF with CRM-197 or blocking its ability to bind EGFR with neutralizing antibodies abolished GPER-dependent ERK activation and proliferation (**Fig 2.5E,F, 2.6B**). It has been reported that membrane tethered pro-HB-EGF can activate the EGFR on adjacent cells in a juxtacrine manner, independent of cleavage by proteases (Dong et al., 2005; Takemura et al., 1997). Pro-HB-EGF signaling has been previously reported in MCF10A cells (Willmarth and Ethier, 2006a), and it is possible this is the mechanism of transactivation of the EGFR downstream of GPER activation in these cells.

In this study, we show for the first time that GPER mediates E2-induced proliferation in normal breast tissue and in normal human tumor tissue. We have also demonstrated a novel mechanism for transactivation of the EGFR in MCF10A cells downstream of GPER. Given the ability of GPER to promote proliferation in normal breast tissue as well as breast cancer cells, and the

correlation between GPER expression and negative outcome in a breast tumor setting, the need to understand the mechanism of E2-induced proliferation and signaling is essential. The demonstrated ability of GPER-selective antagonist G36 to block E2-induced proliferation *in vitro* in cell lines (**Fig 2.3)** as well as in human tissue highlights its potential importance in therapeutic intervention in breast and other E2-responsive tissues

2.5 Materials and Methods

2.5.1 Reagents

Dulbecco's Modified Eagle Medium (DMEM), phenol red-free DMEM, E2, fetal bovine serum (FBS), normal goat serum (NGS), insulin, cholera toxin, transferrin, hydrocortisone and prolactin were from Sigma (St. Louis, MO). Recombinant epidermal growth factor (EGF) and penicillin/streptomycin (P/S) were from Invitrogen (Carlsbad, CA). Bovine serum albumin (BSA) was from Amresco (Solon, OH). Growth factor reduced phenol red-free Matrigel™ was from BD Biosciences (San Jose, CA). G-1 was synthesized as described (Bologa et al., 2006) and provided by Jeffrey Arterburn (New Mexico State University, Las Cruces, NM). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). Small interfering RNA (siRNA) was purchased from Dharmacon RNAi Technologies (Dharmacon, Lafayette, CO): ON-TARGET plus SMARTpool siRNA for GPER (L-005563-00) and ON-TARGETplus siControl Non-Targeting siRNA (D-001810-02).

2.5.2 Inhibitors and antibodies

EGFR inhibitor Tyrphostin AG1478, PI3K inhibitor LY294002, Src inhibitor PP2, MEK inhibitor U0126 and MMP inhibitor GM6001 were from Calbiochem (La Jolla, CA). Diphtheria toxin mutant CRM-197 (Berna Products, Coral Gables, FL) and HB-EGF neutralizing antibody (R&D Systems, Minneapolis, MN) were a gift from Edward Filardo (Brown University, Providence, RI). G36 was synthesized as described (Dennis et al., 2011) and provided by Jeffrey Arterburn (New Mexico State University). Polyclonal antibody against the human GPER (c-

terminus) was used for GPER localization assays. Rabbit p-Histone H3 antibody and mouse β-actin antibody were from Millipore (Billerica, MA). Rabbit antiphospho-p-44/42 MAPK (ERK1/2) (Thr202/Tyr204) antibody was from Cell Signaling (Beverly, MA). Rabbit anti-Ki-67 antibody was from Neomarkers/Lab Vision (Thermo Fisher, Kalamazoo, MI). Mouse anti-α-tubulin antibody was from Sigma (St. Louis, MO). Goat antirabbit IgG-Alexa 488-conjugated secondary antibody and Goat antimouse IgG-Alexa 533-conjugated secondary antibody were from Invitrogen (Carlsbad, CA). Goat antirabbit IgG-HRP-conjugated antibody was from GE Healthcare (Princeton, NJ) and goat antimouse IgG-HRP-conjugated antibody was from Cell Signaling.

2.5.3 Cell Culture

Immortalized, non transformed MCF10A human breast epithelial cells (ATCC, Manassas, VA; catalog number CRL-10317) were maintained in MCF10A complete media (DMEM/F-12 supplemented with 5% dextran-charcoal-stripped fetal bovine serum, 10 μg/mL insulin, 100 ng/mL cholera toxin, 0.5 μg/mL hydrocortisone, 20 ng/mL recombinant epidermal growth factor and 1% penicillin/streptomycin). Cells were cultured in a humidified atmosphere containing 5% CO2 at 37◦C. For proliferation assays, cells were passaged onto 12mm glass coverslips (Electron Microscopy Sciences, Hatfield, PA) and cultured in phenol red-free MCF10A media with all supplements listed above. Overnight cell synchronization for proliferation and immunoblot analysis was performed as previously described (Chou et al., 1999). After overnight synchronization, cells were stimulated for 24 hours with vehicle control, 17-beta

estradiol (1nM to 100nM) (E_2), G-1 (GPER-selective agonist) (1nM to 100nM), and G36 (GPER-selective antagonist; 5nM to 500nM), fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS) for 15 minutes at room temperature. Compounds were diluted in dimethylsulfoxide (DMSO); therefore control tissues were incubated in media supplemented with DMSO vehicle. For some experiments, MCF10A cells were grown in 60mm cell culture dishes and transfected with siRNA using Lipofectamine 2000 as per manufacturer's instructions. For immunoblot analysis, cells were grown on 60mm plates in phenol red-free MCF10A media, synchronized overnight and stimulated with compound.

MCF10A cells were also grown in Growth Factor Reduced phenol red-free Matrigel™ on 8-well chamber slides (BD Falcon, San Jose, CA). Approximately 5,000 MCF10A cells were seeded on 40µL of Matrigel in each chamber on the slide. Cells were suspended in growth media (described above) supplemented with 2% Matrigel. The media was changed every two days, and after four days in culture, various treatment compounds were added to growth media. Cells continued to grow in Matrigel until day 10, whereby they were fixed with 4% PFA in PBS for 15 minutes at room temperature. Immunofluoresence assays were carried out of MCF10A cells in 2D and 3D according to a method previously described (Debnath et al., 2003). Images were captured on either a Zeiss 200M Axiovert inverted microscope (Carl Zeiss Inc., Oberkochen, Germany), using a x400 total magnification (2-D cells) or a Zeiss LSM 510 confocal microscope using x400 total magnification and an optical thickness of 0.7 µM (3-D cells).

2.5.4 Tissue Samples

Human breast tissue was acquired from female patients who were undergoing reduction mammoplasty surgery between November 2007 and January 2011. Normal breast tissue remaining after pathological testing was collected and used in this study. Successive specimens were collected at University of New Mexico hospital (UNMH), and received from the cooperative human tissue network (CHTN Western division- Vanderbilt University, Nashville, TN), a division of the National Cancer Institute. This study protocol was approved by University of New Mexico Health Sciences Center institutional review board (IRB). Tissue collected at UNMH was transported to the laboratory on ice in D-MEM/F-12 medium containing 1% P/S, within 1-2 hours of surgery. Tissue obtained from CHTN was shipped overnight on ice in RPMI medium (Sigma) supplemented with 1% P/S. The tissue was dissected into 3 mm^3 pieces in phenol-red free D-MEM/F-12 medium in order to exclude as much adipose tissues as possible, saving the collagenous connective tissue where the epithelial ducts and lobules are found.

2.5.5 Organ Culture

Breast tissue was incubated according to a previously described method (Eigeliene et al., 2006), in which pieces of breast tissue are placed on sterile lens paper lying on stainless steel grids (our protocol modified this to use nylon grids) atop a 35mm tissue culture dishes inside a 10cm dish. For experiments done in breast tumor tissue, tissue was submerged in media in a 24 well plate. Tissue was incubated overnight in a humidified atmosphere with a mixture of 5% CO_2

and 95% air at 37˚C in phenol-red free D-MEM/F-12 medium supplemented with 1% P/S, 10ug/mL insulin, 3ug/mL prolactin, 4mg/ml transferrin and 1ug/mL hydrocortisone. Following overnight incubation to “rest” the tissue, additions were made to the medium in the inner tissue culture dish; including vehicle control, 17-beta estradiol (1nM to 100nM) (E_2), G-1 (GPER-selective agonist) (1nM to 100nM), and G36 (GPER-selective antagonist; 5nM to 500nM). Compounds were diluted in dimethylsulfoxide (DMSO); therefore control tissues were incubated in media supplemented with DMSO vehicle. Growth media was changed every two days and fresh treatments were added. Tissue was collected 7 days after the addition of treatments and fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight at room temperature.

2.5.6 Indirect Immunofluorescence (Tissue)

For immunofluorescence staining, paraffin sections (5µm) were mounted on Super-Frost Plus slides (Menzel- Gläser, Germany). After rehydrating sections through a graded alcohol series followed by water, the slides were treated for antigen retrieval by boiling in a microwave oven in 0.01 M citrate buffer (pH 6.0) for 20 minutes. After a series of washes the sections were incubated with 0.1% Triton X-100 containing 3% NGS for 30 min at room temperature to permeabilize cells and block non-specific binding antibody. Tissue sections were then incubated with primary antibodies diluted in PBS + 0.1% Tween-20 containing 3% NGS overnight at 4°C in a humid chamber. Following overnight incubation with primary antibody, tissue sections were washed and incubated with species-matched Alexafluor conjugated secondary antibodies

(Invitrogen) for 1 hour at room temperature in a dark chamber. Sections were incubated for 15 minutes with Topro-3 (Molecular Probes) to stain nuclei. Sections were mounted with Vectashield mounting media (Vector Labs) and sealed with nail polish. Images were captured on either a Zeiss 200M Axiovert inverted microscope using a x400 total magnification or a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc., Oberkochen, Germany), using x400 total magnification and an optical thickness of 0.7 µm.

2.5.7 Western Immunoblotting

Cells were lysed in RIPA buffer supplemented with buffer supplemented with sodium fluoride (50 mM), sodium orthovanadate (1 mM), phenylmethylsulfonylfluoride (1 mM), and protease cocktail (1X). Cell lysate protein concentration was determined by performing a Bradford protein assay (Bio-rad, Hercules, CA). Equal protein concentrations per lysate were loaded on a 4-20% SDS-PAGE gel (Thermo-Scientific, Rockford, IL) and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). After blocking in 5% nonfat dry milk for 1 hour at room temp, the membranes were incubated with primary antibodies at a 1:100 to 1:10,000 dilution in 3% BSA overnight at 4˚C with gentle rocking. After a series of washing, the blots were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG at 1:10,000 in 3% BSA for 1 hour at room temperature with gentle rocking. The blots were developed using Supersignal West Pico Chemilumiscent Substrate (Thermo Fisher). Films were then scanned and quantified using ImageJ software (National Institutes of Health, Bethesda, MD)

2.5.8 Quantitation

For Ki-67 and p-histone H3 immunofluorescence assays, cells staining positive for these proliferation markers were expressed as a percentage of the total number of cells in each treatment sample. Blind quantitation was performed, and fields were chosen at random.

2.5.9 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 4.03 (La Jolla, CA). Analysis done with a one way analysis of variance (ANOVA) within Prism estimates the correlation of variables (protein expression, proliferation, etc) with treatment groups (sham, E2, G-1, G-36, etc). Pairwise comparisons of results between different treatment groups were determined using a one-way Analysis of Variance (ANOVA) followed by a Dunnett's test. Data represents the mean ± SEM of three or more separate experiments. P-values less than 0.05 were considered to be significant.

2.6 Figure Legends

Figure 2.1 17-β Estradiol stimulates proliferation in MCF10A cells.

Proliferation was assessed by immunofluorescence using an anti-phospho (ser10) Histone H3 (pH3) antibody in MCF10A cells cultured in the presence of vehicle (sham) or the indicated concentrations of 17-β estradiol (E2) for 24 hours. Data represents the average of three independent experiments. Results are expressed as mean ± SEM and statistical significance ($p \leq .05$) was assessed by one-way ANOVA followed by a Dunnett's test. (*, significantly different relative to sham)

Figure 2.2 MCF10A cells express GPER.

GPER expression was assessed in MCF10A cells by immunofluorescence (A) and western blotting (B), probing with an anti-human GPER peptide antibody. siRNA knockdown of GPER expression was also demonstrated in cells transfected with GPER-specific siRNA, 72hours following transfection (representative experiment shown in B). Cells transfected with non-specific (scrambled) control siRNA express normal levels of protein (B). The histogram displays densitometric quantitation of three independent GPER immunoblots following no transfection (NT), or 72 hr following transfection with control siRNA or GPER-specific siRNA (C). Quantitation is normalized to β-actin immunodetection. Results are expressed as mean ± SEM and statistical significance ($p = .0176$) was assessed by one-way ANOVA followed by a Dunnett's test. (*, statistically significant relative to non-transfected cells)

Figure 2.3 E2 and G-1-induced proliferation is dependent on GPER in MCF10A cells.

Proliferation was assessed in MCF10A cells grown on glass coverslips in the presence of indicated concentrations of GPER agonists (E2, G-1) and antagonist G36 for 24 hours (A, B). Proliferation was also assessed in control and GPER siRNA transfected MCF10A cells following 24 hour stimulation with E2 or G-1 (C). Proliferation was quantified by immunofluorescence using an anti-phospho Histone H3 antibody. Knockdown of GPER was confirmed by western blot with anti-GPER antibody (D). Data is representative of a minimum of three independent experiments. Results are expressed as mean ± SEM and statistical significance ($P \leq .05$) was assessed by one-way ANOVA followed by a Dunnett's test. (*, significantly different relative to sham; #, significantly different relative to E2 or G-1; ns = not significant.)

Figure 2.4 GPER activation induces activation of the MAPK signaling cascade.

MCF10A cells were stimulated with indicated concentrations of E2 or G-1 alone or in combination with GPER antagonist G36, for 15 minutes (A). Lysates were prepared and immunoblotted with antibodies specific to phospho-ERK (p-ERK). Equal protein loading was confirmed by β-actin immunoblotting. Histograms represent fold change (p-ERK relative to actin) in p-ERK protein expression, relative to vehicle-treated cells (sham). p-ERK was also assayed in cells transfected with control or GPER siRNA-treated cells 72 hours after transfection,

and then stimulated with E2 or G-1 for 15 minutes (B). Data are representative of three independent experiments. Results are expressed as mean ± SEM and statistical significance (P≤ .05) was assessed by one-way ANOVA followed by a Dunnett's test. (*, significantly different relative to sham; #, significantly different relative to E2 or G-1)

Figure 2.5 GPER-dependent activation of MAPK (ERK1 and ERK2) is dependent on Src activation but not MMP activation in MCF10A cells.

Signal transduction inhibitors were tested for their ability to block GPER-dependent ERK activation in MCF10A cells. Cells were pre-incubated for 30 minutes with either vehicle (sham), AG1478 (A, 250 nM, inhibitor of EGFR), U0126 (B, 10uM, inhibitor of MEK), PP2 (C, 10 nM, inhibitor of Src), GM6001 (D, 25 uM, inhibitor of MMPs), CRM-197 (E, 0.2mg/mL, inhibitor of HB-EGF or HB-EGF neutralizing antibody (F, 6ng/mL), then stimulated with 10nM EGF, 10nM E2 or 100nM G-1 for 15 minutes. Lysates were western blotted with antibodies specific for phospho-ERK. Equal protein loading was confirmed by β-actin immunoblotting. Histograms represent fold change in p-ERK protein expression. Data are representative of three independent experiments. Results are expressed as mean ± SEM and statistical significance (P≤ .05) was assessed by one-way ANOVA followed by a Dunnett's test. (* significantly different relative to sham)

Figure 2.6 GPER-dependent proliferation requires transactivation of the EGFR.

Signal transduction inhibitors were tested for their ability to block GPER-dependent proliferation in MCF10A cells. Cells were preincubated for 30 minutes with either vehicle (sham, A & B), AG1478 (250nM, EGFR inhibitor, A), U0126 (10uM, MEK inhibitor, A), LY294002 (10uM, PI3K inhibitor, A), PP2 (10nM, Src inhibitor, B), GM6001 (25uM, MMP inhibitor, B), CRM197 (HB-EGF release inhibitor, (0.2mg.mL, B) or HB-EGF neutralizing antibody (6ng/mL, B) and then stimulated with EGF (10 nM), E2 (10 nM) or G-1 (100 nM) for 24 hours. Proliferation was quantified by immunofluorescence using an anti-phospho Histone H3 antibody (pH3). Data are representative of a minimum of three independent experiments. Results are expressed as mean ± SEM and statistical significance ($P \leq .05$) was assessed by one-way ANOVA followed by a Dunnett's test. (* significantly different relative to sham).

Figure 2.7 Illustration of MCF10A 3-D culture method.

Figure taken from Debnath et al, 2003, *Methods,* 30; p 261

Figure 2.8 Estrogen-induced GPER activation stimulates proliferation in a 3-dimensional model of breast morphogenesis.

MCF10A cells were grown in 3D on Matrigel™ basement membrane in the presence of 10nM E2 or 100nM G-1 for six days in culture. Proliferation (B) was quantified by immunofluorescence using an anti-pH3 antibody. An representative

spheroid immunolabeled with pH3 (green) and anti-gamma tubulin (ref) is shown (A; arrow indicates phospho-histone immunolabeled chromatin; arrowhead indicates mitotic spindle). Total cell number per spheroid was quantified for each treatment group (C). Data are representative of three independent experiments. Results are expressed as mean ± SEM and statistical significance (P≤ .05) was assessed by one-way ANOVA followed by a Dunnett's test. (*, significantly different relative to sham).

Figure 2.9 Illustration of human breast tissue culture method.

Figure taken from Eigeliene et al, 2006, *BMC Cancer* 6; p 3

Figure 2.10 E2 and G-1 promote proliferation in human breast tissue.

Breast epithelial proliferation was assessed in the presence of GPER agonists E2 and G-1(A, B) and antagonist G36 (B) in alveolar structures within human breast tissue explants expressing both ERα (C) and GPER (D). Proliferation of luminal epithelial cells was quantified by immunofluorescence using anti-Ki-67 antibody. ERα (C) and GPER (D) protein expression was confirmed by immunohistochemistry analysis using antibodies directed at each protein. Each treatment group consisted of tissue samples from a minimum of five different patients. Results are expressed as mean ± SEM, and statistical significance ($P \leq 0.05$) was assessed by one way ANOVA followed by a Dunnett's t-test. (* significantly different relative to sham, # significantly different relative to E2 or G-1)

Figure 2.11 E2 and G-1 promote proliferation in tumorigenic human breast tissue.

Breast tumor proliferation was assessed in the presence of GPER agonists E2 and G-1 and antagonist G36. Proliferation was quantified by immunofluorescence using anti-Ki-67 antibody. Each treatment group consisted of tissue samples from a minimum of five different patients. Results are expressed as mean ± SEM, and statistical significance ($P \leq 0.05$) was assessed by one way ANOVA followed by a Dunnett's t-test. (* significantly different relative to sham, # significantly different relative to E2 or G-1)

2.7 Figures

Figure 2.1 17-β Estradiol stimulates proliferation in MCF10A cells

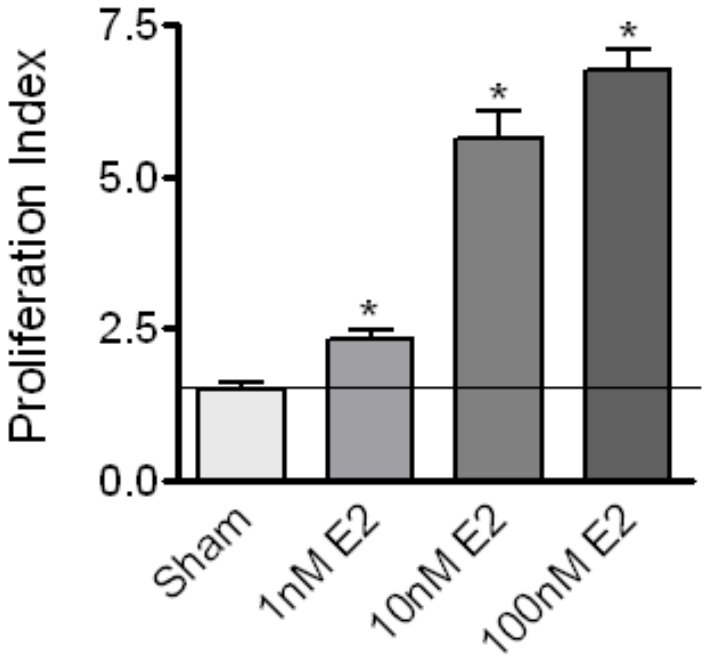


Figure 2.2 MCF10A cells express GPER

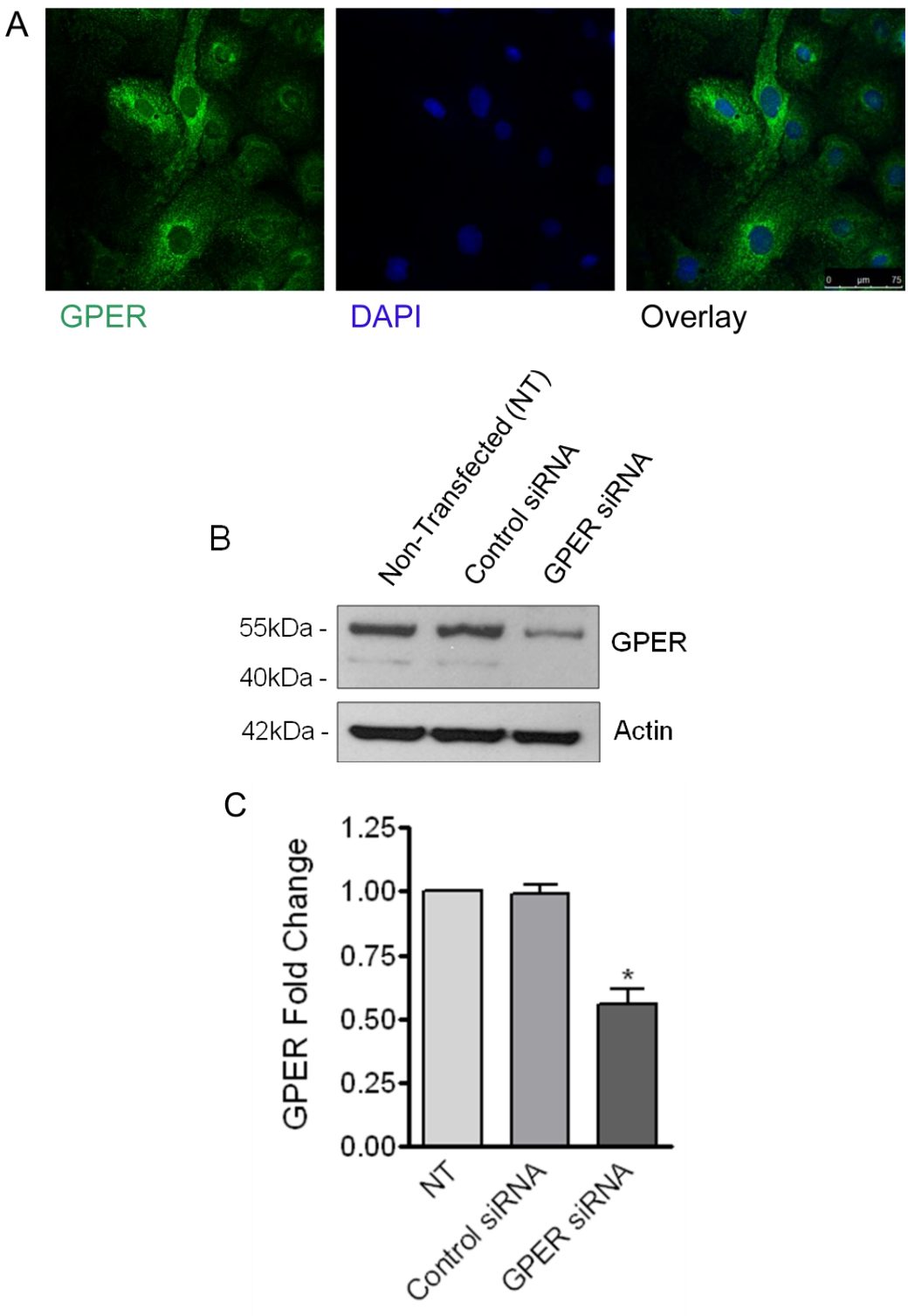


Figure 2.3 E2 and G-1-induced proliferation is dependent on GPER in MCF10A cells

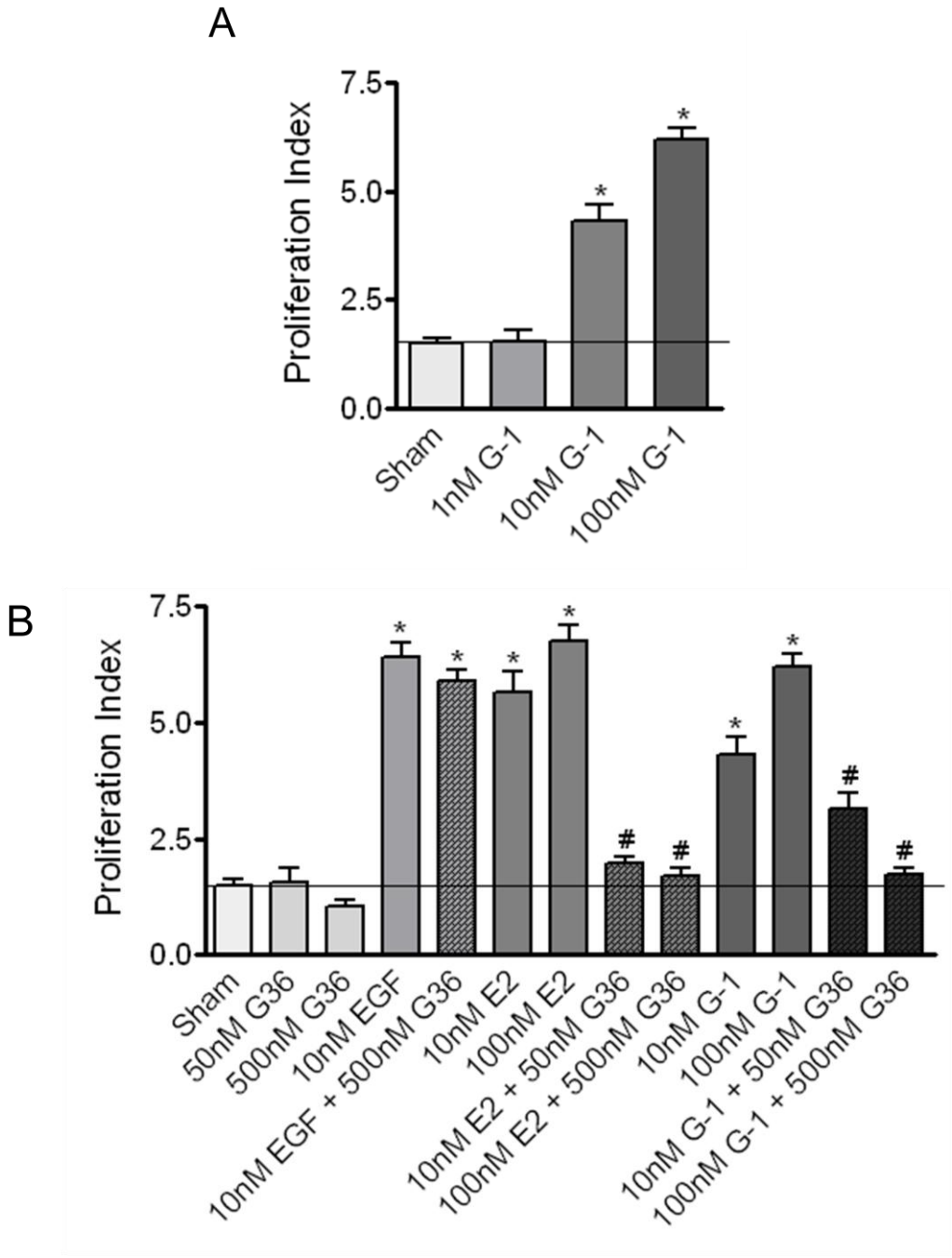


Figure 2.3 E2 and G-1-induced proliferation is dependent on GPER in MCF10A cells

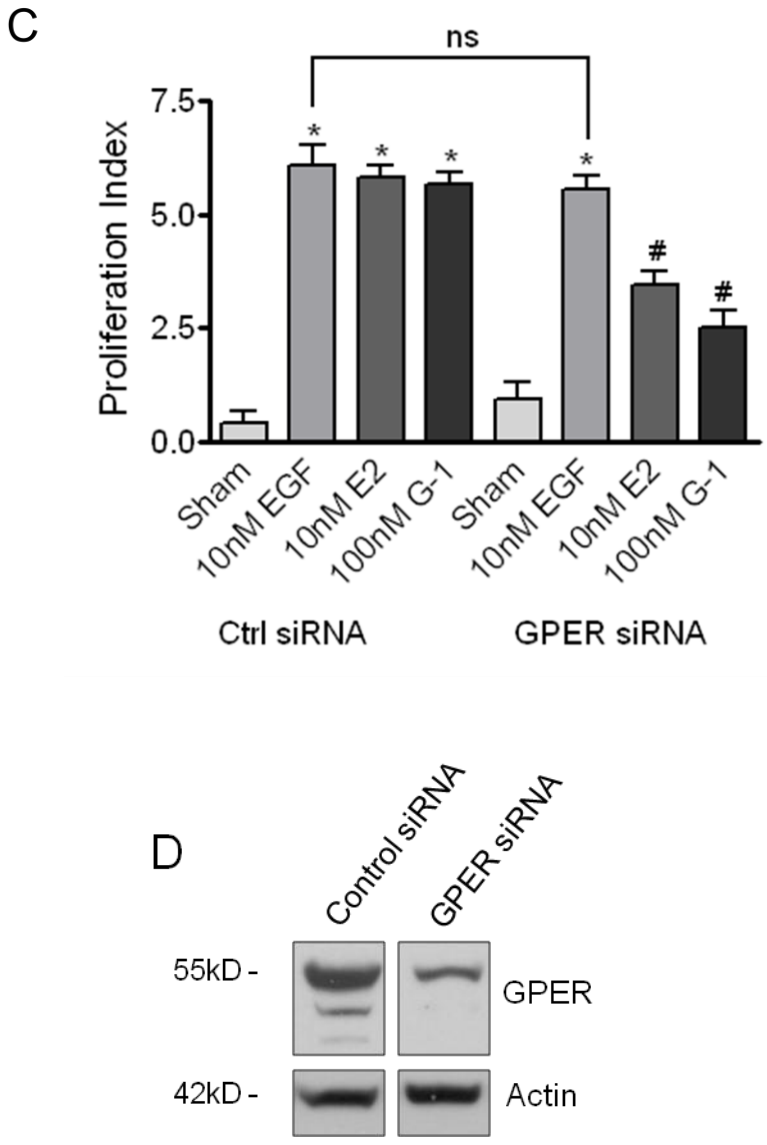


Figure 2.4 GPER activation induces activation of the MAPK signaling cascade

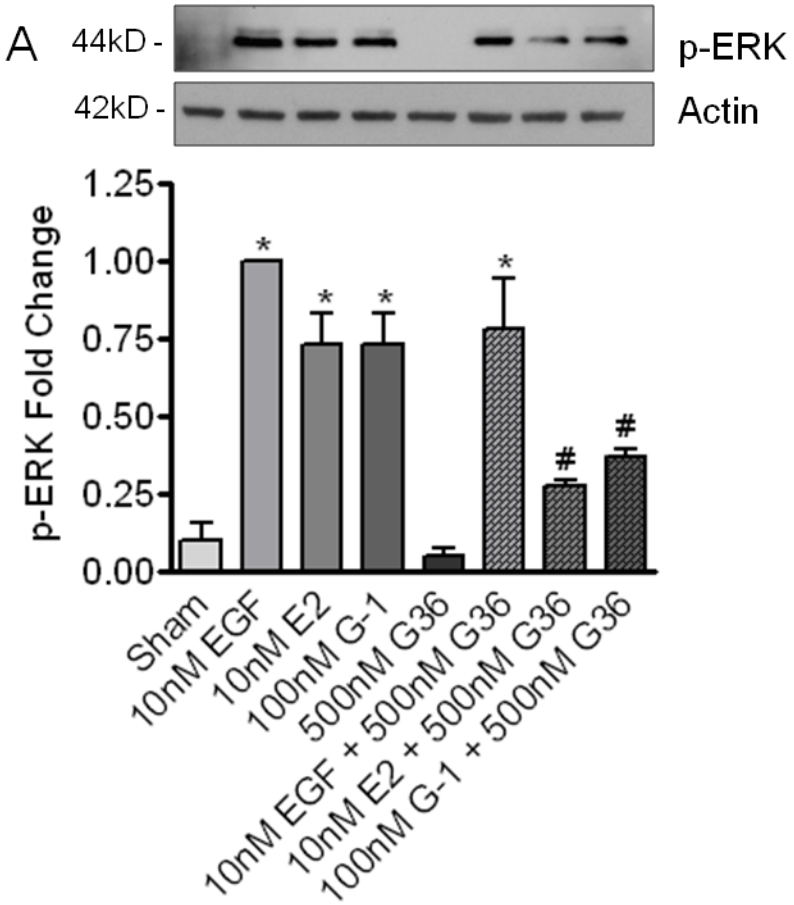


Figure 2.4 GPER activation induces activation of the MAPK signaling cascade

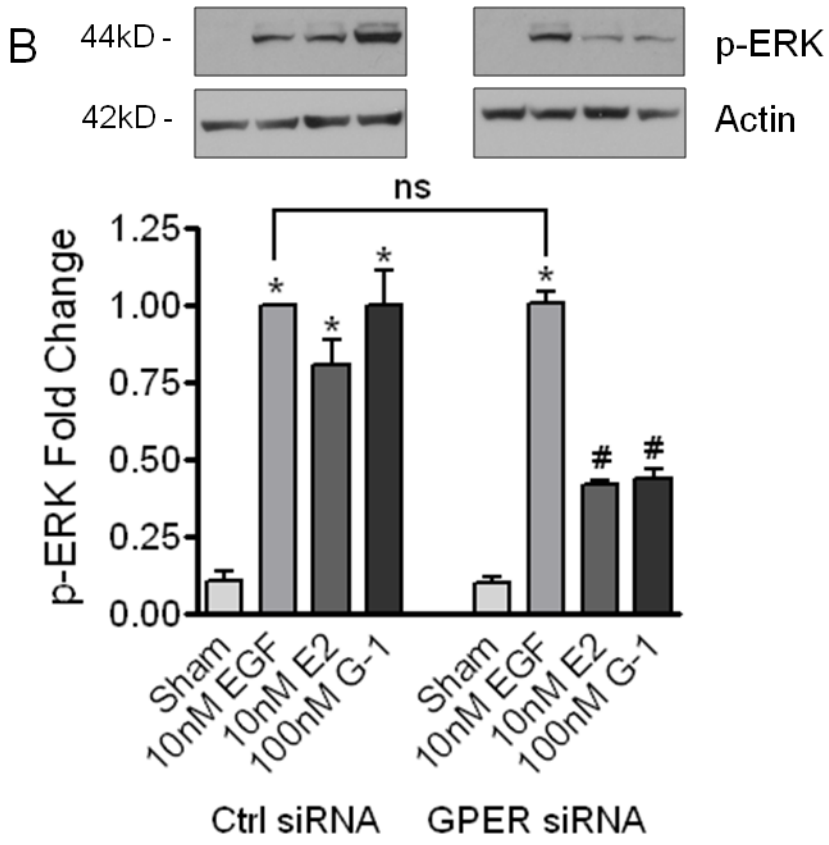


Figure 2.5 GPER-dependent activation of MAPK (ERK1 and ERK2) is dependent on Src activation but not MMP activation in MCF10A cells.

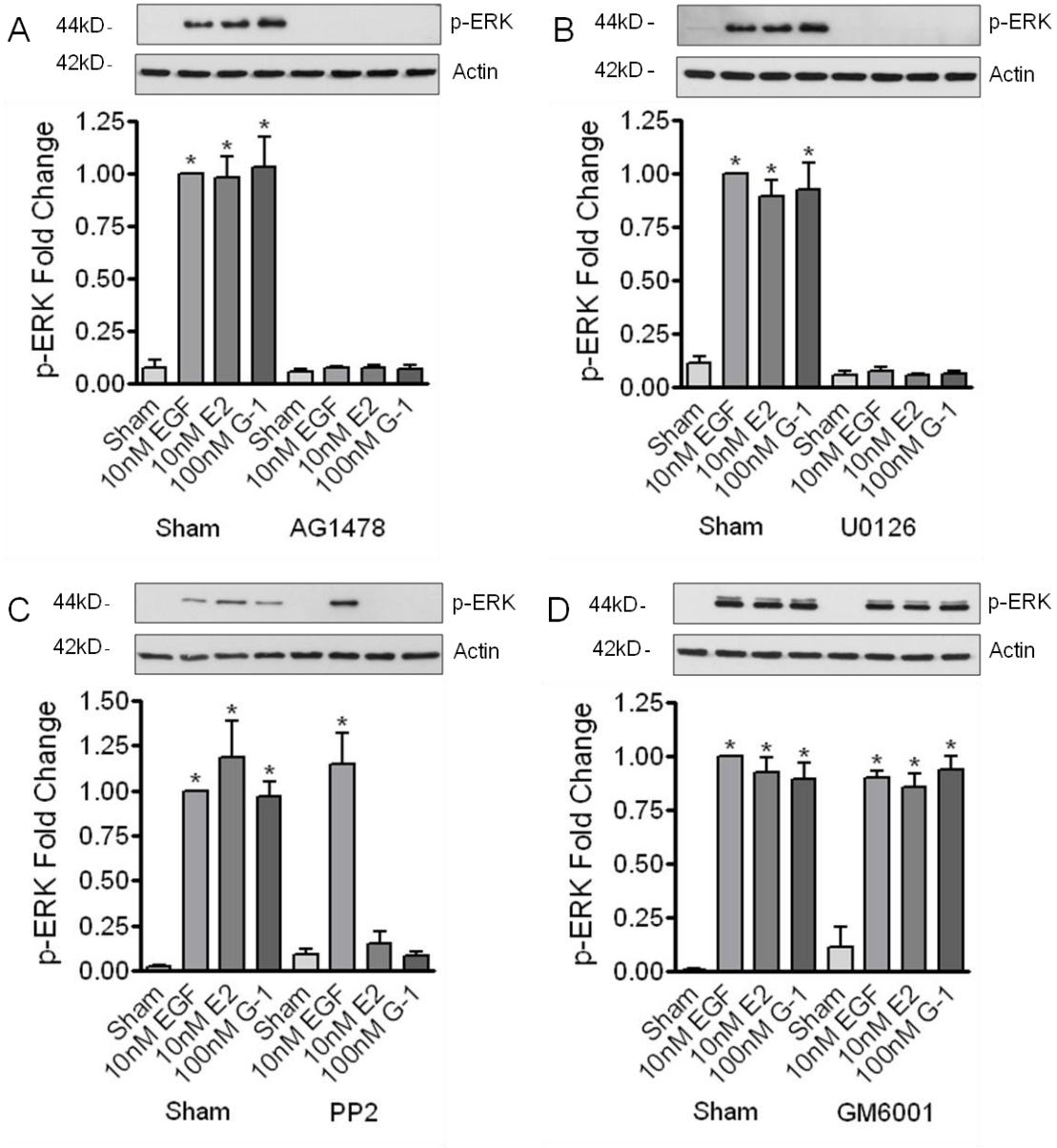


Figure 2.5 GPER-dependent transactivation of the EGFR is dependent on Src activation but not MMP activation in MCF10A cells.

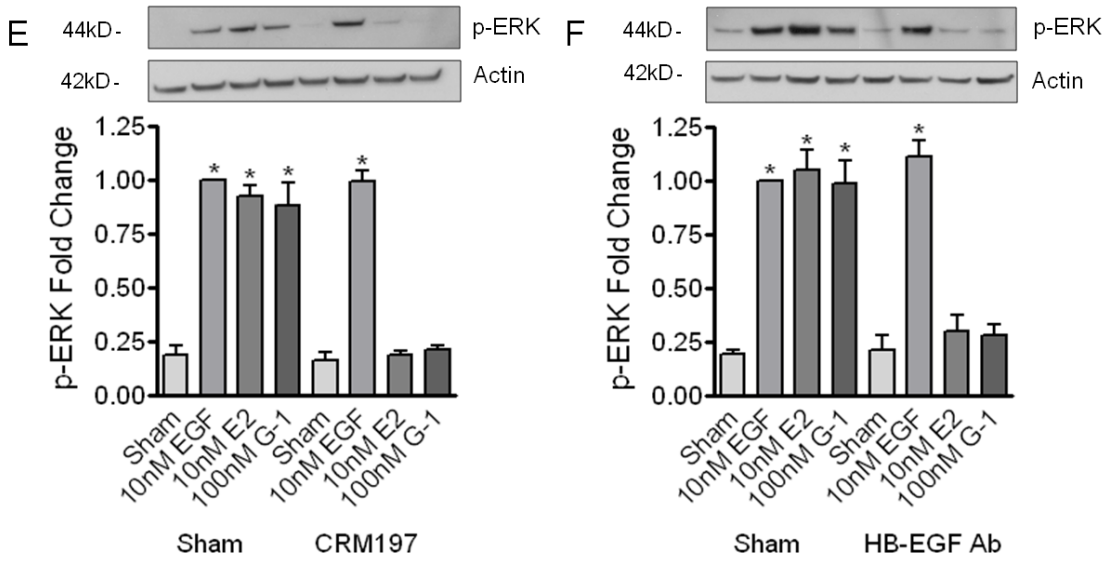


Figure 2.6 GPER-dependent proliferation requires transactivation of the EGFR.

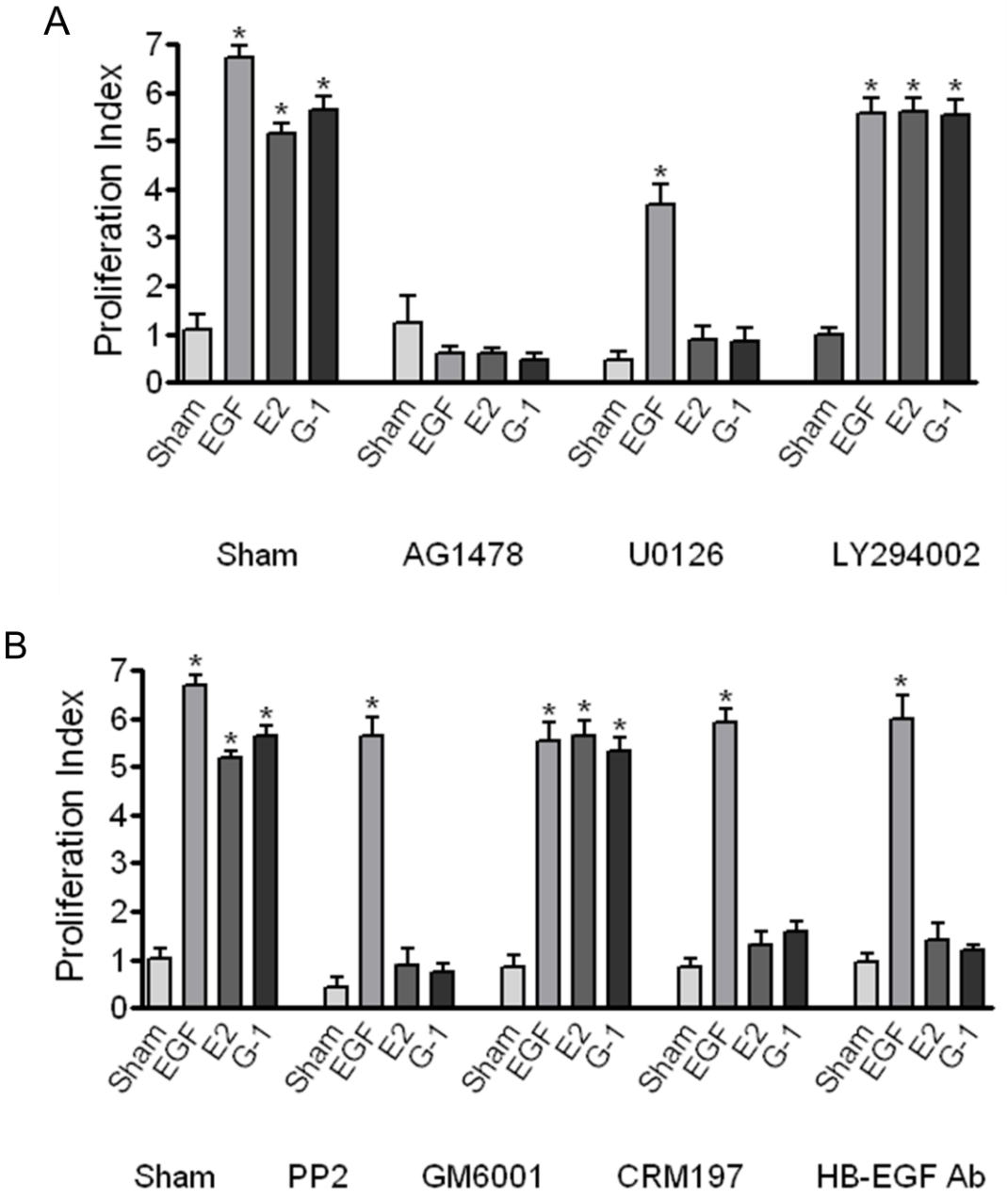


Figure 2.7 Illustration of MCF10A 3-D culture method

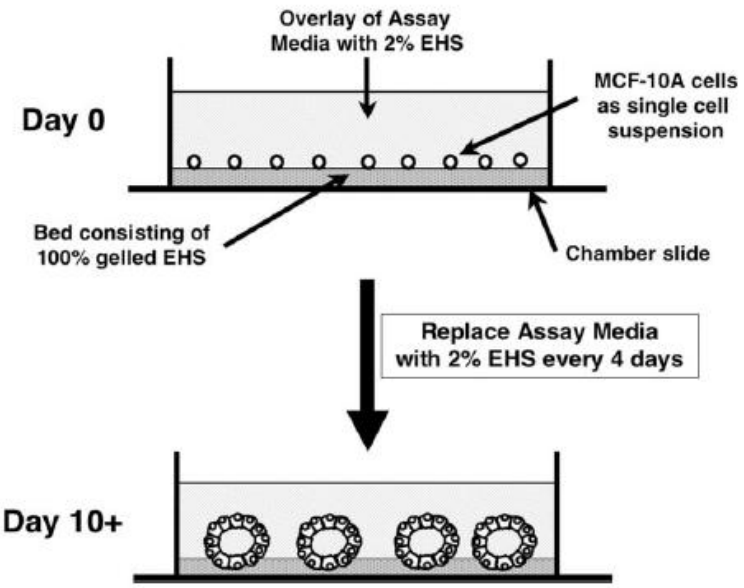


Figure 2.8 Estrogen-induced GPER activation stimulates proliferation in a 3-dimensional model of breast morphogenesis

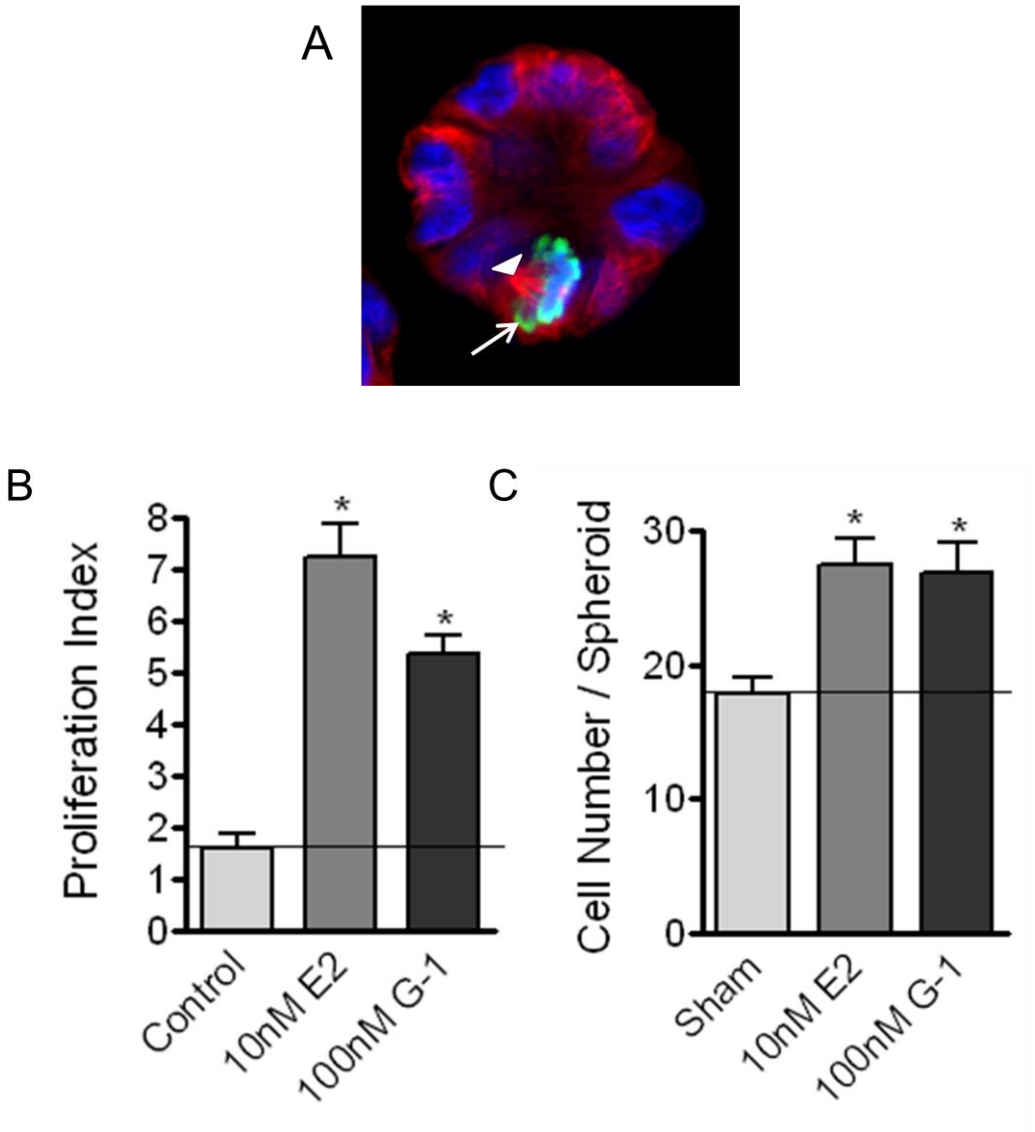


Figure 2.9 Illustration of human breast tissue culture method

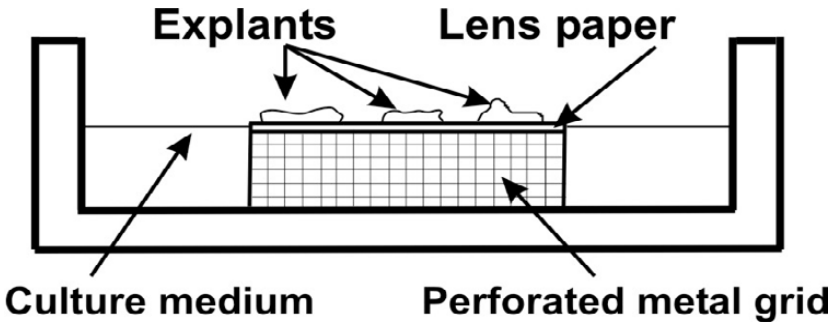


Figure 2.10 E2 and G-1 promote proliferation in human breast tissue

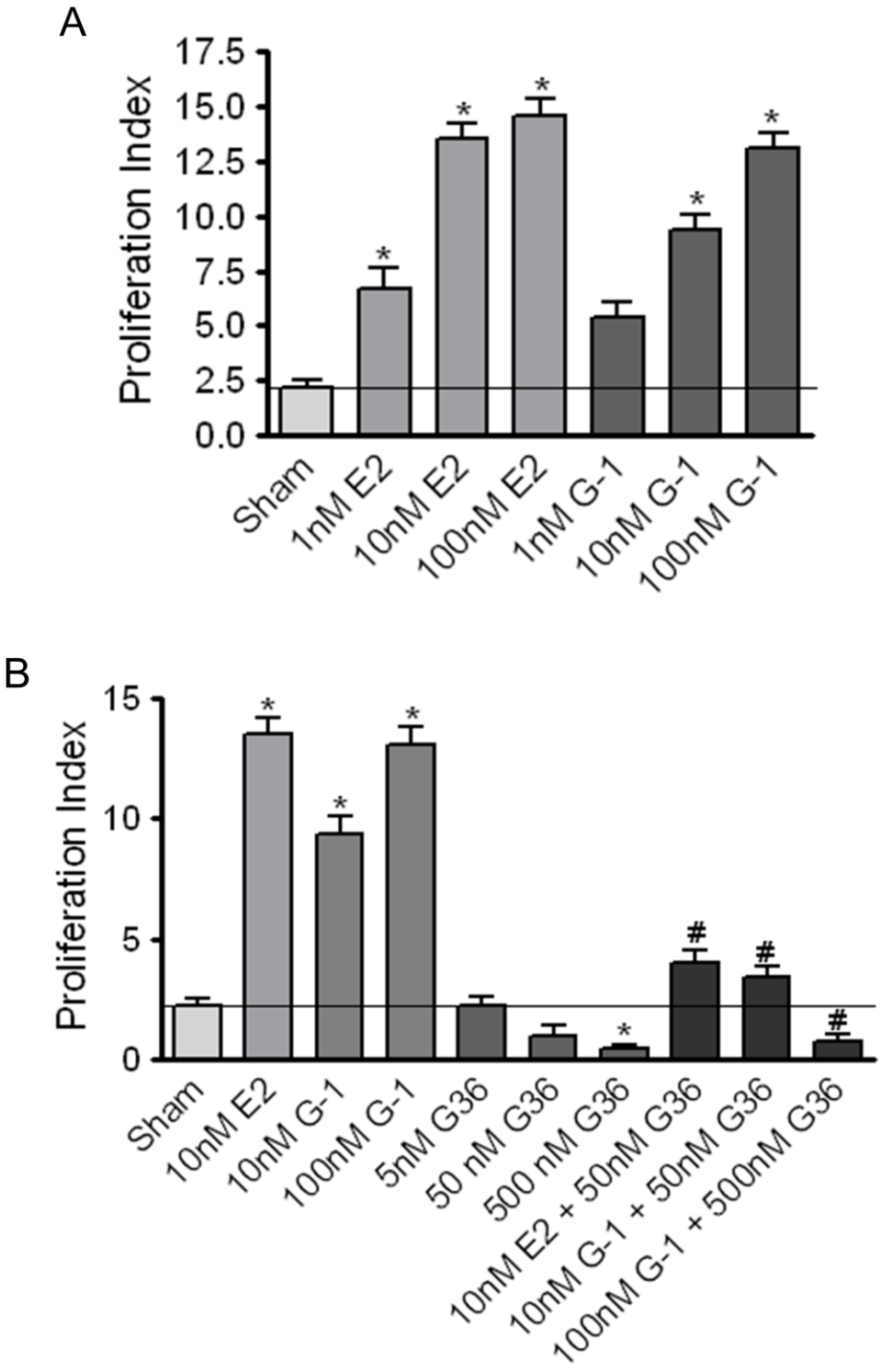


Figure 2.10 E2 and G-1 promote proliferation in human breast tissue

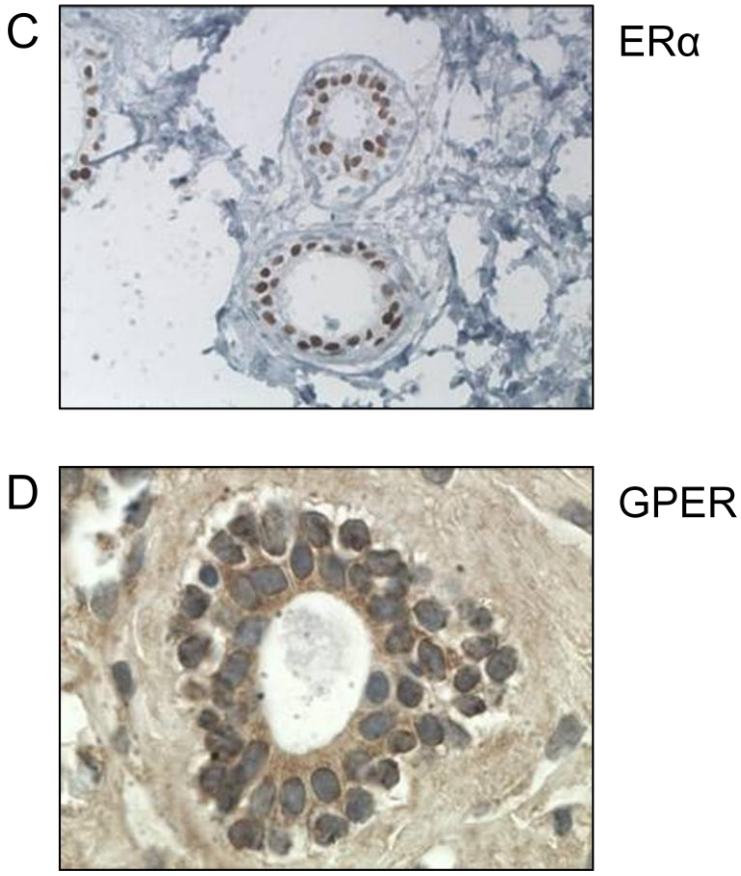
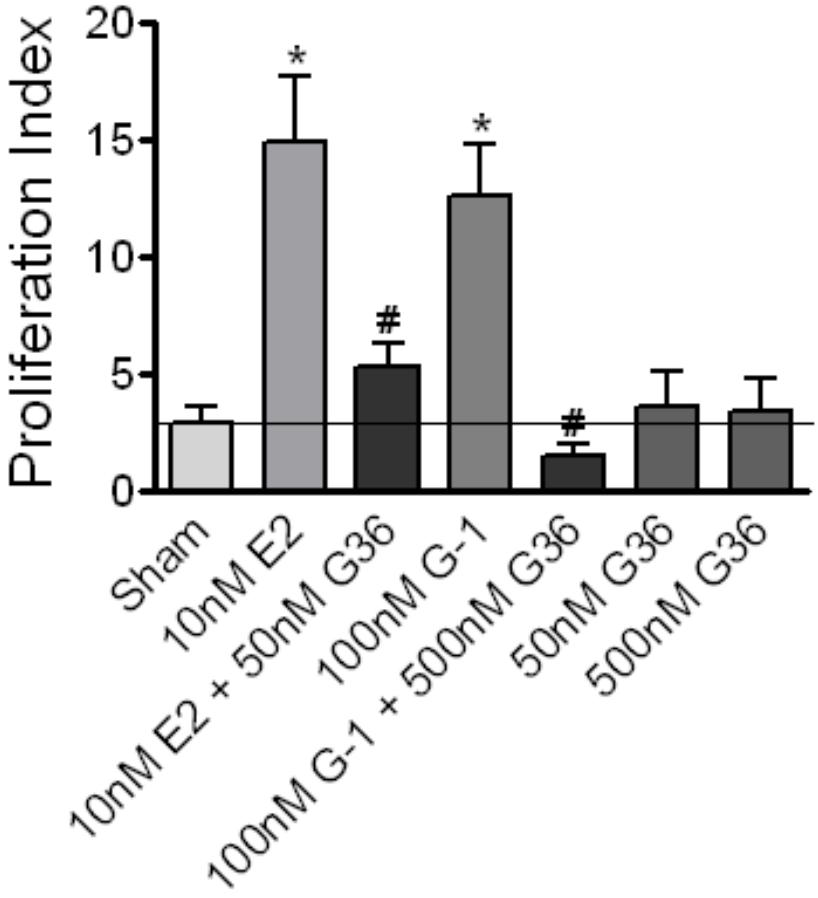


Figure 2.11 E2 and G-1 promote proliferation in tumorigenic human breast tissue



3. ESTROGEN REGULATION OF CELL DIVISON ORIENTATION AND ALVEOLAR MORPHOLOGY IN THE MAMMARY GLAND

3.1 Abstract

Estrogen (17β-estradiol, E2) is a potent mitogen, able to promote proliferation and morphological homeostasis in the mammary gland. Classical estrogen receptors (ERs) ERα and ERβ, are known to mediate the effects of E2-induced morphogenesis in the mammary gland based on studies done with ER null mice; however it is unknown if the novel G protein-coupled estrogen receptor (GPER) mediates the effects E2 on breast epithelial morphogenesis. Since we have previously shown that GPER mediates the proliferative effects of E2 in the breast, and E2-induced proliferation promotes ductal elongation and morphological development of the mammary gland, we hypothesize that GPER is mediating E2-induced morphogenesis in the breast. To determine the involvement of GPER in E2-induced breast epithelial morphogenesis, we utilized an *ex vivo* model to culture human breast tissue in the presence of E2, GPER-selective agonist G-1 and GPER-selective antagonist G36. We also used a 3-D cell culture model of MCF10A breast epithelial cells to answer questions of morphology and regulation of mitotic spindle orientation.

We found that E2 and G-1 stimulation of human breast tissue for seven days led to distinct morphological characteristics within alveolar structures in the mammary gland. E2 led to an increase in luminal epithelial layers, and this effect was not abrogated by G36, suggesting the E2-induced increase in epithelial height is mediated by classical ERs and not GPER. G-1 stimulation of breast

tissue resulted in an increase in the area of the lumen in alveolar structures (luminal area). G36 was able to significantly reduce the increased luminal area promoted by G-1 stimulation, suggesting this phenotype is mediated by GPER.

We also found that MCF10A 3-D spheroids treated with E2 exhibited mitosis in which the mitotic spindle had been rotated to be perpendicular relative to the basement membrane, whereas sham and G-1 treated mitotic MCF10A cells retained mitotic spindle orientation parallel relative to the basement membrane. We investigated E-cadherin expression in the human breast tissue, since adhesion molecules are known to be important for guiding mitotic spindle orientation during proliferation, and we found that E2 and G-1 have different effects on E-cadherin expression. E2 stimulation of breast tissue, after seven days increased E-cadherin expression whereas G-1 stimulation decreased E-cadherin expression at cell-cell junction in alveolar structures. We observed increased FAK activation in G-1 treated MCF10A cells, and since FAK is a known binding partner of Src and Src is activated downstream of GPER, it is possible that FAK/Src phosphorylation of E-cadherin is leading to its downregulation in human breast tissue. The relationship of E2, GPER and mitotic spindle orientation is complex but our results do support a role for GPER in E2-induced breast epithelial morphogenesis.

3.2 Introduction

E2 regulates breast epithelial morphogenesis and maintenance of mammary gland homeostasis. This has been shown through studies in estrogen receptor knockout animals, where the mammary gland remains a rudimentary structure due to the lack of ERα- induced proliferation and subsequent branching morphogenesis, occurring during development (Brisken and O'Malley, 2010). E2 induces changes in human breast tissue cultured *ex vivo* after one week of stimulation (Eigeliene et al., 2006), in which E2 treatment led to a multi-layered luminal epithelium and increased luminal epithelial cell height, concomitant with increased proliferation in ductal/alveolar structures in breast tissue; however control-treated tissue maintained a single layer of luminal epithelial cells (Eigeliene et al., 2006).

A possible mechanism influencing breast morphology is the orientation of cell division. Cells divide in an orientation dependent upon the position of the mitotic spindles, which occurs perpendicular to the final plane of division (**Fig. 3.1**). In the case of simple epithelia, mitotic spindle orientation relative to the underlying basement membrane will determine whether cell division results in two daughter cells lying parallel with or perpendicular to the basement membrane. In order to maintain the single layer of epithelial cells, cell division parallel to the basement membrane predominates (Perez-Moreno et al., 2003). Loss of parallel cell division orientation, e.g., due to signals that promote rotation in the mitotic spindle, would disrupt epithelial tissue morphology and allow for vertical tissue expansion. Morphology such as this is observed in early breast

cancers including Ductal Carcinoma In Situ (DCIS), where epithelial cells fill the lumen, rather than maintaining simple cuboidal epithelial morphology. Other factors, such as evasion of apoptosis in central-located cells, may also be playing a crucial role in this type of dysmorphology; however, it is interesting that E2 is reported to induce a rotation in the mitotic spindle apparatus (perpendicular to the basement membrane) in hormone responsive tissues including the endometrium (Gunin, 2001) and the prostate (Liu et al., 2008), suggesting this could also occur in the mammary gland. Although mechanisms regulating the mitotic spindle are still being elucidated, establishment of epithelial polarity and the formation of cell-cell junctions and cell-ECM interactions seem to be important determinants in mitotic spindle orientation (van Roy and Berx, 2008; Wheelock and Johnson, 2003). Thus, factors such as junctional proteins, specifically E-cadherin in adherens junctions between cells have the potential to contribute to the generation of polarity cues that are important for orienting mitotic spindles during cell division (den Elzen et al., 2009; Inaba et al., 2010; Kunda and Baum, 2009; Toyoshima and Nishida, 2007).

E-cadherin, a component of the adherens junctions, is member of a family of functionally related transmembrane glycoproteins that mediate calcium-dependent cell adhesion and maintenance of epithelial structures. The ectodomain of E-cadherin mediates homophilic ligation between epithelial cells, while the cytoplasmic tails of E-cadherin interact with p120-catenin and β-catenin to link E-cadherin to the actin cytoskeleton (van Roy and Berx, 2008). In the mammary gland, E-cadherin is expressed in luminal epithelial cells, and has

been shown to play a role in the maintenance of differentiation, integrity of luminal cell-cell contacts, assembly of other junctional components including tight junctions and desmosomes, and correct apicobasal cell polarity within alveolar structures (Daniel et al., 1995). Blocking the function of E-cadherin with specific antibodies has been shown to effect cell division and overall integrity of alveolar structures (Daniel et al., 1995), highlighting the importance of E-cadherin in breast epithelial morphogenesis. Adherens junctions, and specifically E-cadherin, are the site of interaction between the cell cortex and the astral microtubules, which are the guiding microtubules during orientation of the mitotic spindle (Ligon and Holzbaur, 2007). A study performed by den Elzen *et al* in 2009 demonstrated that E-cadherin was required for proper planar cell division within mammalian epithelial cells (including in MCF10A breast epithelial cells), and in the absence of E-cadherin, proper mitotic spindle orientation was perturbed (den Elzen et al., 2009). A requirement for E-cadherin in the establishment of proper mitotic spindle orientation has also been demonstrated in the drosophila epithelium (Inaba et al., 2010).

In a tumor setting, disruption of adherens junctions and decreased E-cadherin expression has been associated with and contributes to the epithelial-to-mesenchymal transition, increased proliferation and invasion in many types of cancer including breast cancer and is therefore thought of as a tumor suppressor protein. The vital role E-cadherin plays in the establishment of epithelial polarity and proper mammary gland morphology, its role in regulating mitotic spindle

orientation and its role as a tumor suppressor protein prompted us to inquire into the regulation of E-cadherin, specifically in the mammary gland.

The interaction of E-cadherin with the cytoskeleton, and thus the ability of E-cadherin to form cell-cell adhesions, is tightly regulated by tyrosine phosphorylation (Roura et al., 1999). Until recently, it was thought that regulation of adherens junctions was carried out by phosphorylation of β-catenin and p120-catenin directly by the non-receptor tyrosine kinase Src. Src phosphorylation of β-catenin and p120-catenin was shown to disrupt adherens junctions and cause dissociation of E-cadherin from the cytoskeleton (Behrens et al., 1993; Reynolds et al., 1994) More recently, it has been demonstrated that Src can directly phosphorylate E-cadherin, which leads to the recruitment and binding of the E3-ligase Hakai (Fujita et al., 2002; Shen et al., 2008). Hakai ubiquinates E-cadherin leading to its degradation (Shen et al., 2008), consistent with the hypothesis that Src directs E-cadherin away from a recycling endosome pathway into a lysosomal targeting pathway (Palacios et al., 2005). The ability of Src to mediate de-regulation of E-cadherin is further shown by the fact that inhibition of Src restores e-cadherin-mediated adhesion in breast cancer cells and reduces migration (Nam et al., 2002). Despite what is known, the relationship between Src and E-cadherin is complicated. Src has been shown to have a biphasic effect on E-cadherin function. At low levels of activation, Src exerts a supportive role of E-cadherin function and positively regulates E-cadherin-mediated cell adhesion, yet at higher levels of activation, Src impacts E-cadherin adhesion negatively (McLachlan et al., 2007). Src is found to be elevated in many different types of

cancer (Irby and Yeatman, 2000) and is associated with altered adhesion of the cell to the extracellular matrix (Jones et al., 2002). In colorectal cancer cells, overexpression of Src induced EMT, characterized by disorganization of E-cadherin and enhanced assembly of dynamic αvβ1 integrin-mediated focal adhesion-like structures (Avizienyte et al., 2002; Avizienyte et al., 2004) It was also observed that Src was localized to newly formed matrix adhesion sites (Avizienyte et al., 2002) whereas Src is commonly found to co-localize with E-cadherin at the sites of cell-cell adhesion (Calautti et al., 1998; Owens et al., 2000). Localization of Src at matrix adhesion sites requires Src-dependent tyrosine phosphorylation of focal adhesion kinase (FAK) (Avizienyte et al., 2002), suggesting Src-FAK signaling leads to a switch in adhesion type preference from E-cadherin mediated adhesion to integrin-mediated adhesion, and promotes a more motile phenotype. In accordance, expression of a Src-inactivating kinase in colon cancer cells decreases the number of focal adhesions and promotes E-cadherin-mediated cell-cell adhesion (Rengifo-Cam et al., 2004).

FAK is a protein tyrosine kinase recruited to sites of integrin clustering via interactions between its c-terminal domain and integrin-associated proteins paxillin and talin (Mitra et al., 2005). FAK association with β-integrins facilitates its activation by mediating autophosphorylation of FAK at Y397 (Mitra et al., 2005). Integrin-stimulated FAK phosphorylation at Y397 leads to conformational changes in FAK and creates a high-affinity binding site for the Src-homology 2 domain of Src-family tyrosine kinases (Playford and Schaller, 2004). The Src-FAK signaling complex further phosphorylates FAK on several tyrosine residues,

including Y576, Y861 and Y925 (Mitra et al., 2005; Playford and Schaller, 2004). Activated FAK at sites of αvβ1 integrin clustering has been shown to mediate the process of fibronectin (FN) matrix assembly, a process important for embryogenesis, wound healing and blood vessel formation (Francis et al., 2002; George et al., 1993). Fibronectin matrix assembly occurs when FN, secreted as a soluble dimer, is converted into an insoluble fibrillar structure in the extracellular matrix. Binding of soluble FN to αvβ1 integrin at the cell surface leads to the formation of focal adhesions, allowing for FN dimers to interact with one another to form insoluble FN fibrils in the ECM (Wierzbicka-Patynowski and Schwarzbauer, 2003). Interestingly, it was recently demonstrated by Quinn *et al* that the process of fibronectin matrix assembly is required for GPER-dependent transactivation of the EGFR in MDA-MB-231 breast cancer cells (Quinn et al., 2009). Quinn *et al* demonstrated that E2 activation of GPER lead to Gβγ-subunit protein-dependent activation of αvβ1 integrin, and activation of αvβ1 integrin is a prerequisite event for EGFR transactivation (Quinn et al., 2009). Quinn and colleagues were unable to measure FAK autophosphorylation (Y937) after GPER activation; and they attribute this to the possibility that GPER activation in MDA-MB-231 cells doesn't generate enough αvβ1 integrin activation to permit FAK activation; however, a role for E2 in fibronectin matrix assembly is demonstrated by the requirement for GPER (Quinn et al., 2009). E2-induced upregulation of Src and FAK have been observed in MCF-7 breast cancer cells (Planas-Silva et al., 2006; Planas-Silva and Waltz, 2007). E2 can induce cytoskeletal remodeling and EMT in endometrial cancer cells through activation of Src and FAK, and E2-

dependent migration was blocked with Src inhibitor PP2 in these cells (Acconcia F, Endocrinology, 2006).

These observations taken together (E-cadherin-induced regulation of mammary gland morphology and spindle orientation, Src-FAK-mediated de-regulation of E-cadherin, E2-induced regulation of Src, FAK and E-cadherin, GPER-dependent activation of Src, and the requirement of FN matrix assembly in GPER-dependent transactivation of the EGFR) led us to hypothesize that E2 is mediating breast epithelial morphogenesis through a mechanism involving Src-FAK-mediated downregulation of E-cadherin and altered regulation of mitotic spindle orientation.

To further investigate if E2-induced breast morphogenesis is mediated by GPER, proceeding through alterations in E-cadherin expression and mitotic spindle orientation we employed two models; human breast tissue culture *ex vivo* and MCF10A breast epithelial cells grown in a three-dimensional environment. As we have demonstrated that E2 and G-1 induce proliferation in human breast tissue stimulated for one week *ex vivo* (chapter 2), and evidence from ER -/- transgenic mice demonstrate E2's role in morphologic homeostasis of the mammary gland, we were interested in the effect of E2 and GPER-selective agonist on mammary gland morphology in normal human breast tissue cultured *ex vivo* as previously described (Eigeliene et al., 2006)).

3.3 Results

3.3.1 Estrogen and G-1 induce distinct morphological changes in breast tissue

Human breast tissue was cultured *ex vivo* on a raft culture system, as previously described in chapter 2. Tissue was stimulated for 7 days in culture with control (sham), E2, G-1 and combinations of E2/G-1 and GPER-selective antagonist G36. Tissue was fixed, paraffin embedded and sectioned. Tissue sections were stained with hematoxylin and eosin (H&E) to detect changes in morphology as a result of stimulation with E2 or G-1. Histological examination of human breast tissue revealed that that alveolar and ductal epithelia retained morphologic structure throughout the culture period of seven days when compared to tissue fixed at zero time, before the addition of treatments (**Fig 3.3a)**. The human mammary gland consists of a network of ducts converging at the nipple. A cross section of a mammary duct is shown in **figure 3.2**. In the center of the duct is the lumen, into which milk is secreted and stored during lactation. Surrounding the lumen is a single layer of luminal epithelial cells, responsible for synthesis of milk products during terminal differentiation. Adjacent to the luminal epithelial cells is a single layer of contractile myoepithelial cells, surrounded by the basement membrane.

Treatment of breast tissue with vehicle alone for one week did not induce changes in morphology, and ductal cross sections maintained proper morphology similar to the cartoon structure (**Fig 3.3a A**). Conversely, we observed that treatment with E2 for one week resulted in an increase in alveolar epithelial wall thickness (epithelial height; **Fig 3.3a B**) when compared to control treated

samples (**Fig 3.3a A**). Treatment with GPER agonist G-1 had no effect on epithelial height within alveolar structures; however G-1 did increase the luminal area within alveolar structures compared to control tissue samples (**Fig 3.3a C**). In order to determine if these effects were significant, epithelial height and luminal area measurements were carried out using Metamorph image analysis software. Metamorph quantitation revealed that E2 treatment over seven days in culture does stimulate a significant increase in human breast tissue when compared to control treated tissues (**Fig 3.3b A**). Metamorph quantitation also revealed that G-1 leads to a significant increase in luminal area, an effect that we observed by histological analysis of H&E stained tissue sections (**Fig 3.3b B**) The increase in luminal area seen with G-1 stimulation was not seen in control treated tissue, tissue treated with E2, GPER-selective antagonist (G36) alone, or a combination of E2 and G36 (**Fig 3.3b B**). In breast tissue samples treated with a combination of G36 and G-1, GPER-selective antagonist G36 was able to significantly reduce the luminal area increase induced by treatment with G-1 (**Fig 3.3b B**).

To this point, we have observed E2-induced, GPER-dependent proliferation both in MCF10A cells and in human breast tissue (chapter 2) as well as distinct morphologic changes in breast epithelium as a result of E2 and G-1 stimulation (**Fig 3.3a, b**). A possible mechanism coupling increased proliferation in breast tissue to changes in morphology is an alteration in the mitotic spindle orientation. As described earlier, mitotic spindle orientation determines the plane of cytokinesis, and thus the positioning of daughter cells at the end of mitosis. An

example of parallel versus perpendicular mitotic spindle orientation and the resulting placement of daughter cells is shown in **Figure 3.1**. It was demonstrated that E2 induces a rotation in the mitotic spindle in hormone dependent tissues, including the prostate and endometrium (Gunin, 2001; Liu et al., 2008). We were interested if activation of estrogen receptors in the breast epithelium, including GPER, is able to alter spindle orientation during mitosis, since this could explain the morphologic changes we observe in breast tissue. Increased proliferation in luminal epithelial cells in which the mitotic spindle is predominately oriented parallel to the basement membrane could presumably lead to alveolar or ductal structures in which the lumen is enlarged, similar to what we observe in the G-1 treated breast tissue (**Fig 3.4A**). In contrast, increased proliferation in luminal epithelial cells in which the mitotic spindle has a perpendicular orientation relative to the basement membrane could possibly lead to multiple luminal epithelial cell layers, which is what we observe in the E2 treated breast tissue (**Fig 3.4B**).

To determine the role of GPER activation in mitotic spindle orientation, we used a well characterized model of breast epithelial morphogenesis; MCF10A cells grown in a 3-D environment on Matrigel™ basement membrane. MCF10A cells are non-transformed breast epithelial cells, and when cultured on a basement membrane exhibit morphologic properties similar to alveolar structures in the human mammary gland (Debnath et al., 2003). When seeded on a layer of basement membrane in a chamber slide (**Fig 3.5**), MCF10A cells follow a strict order of events including proliferation to form multicellular structures, polarization,

survival of outer cell layer and apoptosis of cells in the lumen. It was demonstrated in chapter two of this study that E2 and G-1 induce proliferation in MCF10A cells in a 3-D environment. Their ability to proliferate in response to E2 and G-1 stimulation, together with their ability to from hollow, polarized structures similar to the human mammary gland, make MCF10As a good model to study mitotic spindle orientation.

3.3.2 Estrogen and G-1 treatment of MCF10A cells alters mitotic spindle orientation in a 3-D environment

MCF10A cells were grown in 8-well chamber slides as previously described (Debnath et al., 2003). Once cells had formed multicellular structures at day four, E2 and G-1 were added to the culture media. MCF10A spheroid structures were stimulated with E2 and G-1 for six days in culture, fixed and immunolabeled with phospho-histone H3 (green) to distinguish proliferating cells, and co stained with anti-α-tubulin (red), which labels microtubules, components of the spindle apparatus. Images of MCF10A acini containing mitotic cells were captured with confocal microscopy, and mitotic cells were categorized as either “parallel” or “perpendicular” based on their mitotic spindle orientation relative to the basement membrane. Cells in which the mitotic spindle was positioned from 0˚ to 45˚ to the basement membrane of the epithelium were regarded as parallel oriented (**Fig 3.6A, B**). Mitotic cells with poles aligned from 45˚ to 90˚ to the plane of the basement membrane were considered to be perpendicular oriented (**Fig 3.6C, D**). The ratio of mitotic MCF10A cells with either parallel or perpendicular mitotic spindles was quantified per treatment group (**Fig 3.6E**). E2

treatment caused a significant increase of mitotic cells with a perpendicular oriented mitotic spindle apparatus when compared to control treated cells (**Fig 3.6E**). Treatment of MCF10A cells with G-1 did not induce a change the orientation of the mitotic spindle when compared to control treated cells, although there was a slight increase in the number of parallel oriented mitotic cells in the G-1 treated sample (**Fig 3.6E**), thus G-1 stimulation seem to preferentially promote a parallel mitotic spindle orientation in MCF10A cells.

3.3.3 Activation of GPER in human breast tissue decreases E-cadherin protein expression at epithelial junctions in human breast tissue

The molecular regulation of mitotic spindle orientation in polarized epithelial cells remains under investigation, although there is evidence that spindle orientation is guided by cellular adhesion proteins, specifically E-cadherin (den Elzen et al., 2009; Le Borgne et al., 2002). E-cadherin is expressed between luminal epithelial cells in the mammary gland at adherens junctions (**Fig 3.7**). E-cadherin is a calcium dependent cell adhesion molecule that is linked to the actin cytoskeleton through its interaction β-catenin. Antibody-mediated disruption of E-cadherin-mediated adhesion in the mammary gland causes dissociation of the epithelium (Daniel et al., 1995), indicating the importance of this cell adhesion molecule in the maintenance of breast epithelial integrity. Further support for E-cadherin's role in mammary gland morphogenesis comes from a transgenic mouse model in which a dominant negative (DN) E-cadherin was expressed in the mammary gland (Delmas et al., 1999). During lactation in these mice, DN E-cadherin caused a decrease in cell-cell adhesion, discontinuity

of the basement membrane and a loss of epithelial polarity. E-cadherin's ability to regulate morphogenesis and polarity in a hormone responsive tissue indicates that it might also play a role in regulating mitotic spindle orientation.

To investigate the relationship between GPER activation and E-cadherin expression in the mammary gland, human breast tissue was treated for seven days with control, E2 or G-1, fixed, paraffin embedded and sectioned. Sections of breast tissue were immunolabeled with an antibody against E-cadherin. Confocal microscopy was used to capture images of E-cadherin staining in mammary tissue, and E-cadherin fluorescence intensity relative to total area of alveolar structures was determined with Slidebook image analysis software.

As expected, E-cadherin was found to be expressed and localized in breast tissue alveolar and ductal structures at cellular junctions in untreated tissue (**Fig 3.8A**). E-cadherin protein expression, measured by fluorescence intensity, was increased in a dose dependent manner in breast tissue treated with E2 (**Fig 3.8C-E, I**) when compared to sham treated tissue (**Fig 3.8B**). Although the E2-induced increase in junctional E-cadherin staining was not found to be significant ($p = .0588$), the trend is apparent from the images and the quantitation (**Fig 3.8C-E, I**). Treatment of breast tissue with G-1 had an opposite effect on E-cadherin protein expression. G-1 stimulation led to a dose dependent decrease in E-cadherin protein expression (**Fig 3.8F-H**), with 100nM G-1 stimulation leading to a significant decrease in E-cadherin protein expression when compared to control treated breast tissue (**Fig 3.8I**).

It is possible that G-1-induced E-cadherin protein downregulation is mediated by Src-FAK induced phosphorylation of E-cadherin or its binding partners, leading to its internalization and degradation, since this mechanism has previously been reported (Shen et al., 2008). Although there is little evidence directly linking FAK phosphorylation to E-cadherin de-regulation, a study from 2002 revealed that phosphorylation of FAK on Src-specific sites is required for Src-induced downregulation of E-cadherin in colon cancer cells (Avizienyte et al., 2002). It has also been shown that there is a role for Src-FAK-dependent signaling in the transition from E-cadherin mediated cell adhesion to integrin-mediated signaling, thus the promotion of a more motile phenotype of cancer cells (Avizienyte et al., 2002; Rengifo-Cam et al., 2004). This data, coupled with an observed E2-induced FAK upregulation in MCF-7 breast cancer cells (Planas-Silva and Waltz, 2007), and the requirement of fibronectin matrix assembly (of which FAK phosphorylation is an important step (Wierzbicka-Patynowski and Schwarzbauer, 2003)) in GPER-dependent transactivation of the EGFR (Quinn et al., 2009) prompted us to investigate whether GPER activation in MCF10A cells leads to increased FAK phosphorylation.

3.3.4 Activation of GPER increases FAK phosphorylation in MCF10A cells

MCF10A cells were stimulated for 5 or 10 minutes with vehicle, E2, or G-1, lysed and protein extracted for western blot analysis. Western blots containing MCF10A cell lysates were probed for FAK activation; (Y397), the autophosphorylation site that becomes activated on FAK recruitment to focal adhesions (Mitra et al., 2005). Western blot analysis revealed that 100nM G-1 led

to a significant increase in phosphorylated FAK after ten-minute stimulation (**Fig 3.9**). E2 also led to an increase in FAK activation at 10 minutes, although the increase was not significant (**Fig 3.9**). Stimulation with positive control EGF also induced FAK phosphorylation after five minutes of stimulation when compared to control treated cells at the equivalent time point (**Fig 3.9**).

3.4 Discussion

E2 plays an important role in promoting proliferation and branching morphogenesis during development (Brisken and O'Malley, 2010). E2's ability to promote proliferation and influence morphogenesis in the breast apart from a developmental setting has also been demonstrated (Eigeliene et al., 2006). The overall goal of this study was to determine if GPER mediates E2-induced processes in the breast including proliferation and epithelial morphogenesis. Since we demonstrated in chapter 2 that E2-induced proliferation in breast tissue was partially dependent on GPER, we investigated here whether GPER activation promotes morphologic changes in mammary epithelium, and possible mechanisms regulating GPER-dependent morphogenesis.

We used a previously characterized *ex vivo* model ((Eigeliene et al., 2006) to culture human breast tissue for seven days in the presence of control, E2, or GPER-selective agonist, G-1, and antagonist, G-36 We found that after one week of stimulation with E2, alveolar structures had an increased epithelial height (**Fig 3.3a B**) when compared to control treated tissue (**Fig 3.3a A**); an effect previously demonstrated (Eigeliene et al., 2006). G-1 stimulation had no effect on the epithelial height, nor was G36 able to abrogate the E2-induced increase in epithelial height, suggesting this effect is mediated by classical ER's and not GPER. G-1 stimulation did however lead to an increase in the luminal area (**Fig 3.3a C**) when compared to control treated tissue, and this effect was abrogated by GPER-selective antagonist G36, suggesting this effect is mediated by GPER in breast tissue. Human breast tissue does express both classical ER's and

GPER (**Fig 2.10 C, D**), so it is possible that E2 is mediating different morphological effects through both receptors. It is interesting that E2 didn't stimulate an increase in luminal area in addition to an increase in epithelial height, since E2 is able to activate both ERα/β and GPER. This data suggests that E2-induced epithelial height increase (mediated by ERα) is a dominant phenotype relative to increased luminal area mediated by GPER. A future experiment to determine if this hypothesis is correct would be to culture breast tissue with both E2 and G-1, and see the resultant phenotype (increased epithelial height vs. increased luminal area). This experiment could also be modified to include tamoxifen in combination with E2 and G36. Tamoxifen in the breast acts as an anti-ERα yet is also able to activate GPER. I would hypothesize breast tissue treated with E2, tamoxifen and G36 would retain a morphology similar to control treated tissue, since tamoxifen would inhibit E2-induced activation of ERα (presumably inhibiting increased epithelial height) and G36 would inhibit tamoxifen-induced activation of GPER (presumably inhibiting increased luminal area).

We have demonstrated E2- and G-1-dependent proliferation in human breast tissue as well as MCF10A breast epithelial cells (**Figure 2.1, 2.3, 2.10**). We have also observed distinct changes in morphology resultant from E2-and G-1-stimulation of breast tissue. Since mitotic spindle orientation is directly linked to proliferation and to the morphogenesis and organization of epithelial structures, and because it has been observed that E2 promotes a rotation in the mitotic spindle apparatus during proliferation of the hormone-responsive murine uterus,

we chose to investigate whether E2-induced regulation of mitotic spindle orientation is mediated by GPER. Immunofluorescence analysis of MCF10A cells grown in 3-D cultures, labeled with mitosis marker p-histone H3 and an anti-alpha tubulin antibody revealed that E2 promotes a rotation of the mitotic spindle to that of a perpendicular orientation relative to the basement membrane (**Fig 3.6C-D, E**) in a significant number of cells, whereas control treated cells maintained a predominately parallel mitotic spindle orientation relative to the basement membrane (**Fig 3.6A-B, E**). While G-1 didn't stimulate a rotation in the mitotic spindle apparatus, acinar structures with increased G-1-induced proliferation, coupled with a predominately parallel oriented mitotic spindle could presumably result in an increased luminal area, similar to what we observe in G-1 treated breast tissue. Although the spindle orientation results observed in MCF10A cells agree with the morphologic phenotypes we see with E2 and G-1-stimulation of breast tissue, MCF10A cells only express one ER, GPER, unlike human breast tissue, which expresses all three, ERα/β and GPER. Qualitatively distinct GPCR activation through different ligands has previously been described (Mailman, 2007; Violin et al., 2010; Zidar et al., 2009) and it is possible that G-1 and E2 are promoting differential effects on mitotic spindle orientation mediated through the same receptor, GPER, in MCF10A cells.

We next asked if we could identify a cellular event linking E2 and G-1 stimulation to the spindle apparatus. Because there is evidence that E-cadherin can regulate mitotic spindle orientation in epithelial cells, and evidence of E2-induced de-regulation of E-cadherin at cell-cell junctions (Oesterreich et al.,

2003), we inspected E-cadherin expression in human breast tissue by confocal microscopy. E2 and G-1 had dissimilar effects on E-cadherin expression in human breast tissue; not surprising given that this tissue expresses all three estrogen receptors (ERα/β and GPER), and because the tissue responded differently to E2 and G-1 in terms of morphological characteristics. Breast tissue treated with E2 showed increased E-cadherin expression in a dose-dependent manner (**Fig 3.8C-E**), although the difference was not statistically significant relative to control treated tissue (**Fig 3.8B**). Treatment of tissue with G-1 reduced E-cadherin expression in a dose-dependent manner at cell-cell junctions (**Fig 3.8F-H**) when compared to control treated tissue. Our results showing E2-induced E-cadherin downregulation don't agree with previous a previous report of E2-induced upregulation of E-cadherin (Oesterreich et al., 2003); however this study was carried out in breast cancer cells, indicating E-cadherin regulation may be complicated by tissue type and normal vs. tumorigenic state. The G-1-induced downregulation we observe in human breast tissue is interesting in light of the G-1-increased luminal size we also observe in human breast tissue. In mice, disruption of E-cadherin in the mammary gland led to dissociation of the epithelial layer (Daniel et al., 1995). An interesting hypothesis to consider is that similar to blocking E-cadherin function with an antibody, G-1-mediated downregulation of E-cadherin at cell-cell junctions could be causing a slight dissociation, or loosening of the luminal epithelial layer, which when coupled to an increase in proliferation could lead to alveolar structures with enlarged lumens. A way to test this hypothesis would be to determine if GPER-selective antagonist G36 can

restore E-cadherin expression. It would also be interesting to see the effect on E-cadherin protein expression in breast tissue treated with both E2 and G-1, in order to determine which ligand (and/or receptor) has preference in terms of E-cadherin regulation. These experiments could also be carried out in MCF10A cells, where we would expect E2 and G-1 to induce E-cadherin downregulation since MCF10A cells only express GPER; however the differential effects due to E2 and G-1 on mitotic spindle orientation complicate this hypothesis.

A common mechanism to regulate E-cadherin expression is phosphorylation of E-cadherin cytoplasmic domain directly, or its binding partner β-catenin, by the non-receptor tyrosine kinase Src (Owens et al., 2000). Phosphorylation of E-cadherin has been shown to lead to its internalization and ubiquitin-mediated degradation (Fujita et al., 2002; Shen et al., 2008). A well known binding partner of Src, FAK, is also thought to mediate the phosphorylation and downregulation of E-cadherin (Quadri, 2012). In the context of GPER signaling, Src is known to be activated downstream of GPER, and fibronectin matrix assembly (a process that recruits FAK to focal adhesions at the membrane) is required for GPER-dependent EGFR transactivation (Quinn et al., 2009). We demonstrated in chapter two that Src is required for GPER-dependent proliferation in MCF10A cells (**Fig 2.5C**), so we next determined if E2 or G-1 stimulation modulated FAK activation in MCF10A cells. By western blot analysis we demonstrated that E2 and G-1 stimulate an increase in p-FAK (Y397), and the G-1-induced increase in p-FAK is significant at 10 minute stimulation (**Fig 3.9**)

Observations to this point suggest that activation of GPER in the mammary gland leads to an increase in Src and FAK. These kinases could presumably phosphorylate E-cadherin at cell-cell junctions, leading to the de-regulation of E-cadherin and downstream morphological consequences of this could include breast epithelial structures with increased lumen size. Data also suggests that classical ERs are also contributing to breast epithelial morphogenesis by promoting changes in mitotic spindle orientation during cellular proliferation. More work will be needed to elucidate the specific mechanism of E2-induced breast epithelial morphogenesis; however it is apparent that GPER is contributing to this process.

3.5 Materials and Methods

3.5.1 Reagents

Dulbecco's Modified Eagle Medium (DMEM), phenol red-free DMEM, E2, fetal bovine serum (FBS), normal goat serum (NGS), insulin, cholera toxin, transferrin, hydrocortisone and prolactin were from Sigma (St. Louis, MO). Recombinant epidermal growth factor (EGF) and penicillin/streptomycin (P/S) were from Invitrogen (Carlsbad, CA). Bovine serum albumin (BSA) was from Amresco (Solon, OH). Growth factor reduced phenol red-free Matrigel™ was from BD Biosciences (San Jose, CA). G-1 was synthesized as described (Bologa et al., 2006) and provided by Jeffrey Arterburn (New Mexico State University, Las Cruces, NM). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). Small interfering RNA (siRNA) was purchased from Dharmacon RNAi Technologies (Dharmacon, Lafayette, CO): ON-TARGET plus SMARTpool siRNA for GPER (L-005563-00) and ON-TARGETplus siControl Non-Targeting siRNA (D-001810-02).

3.5.2 Inhibitors and antibodies

G36 was synthesized as described (Dennis et al., 2011) and provided by Jeffrey Arterburn (New Mexico State University). Rabbit p-Histone H3 antibody and mouse β-actin antibody were from Millipore (Billerica, MA). Mouse anti-E-cadherin antibody and mouse antiphospho-FAK (Y397) antibody were from BD Biosciences (San Jose, CA). Mouse anti-α-tubulin antibody was from Sigma (St. Louis, MO). Goat antirabbit IgG-Alexa 488-conjugated secondary antibody and Goat antimouse IgG-Alexa 533-conjugated secondary antibody were from

Invitrogen (Carlsbad, CA). Goat antirabbit IgG-HRP-conjugated antibody was from GE Healthcare (Princeton, NJ) and goat antimouse IgG-HRP-conjugated antibody was from Cell Signaling.

3.5.3 Cell Culture

Immortalized, non transformed MCF10A human breast epithelial cells (ATCC, Manassas, VA; catalog number CRL-10317) were maintained in MCF10A complete media (DMEM/F-12 supplemented with 5% dextran-charcoal-stripped fetal bovine serum, 10 µg/mL insulin, 100 ng/mL cholera toxin, 0.5 µg/mL hydrocortisone, 20 ng/mL recombinant epidermal growth factor and 1% penicillin/streptomycin). Cells were cultured in a humidified atmosphere containing 5% CO2 at 37◦C. Overnight cell synchronization for immunoblot analysis was performed as previously described (Chou et al., 1999). After overnight synchronization, cells were stimulated for 24 hours with vehicle control, 17-beta estradiol (1nM to 100nM) (E_2), G-1 (GPER-selective agonist) (1nM to 100nM), and G36 (GPER-selective antagonist; 5nM to 500nM).

MCF10A cells were also grown in Growth Factor Reduced phenol red-free Matrigel™ on 8-well chamber slides (BD Falcon, San Jose, CA). Approximately 5,000 MCF10A cells were seeded on 40µL of Matrigel in each chamber on the slide. Cells were suspended in growth media (described above) supplemented with 2% Matrigel. The media was changed every two days, and after four days in culture, various treatment compounds (described above) were added to growth media. Cells continued to grow in Matrigel until day 10, whereby they were fixed

with 4% PFA in PBS for 15 minutes at room temperature. Immunofluoresence assays were carried out of MCF10A cells in 2D and 3D according to a method previously described (Debnath et al., 2003). Images were captured on a Zeiss LSM 510 confocal microscope (Carl Zeiss Inc., Oberkochen, Germany), using x400 total magnification and an optical thickness of 0.7 µM.

3.5.4 Tissue Samples

Human breast tissue was acquired from female patients who were undergoing reduction mammoplasty surgery between November 2007 and January 2011. Normal breast tissue remaining after pathological testing was collected and used in this study. Successive specimens were collected at University of New Mexico hospital (UNMH), and received from the cooperative human tissue network (CHTN Western division- Vanderbilt University, Nashville, TN), a division of the National Cancer Institute. This study protocol was approved by University of New Mexico Health Sciences Center institutional review board (IRB). Tissue collected at UNMH was transported to the laboratory on ice in D-MEM/F-12 medium containing 1% P/S, within 1-2 hours of surgery. Tissue obtained from CHTN was shipped overnight on ice in RPMI medium (Sigma) supplemented with 1% P/S. The tissue was dissected into 3 mm^3 pieces in phenol-red free D-MEM/F-12 medium in order to exclude as much adipose tissues as possible, saving the collagenous connective tissue where the epithelial ducts and lobules are found

3.5.5 Organ Culture

Breast tissue was incubated according to a previously described method (Eigeliene et al., 2006), in which pieces of breast tissue are placed on sterile lens paper lying on stainless steel grids (our protocol modified this to use nylon grids) atop a 35mm tissue culture dishes inside a 10cm dish. For experiments done in breast tumor tissue, tissue was submerged in media in a 24 well plate. Tissue was incubated overnight in a humidified atmosphere with a mixture of 5% CO_2 and 95% air at 37˚C in phenol-red free D-MEM/F-12 medium supplemented with 1% P/S, 10ug/mL insulin, 3ug/mL prolactin, 4mg/ml transferrin and 1ug/mL hydrocortisone. Following overnight incubation to “rest” the tissue, additions were made to the medium in the inner tissue culture dish; including vehicle control, 17-beta estradiol (1nM to 100nM) (E_2), G-1 (GPER-selective agonist) (1nM to 100nM), and G36 (GPER-selective antagonist; 5nM to 500nM). Compounds were diluted in dimethylsulfoxide (DMSO); therefore control tissues were incubated in media supplemented with DMSO vehicle. Growth media was changed every two days and fresh treatments were added. Tissue was collected 7 days after the addition of treatments and fixed in 4% paraformaldehyde (PFA) in phosphate buffered saline (PBS) overnight at room temperature.

3.5.6 Tissue histology and Image Analysis

Tissue was dehydrated, embedded in paraffin, and sectioned. Paraffin sections (5µm) were mounted on Super-Frost Plus slides (Menzel- Gläser, Germany). After rehydrating sections through graded alcohol series followed by water, tissue was stained with hematoxylin and eosin (H&E) for histological

examination. Images were obtained under light microscopy at x400 magnification with the aid of a Zeiss 200M Axiovert inverted microscope (Carl Zeiss Inc., Oberkochen, Germany) and morphological measurements were made using metamorph image analysis software (Molecular devices, Union CA). Histomorphometric evaluation was carried out to determine the effects of different treatment on epithelial morphology. All morphometric measurements were done on H&E-stained sections. Using a digital drawing system run by Metamorph, acinar structures were measured by outlining luminal epithelial cell layers, and the luminal space on the monitor screen with the computer mouse. Epithelial height (µm) was calculated as an average of 8 cross-sectional lines traced from the within the luminal epithelial cell layer, and luminal area (μm^2) was calculated by tracing the empty space within the luminal epithelial cells.

3.5.7 Indirect Immunofluorescence (Tissue)

For immunofluorescence staining, paraffin sections (5µm) were mounted on Super-Frost Plus slides (Menzel- Gläser, Germany). After rehydrating sections through a graded alcohol series followed by water, the slides were treated for antigen retrieval by boiling in a microwave oven in 0.01 M citrate buffer (pH 6.0) for 20 minutes. After a series of washes the sections were incubated with 0.1% Triton X-100 containing 3% NGS for 30 min at room temperature to permeabilize cells and block non-specific binding antibody. Tissue sections were then incubated with primary antibodies diluted in PBS + 0.1% Tween-20 containing 3% NGS overnight at 4°C in a humid chamber. Following overnight incubation with primary antibody, tissue sections were washed and

incubated with species-matched Alexafluor conjugated secondary antibodies (Invitrogen) for 1 hour at room temperature in a dark chamber. Sections were incubated for 15 minutes with Topro-3 (Molecular Probes) to stain nuclei. Sections were mounted with Vectashield mounting media (Vector Labs) and sealed with nail polish. Images were captured on a Zeiss 200M Axiovert inverted microscope (Carl Zeiss Inc., Oberkochen, Germany) using x400 total magnification.

3.5.8 Western Immunoblotting

Cells were lysed in RIPA buffer supplemented with buffer supplemented with sodium fluoride (50 mM), sodium orthovanadate (1 mM), phenylmethylsulfonylfluoride (1 mM), and protease cocktail (1X). Cell lysate protein concentration was determined by performing a Bradford protein assay (Bio-rad, Hercules, CA). Equal protein concentrations per lysate were loaded on a 4-20% SDS-PAGE gel (Thermo-Scientific, Rockford, IL) and then transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA). After blocking in 5% nonfat dry milk for 1 hour at room temp, the membranes were incubated with primary antibodies at a 1:100 to 1:10,000 dilution in 3% BSA overnight at 4˚C with gentle rocking. After a series of washing, the blots were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG or goat anti-mouse IgG at 1:10,000 in 3% BSA for 1 hour at room temperature with gentle rocking. The blots were developed using Supersignal West Pico Chemilumiscent Substrate (Thermo Fisher). Films were then scanned and quantified using ImageJ software (National Institutes of Health, Bethesda, MD).

3.5.9 Cell Imaging and image analysis

For E-cadherin image analysis, Slidebook version 4.2 (Intelligent Imaging Innovations, Denver, CO) was used to determine fluorescence intensity in human breast tissue. This software measures the intensity of each pixel above a set intensity threshold in a demarcated area. The number of pixels above a certain threshold within this area is expressed as a percentage of the total number of pixels in the selected area.

3.5.10 Statistical analysis

Statistical analysis was performed using GraphPad Prism version 4.03 (La Jolla, CA). Analysis done with a one way analysis of variance (ANOVA) within Prism estimates the correlation of variables (acinar thickness, luminal area, etc) with treatment groups (sham, E2, G-1, G-36, etc). Pairwise comparisons of results between different treatment groups were determined using a one-way Analysis of Variance (ANOVA) followed by a Dunnett's test. Data represents the mean ± SEM of three or more separate experiments. P-values less than 0.05 were considered to be significant.

3.6 Figure Legends

Figure 3.1 Mitotic spindle orientation in adherent epithelial cells.

A: an example of an epithelial cell undergoing mitosis in which the mitotic spindle aligns parallel to the basement membrane. This will result in daughter cells that are next to one another and adjacent to the basement membrane. B: an example of an epithelial cell undergoing mitosis perpendicular to the basement membrane. This will result in stacked daughter cells, with one adjacent to the basement membrane, and the second protruding into the lumen.

Figure 3.2 Illustration of a human mammary gland duct in cross section.

A central lumen is surrounded by a single layer of luminal epithelial cells. Adjacent to these cells are a single layer of contractile myoepithelial cells, surrounded by the basement membrane.

Figure 3.3a E2 and G-1 treatment of human breast tissue results in increased epithelial height and luminal area respectively.

Hematoxylin and eosin staining of human breast tissue cultured *ex vivo* for one week with sham (A), 10nM E2 (B) or 100nM G-1 (C) highlights alveolar morphology. (Scale bar represents 50µM)

Figure 3.3b E2 and G-1 treatment of human breast tissue results in increased epithelial height and luminal area respectively.

Alveolar epithelial wall thickness (height, A) and luminal area (B) were measured on epithelial structures in breast tissue cultured *ex vivo* for seven days in the

presence of E2, G-1, G36, E2 + G36 or G-1 + G36, and compared with control treated tissue. Each group consisted of tissue samples from a minimum of five different patients. Results are expressed as mean ± SEM, and statistical significance ($P \leq 0.05$) was assessed by one-way ANOVA followed by a Dunnett's test-test. (*, significantly different relative to sham; #, significantly different relative to G-1)

Figure 3.4 Hypothetical effect of mitotic spindle orientation on alveolar morphology in breast tissue.

Breast epithelial proliferation under conditions that increase the percentage of mitotic spindles with alignment parallel to the basement membrane could result in alveoli with increased lumen size (A). Proliferation in cells under conditions that increase mitotic spindle rotation to a perpendicular orientation relative to the basement membrane could result in alveoli with increased luminal epithelial layers (B).

Figure 3.5 MCF10A model of breast morphogenesis.

Timeline of events in development of an MCF10A acinar structure grown in a 3-D environment on Matrigel™ basement membrane. (Figure taken from Debnath et al, 2003, *Methods,* 30; p 261)

Figure 3.6 Estrogen leads to a rotation of the mitotic spindle in MCF10A cells grown in Matrigel™

Mitotic cells were assessed by immunostaining with anti-pH3 (green) and spindle orientation was determined by immunostaining with anti-α Tubulin antibody (red). Mitotic spindles were determined to be either oriented parallel to the basement membrane (A,B) or perpendicular to the basement membrane (C,D) in cells grown in the presence of sham, E2 or G-1. Results are expressed as a ratio of parallel to perpendicular spindle orientation per treatment group and are presented as mean ± SEM. Statistical significance ($P \leq 0.05$) was assessed by Student's t-test. (*, significantly different relative to sham perpendicular percentage).

Figure 3.7 E-cadherin in mammary alveolar structures.

E-cadherin, a calcium dependent transmembrane cell adhesion protein, is expressed in breast luminal epithelial adherens junctions. E-cadherin interacts in homotypic fashion with other E-cadherin molecules on adjacent cells. The intracellular domain of E-cadherin is interacts with the actin cytoskeleton through β-catenin and α-catenin.

Figure 3.8. G-1 downregulates E-cadherin expression in human breast tissue.

E-cadherin expression was assessed in untreated human breast tissue (A) or tissue treated for seven days with Sham (B), E2 (C-E) or G-1 (F-H) by

immunofluorescence using an anti-E-cadherin antibody (green). Confocal images were acquired and quantitation of E-cadherin fluorescence intensity was performed using Slidebook™ image analysis software. The graph represents the average fluorescence intensity per area (in pixels) of alveolar structures for each treatment group (I). Each group consisted of tissue samples from a minimum of five different patients. Results are expressed as mean ± SEM, and statistical significance ($P \leq 0.05$) was assessed by one-way ANOVA followed by a Dunnett's test-test. (*, significantly different relative to sham)

Figure 3.9 G-1 induces FAK activation in MCF10A cells.

MCF10A cells were stimulated with indicated concentrations of EGF, E2 or G-1 for 5 or 10 minutes. Lysates were immunoblotted with antibodies specific for phospho-FAK (representative blot shown here). Equal protein loading was confirmed with β-actin. Histogram represents fold change in p-FAK protein. Data are representative of three independent experiments. Results are expressed as mean ± SEM and statistical significance ($P \leq .05$) was assessed by one-way ANOVA followed by a Dunnett's test. (*, significantly different relative to sham)

3.7 Figures

Figure 3.1 Mitotic spindle orientation in adherent epithelial cells

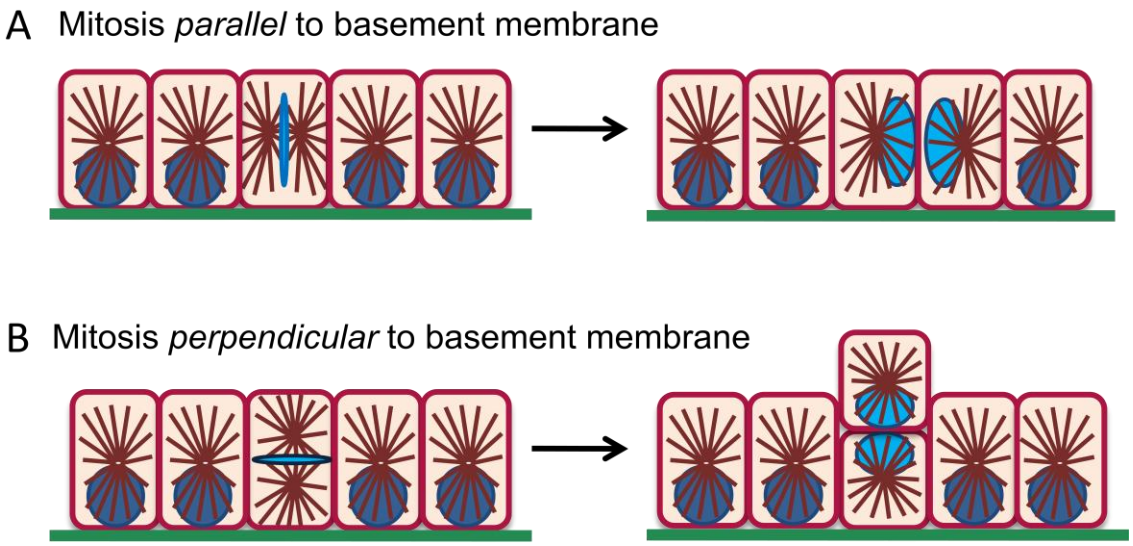


Figure 3.2 Illustration of a human mammary gland duct in cross section

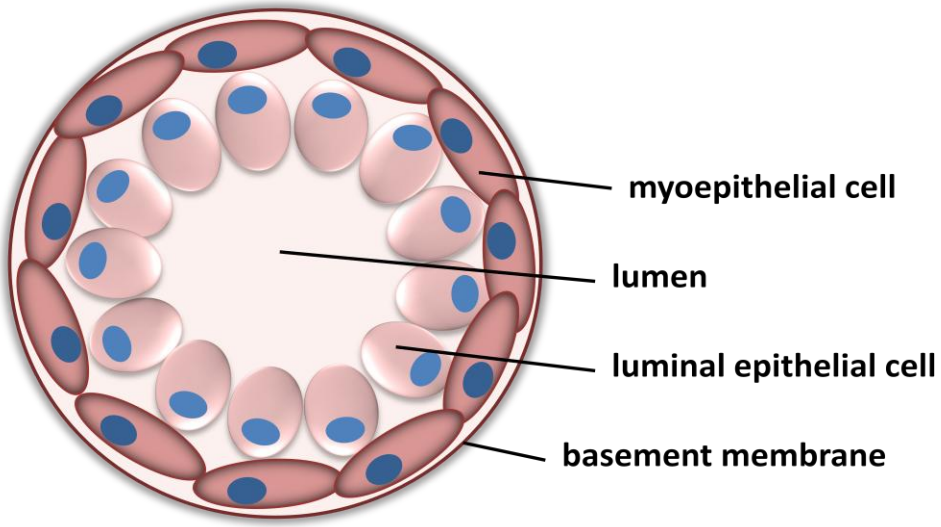


Figure 3.3a E2 and G-1 treatment of human breast tissue results in increased epithelial height and luminal area

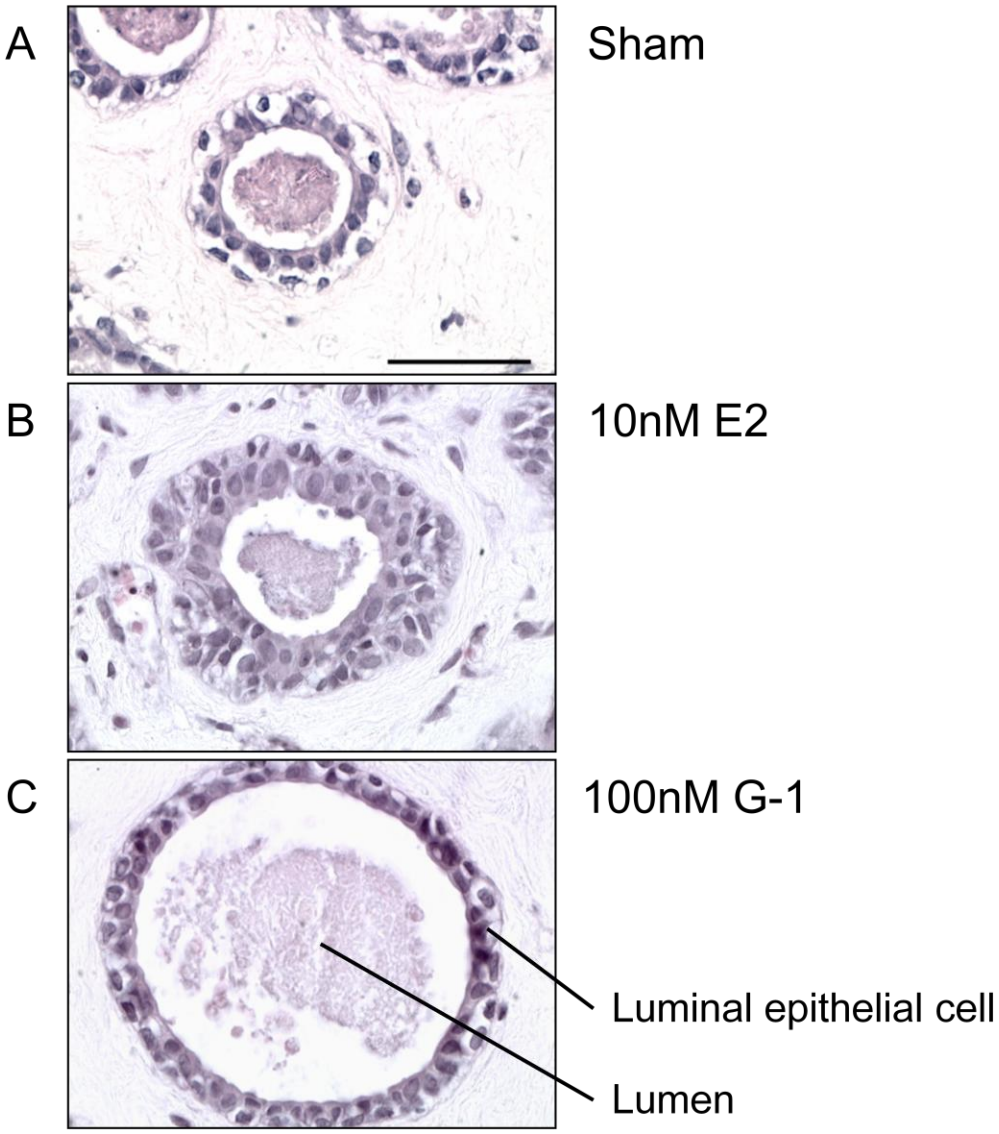
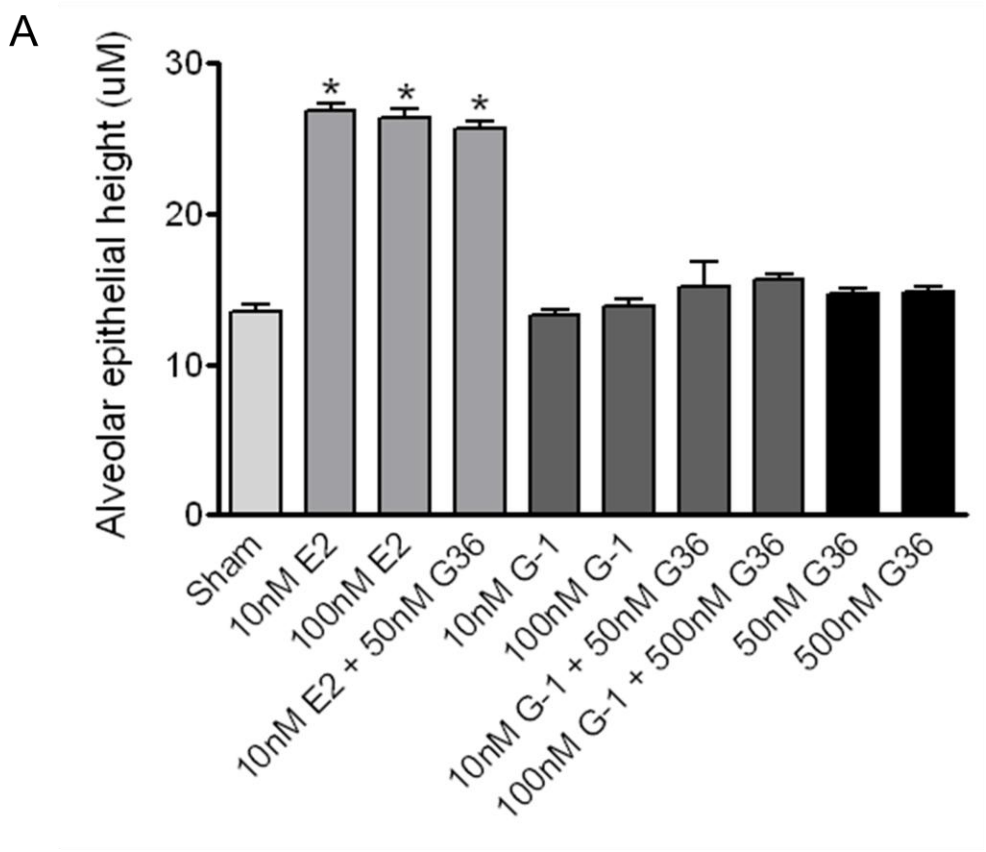
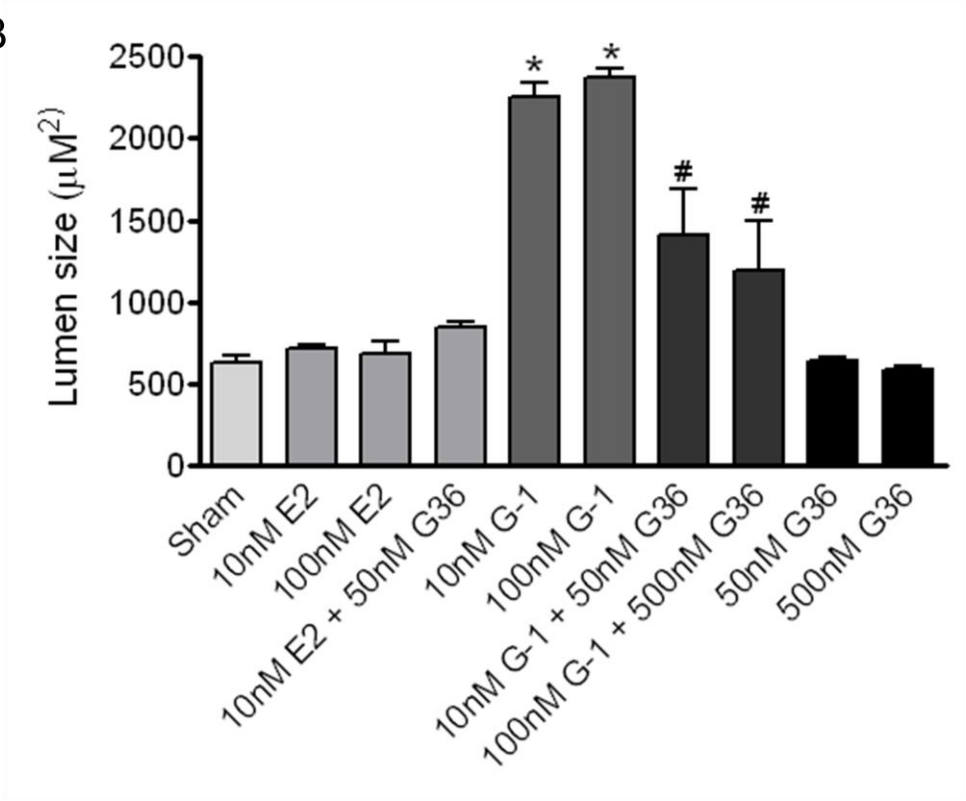


Figure 3.3b E2 and G-1 treatment of human breast tissue results in increased epithelial height and luminal area



B


Lumen size (μM2)
2500
2000
1500
1000
500
0
*
*
#
#
Sham
10nM E2
100nM E2
10nM E2 + 50nM G36
10nM G-1
100nM G-1
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10nM G-1 + 500nM G36
50nM G36
500nM G36

Figure 3.4 Hypothetical effect of mitotic spindle orientation on alveolar morphology in breast tissue

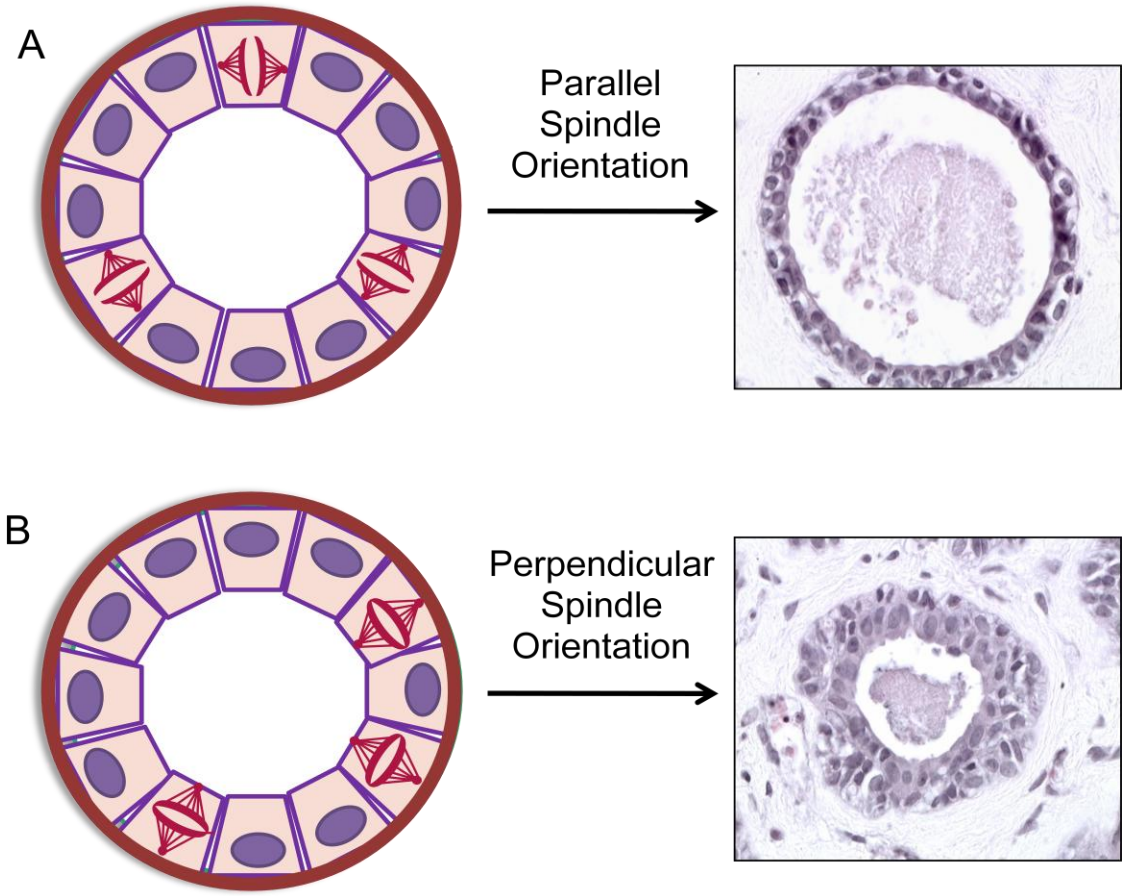


Figure 3.5 MCF10A model of breast morphogenesis

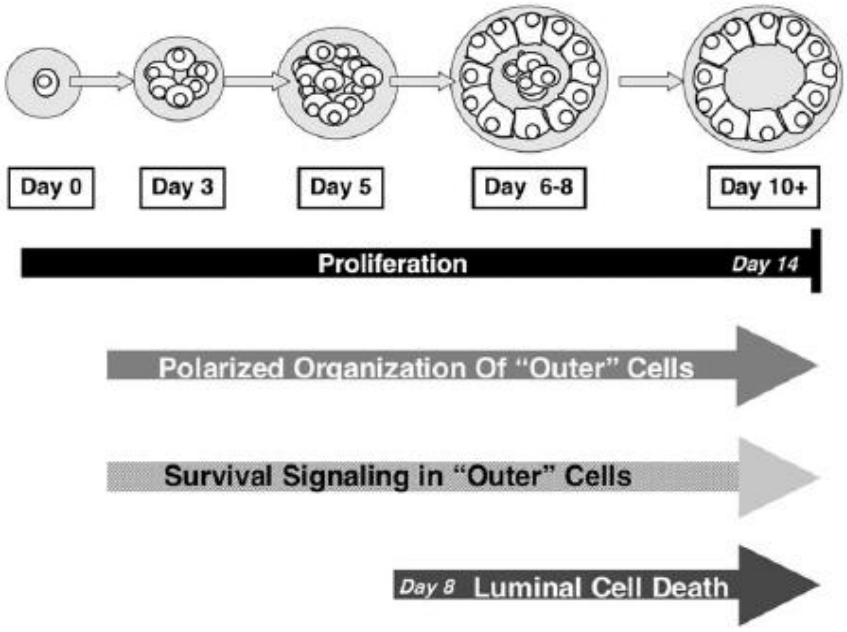


Figure 3.6 Estrogen leads to a rotation of the mitotic spindle in MCF10A cells grown in Matrigel™

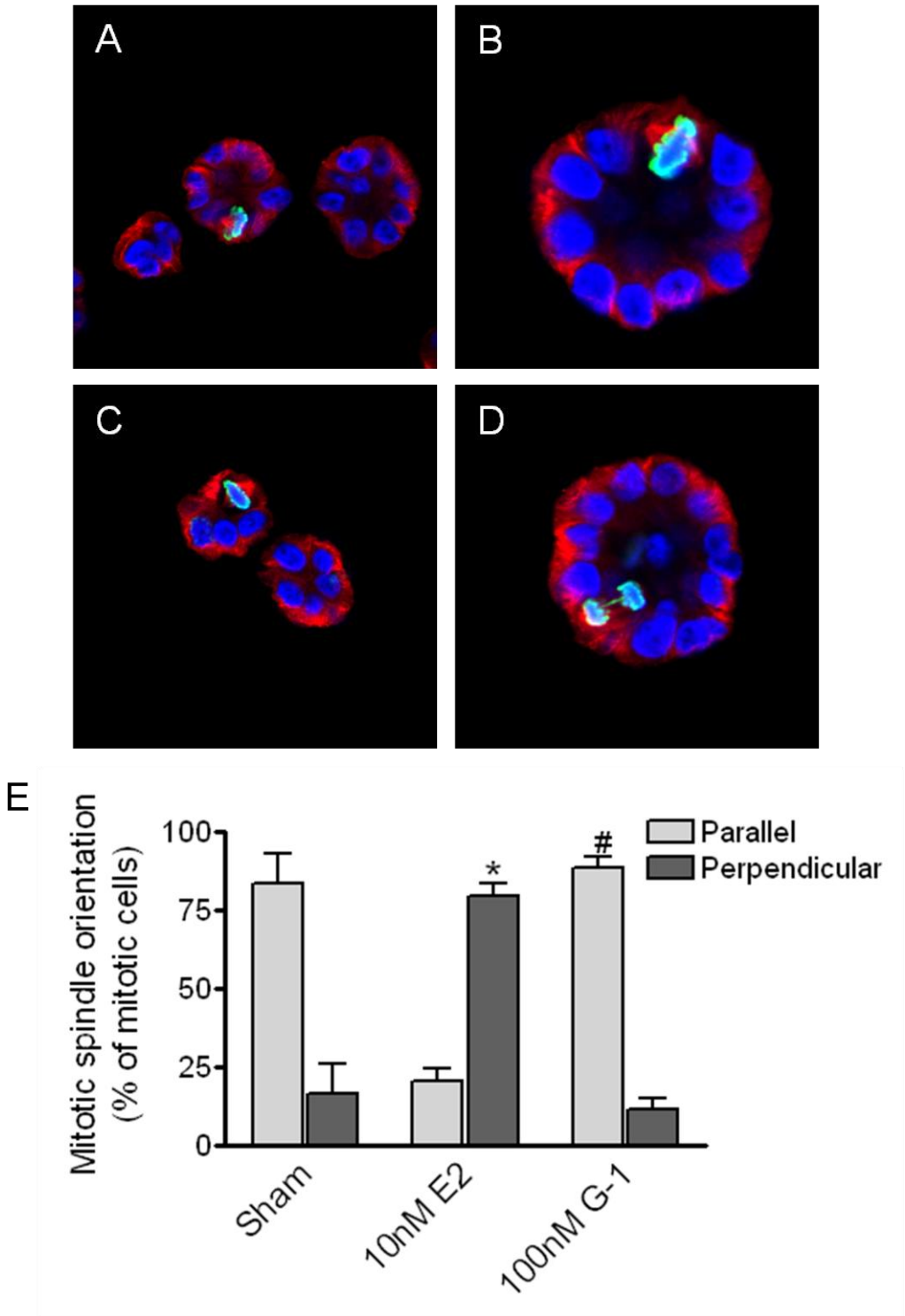


Figure 3.7 E-cadherin in mammary alveolar structures.

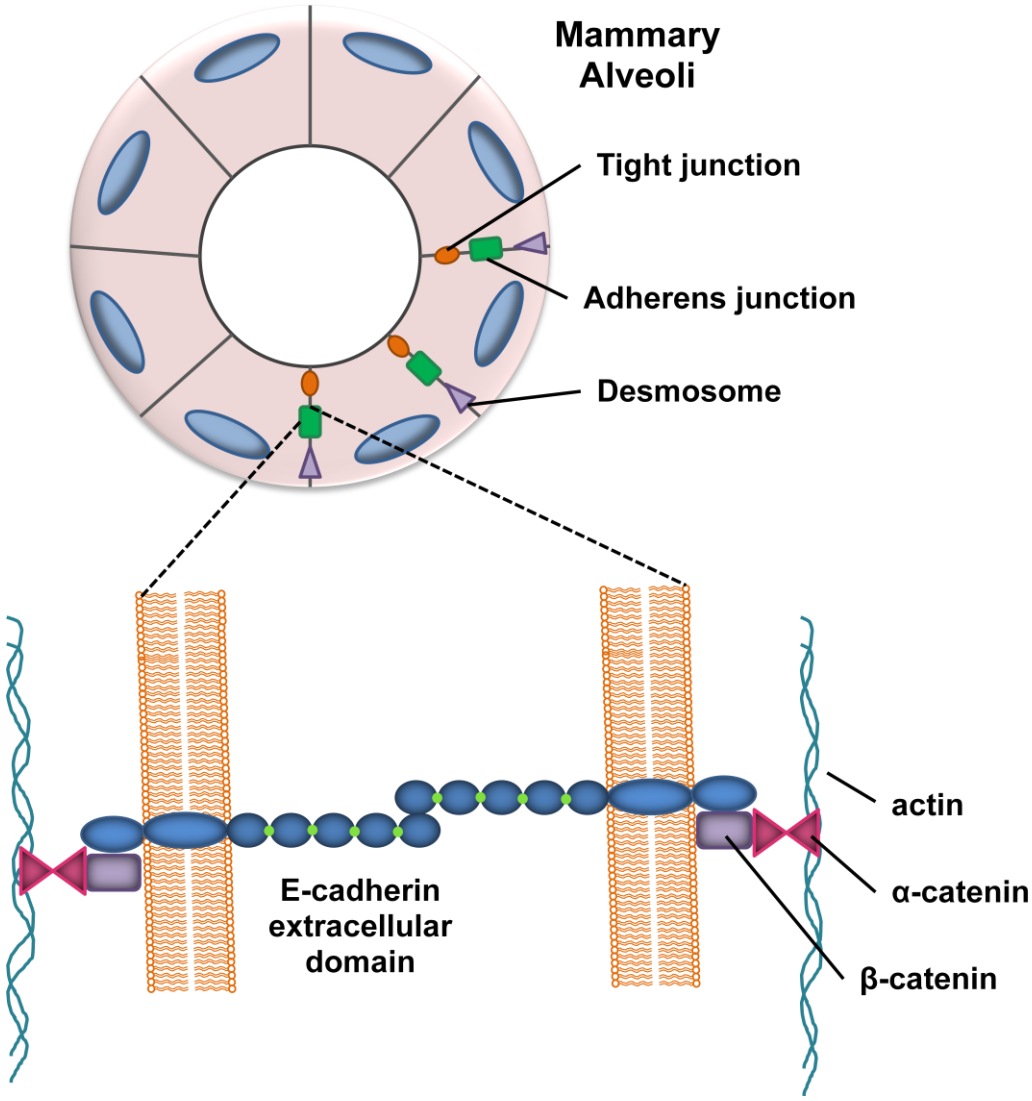


Figure 3.8 G-1 downregulates E-cadherin expression in human breast tissue

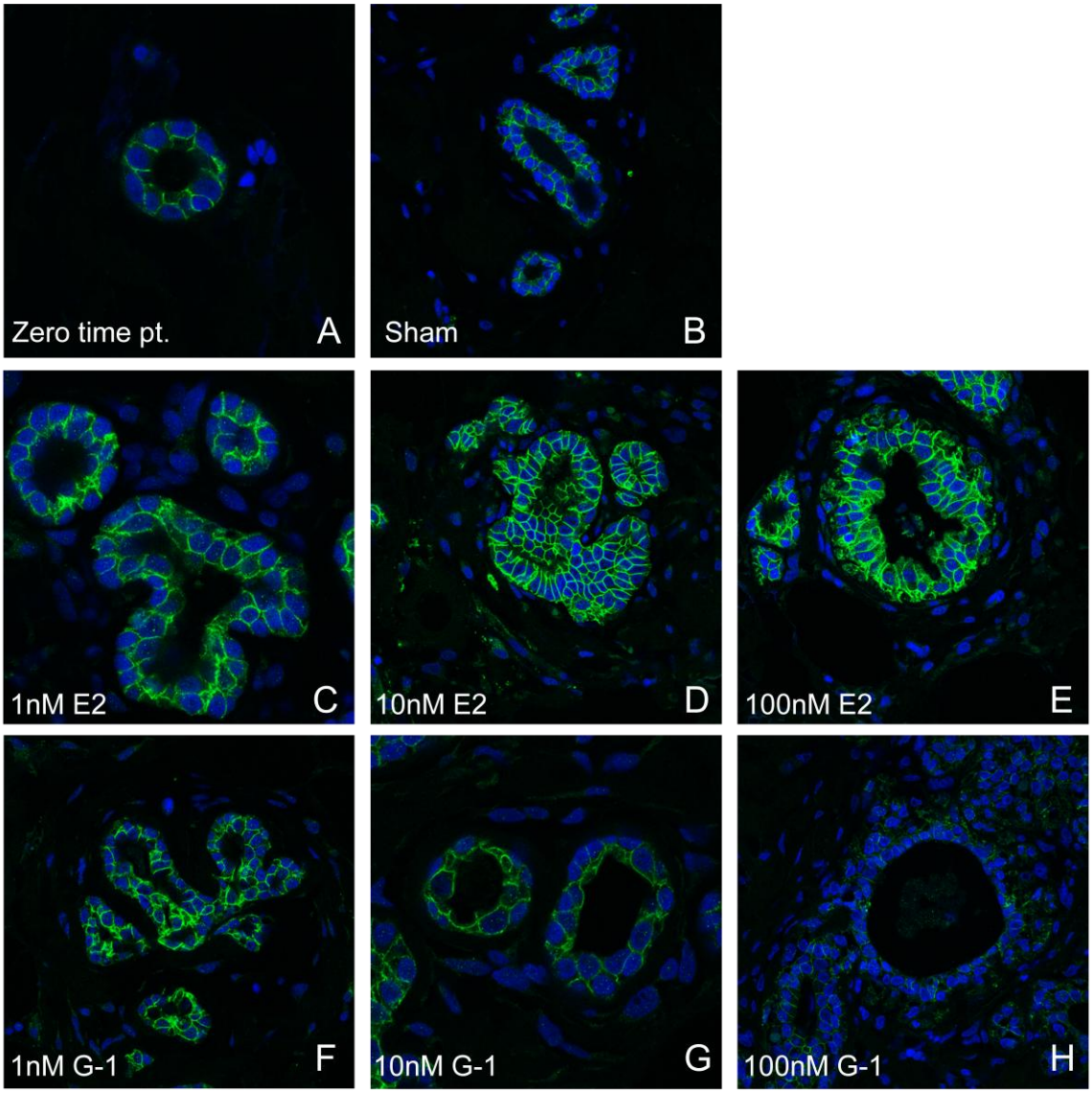


Figure 3.8 G-1 downregulates E-cadherin expression in human breast tissue

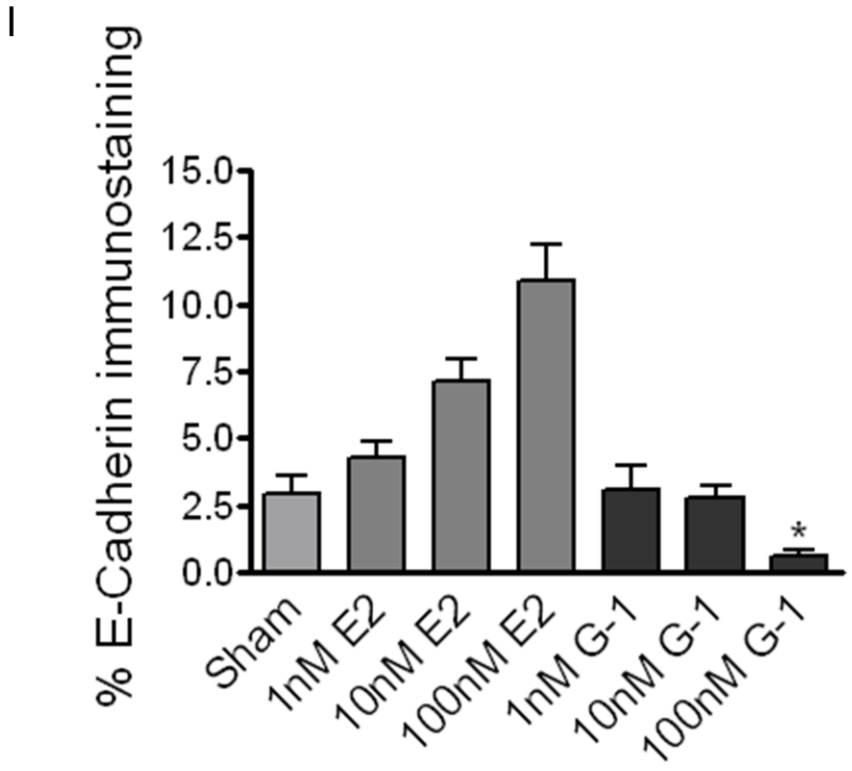
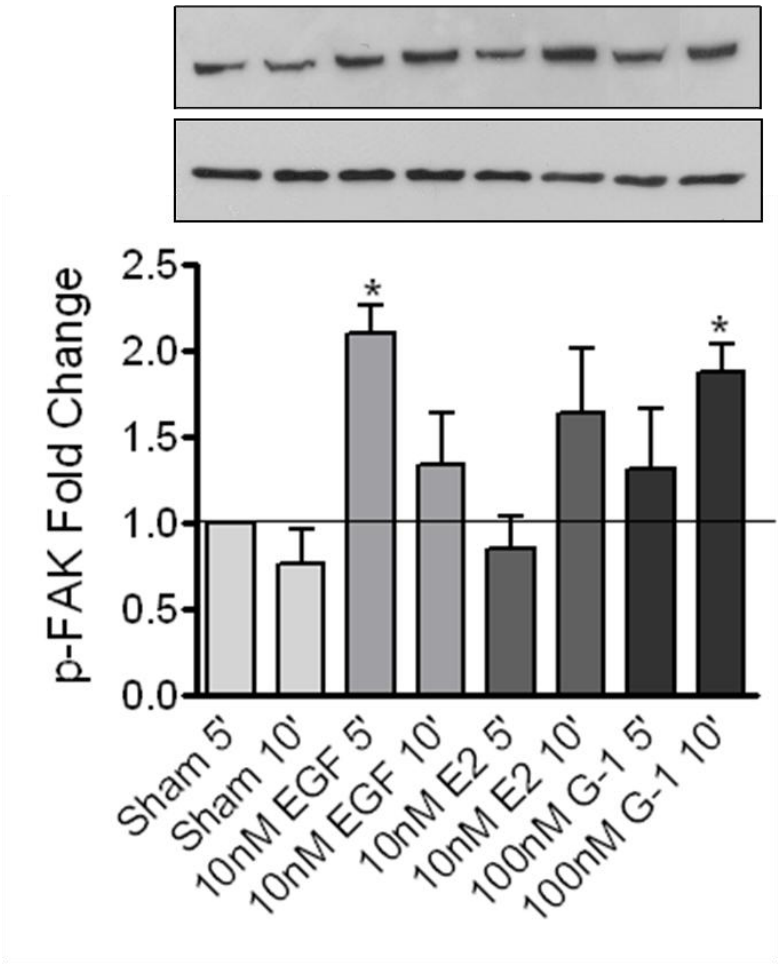


Figure 3.9 G-1 induces FAK activation in MCF10A cells



4. GPER REGULATION OF MALE REPRODUCTIVE PHYSIOLOGY

4.1 Abstract

Estrogen (17β-estradiol, E2) is a complex signaling molecule, regulating physiologic responses in diverse tissues in both male and female reproductive systems. Classically thought of as the female sex hormone, E2 also has a vital role in the male reproductive system, specifically in spermatogenesis. Spermatogenesis (the generation of haploid male gametes from diploid stem cells) occurs in the seminiferous tubules of the testes. Classical ERs (ERα/β) are abundantly expressed in cells of the testes and epididymis, the E2 responsive coiled tube connecting the seminiferous tubules with the vas deferens where mature sperm undergo maturation. Dependence on E2 for proper testicular function and spermatogenesis is demonstrated in studies in mice lacking either ERα or aromatase, the enzyme that converts testosterone into E2. While E2 is necessary for spermatogenesis and testicular function to occur, exposure to high dose E2 during development or exposure to environmental estrogens throughout a man's lifetime results in abnormal spermatogenesis and testicular development, and decreased sperm counts respectively. E2 is able to negatively regulate spermatogenesis by inhibiting gonadrotrophin release from the pituitary, thus interfering with testosterone synthesis.

The novel G protein-coupled Estrogen GPER is expressed in testicular cells, and contributes to E2 induced rapid cell signaling in the testes. For the current study we asked if GPER contributed to E2-dependent regulation of spermatogenesis and epididymal morphology in C57BL/6 mice, which have been

shown to be extremely sensitive to E2 exposure. At 23 days of age, C57BL/6 mice were subcutaneously implanted with 21-day release pellets containing placebo (sham), E2 or G-1. At day 44 mice were euthanized and male reproductive organs were removed for morphological analysis.

We found that while E2 treatment of mice resulted in decreased testes size, wet weight and spermatogenesis (G-1 had no effect on these characteristics), G-1 treatment led to an increase in the size of epididymal lumens. Although the mechanism of this GPER-mediated phenotype was not established, this finding is intriguing, given that we have also observed strikingly similar G-1-induced morphological changes in the mammary gland.

4.2 Introduction

Although long considered to be primarily a female sex hormone, E2 also exerts a variety of effects on the male reproductive system. E2 plays an important role in spermatogenesis and thus fertility, which begins with meiosis and generation of haploid spermatozoa from diploid stem (germ) cells in the seminiferous tubules of the testes, continuing in the epididymis, the highly coiled testicular tube connecting the seminiferous tubules to the vas deferens (Joseph et al., 2011). The testes are located inside a tough membranous coat, the tunica albuginea, and the tightly coiled seminiferous tubules are enclosed within the testes. A cross-sectional view of a mouse seminiferous tubule is shown in figure 4.1 The seminiferous tubules are the site of stem cell (spermatogonia) mitosis, subsequent meiosis (primary and secondary spermatocytes), and finally spermatogenesis, the generation of morphologically distinct spermatozoa (Carreau et al., 2012; McLachlan, 2000). The seminiferous epithelium consists of Sertoli cells, often called “nurse cells”, which are tall columnar cells that extend from the basement membrane into the lumen of the tubule (**Fig 4.1**). Tucked in between the Sertoli cells are the spermatogenic cells. Newly differentiated sperm are transported from the testes by way of the efferent ductuals to the epididymis (**Fig 4.2**). The epididymis is a narrow, tightly coiled epithelial tube located adjacent to the testes. As sperm proceed through the coiled tube of the epididymis, they undergo further motility-enabling maturation before they enter the vas deferens (Carreau et al., 2012).

The testes have a dual role of producing spermatozoa, to promote fertility, and secreting androgens, including testosterone, to promote generation of male secondary sexual characteristics (Carreau et al., 2012). Androgens are produced in the Leydig cells, located in the stroma between seminiferous tubules. These functions are regulated by the hypothalamus-pituitary-gonadal axis, similar to the regulation of E2 production in female reproductive physiology (McLachlan, 2000). Hypothalamic gonadotrophin-releasing hormone (GnRH) drives this process by inducing pituitary gonadotrophin secretion of both follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH acts through its receptors on Sertoli cells in the testes seminiferous epithelium, where it stimulates primary spermatocytes to undergo the first division of meiosis, thus producing secondary spermatocytes (Bliss et al., 2010; Finkelstein et al., 1991). LH acts on Leydig cells (interstitial cells of Leydig) in the testes to promote testosterone secretion. LH regulates testosterone production by regulating the expression of 17-β hydroxysteroid dehydrogenase, the enzyme responsible for converting androstenedione into testosterone in the Leydig cell (Payne et al., 1987). Testosterone activates genes in Sertoli cells, promoting differentiation of spermatagonia into spermatocytes, and thus is required for normal spermatogenesis to occur. Sertoli cells are also induced to produce androgen binding protein, which binds testosterone as it is synthesized and helps to concentrate it in the right place to direct spermatogenesis (McLachlan, 2000).

Similar to the regulation of E2 production in the ovaries, endocrine regulation of testosterone and spermatogenesis involves a negative feedback of

testosterone on LH and FSH secretion from the pituitary, by inhibiting GnRH release from the hypothalamus (Bliss et al., 2010; Finkelstein et al., 1991). The negative feedback of testosterone production is also mediated by inhibin. This protein, stimulated by androgen secretion, is secreted by Sertoli cells, and acts to inhibit FSH secretion, thus creating a negative feedback look to regulate testosterone production (Bliss et al., 2010).

It is known that estrogens, including 17β-estradiol (E2), are involved in the regulation of growth, differentiation and spermatogenesis of normal human testes, mediated by classical ERs, ERα/β (Carreau et al., 2012; O'Donnell et al., 2001). In addition to a supportive and necessary role in testicular function, high dose E2 exposure in mice during development has been shown to have a negative impact on spermatogenesis (O'Donnell et al., 2001). Further support for E2's negative impact on spermatogenesis is shown in humans, where exposure to environmental E2s is correlated with decreased sperm count (Sharpe and Skakkebaek, 1993; Toppari et al., 1996). E2 regulation of testicular function extends to a tumorigenic setting, where it has been shown that classical ERs mediate E2ic responses in testicular germ cell tumors (Pais et al., 2003; Rago et al., 2011).

Expression patterns of classical estrogen receptors ERα/β vary depending on species. As our experiments were carried out in mice, the following description of expression and localization of classical ERs is based on the pattern in rodents. Based on immunohistochemical analysis of the testes, ERα is observed mainly in Leydig cells, whereas ERβ has been visualized in most of the

somatic cells of the testes (Hess et al., 1997). Novel estrogen receptor GPER is also expressed in the testes in both humans (Rago et al., 2011) and rat Sertoli cells (Lucas et al., 2010). In addition to GPER expression in testicular cells, GPER is overexpressed in testicular germ cell tumors (Franco et al., 2011).

There is accumulating evidence that GPER mediates rapid, E2-induced signaling in testicular cells (Chimento et al., 2011; Lucas et al., 2010; Sirianni et al., 2008). Studies with the mouse spermatogonial GC-1 cell line demonstrated E2 rapidly activates the EGFR through a crosstalk between GPER and classical ERs, leading to cell proliferation (Sirianni et al., 2008). In rat Sertoli cells, activation of GPER induces transactivation of the EGFR via Src, MMP-dependent cleavage of HB-EGF, and MAPK activation (Lucas et al., 2010). GPERs role in regulation of apoptosis has also been described (Lazari et al., 2009), and is shown to involve E2-mediated increase in antiapoptotic protein BCL2 and decrease in the proapoptotic protein BAX (Lucas et al., 2010).

Because of the profound effects of E2 on male reproductive function, including in the testes and epididymis, together with the fact that GPER is found to be expressed in the testes (Lucas et al., 2010; Rago et al., 2011), we investigated the role of GPER in mediating physiologic functions of E2 in the testes and epididymis. It has been shown that certain mice strains are more susceptible to E2-induced disruption of male reproductive development. Spearow and colleagues compared the effects of juvenile E2 exposure on testes weight, development and spermatogenesis in three different strains of mice, including CD-1 from Charles River, C57BL/6J (B6) from the Jackson Laboratory, and

several that had been developed from a common base population. C57BL/6 mice showed a large (greater than 16-fold) susceptibility to E2-disruption of testicular function when compared to widely used CD-1 mouse line (Spearow et al., 1999). Based on this study, we chose the C57BL/6 mouse strain to investigate the role of GPER in E2's regulation of testicular function.

C57BL/6 male mice were implanted subcutaneously in the scapular region with slow release pellets (21-day release) containing placebo (sham), E2 (2.1µg), G-1 (21µg) or G36 (105µg) at day 23 of age. A cohort of mice received two pellets (E2 + G36 or G-1 +G36). At 44 days of age (21 days post-pellet insertion), mice were euthanized and male reproductive organs including testes and epididymis were removed and analyzed to determine E2-and G-1-induced changes is weight, morphology and presence of mature sperm.

4.3 Results

4.3.1 Pubertal exposure to estrogen reduces testes size and wet weight in mice.

As E2 is known to induce a reduction in testicular size and wet weight (Spearow et al., 1999), we were interested if GPER mediates this effect. After mice had been euthanized, yet before dissection of the male reproductive organs, mice were weighed, and testes were weighed upon removal. Testes from E2 treated mice appeared much smaller than testes from mice receiving a sham or G-1 pellet (**Fig 4.4**). Testes wet weight fwas significantly reduced in E2-treated mice when compared to sham-treated mice (**Fig 4.5**). E2's effect on wet weight was not inhibited by GPER-selective antagonist G36, nor did G36 alone have an effect on testes wet weight when compared to mice having received a sham pellet. Also, G-1 alone, or in combination with G36 had no effect on testes wet weight when compared to sham treated mice (**Fig 4.5**).

4.3.2 Estrogen disrupts testicular morphology and spermatogenesis in male mice.

We were next interested the role of GPER in spermatogenesis (judged by the presence of mature sperm in the seminiferous tubules at time of death) and regulation of testicular morphology, since E2 adversely affects both of these characteristics (Spearow et al., 1999). Testes were fixed, paraffin embedded and sectioned. Representative images of H&E stained sections from mice receiving sham (A), E2 (B) or G-1 (C) pellets are shown in **figure 4.6**. The presence of sperm in the lumens of these seminiferous tubules is highlighted with arrows (**Figure 4.6 A, C**; bottom panel). From the images it is apparent that E2

abolished sperm production, based on the absence of mature sperm in the seminiferous tubules of mice that received an E2 pellet (**Figure 4.6B**). E2 treatment of C57BL/6 mice also resulted in a disruption in proper testicular morphology (designated with arrowheads in **Fig 4.6B**, top image). Upon quantification of spermatogenesis, we found that E2 significantly reduced the number of sperm per seminiferous tubule lumen compared to sham treated mice, whereas G-1 had no effect on spermatogenesis (**Fig 4.7**). G36 was unable to inhibit the E2-mediated decrease in spermatogenesis. Also, G-1 alone, or in combination with G36 had no effect on spermatogenesis in male C57BL/6 mice (**Fig 4.7**)

4.3.3 G-1 promotes increase epididymal lumen size in male mice

Another E2-responsive organ in the male reproductive system is the epididymis. The structure of the epididymis, shown in **figure 4.3**, is similar in many ways to an alveolar structure found in the mammary gland. Both structures are tubular, with a central lumen surrounded by a layer of luminal epithelial cell (termed principal cells in the epididymis). Since G-1-mediated activation of GPER in human breast tissue resulted in an increase in luminal size in alveolar structures (**Fig 3.3C, 3.4B**), we were interested in the effect of E2 and G-1 on morphologic changes to a similar epithelial structure.

Representative images of H&E stained C57BL/6 epididymal structures from mice receiving sham (A), E2 (B) or G-1 (C) pellets are shown in **Figure 4.8**. E2-induced disruption of morphological structures is seen in the epididymis,

consistent with observation of disrupted morphology in the seminiferous tubules (**Fig 4.6B**); however E2 did not promote changes in luminal area (**Fig 4.8A**) when quantitated and compared to sham treated mice (**Fig .8A, 4.9**). Conversely, epididymal structures from mice that received a G-1 pellet had significantly larger lumens (**Fig 4.8C**) when compared to mice that had received a sham pellet (**Fig 4.8A, 4.9**); this effect was significantly reduced in mice that had received a G-1 pellet together with a G36 pellet (**Fig 4.9**).

4.4 Discussion

The effects of E2 in the male reproductive system have classically been attributed to ligand-dependent transcription factor receptors ERα and ERβ. E2's role on spermatogenesis seems to be a dose dependent effect. E2 is required for development of the male reproductive system and for proper spermatogenesis, demonstrated by the lack of proper development and sperm production in aromatase null and ER-/- mice (Korach, 1994; Lubahn et al., 1993). At high concentrations; however, E2 has a detrimental effect on spermatogenesis based on E2's ability to negatively regulate the hypothalamic-pituitary-testicular axis, specifically inhibiting FSH and LH secretion from the pituitary (Finkelstein et al., 1991; Handelsman et al., 2000). The confounding roles of E2 in the male reproductive system are further complicated by the identification and expression of a third estrogen receptor, GPER, in the testes (Rago et al., 2011). While some signaling mechanisms have been characterized in terms of E2-induced activation of GPER in the testes (Chimento et al., 2011; Lucas et al., 2010; Lucas et al., 2010), a direct mechanism for GPER-dependent regulation of E2-induced responses has yet to be elucidated.

For the present study, we were interested in determining GPER's role in E2-mediated regulation of testes size and spermatogenesis. Consistent with a previous report (Spearow et al., 1999), we found that mice receiving E2 pellets had significantly reduced testes size (**Fig 4.4B**), wet weight (**Fig. 4.5**) and spermatogenesis (**Fig 4.6B, 4.7**) when compared to control treated mice, and these effects were not affected by the presence of GPER-selective antagonist

G36. This suggests that E2-induced reduction in testes size and spermatogenesis is mediated through classical ERs in the testes and not GPER. This is confirmed by the fact that G-1 was not able to stimulate a decrease in testes wet weight (**Fig 4.5**) or a decrease in spermatogenesis (**Fig 4.7**) in C57BL/6 mice that received a G-1 pellet.

In addition to regulation of testicular function, we were also interested in determining the role of GPER in epididymal regulation by E2. Since the structure of the epididymis is similar to alveolar structures in the mammary gland, and G-1 stimulated a change in morphology resulting in larger lumens in alveolar structures in human breast tissue, we used same morphological measurements to characterize epididymal luminal area in mice that had received sham, E2 and G-1 pellets. Our results demonstrate that while E2 had no effect on luminal area within epididymal structures in C57BL/6 male mice, mice receiving G-1 pellets had statistically significantly larger lumens. The G-1-induced increase in luminal area was inhibited in mice that received a G-1 pellet in combination with a G36 pellet (**Fig 4.9**), suggesting that this effect is mediated by GPER.

The ability of G-1 to promote a similar effect (increased lumen size) in two diverse tissue types (the mammary gland and the epididymis) is not surprising, since the signaling mechanism downstream of GPER activation is also similar. It has been shown in breast cancer cells, as well as in normal breast epithelial cells (Chapter 2) that GPER activation leads to transactivation of the EGFR and downstream activation of MAPK and PI3K signaling cascades (Filardo et al., 2000). Similarly, in rat Sertoli cells, GPER has been shown to mediate rapid E2

signaling, by transactivation of the EGFR through activation of Src, MMPs, and cleavage of HB-EGF at the cell surface. Our results demonstrate that while GPER isn't involved in certain E2-induced processes in the male reproductive physiology of C57BL/6 mice, including E2-induced decreased testes size and E2-induced decreased spermatogenesis, it does play a role in morphological regulation of the epididymis, similar to its role in regulation of breast epithelial morphology. Homeostasis of epithelial structures is important in the maintenance of function of epithelial structures. The epididymis is the final location of sperm maturation, and thus is a very important organ in male fertility. The ability of GPER to modulate epididymal morphology suggests that E2-induced activation of GPER occurs in the epididymis, and may be indicative of a possible role for GPER in sperm maturation.

4.5 Materials and Methods

4.5.1 Mouse Models

C57BL/6 male mice were used in this study due to their high sensitivity to E2 (Spearow et al., 1999). At 23 days of age, slow release pellets containing placebo (sham), 2.1µG E2, 21µG G-1, or 105 µG G36 were implanted with a trocar subcutaneously in the scapular region of male C57BL/6 mice. A subset of mice received two pellets (2.1µ E2 + 105µG G36; 21µG G-1 + 105µG G36). Pellets were obtained from Innovative Research of America (Sarasota, FL). At 44 days of age, mice were euthanized tissue was collected from mice, including testes and epididymis. Tissue was fixed with 4% paraformaldehyde.

4.5.2 Histology

Tissue was dehydrated and embedded in paraffin, and sectioned. Paraffin sections (5µm) were mounted on Super-Frost Plus slides (Menzel- Gläser, Germany). After rehydrating sections through graded alcohol series followed by water, tissue was stained with hematoxylin and eosin (H&E) for histological examination. Images were obtained under light microscopy at x400 magnification with the aid of a Zeiss 200M Axiovert inverted microscope (Carl Zeiss Inc., Oberkochen, Germany) and morphological measurements were made using metamorph image analysis software (Molecular devices, Union CA).

4.5.3 Morphologic evaluation of Epididymal Lumens

Histomorphometric evaluation was carried out to determine the effects of different treatments on testicular and epididymal morphology. All morphometric

measurements were done on H&E-stained sections. Using a digital drawing system run by Metamorph, cross-sections of epididymal tubules were measured by outlining epididymal luminal space on the monitor screen with the computer mouse. Luminal area (μm^2) was calculated by tracing the empty space within the luminal epithelial cells. In each treatment sample, a minimum of 15 epididymal structures were randomly selected and measured.

4.5.4 Testes Wet Weight/Sperm Quantification

Before tissue collection, mice were weighed. Testes were weighed upon removal from mice. To determine the testes wet weight measurement, testes weight was expressed as a percentage of total body weight per mouse. To determine effects of pellets on spermatogenesis in mice, the number of mature sperm was counted per each testicular lumen per H&E stained sections. A minimum of 15 testicular lumens were included per treatment group and is expressed as an average.

4.5.5 Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 4.03 (La Jolla, CA). Analysis done with a one-way Analysis of Variance (ANOVA) within Prism estimates the correlation of variables (spermatogenesis, testes wet weight and epididymal luminal area) with treatment groups (sham, E2, G-1, G-36, etc). Pairwise comparisons of results between different treatment groups were determined using a Dunnett's test following the one-way ANOVA. Data represents the mean ± SEM of three independent experiments. P-values less than 0.05 were considered to be significant.

4.6 FIGURE LEGENDS

Figure 4.1 Endocrine regulation of testicular cells by the hypothalamic-pituitary-gonadal axis.

Gonadotrophin releasing hormone (GnRH) is released from the hypothalamus which stimulates cells in the anterior pituitary to secrete luteinizing hormone (LH) and follicle-stimulating hormone (FSH). LH and FSH act on cells in the testes to stimulate testosterone production and initiation of spermatogenesis. Testosterone produced in Leydig cells inhibits the hypothalamus from releasing GnRH, and the pituitary from releasing FSH and LH. Inhibin, produced by Sertoli cells also inhibits FSH synthesis and secretion.

Figure 4.2 Cross-sectional structure of a mouse seminiferous tubule. Spermatogenesis, the generation of haploid, mature spermatozoa from diploid germ cells (spermatogonia), occurs within seminiferous tubules in the testes. The tubule epithelium consists of Sertoli cells which nurse the maturing sperm, coordinate spermatogenesis, and synthesize androgen binding hormone which binds androgens, concentrating it in the tubules. Enveloped by the Sertoli cells are spermatogenic cells, with the diploid spermatogonia along the external basal lamina, and successive differentiative stages (primary spermatocytes > secondary spermatocytes > spermatids > spermatozoa) arrayed toward the lumen. Leydig cells reside in the interstitial space surrounding seminiferous tubules and are the site of testosterone synthesis.

Figure 4.3 Illustration of mouse epididymis.

The epididymis consists of a two-layered pseudostratified epithelium made up of principal cells and basal cells, thought to be stem or progenitor cells. The epithelial layer is separated from the underlying connective tissue by a basement membrane

Figure 4.4 Estrogen reduces testes size in male C57BL/6 mice.

C57BL/6 mice (23 days of age) were implanted subcutaneously with slow release pellets containing sham (A), 2.1µg E2 (B) or 21µg G-1 (C). After 21 days, testes were removed. Representative light microscopy images of testes from mice receiving indicated pellets are shown here.

Figure 4.5 Estrogen reduces testes wet weight in male C57BL/6 mice.

C57BL/6 mice (23 days of age) were implanted subcutaneously with slow release pellets containing sham, 2.1µg E2, 21µg G-1, 105µg G36, or with both E2 and G36 or G-1 and G-36. After 21 days, testes were removed. Testes wet weight (D) was calculated by the weight of the testes upon removal relative to the body weight of the intact mouse before dissection. Data are representative of three independent experiments. Results are expressed as mean ± SEM and statistical significance ($P \leq .05$) was assessed by one-way ANOVA followed by a Dunnett's test. (*, significantly different relative to sham)

Figure 4.6 Estrogen disrupts testicular morphology and spermatogenesis in male C57BL/6 mice.

C57BL/6 mice (23 days of age) were implanted subcutaneously with slow release pellets containing sham (A), 2.1µg E2 (B) or 21µg G-1 (C). After 21 days, testes were prepared for histology (H&E stain). Arrowheads in B (top image) represent a disruption in proper testicular morphology that is not seen in sham (A) or G-1 (B) treated mice. Arrows in A and C (bottom images) represent the presence of mature sperm (dark purple structures are sperm heads) in the testes; not observed in E2 treated mice (B).

Figure 4.7 Estrogen disrupts spermatogenesis in male C57BL/6 mice.

C57BL/6 mice (23 days of age) were implanted subcutaneously with slow release pellets containing sham, 2.1µg E2, 21µg G-1, 105µg G36, E2 + G36, or G-1 + G-36. After 21 days, testes were prepared for histology. Spermatogenesis was quantified as the average number of sperm per testicular structure in a minimum of 15 structures per treatment group (D). Data are representative of three independent experiments. Results are expressed as mean ± SEM and statistical significance ($P \leq .05$) was assessed by one-way ANOVA followed by a Dunnett's test. (*, significantly different relative to sham)

Figure 4.8a G-1 induces an increase in lumen size in male C57BL/6 epididymis.

Luminal area was measured on epididymal epithelial structures from male C57BL/6 mice treated with pellets containing sham (A), 2.1µg E2 (B) or 21µg G-1(C). After 21 days, epididymal tissue was prepared for histology (H&E stain).

Figure 4.8b G-1 induces an increase in lumen size in male.

C57BL/6 epididymis mouse epididymis. Luminal area was measured on epididymal epithelial structures from male C57BL/6 mice treated with pellets containing sham, 2.1µg E2, 21µg G-1, 105µg G36, E2 + G36, or G-1 + G-36. After 21 days, epididymal tissue was prepared for histology. Data are representative of three independent experiments. Results are expressed as mean ± SEM, and statistical significance ($P \leq 0.05$) was assessed by one-way ANOVA followed by a Dunnett's test-test. (*, significantly different relative to sham; #, significantly different relative to G-1)

Figure 4.8b G-1 induces and increase in lumen size in male C57BL/6 epididymis mouse epididymis.

Luminal area was measured on epididymal epithelial structures from male C57BL/6 mice treated with pellets containing sham, 2.1µg E2, 21µg G-1, 105µg G36, E2 + G36, or G-1 + G-36. After 21 days, epididymal tissue was prepared for histology. Data are representative of three independent experiments. Results are expressed as mean ± SEM, and statistical significance ($P \leq 0.05$) was assessed

by one-way ANOVA followed by a Dunnett's test-test. (*, significantly different relative to sham; #, significantly different relative to G-1)

4.7 Figures

Figure 4.1 Endocrine regulation of testicular cells by the hypothalamic-pituitary-gonadal axis

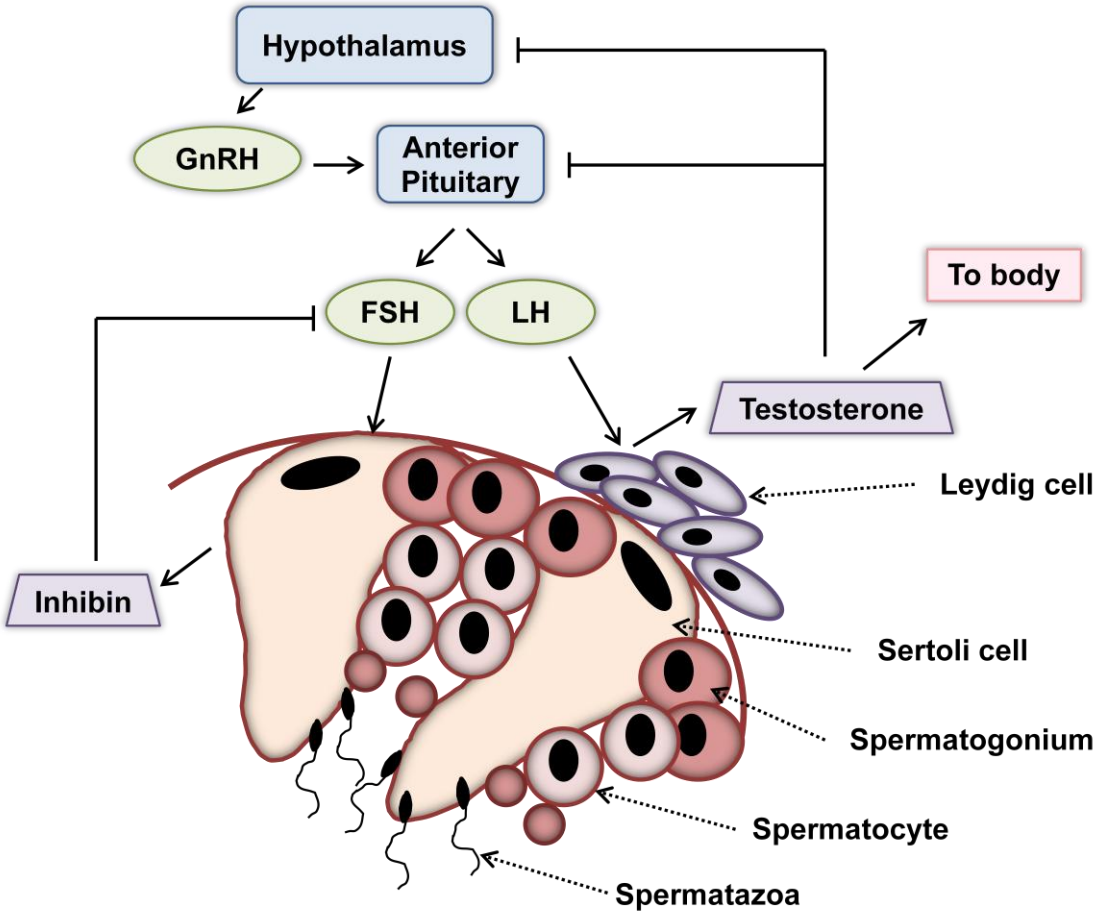


Figure 4.2 Cross-sectional structure of a mouse seminiferous tubule

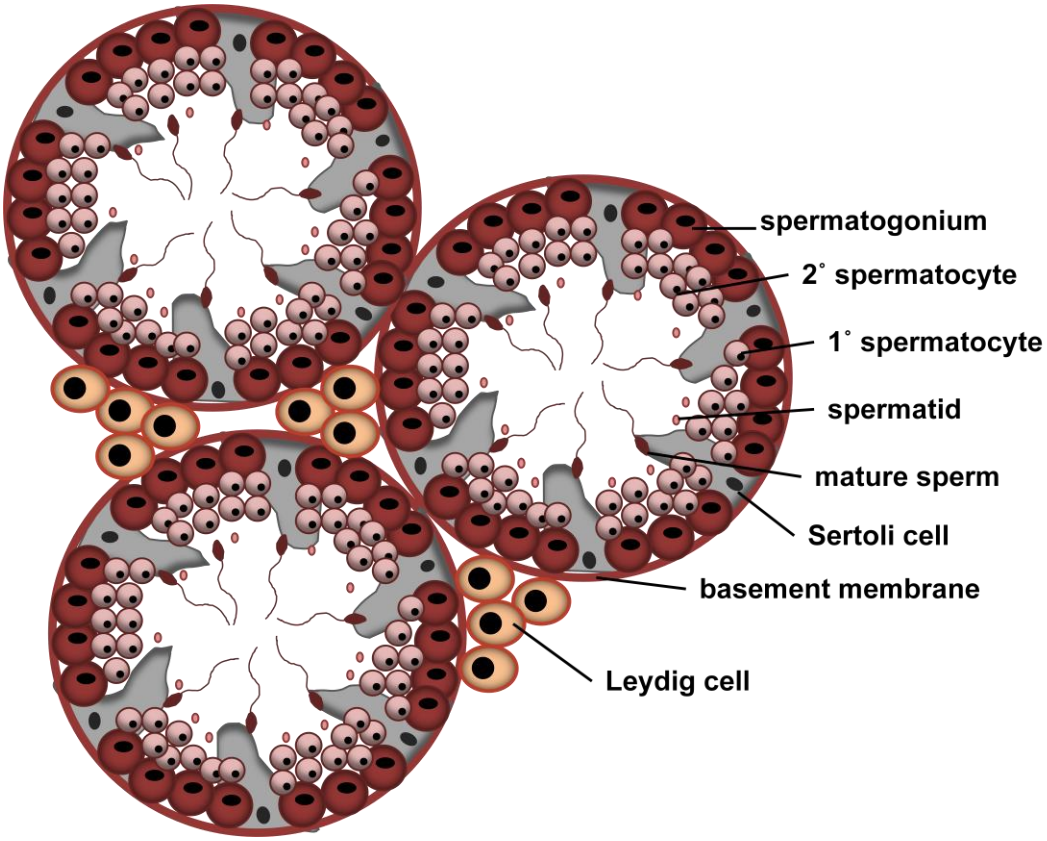


Figure 4.3 Illustration of mouse epididymis

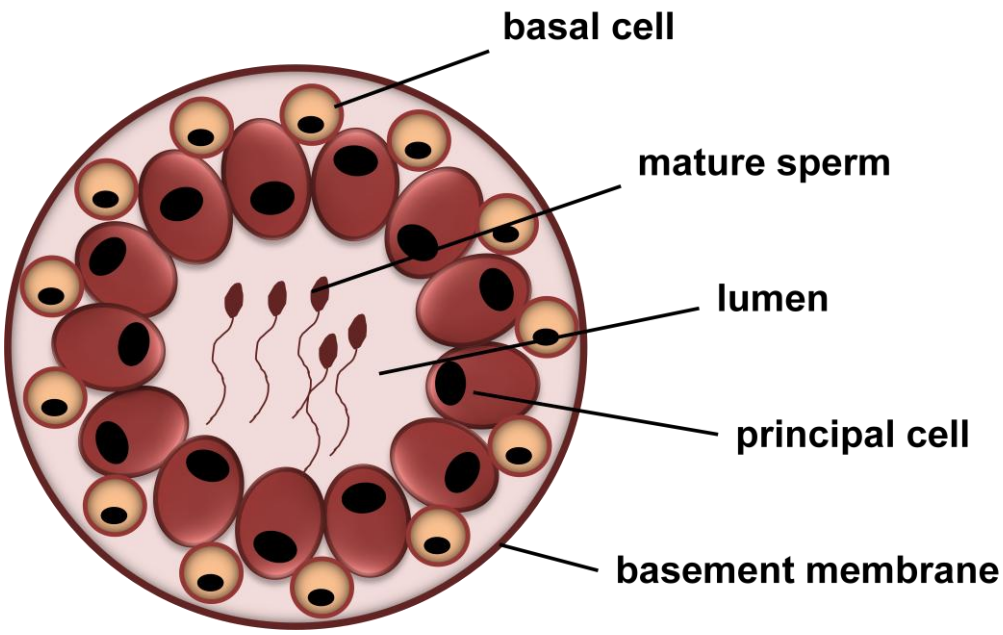


Figure 4.4 Estrogen reduces testes size in male C57BL/6 mice

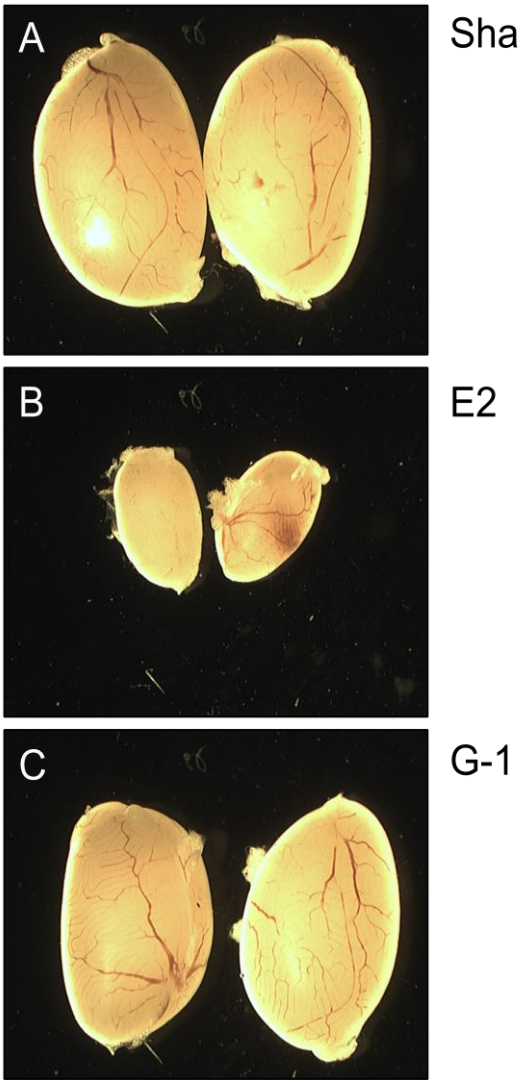


Figure 4.5 Estrogen reduces testes wet weight in male C57BL/6 mice

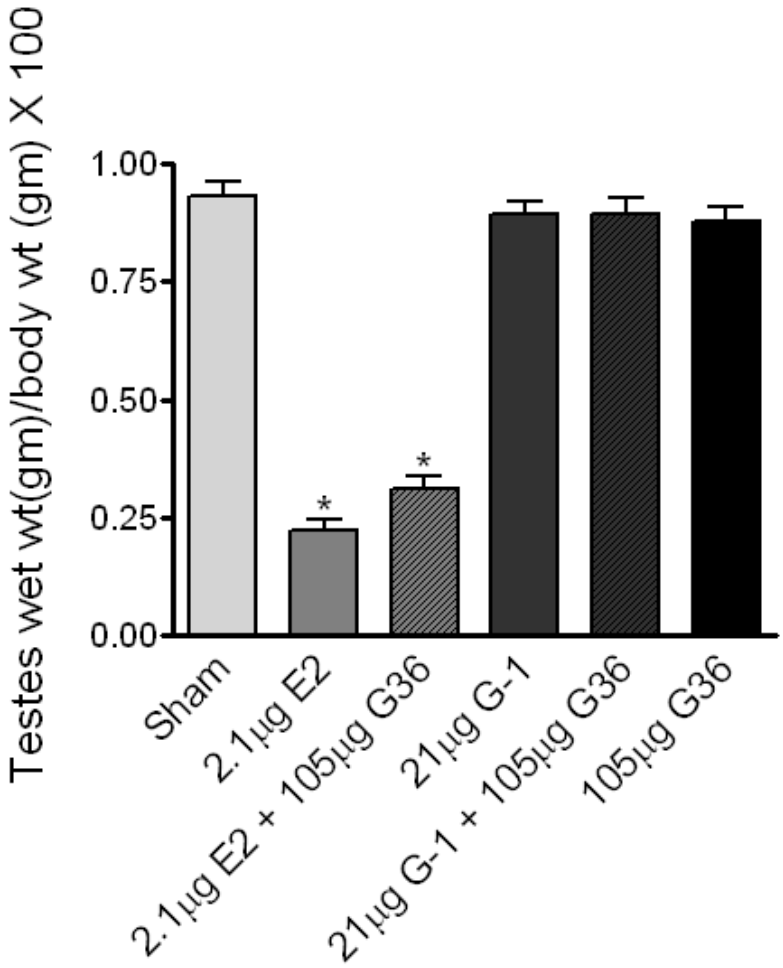


Figure 4.6 Estrogen disrupts testicular morphology and spermatogenesis in male C57BL/6 mice

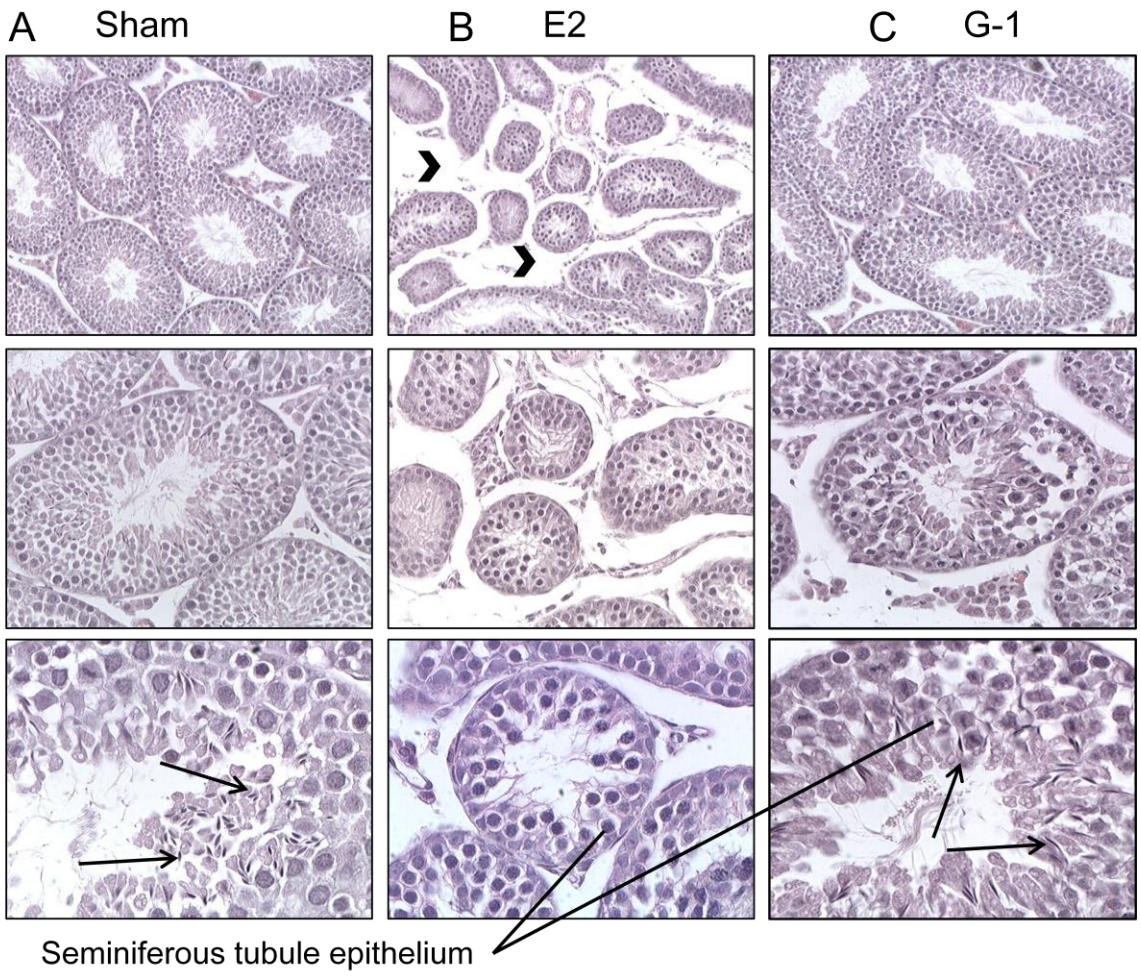


Figure 4.7 Estrogen disrupts spermatogenesis in male C57BL/6 mice

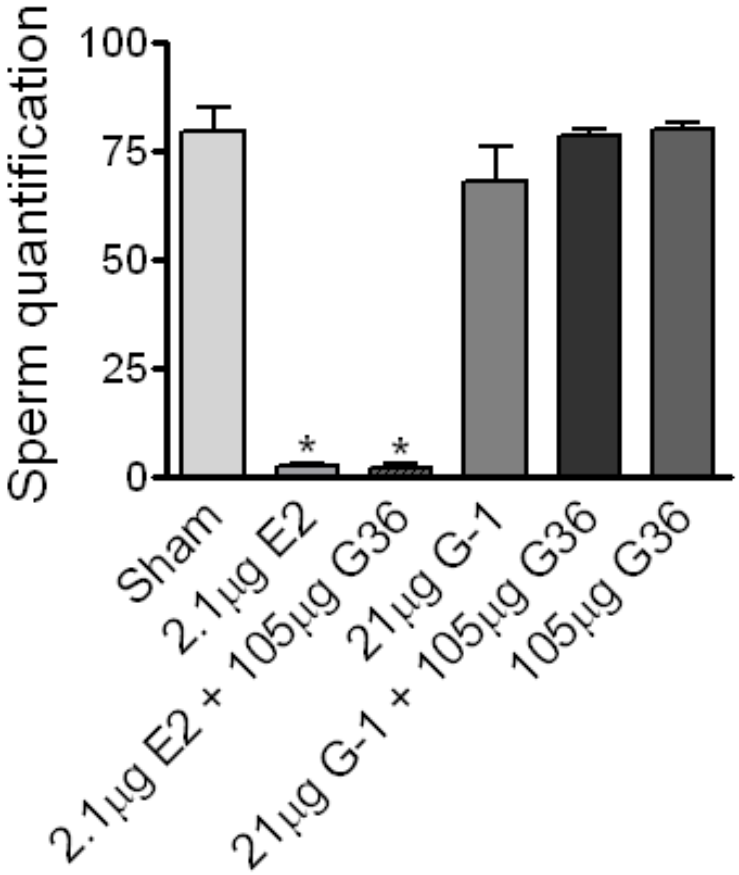


Figure 4.8a G-1 induces an increase in lumen size in male C57BL/6 epididymis

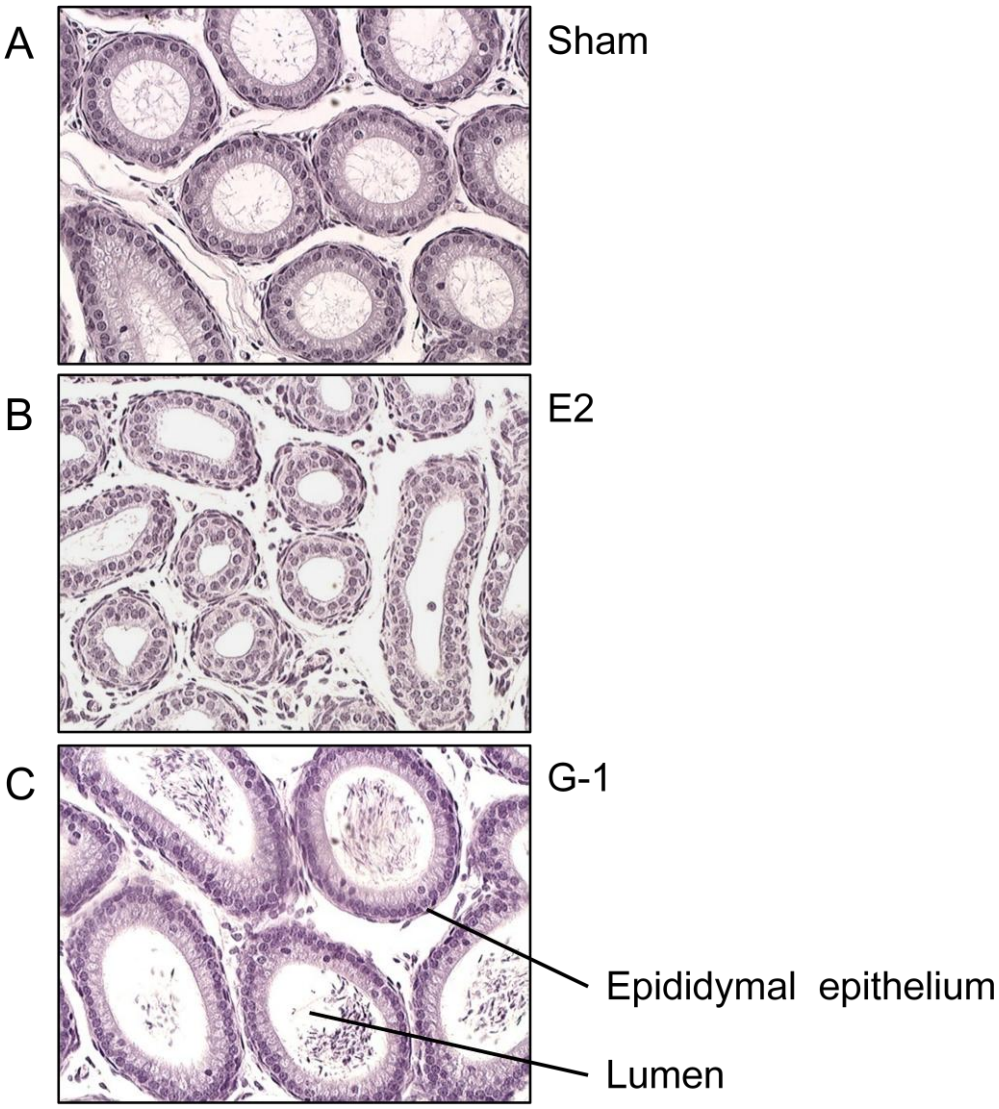
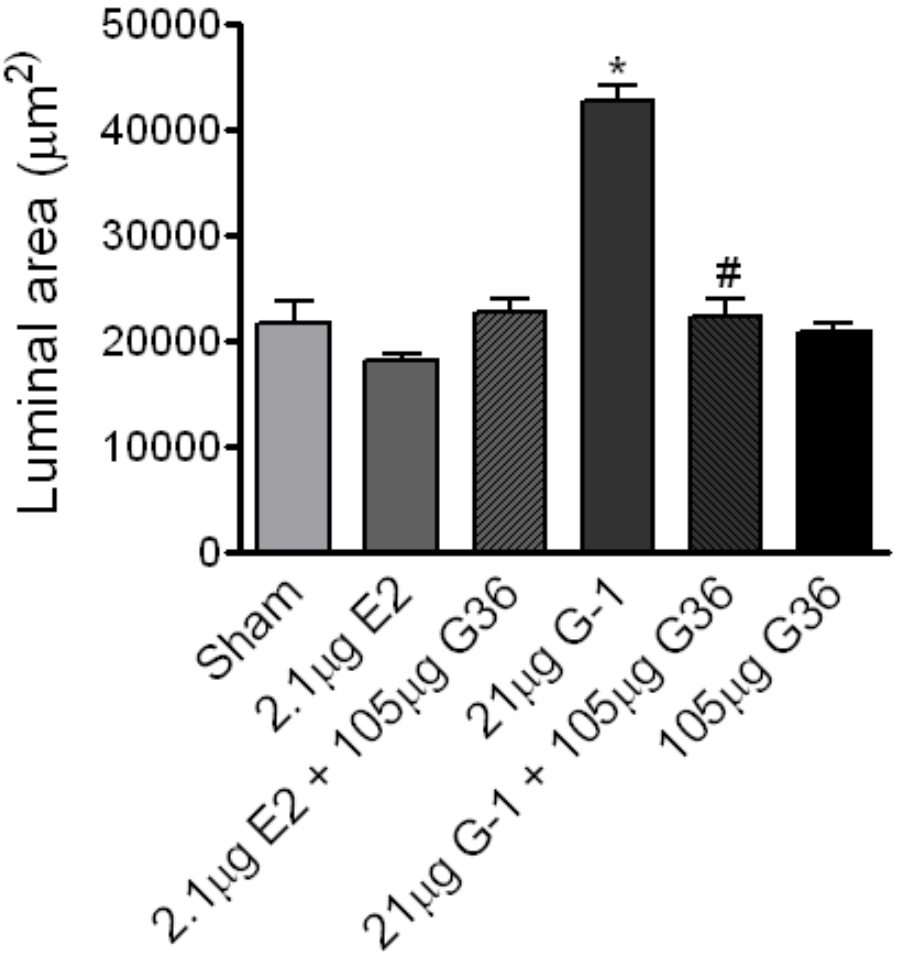


Figure 4.8b G-1 induces an increase in lumen size in male C57BL/6 epididymis mouse epididymis



5. SUMMARY AND FUTURE DIRECTIONS

5.1 Summary

In this study, our aim was to determine if GPER contributes to E2-induced physiologic processes in the breast, including regulation of breast epithelial proliferation and morphogenesis. E2's effects mediated through classical ERs ERα and ERβ are well characterized, with much knowledge acquired through studies done in ER-/- mice (Couse and Korach, 1999; Feng et al., 2007; Forster et al., 2002a; Korach, 1994). ERα mediates E2-induced proliferation and subsequent branching morphogenesis of the mammary gland at puberty, upon initial exposure to circulating E2 (Brisken and O'Malley, 2010), and ERβ mediates E2-induced terminal differentiation of the gland in preparation for lactation at pregnancy (Forster et al., 2002a). The identification and characterization of a third estrogen receptor has complicated the views of E2 signaling, since this receptor, a G protein–coupled estrogen receptor (GPER), shares no homology with the classical ERs, which generally function as ligand-dependent transcription factors [although evidence of rapid E2 signaling mediated through classical ERs does exist (Kelly and Levin, 2001; Levin, 2002)]. Our goal for this work was to provide evidence for a role for GPER mediation of the physiologic effects of E2 in female and male reproductive organs; a summary for each chapter follows.

5.1.1 Chapter 2

As the chief role of E2 in the mammary gland is stimulation of cellular proliferation, we were interested in the function of GPER in mediating this

proliferative effect. We demonstrated GPER-dependent, E2-induced proliferation in three different models, normal human breast tissue cultured *ex vivo*, tumorigenic human breast tissue cultured *ex vivo*, and in a non-transformed breast epithelial cell line, MCF10A. Comparing normal human breast tissue and MCF10A cells, one key difference between the two is the expression of ERs. MCF10A cells only express GPER, thus the observed increased in proliferation with E2 and G-1 treatment can be attributed to GPER alone, whereas breast tissue expresses all three ERs (ERα/β and GPER). The ability of GPER-selective ligand G36 to partially abrogate E2-induced proliferation indicated GPER is responsible in part for E2-induced proliferation in the breast. We demonstrated that GPER-induced proliferation is dependent on transactivation of the EGFR by activation of Src; however MMPs and cleavage of HB-EGF is not required for E2- or G-1-induced ERK activation or proliferation in MCF10A cells, suggesting a possible mechanism for direct intracellular EGFR transactivation by Src, downstream of GPER. Alternatively, these results can be explained by juxtracrine activation of EGFR via pro-HB-EGF on the cell surface.

In the third model, the ability of GPER to mediate E2-induced proliferation in tumorigenic breast tissue is exciting in light of the correlation between GPER expression in tumors and increased tumor size (Filardo et al., 2006). Based on our *ex vivo* results, it is possible to hypothesize that E2 or tamoxifen is promoting proliferation in tumors *in vivo*, since tamoxifen is a known agonist for GPER (Pandey et al., 2009). There is evidence that long term tamoxifen treatment of breast cancer cells *in vitro* increases E2 sensitivity, which leads to increased

GPER expression. Tamoxifen can then act as an agonist for GPER in these cells, causing transactivation of the EGFR, and ERK phosphorylation, which in turn promotes the sensitivity of these cells to E2, creating a positive feedback mechanism (Ignatov et al., 2010). Ignatov and colleagues also observed a positive correlation between GPER expression and tamoxifen resistance in primary breast tumors (Ignatov et al., 2011). These observations, taken together with our own data from chapter 2 (E2-induced proliferation in tumors) suggest that GPER could be promoting proliferation in breast tumors in response to E2, and could also be activated by tamoxifen in women who are receiving it as an anti ER therapy for breast cancer.

5.1.2 Chapter 3

Coupled to E2's primary role in proliferation is the role of E2 in morphological regulation of the mammary gland. Morphologic changes in the gland are initiated by E2-induced, ERα-mediated proliferation (Brisken and O'Malley, 2010). Since we demonstrated in chapter 2 that GPER-mediates E2-induced proliferation in the mammary gland, we sought to determine if E2 and G-1-dependent increases in proliferation affect mammary gland morphology. We observed that E2 leads to increased luminal epithelial cell layers (in normal alveolar structures there is a single layer) and G-1-led to an increase in the luminal area within alveolar epithelial structures. In an effort to link proliferation to changes in morphology, we investigated E2's and G-1's ability to regulate mitotic spindle orientation in MCF10A breast epithelial cells cultured in 3D. We observed that E2, but not G-1 induced a rotation of the mitotic spindle in proliferating cells,

therefore E2-promoted a plane of cell division that was perpendicular to the basement membrane. This could presumably produce alveolar structures with multiple epithelial layers, consistent with what is observed in E2-treated tissue. Although G-1 did not alter mitotic spindle orientation, if the majority of the proliferating mitotic spindles are oriented parallel to the basement membrane, and proliferation is enhanced due to G-1 treatment, larger lumens could result, albeit with a single epithelial layer. We also observed in chapter 3 that G-1-induces downregulation of E-cadherin in human breast tissue. One mechanism to regulate E-cadherin-mediated cell-cell adhesion is by phosphorylation of E-cadherin, and this leads to internalization and degradation of E-cadherin (Shen et al., 2008). FAK and Src are known to interact with one another to mediate phospho-regulation of cellular targets (Hiscox et al., 2007; Planas-Silva et al., 2006), which is interesting because we have shown Src to be required for GPER-dependent proliferation (chapter 2) and we have also seen a significant increase in FAK activation with G-1 treatment of MCF01A cells. Based on these results, it's not implausible to hypothesize that E2-induced GPER activation and subsequent Src activation lead to FAK activation, and the Src/FAK kinases induced downregulation of E-cadherin in alveolar structures, thus loosening the cell-cell adhesions, allowing for a single-layered epithelial structure that can accommodate increased proliferation.

5.1.3 Chapter 4

At first glance, chapter four might seem like an outlier in this study, since it focuses on E2 regulation of a completely different male organ in mice, whereas

the rest of the thesis work is carried out in female breast tissue and breast epithelial cells. There are many similarities; however, that can be used to draw conclusions between chapter 3 and 4. For example, both breast tissue and testes/epididymal tissue are under tight endocrine control regulated by the hypothalamus-pituitary-gonad axis (Bliss et al., 2010). Next, there is evidence that these tissues are subject to regulation by E2, mediated by all three estrogen receptors (ERα/β and GPER), and lastly, the structure of mammary alveoli is similar to the structure of the epididymal epithelium (highlighted in chapter 3 discussion); specifically the epididymis consists of an epithelial duct with a single layer of cuboidal/columnar epithelia surrounding a central lumen. Accordingly, it is intriguing that G-1 induces a similar effect, increased lumen size, in both the mammary alveolar epithelium and the epididymal epithelium. Although little has been reported in terms of GPER-dependent signaling cells in the male reproductive system, initial observations suggest GPER-dependent transactivation of the EGFR in testicular cells is mediated by activation of Src, similar to our own data (chapter 2) and previous reports in breast cancer cells (Filardo et al., 2000).

It is conceivable that GPER exerts other effects in the epididymal epithelium, for instance downregulation of E-cadherin (similar to the breast tissue; chapter 3), since E-cadherin is expressed at cell-cell junctions between the lumen-lining principal cells in the epididymis (Andersson et al., 1994; Marin-Briggiler et al., 2008). E2 also plays a role in fluid resorption in the epididymis and the efferent ductuals. Studies with ER-/- mice reveal that inhibition of fluid

resorption in the efferent ductuals and the epididymis can lead to inefficient spermatogenesis because the tubules are full of fluid (Hess et al., 1997). It is possible that GPER is mediating its effects on epididymal luminal morphology by inhibiting proper fluid resorption in the male gonads, although the molecular mechanisms by which GPER elicits effects in the male reproductive tract await further investigation.

5.2 OVERALL CONCLUSIONS

In this work, we were able to show a specific requirement for GPER function in E2-induced proliferation in human breast tissue. This is the first demonstration of GPER-mediated proliferation in a human tissue. We also described a novel signaling mechanism in MCF10A cells for GPER-dependent transactivation of the EGFR, independent of MMP activation and cleavage of cell surface-bound HB-EGF (which had been previously reported in breast cancer cells (Filardo et al., 2000; Filardo, 2002). The ability of GPER to mediate E2-induced proliferation in normal and tumorigenic breast tissue (chapter 2), together with its widely reported expression in breast tumors and breast cancer cell lines (Filardo et al., 2006; Kuo et al., 2007; Luo et al., 2011), and our observations of GPER-mediated morphological regulation and E-cadherin downregulation in the mammary epithelium leads to the postulation that this receptor is certainly involved in physiology regulation of hormone-dependent tissue and will someday be an effective target in patients with breast or other hormone responsive tumors.

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