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GPER-MEDIATED REGULATION OF NUCLEAR AKT/FOXO3A SIGNALING

Erin Zekas

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**GPER-MEDIATED REGULATION OF NUCLEAR
AKT/FOXO3A SIGNALING**

by

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B.S. Biology,
Temple University, 2005

DISSERTATION

Submitted in Partial Fulfillment of the
Requirements for the Degree of

**Doctor of Philosophy
Biomedical Sciences**

The University of New Mexico
Albuquerque, New Mexico

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ABSTRACT

17 β -estradiol (estrogen) has been demonstrated to regulate survival in breast cancer cells, which is partially mediated by its nuclear receptors ER α and ER β and the G protein-coupled estrogen receptor (GPER). We previously established that estrogen can activate the phosphoinositide 3-kinase (PI3Kinase) prosurvival pathway via GPER stimulation resulting in PIP3 generation within the nucleus of breast cancer cells; the mechanism for this is still unclear. PIP3 generation results in Akt activation, which is known to inactivate FOXO3a, a proapoptotic transcription factor that translocates from the nucleus to the cytoplasm upon inactivation resulting in a decrease in proapoptotic gene expression and consequently an increase in cell survival. Here, utilizing a FoxO3-GFP construct, we report FOXO3a inactivation as a result of GPER stimulation by E2 and the GPER-selective agonist G-1 in the estrogen-responsive breast cancer cell line MCF7 and that ER α is not required. The p110 α catalytic subunit of PI3Kinase,

and the transactivation of the EGFR constitute the mechanism by which GPER inactivation of FOXO3a occurs. Additionally, E2 and G-1 stimulation of MCF7 cells results in a decrease in caspase activation compared to negative control. This suggests, in part, that GPER stimulation is required for survival of breast cancer cells and that GPER expression and FOXO3a localization should be utilized as prognostic markers in breast cancer treatments. Furthermore, our results indicate a need for GPER antagonists in GPER positive breast cancers in order to counteract GPER related prosurvival effects in combination with chemotherapeutic drug treatments.

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LIST OF ABBREVIATIONS

- AC—adenyl cyclase
- ADAM—a disintegrin and metalloprotease
- AF—activation function
- AI—aromatase inhibitors
- Akt—protein kinase B
- AP1—activating protein 1
- AR—androgen receptor
- ATCC—American type culture collection
- Bad—Bcl-2-associated death promoter
- BCL2—B-cell lymphoma 2
- Bim—Bcl-2 interacting mediator of cell death
- BSA—bovine serum albumin
- cAMP—cyclic adenosine monophosphate
- cdk—cyclin-dependent kinase
- cDNA—complementary deoxyribonucleic acid
- DBD— deoxyribonucleic acid binding domain
- DMEM—Dulbecco's modified eagle medium
- DNA— deoxyribonucleic acid
- E1—estrone
- E2—17 β -estradiol
- E3—estriol
- EGF—epidermal growth factor
- EGFR—epidermal growth factor receptor

ERE—estrogen response element
ER—estrogen receptor
ERK—extracellular signal-regulated kinases
ER α —estrogen receptor α
ER β —estrogen receptor β
FasL—Fas ligand
FBS—fetal bovine serum
FHIT—fragile histidine triad protein
FOX—forkhead box
FSH—follicle stimulating hormone
G15—GPER-selective compound 15 (antagonist)
G1—Gap 1 phase of mitosis cell cycle
G-1—GPER-selective compound 1 (agonist)
G36—GPER-selective compound 36 (antagonist)
GDP—guanosine diphosphate
GEF—guanine nucleotide exchange factor
GFP—green fluorescent protein
GnRH—gonadotropin releasing hormone
GPCR—G protein-coupled receptor
GPER—G protein-coupled estrogen receptor
GPR30—G protein-coupled receptor 30
GR—glucocorticoid receptor
GTP—guanosine triphosphate
HB-EGF—heparin-bound epidermal growth factor
HDL—high density lipoprotein

HER2—human epidermal growth factor receptor 2

hr—hour

HSP—heat shock protein

IKB—Inhibitor of κ B

IKK β — Inhibitor of nuclear factor kappa-B kinase subunit β

JNK— c-Jun NH2-terminal kinase

kDa—kilodaltons

ki—inhibition constant

LBD— ligand binding domain

LDL—low density lipoprotein

LH—luteinizing hormone

MAPK—mitogen-activated protein kinase

min—minute

MMP—matrix metalloprotease

MOMP—mitochondrial outer membrane permeabilization

MR—mineralocorticoid receptor

mRNA—messenger ribonucleic acid

NCBI—National Center for Biotechnology Information

NF-KB— nuclear factor kappa-light-chain-enhancer of activated B cells

NGF—nerve growth factor

NIH—National Institutes of Health

p21—cyclin-dependent kinase inhibitor 1

p27—cyclin-dependent kinase inhibitor 1B

PBS—Phosphate buffered saline

PDK—3-phosphoinositide-dependent kinase
PFA—paraformaldehyde
PH-FP—pleckstrin homology conjugated fluorescent protein
PH—pleckstrin homology
PI3K—phosphoinositide 3-kinase
PIP2— phosphatidylinositol 4,5-bisphosphate
PIP3--phosphatidylinositol (3,4,5)-trisphosphate
PR—progesterone receptor
PTEN—Phosphatase and tensin homolog
rb—retinoblastoma
RFP—red fluorescent protein
S phase—synthesis phase
SALL4— sal-like protein 4
SERM—selective estrogen receptor modulator
Ser—Serine
SGK— serum and glucocorticoid-inducible kinase
SH3—src homology 3 domain
siRNA—small interfering ribonucleic acid
Src—Proto-oncogene tyrosine-protein kinase
STAR— Study of Tamoxifen and Raloxifene
TBS—tris buffered saline
TBST—tris buffered saline with .01% Tween-20
TCGA—The Cancer Genome Atlas
TGF— β transforming growth factor beta

Thr—Threonine

TM—triple mutant

TNBC—triple negative basal-like breast cancer

TRAIL—TNF-related apoptosis-inducing ligand

CHAPTER 1

1. Introduction

1.1. Estrogen Synthesis

Estrogens are members of the steroid family of hormones that includes testosterone, glucocorticoids, mineralcorticoids, and progesterone. These hormones regulate physiological processes involved in the development and maintenance of an array of tissue types in both males and females. Estrogens function as the primary female sex hormone and occur in three forms in nature: estriol (E3), estrone (E1) and the most biologically active 17β -estradiol (E2) (Figure 1.1). In females, estrogens are primarily produced by the ovaries in response to Follicle Stimulating Hormone (FSH) stimulation but are also excreted in small amounts by the liver, the breasts, the adrenal glands and fat cells. GnHR, the gonadotropin releasing hormone, is secreted from the hypothalamus and binds to the GnHR receptor in the anterior pituitary, triggering the release of FSH and Luteinizing Hormone (LH) (Bliss et al., 2010). LH goes on to stimulate production of androgens in females. It does this by acting 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. FSH binds to receptors on the granulosa cells, stimulating the expression of aromatase enzymes that convert androstenedione to testosterone. Finally, testosterone is converted via aromatase into 17β -estradiol (E2). E2 plays a critical role in the development of the female reproductive organs and

secondary sex characteristics. In males, it is primarily produced by the testes and is essential for reproductive development and function.

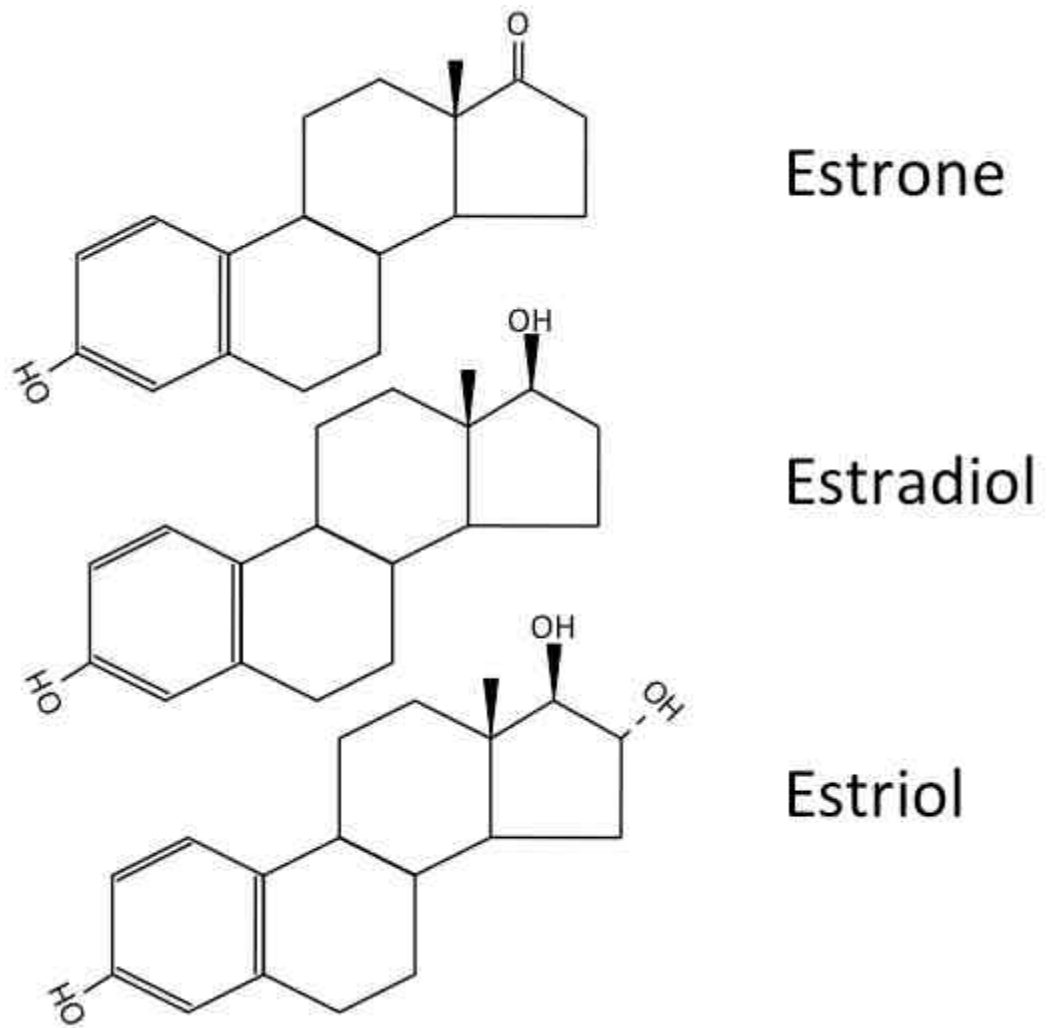


Figure 1.1. Physiological Estrogens. Chemical structures of the three naturally occurring estrogens.

1.2. Estrogen Function

E2 functions as the main female sex hormone; however there are additional targeted effects of E2 throughout the body other than its role in development and maintenance of female reproductive organs. E2 is lipophilic and can cross both the plasma membrane and blood brain barrier. Its concentration within the brain was initially discovered using tritium-labeled steroid hormones that revealed estrogen-concentrating cells in the pituitary gland, the hypothalamus and other brain regions (Lee and Pfaff, 2008). Some of estrogen's effects within the brain include neuronal cell proliferation and survival, as well as synaptogenesis during a sensitive developmental period that establishes the sexually differentiated brain (Lee and McEwen, 2001).

Estrogen has also been shown to have cardiovascular effects. One instance of these effects has been demonstrated in cardiomyocytes, where estrogen was found to prevent cardiac myocyte death. Estrogen is able to prevent apoptosis in these cells by modulating the two major isoforms of p38 MAPK (α inactivation and β activation), and also by activation of PI3Kinase, which initiates prosurvival pathways (Kim et al., 2006). Another example of estrogen having cardiovascular effects is its ability to modulate the lipid profile by decreasing low-density lipoprotein (LDL) and increasing high-density lipoprotein (HDL). Higher levels of HDL have been associated with improved cardiac health (although the mechanism is not entirely clear), and the presence of estrogen and these increased HDL levels have been shown to be cardioprotective in pre-menopausal women. However, if cardiac damage is already present in post-menopausal

women, estrogen hormone therapy should be administered with statins (Barton, 2013).

Despite these diverse effects, estrogen's main function occurs within the estrous cycle. Mammogenesis (mammary growth) occurs minimally until puberty is reached. The onset of puberty triggers rapid activation of ductal elongation and branching, which is under strict control of hormones. As the estrous cycle continually repeats, in addition to during pregnancy, the complexity of the structure of the mammary glands increases (Pelekanou and Leclercq, 2011). When these normal growth pathways are not regulated and additionally because of genetic mutations, breast cancer can occur. Estrogen's regulation of mammary growth events, as well as its other effects in the body is mediated by specific estrogen receptors.

1.3. Estrogen Receptors and Genomic Signaling

In the late 1950s, Elwood Jenson discovered and began characterizing a protein that was able to bind E2 (Toft and Gorski, 1966) (Nilsson et al., 2001). E2 was known to stimulate growth; however the accepted mechanism at the time was that E2 was an enzyme cofactor in a reversible oxidation/reduction reaction leading to hydrogen transfer from NADH or NADPH and that this biochemical reaction resulted in growth (Jensen et al., 2010). In order to determine estrogen's distribution in tissues, physiological concentrations of tritium-labeled estrogen was administered to immature female rats resulting in uptake in the liver and the kidneys, as well as prolonged uptake in the uterus and vagina. The

tritiated E2 was determined not to be chemically altered, contrary to the proposed mechanism of action, suggesting that E2's effects were a result of binding to another protein. This protein was originally called estrophillin, and suggested to bind E2 resulting in the growth response (Jensen et al., 2010). Jensen et al. were eventually able to clone the receptor by first producing the antibody to the receptor, then adding the tritiated E2 hormone marker to form a radioactive immune complex. This would yield a larger complex that was able to be separated out utilizing sucrose density centrifugation. The fractions obtained from the sucrose gradient centrifugation contained high levels of ER mRNA, which enabled the production of cDNA libraries (Green et al., 1986). Another estrogen binding protein was later cloned from a rat prostate cDNA library (Kuiper et al., 1996). The first receptor became known as ER α , while the latter was named ER β .

In the absence of ligand, ERs are localized predominantly within the nucleus, while a small percentage is localized to the cytoplasm. Heat shock protein (HSP) chaperones keep the receptors in an inactive conformation as well as stabilize them from degradation. Estrogen is able to diffuse through the plasma membrane, enter the cell and diffuse into the nucleus where it can bind ER α and ER β . Upon E2 binding, conformational changes in these receptors occur, leading to the dissociation of inactivating proteins, dimerization and translocation of cytoplasmic receptors to the nucleus and/or nuclear receptors to DNA. ERs bind to estrogen response elements (EREs) in DNA and act as transcription factors. ER α has been shown to modify genes that encode for proteins involved

in cell growth and proliferation. ER β 's function is less well characterized, but it has been suggested in many cases to oppose the functions of ER α (Sanchez et al., 2013).

ER α and ER β share similar structures and belong to the steroid/thyroid binding family of nuclear receptors (Figure 1.2). The most conserved central binding domain of the estrogen receptors is the DNA binding domain (DBD), which recognizes its target sequences within DNA and is also important in receptor dimerization upon ligand binding. The ligand-binding domain (LBD) is located near the COOH-terminus and mediates ligand binding, receptor dimerization, nuclear translocation and transactivation of target gene expression. The NH₂-terminal domain is the least conserved and is variable with respect to both sequence and length. In nuclear receptors, this domain encodes for a ligand-independent activation function (AF-1), which is involved in protein-protein interactions and transcriptional activation. The AF-1 of ER α is known to be active in stimulating receptor gene expression; however the AF-1 of ER β has been suggested to have negligible activity. In addition to sequence homology within the DBD and the LBD, the two ERs have similar affinities for E2 and also bind the same response elements within DNA.

Estrogen binding to its nuclear receptors leads to receptor dimerization, and subsequently translocation to DNA where the receptors function as transcription factors that can bind estrogen response elements (EREs) and modify gene expression. Which genes are affected by this transcriptional regulation depends on cell type, the presence or absence of coregulatory proteins, and which ER is

involved. E2-bound ERs can also bind to other transcription factor complexes such as Fos/Jun and modulate gene expression of promoters that do not contain EREs.

In addition to full length ER α , splice variants have been described (Taylor et al., 2010). These include a 46 kDa protein that is the product of an N-terminal truncation as well as a 36 kDa variant that has the same N-terminal truncation with an additional C-terminal truncation (Kim and Bender, 2009) (Chaudhri et al., 2014). In the absence of the N-terminal transcription activation domain, these splice variants have been demonstrated to act as inhibitors of ER α -mediated transcription and to mediate rapid signaling pathways. Although ER β has many known splice variants, they are not yet characterized.

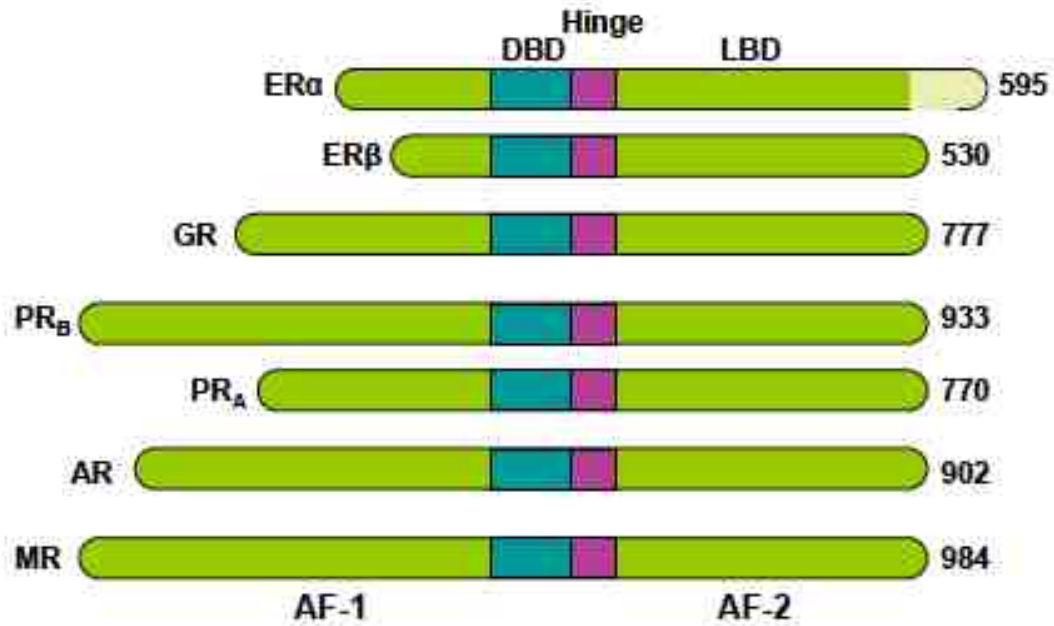


Figure 1.2. Steroid hormone receptor family. The domains starting from the N-terminus (left) to C-terminus (right). NTD = N-terminal domain, DBD = DNA binding domain. LBD = ligand binding domain. AF = activation function. The steroid hormone receptor abbreviations are ER – estrogen receptor, GR – glucocorticoid receptor, PR – progesterone receptor, AR – androgen receptor, and MR – mineralocorticoid receptor. The numbers to the right are the lengths in amino acid residues.

1.4. Estrogen Receptors and Nongenomic Signaling

Estrogen's earliest cellular effects were in fact described as rapid signaling events such as second messenger production (cAMP) and regulation of ion channels (Ca^{++}). In 1975, a Nature article demonstrated rapid calcium uptake in endometrial cells in response to estrogen (Pietras and Szego, 1975). It was originally assumed that ER α was responsible for these rapid signaling effects of estrogen.

In 1997, using differential cDNA library screening, the cDNA library of MCF7 cells, an estrogen receptor positive breast carcinoma cell line, was compared to the cDNA library of MDA-MB-231 cells, an estrogen receptor negative breast carcinoma cell line (Carmeci et al., 1997). This technique is utilized to determine the differential expression of genes in one cell type compared to another. One cDNA that was more highly expressed in MCF7 cells compared to MDA-MB-231 cells was isolated and the sequence analyzed. When compared to the nucleic and amino acid sequences found in GenBank/EMBL at NCBI, it was determined that the open reading frame of the isolated cDNA shared extensive sequence homology with previously described G protein-coupled receptors (GPCRs). This orphan GPCR was initially named GPR30 and much later renamed the G protein-coupled estrogen receptor (GPER). Due to its pattern of expression, GPER was hypothesized to be involved in physiological responses specific to hormonally responsive tissues. However at this point, its physiological ligand(s) were not known and further characterization of this receptor was required.

GPER has since been more clearly defined as a G protein-coupled receptor belonging to the family of 7 transmembrane spanning receptors. Most GPCRs are localized to the plasma membrane. However, our group was able to demonstrate that GPER is localized predominantly to intracellular membranes associated with the endoplasmic reticulum and the Golgi apparatus in multiple cell types (Revankar et al., 2005). This was determined utilizing fluorescence microscopy techniques to visualize labeled GPER colocalizing with labeled markers of the endoplasmic reticulum and the Golgi. However, it has more recently been suggested that in other tissues, such as renal epithelia, GPER is significantly expressed at the plasma membrane (Cheng et al., 2011). This study proposed that some cells may regulate GPER's action by modulating the subcellular distribution of the receptor between the plasma membrane and the perinuclear compartment and that this occurs by the process of endocytosis, with accumulation of GPER in the perinuclear compartment.

There are regions of GPCR receptors that are exposed to the extracellular space or, as in the case of GPER, to the intracellular cytoplasmic space since it is localized to the ER. The portions of the receptor flanking the surface of the membrane along with the transmembrane domains contribute to ligand binding. The structural and chemical composition of these regions is dependent on the receptor and this composition essentially forms a binding pocket for its corresponding ligand. Ligand binding results in receptor activation and transmission of signals through the membrane via the transmembrane domains. GPCRs are associated with their canonical heterotrimeric G proteins consisting

of α and $\beta\gamma$ subunits that are bound to the cytoplasmic region of the receptor. GPCRs essentially function as guanine nucleotide exchange factors (GEFs) that can activate an associated G-protein by exchanging its bound GDP for a GTP. The G-protein's α subunit, together with the bound GTP, can then dissociate from the β and γ subunits to further affect intracellular signaling proteins or target functional proteins directly depending on the α subunit type. The $\beta\gamma$ subunits together have also been demonstrated to have their own signaling effects. GPCR stimulation can lead to initiation of such downstream events as the activation of adenylate cyclase, which results in the production of the second messenger cAMP (cyclic adenosine monophosphate). cAMP activates multiple kinases including protein kinase A (PKA). The stimulation of GPCRs and subsequent dissociation of its G-proteins can also be coupled to effector molecules such as phospholipases, phosphodiesterases and ion channels (Prossnitz et al., 2008). Additionally, GPCRs have been demonstrated to appropriate crosstalk with receptor tyrosine kinases, particularly the EGFR (George et al., 2013). There is evidence that suggests GPER is coupled to various G proteins resulting in the activation of multiple downstream signaling cascades.

It had been previously established that estrogen promotes rapid activation of the MAPKs ERK-1/2; however, it was unclear whether ER α or ER β were required. In 2000, the Filardo group determined that E2 activation of ERK was due to GPER stimulation through transactivation of the EGFR in an ER α -independent manner (Filardo et al., 2000). The effect was established in SKBR3 cells that express

GPER but not ER α or ER β . It was determined that GPER-stimulated ERK-1/2 activation was inhibited by Src family tyrosine kinase inhibitors and heparin-bound-EGF (HB-EGF)-neutralizing antibodies. Additionally, pertussis toxin and a G $\beta\gamma$ -sequestering peptide inhibited GPER-dependent ERK-1/2 activation.

Therefore, it was concluded that GPER signaled through G $\beta\gamma$ -dependent Src activation ultimately resulting in transactivation of the EGFR following cleavage of pro HB-EGF by matrix metalloproteinases (Filardo et al., 2000). Although GPER induces ERK-1/2 activation downstream of EGFR, it also inhibits this effect via cAMP production resulting from G α_s activation. This dual action of GPER tightly regulates ERK-1/2 activation, which ensures the potent actions of the MAPKs are not sustained (Filardo et al., 2002).

Subsequently, our group discovered in 2005 that E2 also activates PI3Kinase as a consequence of transactivation of the EGFR (Revankar et al., 2005). Using the PH domain of the downstream effector Akt as a reporter, we concluded PI3Kinase activation led to nuclear accumulation of its phosphorylated substrate PIP3. The mechanism for this activity is however still unclear (Figure 1.3).

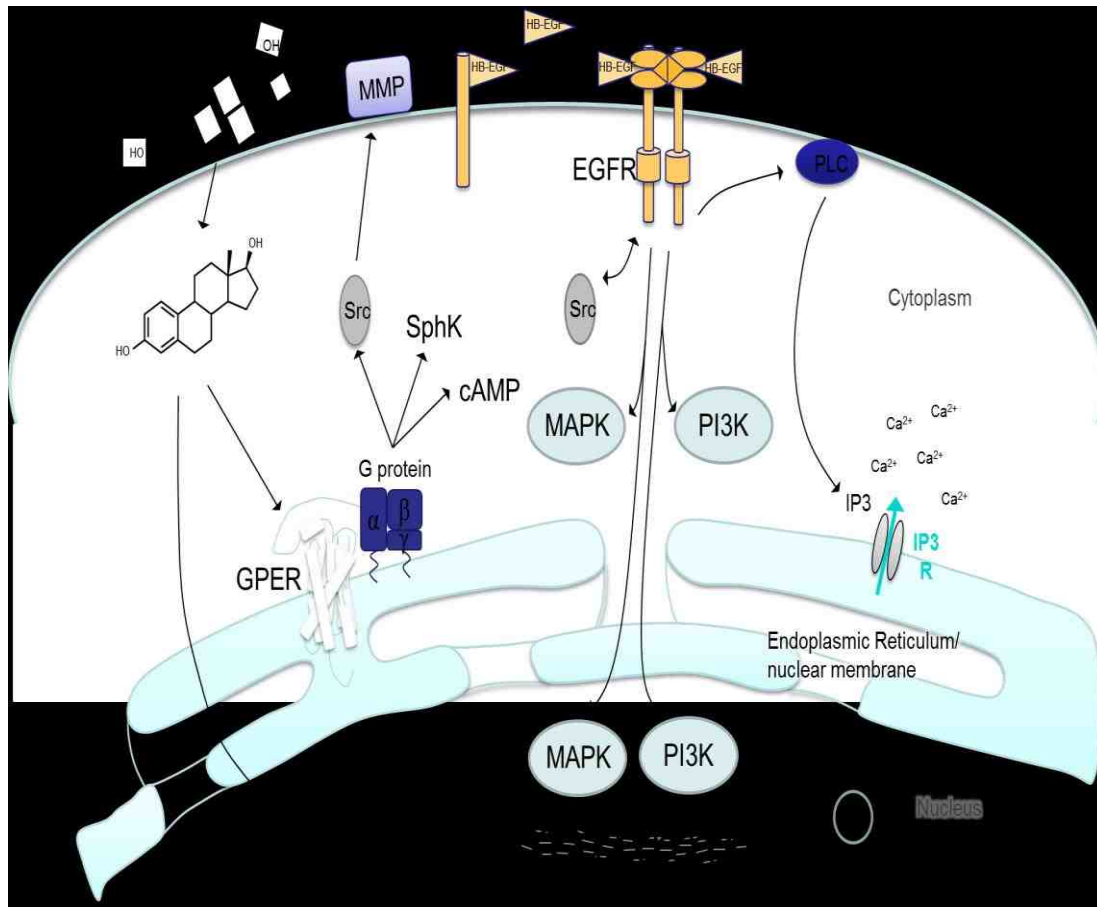


Figure 1.3. Estrogen receptor signaling summary. Estrogen is able to diffuse through the plasma and nuclear membrane in order to bind its canonical ERs within the nucleus. Estrogen can also bind GPER, which leads to transactivation of the EGFR and downstream signaling including PI3Kinase activation.

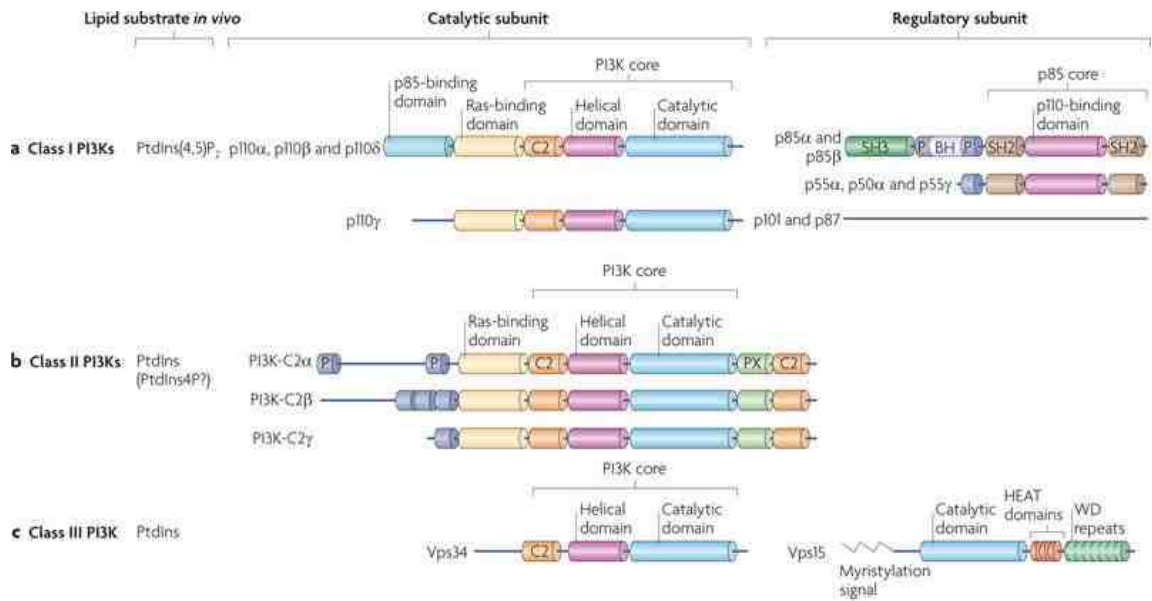
1.5. PI3Kinase

Lipid kinases have become highly scrutinized because of their association with growth factor-stimulated pathways, whose overstimulation can result in cancerous phenotypes when not properly regulated. In particular, the phosphoinositide 3-kinase (PI3K) family has been extensively investigated. The PI3K family is divided into four groups (I_A, I_B, II, and III) according to structural features and substrate specificity (Figure 1.4) (Vanhaesebroeck et al., 2010). Class I_A of PI3Ks are enzymes that consist of a catalytic p110 domain (p110 α , p110 β , and p110 δ) and a regulatory p85 domain (p85 α , p85 β , and p55 γ). The two ubiquitously expressed catalytic domains are p110 α and p110 β , which are usually coupled to their respective regulatory subunits p85 α and p85 β .

PI3Kinase activation occurs when the SH2 domains of the p85 subunit can bind to phosphorylated tyrosine residues generated by an upstream kinase in order to release the active catalytic p110 subunit. p110 α has been demonstrated to have a role in growth factor and metabolic signaling as well as being selectively mutated and overexpressed in a variety of cancers (Foukas et al., 2006). p110 β has been reported to be involved in DNA replication, S phase progression, and DNA repair (Marques et al., 2009) (Kumar et al., 2010) (Kumar et al., 2011). It has been suggested that, despite their similar structures and ubiquitous expression, the differing functions of p110 α and p110 β are related to their distinct subcellular localization. p110 α has been shown to be primarily localized to the cytoplasm while p110 β is primarily localized to the nucleus.

When a Class I_A PI3K is activated by an upstream signaling event, such as growth factor receptor activation, the phosphotyrosine residues generated within the receptor tail can bind to the p85 subunit and free the catalytic domain to function as a kinase. Activated PI3K converts the membrane phospholipid, phosphatidylinositol 4,5-bisphosphate (PIP₂) into phosphatidylinositol (3,4,5)-triphosphate (PIP₃) (Vanhaesebroeck et al., 2010). PIP₃ can then bind to the PH domains of Protein Kinase B (Akt) and 3-phosphoinositide-dependent kinase (PDK), recruiting these proteins to the membrane. The accumulation of PIP₃ is negatively regulated by the protein phosphatase PTEN, which dephosphorylates PIP₃ back to PIP₂. At the membrane, PDK can only phosphorylate the Thr308 residue of Akt. In order to phosphorylate Akt's additional activation residue Ser473, it has been suggested that PDK forms a complex with another kinase, PRK (Datta et al., 1999). The actions of activated Akt include regulating cell survival by interacting with multiple proteins. One such protein is Bad, a member of the proapoptotic Bcl-2 family, which has been shown to be phosphorylated and inactivated by Akt. The human caspase-9 which executes apoptosis was also shown to be phosphorylated and inactivated by Akt (Datta et al., 1999). A number of transcription factors, which include members of the forkhead box (FOX) family, can also be regulated by Akt. The FOX class of transcription factors are involved in cell fate decisions, proliferation and metabolism. In particular, FOXO3a, a transcription factor that aids in the production of proapoptotic genes, is known to be phosphorylated by Akt (Brunet et al., 1999).

This leads to the exclusion of FOXO3a from the nucleus and subsequently a decrease in proapoptotic gene expression.



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Figure 1.4. Phosphoinositide 3-kinases (PI3Ks) are divided into three classes based on their structural and biochemical features. All PI3K catalytic subunits have a core structure consisting of a C2 domain, a helical domain and a catalytic domain (Vanhaesebroeck et al., 2010). (a) Class I use phosphatidylinositol-4,5-bisphosphate (PtdIns(4,5)P₂) as their substrate and exist in complex with a regulatory subunit, either a p85 isoform (for p110α, p110β and p110δ) or p101 or p87 (for p110γ). All p85 isoforms have two Src homology 2 (SH2) domains. p101 and p87 lack SH2 domains. (b) Class II PI3Ks use PtdIns as a substrate, but might also use PtdIns-4-phosphate (PtdIns4P) under certain conditions. They lack regulatory subunits but have amino- and carboxy-terminal extensions to the PI3K core structure. (c) Class III PI3K has one catalytic member, vacuolar protein sorting 34 (Vps34) which uses PtdIns as a substrate and binds Vps15.

1.6. FOX Proteins

In 1990, when the DNA binding motif of the hepatocyte enriched HNF-3 transcription factors was initially being described, it did not resemble any known binding motif (Lai et al., 1990). However, once the sequence was analyzed, its 110-amino-acid DNA binding domain was discovered to be almost perfectly conserved with the *Drosophila melanogaster forkhead* gene (Weigel and Jackle, 1990). Proteins with this conserved domain are now referred to as forkhead box (FOX) proteins and consist of a large family of more than 100 transcriptional regulators.

The mammalian forkhead domain is a monomeric DNA-binding domain, approximately 100 amino acids long, and consists of three α helices and two characteristic large loops or butterfly-like 'wings' (Burgering, 2008). There are more than 100 members of the FOX family and they are involved in regulating a number of cellular processes, with roles in development, differentiation, proliferation, apoptosis, stress resistance and metabolism. They are divided into 19 different subgroups and the 39 human Forkhead proteins are designated by a capital letter after FOX (FOX for 'Forkhead Box' A to S). The FOXO subfamily of transcription factors is involved in cell fate decisions, proliferation and metabolism. Other examples of FOX proteins that have a role in development are FOXC, which is involved in organogenesis and FOXP, involved in language acquisition and organogenesis (Lehmann et al., 2003).

The FOXO subgroup consists of four members: FOXO1, FOXO3a, FOXO4 and FOXO6. These proteins are ubiquitously expressed, but vary in their levels of

expression in different cell types. FOXO1 is more highly expressed in adipose tissue, while FOXO4 is found in the heart and muscle. FOXO3a has elevated levels in the brain and liver, whereas FOXO6 is highly expressed in the developing brain, suggesting a role in neural development. FOXO proteins have the same DNA binding-domain, which suggests that they should be able to bind to similar sequences within DNA. The core consensus sequence for FOXO binding is (5'TTGTTTAC3') (Furuyama et al., 2000). FOXO3a, 1, and 4 initially became of interest when they were found at the chromosomal translocations of tumors. This localization suggested that FOXO proteins may play a role in tumor development.

FOXO3a, 1 and 4 can be localized either to the nucleus or the cytoplasm depending on their phosphorylation status, while FOXO6 is predominantly nuclear and its phosphorylation status only affects its function. When FOXO proteins are nuclear and functional, they act as transcription factors that regulate expression of their target genes. FOXO proteins are able to induce cell cycle arrest by upregulating the cdk inhibitors p27kip and p21, and the Rb family member p130, which block G1/S phase progression. FOXO proteins can also promote apoptosis by inducing expression of the proapoptotic Bcl-2 family member Bim. Bcl-2 family members are involved in MOMP (mitochondrial outer membrane permeabilization), which induces cytochrome C release and subsequent events leading to cell death. FOXO proteins can also induce apoptosis by increasing expression of the death receptor ligand FasL and the cytokine TRAIL (Accili and Arden, 2004). FOXO protein regulation of gene

expression that results in inhibition of the cell cycle and apoptosis suggests that this subgroup of forkhead proteins represents *bona fide* tumor suppressors. FOXO proteins are regulated/phosphorylated by Akt. Once Akt is activated by the PI3Kinase pathway in the presence of growth factor signals, it is presumed to translocate from the plasma membrane to the nucleus where it phosphorylates FOXO proteins at specific residues. Akt can phosphorylate FOXO3a, 1, and 4 on three key regulatory sites Ser253, Ser315 and Thr32 though Ser253 and Thr32 are the preferred sites. FOXO proteins can also be phosphorylated by SGK (serum and glucocorticoid-inducible kinase), which prefers the combination of Ser315 and Thr32. In the absence of growth factor signals/activation of the PI3Kinase pathway, FOXO proteins are localized predominantly to the nucleus, where they carry out their functions as proapoptotic transcription factors. However, when the PI3Kinase pathway is stimulated, phosphorylated FOXO proteins translocate from the nucleus to the cytoplasm. A leucine-rich region within their C-terminal domains functions as a nuclear export sequence, for which the 14-3-3 protein is required. 14-3-3 proteins are α -helical molecules that regulate intracellular signal transduction. The Akt phosphorylation sites at the N terminus and in the forkhead domain of FOXO proteins create two 14-3-3 binding motifs and induce FOXO binding to nuclear 14-3-3 proteins (Brunet et al., 2002). Akt phosphorylation and subsequent binding of the 14-3-3 protein results in FOXO's exclusion and transport from the nucleus to the cytoplasm. Once FOXO proteins translocate to the cytoplasm, they are targeted for degradation by the ubiquitin-proteasome pathway.

In addition to Akt, the inhibitor of nuclear factor kappa-B kinase subunit β (IKK β) also causes the proteasome-dependent degradation of FOXO factors, specifically FOXO3a, via phosphorylation at Ser644 (Hu et al., 2004). Since IKK β positively regulates NF-KB by inactivating its negative regulator IKB, and phosphorylates FOXO3a for degradation, IKK β can promote uncontrolled cell proliferation leading to tumorigenesis.

1.7. PI3Kinase/Akt/FOXO and Cancer

Since the PI3Kinase/Akt/FOXO pathway leads to regulation of cell proliferation, control of the cell cycle and apoptosis, it is not surprising that dysregulation of this pathway can result in tumor growth and cancer. The most common irregularity in this signaling pathway promoting cancer is mutations in *PI3KCA*, which is the gene encoding the p110 α catalytic domain of PI3Kinase (Janku et al., 2014). Three different mutations have been attributed to *PI3KCA*-induced cancer. The first, H1047R, occurs in the activation loop of the catalytic domain and results in constitutive kinase activity. The other two, E542K and E545K, are located in the helical domain of the catalytic subunit and have been determined to block the ability of the regulatory domain to keep the catalytic domain in an inactive state. All three of these mutations result in the over-activation of PI3Kinase, leading to uncontrolled cell proliferation, survival and tumorigenesis. The Cancer Genome Atlas (TCGA) reports that the *PI3KCA* gene is mutated in 53% of endometrial cancers, 35% of breast cancers, 23% of cervical cancers, 21% of gastric cancers, 20% of head and neck cancers, 20% of colorectal

cancers, 15% of lung squamous cell cancers, and 10% of glioblastomas as well as in other tumor types (Polivka and Janku, 2014). There are many selective inhibitors for PI3Kinase currently in clinical trials.

Another constituent of this signaling pathway whose function can be disrupted and can cause cancer is PTEN (phosphatase and tensin homolog). PTEN has been deemed a tumor suppressor because of its ability to dephosphorylate PIP3, which prevents Akt binding and subsequent activation. The disruption of PTEN's tumor suppressor ability can be attributed to mutations, deletions, transcriptional silencing and epigenetic changes. For example, the zinc-finger transcription factor sal-like protein 4 (SALL4) represses PTEN transcription by recruiting an epigenetic repressor complex to the PTEN locus. Additionally, hypermethylation of PTEN's promoter causes it to be epigenetically silenced and this pathological feature is observed in many cancers (Song et al., 2012). Furthermore, PTEN splice variants retaining introns 3 and 5 have been found in sporadic breast cancer. This result suggests that alternative splicing of PTEN could also contribute to the development of breast cancer.

Downstream of PI3Kinase activation, Akt activation results in cell proliferation and survival through inactivation of FOXO and many other proteins. The 3 different isoforms of Akt (1, 2, and 3) exhibit approximately 80% sequence homology. Akt was initially discovered to be overexpressed in ovarian cancers, as well as in pancreatic tumors and other human malignancies (Polivka and Janku, 2014). In addition to overexpression, there are known activating mutations that consist of the amino acid substitution of E17K in Akt's lipid binding

domain that keep it localized to the membrane, therefore increasing its ability to be activated by membrane localized PDK. These mutations have been found in ovarian, breast and colorectal cancers. Akt inhibitors are being developed that allosterically inhibit this kinase.

Activated FOXO3a is a proapoptotic transcription factor within the nucleus, and it has been referred to as a tumor suppressor. FOXO3a is a downstream target of PI3Kinase/Akt, which makes it an attractive prognostic marker in cancers. As discussed above, PI3Kinase hyperactive mutations are found in breast cancer and would result in cytoplasmic localization and inactivation of FOXO3a. Chen et al. suggested that nuclear FOXO3a is correlated with poor prognosis in breast cancer. They propose that the uncoupling of sustained nuclear FOXO3a and PI3Kinase/Akt signaling results in an inactive nuclear FOXO3a which results in cancer progression (Chen et al., 2010). In contrast, Hashaby et al. correlated poor prognosis with cytoplasmic localization of FOXO3a in breast cancer patient tissue samples and nuclear localization with good prognosis (Habashy et al., 2011). FOXO proteins have been shown to interact with a well-known tumor suppressor, p53, within the nucleus. This interaction has been suggested to lead to coordinated tumor suppression because p53 and FOXO proteins can regulate some of the same genes, such as p21, a cell cycle inhibitor (van der Horst and Burgering, 2007). FOXO proteins also interact with SMADs to regulate target gene expression leading to tumor suppression. SMADs respond to transforming growth factor beta (TGF- β) and translocate to the nucleus in order to regulate their antiproliferative target genes. Additionally, FOXO proteins can interact with

β -catenin and this association is suggested to sequester β -catenin, inhibiting cellular adhesion, which promotes tumor growth.

1.8. Breast Cancer

Breast cancer is one of the leading causes of cancer-related deaths in women. In recent years, the incidence has decreased due to early detection and more specialized treatment methods. Characterization of the receptor status of a patient's tumor enables physicians to decide the best course of treatment on a more individual basis. It has been recommended by the American Society of Clinical Oncology and the College of American Pathologists that all invasive breast cancer patient samples be tested for their ER, PR and HER2 receptor status (Anderson et al., 2014). The major tumor subtypes can be classified into four groups based on their receptor status. Luminal A is defined as ER+, and/or PR+, and HER2- and is considered to be the least invasive with a generally higher survival rate while Luminal B (ER+, and/or PR+, HER2+ or HER2- with high Ki67) is characterized by higher proliferation rates and a poorer survival prognosis. Both Luminal A and B are able to respond to endocrine therapy. HER2-overexpressing tumors have a higher grade, spread more aggressively, even to lymph nodes, and often respond to HER2-targeted therapies. Lastly, triple negative or basal-like breast cancer (TNBC) does not express any of the above receptors and because of this has no targeted therapies.

Estrogen's role in breast cancer is linked to its receptor status. If estrogen levels are elevated, it can lead to overstimulation of the canonical ER α receptor when

present, resulting in uncontrolled cell proliferation, i.e. cancer. However, ER α 's expression in certain breast cancers can also allow it to be targeted by the anti-estrogen, tamoxifen. Tamoxifen, a SERM (Selective Estrogen Receptor Modulator), was introduced in the 1970s and is frequently used in pre-menopausal woman diagnosed with ER-positive cancer. Tamoxifen is able to antagonize ER α by inhibiting its AF2 domain and therefore reducing genomic signaling that leads to uncontrolled proliferation (Johnston and Yeo, 2014). In post-menopausal women it is becoming more common to use aromatase inhibitors (AIs), which block the overall production of estrogen. However, SERMS such as tamoxifen have also been shown to act as agonists for the more recently discovered estrogen receptor, GPER, which could have the opposite effect on an estrogen-responsive tumor that is ER-negative.

It had been previously established that approximately one in four patients did not respond to tamoxifen therapy with various reasons implied such as ER expression heterogeneity, etc. As of 2002, GPER had been demonstrated to be an estrogen-responsive receptor that induced rapid signaling effects resulting in proliferation of cells in the absence of the classical ERs (Filardo, 2002). In 2006, to determine whether GPER could be related to unsuccessful hormone therapy in breast cancer, a study was performed to assess the relative tissue distribution of GPER, ER, and PR in intraductal and invasive ductal carcinoma, correlated with other known histopathologic markers of disease (Filardo et al., 2006).

Approximately 60% of the samples expressed GPER, with roughly 50% of ER α -positive tumors expressing GPER. This would indicate an intact E2 signaling

pathway is also present in ER α -negative tumors that express GPER. This study also demonstrated increased tumor size and the presence of distant metastases correlated with increased GPER expression. In 2010, it was established that tamoxifen resistance in MCF-7 breast cancer cells, which are ER+ and GPER+, was dependent on GPER expression (Ignatov et al., 2010). Additionally, this study confirmed a role for tamoxifen as a GPER agonist. This would suggest that GPER expression could be a marker for the occurrence of tamoxifen resistance in ER+ breast cancer and that targeting GPER in addition to ERs could rectify this problem.

1.9. GPER-selective ligands

Estrogen binds to the classical canonical receptors, ER α and ER β . It has also been established that GPER is an estrogen receptor that can bind E2.

Furthermore, GPER and ER β can bind the ER α antagonists ICI 182,780, tamoxifen and raloxifene (Barkhem et al., 1998)(Prossnitz and Barton, 2014).

This makes it more challenging to delineate GPER's response when all three receptors are present. In order to determine the cellular effects in response to stimulation of GPER, a GPER-selective agonist was generated. This was accomplished by screening a large library of chemical compounds to discover those that could bind selectively to GPER (Bologa et al., 2006).

A virtual screen of a library of approximately 10,000 GPCR-associated compounds was analyzed 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 (Figure 1.5), was subsequently identified as a selective GPER agonist. G-1 competitively displaced binding of fluorescent E2 (E2-Alexa) in GPER-transfected cells. G-1 was determined to have an inhibition constant (K_i) of 11nM for GPER, whereas the K_i for E2 binding GPER is 5.7 nM (Bologna et al., 2006). No significant affinity of G-1 for ER α or ER β was observed. The specificity of G-1 as an agonist was confirmed by its ability to promote intracellular calcium immobilization in COS-7 cells transfected with GPER, but not in COS-7 cells transfected with the canonical ERs.

A GPER-selective antagonist was later identified and named G15 (Figure 1.5). G15 antagonizes E2-dependent GPER activation in vitro and in vivo, based on data from a study using an established assay of E2-induced murine uterine proliferation (Dennis et al., 2009). G15 exhibits low-affinity cross reactivity to ER α at concentrations of 10 μ M and above. G36 (Figure 1.5) was subsequently synthesized and exhibited a higher selectivity for GPER over ER α than G15 (Dennis et al., 2011). These selective agonist and antagonists are essential tools for studying GPER specific effects in cells and in vivo.

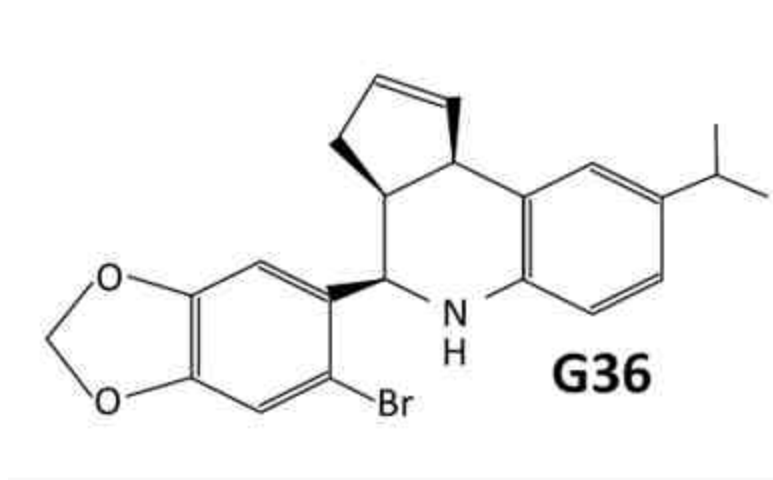
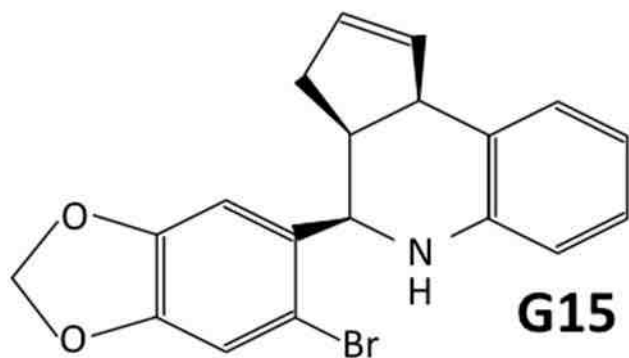
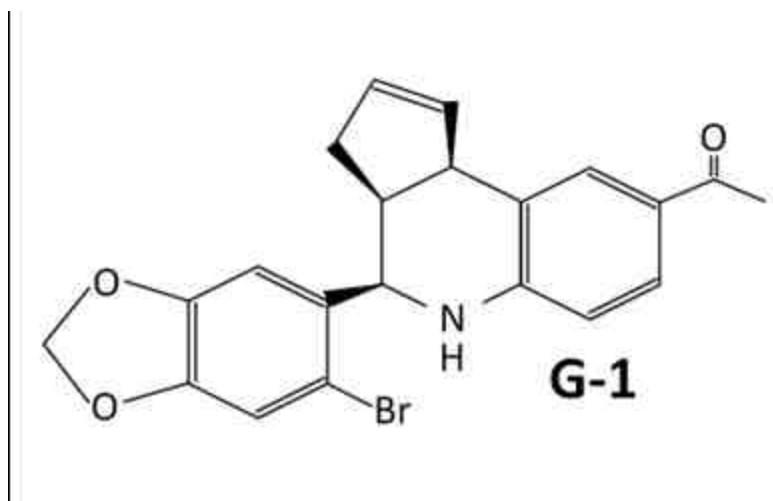


Figure 1.5. GPER-selective ligands. Chemical structures of GPER-selective agonist (G-1) and antagonists (G15 and G36).

Rationale

In addition to its genomic effects, estrogen has been shown to mediate rapid signaling events in diverse cell types, including breast cancer cells. These effects are, in part, mediated through its classical soluble nuclear receptor, ER α . However, in 2000 a novel estrogen receptor known as GPR30 (currently named GPER) was demonstrated to activate ERK-1/2 in response to estrogen. In 2005, our lab demonstrated PI3Kinase activation was also initiated by estrogen activation of GPER. The mechanism of this activation and its downstream effects are currently not well understood.

Hypothesis

Our lab has previously demonstrated that the estrogen receptors ER α and GPER can activate PI3Kinase when exogenously and endogenously expressed. FOXO3a has been shown to be inactivated by PI3Kinase-activated Akt via a phosphorylation event leading to the exclusion of FOXO3a from the nucleus. Therefore, we hypothesize that estrogen receptor stimulation in MCF7 cells expressing both ER α and GPER can inactivate FOXO3a through activation of PI3Kinase by GPER.

Specific Aims

Aim 1: Determine the effects of estrogen induced PI3Kinase activation.

Aim 2: Determine the mechanisms responsible for estrogen regulation of FOXO3a.

Aim 3: Investigate the resulting effects of FOXO3a regulation by estrogen receptors.

CHAPTER 2

2. GPER activation leads to FOXO3a inactivation in MCF7 breast cancer cells.

2.1. Abstract

In addition to its genomic effects, the steroid hormone estrogen has been shown to mediate rapid signaling events in diverse cell types, including breast cancer cells. These effects are, in part, mediated through its canonical soluble nuclear receptor, estrogen receptor α (ER α). However, a novel estrogen receptor known as GPER (G-protein coupled estrogen receptor, previously termed GPR30) has been demonstrated to activate multiple signaling cascades in response to estrogen. We previously established that PI3Kinase-mediated production of PIP3 in the nucleus is initiated by estrogen-mediated stimulation of GPER. The mechanism of this activation and its downstream effects are not well understood. Here we describe that estrogen and the GPER-selective agonist G-1 induce rapid signaling through GPER to activate PI3Kinase. This activation occurs via the transactivation of EGFR leading to the activation of the p110 α subunit of PI3Kinase resulting in the subsequent Akt-mediated phosphorylation of the transcription factor FOXO3a. PI3Kinase inactivates the proapoptotic protein FOXO3a, resulting in its translocation from the nucleus to the cytoplasm. The nuclear exclusion of FOXO3a by GPER activation suggests GPER signaling results in prosurvival cellular effects in breast cancer cells.

2.2. Introduction

Estrogen is the main female sex hormone involved in an array of physiological processes. The most biologically active form of estrogen, 17β -estradiol (E2), is primarily produced in the ovaries of females and the testes of males, and is involved in the development, maintenance and function of the reproductive organs in both. In females, estrogen also regulates mammary growth and this is dependent on the estrous cycle. When normal mammary growth pathways are not properly regulated, and/or genetic mutations occur, breast cancer can develop.

Estrogen's actions are mediated by its classical nuclear receptors ER α and ER β . These actions include, but are not limited to, E2 binding to its receptors, which then dimerize and function as transcription factors that bind to EREs (Estrogen Response Elements) within DNA to modulate gene expression (Kumar and Chambon, 1988). Activated ER α modifies the production of genes, which results in the expression of proteins that can promote cell growth and proliferation. ER β 's function is less well characterized, but it has been suggested in many cases to oppose the functions of ER α (Sanchez et al., 2013). More recently, another estrogen receptor was discovered, originally named GPR30 and now GPER (G-protein coupled estrogen receptor) (Carmeci et al., 1997). GPER has been demonstrated to be responsible for estrogen's activation of the MAP Kinases ERK-1/2 in ER α - and ER β - negative cells (Filardo et al., 2000). Estrogen's activation of ERK-1/2 is dependent on the epidermal growth factor receptor (EGFR), which is transactivated by GPER stimulation. Transactivation

of EGFR by GPER stimulation occurs in a Src-dependent manner via the release of Heparin-bound epidermal growth factor (HB-EGF), which binds and activates the EGFR. Subsequently, the Prossnitz laboratory discovered in 2005 that E2 can activate PI3Kinase, also as a consequence of transactivation of the EGFR (Revankar et al. 2005). Using the PH domain of the downstream effector Akt (Protein Kinase B) as a reporter, they concluded PI3Kinase activation led to nuclear accumulation of its phosphorylated substrate PIP3. The mechanism for this is still unclear.

The enzyme PI3Kinase converts the membrane phospholipid, phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol-(3,4,5)-triphosphate (PIP3). PI3Kinase consists of a catalytic domain and a regulatory domain. The two ubiquitously expressed catalytic domains are p110 α and p110 β and are usually coupled to their respective regulatory subunits p85 α and p85 β , respectively (Vanhaesebroeck et al., 2010). PI3Kinase activation occurs when the SH2 domains of the p85 subunit bind to phosphotyrosine residues generated by an upstream kinase in order to release the active p110 catalytic subunit.

p110 α has been demonstrated to have a role in growth factor and metabolic signaling as well as being selectively mutated and overexpressed in a variety of cancers (Foukas et al., 2006). p110 β has been reported to be involved in DNA replication, S phase progression, and DNA repair (Kumar et al., 2011).

Downstream of PI3Kinase activation, PIP3 recruits Akt leading to Akt phosphorylation and activation by PDK. Akt has many substrates such as the proapoptotic protein Bad and glycogen synthase kinase-3 (GSK-3). The

phosphorylation of these substrates by Akt can lead to cellular responses including cell survival by inactivating Bad and the regulation of metabolism by inhibiting GSK-3 (Datta et al., 1999). Additionally, the proapoptotic transcription factor, FOXO3a is inhibited by Akt to promote cell survival (Zheng et al., 2000). The forkhead box O (FOXO) class of transcription factors is involved in cell fate decisions, proliferation, and metabolism. The FOXO transcription factors are characterized by their 'winged helix' DNA binding domains. Since their functions are regulated by pathways found to be deregulated in cancer, FOXO proteins are generally characterized as tumor suppressors (Burgering, 2008). FOXO proteins can be regulated by Akt, specifically FOXO3a. FOXO3a is a transcription factor that aids in the production of proapoptotic genes, and is phosphorylated by Akt (Zheng et al., 2000). Akt preferentially phosphorylates FOXO3a at Ser253 and Thr32, leading to the exclusion of FOXO3a from the nucleus and a decrease in proapoptotic gene expression (Brunet et al., 1999). FOXO3a localization has been utilized to determine whether a cell is in a prosurvival or proapoptotic state (Jacobs et al., 2003) (Wen et al., 2011). Nuclear FOXO3a suggests that signaling pathways such as the PI3Kinase/Akt pathway are inactive and FOXO3a is acting as a proapoptotic transcription factor within the nucleus. Bim and p27 are examples of proteins whose gene expression is upregulated by nuclear localized FOXO3a. When the PI3Kinase/Akt signaling pathway is activated, FOXO3a is phosphorylated by Akt and excluded from the nucleus, rendering it inactive. FOXO3a has been shown to localize to the nucleus in response to chemotherapeutic drugs, such as Doxorubicin, in a breast cancer cell line (Ho et

al., 2012). Furthermore, in patient tissue samples, nuclear localization in luminal-like breast cancers has been associated with a good prognosis (Habashy et al., 2011). Direct effects of estrogen receptor stimulation on FOXO3a phosphorylation have not been investigated.

Previous studies in our lab have demonstrated that stimulation of GPER and ER α results in the accumulation of the PH-RFP reporter in the nucleus (Revankar et al., 2005). These studies were performed in COS-7 cells cotransfected with GPR30-GFP/ER α -GFP and PH-RFP, and nuclear localization of PH-RFP in response to receptor stimulation was monitored using confocal microscopy. The PH domain of Akt binds to phosphatidylinositol (3,4,5)-triphosphate (PIP3), which is generated from the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) by the upstream PI3Kinase. PI3Kinase inhibition abolished the nuclear accumulation of PIP3 in this assay, suggesting that GPER and ER α stimulation results in the activation of PI3Kinase. Furthermore, our previous studies also demonstrated that in the human breast cancer cell line SKBR3, which express GPER and are ER α -/ER β - negative, estrogen and the GPER specific agonist G-1 were able to stimulate PIP3 production in the nucleus. The mechanism and downstream effects of estrogen receptor stimulated PI3Kinase activation are not well understood. In the present study, we utilized the human breast cancer cell line MCF7, which express all three estrogen receptors (ER α , ER β and GPER) in order to provide a more physiologically relevant setting to investigate estrogen receptor activation of the PI3Kinase pathway.

2.3. Materials and Methods

Cell Culture

The human breast cancer cell line, MCF7 (ATCC) were maintained in Dulbecco's modified eagle's medium (DMEM) (Sigma) with 1% Penicillin/Streptomycin/Glutamine Solution (100X) (Thermo Scientific) and 10% Fetal Bovine Serum (Thermo Scientific). Where serum starvation is indicated, DMEM was replaced with DMEM/F-12 50/50 without phenol red (Cellgro, Mediatech) with 1% Penicillin/Streptomycin/Glutamine Solution.

Inhibitors and Antibodies

LY 294002 (LY) (CalBiochem) is used as a broad spectrum inhibitor of all PI3Kinases. PIK-75 (Chemdea, New Jersey) is used to inhibit the p110 α isoform of the PI3-Kinase catalytic subunit, while TGX-221 (Chemdea, New Jersey) inhibits the p110 β isoform. EGFR inhibitor Tyrphostin AG1478 (Calbiochem) is used to inhibit the EGFR receptor's tyrosine kinase activation. Primary antibodies used were Bim, p27, phospho-Akt and Total Akt (Cell Signaling), ER α (Santa Cruz Biotechnology, Inc.), and GPER polyclonal antibody against a C-terminal peptide in the human GPER protein previously described (Revankar et al., 2005).

Transfections/Assays

PH-RFP Translocation

The PIP3 binding domain of Akt fused to mRFP1 (PH-mRFP1) was used to localize cellular PIP3. MCF7 cells were seeded at ~20,000K cells per well on 12 mm coverslips in a 24 well plate 24 hours prior to transfection. Cells were

transfected with 0.6 µg of PH-mRFP1 using the Lipofectamine 2000 Transfection reagent (Invitrogen) following the manufacturer's protocol. Approximately 24 hours after transfection cells were serum starved for 24 hours followed by stimulation with ligands as indicated. The cells were fixed with 2% PFA in PBS, washed, mounted in Vectashield (Vector Laboratories, Inc.) and analyzed by confocal microscopy on the Leica SP5 microscope. Images were analyzed using Slidebook. A mask for the nucleus alone and for the entire cell including the nucleus was created. The average nuclear intensity of the cy3 channel divided by the average intensity of the entire cell indicates the amount of PH-RFP that is localized to the nucleus after stimulation.

FoxO3-GFP Translocation

The FoxO3-GFP plasmid was a generous gift from Dr. Marten P. Smidt (University of Amsterdam) and was generated as described (Jacobs et al., 2003). MCF7 cells were seeded at ~20,000K cells per well on 12 mm coverslips in a 24 well plate 24 hours prior to transfection. Cells were transfected with 0.6 µg of FoxO3-GFP using the Lipofectamine 2000 Transfection reagent (Invitrogen) following the manufacturer's protocol. Approximately 24 hours after transfection cells were serum starved for 24 hours followed by stimulation with ligands/inhibitors as indicated. The cells were fixed with 2% PFA in PBS, washed, mounted in Vectashield and analyzed by confocal microscopy on the Leica SP5 microscope. Approximately 50 cells per treated coverslip were counted through the eyepiece and the localization of FoxO3-GFP was assessed.

Localization status was separated into three categories: predominantly nuclear, partially nuclear, and cytoplasmic.

siRNA and FOX3-GFP Translocation

MCF7 cells were seeded at ~20,000K cells per well on 12 mm coverslips in a 24 well plate 24 hours prior to transfection. Small-interfering RNA (siRNA) was from Dharmacon RNAi Technologies (Lafayette, CO, USA). 50 pmol per well of siGPER (ONTARGET plus SMARTpool siRNA (L-005563-00)), siER α (ONTARGET plus SMARTpool siRNA (L-003401-00) Human ESR1), and siControl (ON-TARGETplus siControl Non-Targeting siRNA (D-001810-02)) were transfected using Lipofectamine 2000. 24 hours after transfection, 0.6 μ g of FoxO3-GFP was transfected into each well. The next day, cells were serum starved for 24 hours and treated as specified. The cells were fixed with 2% PFA in PBS, washed, mounted in Vectashield and analyzed by confocal microscopy on the Leica SP5 microscope. Approximately 50 cells per treated coverslip were counted and the localization of FoxO3-GFP was assessed.

Immunofluorescence

Akt Antibody Staining and Localization

MCF7 cells were seeded onto 12 mm coverslips in a 24 well plate, serum starved for 24 hours, then treated with ligands as indicated. Cells were fixed in 2% PFA, permeabilized with 0.1% Triton-X100 for 10 minutes, and 1% BSA blocking for 1 hour. Coverslips were then incubated with 1:200 Akt antibody in 1% BSA overnight in a moist chamber at 4°C. Cells were then washed with PBS and incubated with Goat anti-rabbit Alexa 533 secondary antibody for 1 hour at room

temperature in the dark. After washing with PBS, coverslips were mounted with Vectashield and analyzed by confocal microscopy. Akt cellular distribution was analyzed using Slidebook. The nucleus was isolated by masking and the average nuclear intensity of the Cy3 channel was determined.

Caspase Activation

Magic Red® Caspase 3/7 Detection Kit (Immunochemistry Technologies) was utilized in order to monitor Caspase 7 activation in MCF7 cells. MCF7 cells were seeded onto 12 mm coverslips in a 24 well plate. Approximately 24 hours after seeding, treatments were added for 1, 2, 3 and 5 days. At the end of each time point, cells were incubated with the Magic Red substrate solution diluted as directed by manufacturer's protocol for 1 hour at 37°C, washed twice with PBS and fixed with 4% PFA. Cells were washed twice and stained with TO-PRO®-3 (Life Technologies) for 10 minutes at room temperature in the dark then washed twice again with PBS. The coverslips were mounted in Vectashield and analyzed by confocal microscopy on the Leica SP5 microscope.

Western Blotting

MCF7 cells were seeded to 60-80% confluency in 60 mm dishes and serum starved 24 hours prior to indicated treatments. After treatment, cells were washed twice with cold PBS and scraped into lysis buffer. Cells were lysed in RIPA buffer containing NP-40 supplemented with sodium fluoride (50 mM), sodium orthovanadate (1 mM), phenylmethylsulfonyl fluoride (1 mM), 0.1% SDS, 0.5% sodium deoxycholate and protease cocktail (1X). Cell lysate protein concentration was determined by Bradford protein assay (Bio-Rad, Hercules,

CA). Equal protein concentrations per lysate were loaded on a 4-20% Precise Tris-Glycine Gels (Thermo Scientific) and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked in 5% Blotting Grade Blocker Non-Fat Dry Milk (Bio-Rad) for 1 hour at room temperature and then incubated with primary antibodies (1:1000 dilution for Bim, p27 and pAkt) (1:500 for ER α) (1:5000 for GPER) (1:10,000 for actin) in 3% BSA overnight at 4°C with gentle rocking. After a series of washes, the blots were then incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG (1:3000) or goat anti-mouse IgG for actin (1:5000) in 3% BSA for 1 hour at room temperature with gentle rocking. The blots were developed using Supersignal West Pico Chemiluminescent Substrate (Thermo Fisher). Films were scanned and quantified using ImageJ software (National Institutes of Health).

Image Analysis

Statistical analysis

Statistical analysis was performed using GraphPad Prism version 5. Analysis was done with a one way analysis of variance (ANOVA) within Prism estimates the correlation of variables with treatment groups (DMSO, EGF, G-1, E2, etc).

Pairwise comparisons of results between different treatment groups were determined using a one-way Analysis of Variance (ANOVA) comparing all values to control followed by a Dunnett's posttest. Data represents the mean \pm SEM of three or more separate experiments. P-values less than .05 were considered to be significant. In order to determine significance in siRNA experiments between

siControl and siGPER or siER α as well as EGF time points, a two way ANOVA analysis was performed utilizing Bonferroni posttest.

2.3. Results

PH-RFP localization in MCF7 cells.

In order to determine whether estrogen receptor stimulation leads to PI3Kinase activation, we utilized the PH reporter assay previously described. EGF is utilized as a positive control because it is known to activate the PI3Kinase pathway (Burgering and Coffey, 1995). Although EGF induced a small but significant increase in PH-RFP in the nucleus, there was no detectable difference in PH-RFP within the nucleus of MCF7 cells upon stimulation with E2 and G-1 compared to DMSO vehicle control (Figure 2.1). There is an observable amount of PH-RFP within the nucleus under non-treated, starved conditions (Figure 2.1a). This potentially suggests that the difference between residual PIP3 generation and the amount that occurs following estrogen receptor stimulation is below the detection limit.

Estrogen receptor stimulation induces phosphorylation of Akt in MCF7 cells.

Downstream of PI3Kinase activation, Akt is recruited to PIP3 and phosphorylated by PDK. Therefore we monitored the phosphorylation of Akt at the Ser473 position in response to estrogen receptor stimulation (Figure 2.2). E2 induces a slight increase in phospho-Akt compared to DMSO vehicle control. However, the GPER-selective ligand G-1 significantly enhances the phosphorylation of Akt

compared to control. This suggests that GPER is the receptor responsible for Akt activation in response to E2 and that E2's activation of this pathway via GPER in MCF7 cells may be counteracted by E2 binding to ER α and ER β as well resulting in alternative cellular effects. When cell lysates were preincubated with the PI3Kinase inhibitor LY 294002 (LY), phosphorylation of Akt by all ligands was inhibited, suggesting that GPER activation resulting in Akt phosphorylation requires PI3Kinase.

Estrogen receptor stimulation induces translocation of Akt in MCF7 cells.

We have previously demonstrated PIP3 accumulation in response to E2 and G-1 in the nucleus of COS-7 cells with exogenously expressed estrogen receptors (Revankar et al., 2005). In order to investigate the downstream effects of this in cells with endogenous estrogen receptors, we examined whether Akt would translocate to the nucleus in response to E2 and G-1 in MCF7 cells. MCF7 cells were treated with DMSO vehicle control, EGF, E2 and G-1, followed by staining with an Akt antibody (Figure 2.3). The average nuclear intensity per cell was assessed by masking the nucleus to determine the average fluorescence intensity within the nucleus. While E2 did not induce a significant increase in the translocation of Akt to the nucleus compared to control (Figure 3c), EGF and G-1 (Figure 2.3b and 2.3d) treatments led to a significant increase in total Akt within the nucleus. This suggests that G-1 activation of GPER causes Akt to translocate to the nucleus. It can be hypothesized that E2 stimulation of GPER activates the Akt already present within the nucleus or that the response would potentially increase with time.

Estrogen receptor activation leads to FOXO3a inactivation.

Akt has many known targets within the nucleus (Datta et al., 1999). In particular, Akt is known to phosphorylate the proapoptotic transcription factor, FOXO3a. When FOXO3a is active (i.e. unphosphorylated), it resides within the nucleus functioning as a transcription factor modulating the expression of proapoptotic genes. In the presence of growth factor signaling that activates the PI3Kinase pathway, FOXO3a is phosphorylated by Akt and translocates from the nucleus to the cytoplasm, where it is subsequently degraded. In order to investigate estrogen receptor activation of the PI3Kinase pathway, we employed a FoXO3-GFP fusion protein obtained from the Smidt laboratory (Jacobs et al., 2003). In humans, FOXO3a is a member of the FOXO class of forkhead transcription factors. In mice, FoxO3 is FOXO3a's orthologue. Human FOXO3a and mouse FoxO3 share a high sequence homology as well as the same Akt phosphorylation sites (Jacobs et al., 2003). Therefore, we transfected MCF7 cells with FoxO3-GFP and monitored its localization in response to E2 and G-1 to determine estrogen receptor activation's effects on FoxO3-GFP. Serum was utilized as a positive control because it contains multiple growth factors that can activate the PI3Kinase pathway which would inactivate FoxO3-GFP. EGF was also a positive control because EGFR activation is known to lead to PI3Kinase activation (Burgering and Coffey, 1995). DMSO (vehicle) was the negative control. Depending on the treatment, we observed FoxO3-GFP's localization in three distinct patterns: predominantly nuclear, partially nuclear, and predominantly cytoplasmic (Figure 2.4a and 2.4b). Predominantly nuclear

(Figure 2.4a cell on right) refers to cells where nuclear FoxO3-GFP is greater than cytoplasmic, while partially nuclear (Figure 2.4a cell on left) has greater cytoplasmic localization, but with a visible amount of FoxO3-GFP in the nucleus. Predominantly cytoplasmic cells do not have any visible FoxO3-GFP in the nucleus. The percentage of each localization pattern counted per treatment was plotted (Figure 2.4c). The majority of DMSO treated cells are predominantly nuclear, with a very low percentage of cells that have a predominantly cytoplasmic pattern. A high percentage of the serum and EGF treated cells exhibited a predominantly cytoplasmic pattern. E2 and G-1 treated cells had a slightly higher percentage of cells that were predominantly cytoplasmic as opposed to predominantly nuclear. In order to interpret the data based on FoxO3-GFP's inactivation by Akt, the predominantly cytoplasmic fraction of each treatment was analyzed (Figure 2.5). DMSO has a low percentage of cells with inactive FoxO3-GFP. The positive controls, serum and EGF induced a substantial increase in cells where FoxO3-GFP was predominantly cytoplasmic and essentially inactive. E2 and G-1 had a significant percentage of cells with predominantly cytoplasmic FoxO3-GFP compared to control suggesting that estrogen receptor stimulation can inactivate FOXO3a. To determine the dependence of FoxO3-GFP's inactivation on concentration, increasing amounts of E2 and G-1 were added (Figure 2.6). The maximum response for E2 was obtained at 50 nM, while the maximum response for G-1 was at 100 nM.

Estrogen receptor activation leads to rapid FOXO3a inactivation.

Since rapid signaling events typically occur within 30 minutes, FoxO3-GFP's inactivation by estrogen receptor activation was monitored over a 30 minute period. The maximum response to E2 and G-1 compared to control was observed at 15 minutes (Figure 2.7). While a sustained response is still observed at 30 minutes, this is the first report of rapid inactivation of FOXO3a in response to estrogen receptor stimulation in a breast cancer cell line.

GPER is the estrogen receptor responsible for inactivation of FoxO3-GFP.

MCF7 cells express three estrogen receptors (GPER, ER α and ER β), all of which are capable of binding E2. Because FoxO3-GFP can be inactivated by E2 and G-1, we next sought to determine which estrogen receptor was responsible for E2 inactivation of FoxO3-GFP. MCF7 cells were consecutively transfected with GPER siRNA and FoxO3-GFP, and FoxO3-GFP inactivation in response to E2 and G-1 was assessed. Knockdown of GPER significantly reduced E2 and G-1's ability to inactivate FoxO3-GFP (Figure 2.8a) indicated by a significant decrease in the percentage of cells with cytoplasmic FoxO3-GFP. This suggests that GPER is the estrogen receptor responsible for E2's inactivation of FoxO3-GFP. Furthermore, knockdown of GPER did not affect EGF's ability to inactivate FoxO3-GFP (Figure 2.8b). GPER knockdown was confirmed by western blot (Figure 2.8c).

To further establish GPER's role in FoxO3-GFP's inactivation, ER α was knocked down in MCF7 cells. Utilizing cells transfected with ER α siRNA with FoxO3-GFP had no effect on E2's ability to inactivate FoxO3-GFP (Figure 2.9a). EGF was

also able to inactivate FoxO3-GFP, and ER α knockdown was confirmed by Western blot (Figure 2.9b and 2.9c).

FOXO3-GFP inactivation requires PI3-Kinase and the transactivation of EGFR in MCF7 cells.

It has been previously established that GPER stimulation leads to PI3Kinase activation and that EGFR transactivation was required as an intermediate in this signaling pathway (Revankar et al., 2005). To confirm PI3Kinase's role in GPER's inactivation of FoxO3-GFP in our current model, MCF7 cells with FoxO3-GFP were preincubated with the broad spectrum PI3Kinase inhibitor LY 294002 (LY) and subsequently treated with EGF, E2 and G-1 (Figure 2.10a). LY 294002 was able to abrogate FoxO3-GFP's inactivation by these ligands establishing that PI3Kinase is required (Figure 2.10a). EGFR is also essential because the EGFR inhibitor, AG1478 was also capable of significantly reducing FoxO3-GFP's inactivation by EGF, E2 and G-1 (Figure 2.10a). A representative image of the predominantly nuclear localization pattern that was observed with LY 294002 and AG1478 treatments is shown (Figure 2.10b).

The p110 α subunit of PI3Kinase is responsible for FoxO3-GFP inactivation while p110 β inhibition enhances p110 α activity.

The Class I_A subset of PI3Kinases employs PIP2 as their substrate and consists of a catalytic subunit (p110) and a regulatory subunit (p85). The two ubiquitously expressed PI3Kinase isoforms, p110 α and p110 β , have differing functions (Kumar et al., 2011). In order to determine which PI3Kinase isoform is responsible for inactivation of FoxO3-GFP as a result of GPER stimulation, cells

were preincubated with either PIK-75 which inhibits p110 α , or the p110 β inhibitor, TGX-221 (Figure 2.11a). PIK-75 inhibited FoxO3-GFP inactivation by EGF, E2 and G-1 suggesting that p110 α is the PI3Kinase subunit responsible. TGX-221 surprisingly enhanced FoxO3-GFP inactivation, even when it was added to cells alone as a control. We hypothesized that there may exist a balance or cross-interaction between the two p110 isoforms such that inhibiting p110 β may increase p110 α activity. To test this, we incubated MCF7 cells with FoxO3-GFP in the presence of both TGX-221 and PIK-75, with the goal that if TGX-221 inhibition of p110 β is resulting in the activation of p110 α , then this activity should be inhibited by the p110 α -specific inhibitor (Figure 2.11b). Indeed, inhibiting both p110 catalytic subunits ablated the inactivation of FoxO3-GFP, suggesting that inhibiting p110 β does cause an upregulation in the activity of p110 α .

Estrogen receptor stimulation does not significantly affect Bim or p27 levels.

FOXO3a is a transcription factor responsible for the expression of proapoptotic genes that encode proteins such as Fas ligand, TRAIL, Bim and the cell cycle inhibitor, p27kip. In order to determine if E2 and/or G-1 stimulation of GPER leading to inactivation of endogenous FOXO3a can decrease the protein levels of Bim and p27, MCF7 cells were treated with ligands, lysed and separated by gel electrophoresis. Western blot analysis of Bim and p27 levels in cells treated with DMSO, EGF, E2 and G-1 over 24 and 48 hours revealed no significant difference between samples (Figure 2.12). One possible explanation is that E2 and G-1 are able to inactivate FOXO3a in approximately 20 percent of the cells, so measuring

Bim and p27 level changes in whole cell lysates could potentially be difficult to detect.

E2 and G-1 reduce Caspase 7 activation in MCF7 cells.

It can be hypothesized that E2 and G-1 inactivation of FoxO3-GFP would shift cells towards a more pro-survival state since FOXO3a is a pro-apoptotic transcription factor. Caspase activation is an indicator of the initiation of apoptosis, which eventually results in cell death. To test this, MCF7 cells were incubated with DMSO, serum, E2 and G-1 for 1, 2, 3 and 5 days. Before fixing, cells were then incubated with the Magic Red Caspase substrate for 1 hour. The Magic Red substrate can be cleaved by activated caspase 3 or 7, generating a fluorescent product that can be monitored utilizing confocal microscopy. MCF7 cells are caspase 3 deficient, suggesting that the Magic Red substrate would be cleaved by any activated Caspase 7 (Janicke et al., 1998). After 1 or 2 days, there is not an observable difference in 10 nM E2 or 100 nM G-1 treated cells compared to DMSO vehicle control (Figure 2.13a). However at these shorter time points, 10 nM G-1 and 50 nM E2 displayed an observable decrease in caspase activation. Prolonged treatments (3 and 5 days) indicate that 10 nM E2, 10 nM G-1 and 50 nM E2 result in decreased caspase activation as compared to DMSO control (Figure 2.13b). These results suggest that prolonged exposure to E2 can reduce caspase activation in MCF7 breast cancer cells. Because G-1 can also reduce caspase activation at longer time points, we hypothesize that GPER is partially responsible for this decrease.

2.4. Discussion

Several reports have demonstrated in MCF7 cells, the ability of chemotherapeutic drugs to induce FOXO3a activation (i.e. accumulation of FOXO3a in the nucleus) (Sunters et al., 2006) (Ho et al., 2012). FOXO3a activation results in an increase in proapoptotic protein expression and provides a mechanism for how these drugs can induce apoptosis in breast cancer cells, specifically MCF7 cells. The steroid hormone estrogen (E2) is known to cause progression of hormone-sensitive tumors, including breast cancer, through its classical nuclear receptors by transcriptional regulation resulting in cell proliferation and survival (Lappano et al., 2014). However, E2 has also been demonstrated to have rapid signaling growth effects through an additional receptor, GPER. These signaling effects include the activation of MAPKs as well as activation of the PI3Kinase pathway (Filardo et al., 2000) (Revankar et al., 2005). The downstream effects of PI3Kinase activation by E2 have not been extensively studied. However, it has been established that PI3Kinase activation by growth factor receptors in general can stimulate AKT to phosphorylate and inactivate FOXO3a thereby excluding it from the nucleus (Brunet et al., 1999). Here we provide a mechanism by which the activation of GPER by E2 and the GPER-selective agonist, G-1, can lead to PI3Kinase/AKT activation and subsequently the inactivation of FOXO3a.

It was previously established by our lab that activation of GPER by E2 and G-1, as well as E2 activation of ER α leads to the accumulation of PIP3 in the nucleus (Revankar et al., 2005). PIP3 nuclear accumulation required PI3Kinase

activation and the EGFR. This assay was performed in COS-7 cells transiently expressing these receptors and SKBR3 cells which only express GPER. In order to investigate this pathway utilizing a more physiologically relevant model of ER α -positive breast cancer, we employed MCF7 cells, which express all three estrogen receptors (ER α , ER β and GPER), throughout this study. First, we sought to determine whether E2 and/or G-1 stimulation of MCF7 cells would result in the nuclear accumulation of PIP3. MCF7 cells transfected with the PH-RFP reporter did not show significant translocation of the PH-RFP reporter compared to vehicle control (Figure 2.1). Under serum starved conditions in the presence of the negative control, there is still an observable amount of PH-RFP in the nucleus (Figure 2.1a). Therefore, one possibility could be that there is a basal level generation of PIP3 within the nucleus of MCF7s. Because MCF7 cells are a breast cancer cell line, they could potentially have heightened activation of one or more growth factor pathways which could possibly be the reason for the basal level of PIP3 within the nucleus. Basal level PIP3 could make it difficult to detect any significant changes in PIP3 generation within the nucleus upon estrogen receptor stimulation. Additionally, PH-RFP is overexpressed and its localization under serum starved conditions may or may not reflect where PIP3 is actually being generated, again making it difficult to detect an increase in nuclear PH-RFP localization upon estrogen receptor stimulation.

To evaluate activation of Akt as a consequence of estrogen receptor activation, we monitored the total amount of phospho-Akt in MCF7 cells (Figure 2.2). We

utilized EGF as a positive control since it is known to activate the PI3Kinase/Akt pathway (Burgering and Coffey, 1995). EGF and G-1 are able to significantly increase phospho-Akt levels compared to control, while E2 only showed a trend towards increased phospho-Akt levels. Previous results demonstrate that E2 treatment of MCF7 cells only slightly increased phospho-Akt levels between 0 and 24 hours with a maximum, significant increase at 72 hours (Lee et al., 2005). Our experiments were performed in 20 minutes to monitor rapid signaling effects; therefore we might not be able to detect increased phospho-Akt level effects if they take longer to occur. Furthermore, since E2 binds to all three estrogen receptors in MCF7s, we propose that knockdown of ER α and ER β , isolating E2 stimulation of GPER would result in the same significant rapid increase of phospho-Akt levels as G-1 in MCF7 cells because it is possible that ER α and ER β are potentially counteracting E2's effects as a result of GPER stimulation. An additional experiment would be to measure phospho-Akt levels with E2 and G-1 treatment over a 24 hour time period to determine if the rapid phosphorylation of Akt by GPER would be sustained.

Previous results from our lab have suggested nuclear signaling in response to estrogen receptor stimulation by nuclear pip3 accumulation (Revankar et al., 2005). Other results observed in endometrial cancer cells suggest Akt activation within the nucleus as an outcome of estrogen stimulation (Abe et al., 2011). In this particular study they monitored phospho-Akt and total Akt levels within the nucleus of endometrial cancer cells overexpressing ER α . A significant difference in both phospho-Akt and total Akt nuclear levels was reported following 1 to 3

hours of E2 treatment. They also correlated poor prognosis to nuclear phospho-Akt. In the current study we are able to demonstrate rapid translocation (15 minutes) of total Akt in response to EGF and G-1, but no significant difference in E2-mediated translocation of total Akt at this time point (Figure 2.3). This suggests that GPER is responsible for rapid translocation of Akt in MCF7 cells. Based on the previously published results in endometrial cancer cells, longer time points might be necessary to determine E2's effects on total Akt translocation in MCF7 cells.

Downstream of its translocation and activation, Akt has specific kinase functions within the nucleus that regulate cell survival (Datta et al., 1999). One well-established function is the phosphorylation of FOXO3a, resulting in its inactivation and subsequent exclusion from the nucleus (Brunet et al., 1999). Others have utilized the shuttling mechanism of FOXO3a inactivation by transfecting a GFP-construct into PC12 cells and monitoring FoxO3-GFP localization in response to NGF (Wen et al., 2011). Because we were able to detect rapid activation of phospho-Akt and translocation of total Akt to the nucleus in MCF7 cells, we sought to elucidate the downstream effects of this by monitoring FOXO3a inactivation. FoxO3-GFP, a mouse orthologue construct with sequence homology to human FOXO3a, was transfected into MCF7 cells. FoxO3-GFP's localization after treatments was assessed by microscopy and separated into three categories, ranging from predominantly nuclear to predominantly cytoplasmic (Figure 2.4). Ultimately, the extent of cytoplasmic localization was used as an indicator of FoxO3-GFP inactivation (Figure 2.5).

We observed that not only did E2 inactivate FoxO3-GFP significantly as compared to control, but so did the GPER-selective agonist G-1. It is not clear whether the partially nuclear state indicates the cell is in a more proapoptotic or prosurvival state. Further studies to assess this may include microscopy analysis, on a cell to cell basis, of the downstream product of FOXO3a, Bim. Bim levels would be an indicator of FOXO3a's activity and we predict that there would be a detectable decrease in cells with cytoplasmic FoxO3-GFP and potentially partially nuclear cells compared to predominantly nuclear cells (Kim et al., 2014). Furthermore, we were able to determine that inactivation of FoxO3-GFP is dependent on concentration as well as time (Figures 2.6 and 2.7). The maximum response for E2 was 50 nM and 100 nM for G-1 at 15 minutes. 10 nM E2 and 10 nM G-1 also significantly increased FoxO3-GFP inactivation but the effect was beginning to decrease by 300 nM. Higher doses of G-1 have been reported to exhibit antiproliferative effects in MCF7 cells which could potentially explain the decrease in inactivation of FOXO3a at high doses of E2 and G-1 (Ariazi et al., 2010).

GPER is known to be responsible for at least some of the rapid signaling effects of E2 (Filardo et al., 2000) (Revankar et al., 2005). Because the GPER selective agonist G-1 had a significant effect on phospho-Akt levels and the translocation of Akt to the nucleus, we hypothesized that GPER was also responsible for the inactivation of FoxO3-GFP in response to E2. Knockdown of GPER significantly decreased FoxO3-GFP inactivation compared to siControl (Figure 2.8). However, knockdown of ER α had no significant effect (Figure 2.9). It was

recently published that FoxO3a overexpression decreases motility, invasiveness, and anchorage-independent growth in MCF7 cells and that this is dependent on ER α expression (Sisci et al., 2013). Here it was suggested that E2 treatment of MCF7 cells allowed for a synergistic activation of FOXO3a, dependent on ER α and leading to Caveolin-1 expression, which was suggested to decrease invasiveness and migration. There was no account for GPER's effect in this study. E2 binds GPER in MCF7 cells as well so there is likely a balance between the two receptor pathways. We propose that knockdown of endogenous ER α would reverse this effect because E2 stimulation of GPER in MCF7 cells would inactivate FOXO3a and exclude it from the nucleus, decreasing Caveolin-1 expression. We did not determine a role for ER β in these studies; however ER α and ER β have been reported to interact with FOXO3a in MCF7 cells (Zou et al., 2008). This raises the possibility that if ER α and ER β interact with FOXO3a and potentially keep it localized to the nucleus, this could result in constitutive FOXO3a activity and proapoptotic effects.

To delineate the pathway by which E2 and G-1 inactivate FoxO3-GFP in MCF7 cells we employed inhibitors and monitored their effects on FoxO3-GFP inactivation (Figure 2.10a). As expected, the PI3Kinase inhibitor, LY 294002, blocked FoxO3-GFP inactivation by EGF, E2 and G-1, which is evidenced by the strong nuclear localization of FOXO3-GFP in cells treated with LY 294002 (Figure 2.10a and 2.10b). It has been previously determined that PI3Kinase can activate Akt in MCF7 cells and that this effect is inhibited by LY 294002 (Jordan et al., 2004). It has also been reported that the p85 subunit of PI3Kinase can

interact with ER α in the presence of estrogen and that this interaction mediates protection from apoptosis in MCF7 cells (Greger et al., 2007). Additionally, the p110 α subunit of Class I_A PI3Kinases has a known mutation in its gene that results in constitutive activation and that is found to be mutated in 35% of breast cancers (Janku et al., 2014) (Polivka and Janku, 2014). Here we provide the first report that implicates the p110 α subunit of PI3Kinase is responsible for GPER-mediated inactivation of FOXO3a (Figure 2.11a). The p110 β subunit has been described to have a nuclear localization and has also been deemed to be involved in certain nuclear processes, including DNA replication and repair (Marques et al., 2009) (Kumar et al., 2010) (Kumar et al., 2011). Our results indicated that the p110 β subunit PI3Kinase is not responsible for EGF, E2, or G-1 induced inactivation of FOXO3a (Figure 2.11a). Furthermore, inhibition of p110 β actually enhances p110 α activity in MCF7 cells (Figure 2.11b). One possible explanation for the enhanced p110 α activity as a result of p110 β inhibition could be that activated p110 β can associate with a fraction of activated p110 α sequestering it from its substrates. Therefore, when p110 β is inhibited and can potentially no longer interact with this fraction of p110 α , p110 α is no longer associated with p110 β which results in its increased activity.

Generally, GPCRs are known to transactivate the EGFR through a signaling cascade involving the activation of Src which results in the activation of membrane-bound MMPs such as the ADAM family members (George et al., 2013). These MMPs can cleave EGF ligands that in turn bind to the EGFR and other EGFR family members and promote dimerization and activation of these

receptors. It was first demonstrated in 2000, that estrogen transactivates the EGFR to the MAPK signaling axis via GPER (Filardo et al., 2000). This was found to occur through the release of surface-bound proHB-EGF in SKBR3 cells, which are an ER-negative human breast cancer cell line. The transactivation of EGFR by GPER is mediated by G β γ -subunit/Src family kinase-dependent intracellular signals (Filardo, 2002). Later, the Prossnitz laboratory reported in 2005 that PI3Kinase activation of GPER required EGFR (Revankar et al., 2005). Additionally, in ovarian cancer cells transiently expressing GPER, the activation of the EGFR and Akt could be significantly enhanced by G-1 and inhibited by a Src family kinase inhibitor (Fujiwara et al., 2012). This study also correlated poor outcome with GPER and EGFR expression in ovarian cancer. Here we conclude that E2 and G-1 inactivation of FoxO3-GFP requires the EGFR (Figure 2.10a). To further outline EGFR's mechanism in this pathway, we would need to investigate whether HB-EGF is cleaved as a result of GPER activation, also whether Src activation is required.

FOXO3a is a transcription factor which regulates the expression of proapoptotic proteins. The Bcl-2 proapoptotic family member, Bcl-2 interacting mediator of cell death (Bim), contains a BH3 domain, allowing it to bind pro-survival Bcl-2 molecules, neutralizing their function. Bim's association with the PI3Kinase/Akt pathway was originally investigated in lymphocytes where inhibition of PI3Kinase by LY 294002 elevated Bim levels (Dijkers et al., 2000). An inducible FOXO3a construct was utilized and Bim levels were monitored upon induction. In the presence of prosurvival signals, increasing FOXO3a expression was able to

elevate Bim. Bim levels were also found to be regulated by FOXO3a in sympathetic neurons, with higher Bim resulting in apoptosis (Gilley et al., 2003). FOXO3a can also mediate G1 cell cycle arrest by upregulating expression of the cell cycle inhibitor p27kip1 (p27) (Medema et al., 2000). p27 blocks the activity of the cyclin E-cyclin-dependent kinase 2 (CDK2) complex and this prevents cell cycle progression. There are FOXO binding sites within the p27 promoter, which suggests that it is directly regulated by activated FOXO3a. Trotman et al. were able to demonstrate that Pml nuclear bodies can promote apoptosis in prostate cancer cells by inactivation of nuclear Akt, which renders FOXO3a active and results in the production of proapoptotic proteins p27 and Bim (Trotman et al., 2006). Since it has been established that FOXO3a can directly regulate Bim and p27 expression levels, we sought to determine whether E2 and G-1 inactivation of FOXO3a would decrease Bim and p27 levels in MCF7 cells (Figure 2.12). When analyzing the data, it appears that G-1 is trending towards a reduction of Bim after 48 hours. Otherwise, no significant difference in Bim protein levels was detected. Additionally, it appears that p27 levels were trending towards a decrease when treated with E2 after 48 hours. We hypothesize that detecting Bim and p27 level changes in a whole cell population may mask the effect of FOXO3a inactivation by GPER stimulation that we are able to observe on the single cell level. A potential reason for not being able to detect a significant decrease in Bim and p27 protein levels is that only approximately 20 percent of the cells treated exhibited a predominantly cytoplasmic pattern of FoXO3-GFP. A decrease in protein levels as a result of this inactivation may be too difficult to

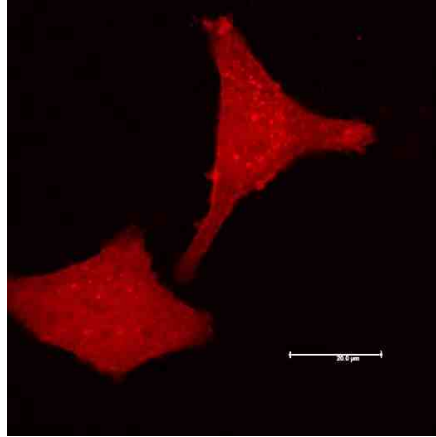
detect in a whole cell population over this time period. Additionally, since our positive control, EGF was not able to induce a decrease in Bim or p27 levels, it is possible that serum starvation prior to treatment pushed the cells to a proapoptotic state that was not able to be reversed by EGF, E2 or G-1 treatment. The long term effects on Bim and p27 levels in the presence of E2 and G-1 should be further examined to determine whether there is a decrease over time with extended exposure to estrogen receptor ligands.

Although we were not able to observe a detectable decrease in the proapoptotic protein levels of Bim and p27, we still sought to determine whether E2 and G-1 inactivation of FOXO3a could result in decreased apoptosis of MCF7 cells. In order to investigate this on a cellular level, a cleavable fluorescent substrate was incubated with MCF7 cells after 1, 2, 3 and 5 days of treatment. It can be concluded that prolonged exposure to E2 results in an observable decrease in caspase activation (Figure 2.13b). A lower concentration of G-1, but not E2 is able to decrease caspase activation at earlier time points (Figure 2.13a). We hypothesize that this is a result of rapid signaling effects of GPER stimulation that are still observed after 3 and 5 days. We also predict that based on our results, inactivation of FOXO3a is partially responsible for decreased caspase activation. Further experiments to delineate FOXO3a's role in decreased caspase activation in MCF7 cells are required.

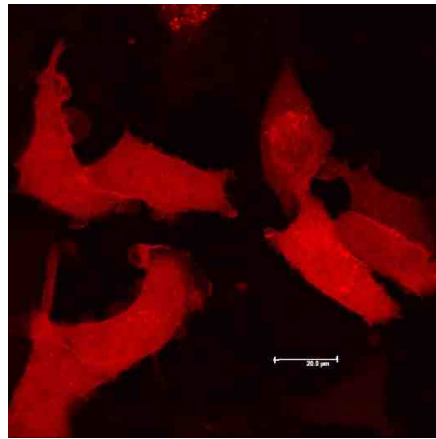
There are conflicting studies regarding the prognosis of breast cancer tissue samples with nuclear localized FOXO3a (Chen et al., 2010) (Habashy et al., 2011). Our results demonstrating FOXO3a inactivation and nuclear exclusion by

E2 and G-1 suggest this is one possible mechanism by which tumor cells evade apoptosis. Therefore we hypothesize, that in a tumor environment, nuclear FOXO3a would be more beneficial because it would shift the cells towards a more proapoptotic state.

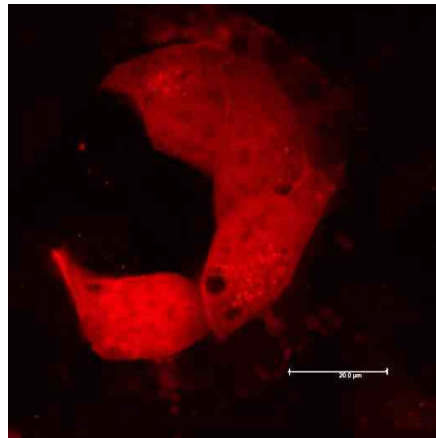
a.) DMSO



b.) EGF



c.) E2



d.) G-1

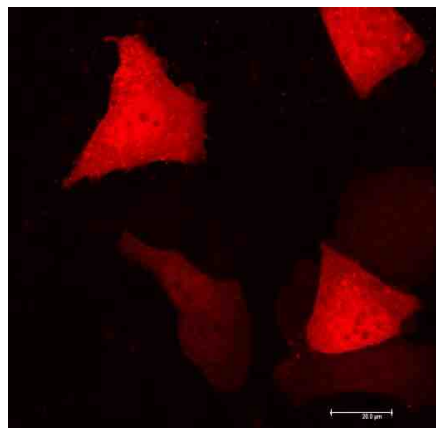


Figure 2.1

PH-RFP

Localization

e.)

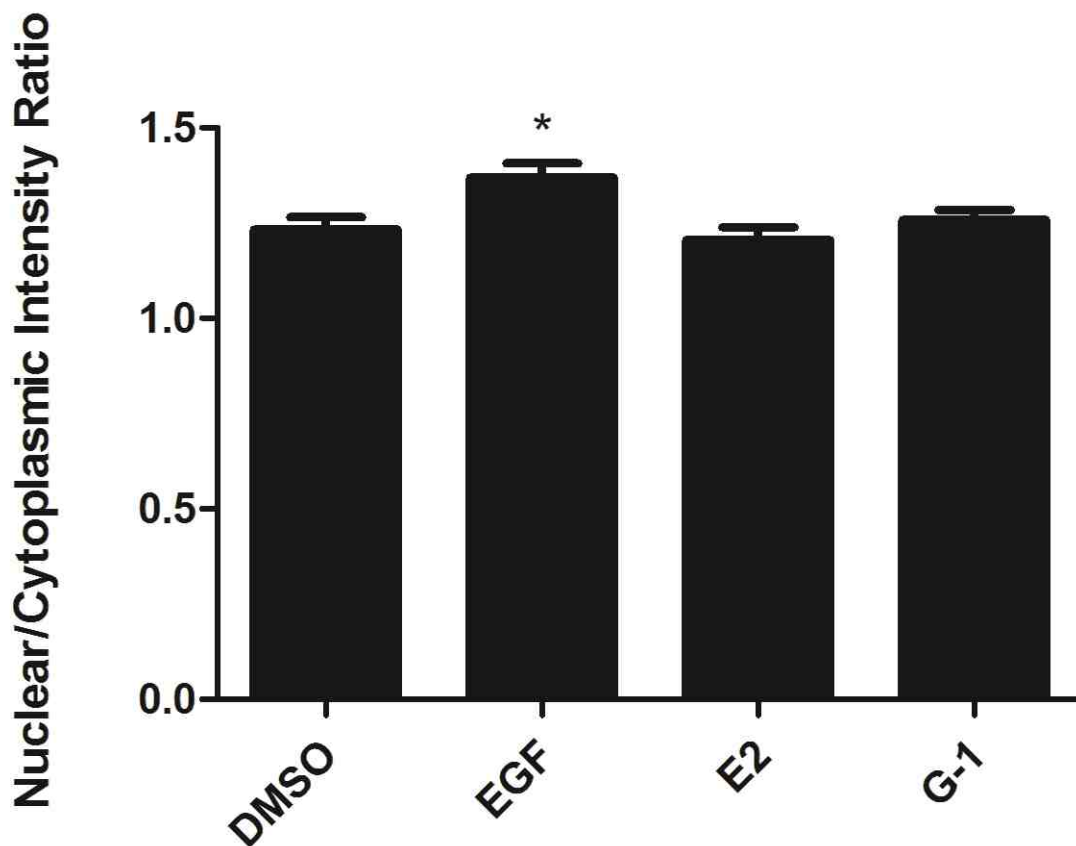


Figure 2.1. PH-RFP localization in MCF7 cells. (a-d) MCF7 cells were transfected with PH-mRFP1 and serum starved for 24 hours prior to treatments. Cells were treated with 0.1% DMSO, 50 ng/ml EGF, 50 nM E2 and 100 nM G-1 for 15 min. (e) Images were analyzed with Slidebook by creating a nuclear mask and a mask of the whole cell. Each cell's nuclear mean intensity was divided by its total cell mean intensity and was plotted as a data point. [Relative to control (DMSO) $p = <.05$ for EGF (*)].

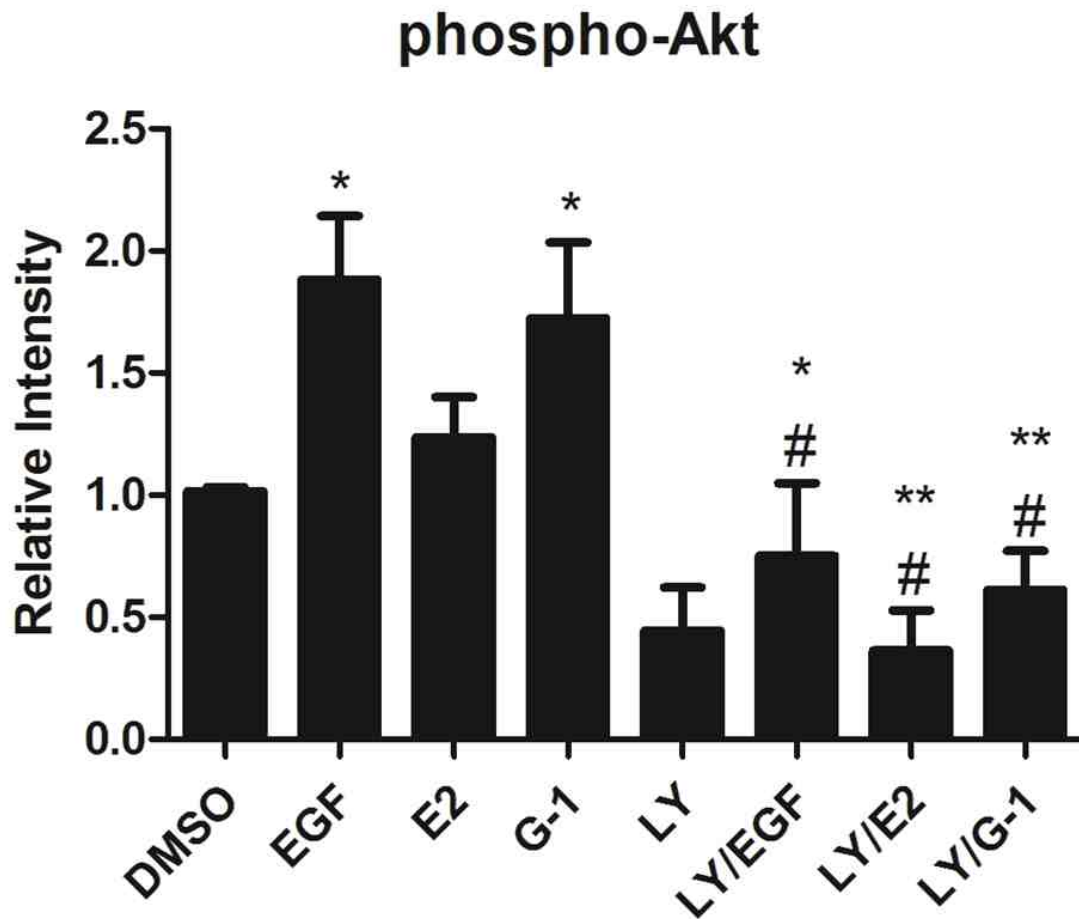
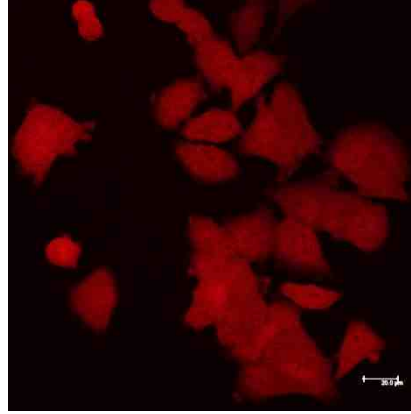


Figure 2.2. Estrogen receptor stimulation induces phosphorylation of Akt in MCF7 cells. MCF7 cells were serum starved for 24 hours prior to treatments. Cells were treated with 0.1% DMSO, 50 ng/ml EGF, 50 nM E2 and 100 nM G-1 for 20 min. Where indicated cells were pretreated with 10 μ M LY 294002 (PI3-Kinase inhibitor) for 30 min. Cells were lysed in RIPA buffer, separated by gel electrophoresis, transferred by Western blot and quantitated using ImageJ software. Results were analyzed using a one way ANOVA with Dunnett's Multiple Comparison post hoc test. [Relative to control (DMSO) $p < .05$ for EGF and G-1 (*). Relative to ligand without inhibitor (#) $p < .01$ for E2 and G-1(**), $p < .05$ for EGF (*)].

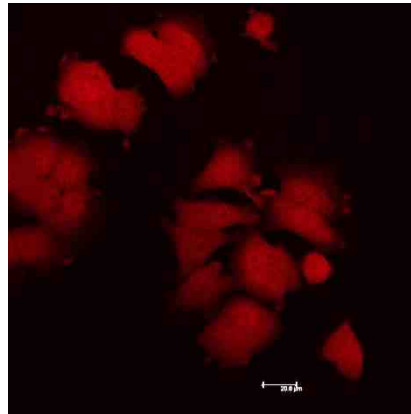
a.) DMSO



b.) EGF



c.) E2



d.) G-1

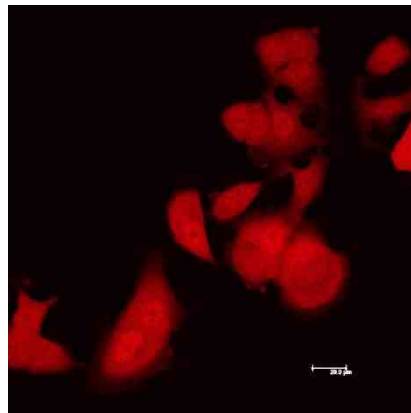


Figure 2.3
Total Akt Ab
Localization

e.)

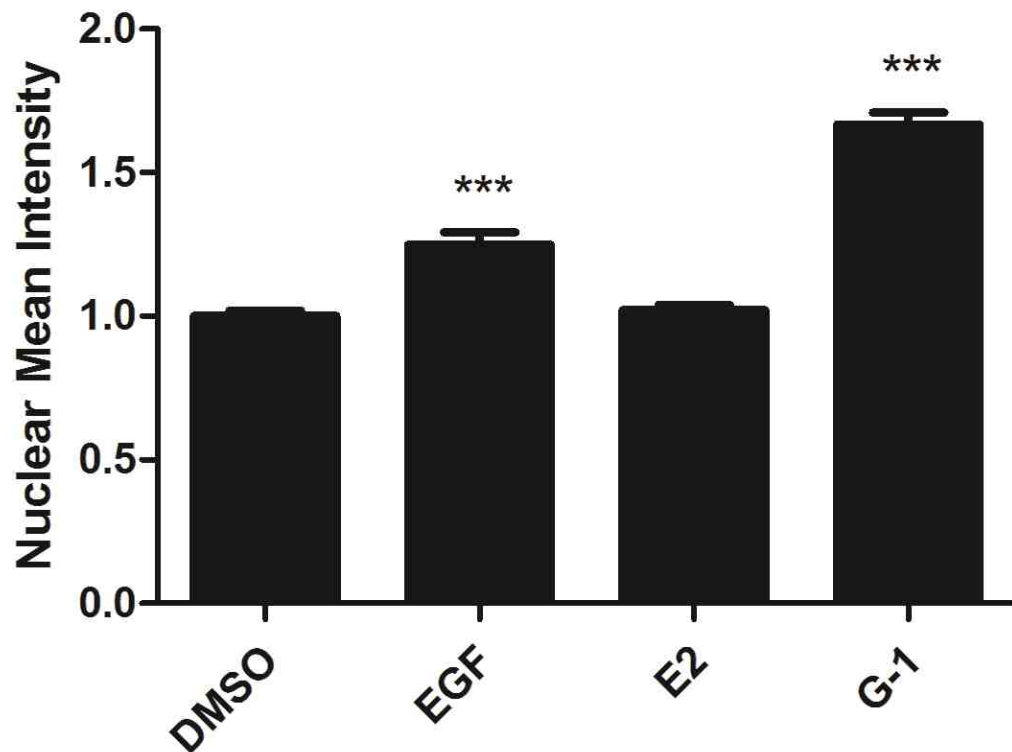


Figure 2.3. GPER stimulation induces nuclear translocation of Akt in MCF7 cells. (a-d) MCF7 cells were serum starved for 24 hours prior to treatments. Cells were treated with 0.1% DMSO, 50 ng/ml EGF, 50 nM E2 and 100 nM G-1 for 15 min. (e) Images were analyzed with Slidebook by creating a nuclear mask in order to measure the mean intensity of that area. Each cell's nuclear mean intensity was plotted as a data point. [Relative to control (DMSO) $p = <.001$ for EGF and G-1 (***)].

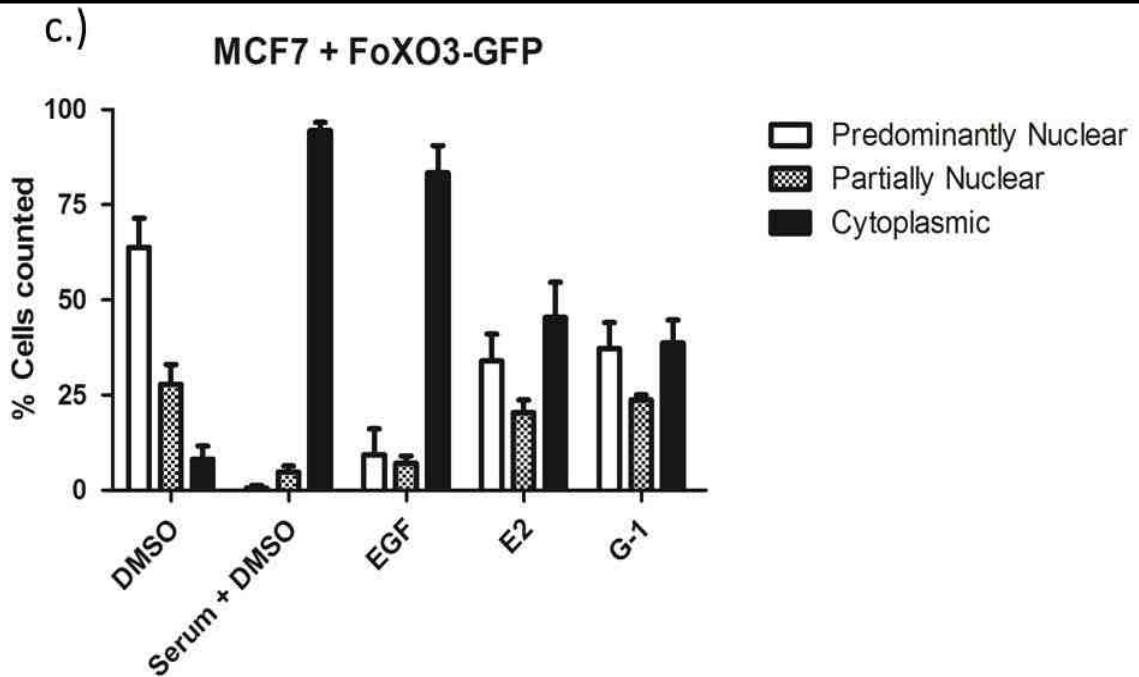
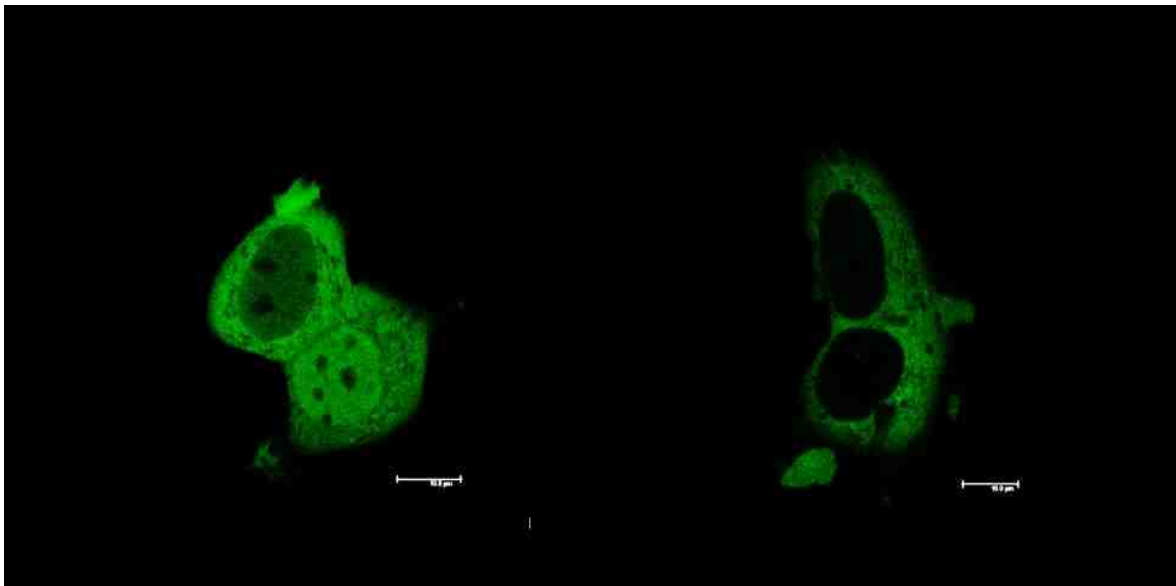
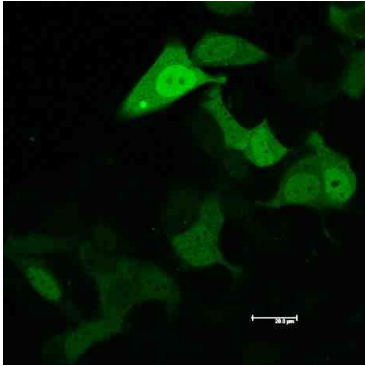
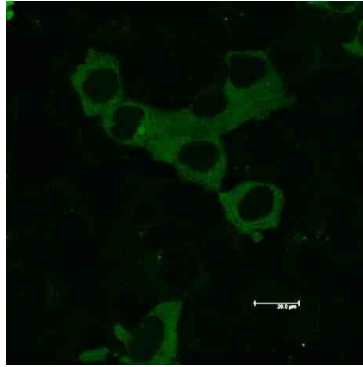


Figure 2.4. FoxO3-GFP localization. (a) MCF7 cells transfected with FoxO3-GFP and supplemented with serum free media for 24 hr. (b) MCF7, same as Fig 2a., treated with 50ng/ml EGF for 15 min. (c) Cells were treated with 0.1% DMSO, 0.1% DMSO and serum, 50ng/ml EGF, 50nM E2 and 100nM G-1 for 15min. Based on the images in (a) and (b) cells were separated into the indicated categories and approximately 50 cells were counted per treatment.

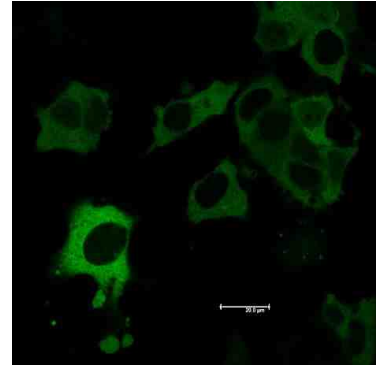
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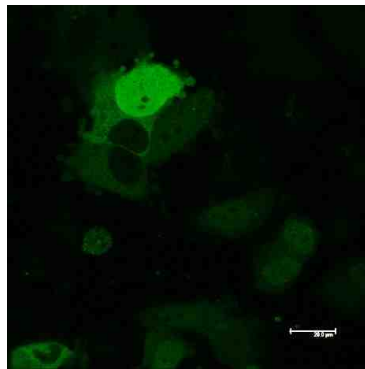
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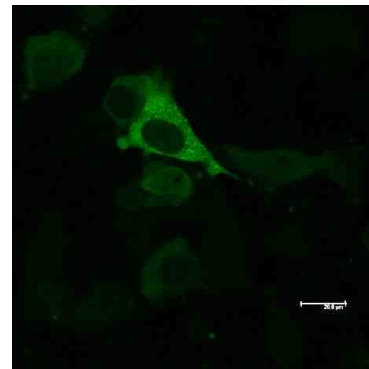
c.) EGF



d.) E2



e.) G-1



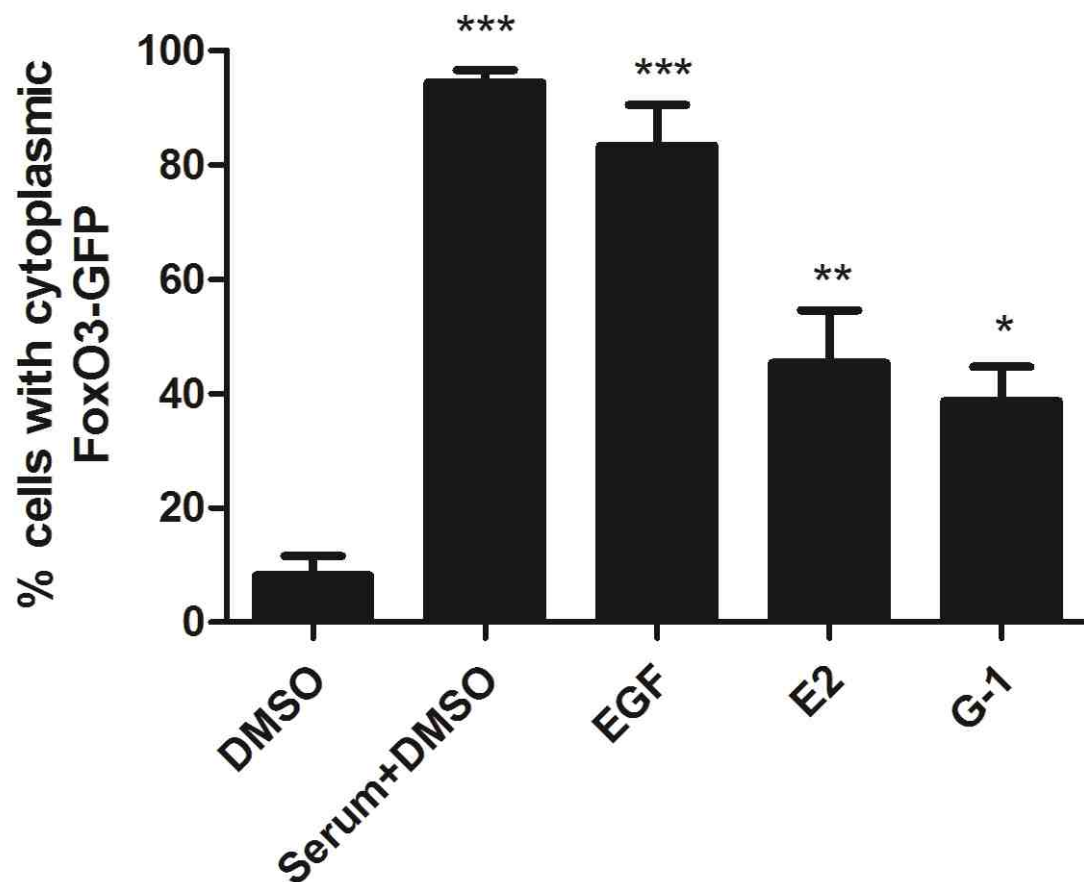


Figure 2.5. Estrogen receptor activation leads to FOXO3a inactivation. MCF7 cells transfected with FoxO3-GFP were serum starved for 24 hours and treated as indicated in Figure 4c for 15 min. (a-e) Representative images. f.) Results were analyzed using a one way ANOVA with Dunnett's Multiple Comparison post hoc test. [Relative to control (DMSO) $p = <.001$ for serum, EGF (***) , $p = <.01$ for E2 (**), $p = <.05$ for G-1 (*)].

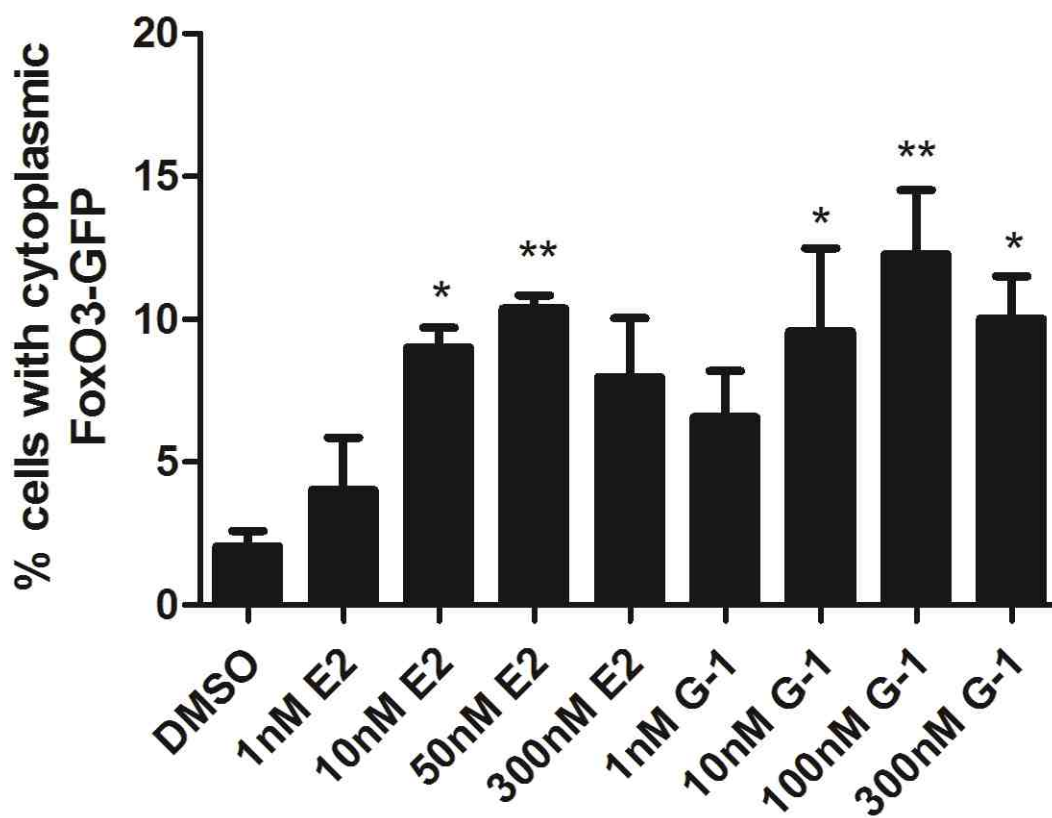


Figure 2.6. Estrogen receptor inactivation of FOXO3a is concentration dependent. MCF7 cells transfected with FoxO3-GFP were serum starved for 24 hours and treated as indicated with E2 and G-1. Results were analyzed using a one way ANOVA with Dunnett's Multiple Comparison post hoc test. [Relative to control (DMSO) at each concentration $p = <.01$ for 50 nM E2 and 100 nM G-1 (**), $p = <.05$ for 10 nM E2, 10nM G-1 and 300 nM G-1 (*)].

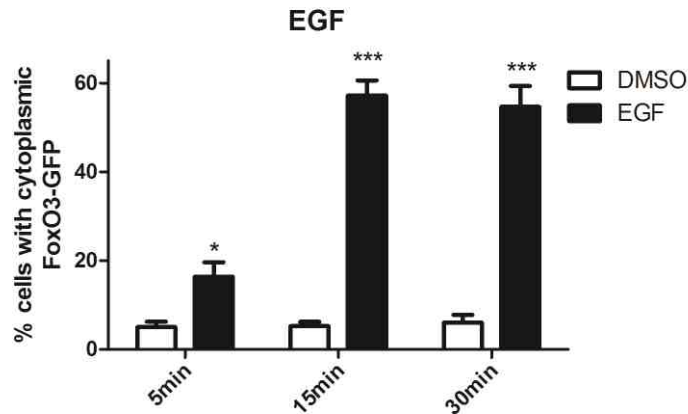
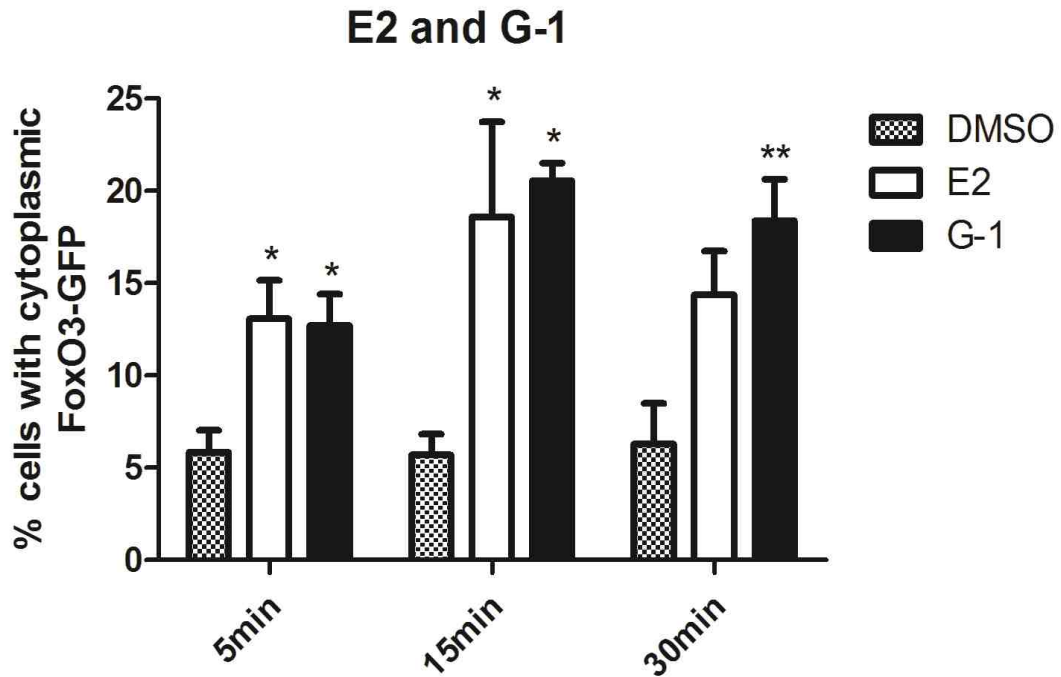


Figure 2.7. Estrogen receptor activation leads to rapid FOXO3a inactivation. MCF7 cells transfected with FoxO3-GFP were serum starved for 24 hours and treated with 0.1% DMSO, 50 nM E2, 100 nM G-1 or 50 ng/ml EGF for 5, 15 and 30 min. Results were analyzed using a one way ANOVA with Dunnett's Multiple Comparison post hoc test. [Relative to control (DMSO) at each time point $p = <.001$ for EGF (***) , $p = <.01$ for G-1 (**), $p = <.05$ for E2, G-1 and EGF (*)].

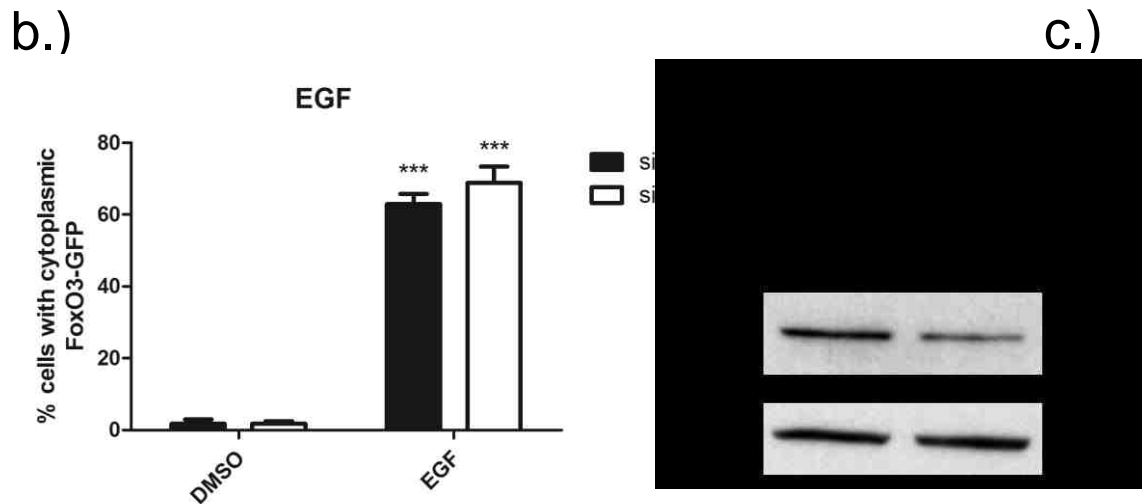
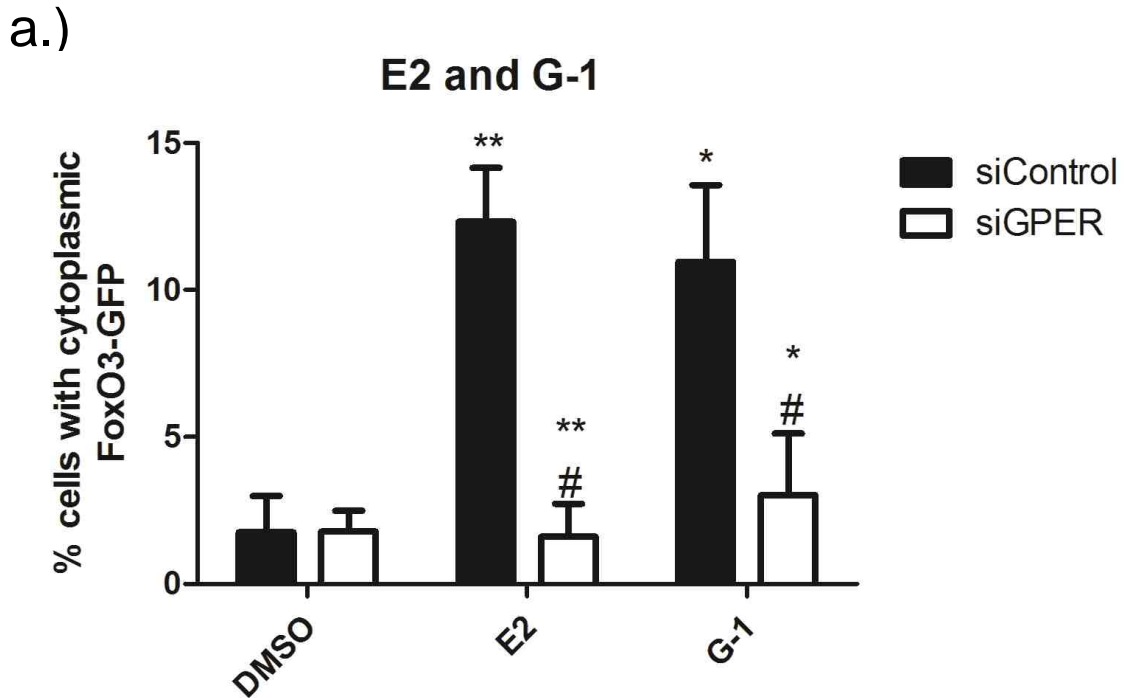


Figure 2.8. GPER is required for E2 and G-1 mediated inactivation of FoxO3-GFP. MCF7 cells were transfected with siGPER and FoxO3-GFP then serum starved for 24 hours prior to treatments. a.) Cells were treated with 0.1% DMSO, 50 nM E2 and 100 nM G-1 for 15 min. b.) Cells were treated with 0.1% DMSO and 50 ng/ml EGF for 15 minutes. c.) Representative Western blot of cell lysates that were collected at the same time as a.) and b.) treatments. a.) and b.) Results were analyzed using a one way ANOVA with Dunnett's Multiple Comparison post hoc test. [Relative to control (DMSO) $p = <.001$ for EGF (***)], $p = <.01$ for siControl E2 (**), $p = <.05$ for siControl G-1 (*)]. siGPER and siControl were analyzed using a two way ANOVA with Bonferroni post hoc tests. [Relative to siControl (#) $p = <.01$ for siGPER E2 (**), $p = <.05$ for siGPER G-1 (*)].

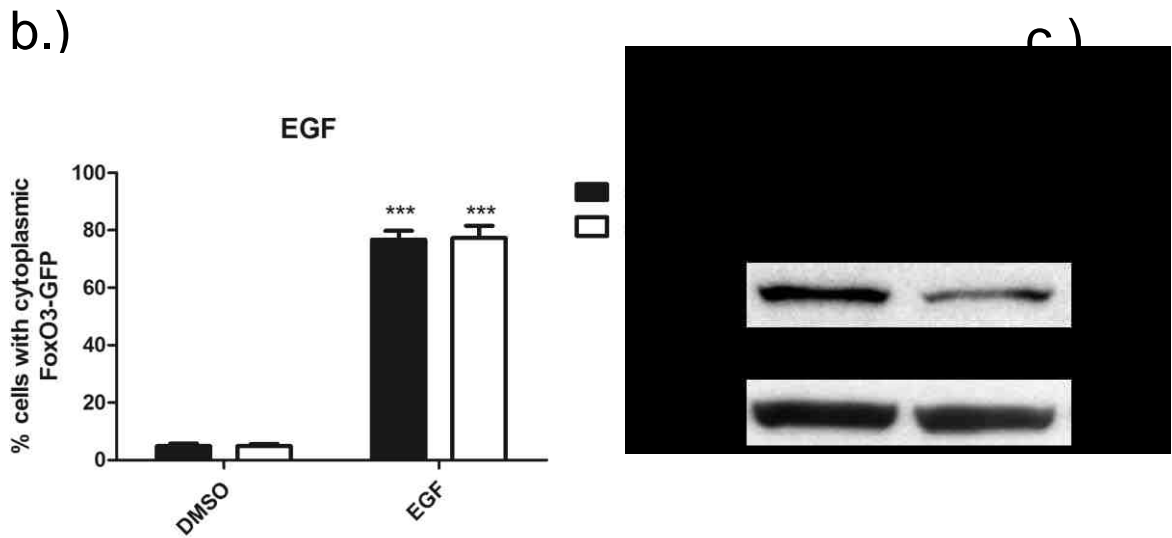
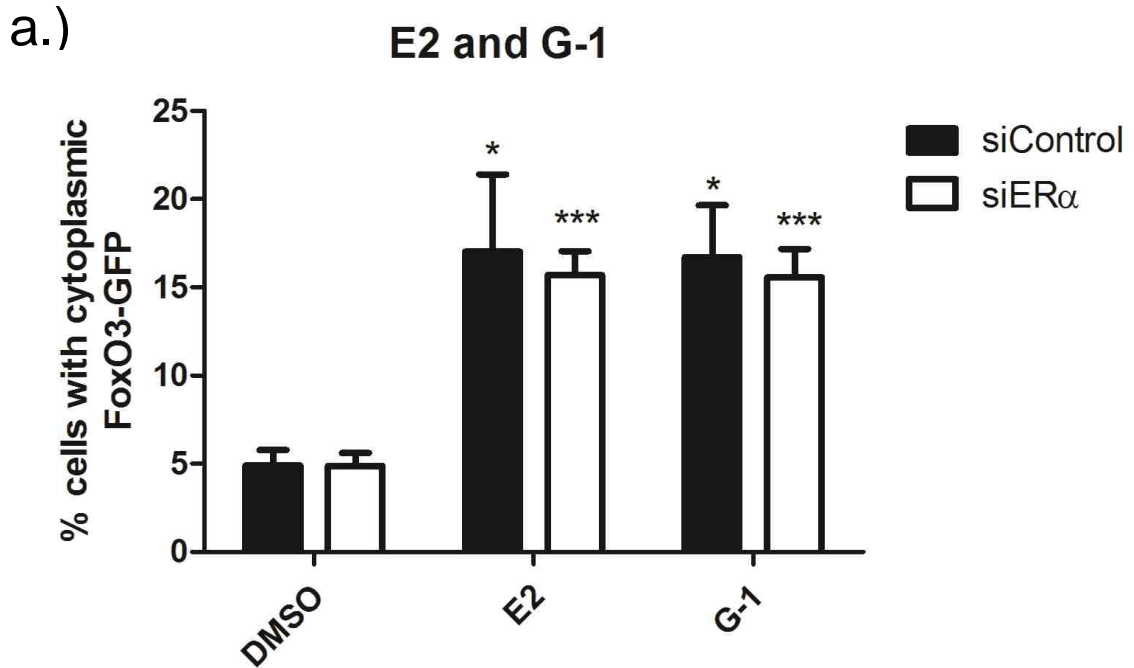
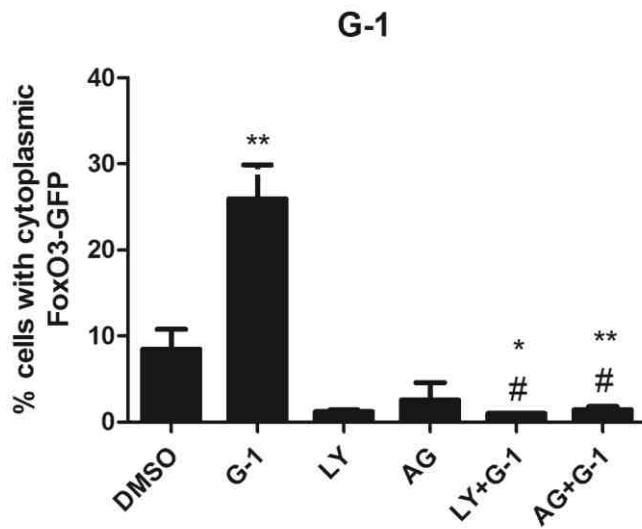
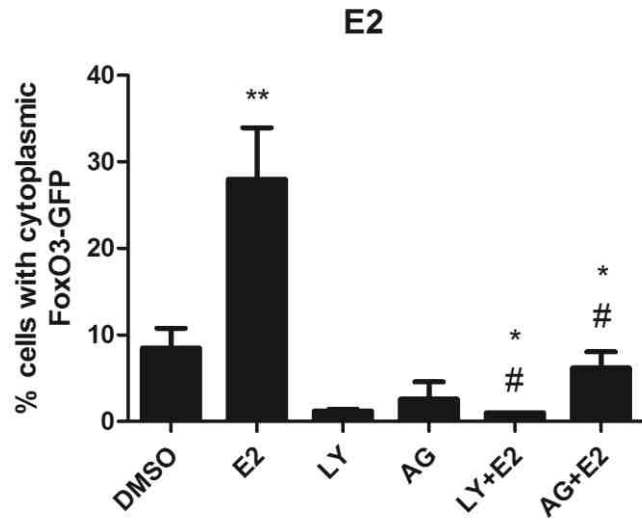
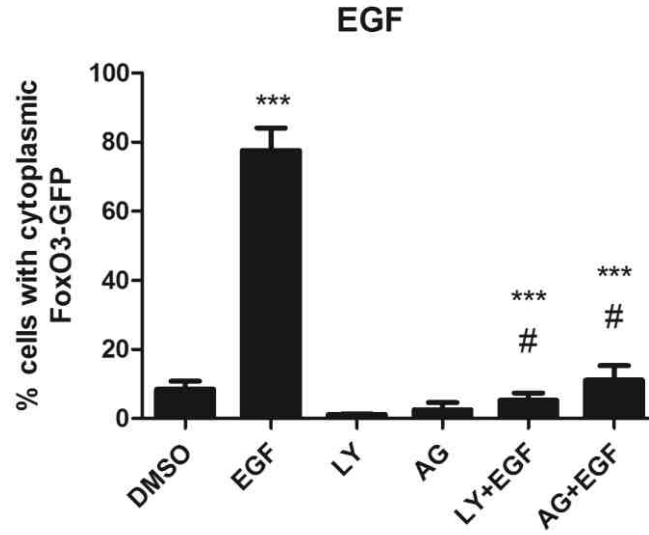


Figure 2.9. FoxO3-GFP inactivation does not require ER α . MCF7 cells were transfected with siER α and FoxO3-GFP then serum starved for 24 hours prior to treatments. a.) Cells were treated with 0.1% DMSO, 50 nM E2 and 100 nM G-1 for 15 min. b.) Cells were treated with 0.1% DMSO and 50 ng/ml EGF for 15 minutes. c.) Representative Western blot of cell lysates that were collected at the same time as a.) and b.) treatments. a.) and b.) Results were analyzed using a one way ANOVA with Dunnett's Multiple Comparison post hoc test. [Relative to control (DMSO) $p = <.001$ for siER α E2, siER α G-1, EGF (***) , $p = <.05$ for siControl E2, siControl G-1 (*)].

a.)



b.)

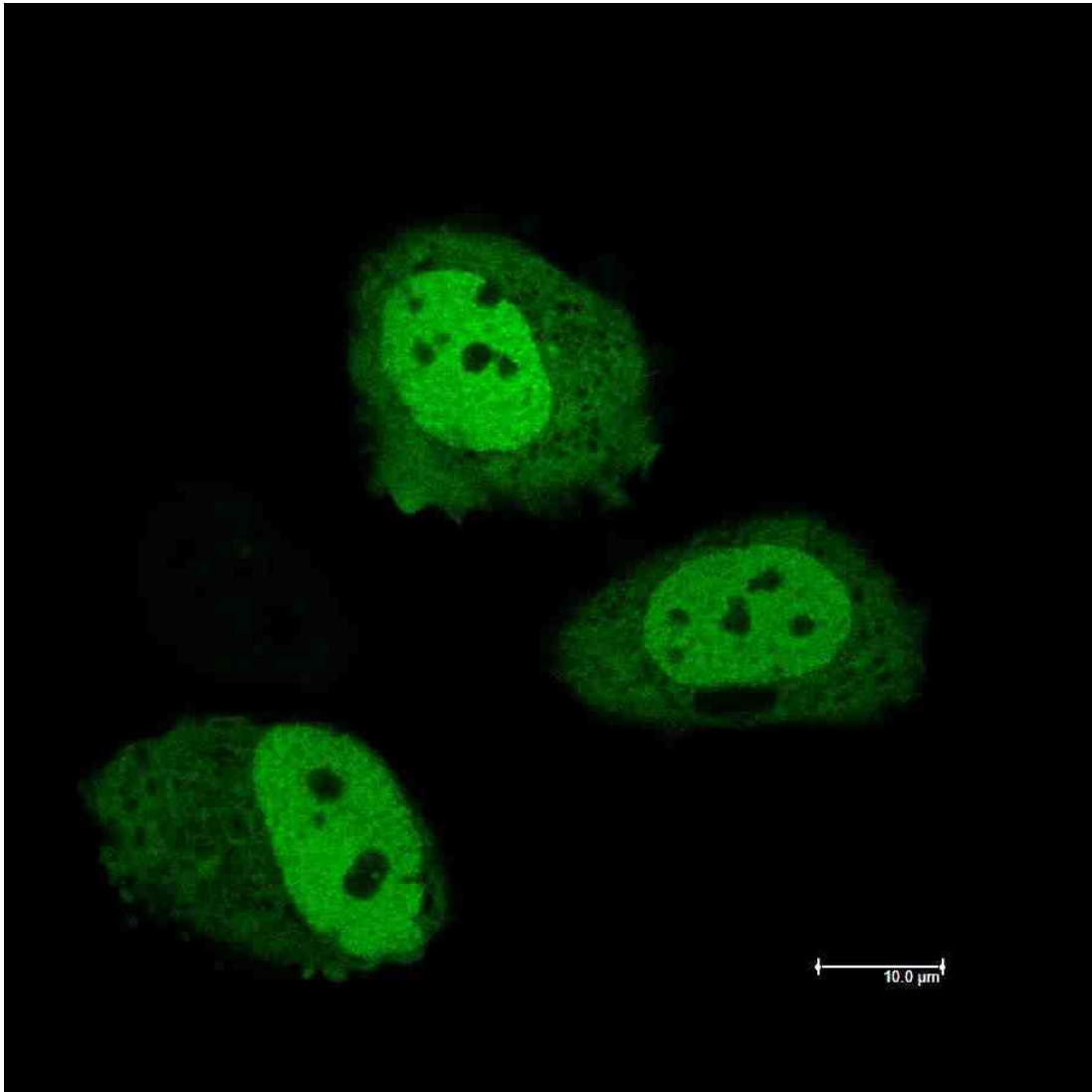
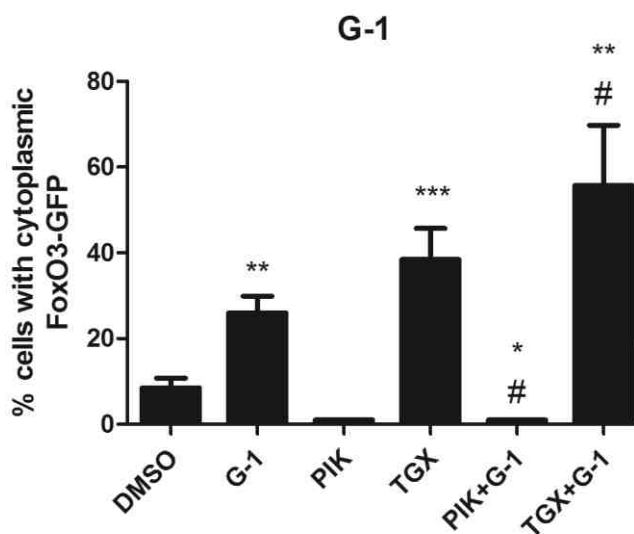
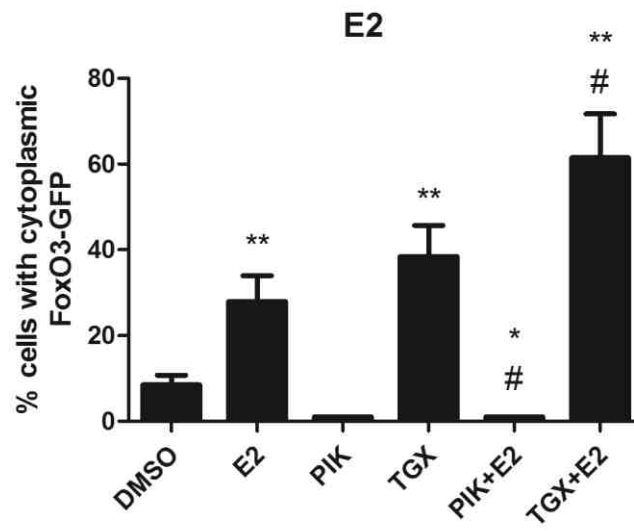
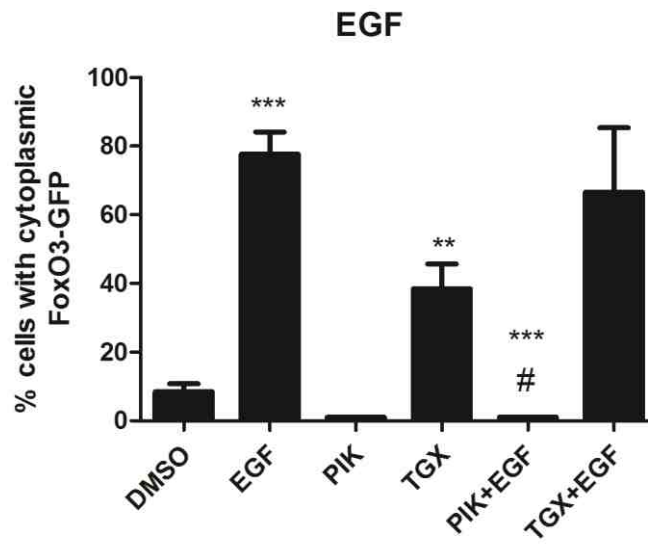


Figure 2.10. FOXO3-GFP inactivation requires PI3-Kinase and the transactivation of EGFR in MCF7 cells. a.) MCF7 cells were serum starved for 24 hours prior to treatments. Cells were treated with 50 ng/ml EGF, 50 nM E2 and 100 nM G-1 for 15min. Where indicated cells were pretreated with 10 μM LY 294002 (PI3-Kinase inhibitor), 250 nM AG1478 (EGFR inhibitor) for 30 min. Results were analyzed using a one way ANOVA with Dunnett's Multiple Comparison post hoc test. [Relative to control (DMSO) $p = <.001$ (***) , $p = <.01$ (**), $p = <.05$ (*), Relative to ligand without inhibitor (#)]. b.) Representative image of MCF7 cells treated with 10 μM LY 294002.

a.)



b.)

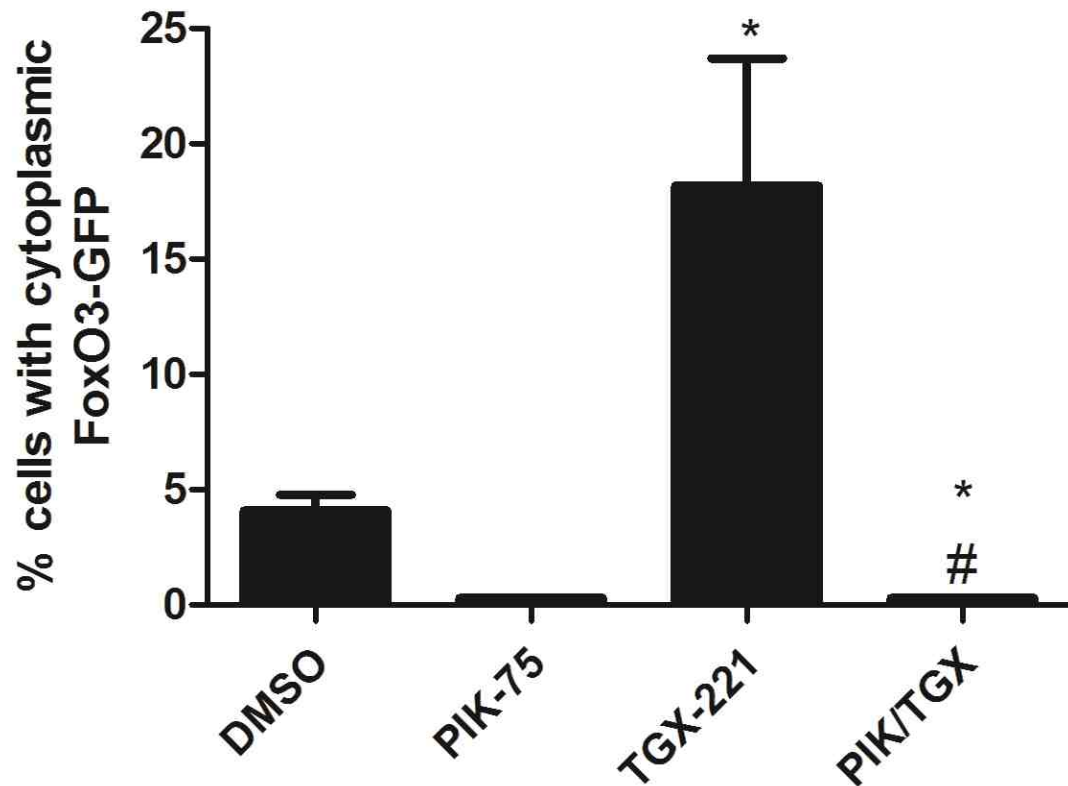


Figure 2.11. The p110 α subunit of PI3Kinase is responsible for FoxO3-GFP inactivation while p110 β inhibition enhances p110 α activity. a.) MCF7 cells were serum starved for 24 hours prior to treatments. Cells were treated with 50 ng/ml EGF, 50 nM E2 and 100 nM G-1 for 15 min. Where indicated cells were pretreated with 100 nM PIK-75 (p110 α inhibitor) and 100 nM TGX-221 (p110 β inhibitor) for 30 min. Results were analyzed using a one way ANOVA with Dunnett's Multiple Comparison post hoc test. [Relative to control (DMSO) $p = <.001$ (***) , $p = <.01$ (**), $p = <.05$ (*), Relative to ligand without inhibitor (#)]. b.) MCF7 cells were serum starved for 24 hours prior to treatments. Cells were treated with 100 nM PIK-75 (p110 α inhibitor) and 100 nM TGX-221 (p110 β inhibitor) for 45 min. Results were analyzed using a one way ANOVA with Dunnett's Multiple Comparison post hoc test. [Relative to control (DMSO) $p = <.05$ (*), # = TGX-221 vs. PIK/TGX].

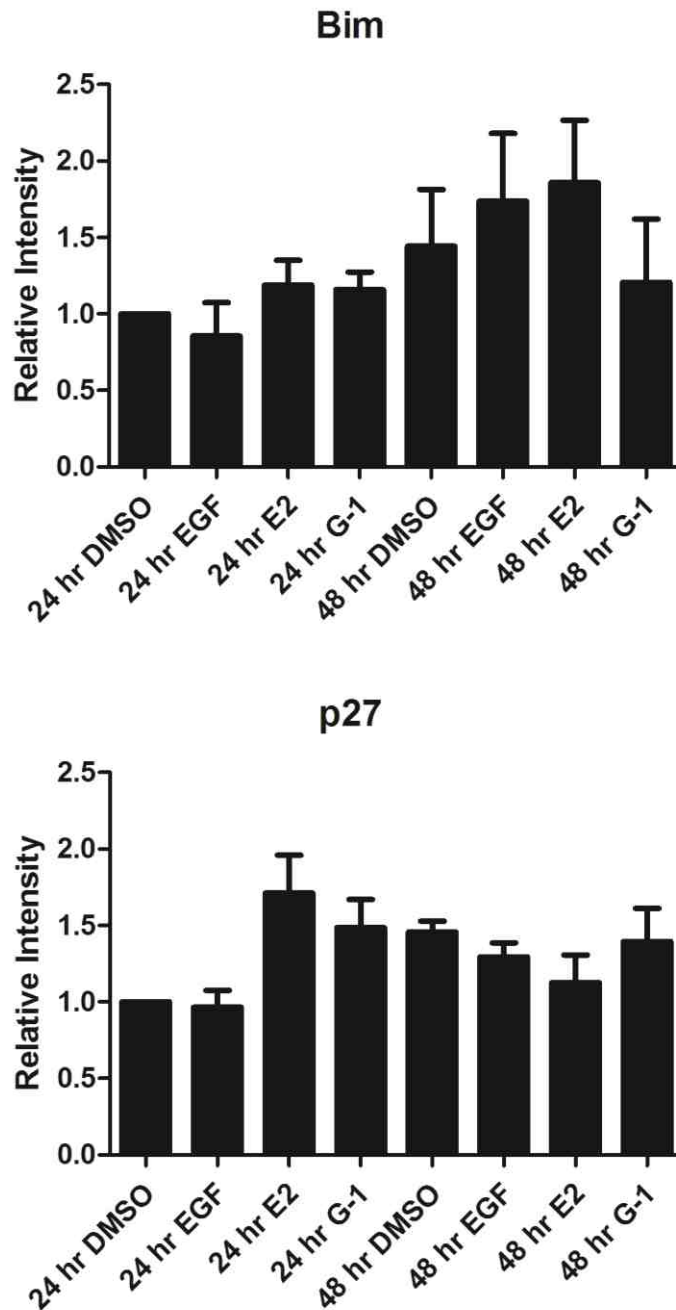
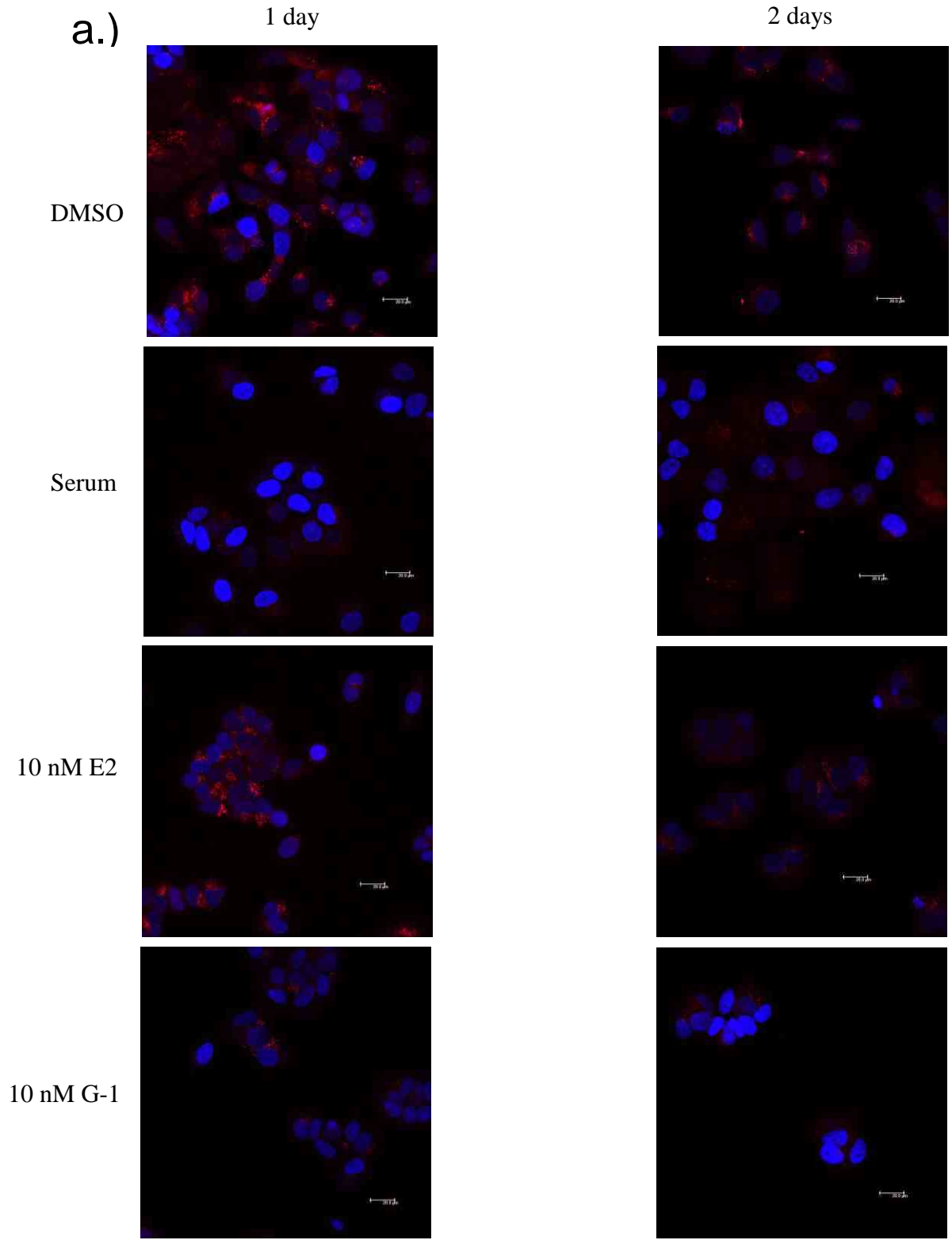


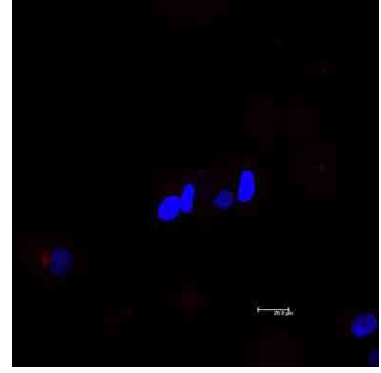
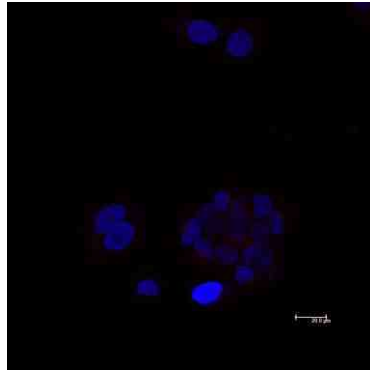
Figure 2.12. Estrogen receptor stimulation does not significantly affect Bim or p27 levels. MCF7 cells were serum starved for 24 hours prior to treatments. Cells were treated with 0.1% DMSO, 50 ng/ml EGF, 50 nM E2 and 100 nM G-1 for indicated times. Cells were lysed in RIPA buffer, separated by gel electrophoresis, transferred by Western blot and quantitated using ImageJ software.



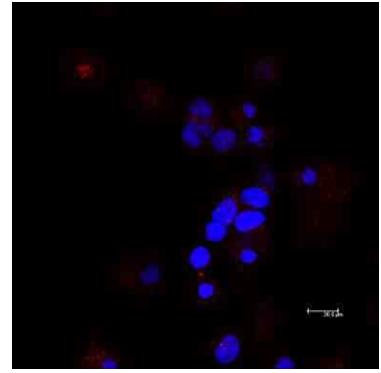
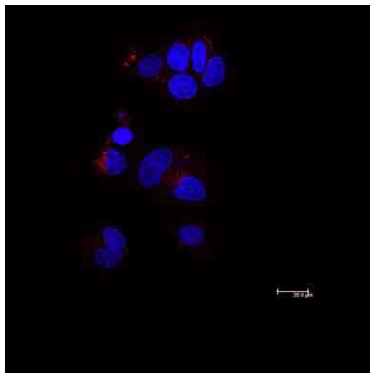
1 day

2 days

50 nM E2



100 nM G-1

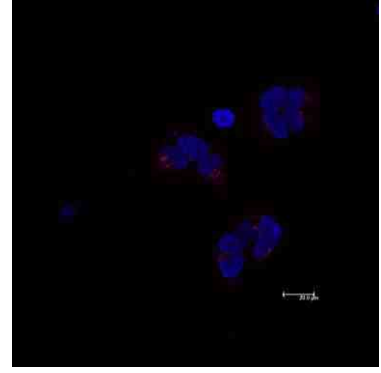
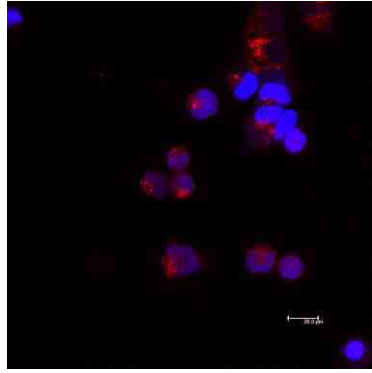


b.)

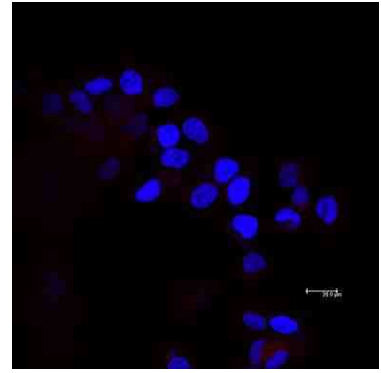
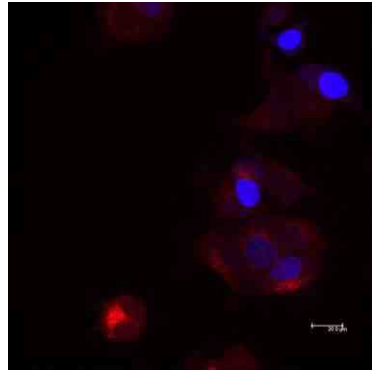
3 days

5 days

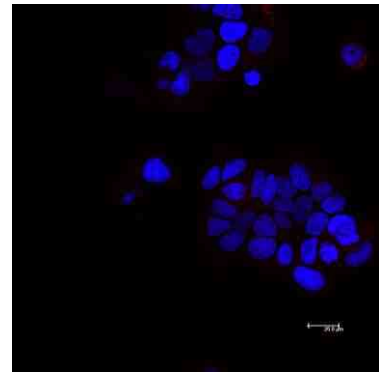
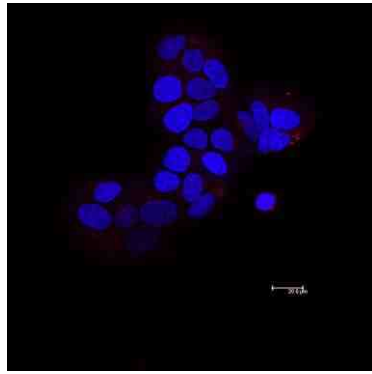
DMSO



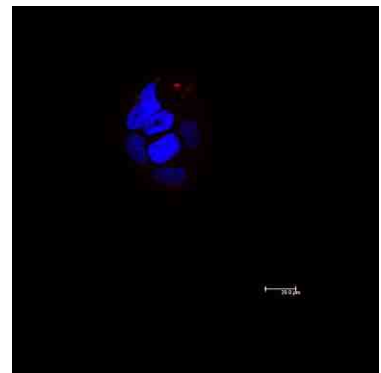
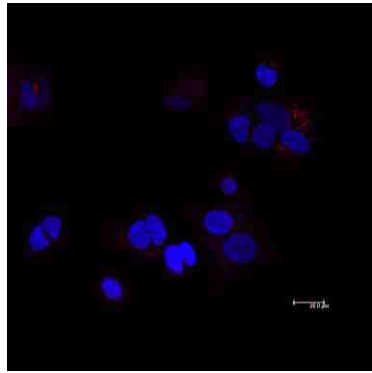
Serum



10 nM E2



10 nM G-1



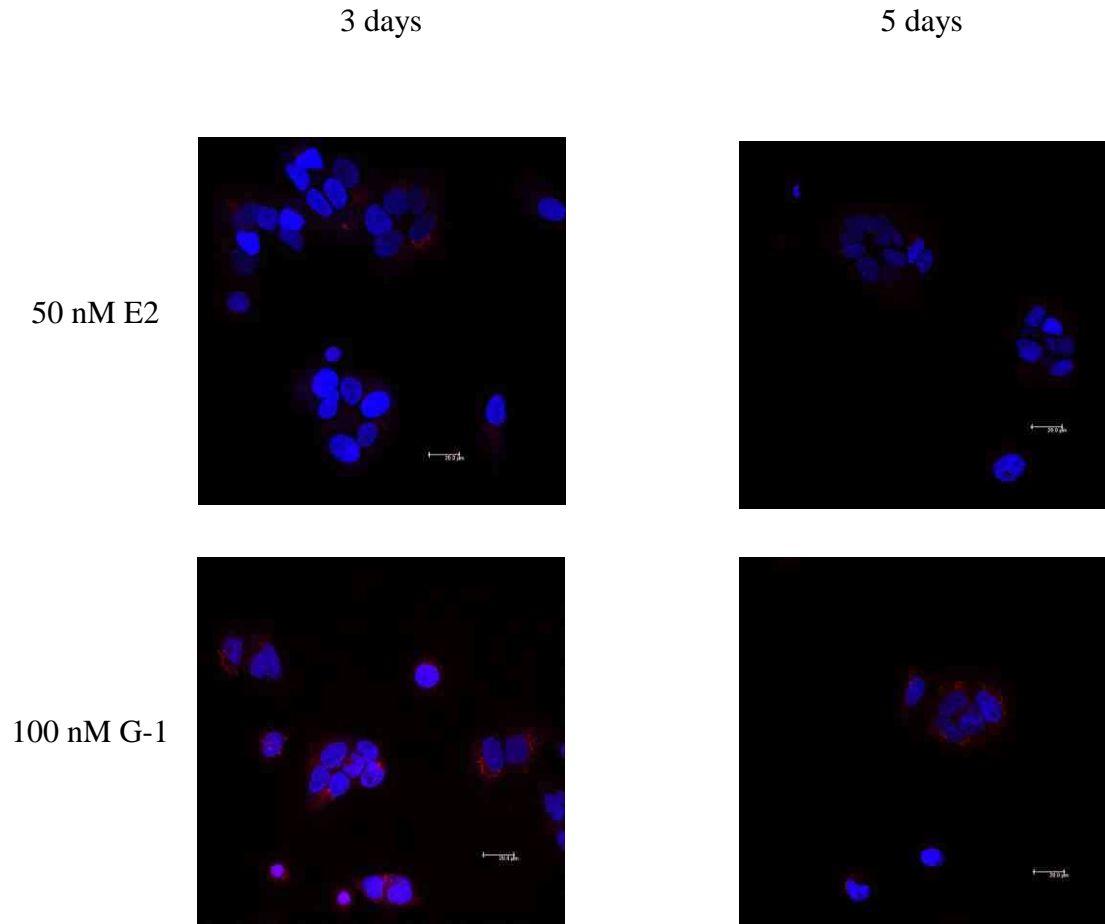


Figure 2.13. Estrogen receptor stimulation and caspase activation. MCF7 cells were treated as indicated for 1 and 2 (a), 3 and 5 (b) days. After treatment, cells were incubated with Magic Red caspase substrate solution for 1 hour at 37°C. Images are representative of each treatment at each time point. Accumulation of the cresyl violet fluorescent product is indicative of caspase activation.

CHAPTER 3

3. Conclusions, significance and future directions

3.1. Conclusions

The current studies sought to elucidate the mechanism by which PI3Kinase activation by the G-protein coupled estrogen receptor, GPER, can lead to prosurvival effects that allow breast cancer cells to evade apoptosis. Since our previous study in 2005, the literature investigating PI3Kinase and GPER in a breast cancer cell environment is scarce. Traditionally, PI3Kinase converts the membrane phospholipid PIP2 to PIP3 at the cytoplasmic plasma membrane (Czech, 2000). Our results indicating that PIP3 is generated in the nucleus upon estrogen receptor stimulation was a novel concept and the mechanism by which this occurs is still unknown. Here, I sought to elucidate this mechanism in MCF7 cells, a breast cancer cell environment that includes all three estrogen receptors, to provide a more physiologically relevant setting of a typical ER α^+ breast cancer cell.

The first approach to understanding this pathway was to transfect the PH-RFP reporter into MCF7 cells to monitor nuclear PIP3 generation in response to estrogen receptor stimulation. We were not able to detect a consistent, overall difference in the amount of PH-RFP that translocates to the nucleus in response to E2 and G-1 compared to DMSO vehicle control (Figure 2.1). However, there is an observable amount in the nucleus, even under serum starved conditions (Figure 2.1a). Nuclear PH-RFP under starved conditions could be the result of

residual activation of signaling pathways that activate PI3Kinase. There could also be competition between endogenous proteins and the PH reporter, which would make any response to stimulus difficult to detect (Czech, 2000). Additionally, because we transfected MCF7 cells with the PH-RFP reporter, its localization prior to stimulation may not be indicative solely of PIP3 localization. Therefore, when the cells are stimulated with ligand, the PH-RFP that is already localized to the nucleus may bind to PIP3 generated there, masking a translocation effect. This is in contrast to what we previously observed with SKBR3 cells, where under unstimulated conditions the PH-RFP reporter is localized to the plasma membrane due to constitutive EGFR activation from overexpressed Her2. In order to eliminate any possible expression level localization effects, we treated cells and then stained them using a PIP3 antibody (Echelon) to detect endogenous PIP3 generation. The antibody exhibited a strong nuclear localization even under serum starvation conditions (data not shown). Previous results were published that describe E2 treatment of MCF7 cells leading to accumulation of the PIP3 antibody at the plasma membrane (Lee et al., 2005). However, they did not permeabilize the cells and in our experiments we used 0.1% Triton-X to permeabilize before blocking. Initially, we blocked and then incubated the cells with the PIP3 antibody without permeabilization, but saw a more punctate, nonspecific pattern. Because of our conflicting results with this previously published work, the methods for this experiment should be further investigated.

PIP3 is able to bind the PH domain of Akt, which anchors Akt to the membrane resulting in phosphorylation and activation by PDK. GPER stimulation in MCF7 cells by G-1 was able to induce rapid phosphorylation of Akt (Figure 2.2). We attribute E2's slight increase in Akt activation compared to control to GPER stimulation and propose longer time points based on previous results to show a more significant increase of phospho-Akt in response to E2 (Lee et al., 2005). ER α 's contribution to Akt activation in MCF7 cells needs to be determined. We also examined phospho-Akt on a cellular level by immunofluorescence and microscopy, but endogenous phospho-Akt levels were more difficult to detect. One possible way to enhance visualization of phospho-akt levels on a cell to cell basis, would be to utilize a biotinylated anti-streptavidin antibody. Consecutive steps result in binding of more fluorochromes at the target site, thereby amplifying the signal that results from phospho-Akt generated upon estrogen receptor stimulation in each cell. Phospho-akt detection on a cellular basis would also delineate Akt localization upon activation.

Akt functions in the cytoplasm as well as the nucleus. Because we hypothesize that GPER downstream signaling events occur in the nucleus, total Akt translocation was monitored in response to E2 and G-1. G-1 was able to significantly induce translocation of Akt to the nucleus compared to control (Figure 2.3d and 2.3e). No detectable increase in nuclear Akt was observed with E2, but we hypothesize that since E2 is binding to all three estrogen receptors, it is likely that Akt's response is diminished due to competing effects. Also, as we observed with Akt activation, E2 may not induce rapid translocation of Akt.

The proapoptotic transcription factor, FOXO3a, is a known nuclear target of Akt (Brunet et al., 1999). In the presence of growth factor signals, Akt is activated and phosphorylates FOXO3a. FOXO3a phosphorylation results in its inactivation and exclusion from the nucleus. We utilized a FoXO3-GFP construct to monitor its rapid inactivation and translocation from the nucleus to the cytoplasm as a result of E2 and G-1 treatment. Serum and EGF were positive controls initially, but EGF was chosen to be the positive control in all subsequent experiments because it is presumably only acting through the EGFR. Serum contains many different growth factors such as insulin, which would not allow us to isolate the signaling effects of the EGFR pathway in order to compare it to the transactivation of the EGFR by E2 and G-1 GPER stimulation. Compared to the vehicle DMSO negative control, E2 and G-1 significantly inactivated FoxO3-GFP (Figure 2.5). This inactivation is concentration dependent and occurs rapidly (in 15 minutes) (Figure 2.6 and 2.7).

Because MCF7 cells express all three estrogen receptors, G-1 is an important tool in delineating GPER's role in this pathway. It is also essential to determine which receptor is mediating E2's effects. Knockdown of GPER significantly decreased E2's ability to inactivate FoxO3-GFP, while ER α knockdown had no effect (Figure 2.8 and 2.9). These results are the first to demonstrate that GPER is the estrogen receptor responsible for E2 inactivation of FOXO3a. Zou et al. reported that FOXO3a functions as a negative regulator of ER α and ER β by directly associating with the receptor (Zou et al., 2008). This binding decreases the transcription of ER related genes and has antiproliferative outcomes. Sisci et

al. described that overexpression of FOXO3a has antiproliferative effects and that these effects are dependent on ER α and E2 (Sisci et al., 2013). Neither of these studies examined GPER's contribution. Based on our results, if E2 promotes FOXO3a's apoptotic effects through ER α , then it is possible that inhibiting E2 binding to GPER could enhance this effect.

p110 α is the PI3Kinase catalytic subunit responsible for E2 and G-1 inactivation of FoxO3-GFP (Figure 2.11a); EGFR is required for this activity (Figure 2.10a). This is the first report that links the activation of a specific PI3Kinase isoform to GPER stimulation leading to prosurvival effects in breast cancer cells. GPER's involvement in this pathway makes it an attractive target for antagonists such as G36 and G15 in breast cancers where GPER expression is confirmed and/or overexpressed.

FOXO3a is a proapoptotic transcription factor that has been described to regulate the transcription of Bim, p27, Fas Ligand and TRAIL. We attempted to correlate FOXO3a inactivation by GPER with a change in Bim and p27 proteins expression levels. A significant decrease over 24 and 48 hours was not detectable with EGF, E2 or G-1 compared to DMSO vehicle control (Figure 2.12). MCF7 cells were serum starved for 24 hours before treatments. An alternative approach might be not to serum-starve the cells and add treatments 24 hours after cells are seeded. This could potentially decrease the high levels of Bim we observe at the 24 hour time point, since the cells could already be shifted too far towards a proapoptotic state that cannot be rescued even by our positive control

EGF. Then, we anticipate that we may see more detectable differences at the 48 hour time point.

Caspase activation is an important step in the initiation of apoptosis. We hypothesized that GPER stimulation resulting in the inactivation of FOXO3a would decrease caspase activation in MCF7 cells. Employing the Magic Red caspase activation kit, we were able to observe a decrease in caspase activation with E2 and G-1 compared to DMSO vehicle control at multiple time points following serum starvation (Figure 2.13). In order to determine if FOXO3a inactivation is responsible for this decrease in caspase activation, we will utilize a phospho-FOXO3a antibody. We would predict that since E2 and G-1 treatment result in a decrease in caspase activation, that there would be a concomitant increase in phospho-FOXO3a in the cytoplasm.

3.2. Significance

The rapid signaling effects of estrogen were observed in cells in the mid-1970s, even before its genomic effects had been characterized (Pietras and Szego, 1975). It wasn't until 2000 that some of these effects were attributed to the novel G-protein coupled estrogen receptor (GPR30), which is now termed GPER (Filardo et al., 2000). In 2005, our group provided evidence for PI3Kinase activation via GPER (Revankar et al., 2005). Stimulation of GPER has been shown to activate proliferative/prosurvival pathways that eventually result in changes in gene expression. Although these pathways are utilized by other receptors such as the EGFR, and have been characterized, the rapid signaling effects that occur immediately after GPER stimulation by estrogen and the components involved are still being characterized.

Estrogen and its canonical receptors are responsible for the growth of hormone-responsive breast cancer tumors. Targeting these receptors in order to inhibit their actions has been a major goal of breast cancer treatment. Selective estrogen receptor modulators (SERMs) were developed for this purpose and have been widely used. The SERM tamoxifen is an estrogen receptor (ER) antagonist used to treat ER-positive breast cancers. In a recent study, the efficacy of tamoxifen treatment in ER-positive breast cancers was analyzed and results show that recurrence rates after 5 years of tamoxifen treatment were reduced substantially compared to non-adjuvant treatment over a ten year period (Early Breast Cancer Trialists' Collaborative et al., 2011). However, tamoxifen treatment of ER-negative tumors had little or no effect on recurrence rates. More

recently, another SERM, raloxifene, was developed to decrease the unwanted toxicity side effects of tamoxifen. In the STAR (Study of Tamoxifen and Raloxifene) study, although raloxifene had fewer side effects, it did not appear to be as effective as tamoxifen in reducing the incidence of noninvasive breast cancer. The raloxifene-treated group from this study also had a nonsignificant reduction in endometrial cancer. Aromatase Inhibitors (AIs), which inhibit the aromatase enzyme responsible for the biosynthesis of estrogen, are an alternative strategy to SERMs. It has been shown that aromatase inhibitors are highly effective in preventing invasive ER-positive breast tumors in high-risk women. However, significant side-effects have been observed in follow-up studies (den Hollander et al., 2013). The side effects of these drugs, as well as the difficulties in treating ER-negative tumors, suggest that other targets need to be elucidated in order to more effectively and safely treat breast cancer.

Estrogen has been demonstrated to have rapid signaling effects in breast cancer cells, such as the activation of adenylyl cyclase (AC) resulting in cAMP production as well as MAP-Kinase activation (Aronica et al., 1994) (Improta-Brears et al., 1999). In 2000, the antiestrogen ICI 182,780, which can antagonize these effects in certain ER-positive cells, was discovered to increase MAP-Kinase activation in MCF7 cells, which are ER-positive, and the ER-negative cell line SKBR3 (Filardo et al., 2000). This suggested that mitogen activated protein kinase (MAPK) activation and other rapid signaling effects of E2 could potentially occur through a non-ER dependent mechanism. This work concluded that GPER was responsible for the rapid signaling effects of E2 and ICI 182,780 and

that this signaling required the transactivation of the EGFR. Therefore, GPER was proposed to be responsible for the lack of effectiveness of antiestrogen treatments in some ER-negative breast cancers.

Because long-term treatment with tamoxifen was associated with an increased risk of endometrial cancer, the mechanism for this agonistic effect of tamoxifen was investigated (Vivacqua et al., 2006). MAPK activation by GPER was described in endometrial cancer cells and these cells were found to have a downstream proliferative response to the active metabolite of tamoxifen, 4-hydroxytamoxifen (OHT), by upregulating *c-fos* gene expression. *c-fos* is a proto-oncogene that expresses a protein which can heterodimerize with *c-jun* and together function as the activating protein 1 (AP1) transcription factor for regulating proliferative gene expression. This suggests that GPER may be involved in tamoxifen's induction of endometrial cancers and that GPER expression should be considered a factor when determining the most effective endocrine therapy treatments.

GPER has been suggested to be responsible for tamoxifen resistance, the ability of tamoxifen to cause proliferative signaling in endometrial cancer cells, as well as the proliferative signaling in response to the ER antagonist, ICI 182,780.

There are other explanations that have been offered to account for nonresponsiveness to endocrine therapy: intratumoral heterogeneity in ER expression, drug resistance as a result of evolution of mutant ERs with reduced affinity for ER antagonists, partial receptor antagonism, and the presence or absence of trans-acting factors that influence ER functionality (Filardo et al.,

2006). Because of these possibilities, more strategies have been employed to determine ER activity and develop more effective antagonists. One example of a more complete ER antagonist, fulvestrant, is being assessed in clinical trials for patients with primary and advanced breast cancer (Ciruelos et al., 2014).

Additionally, progesterone receptor (PR) has become a comarker because its gene transcription is regulated by ER-dependent gene transactivation, meaning that if there is high PR expression then there would be an improved response to tamoxifen. Additionally, a study was performed to acquire baseline information regarding the potential role of GPER as an independent factor in human breast cancer (Filardo et al., 2006). The aim of this study was to determine the relative tissue distribution of GPER, ER and PR in intraductal and invasive ductal carcinoma. GPER expression varied in breast tumors, in which 62% of invasive tumors and 42% of intraductal tumors were positive. Codistribution of ER and GPER was detected in 43% of invasive breast tumors, which indicated a significant association between ER and GPER. Coexpression of GPER and ER was linked to PR positivity. Additionally, GPER positively correlated with HER-2/neu, tumor size, and metastasis. These results provide further evidence that GPER has an independent influence on breast cancer and should be utilized as a marker for treatment and prognosis.

Taxanes, such as paclitaxel and its derivatives, are an alternative class of drugs utilized in the treatment of breast cancer (Blagosklonny and Fojo, 1999). These drugs interact with cellular microtubules associated with the spindle apparatus during mitosis. The taxanes cause cell death through the interruption of mitosis,

but the exact mechanism by which apoptosis occurs had not been elucidated in breast cancer cells treated with paclitaxel. Sunter et al. demonstrated a role for nuclear FOXO3a in the induction of paclitaxel-induced apoptosis by FOXO3a's upregulation of the proapoptotic protein, Bim (Sunter et al., 2003). In order to investigate this mechanism further, the same group utilized MCF7 cells treated with paclitaxel and demonstrated a dose-dependent increase in nuclear localization of FOXO3a (Sunter et al., 2006). The nuclear localization of FOXO3a was accompanied by decreased Akt signaling but increased c-Jun NH2-terminal kinase 1/2 (JNK1/2) and p38 activity. Akt is a prosurvival factor while p38 and JNK are both implicated in apoptosis. This allowed them to conclude that breast cancer cell death initiated by paclitaxel is dependent on JNK activation, which results in Akt inhibition and an increase in nuclear/activated FOXO3a.

Additionally, FOXO3a is suggested to induce apoptosis in breast cancer cells in response to the cancer treatment drug doxorubicin (Ho et al., 2012). In cases of breast cancer that are advanced or metastatic, anthracycline derivatives such as doxorubicin or epirubicin have been utilized as a more aggressive option over hormone targeted therapies. These drugs induce cell cycle arrest and cell death by apoptosis, but long-term use leads to an acquired drug resistance (Wong and Goodin, 2009). Doxorubicin functions by inhibiting topoisomerase II, which functions in DNA replication, and also by producing free radicals. Both these effects result in apoptosis. Doxorubicin was shown to induce cell death in MCF7 cells through phosphorylation of FOXO3a on Ser7 (Ho et al., 2012).

Phosphorylation at this site causes FOXO3a to be localized to the nucleus and function as a proapoptotic transcription factor. Previous data demonstrated that paclitaxel induced JNK activation and Akt inactivation in MCF7 cells results in nuclear localization of FOXO3a (Sunters et al., 2006). p38 was implicated because of its increased activity; however its exact role in FOXO3a nuclear localization had not been elucidated. p38 MAPKs are responsive to stress stimuli, such as cytokines, ultraviolet irradiation, heat shock, and osmotic shock, and are involved in cell differentiation, apoptosis and autophagy. Ho et al. demonstrated a direct interaction between p38 and FOXO3a, and that p38 binds and phosphorylates a recombinant FOXO3a in vitro (Ho et al., 2012). Utilizing HPLC and mass spectrometry, Ser7 was identified as the site for p38 phosphorylation and that this site was phosphorylated in response to doxorubicin. Importantly, they also demonstrated FOXO3a nuclear localization in response to doxorubicin, and that p38 and Ser7 phosphorylation were required for nuclear localization by p38. These examples demonstrate that the proapoptotic transcription factor FOXO3a is activated by certain chemotherapeutic drugs utilized in breast cancer treatments.

Nuclear exclusion and inactivation of FOXO3a is a consequence of the activation of prosurvival pathways such as the PI3Kinase/Akt pathway (Brunet et al., 1999). A study in 2004 attempted to correlate Akt activation with FOXO3a cytoplasmic localization in primary tumors (Hu et al., 2004). The levels of FOXO3a and phospho-Akt were examined in 131 human primary breast tumor specimens using IHC staining and found that FOXO3a was mostly cytoplasmic in tumor

tissues with high levels of phospho-Akt. Additionally, in tumors with negative phospho-Akt, FOXO3a was mostly nuclear. Surprisingly, they also detected FOXO3a cytoplasmic localization in tumor samples that were not positive for phospho-Akt, suggesting an additional mechanism for the nuclear exclusion of FOXO3a in these samples. The I κ B kinase (IKK) signaling pathway has been established as an anti-apoptotic pathway. IKK phosphorylates the inhibitory I κ B α protein, resulting in the dissociation of I κ B α from NF- κ B. NF- κ B can then translocate to the nucleus and activate gene expression, including the upregulation of certain tumorigenic or angiogenic factors, chemokines, adhesion proteins, as well as inhibitors of apoptosis. Constitutive activation of IKK and NF- κ B in human breast cancer cell lines and other primary tumors has confirmed its role in tumorigenesis (Karin et al., 2002). Because of the IKK/NF- κ B pathway's role in tumorigenesis, it was thought to be potentially involved in FOXO3a regulation. Hu et al. found that high levels of nuclear FOXO3a are correlated with low levels of IKK β in the human breast tumor specimens and that this positively correlates with the survival rate in breast cancer (Hu et al., 2004). This work demonstrated that IKK, independent of Akt, can phosphorylate FOXO3a, excluding it from the nucleus which promotes cell proliferation and tumorigenesis in breast cancer cells.

Because FOXO3a localization can predict whether a cell is in a prosurvival or proapoptotic state, its regulation has been more extensively investigated and its relation to cancer has become increasingly important. However, there has been conflicting evidence in recent studies with respect to FOXO3a's localization and

breast cancer prognostic outcomes (Chen et al., 2010; Habashy et al., 2011) (Jiang et al., 2013). In 2010, Chen et al. showed that nuclear FOXO3a was associated with lymph node positivity, poor prognosis, and phospho-Akt expression in invasive ductal carcinoma (Chen et al., 2010). They also reported nuclear FOXO3a in doxorubicin-resistant cells, and that overexpression of FOXO3a enhances PI3Kinase/Akt activity and promotes cell proliferation. Activated Akt failed to inactivate and re-localize FOXO3a to the cytoplasm, and nuclear FOXO3a did not function as a proapoptotic transcription factor. In contrast, Hashaby et al. were able to correlate poor prognosis with cytoplasmic FOXO3a which was also associated with PIKC3A, a constitutively active PI3Kinase mutation linked to breast cancer (Habashy et al., 2011). They proposed that localization is indicative of function and evidence was provided that nuclear localization correlates with good prognosis as well as increased expression of the cell cycle inhibitor, p27. Nuclear localization was also associated with markers of good prognosis such as FHIT (fragile histidine triad protein) and PR (progesterone receptor). Additionally, luminal-like breast cancers expressing nuclear FOXO3a were characterized by low proliferation as indicated by low mitotic frequency and low MIB1 (proliferation marker) expression. In a later study, FOXO3a expression was examined by IHC in breast cancer patient tissue samples and correlated with disease markers such as tumor size, histologic grade, receptor status and overall patient survival (Jiang et al., 2013). To determine if FOXO3a expression itself could be a useful prognostic marker, a Kaplan-Meier analysis was employed to correlate FOXO3a

expression in tumors with overall survival. It was concluded that patients with higher FOXO3a expression showed increased overall survival compared with those who had low FOXO3a-expressing tumors. ER+/FOXO3a+ patients also had better prognoses than those who were ER-/FOXO3a+, which agreed with a previous study that indicated that ER is required for FOXO3a inhibition of cell proliferation (Zou et al., 2008). These conflicting results suggest the need for further studies investigating the relationship between FOXO3a localization/expression and its prognostic utility in breast cancer.

In the current study, we provide evidence indicating that E2 and G-1 decrease apoptosis in MCF7 breast cancer cells over longer time periods in vitro. We hypothesize that this evasion of apoptosis is a result of inactivation of FOXO3a and that GPER is responsible. These results provide further indication that GPER expression should be utilized as a prognostic factor and a target in breast cancer treatment. Targeting FOXO3a and enhancing its activity could also be a potential treatment option.

3.3. Future directions

Our results suggest that the rapid signaling effects of E2 in MCF7 cells are attributed at least in part to the G-protein coupled estrogen receptor (GPER). To determine E2's maximum rapid signaling effects of GPER stimulation on Akt activation, phospho-Akt levels in MCF7 cells should be evaluated at longer time points (30 minutes, 45 minutes and 1 hour or more after E2 exposure). We hypothesize that there may be a peak rapid accumulation of phospho-Akt in response to E2 before the previously published significant increase at 72 hours (Lee et al., 2005). Rapid activation of Akt by E2 can lead to prosurvival signaling and potentially to changes in gene expression. Elucidating the downstream genomic effects that occur as a result of rapid signaling by E2 can provide potential targets for inhibiting cell proliferation and survival of breast cancer cells expressing GPER. Furthermore, we propose that siRNA knockdown of ER α in MCF7 cells can delineate GPER's role in rapid activation of Akt, and this would help to further elucidate its downstream prosurvival effects in response to rapid activation by E2.

Phospho-Akt levels were monitored in whole cell lysates by Western Blot analysis, which makes it difficult to elucidate Akt activation on a cellular and subcellular level. In order to further define the localization of phospho-Akt in response to estrogen receptor stimulation, Akt activation would need to be monitored on a cellular level. Localization of phospho-Akt upon activation would confirm our total Akt localization results and aid in interpreting the mechanism by which E2 activates this prosurvival pathway in MCF7 cells.

Activated Akt can phosphorylate FOXO3a at three specific sites: Thr32, Ser253 and Ser315. A constitutively active mutant where all three Akt phosphorylation sites are mutated to alanine (FOXO3a-TM) exists to monitor FOXO3a's function as a proapoptotic transcription factor under any conditions. To further confirm that GPER stimulation leads to Akt activation resulting in FOXO3a phosphorylation and inactivation, FOXO3a-TM could be utilized. FOXO3a-TM cannot be phosphorylated by Akt, therefore we would expect that E2 would not be able to lead to inactivation of FOXO3a-TM, leading to the inability of E2 to reverse the pro-apoptotic effects of FOXO3a.

We provided evidence for GPER activation resulting in the inactivation of FoxO3-GFP in MCF7 cells. To determine whether this affects protein expression levels of Bim and p27, which are regulated by activated FOXO3a, we monitored Bim and p27 protein levels by Western blot analysis. There was no detectable decrease in Bim or p27 in response to GPER stimulation. To correlate a decrease in these protein levels with inactivation of FOXO3a on a cellular level, we propose monitoring Bim and p27 levels by utilizing immunofluorescence techniques in combination with FoxO3-GFP expression. In MCF7 cells that have cytoplasmic/inactivated FoxO3-GFP in response to E2, we hypothesize that less Bim and p27 would be expressed. This would provide direct evidence for GPER inactivation of FoxO3-GFP resulting in a decrease of the proapoptotic protein Bim and the cell cycle inhibitor p27. The expected results could further demonstrate the benefit of inhibiting GPER and thereby blocking its ability to inactivate FOXO3a.

Doxorubicin is a chemotherapeutic drug frequently used to treat breast cancer and has been previously demonstrated to utilize FOXO3a in order to initiate apoptosis in MCF7 cells (Ho et al., 2012). Doxorubicin targets rapidly dividing cells by inhibiting topoisomerase II, which results in cancer cell death as well as healthy cell death leading to extensive side effects. We hypothesize that GPER is potentially antagonizing doxorubicin's function by promoting cell survival through inactivation of FOXO3a. In order to investigate this hypothesis, we would need to determine whether estrogen opposes the effects of doxorubicin or other chemotherapy drugs in breast cancer cells via FOXO3a inactivation. Utilizing FOXO3a's translocation properties, varying concentrations of E2 would be added to MCF7 cells in the presence of varying concentrations of doxorubicin. We would determine the lowest concentration of Doxorubicin where FOXO3a is still localized to the nucleus in the presence of E2. If a lower dose of doxorubicin could be utilized in combination with an antagonist of GPER for treatment and still be effective in FOXO3a nuclear activation, this could concomitantly decrease cancer drug therapeutic side effects.

To further investigate the clinical relevance of GPER's inactivation of FOXO3a, we propose analyzing FOXO3a localization in normal human breast tissue samples in response to estrogen receptor activation. We would utilize normal human breast tissue that was previously treated with EGF, G-1, and E2 in organ culture, followed by fixation with paraformaldehyde (4%). After fixation, the tissue is paraffin embedded and sectioned. FOXO3a localization would be

analyzed with IHC and IF in these treated sections to determine whether EGF, G-1 and E2 inactivate FOXO3a in human tissue.

Based on the results we provide in this study, further studies correlating GPER with FOXO3a expression, and FOXO3a localization could potentially demonstrate the necessity for including these proteins as prognostic factors in cancer. Although 60% of ovarian cancers are ER α positive, this type of cancer has a lower response to antiestrogen therapies compared to breast cancer (Ho, 2003). This suggests that estrogen mediates some of its effects in ovarian cancer through an alternative mechanism. Ovarian tumor samples have been previously characterized in our lab for their GPER expression, which was correlated with poor survival (Smith et al., 2009). In our current studies we provide evidence for GPER activation resulting in FOXO3a inactivation and cytoplasmic localization. Analyzing these ovarian tumor samples for FOXO3a localization would help us to determine whether cytoplasmic, inactive FOXO3a is correlated with GPER expression in human tumor tissue. By analyzing FOXO3a in tissue and tumor samples, we hope to correlate its localization with patient outcome. Based on our hypothesis, we would expect nuclear FOXO3a to be correlated with better outcome and decreased GPER expression.

In summary, our findings indicate one mechanism by which breast cancer cells can evade apoptosis. Our model proposes that FOXO3a is inactivated by GPER activation which downregulates the production of proapoptotic genes and results in cell survival (Figure 3.1).

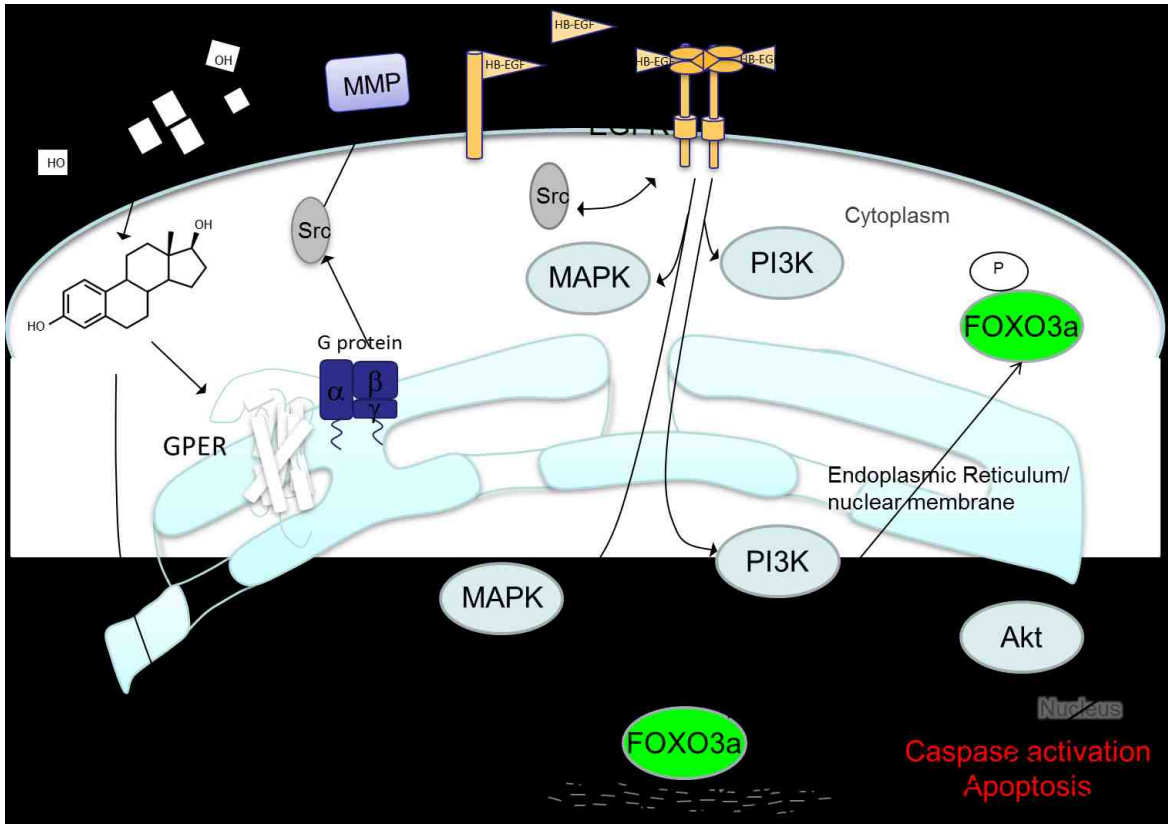


Figure 3.1. Proposed model of FOXO3a inactivation by estrogen. Estrogen stimulates GPER leading to Akt activation. Akt phosphorylates FOXO3a causing its inactivation, translocation to the cytoplasm and subsequent degradation.

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