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The Effects of 17-Beta Estradiol on G-Protein Inwardly Rectifying Potassium Channels (GIRKs) in Breast Cancer

A Dissertation Presented for the Doctor of Philosophy Degree
The University of Tennessee, Knoxville

Michael W. Hance
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DEDICATION

This thesis is dedicated to my mother, Patricia A. Hance, a breast cancer survivor for over twenty years and my late grandfather, William J. Hance. Their courage and strength have provided the inspiration require for completing this project.

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I would like to thank all those whose assistance has made the completion of my Doctorate of Philosophy degree in the Comparative and Experimental Medicine program a possibility. I thank Dr. Madhu Dhar for her valuable, encouraging guidance and supplemental teaching of protein preparation, Western blot analysis, and immunofluorescence microscopy. I would to thank Julia Gouffon and the University of Tennessee Affymetrix core facility for their services and guidance. I would like to thank Dr. Robert Moore whose support and subtle encouragement made this success possible, even under what seemed to be impossible odds. I would also like to acknowledge my gratitude and heartfelt thanks to my advisor, Dr. Howard K. Plummer III, and my graduate committee.

Lastly, I thank my wife, friends and loved ones whose immense encouragement and support helped me keep my chin up, my feet on the ground and ultimately made this possible.

Abstract

Breast cancer is a leading cause of cancer death and in 2009, the American Cancer Society estimates that over 192,000 new cases of breast cancer will be diagnosed, and over 40,000 women will die from breast cancer. Estrogen (E2) is required for normal female development and reproduction, but long-term exposure is carcinogenic and considered a risk factor for breast cancer. Membrane ion channels are essential for cell proliferation and are suggested to have a role in cancer, especially potassium channels. In the present study, we investigate the effects of estrogen and the estrogen antagonist ICI182780 on G-protein inwardly rectifying potassium channels (GIRK) in estrogen responsive MCF-7 breast cancer cells. GIRK1 and GIRK2 specific channels are thought to play a major role in rapid channel activation. We found increases in GIRK1 and GIRK2 membrane protein levels in response to estrogen treatment, as well as increases in intracellular potassium concentrations and cellular proliferation. ICI182780 treatment increased GIRK1, GIRK2 and GIRK4 membrane protein levels but resulted in an initial decrease in intracellular potassium concentration and decreased cell proliferation. GIRK1 RNAi knockdown decreased estrogen receptor alpha protein levels and activation. In addition, estrogen treatment resulted in increased phosphorylation of specific members of GPCR and MAPK signaling pathways that have been shown to be responsive to GIRK1 knockdown. Using microarray analysis of nontreated and E2 treated MCF7 cells, we observed 489 differentially expressed genes (283 upregulated and 206 downregulated) that were comprised largely of transcription and cell cycle associated genes. This study identified several human cell cycle associated genes that are both responsive to E2 treatment and are functionally correlated with GIRKs. Five genes were selected for further analysis by real time PCR. Our data suggests that specific GIRK channel composition at the cell membrane may be stimulated by

estrogen exposure or downstream targets of estrogen signaling and may contribute to increased cell proliferation in MCF-7 breast cancer cells. Taken together, these data add further support of GIRK involvement in cancer progression and identify some potential biological roles of GIRKs in cancer biology beyond the initilal findings of GIRK1 assciation in metastatic breast cancer.

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List of Symbols and/or Abbreviations

ACS	**American Cancer Society**
ADRB2	**β_2 adrenergic receptor**
BK_{Ca}	**large-conductance, calcium- and voltage-activated potassium channel**
BrdU	**5-Bromo-2' deoxy-uridine**
cAMP	**cyclic AMP**
CDC6	**Cell division cycle homolog 6**
CNS	**central nervous system**
CREB	**cyclic AMP response element binding protein**
DSCC1	**Defective in sister chromatid cohesion 1 homolog**
E2	**17β estradiol**
EAG	**ether a go-go voltage gated K+ channel**
E_K	**K^+ equilibrium potential**
ERα	**estrogen receptor α**
ERβ	**estrogen receptor β**
FOXM1	**Forkhead box M1**
GC RMA	**GeneChip Robust Multiarray Averaging**
GIRK	**G protein inwardly rectifying K^+ channels**
GnRH	**gonadotropin-releasing hormone**
GPCR	**G protein coupled receptor**
Kv1.3	**voltage-gated potassium channel 1.3**
MELK1	**Maternal embryonic leucine zipper kinase**
NNK	**4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone**
NSCLC	**non-small cell lung cancer**
PBK	**PDZ binding kinase**
PCA	**principle components analysis**
PCR	**polymerase chain reaction**
PKA	**protein kinase A**
PTX	**pertussis toxin**
RFC	**replication factor C**
SCLC	**small cell lung cancer**
siRNA	**short interfering RNA**
TEA	**tetraethylammonium**
U5	**U50488H (trans-(±)3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]cyclohexyl-benzeneacetamide)**

Introduction: A potential Correlation between G Protein Inwardly Rectifying Potassium Channels (GIRKs) and Estrogen signaling in Breast Cancer

CHAPTER I

Background

Breast cancer is one of the most common cancers in women and is reported as the second highest cause of cancer death after lung cancer (2). In addition, men are at risk in rare cases (2). Multiple risk factors contribute to the onset of breast cancer such as lifestyle (including smoking, drinking, and diet), family history, age, race, geographical location, age of menarche, age of parturition, and age of menopause (2). Broadly defined breast cancer is malignant cell growth originating from the ductal and lobular tissue of the breast (2). The American Cancer Society (ACS) estimates that 192,000 women will be diagnosed with breast cancer in 2009 and approximately 40,000 women will die from breast cancer in 2009 (2). Approximately 13% of women will have invasive breast cancer at some point in their lives (2). It has been suggested that the US and Europe account for 16% of the world population and approximately 60% of the worldwide incidence of breast cancer (87,88). Today, breast cancer accounts for 22% of all female cancers and 1 in 35 cases will be fatal (2, 87). Fortunately, breast cancer death rates are decreasing, which may be attributed to improved diagnostic abilities and better treatment options (2).

The American Cancer Institute broadly defines breast cancer as a disease characterized by unregulated cell growth that can invade surrounding tissues, specifically the dutal tissues responsible for the transport of milk and the lobular tissues that produce milk, however metastatic breast cancer is capable of spreading to other tissues in the body (77). The development of the mammary gland is coordinated by the actions of hormones and growth factors including estrogen signaling via estrogen receptor α (ERα) (46). The action of estrogen or 17β estradiol (E2) is involved in cellular proliferation and differentiation that initiate both the terminal end-bud formation and duct development (46). E2 is a key regulator in the

differentiation and the functional capacity of multiple tissues, especially the mammary gland, where E2 is considered one of the leading causes of sporadic breast cancer or breast cancer that arises from genetic changes that occur during a women's life rather than from a germline mutation (19, 20, 77, 132). The relationship between both endogenous and exogenous estrogen (E2) and breast cancer pathogenesis is well documented (19, 20, 46, 77, 132).

Considerable advancements have been made in diagnosis and treatment of breast cancer but there are still many poorly understood biological and clinical aspects of the disease. These include our understanding of tumor progression and reoccurrence, therapeutic resistance, treatment and prevention, and diagnosis (96). In the past, breast cancer was thought as a singular disease, but evidence suggests that breast cancer is a heterogeneous group of complex diseases that differ at the clinical and molecular levels (91, 117). The use of comprehensive gene expression profiling has enabled researchers to identify five major molecular subtypes of breast cancer: 1) basal-like, 2) luminal A, 3) luminal B, 4) HER2+/ estrogen receptor α negative (ERα(-)) and 5) normal breast–like (96). These subtypes demonstrate distinct biological differences that affect their clinical responses to treatment (96). It is generally thought that basal-like and HER2+/ERα (-) breast cancers have a poorer prognosis, and luminal A tumors have the best prognosis (38, 96, 99, 117). The presence of ERα is one criteria used to classify invasive breast cancer because the presence of the ERα is generally associated with a better prognosis and response to clinical intervention (98). Additionally, the lack of ERα (in basal-like and HER2+/ERα(-) tumors) correlates with a poor prognosis and limited response to current clinical therapies, which generally target ERα (36, 98).

Estrogen Carcinogenesis and Cancer

Accumulating evidence indicates that E2 is a carcinogen and there is a correlation between E2 exposure and its potential roles in the development and progression of breast cancer (132). The role of E2 in carcinogenesis is unclear, but it is thought to be the consequence of two complimentary pathways, specifically E2 metabolism and ER signaling (132). Figure 1.1 illustrates these two estrogen carcinogenesis pathways (132). In E2 metabolism, estrone (E1) and E2 undergo oxidative metabolism to yield two major products: 2-hydroxycatechol estrogen by cytochrome P-450 1A1, 1A2 and 1A3 or to 4-hydroxycatechol estrogen by cytochrome P-450 1B1 (35, 45, 67). These oxidative E1 and E2 species result in the production of E2 3,4 quinone, which can lead to oxidative DNA damage by depurination and mutation though the formation of unstable adducts with adenine and guanine (15, 16, 17, 23, 132). Reactive oxygen species can be produced by redox cycling of the estrogen quinones to hydroquinones and catechols, which contributes to DNA and lipid oxidative damage (15, 67, 132). Additionally, oxidative metabolism of E2 can produce 16α-hydroxyestrone that contributes to covalent binding to proteins and DNA (132). Yager et al. theorizes that oxidative metabolites of estrogen have genotoxic, mutagenic, transforming, and carcinogenic effects on cells; thus suggesting that E2 may initiate and/or contribute to the progression of carcinogenesis (132). It has been shown using MCF-7 breast cancer cells that there is an accumulation of oxidative damage after being treated with E2 (60, 80, 81, 82).

Samuni et al. has shown that estrogen metabolites can induce genetic mutations in the thymidine kinase gene in MCF-7 breast cancer cells and this data provides evidence of the mutagenic properties of E2 and E2 associated metabolites (109). Russo et al. demonstrated neoplastic transformation of non-turmorgenic MCF-10F breast cells treated with E2 (108). E2 treatment of MCF-10F breast cells induced anchorage independent growth, colony formation,

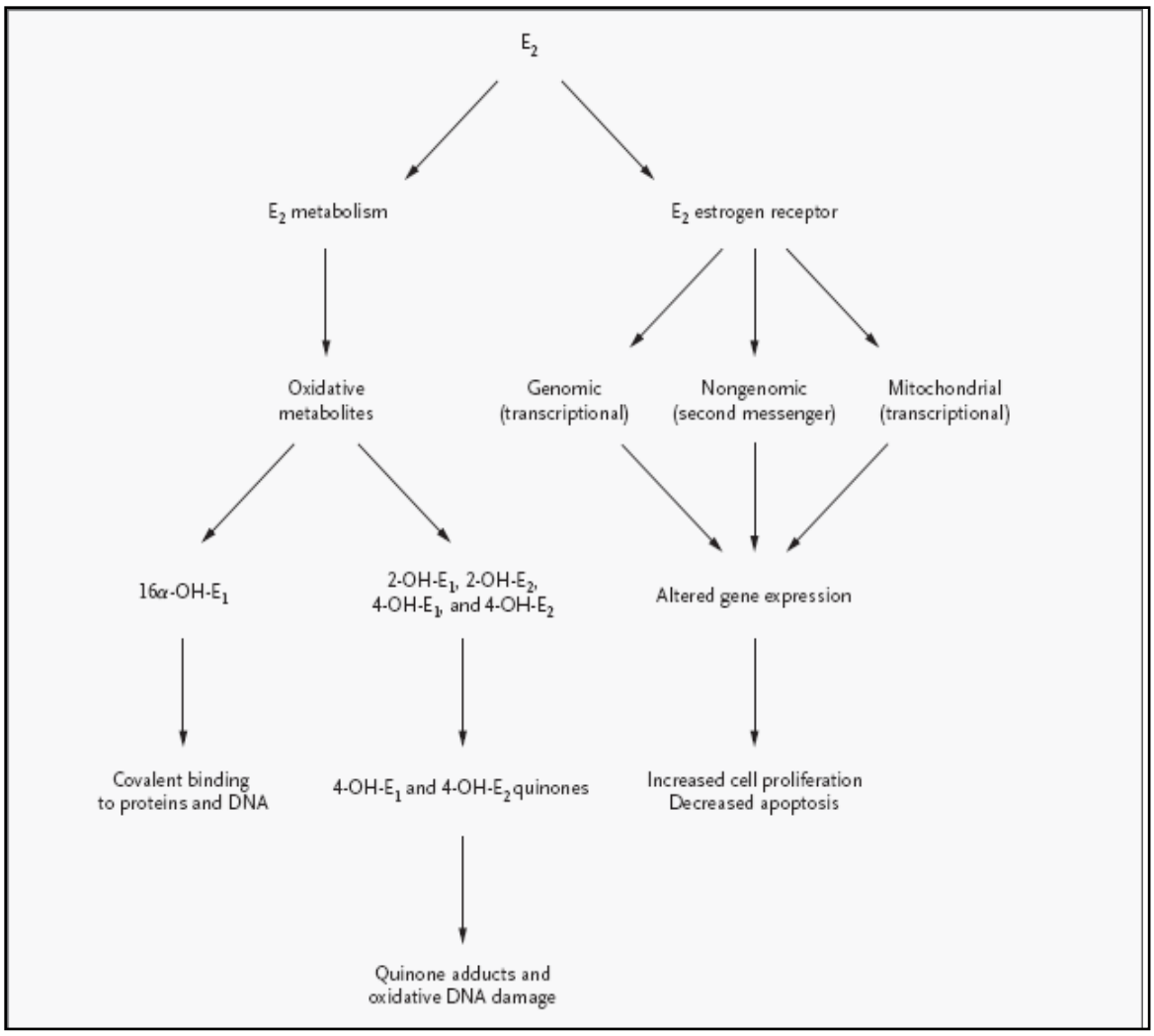

Figure 1.1: Estrogen Carcinogenesis. Carcinogenicity of estrogen occurs by two complementary pathways, E2 metabolism and E2-ER signaling, that contribute to the initiation, promotion and/or progression of breast cancer. Both estrone and estrogen can undergo oxidative metabolism that results in oxidative DNA damage or covalent modifications to proteins and DNA. Additionally, E2-ER mediated signaling modulates genomic, nongenomic and mitochondrial expression that can lead to increased cell proliferation and decreased apoptosis. Abbreviations: E_1 (estrone), E_2 (estradiol), 2-OH-E_1 (2-hydroxyestrone), 2-OH-E_2 (2-hydroxyestradiol), 4-OH-E_1 (4-hydroxyestrone), 4-OH-E_2 (4-hydroxyestrone) and 16α-OH-E_1 (16α-hydroxyestrone). (Figure from reference 132)

reduced ductulogenic capacity, which are all considered signs of neoplastic transformation (108).

The carcinogenic action of E2 modulates signal transduction pathways that are correlated with proliferation, inhibition of apoptosis, cell migration, and induction of angiogenic factors (132, 45, 67, 108, 112). Figure 1.2 illustrates multiple E2-ER signaling pathways that are associated with induced proliferation and inhibition of apoptosis, which are characteristic events in cancer progression (132). Direct action of E2 on ERs initiates dimerization and binding to estrogen-responsive elements in the regulator portions of estrogen responsive genes (132). These associations with transcription factors, co-activators and repressors can all have a direct effect on gene expression and contribute to tumorgenesis (132). E2 can also act directly on the mitochondria to suppress mitochondrial reactive oxygen species that are required to initiate apoptosis via JNK protein kinases and Bax protein (90, 132). Additionally, E2-ER induced activation of G-protein coupled receptors can modulate PI3K/AKT protein kinases, via the MAPK kinase family and the epidermal growth factor receptor (EGFR), which can suppress Bax translocation to the mitochondria and inhibit cytochrome C release from the mitochondria (27, 72, 90, 102, 122, 132). The suppression of Bax and cytochrome C activity inhibits apoptosis, contributes to abnormal cell cycle regulation, and supports abnormal cell proliferation (27, 72, 90, 102, 122, 132).

The Estrogen Receptors

ERβ was discovered much later than ERα, which increased the complexity of dissecting the actions of both of these receptors in association with the mitochondria, nucleus and cell membrane (31, 64, 65, 89, 93, 128). Both receptors bind E2 and similar compounds; additionally ERs functionally recognize and alter gene expression via estrogen responsive elements (EREs), which are palindromic DNA sequences in the regulatory regions of E2 responsive genes (89, 93,

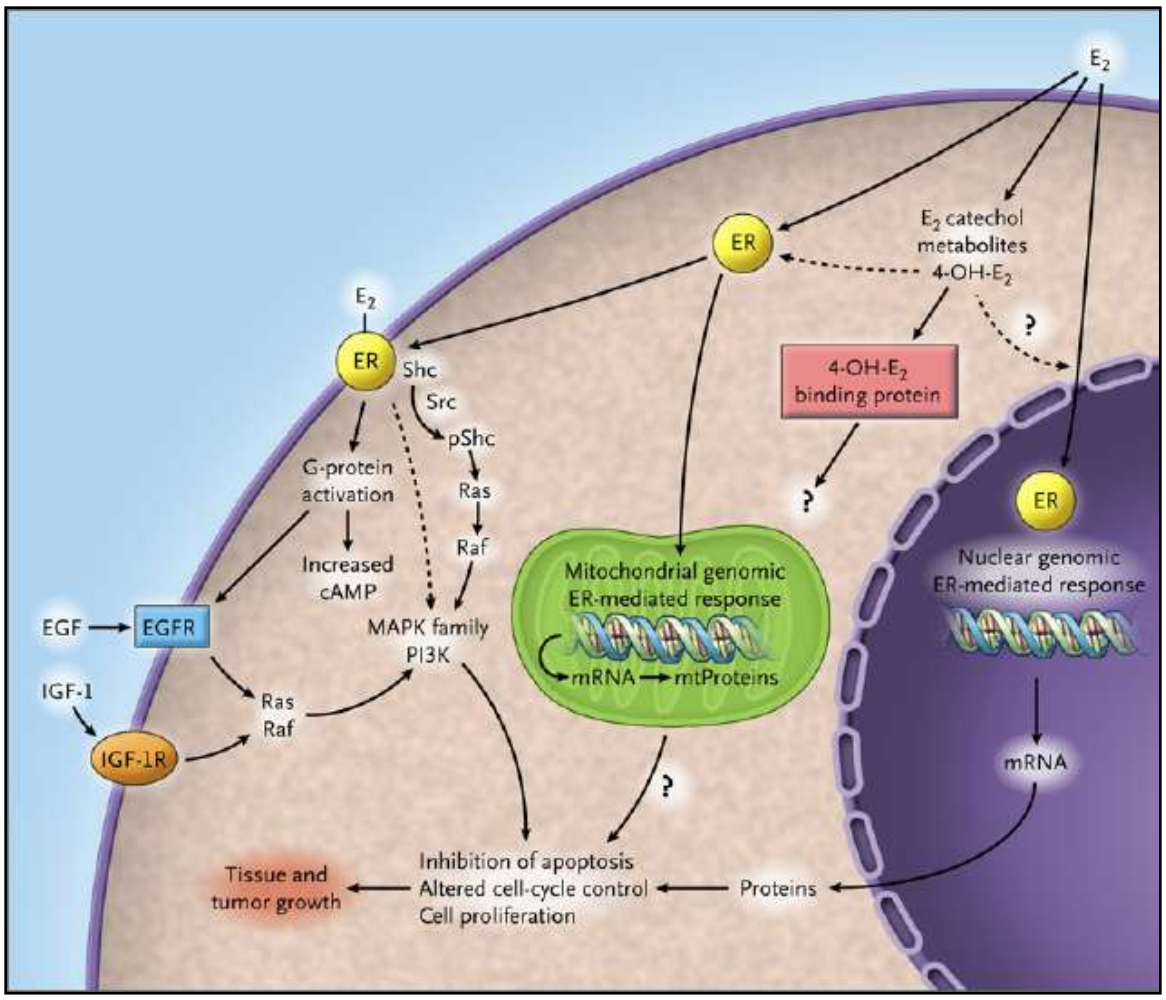

Figure 1.2: Estrogen Signaling Pathways. E2-ER signaling pathways can act directly on the nucleus, mitochondria and as a second messenger in signaling pathways. Additionally, E2 metabolites can potentially interact with these events, which increase the carcinogenic potential of E2 in cancer. Dashed lines represent putative signaling pathways. Abbreviations: cAMP (cyclic AMP), E_2 (estradiol), 4-OH-E_2 (4-hydroxyestrone), ER (estrogen receptor), EGF (epidermal growth factor), EGFR (epidermal growth factor receptor), IGF-1 (insulin-like growth factor 1), IGF-1R (insulin-like growth factor 1 receptor), MAPK (mitogen-activated protein kinase), PI3K (phosphoinositide 3 kinase). (Figure from reference 132)

132). EREs are able to interact with basal transcription factors, co-activators, and co-repressors (89, 93, 132). Evidence has shown that ERβ demonstrates a less robust transcriptional response to E2 compared to ERα (93, 132). Additionally, Both ERs bind tamoxifen, a well-known therapeutic antiestrogenic agent, with similar affinity (93, 132). It has been reported that tamoxifen has an agonistic and antagonist action on ERα expression but only an antagonist effect on ERβ expression (93, 132). Vanacker et al. was able to show that ERα, but not ERβ, can bind and activate transcription from Steriodogenic Factor 1 (SF1) response element, which indicates functional differences between the two receptors (124). ERα protein is composed of 595 amino acids transcribed from the ESR1 gene at the location of 6q25.1 and ERβ protein is composed of 530 amino acids transcribed from the ESR2 at the location of 14q.23.2 (22, 28, 137). Figure 1.3 illustrates a schematic representation of the full-length human ERα and ERβ proteins (138). Both ERs share similar structures that are characteristic of members in the nuclear receptor subfamily (22).

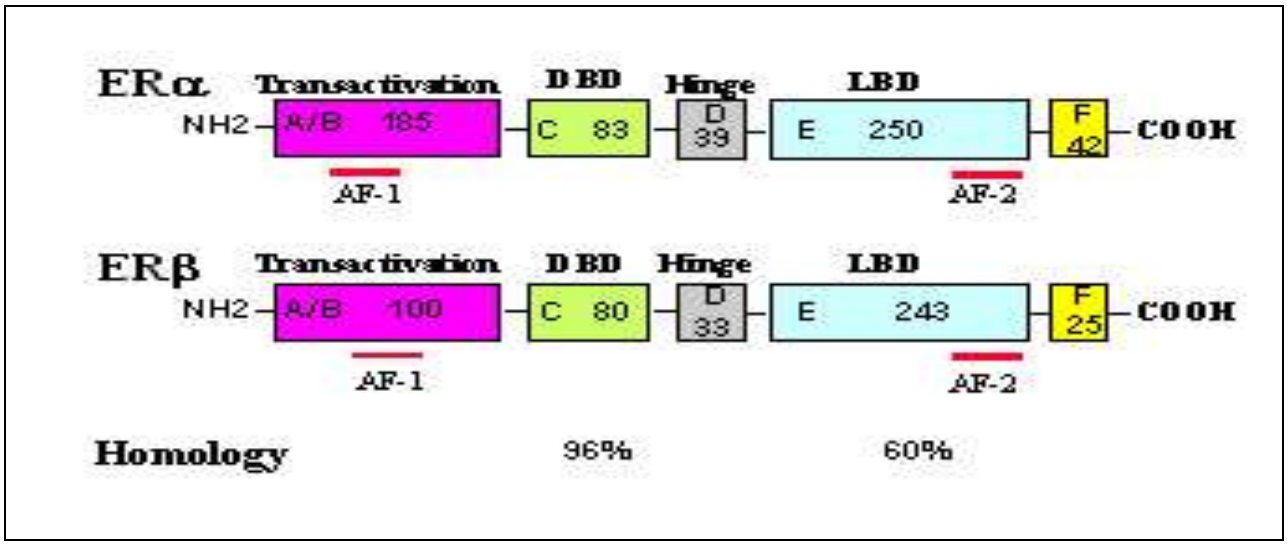

Figure 1.3. Diagrams of Human ERα and ERβ proteins. Colored boxes identify specific domains and approximate size of each domain is included. The percent homology is similar in the DBD but significantly different in the LBD. Abbreviations: DBD (DNA binding domain), LBD (ligand binding domain). (Figure from Reference 138)

ERα and ERβ have 96% amino acid identity in their DNA binding domains (domain C; DBD) but only share approximately 60% amino acid identity in their ligand-binding domains (domain E; LBD), and this difference in the ligand-binding domain could account for the differences between the responses of the two receptors to various ligands (22, 89, 93). Additionally, the hinge region and the carboxyl terminal (domain D and domain F) are poorly conserved, and significantly shorter in ERβ when compared to ERα (22). ERα and ERβ are thought to have related as well as unique downstream targets based on genomewide microarray studies, which suggests that these two receptors have distict biological functions (137). These views are further supported by differences in transcriptional acitivity in response to specific ligands, differneces in pattern expression, and the differences in phenotypes seen from ER isoform knockout mice (137). Additionally, there are some cases where ERβ, specifically ERβ2 isoform, can induced proteosomal degradation of ERα or suppress the activation of ERα responsive genes by inhibiting the recruitment of ERα to EREs (57, 63, 64, 137). These observations support the suggestion that there are functional differences between the two ER subtypes on multiple levels.

The presence of ERα is a powerful predictor of breast cancer prognosis and ERα is a standard therapeutic target in breast cancer treatment (2, 20, 46, 132). Both ERα and ERβ are reported to have cytoplasmic, membrane, mitochondrial and nuclear pools (90, 63). Membrane localized ER act through kinase cascades and other second messengers that affect transcriptional activities in the nucleus (63). One reported example is promoting G1/S cell cycle progression via E2 stimulation of cyclin D1 by ERK or PI3K/Akt activation (27, 63, 72, 90, 102, 122, 132). In addition to the extra-nuclear actions of estrogen and the ER, it was reported that some ERs

localize to the mitochondria in MCF7 breast cancer cells (18, 90). This is significant because release of cytochrome C from the mitochondrial transition pores activates caspase 9 and triggers the effector caspace cascade, which activates apoptosis (18, 90). Pedram et al. demonstrated that E2 treatment could inhibit cytochrome C release, the formation of mitochondrial reactive species, activation of JNK, translocation of Bax to the mitochondria, which decreased apoptotic cell death in MCF7 breast cancer cells (90). These findings suggest a novel function of mitochondrial ERs in tumor cell survival (90). The interaction of E2 with nuclear ERs can activate and suppress gene transcription via ER complex binding directly to EREs in the promoters of target genes; additionally, nuclear ERs can act on alternative response elements to mediate transcriptional activity (57, 63, 64). Direct and indirect actions of ERs can modulate gene and protein expression via the association of co-activators or co-repressors (63).

Roles of Estrogen Regulation of Ion Channels

Estrogen signaling is a key regulatory component in multiple tissues including brain, breast, cardiovascular system, uterus and bone (8, 9, 48, 74, 92, 101, 121, 123). In the brain, estrogen is associated with homeostatic parameters (such as intracellular Ca^{2+}, cytosolic pH and/or cell volume) the neuroendocrine feedback loop, regulation of synaptic plasticity/cognition and estrogen mediated neuroprotection (8, 9, 48, 74, 92, 101, 121, 123). Likewise, estrogen regulates homeostatic parameters and estrogen mediated protection in the cardiovascular system (29, 51, 53). Many of the actions of estrogen signaling, exerted in both of these tissues, are mediated by the classical genomic mechanism of regulation of transcription of genes and/or rapid non-genomic effects including kinase activation, modulation of ion channel activity, and cell to cell communication (8, 9, 29, 48, 51, 53, 74, 92, 101, 121, 123).

Evidence suggests that E2 can exert rapid regulation of calcium and potassium channels in neurons from different areas of the brain (101). G-coupled protein receptors and cAMP-dependant phosphorylation are involved with E2 regulation of K^+ channels in the brain (47, 50). It has been demonstrated that E2 can regulate output of gonadotropin-releasing hormone (GnRH) from the mediobasal hypothalamus thus regulating the reproductive cycle (69). In contrast, direct actions of E2 can also inhibit GnRH neuronal activity through ERs in the GnRH neurons via the negative feedback loop (13, 40, 49, 58, 69, 116). It was demonstrated using in vitro slice preparation that μ-opioid receptor-mediated inhibition of GnRH neurons arise from the activation of G protein inwardly rectifying K^+ channels (GIRK 1-4 or Kir 3.1-3.4) and the activation of these channels produces a strong hyperpolariztion (44, 58, 68, 69). E2 has been shown to play an important role in ventricular repolarization via modulation of rectifying K^+ channels (53). Additionally, there have been reports that estrogen can rapidly inhibit ion currents of slowly and rapidly activating delayed rectifier K^+ channels currents, transient outward K^+ currents and inwardly rectifying K^+ currents in the cardiovascular system (53). The exact mechanism of how E2 influences ion channel expression and function in the cardiovascular system is unknown (29, 53). These findings are significant in relation to understanding ion channel function in cancer because they demonstrate a functional correlation between estrogen signaling and ion channels activity, specifically K^+ channels, in the brain and cardiovascular system. Additionally, the functional roles of ion channels in the cardiovascular system and brain may provide some valuable information of the potential roles of ion channel involvement in cancer but these roles in cancer are unclear at present (56).

Ion Channels in Cancer

Ion channel involvement in cancer, as a whole, has not been studied in detail but accumulating data suggests ion channels are not only vital for cell survival and function but are crucial for tumor development and cancer progression (56). Membrane ion channels are essential for cell proliferation and ion channels are involved in cellular homeostatic functions including: intracellular ion concentration, cytosolic pH regulation, cell volume, and membrane voltage (56). It is thought that the changes that occur in normal cells that lead to conversion to cancer cells are the result of a series of genetic alterations that may include alteration of ion channel activity and this abnormal ion channel activity supports proliferation (56, 130). Figure 1.4 illustrates the generalized stages during the progression of normal epithelium to metastasis in colorectal cancers and the potential stages that abnormal ion channel activity may appear, which is likely similar in may other cancer types (56). Unfortunately, it is not clear at what stage of cancer that the altered channels appear or if the ion channels are expressed differentially in healthy tissue

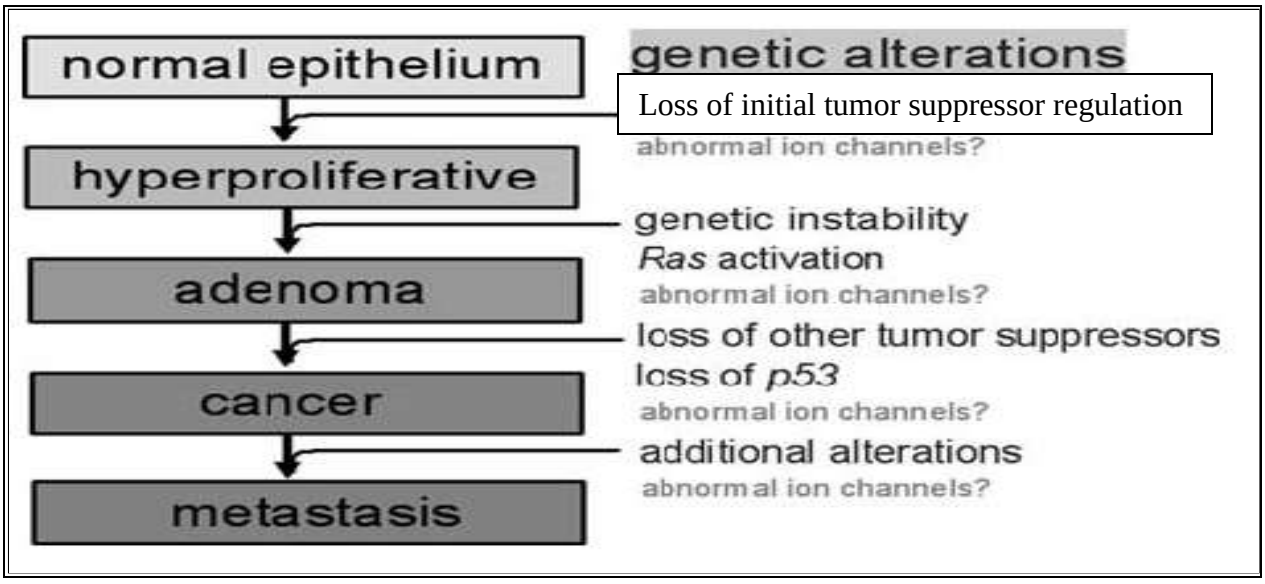

Figure 1.4 Genetic Alterations that occur in the development of cancer. The potential of abnormal ion channel activity exists at each stage in the progression of cancer. (Figure is revised from reference 56)

than in cancer (56). Accumulating evidence indicates that tumor cells contain multiple K^+ channels that are suggested to be required for proliferation of normal healthy tissue such as the over expression of GIRK1 in breast cancer (56). Normally, GIRK1 expression is primarily restricted to tissues of the CNS and cardiovascular system (118). Many of these K+ channels require very different cellular conditions to be functional and it is not clear how these channels interact with other ion channels to drive proliferation or maintain the homeostatic parameters required during cancer progression (56).

It is thought that tumor development is initiated by genomic instability that drives increased cell proliferation and decreased apoptosis (77). Evidence from multiple laboratories suggest that ion channels have a role in the development of cancer, cell proliferation and apoptosis (1,5, 6,7,10,19,24,26, 33, 34, 41, 56, 59, 83, 84, 85, 86, 94, 95, 100, 104, 118,120,126,129,130,131,136). Figure 1.5 illustrates differences seen in cells undergoing proliferation and apoptosis (56).

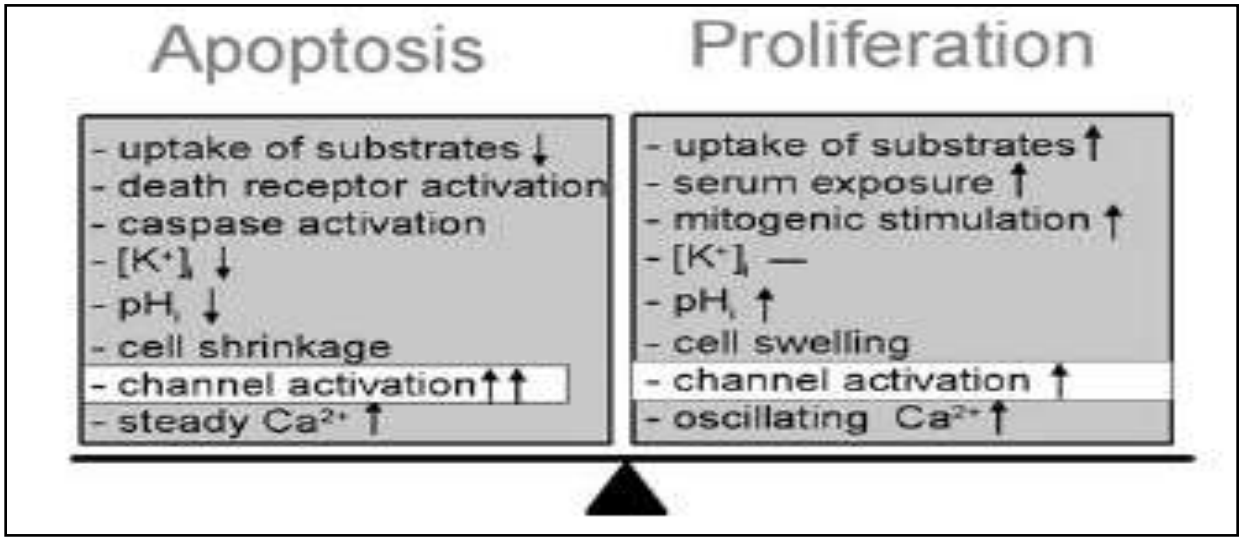

Figure 1.5. The Differences seen in cells undergoing apoptosis and proliferation. There are various factors that support the cellular environment that drives apoptosis and proliferation including intracellular K+ concentration, Ca+ concentration, and cell volume. (Figure is from reference 56)

Ether a go-go voltage gated K+ channel (EAG or hEAG) is a well studied ion channel, and is a cell cycle-regulated K+ channel normally restricted to the brain (26, 56, 83, 86, 85). It has been reported that hEAG mRNA is tightly regulated during cell cycle progression in MCF7 breast cancer cells (85). Activation of hEAG channels induces hyperpolorization of the membrane and drives progression through early G1 phase of the cell cycle (5, 84, 85). Conversely, treatment of MCF7 cells with an tetraethylammonium (TEA; a potassium selective ion channel blocker), astemizole (EAG specific inhibitor), or EAG specific siRNA reduces proliferation, increases the number of cells in early G1 phase and reduces the numbers of cells in S phase of the cell cycle (5, 84, 85). These findings demonstrate the need for EAG K^+ channels in cell cycle progression in multiple cell types including MCF7 (5, 12, 14, 56, 84). Interestingly, it has been suggested that hERG overexpression may be regulated by growth factors and/or estrogen (85). Previous studies have shown increased cell proliferation and the overexpression of several K^+ channels, including EAG K^+ channels, are correlated to growth factors such as insulin, insulin-like growth factor 1 and EGF (30, 33, 85, 106, 131).The exact mechanisms that regulate ion channel involvement in proliferation and tumor progression are not clear. Kunzelmann suggests that ion channels may affect proliferation in two different ways: 1) cellular homeostatic parameters (ie intracellular Ca^{2+} concentrations, cytosolic pH, cell volume) and inhibition of these channels will lower proliferation without direct interference of the cell cycle. 2) ion channel activity such as the ability of K+ channels to hyperpolarize membrane is required at specific points during the cell cycle suggesting a direct role in proliferation (56).

Apoptosis or programmed cell death is directly correlated to an early activation of K^+ channels and decease of intracellular K^+ (56, 126, 135). The roles ion channels play in apoptosis are summarized in figure 1.5. As a result, cells undergoing apoptosis exhibit a loss of volume

and activation of metabolic enzymes such as caspases and nucleases that drive the death signal pathway (56). Intracellular ion concentrations are dynamic during apoptosis and proliferation, but it has been suggested that intracellular K^+ concentrations play a significant role in death signaling and K^+ efflux seen in apoptosis is thought to be triggered by various K^+ channels including some located in the mitochondrial membrane (6, 7, 32, 41, 56, 104, 119, 126). Cell shrinkage is thought to affect the proliferative state of the cell because growth and mitosis require a minimal K^+ concentration (56). The roles of K^+ are closely associated with the actions of other ion channels in regulating cellular homeostatic parameters including volume regulation that support proliferation within a certain range, otherwise apoptosis is triggered (32, 56, 59, 104). It is thought that K^+ influx is linked to proliferation whereas K^+ efflux is linked to apoptosis (56).

The role of ion channels in carcinogenesis is a complex process but there is considerable evidence that indicates that the activity of K^+, Cl^- and Ca^{2+} channels in tumor cells support proliferation. Figure 1.6 illustrates the cellular effects of ion channels in a normal proliferating cell and the potential contributions these ion channels play in tumor development, and cancer progression (56). Mitogenic signaling through downstream kinase cascades are capable of inducing cell survival, growth and differentiation, as well as cellular metabolic changes. K^+ channels have been shown to respond to mitogenic stimulation in various tissues including the CNS and cardiovascular system (13, 29, 30, 33, 40, 44, 47, 49, 50, 53, 56, 58, 68, 69, 101, 106, 116, 126, 131). Mitogenic stimulation of resting cells in the G_0 phase of the cell cycle activates K^+ channels, which push the cells into G_1 phase or initiate proliferation (56). It has been shown that mitogenic stimulation of voltage gated K^+ channels are required for progression of cell cycle in ML-1 acute myeloid leukemia cells and rabbit corneal epithelial cells (33, 56, 106). It is

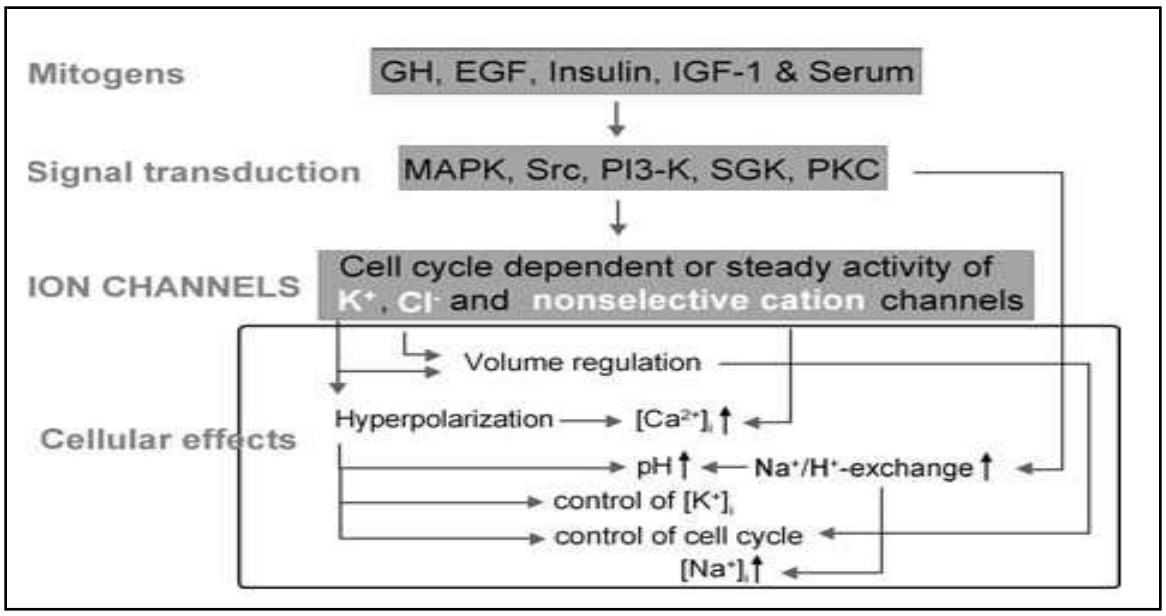

Figure 1.6. Cellular effects of ion channels in a proliferating cell. Mitogens can trigger signal transduction pathways via kinase signaling that induce ion channels and the Na^+/ H^+ exchanger. Ion channel activation initiates various cellular effects as shown. (Figure is from reference 56)

thought that enhanced K^+ conductance can trigger K^+ efflux that results in membrane hyperpolarization, which promotes Ca+ influx and swelling of the cell (56). During the cell cycle progression, cell volume is dynamic because of the accumulation of substrates, synthesized proteins, DNA doubling, cell swelling, mitosis and cell migration (56). Kunzelmann suggests that there is early involvement of K^+ channels in mutagenic signaling events and that activation of K^+ channels can facilitate transmembrane signaling though multiple pathways including ERK and Akt pathways (56). There is a clear correlation between membrane voltage and proliferation. It has been shown that mitogenic stimulation induces a short hyperpolarization peak at early G_1 followed by depolarization (56). Additionally, apoptosis-inhibiting protein (Mcl-1) has been shown to induce membrane hyperpolarization or outward current by activating K^+ channels and Mcl-1 assumes an regulatory role in dynamic cellular conditions (56, 125).

These findings suggest proper membrane voltage during cell cycling is critical and can be regulated via K^+ channels. Membrane voltages are also associated with the transport of substrates across the membrane, regulating cytosolic Ca^{2+} levels, and cytosolic K^+ levels, which directly affect the cytosolic pH (56, 129, 130). Additionally, K^+ channels are thought to have a role in cell migration and metastasis; evidence has shown that during intestinal wound healing there are increased intracellular Ca^{2+} and activation of voltage gated K^+channels (25, 56, 100, 110, 111).

G-Protein Coupled Inwardly Rectifying Potassium Channels (GIRKs) and Cancer

G-Protein coupled inwardly Rectifying Potassium channels (GIRKs) are members of a superfamily of inward rectifying potassium channels, which contains four subunits that are designated GIRK 1-4 or K^+ inward rectifying channels (Kir)3.1-3.4 (71). GIRK1 protein is coded for by the gene KCNJ3 on 2q24.1, GIRK 2 protein by the gene KCNJ6 on 21q22.1, GIRK 3 protein by KCNJ9 on 1q21 and GIRK 4 protein by KCNJ5 on 11q24 (78). GIRKs are found in many tissues including neurons, endothelial cells, and both skeletal and cardiac muscle tissue but are primarily found in abundance in the CNS and cardiovascular system (52). The main role of GIRKs are to initiate inward passage of K^+ ions into the cell at membrane potentials below K^+ equilibrium potential(E_K) but outward conductance is limited to potentials above E_K (52, 55). Therefore the primary role of these channels is to maintain membrane potential, cell volume, regulation of cellular excitability, and intracellular K^+ homeostasis; suggesting that these channels may have a role in cell proliferation (52, 55, 103). All four of the channels have been identified in humans and other mammals (21, 52, 54, 55, 62, 103). Successful cloning of GIRK channels provided important information regarding distribution, function and structure (52). The main sources of these K^+ channels are found in the heart and brain (21, 52, 54, 55, 62, 103).

In cardiovascular tissue, GIRK channels are triggered by M_2 muscarinic and A_1 adenosine receptors that are coupled to pertussis toxin (PTX)-sensitive G proteins, and the $G_{i/o}$ protein family. In the heart, GIRK channels are composed of heteromultimers composed of GIRK1 and GIRK4 (52, 54, 133). In the CNS, GIRK channels are triggered by multiple $G_{i/o}$ protein-coupled receptors and neuronal GIRK channels are predominately composed of GIRK1 and 2 subunits (11, 42, 43, 52, 62, 66, 73, 79, 114, 127). GIRK1 cannot form homodimers and as a result requires other subunits to form a functional channel but GIRK2 and GIRK4 are capable of forming homodimers (11, 42, 43, 52, 54, 62, 66, 73, 79, 114, 127, 133). The GIRK3 subunit is thought to play a regulatory role by inhibiting membrane expression of GIRK1 by inducing lysosomal degradation of these channels but this action is poorly understood at present(52). The primary structure of GIRK subunits is analogous, two transmembrane domains, a pore forming region, cytoplasmic amino terminal end and carboxyl terminal end (37, 52, 103). GIRKs are of clinical significance specifically in pain control and several neurological disorders (3, 52, 75, 76, 105, 115). Additionally, GIRKs have a suggested role in several other pathologies including cardiac arrhythmias, epilepsy, ischemia, diabetes mellitus, and cancer (39, 52, 61, 70, 85, 94, 97, 107, 113, 115, 118, 134).

Stinger et al. measured the expression of GIRK1 mRNA isolated from benign breast tumors, primary invasive breast carcinomas and metastatic breast carcinomas from auxiliary lymph nodes using reverse transcription polymerase chain reaction (PCR), and correlated these data with clinical samples (118). They compared lymph node metastasis, tumor size, tumor grade, and estrogen receptor expression with the occurrence of GIRK1 overexpression; only the presence of lymph node metastasis strongly correlated with GIRK1 overexpression (118). Additionally, overexpression was greatest in tumors isolated from patients with more than one

metastatic lymph node (118). Additionally, Takanami et al. demonstrated that 69% of 72 non-small cell lung cancer (NSCLC) biopsies expressed high levels of GIRK1 expression and this overexpression was associated with lymph node metastasis and tumor stage (120). Their results signify that GIRK1 may have potential uses as a biomarker for clinically aggressive cancers, including breast and NSCLC, with lymph node metastasis and may have potential as a therapeutic target (118, 120).

Overexpression of voltage-gated potassium channel 1.3 (Kv1.3) has been shown in carcinoma tissue via immunohistochemistry (1). Specific K^+ channels, such as Kv1.1 and Kv1.3, have been associated with regulation of apoptosis; Kv1.3 has been shown to have a role in restoring sensitivity of actinomycin D-triggered apoptosis after being transfected into apoptosis-resistant Kv1.3 deficient CTLL-2 T lymphocytes (4, 10, 126, 136). Brevet et al. showed significant GIRK1 overexpression in malignant tissue, when compared to normal breast tissue, with an apical cytoplasmic localization (10, 85). Apoptotic associated K^+ channels, Kv1.1 and Kv1.3, were less expressed in malignant tissue that overexpressed GIRK1, possibly because of their regulatory role in apoptosis (10, 85). These data may suggest that GIRK1 is potentially correlated to the inhibition of apoptosis as well has supporting a pro-tumorgenic environment.

Previous work from our laboratory identified GIRK(1-4) mRNA expression in both NSCLC and small cell lung cancer (SCLC) (94). Additionally, GIRK1 protein expression was identified in three SCLC cell lines (94). We hypothesize that GIRK channels may be functional based on the presence of mRNA expression for GIRK2 and 4 in these cell lines, which would be required to form functional channels because GIRK1 can not form homodimers (94, 71). This work also identified a potential correlation between GIRK expression and β-adrenergic signaling (94). This report concluded that GIRK1 and/or GIRK2 channels may be associated with β-

adrenergic signaling and these actions could be involved in lung and breast cancer progression (94, 95). Additionally, Plummer et al. showed that multiple breast cancer cell lines express GIRK1, GIRK2 and GIRK4 mRNA and proteins, which suggested that the channels were functional (95). They found decreases in β_2-adrenergic receptor mRNA and increased GIRK1 expression using real time PCR after treatment with 1μM propanolol , a β-adrenergic antagonist (95). Treatment with formoterol hemifumarate, a β-adrenergic agonist, led to increased K^+ flux that was suppressed by treatment with clozopine, a GIRK channel inhibitor (95). Treating breast cancer cells with 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK), a high affinity β-adrenergic agonist, increased phosphorylation of Erk1/2 (95). These results suggest a correlation between β-adrenergic signaling and GIRKs, and their potential involvement in breast cancer (24, 95).

The use of charcoal stripped media and serum free media in the culture of multiple breast cancer cell lines has been reported to lead to decreases in GIRK1 expression, which was rescued by the use of serum free media treated with E2 suggesting that GIRKs could be responsive to E2 in breast cancer (24, 34). Additional work from our laboratory, used GIRK1 siRNA mediated knockdown to investigate signaling pathways associated with the action of GIRK channels (34). GIRK1 knockdown lead to significant decreases in β-adrenergic signaling, MAP kinase signaling, and PI3K/Akt signaling in MDA-MB453 breast cancer cells. All three of these pathways have been previously suggested to be estrogen responsive and significant in breast cancer (132). These data contribute further support to the involvement of GIRK action in cancer progression (34).

Hypothesis and overview of results

The role of ion channel involvement in tumor development and cancer progression requires more extensive investigation but increasing evidence has demonstrated that these channels are essential for cell proliferation and likely have a significant role in cancer progression. It has been suggested that these channels may have clinical value as biomarkers and as therapeutic targets. E2 is a key regulatory growth factor that has very diverse roles and extensive data has established E2 as a major risk factor in breast cancer development and progression through classical genomic and non-genomic actions. E2 signaling can exert actions on K^+ channels in the CNS, cardiovascular system, and multiple other tissues. The above reviewed literature demonstrates that ion channels, especially K^+ channels, are essential for cell proliferation and suggests an essential role for ion channels in cancer progression. Multiple studies have outlined mitogens, including estrogen, involvement in cancer progression, the subsequent activation of ion channels and ion channel involvement in cancer progression. Data from our lab demonstrates rescued GIRK1 expression in MDA-MB453 ER(-) breast cancer cells in E2 supplemented charcoal striped media compared to media in serum free or charcoal striped media alone. Therefore, it is logical to hypothesize that the direct and indirect actions of E2 may modulate GIRK expression and function in breast cancer cells. The goal of the studies discribed here are to investigate the role E2 and ERα may play on GIRK expression in ER(+) MCF-7 breast cancer cells. Additionally, the data discribed will suggest that targeting therapeutics toward GIRK1 could contribute to clinical therapies and generating GIRK specific inhibitors may be valuable to overcoming shortcomings in current clinical approaches.

We investigated the effects of estrogen and ICI 182780 (an ER alpha specific antagonist) on GIRK membrane protein expression in ER alpha (+) MCF7 to determine the role estrogen

plays in GIRK expression in ER (+) breast cancer cells (chapter 2). Our data suggests, that E2 treatment appears to increase GIRK1 and GIRK2 protein expression Additionally, we found increased protein phosphorylation of ER mediated signaling pathways including members of Akt, GPCR, and MAPK signaling pathways. We found that cell proliferation was increased in cells treated with E2 and decreased in ICI treated cells. It is not currently known which GIRK subunits are correlated with GIRK1 expression/function in cancer and this is significant because GIRK1 requires other subunits to form functional channels. We used immuno-fluorescent microscopy to evaluate GIRK1 and GIRK2 protein localization in response to treatment with E2. We found that E2 treatment resulted in membrane and cytoplasmic localization of GIRK1 and GIRK2 subunits. These findings suggest that GIRK1 and GIRK2 subunits may be responsive to E2 treatment. Increased GIRK1 and GIRK2 membrane protein expression was also correlated with increased cell proliferation, membrane localization, and intracellular potassium concentrations. Taken together, specific GIRK channel compositions may be responsive to E2 and play a role in breast cancer progression.

We transfected MCF7 breast cancer cells with a GIRK1 siRNA to determine if decreased GIRK1 mRNA had an effect on ER α (chapter 3). We report that ER α protein and gene expression is decreased with knocked down GIRK1 gene expression in MCF7 breast cancer cells after 24 hrs. In addition, knock down of GIRK1 in MCF7 cells demonstrated decreases in phosphorylation of intermediates in G-protein couple receptor signaling, MAPK signaling, and Akt signaling pathways. Our lab has previously identified these pathways to be associated with GIRK1 expression and they are known to be associated with E2-ER signaling (27,34,72,90 132). These findings provide further support that GIRKs may play a role in cancer and could have potential as a theraputic target.

Kunzelmann states that membrane bound potassium channels play a direct role in cell proliferation but there is currently no clear understanding how these channels promote proliferation (56). We used microarray analysis of 24 hour non-treated and E2 treated MCF7 cells to identify possible candidate genes that may be associated with GIRK expression, in response to E2 treatment (chapter 4). We identified four differntially up-regulated cell cycle assciated genes in E2 MCF7 cells, which were also decreased by GIRK1 knock down in MCF7 cells. This data will provide useful information about the poorly understood functions of GIRK expression and function in cancer, and the potential uses of ion channels as specific therapeutics targets.

Based on our above findings , we hypothesize the role of GIRKs in MCF7 breast cancer cells in figure 1.7. GIRK channels are suggested to play a significant role in regulating membrane charge and in the regulation of cell volume (42, 43, 44, 47, 48, 49, 50, 52, 54, 55, 56, 58, 66, 70, 75, 115) The activation of GIRKs occurs by various GPCRs including (opioid, α and β adrenergic receptors etc) and various neurotransmitters, and hormones (1, 3, 11, 21, 24, 29, 42, 43, 44, 47, 48, 49, 50, 52, 54, 55, 56, 58, 66, 70, 75, 115). We suggest that E2 increases GIRK membrane expression and may have a role in stimulating the translocation of GIRK channel proteins to the cell surface. Unfortunately, it is not clear at present if E2 mediated increases in GIRK1 and GIRK2 is directly or through second messenger effects on signal transduction pathways. The second messenger effects of E2 on multiple pathways including GPCR signaling are well established in primary tissues and many cells lines (15, 16, 27, 28, 45, 46, 47, 48, 49, 50, 58, 63, 64, 65, 72,74, 89, 90, 101, 112, 128, 132). We hypothesize that the increased GIRK1 and GIRK2 expression contributes to increases in intracellular potassium concentrations that may promote increased cellular proliferation via positive regulation at specific cell cycle checkpoints

and promote cancer progression. GIRK1 knock down resulted in decreased ERα mRNA and protein expression. Additionally, GIRK1 knock down resulted in reduced phosphorylation of specific E2 responsive signaling pathways and reduced mRNA levels of cell cycle regulatory genes. These studies further our current understanding of the biology of GIRKs in cancer and suggest that GIRKs may have potential as a therapeutic target, but more intense investigation is require to fully elucidate the complex functions of GIRKs in breast cancer.

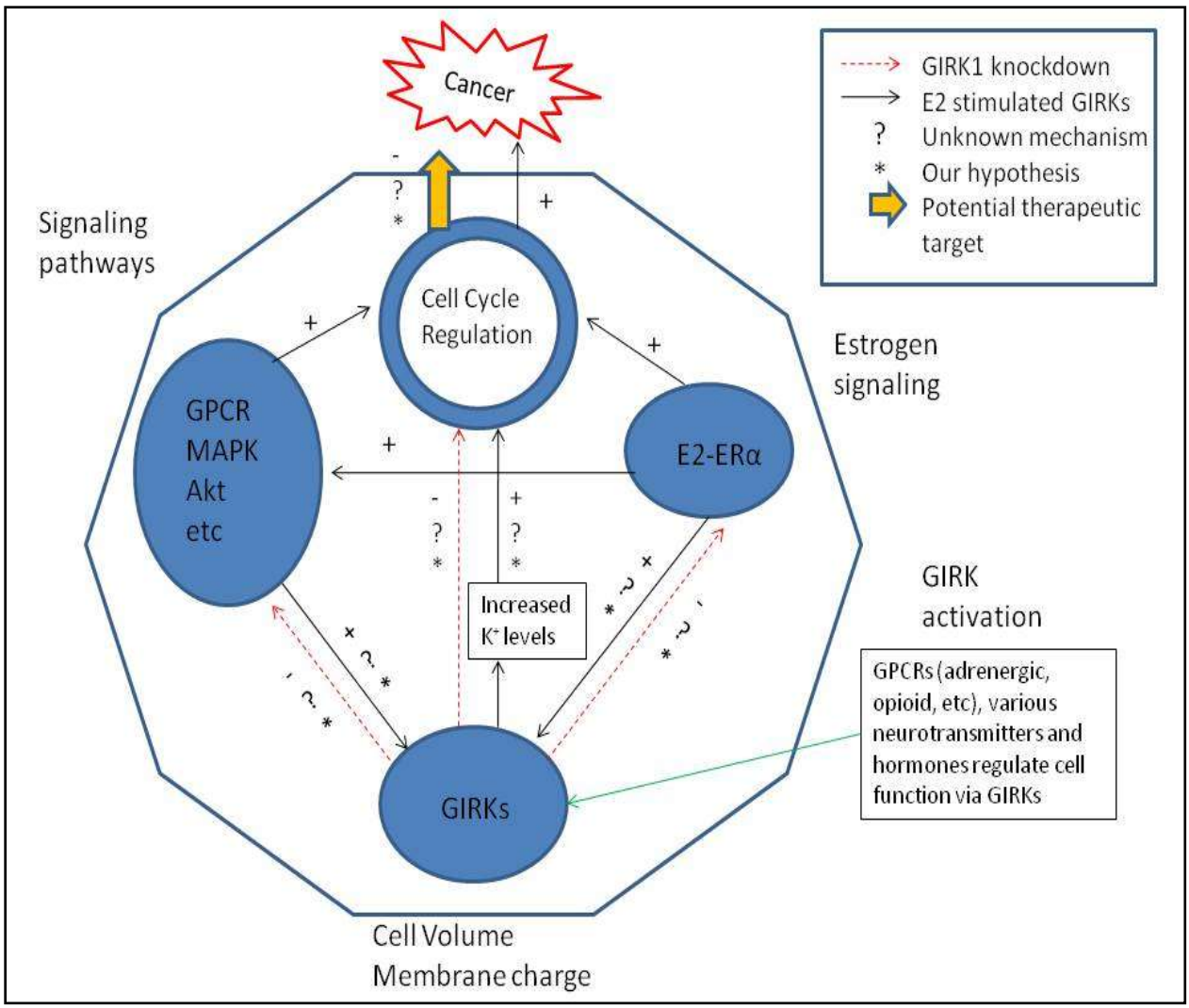

Figure 1.7. Potential mechanisms of GIRKs in MCF7 breast cancer cells.

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17β-Estradiol Functionally Activates G Protein Inwardly Rectifying Potassium Channels in Estrogen Responsive MCF7 Breast Cancer Cells

Chapter 2

Abstract

In 2009, the American Cancer Society estimated that over 192,000 new cases of breast cancer will be diagnosed and over 40,000 women will die from breast cancer, which makes breast cancer the second leading cause of cancer death. Estrogen (E2) is required for normal female development and reproduction but long-term exposure is carcinogenic and considered a risk factor for breast cancer. E2 acts as a mitogen and has been shown to modulate the action of various ion channels throughout the body. Accumulating evidence has shown that many of these ion channels are essential for cell proliferation and cell survival, which are key events in cancer progression. In the present study, we investigate the effects of E2 and the anti-estrogen compound, ICI182780 (ICI), exposure on G-protein inwardly rectifying potassium channels (GIRKs) in estrogen responsive MCF7 breast cancer cells. We found a noticeable increase in GIRK1 and GIRK2 protein levels in response to E2 treatment. Additionally, we saw increases in intracellular potassium concentrations and increased cellular proliferation in response to E2 treatment. ICI treatment also resulted in initial decreases in intracellular potassium concentration and decreased cell proliferation. Previous research has shown that GIRK channel composition may have a distinct role in the specific function of these channels in native tissue. GIRK1 and GIRK2 specific channels are thought to play a major role in rapid channel activation. E2 treatment resulted in increased phosphorylation of specific members of GPCR and MAPK signaling pathways, which previously published results from our lab have been shown to be responsive to GIRK1 knockdown. Our data suggests that GIRK1/2 specific channels are stimulated by estrogen exposure and may play a role in cell proliferation in MCF7 breast cancer cells.

Introduction

Breast cancer is one of the most common cancers in women and is reported as the second highest cause of cancer death after lung cancer (1). In addition, men are at risk in rare cases (1). Multiple risk factors contribute to the onset of breast cancer such as lifestyle (including smoking, drinking, and diet), family history, age, race, geographical location, and estrogen exposure (including age of menarche, age of parturition, and age of menopause) (1). The American Cancer Society (ACS) approximates that 192,000 women will be diagnosed with breast cancer in 2009 and estimates 40,000 women will die from breast cancer in 2009 (1). It is thought that one million new cases of breast cancer are diagnosed each year worldwide and there is a higher incidence in western countries (3). It has been suggested that the US and Europe account for 16% of the world population and approximately 60% of the worldwide incidence of breast cancer (41, 42). Today, breast cancer accounts for 22% of all female cancers (41). ACS suggests that approximately 13% of women will develop invasive breast cancer during their lives and approximately 3% of those cases will be fatal (1).

Stringer et al. suggests that roughly 40% of human primary cancer tissues express G-protein inwardly rectifying potassium channel 1 (GIRK1) mRNA and the expression of GIRK1 mRNA is significantly correlated to lymph node metastasis, and as a result tumors that express GIRK1 tend to have a poor clinical prognosis (51). GIRK expression is normally restricted to the heart and brain (51). Previous data from our lab has demonstrated that a functional link exists between GIRK1 expression and β_2 adrenergic receptor (ADRB2) function (6, 44). ADRB2 is part of the G-protein couple receptor (GPCR) family (7, 46). Other investigators have shown that ovarian cancer invasiveness can be mediated through ADRB2 activation of cell cyclic AMP (cAMP) and protein kinase A (PKA) signaling pathway, which stimulates angiogenesis and

tumor growth. (54, 61). Our lab has demonstrated that estrogen receptor positive (ER (+)) cell lines MCF7, MDA-MB-361, and ZR-75-1 and the ER negative (–) cell line MDA-MB-453 expressed mRNA for the GIRK1 channel while the ER (–) cell lines MDA-MB-468 and MDA-MB-435S did not express GIRK1 (44). Our lab also demonstrated GIRK 1, 2 and 4 protein expression in multiple breast cancer cell types, which was the first report of GIRK protein expression in multiple breast cancer cell lines (10). Data from multiple laboratories including our own suggest that GIRK is associated with proliferation in multiple cancers, and as a result may be a valuable therapeutic target (4, 6, 7, 10, 19, 44, 46, 51). These collective data indicate that GIRK1 is a potential biomarker of clinically aggressive breast cancer and may have therapeutic potential.

Estrogen (E2) is a well documented risk factor associated with breast cancer and is considered a carcinogen (61). The role of E2 in carcinogenesis is unclear, but it is thought to be the consequence of two complimentary pathways, specifically E2 metabolism and ER signaling (61). Yager et al. states that oxidative metabolites of estrogen have genotoxic, mutagenic, transforming, and carcinogenic effects on cells, suggesting that E2 may initiate and/or contribute to the progression of carcinogenesis (61). Previous studies have shown that estrogen metabolites can induce genetic mutations in MCF7 breast cancer cells and initiate neoplastic transformation of non-turmorgenic MCF-10F breast cells treated with E2, providing evidence of the mutagenic properties of E2 and E2 associated metabolites (47,48). Carcinogenic actions of E2 modulate signal transduction pathways that are correlated with proliferation, inhibition of apoptosis, cell migration, and induction of angiogenic factors. E2 is also able to mediate carcinogenic action through non-genomic effects (mitogenic actions) through the estrogen receptor (ER) (11, 17, 29, 35, 43, 45, 47, 49, 57, 61). The presence of ERα is used as a predictor of breast cancer prognosis

and therapeutic targeting of ERα is a standard strategy in current treatment protocols (1, 9, 18, 61).

Previous research has indicated that estrogen may have effects on potassium channels. Specifically, the activation of multiple potassium channels, including GIRKs, are responsive to the effects of E2 in the brain and heart (13, 22, 23, 25, 37, 56). E2 has been shown to induce rapid depolarization of inward rectifying potassium channels in hypothalamic neurons as part of the regulation of the female reproductive cycle (25). Additionally, Coiret et al 2005 demonstrated that E2 can activate maxi-K potassium channels in breast cancer cells, which was associated with increased cell proliferation (8). Evidence from our lab has demonstrated that GIRK protein and gene expression decreased in cells cultured in estrogen stripped media, possibly due to lack of estrogen in the media (10). Taken together, these studies suggest that GIRK channels in human breast cancer cells may be responsive to E2.

The primary focus of this study was to investigate the effects of E2 and the anti-estrogen compound ICI182780 on GIRK protein expression and function in ER(+) MCF7 breast cancer cells. Using a 5-Bromo-2' deoxy-uridine (BrdU) assay, we evaluated cell proliferation in response to these treatments. Additionally, we examined phosphorylation of GPCR, MAPK, and Akt signaling pathways in response to these treatments. Our lab has previously shown that GIRK1 knockdown in the ER (-) MDA-MB453 breast cancer cell line, resulted in decreased protein phosphorylation in these pathways (14). The GPCR, MAPK, and Akt signaling pathways have been shown to be responsive to the second messenger effects of E2-ERα signaling and possibly other non genomic actions associated with E2 signaling (61). Our results demonstrate that GIRK1 and GIRK2 membrane protein levels dramatically increase in response to E2 treatment. Interestingly, we found that ICI treatment increases membrane protein expression of

GIRK1, GIRK2 and GIRK4 subunits, which may the result of secondary effects of ICI. Our results suggest that GIRK expression appears to correlate with ERα function in MCF7 cells, which links GIRK function to a major breast cancer risk factor and suggests that GIRK may have relevance as a therapeutic target in breast cancer.

Materials and Methods

Cell Culture

The human estrogen responsive MCF7 breast cancer cell line was purchased from American Type Culture Collection (ATCC; Rockville, MD, USA) and was maintained RPMI 1640 media supplemented with fetal bovine serum (10% v/v), L-glutamine (2mM), 100U/ml penicillin and 100ug/ml streptomycin at 37 $^{\circ}$C in an environment of 5% CO_2. Cells were treated with 10nM 17β-estradiol (E2) (Sigma, St. Louis, MO) that was dissolved in 100% ethanol (AAPER Shelbyville, KY) and diluted with water, and the E2 was added to charcoal stripped serum enriched media. Cells treated with 100nM ICI182780 (Tocris, Ellisville, MO) that was dissolved in 100% ethanol and diluted with water, which was added to normal media and serum. The final concentrations of ethanol in the media were 0.001% for estradiol treatment and 0.005% for ICI182780 treatment. Both estradiol and ICI182780 were replaced daily for long-term experiments. Controls were treated with vehicle and appropriate media.

Assays

The PepTag Non-Radioactive Protein Kinase Assay kit was used to measure protein kinase A activity in total cellular lysates as per instructions of the manufacturer (Promega, Madison, WI). Briefly, the use of fluorescent peptide substrates highly specific for PKA (PepTag® A1 Peptide-LRRASLG) results in phosphorylation of the peptide which alters the net

charge from +1 to –1. The phosphorylated and nonphosphorylated versions of the substrate can then be rapidly separated on an agarose gel at neutral pH. The assay was performed at least twice with a representative blot used in the figure.

Cell proliferation was measured using a 5-Bromo-2' deoxy-uridine (BrdU) assay (Roche, Indianapolis, IN) according to manufacturer's specifications. Cells were treated in time course (24, 48, and 72 hour) with estradiol, ICI182780 and vehicle in replicates of 5.

The potassium flow assay was revised for our cells from the protocol by Andersson et al (2). The 96 well plates (Costar 96 well white clear bottom plates, Fisher Scientific, Pittsburgh, PA) were loaded with 15,000 cells per well 24 hours prior to an experiment, then the cells were treated from 24-72 hours, in replicates of 6. After treatment, cells were rinsed with serum-free RPMI media and then exposed to fresh serum-free RPMI for 45 min. PBFI potassium specific florescent dye (Molecular Probes/Invitrogen, Carslbad, CA) was prepared by adding 65µl of DMSO to 50 µg dye, then this solution was diluted to 4ml with PBS and 35µl of pluronic F-127 (Invitrogen) was added. Of this dye solution, 50 µl (610 ng) was added to each well for 4 hours after removal of the serum-free media. After the dye treatment, each well was washed with 200 µl 0.9% NaCl, 3 times and then 200 µl 0.9% NaCl was added for the analysis step. As in the original protocol, excitation ratios of 344 nM and 400 nM measured at emission of 500 nM were used to estimate intracellular content of potassium. This was analyzed using a BioTek FLx800 florescent microplate reader (BioTek, Winooski, VT).

Analysis of Protein Expression by Western Blots

Cells were washed twice with phosphate buffered saline and lysed with cold RIPA lysis buffer containing Halt protease inhibitor (Thermo Scientific Pierce Protein Research Products, Rockford, IL) (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 1% Triton × 100, 0.1% SDS, 1%

sodium deoxycholate, 1 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 0.5 mM DTT). Cell lysates were collected from culture plates using a cell scraper, and protein collected by centrifugation. Protein concentrations determined by BCA protein assay (Pierce, Rockford, IL). Additional cell pellets were collected and membrane protein was isolated with the ReadyPrep protein extraction kit (signal) (Biorad, Hercules, CA). Protein levels of membrane proteins were determined using the RCDC kit (Biorad). Aliquots of 20 μg protein were boiled in 2x loading buffer (0.1 M Tris-Cl, pH 6.8, 4% SDS, 0.2% Bromophenyl blue, 20% glycerol) for 4 minutes, then resolved by electrophoresis on 12% SDS-PAGE, transferred to nitrocellulose membranes, blocked for 1 hour in 1% (W/V) non-fat dry milk, and immunoblotted overnight with the specific primary antibodies. Primary antibodies were detected with horseradish peroxidase-coupled secondary antibody and enhanced chemiluminescence (Pierce, Rockford, IL). Antibodies for GIRK1, GIRK2 (Lifespan Biosciences, Seattle, WA), GIRK4 (Sigma, St. Louis, MO, USA), phospho-β2 (Ser 355/Ser 356) (Santa Cruz, Santa Cruz, CA), ERα (Upstate, Temecula, CA), GAPDH, phospho-CREB (Ser133), phospho-Akt (Ser473), phospho-c-Jun (Ser63), phospho-ERα (Ser104/106), phospho-ERK (Thr202/Tyr204) (Cell Signaling, Danvers, MA) were used in these studies. Membranes were probed with an antibody for GAPDH (Cell Signaling) to ensure equal loading of protein between samples. GAPDH has been previously used as a loading control (10, 14, 44). Actin was not used because GIRKs may affect actin (16, 38). Protein bands on the immunoblots were measured using Scion Image software, release Alpha 4.0.3.2 (http://www.scioncorp.com) using three separate measurements of each band on the gel, and each western blot was done at least twice with representative blot used in the figure (10, 14, 44). The protein bands were standardized by GAPDH loading control and converted to a percent based on the control.

Immunofluorescence Microscopy

Approximately 50,000 cells were seeded onto a sterile non coated 13mm round cover slip (Fisher Scientific, Pittsburg, PA) in a sterile 24 well plate (BD Biosciences, San Jose, CA) with antibiotic free RPMI 1640 media supplemented with fetal bovine serum (10% v/v) and grown at at 37 °C in an environment of 5% CO_2. On the second day, cells were treated with 10nM 17β-estradiol. Cells were fixed by 4% paraformaldehyde and subsequently incubated with primary antibodies over night at 4 °C. Fluorescently labeled secondary antibodies were used for detection (Molecular Probes). DAPI (Vector Labs, Burlingame, CA) staining was used to visualize the nucleus. Fluorescent images were taken at a magnification of 40x on a Nikon Eclipse 80I with an attached Nikon digital camera (Nikon Instruments Inc., Melville, NY). For epi-fluorescence of the fluorophors (FITC and TRITC) excitation an argon ion laser with emission at 488 and 547 nm was used and data was collected from 501-587nm.

Statistics

Graphs and statistics created using Microsoft Excel 2003 (Microsoft Corporation, NorthAmerica). ANOVA was employed to determine whether significant differences ($p<0.05$) were present among treatment groups. Significant differences in means of parameters between experimental groups were further analyzed by one sided *t* test to compare means ($p<0.05$).

Results

G-Protein Inwardly Rectifying Potassium Channel Membrane Protein Expression

As in previous research, we only determined GIRK1, GIRK2 and GIRK4 membrane protein expression (10, 14, 44) since the predominant GIRK heterotetramers are GIRK1/GIRK2 or GIRK1/GIRK4 (23). After treatment of MCF7 breast cancer cells with estrogen, GIRK1,

GIRK2 and GIRK4 protein expression increased at 24 and 48 hours followed by a decrease at 72 hours (figure 2.1). The protein expression for GIRK1 and GIRK2 showed large increases compared to GIRK4. After treatment of MCF7 breast cancer cells with ICI182780, GIRK1 expression was increased at 24 and 48 hours followed by a decreased at 72 hours (figure 2.2). GIRK2 and GIRK 4 were increased at all time points (figure 2.2). The increases in GIRK1 after ICI treatment were much less dramatic than the changes seen after E2 treatment (figures 2.1 and 2.2).

Potassium Flow

Intracellular potassium concentrations were measured after E2 and ICI treatment of MCF7 breast cancer cells after 24 hours, 48 hours and 72 hours. At 24 hours, potassium flow induced by ICI treatment is reduced and potassium flow induced by estrogen treatment is increased (figure 2.3). At 48 hours and 72 hours, ICI potassium concentration returned to control levels while potassium flow induced by estrogen treatment is significantly increased (figure 2.3).

Cell Proliferation

Using a 5-Bromo-2' deoxy-uridine (BrdU) assay, proliferation was measured after estrogen (10 nM) or 100 nM ICI treatment. Treatment with ICI significantly decreased cell proliferation at all times (24, 48, and 72 hours) (figure2.4). Conversely, estrogen treatment significantly increased cell proliferation at all time points (24, 48, and 72 hours) (figure2.4).

Estrogen Receptor α Expression and Phosphorylation

ERα expression is increased at all time points after E2 treatment and ERα phosphorylation was increased at 24 hours and 48 hours but decreased at 72 hours(figure 2.5). Similar to the effects on cell proliferation, we see opposite effects of 100nM ICI on ERα. ERα

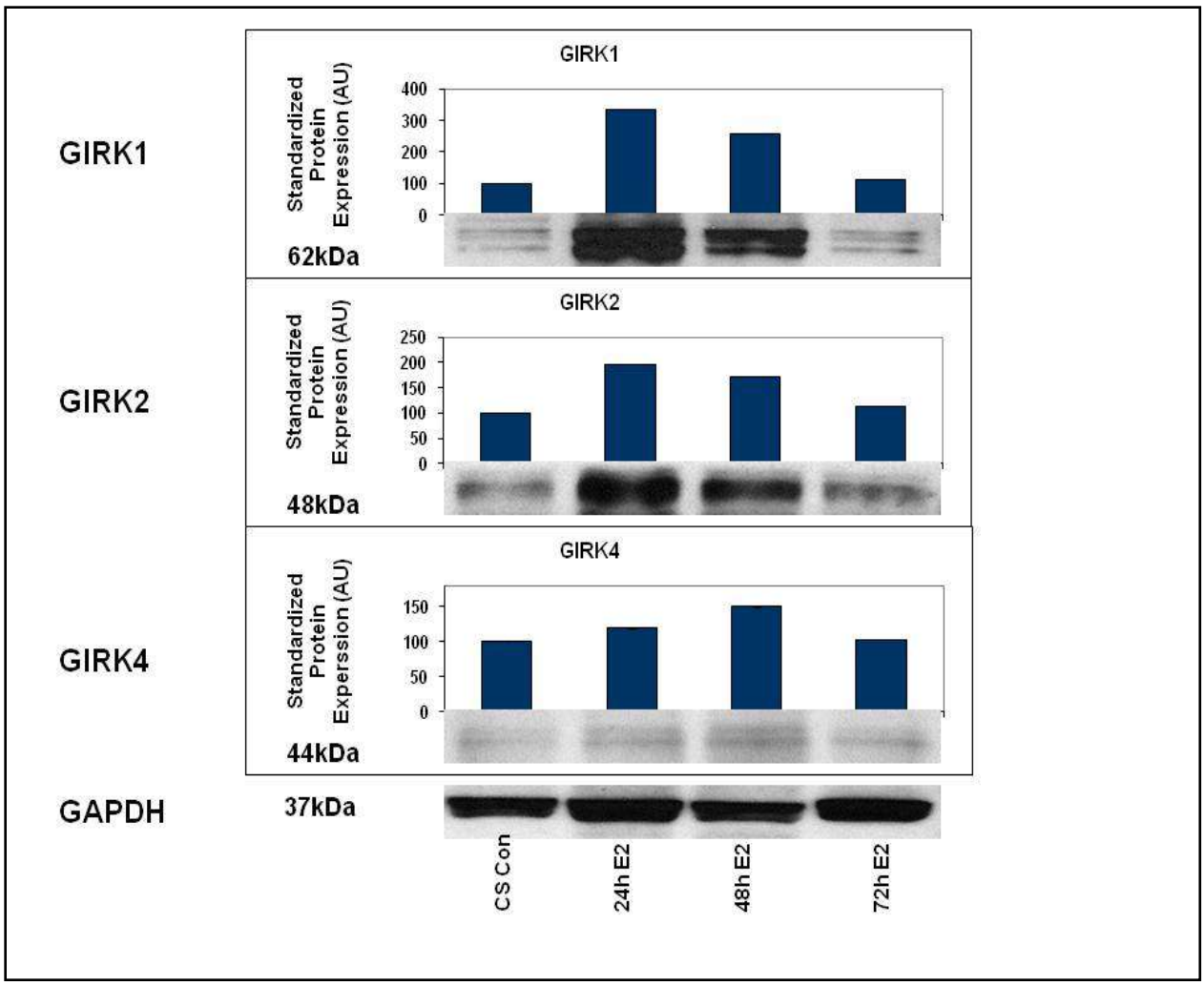

Figure 2.1 Increased GIRK membrane protein expression after 10nM estrogen treatment. The bands are consistent with the expected size: GIRK1 (62 kDa); GIRK2 (48 kDa); GIRK4 (44 kDa); GAPDH (37 kDa). These are gels representative of two separate experiments. Graphs indicate densitometry of the Western blots. The densitometry of GIRK was divided by the densitometry of the control GAPDH (N=1). Each western blot was done at least twice with representative blot used in the figure. AU represents (arbitrary units), CS Con represents (charcoal stripped media control) , 24hE2 represents (24 hour treatment with estrogen), 48hE2 represents (48 hour treatment with estrogen), and 72hE2 represents (72 hour treatment with estrogen).

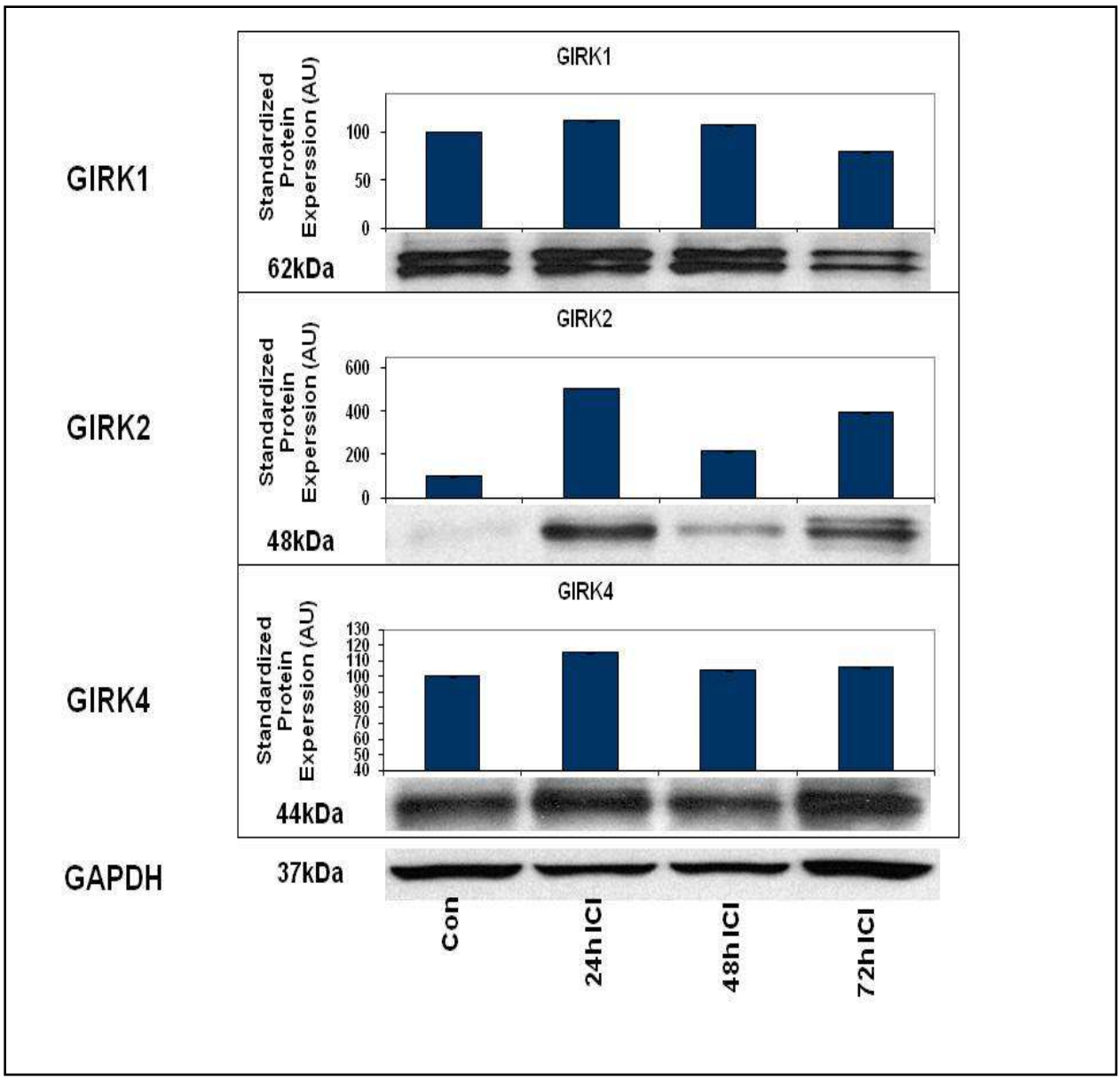

Figure 2.2 Increased GIRK membrane protein expression with 100nM ICI182780 treatment. The bands are consistent with the expected size: GIRK1 (62 kDa); GIRK2 (48 kDa); GIRK4 (44 kDa); GAPDH (37 kDa). These are gels representative of two separate experiments. Graphs indicate densitometry of the Western blots. The densitometry of GIRK was divided by the densitometry of the control GAPDH (N=1). Each western blot was done at least twice with representative blot used in the figure.. Abbreviations: AU represents (arbitrary units), Con represents (control), 24hICI represents (24 hour treatment with ICI182780), 48hICI represents (48 hour treatment with ICI182780), and 72hICI represents (72 hour treatment with ICI182780).

protein expression and phosphorylation decreased at 24 hours but increased at 72 hours (figure 2.6).

G-Protein Couple Receptor Signaling

Previous data indicates a link between GIRK and the β-adrenergic GPCR signaling pathway (6, 44). The downstream targets of this pathway are the cyclic AMP (cAMP) response element binding protein (CREB) and protein kinase A (PKA) (54). β_2-adrenergic and CREB activation was determined by western blot, and PKA levels were determined by a specific PKA assay. There are increases in β_2 adrenergic phosphorylation in MCF cells after E2 treatment at all time points (figure 2.7).There are also increases in CREB phosphorylation at 48 hours and 72 hours after E2 treatment (figure 2.7). Conversely treatment of MCF7 with 100nM ICI 182780, resulted in decreases in β_2 adrenergic phosphorylation and CREB phosphorylation at all time points (figure 2.8). Similarily, there was a decrease in PKA phosphorylation in the ICI treatment and estrogen treatment resulted in an increase in PKA activation (figure 2.9). In summary, estrogen caused an increase in all three indicators of GPCR signaling, whereas ICI182780 treatment caused a decrease in all three indicators of the GPCR system.

Akt and MAPK signaling

After treatment of MCF7 breast cancer cells with 10nM E2, Akt levels remained constant at all time points (figure 2.10). Treatment with E2 increased ERK phosphorylation at all time points (figure 2.10). Levels of c-jun phosphorylation were increased at 24h but decreased at 72 hours (figure 2.10). ICI resulted in decreased Akt phosphorylation at 24 and 48 hours but Akt phosphorylation increased at 72 hours (figure 2.11). Both ERK and c-jun phosphorylation were decreased with ICI treatment (figure 2.11).

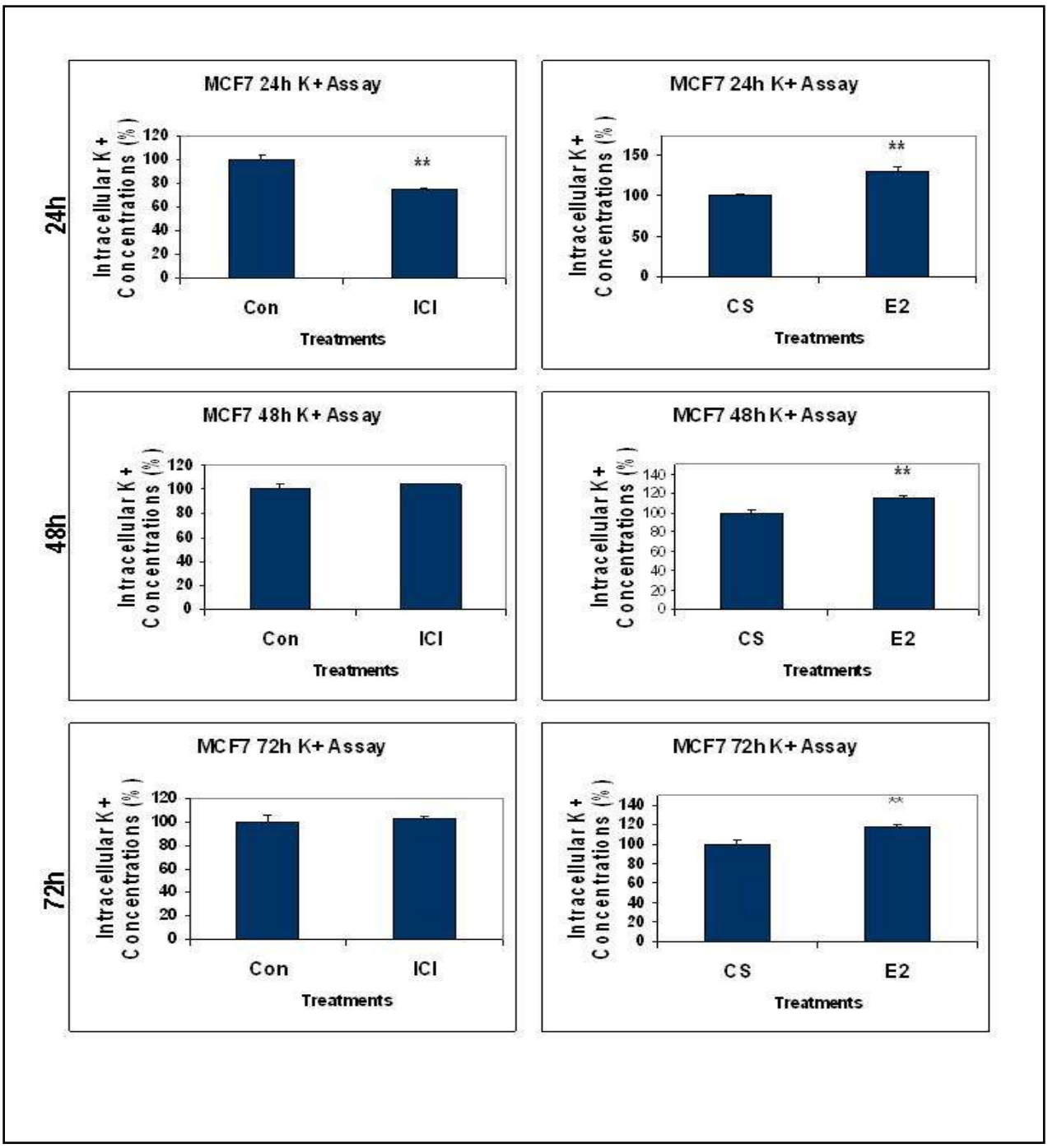

Figure 2.3 Intracellular potassium concentration after treatment with ICI or estrogen. Graphs indicate PBFI fluorescence with control standardized to 100 (control ± standard error). Asterisk (**) indicates significance ($p<0.05$) from control (N=6). Abbreviations: Con represents (control), CS represents (charcoal stripped media control) with vehicle, ICI represents (treatment with 100nM ICI182780), and E2 represents (treatment with 10nM estrogen).

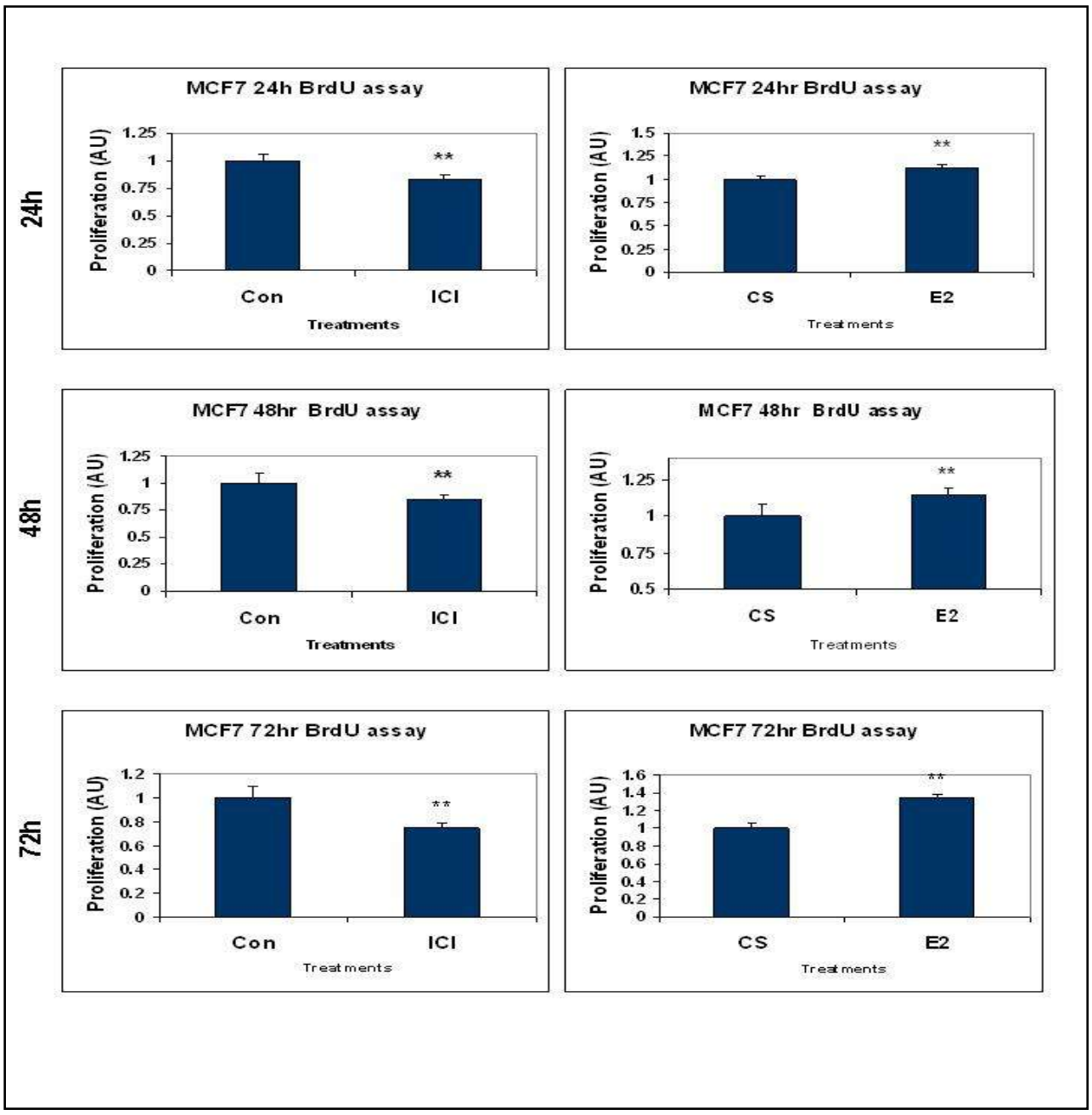

Figure 2.4 Changes of cell proliferation stimulated by estrogen or ICI182780 in MCF-7 cells. Graphs indicate BrdU absorbance with control standardized to 1 (control ± standard error). Asterisk (**) indicates significance ($p<0.05$) from control (N=5). Abbreviations: Con represents (control), CS represents (charcoal stripped media control) with vehicle, ICI represents (treatment with 100nM ICI182780), and E2 represents (treatment with 10nM estrogen).

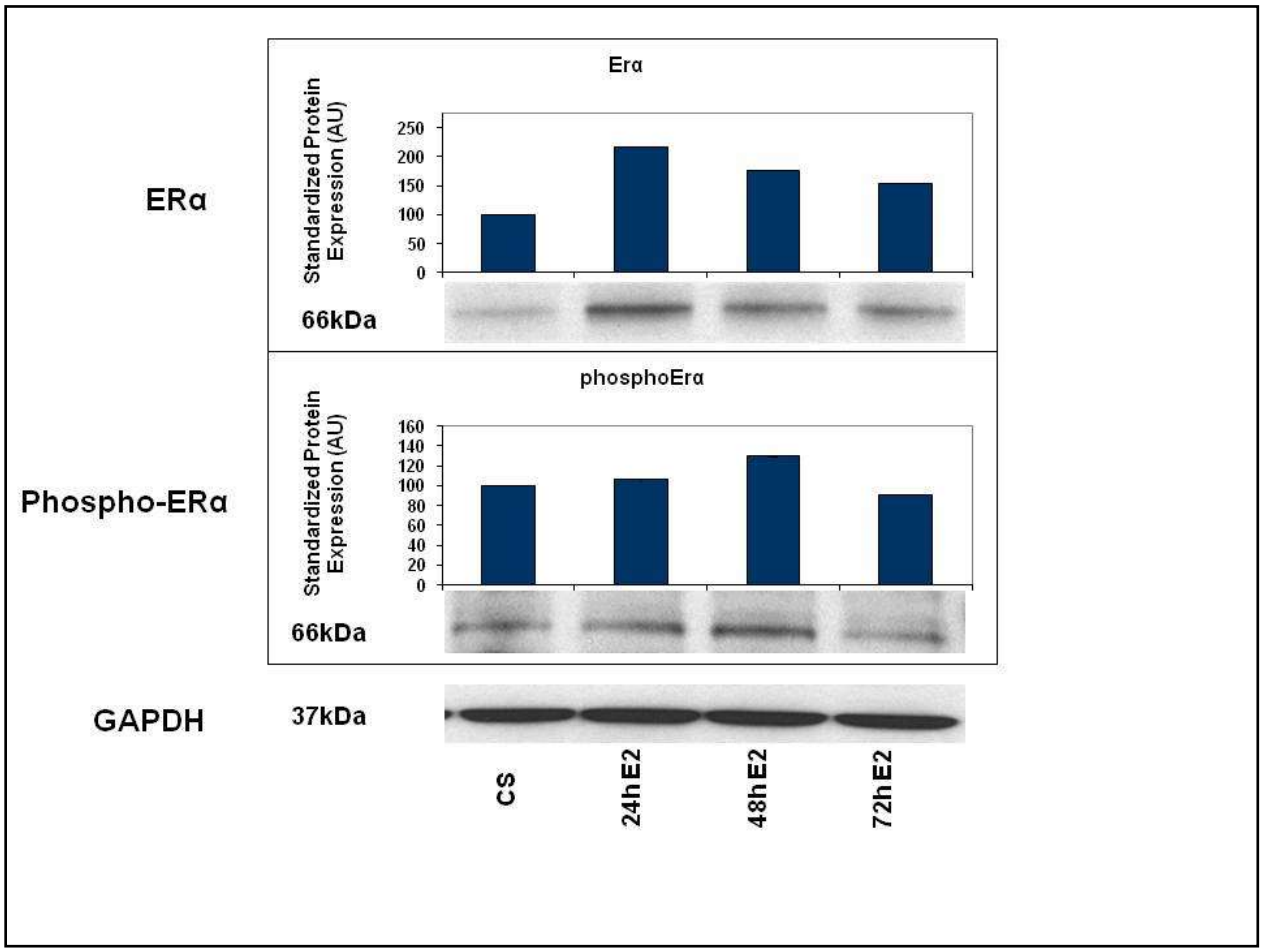

Figure 2.5 Effects of 10nM estrogen treatment on estrogen receptor expression and phosphorylation. The bands are consistent with the expected size: ERα (66 kDa); GAPDH (37 kDa). These are gels representative of two separate experiments. Graphs indicate densitometry of the Western blots. The densitometry of ERα was divided by the densitometry of the control GAPDH (N=1). Each western blot was done at least twice with representative blot used in the figure. Abbreviations: AU represents (arbitrary units), CS represents (charcoal stripped media control), 24hE2 represents (24 hour treatment with estrogen), 48hE2 represents (48 hour treatment with estrogen), and 72hE2 represents (72 hour treatment with estrogen).

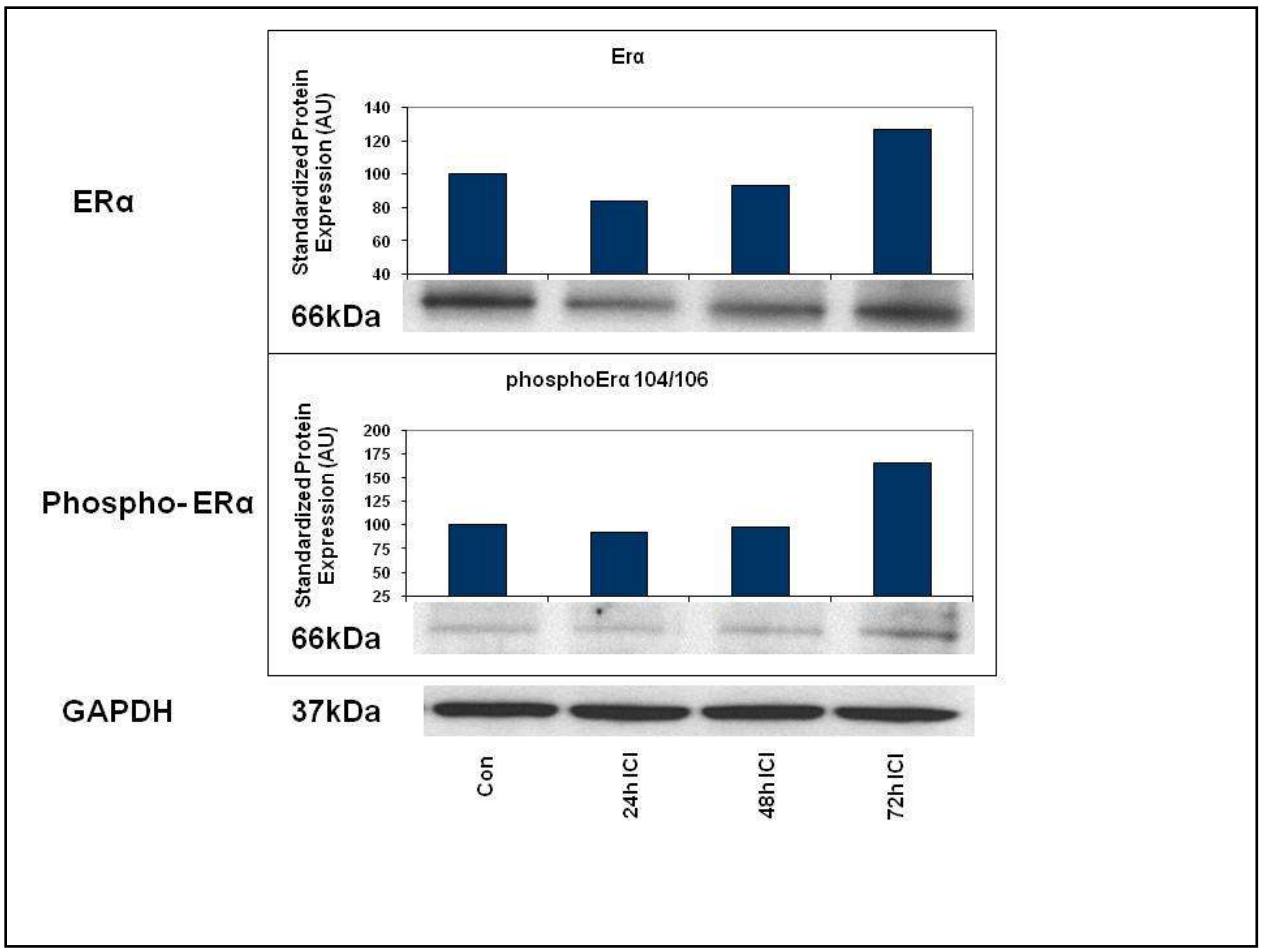

Figure 2.6 Effects of 100 nM ICI182708 treatment on estrogen receptor expression and phosphorylation. The bands are consistent with the expected size: ERα (66 kDa); GAPDH (37 kDa). These are gels representative of two separate experiments. Graphs indicate densitometry of the Western blots. The densitometry of ERα was divided by the densitometry of the control GAPDH (N=1). Each western blot was done at least twice with representative blot used in the figure. Abbreviations: AU represents (arbitrary units), Con represents (control), 24hICI represents (24 hour treatment with ICI182780), 48hICI represents (48 hour treatment with ICI182780), and 72hICI represents (72 hour treatment with ICI182780).

Localization of GIRK 1 and 2 using Immuno-florescence

Immunofluorescence microscopy was used to evaluate the distribution and subcellular localization of GIRK 1 and GIRK2 inMCF7 cells treated with 10nM E2 or cultured in charcoalstripped media (estrogen-deprived) for 24 hours. GAPDH was used as a control and shows localization in the cytoplasm in MCF7 cells cultured in charcoal stripped media and MCF7 cells treated with E2 for 24 hours (figures 2.12 and 2.13). MCF7 cells cultured in charcoal stripped media demonstrate that GIRK1 and GIRK2 localize primarily in the cytoplasm, but co-localization of both GIRK subunits indicates some dual localization in the membrane (figure 2.12). MCF7 cells treated with 10nM estrogen shows GIRK 1 localization in the membrane and GIRK2 localizes both in the cytoplasm and at the membrane supported by the co-localization of GIRK1 and GIRK2 (figure 2.13). These observations support our findings that E2 exposure increases membrane protein concentrations of GIRK1 and GIRK2 in MCF7 cells (figure 2.1)

Discussion

The findings of this study demonstrate the responsiveness of GIRK channel expression and function to E2 exposure in ER (+) MCF7 human breast cancer cells. This is the first report to identify a potential correlation between GIRK1/2 protein expression, estrogen mediated membrane localization of GIRK1/2, and not only E2 mediated proliferation, but signaling pathways known to respond to estrogen stimulation. These data support previously published reports from our laboratory that suggest functional GIRK channels are expressed in breast cancer cell lines and support our hypothesis that GIRK function is associated with several different E2 responsive signaling pathways (6, 9, 10, 18, 20, 35, 43, 14, 44, 61, 62, 63).

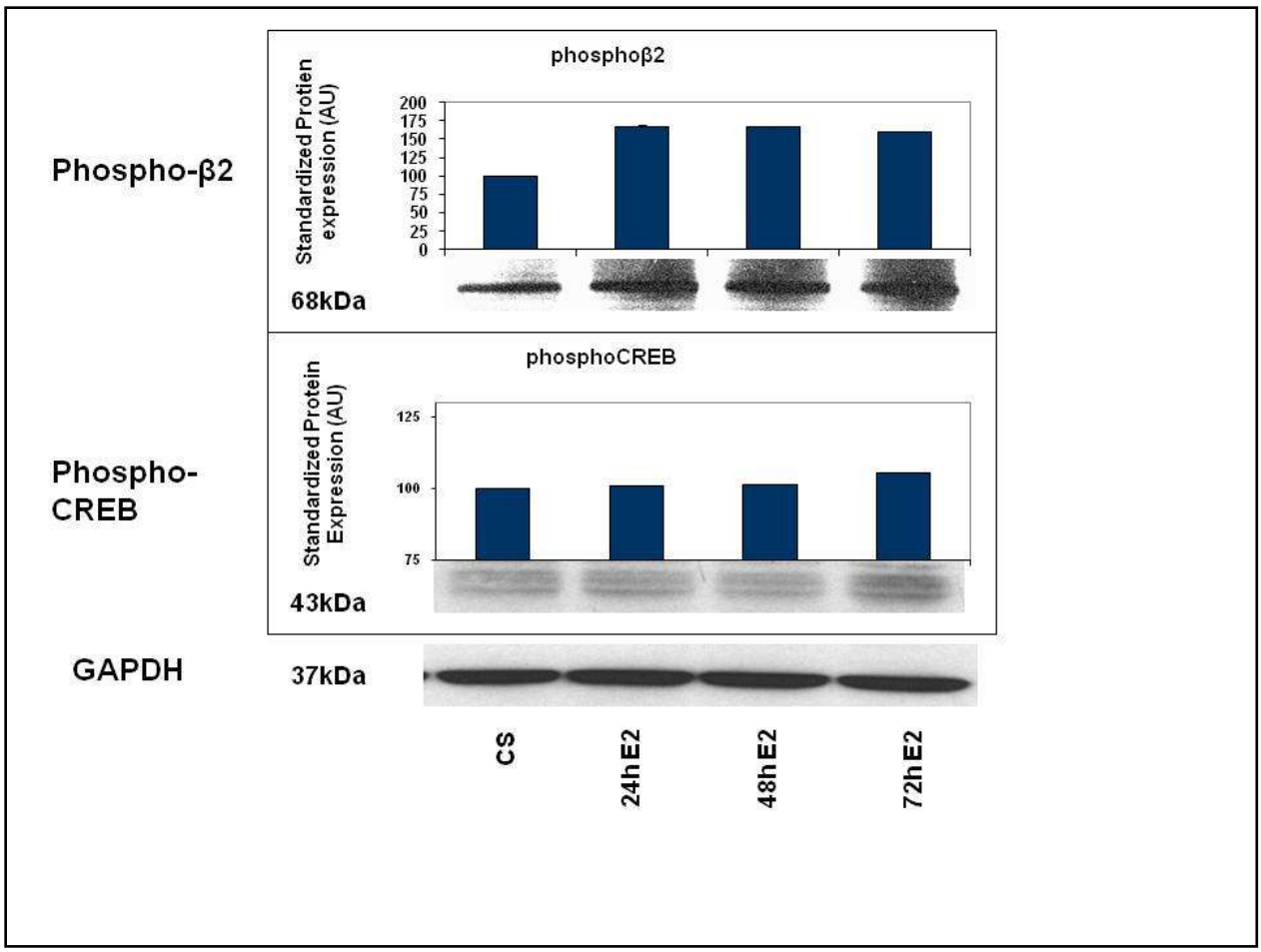

Figure 2.7 Effects of estrogen treatment on G-protein signaling in MCF7 cells. The bands are consistent with the expected size: phospho-β_2 (68 kDa); phospho-CREB (43 kDa); GAPDH (37 kDa). These are gels representative of two separate experiments. Graphs indicate densitometry of the Western blots. The densitometry of phospho-β_2 or phospho-CREB was divided by the densitometry of the control GAPDH (N=1). Each western blot was done at least twice with representative blot used in the figure. Abbreviations: AU represents (arbitrary units), CS represents (charcoal stripped media control), 24hE2 represents (24 hour treatment with 10nM estrogen), 48hE2 represents (48 hour treatment with 10nM estrogen), and 72hE2 represents (72 hour treatment with 10nM estrogen).

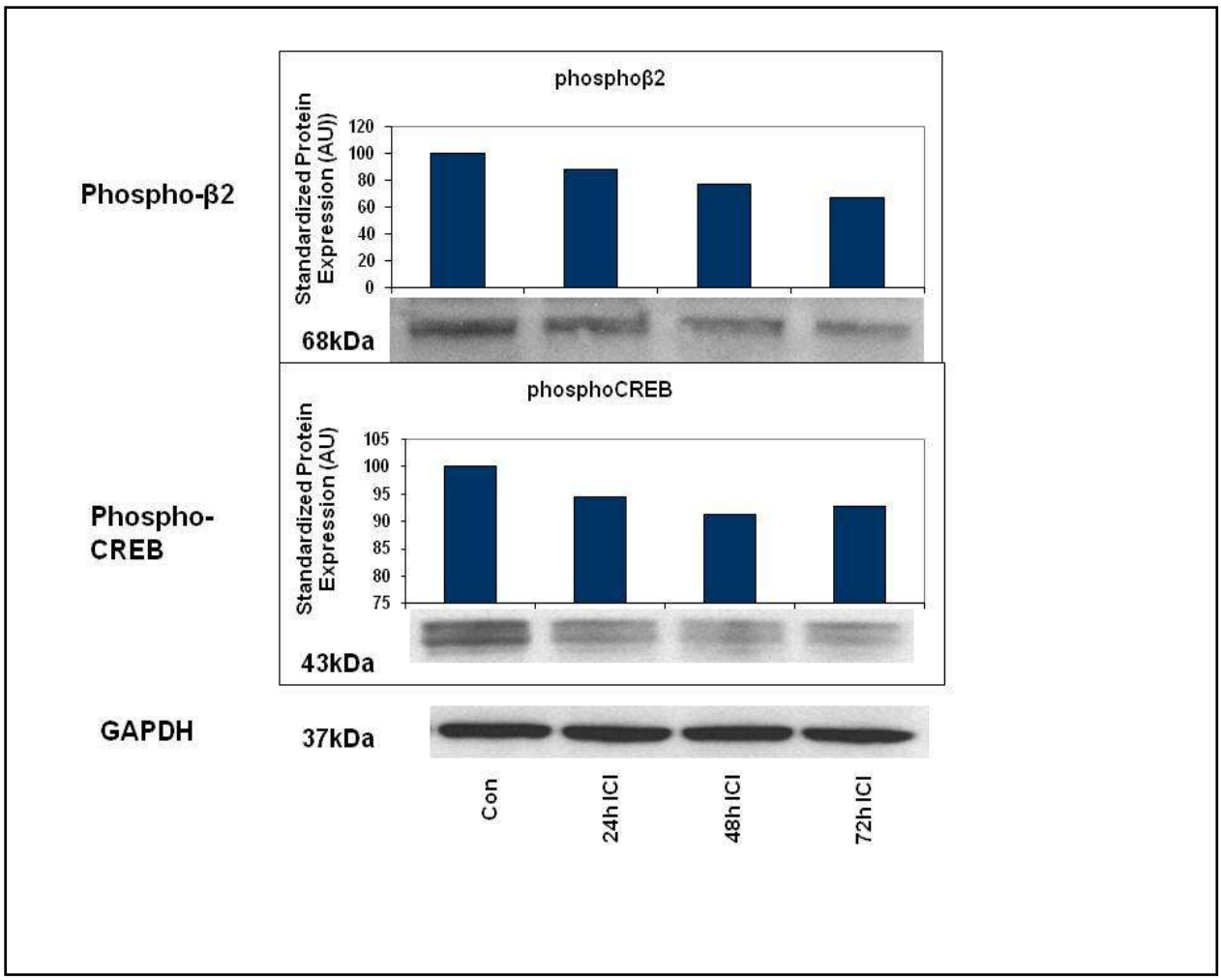

Figure 2.8 Effects of ICI182780 treatment on G-protein signaling in MCF7 cells. The bands are consistent with the expected size: phospho-β_2 (68 kDa); phospho-CREB (43 kDa); GAPDH (37 kDa). These are gels representative of two separate experiments. Graphs indicate densitometry of the Western blots. The densitometry of phospho-β_2 or phospho-CREB was divided by the densitometry of the control GAPDH (N=1). Each western blot was done at least twice with representative blot used in the figure. Abbreviations: AU represents (arbitrary units), Con represents (control), 100nM 24hICI represents (24 hour treatment with 100nM ICI182780), 48hICI represents (48 hour treatment with ICI182780), and 72hICI represents (72 hour treatment with 100nM ICI182780).

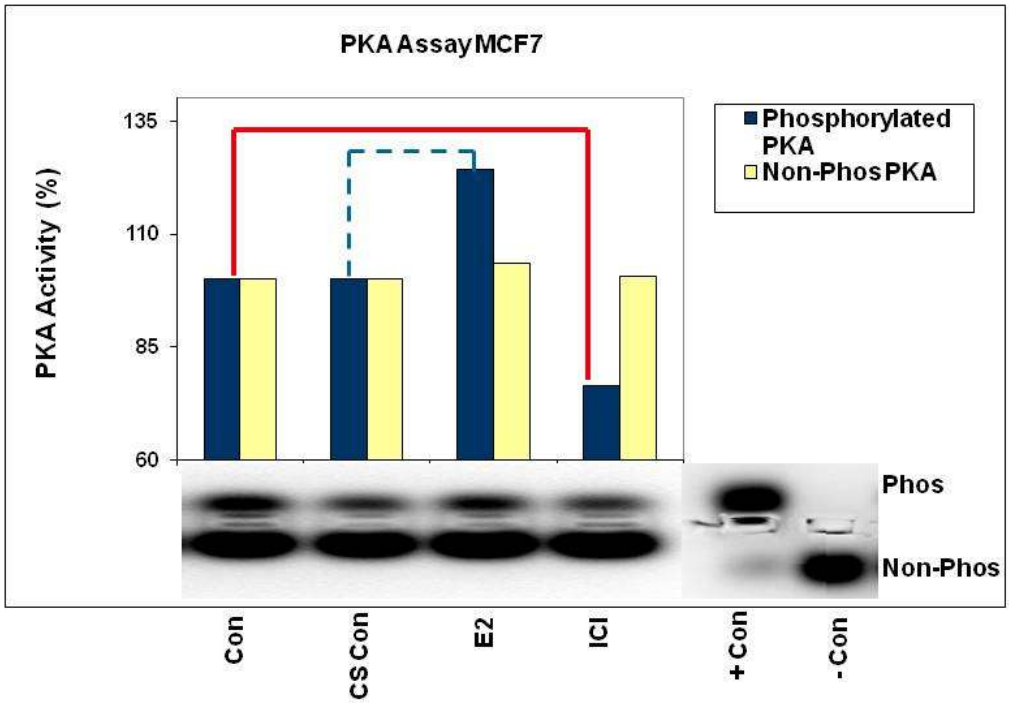

Figure 2.9 Protein kinase A activation after 24 hour treatment with either estrogen or ICI182780. PKA activation was measured by a specific PKA kit (Promega). Assay was done at least twice with representative blot used in the figure. Red line indicates ICI treatment compared to control with vehicle. Broken blue line indicates E2 Treatment and control. Abbreviations: Con represents control with vehicle, CS con represents charcoal stripped media control with vehicle, ICI represents treatment with 100nM ICI182780, and E2 represents treatment with 10nM estrogen

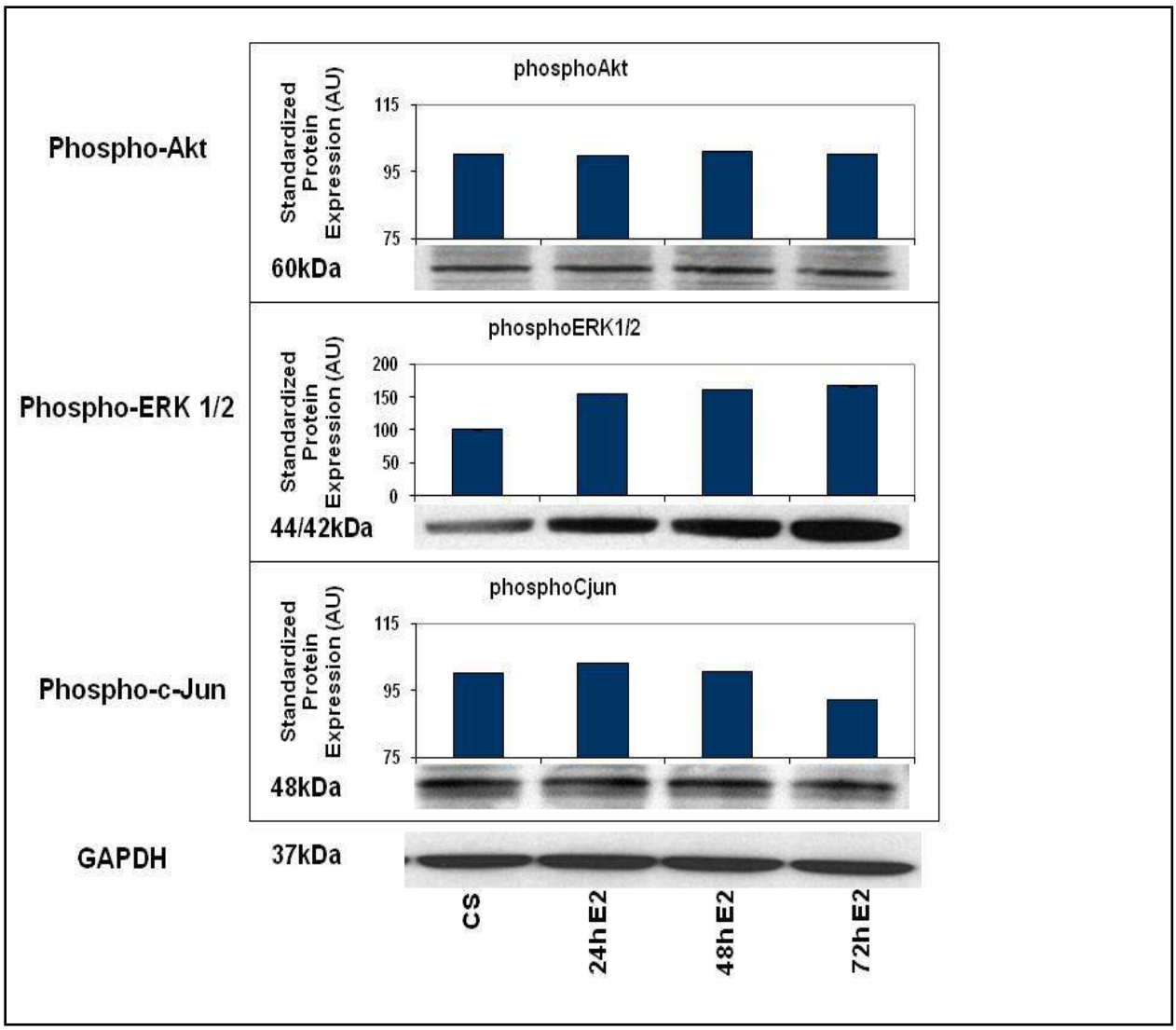

Figure 2.10 Effects of estrogen treatment on Akt, ERK, and c-jun signaling in MCF-7 cells. The bands are consistent with the expected size: phospho-Akt (60 kDa); phospho-ERK (42/44 kDa); phospho-c-jun (48 kDa); GAPDH (37 kDa). These are gels representative of two separate experiments. Graphs indicate densitometry of the Western blots. The densitometry of phospho-Akt or phospho-ERK or phospho-c-jun was divided by the densitometry of the control GAPDH (N=1). Each western blot was done at least twice with representative blot used in the figure. Abbreviations: AU represents (arbitrary units), CS represents (charcoal stripped media control), 24hE2 represents (24 hour treatment with 10nM estrogen), 48hE2 represents (48 hour treatment with 10nM estrogen), and 72hE2 represents (72 hour treatment with10nM estrogen).

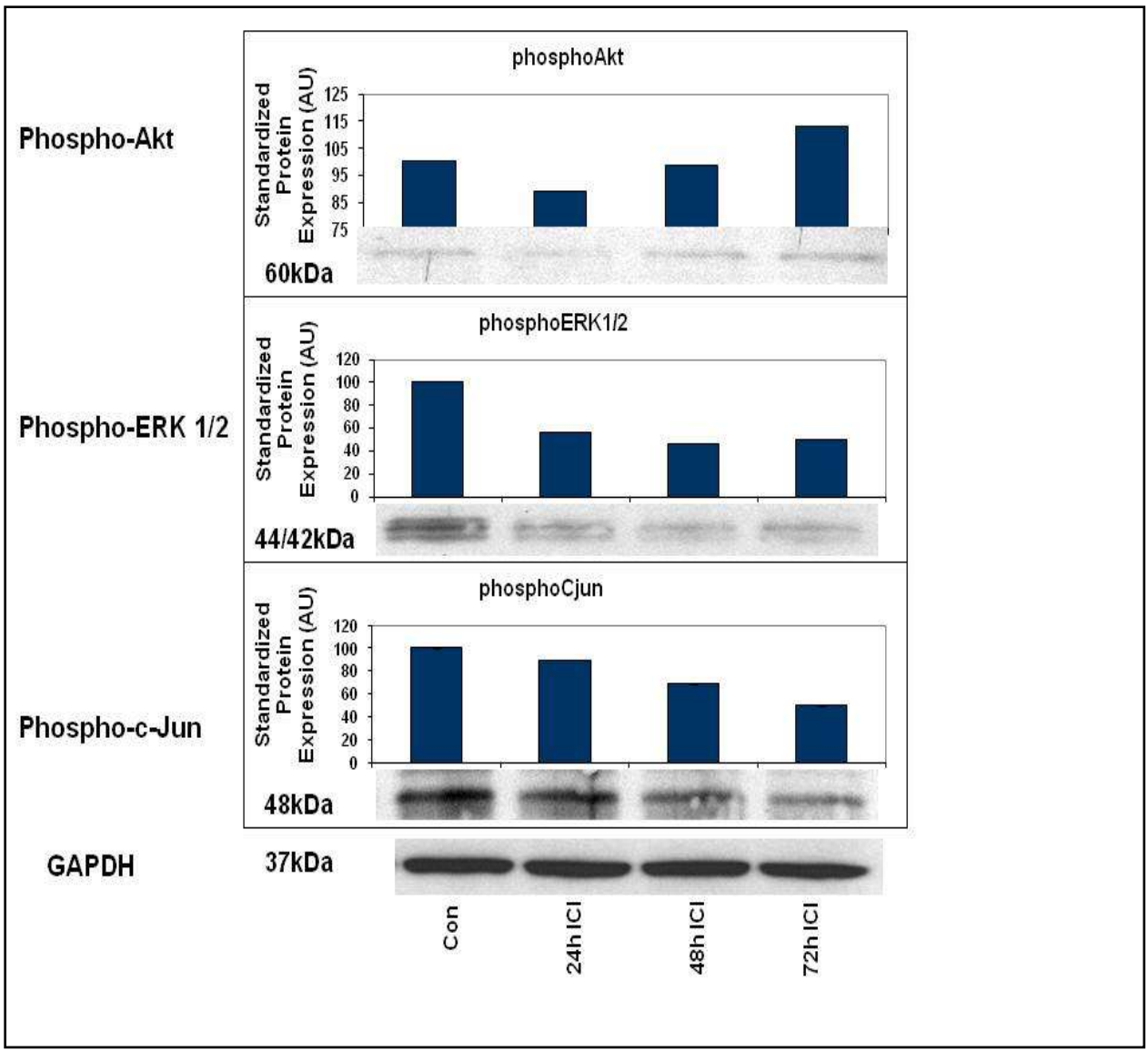

Figure 2.11 Effects of ICI182780 treatment on Akt, ERK, and c-jun signaling in MCF-7 cells. The bands are consistent with the expected size: phospho-Akt (60 kDa); phospho-ERK (42/44 kDa); phospho-c-jun (48 kDa); GAPDH (37 kDa). These are gels representative of two separate experiments. Graphs indicate densitometry of the Western blots. The densitometry of phospho-Akt or phospho-ERK or phospho-c-jun was divided by the densitometry of the control GAPDH (N=1). Each western blot was done at least twice with representative blot used in the figure. Abbreviations: AU represents (arbitrary units), Con represents (control), 24hICI represents (24 hour treatment with 100nM ICI182780), 48hICI represents (48 hour treatment with 100nM ICI182780), and 72hICI represents (72 hour treatment with 100nM ICI182780).

Kobayashi et al suggests that GIRK channels may be responsive to various neurotransmitters and hormones, which may have the ability to modulate various cellular activities (23). E2 has been shown to regulate the secretion of gonadotropin-releasing hormone (GnRH) from the mediobasal hypothalamus thereby regulating the reproductive cycle which can be achieved by rapid hyperpolarizaion of GIRK channels (5, 15, 22, 25, 33, 50). In these studies reported here, E2 treatment significantly increased the protein expression of GIRK 1 and GIRK 2 dramatically but not GIRK4. Whereas, ICI treatment increased the membrane protein expression of all three GIRK subunits possibly through secondary effects associated with ICI such has a potential stimulatory role on an orphan GPCR GPR30 (12, 27, 40). We think that the increased expression of GIRK1/2 channels may be mediated through ERα because treatment of ER(-) MDA MB453 cells with 10nM E2 for 24-72 hours had consistent levels of GIRK1/2 expression that did not increase with treatment and appear to localize heavily in the cytoplasm (data not shown). Han et al. 2005 demonstrated that the ERα isoform specifically mediates E2 induced stimulation of the large-conductance, calcium- and voltage-activated potassium (BK_{Ca}) channel in human coronary artery smooth muscle cells (13). Kobayashi et al suggests that the structure and distribution of GIRK channels is highly associated with the function of the channels (23). Specifically, neuronal GIRK channels, as well as GIRK channels found in the testis, are composed of heteromultimers composed of GIRK 1 and GIRK 2 compared to atrial GIRK channels that are composed of GIRK1 and GIRK4 (23). Furthermore, Mark et al 2000 suggests, from work in transgenic mouse models, that GIRK2 subunits must be present in mediating excitability or hyperpolorization compared to channels composed of GIRK1/4 that support the negative chronotropic effects associated with cardiac physiology (34). Taken together, GIRK 1/2 specific channels appear to respond to estrogen stimulation.

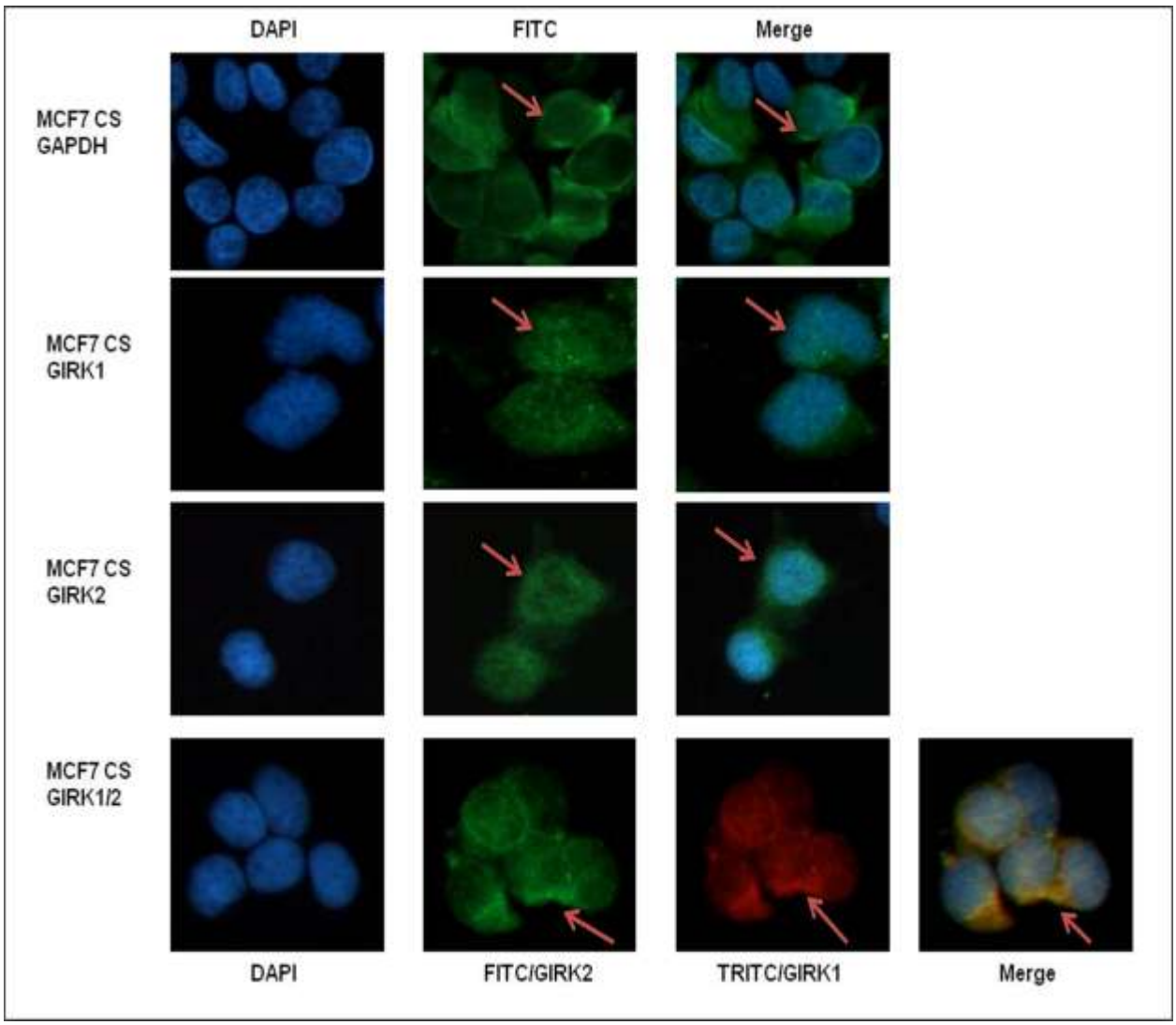

Figure 2.12 Localization of GIRKs after 24 hours of charcoal stripped media treatment using immunofluorescence. Red arrows added to indicate points of localization. Cells were fixed by 4% paraformaldehyde. DAPI staining was used to visualize the nucleus. Fluorescent images at a magnification of 40x on a Nikon Eclipse 80I fluorescence microscope. For epi-fluorescence of the fluorophors (FITC and TRITC) excitation an argon ion laser with emission at 488 and 547 nm was used and data was collected from 501-587nm. FITC image refers to protein localization(GAPDH, GIRK1, or GIRK2) except in the GIRK1/2 co-localization experiment at the bottom where FITC refers to GIRK2 localization and TRITC refers to GIRK1 localization.

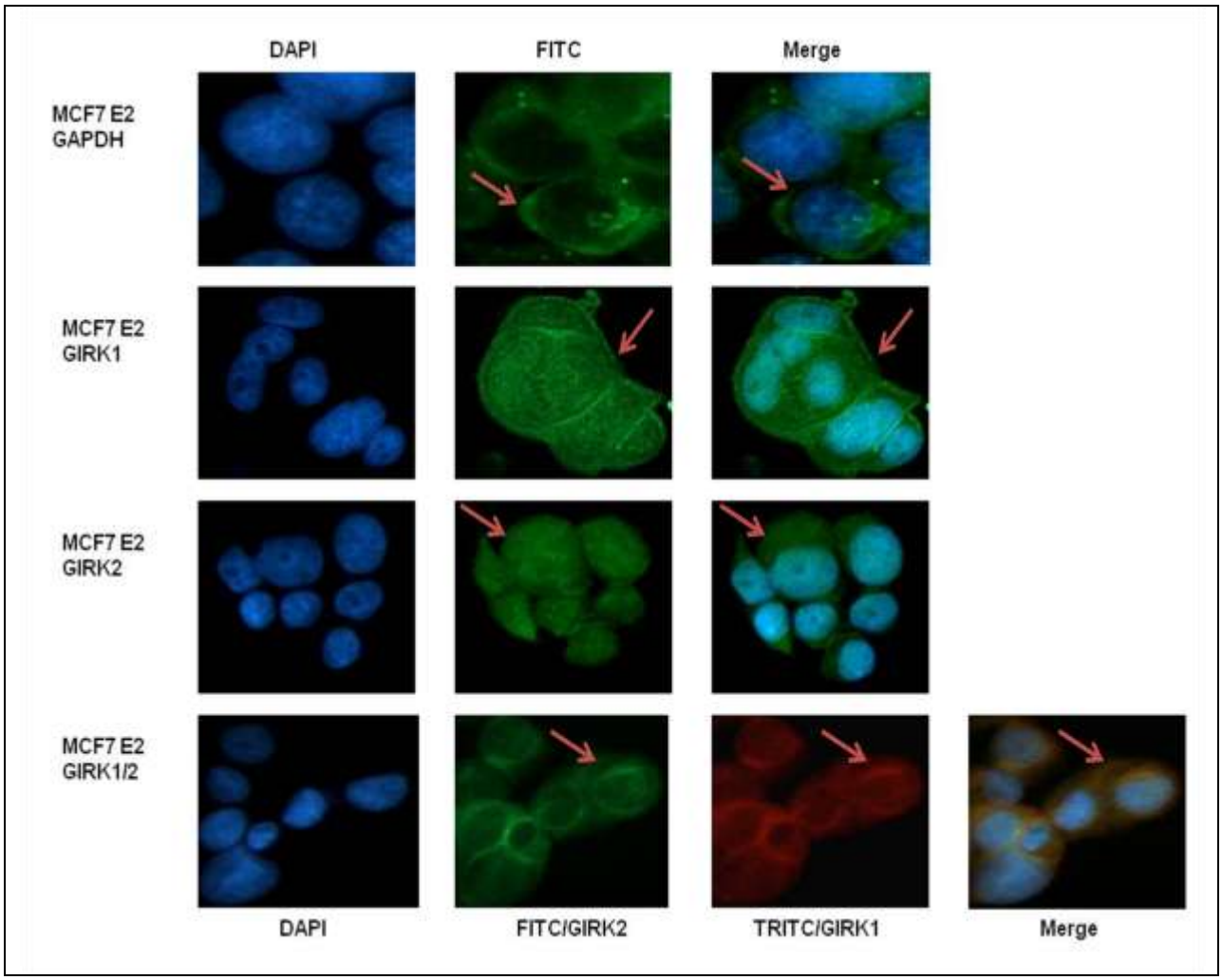

Figure 2.13 Localization of GIRKs after 24 hours of estrogen treatment using immunofluorescence. Red arrows added to indicate points of localization. Cells were fixed by 4% paraformaldehyde. DAPI staining was used to visualize the nucleus. Fluorescent images at a magnification of 40x on a Nikon Eclipse 80I fluorescence microscope. For epi-fluorescence of the fluorophors (FITC and TRITC) excitation an argon ion laser with emission at 488 and 547 nm was used and data was collected from 501-587nm. FITC image refers to protein localization(GAPDH, GIRK1, or GIRK2) except in the GIRK1/2 co-localization experiment at the bottom where FITC refers to GIRK2 localization and TRITC refers to GIRK1 localization.

Intracellular potassium concentration in response to E2 and ICI treatment was measured by a modified protocol from Andersson et al 2006 (2). Kunzelmann 2005 and Takagi et al 1986 suggest that high intracellular concentrations of potassium are seen in rapidly dividing cellsbut cells that are terminally differentiated (G_0 phase) have lower levels of intracellular potassium (24, 53). Additionally, many growth factors and mitosis related enzymes require a minimum intracellular potassium concentration and levels below this point result in reduced proliferation (24). We found a significant increase in intracellular potassium concentration in cells treated with estrogen at all time periods (figure 2.3). There was an increase in cell proliferation in E2 treated MCF7 cells at all time points (figure 2.4). Our results are consistent with previously published data about estrogen mediated proliferation in cancer and the association of increased intracellular potassium concentrations with increased proliferation (20, 24, 53, 61). Our results suggest that increases in GIRK subunits 1 and 2 are associated with increased cell proliferation, which may be in part due to or correlated with increased intracellular potassium concentrations. Treatment with ICI resulted in a significant decrease of intracellular potassium concentration only at 24 hours (figure 2.3). At the same time, we found significant reductions in cell proliferation in ICI treated cells, which agree with the previous findings (28, 59). Liu et al 2003 demonstrated that ICI treatment decreased potassium amplitude of outward current in human coronary artery smooth muscle cells and reduced the proliferative ability of these cells (30). Our data demonstrates that E2 treatment increased cell proliferation and intracellular potassium concentration, which was associated with increased expression of GIRK 1 and 2 subunits. This findings could indicate that GIRK channel function and composition may play a direct role in estrogen mediated proliferation and may also have a role in cell survivability in MCF7 breast cancer cells.

Treatment with E2 increased ERα protein expression and phosphorylation at 24 and 48 hours whereas, ICI treatment of MCF7 cells at 24 and 48 hours resulted in decreased levels (figure 2.5 and 2.6). ICI is a derivative of estradiol and considered a pure anti-estrogen compound that competitively inhibits ER transactivation leading to ER degradation (39). The increase in ERα at 72hrs may be the result of overcompensation or secondary effects of ICI treatment. More work is required to fully understand our findings.

In the present research, estrogen treatment resulted in an increase in β_2 adrenergic phosphorylation that leads to an increase in PKA phosphorylation but only a slight increase in CREB phosphorylation. Previous data indicates a link between GIRK and the β-adrenergic GPCR signaling pathway (6, 14, 44). Part of this pathway is CREB and PKA (54). Estrogen treatment also resulted in consistent levels of Akt phosphorylation, and increase in ERK phosphorylation and an increase in cJun phosphorylation. Our results differ significantly from Zivadinovic et al 2005 who found that cAMP-activated PKA activity is associated with reduced cell proliferation (63). However, these investigators cultured the MCF-7 cells in charcoal-stripped serum for 4 days prior to an experiment (63). Our results also differ from Filardo et al 2002 suggested that PKA activation may suppress ERK1/2 phosphorylation at short time shorter treatment periods through interactions with GPR30 (12). These investigators cultured the cells in serum free media for three days. Previous data from our laboratory indicated growth in serum-free media led to large reductions in both GIRK mRNA and protein expression (10). In addition, in the ER(-) MDA-MB453 cell line, our previous data indicated that GIRK1 RNA interference knockdown led to decreases in beta-adrenergic, MAP kinase and Akt signaling (14). The data from both laboratories (12, 63) are supportive of our hypothesis that many of the effects of estrogen, especially on cell proliferation, mediate GIRK channels and the effects of GIRK on ER

signaling. Further research with stable GIRK knockdowns needs to be done in order to confirm this data.

The results seen in our estrogen treated MCF-7 cells are consistent with findings that suggest hormone induced increases in cAMP can stimulate PKA activation that is capable of positive regulation of activation of ERK1/2 via RAP1 and B-Raf though β-adrenergic receptors (52). PKA activation enhances ERα mediated transcription and PKA likely has a significant role in cAMP activation of ERα mediated transcription (26). Additionally, these authors suggested that the conflicting reports about estrogen activation of MAPK in MCF-7 breast cancer cells may be due to unexplained differences between MCF-7 sublines (26). Future studies should investigate the expression level of B-Raf to better understand the mechanism of action of cAMP in relation to GIRK expression and function in breast cancer.

ICI treatment decreased β_2 adrenergic phosphorylation, which lead to a decrease in PKA phosphorylation and a decrease in CREB phosphorylation. Treatment with ICI also resulted in decreased levels of Akt phosphorylation, a decrease in ERK phosphorylation and a decrease in c-jun phosphorylation, indicating opposite effects on the MCF-7 cells from estrogen treatment. This ICI data (opposite of our estrogen data) also indicates that there is a correlation between GIRK and estrogen-mediated functions of breast cancer.

Mirshahi et al 2002 state that ion channel proteins are important regulators of excitation of cells and their activation is a highly regulated process (36). Largely, ion channel research has focused on the signaling mechanism that regulate the function of these integral membrane proteins. However these studies only address proteins that have been recruited to the membrane suggesting that there may also be value in investigating the processes that regulate translocation of ion channel proteins, especially channels like GIRKs that have multiple subunits, and likely

require a complex trafficking mechanism (31, 32, 36, 55, 60, 62). MCF7 cells cultured in charcoal stripped media for 24 hours demonstrated both cytoplasmic and membrane localization of GIRK1 and GIRK2 (Figure 2.12). Treatment with E2 for 24 hours resulted in membrane localization of GIRK1 but GIRK 2 was localized in the cytoplasm and in the cell membrane (figure 2.13). These observations could suggest that E2 signaling may be associated with GIRK trafficking and membrane localization of GIRK1 and GIRK2. However, more extensive work is require to investigate the processes regulating translocation of GIRKs to the membrane and how the composition of these channels affect the cellular processes.

The results of this study suggest that GIRK channel expression and function are responsive to estrogen stimulation in MCF-7 breast cancer cells, specifically GIRK subunits 1 and 2. Additionally, this study suggests that GIRK channels composed primarily of GIRK 1 and GIRK2 subunits may be associated estrogen mediated proliferation and these findings are outline in our hypothetical mechanism of GIRK funtion in E2 treated MCF7 breast cancer cells (chapter 1; figure 1.7).

ICI treatment resulted in increased expression of all three GIRK channels, these cells demonstrated lower rates of proliferation, which suggests that increased GIRK channel heterogeneity may be associated with maintenance of cellular homeostasis. This is confirmed by the results in the ICI treated cells of decreased intracellular potassium concentration. Another plausible explanation for why we see an increase in GIRK channel expression in ICI treated cells as well as the increase in estrogen treated cells may be the result of ICI activating an orphan GPCR, GPR30, that has been suggested to activate several downstream targets including PI3K/Akt (27, 40). Futhermore, we found that100 nM ICI treatment of ER(-) MDA-MB453 and ER(+) MDA-MB361 breast cancer cells resulted in an increase in GIRK membrane protein

expression, which could idicate that these unexpected secondary actions of ICI are not mediated through ERα (data not shown). We plan future investigations to determine if GPR30 can modulate GIRK channels protein expression or if GPR30 has a regulatory role on GIRK channel translocation to the membrane. However, the action of GRP30 is greatly debated and not clearly understood at present (27, 40), and needs to be studied further especially in relation to ICI stimulation of GIRK channels.

In conclusion, our data suggests that GIRK channels composed of GIRK subunits 1 and 2 may play a role estrogen mediated proliferation in MCF7 breast cancer cells. These data further contribute to the accumulating evidence that ion channel involvement in cancer is essential for proliferation and metastasis, which indicates potential value for targeted therapy. More extensive investigation of GIRK involvement in cancer progression is needed to evaluate the role of GIRKs in estrogen mediated proliferation, the potential functional correlation between GIRKs and ERα, the actions of GIRK1/2 specific channels in cancer progression, and potential candidate genes associated with GIRKs in cell proliferation. Our data suggests GIRK function is correlated to estrogen signaling, a major breast cancer risk factor, and may contribute to improving our current understanding of GIRK channel involvement in breast cancer.

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G-Protein Inwardly Rectifying Potassium Channel 1 (GIRK1) Knockdown Reduces Estrogen Receptor *α*, Beta-Adrenergic, MAP Kinase, and Akt Signaling in Estrogen Responsive MCF7 Breast cancer cells.

Chapter 3

Abstract

In 2009, the American Cancer Society estimates that over 192,000 new cases of breast cancer will be diagnosed and over 40,000 women will die from breast cancer, which makes breast cancer the second leading cause of cancer death. Previous data from our lab has indicated that there is a correlation between the beta-adrenergic receptor signaling pathway and G-protein inwardly rectifying potassium channel (GIRK1) in several breast cancer cell lines. We also found that GIRK1 knockdown reduced beta-adrenergic, MAP kinase and Akt signaling in estrogen nonresponsive (ER(-)) MDA-MB453 breast cancer cells. We hypothesize that GIRK channels composed of GIRK subunits 1 and 2 may play a role estrogen mediated proliferation in MCF7 breast cancer cells from previous studies in our lab. In the present study, to further define the signaling pathways involved with GIRK expression and function, we used RNA interference (siRNA) to knockdown GIRK1 in ER(+) MCF7 breast cancer cells to evaluate ER alpha function. Additionally, we used U50488H, known to stimulate GIRK1/2 channels at low doses (nM concentration), to determine functionality of these channels in breast cancer. MCF7 cells treated with U50488H resulted in increased levels of GIRK1 and GIRK2 subunits, which supports our previous hypothesis that functional GIRK channels do exist in breast cancer cells. In the present study, we screened multiple siRNA constructs that have been previously validated in our lab. The greatest GIRK1 knockdown was seen with the siRNA construct starting at base pair 1315, which knocked down GIRK1 mRNA levels 1.8 fold (85%) at 24 hours. Additionally, GIRK1 knockdown also led to decreased protein phosphorylation of beta-adrenergic, MAP kinase and Akt signaling pathways. In conclusion, these data suggest that functional GIRK channels do exist in breast cancer cells and that disruption of GIRK1 may affect multiple signaling pathways associated with cellular proliferation.

Introduction

Breast cancer is the one of the most common cancers in women and is reported as the second highest cause of cancer death after lung cancer (2). Multiple risk factors contribute to the onset of breast cancer such as lifestyle (including smoking, drinking, and diet), family history, age, race, geographical location, age of menarche, age of parturition, and age of menopause (2). The American Cancer Society (ACS) approximates that 192,000 women will be diagnosed with breast cancer in 2009 and estimates 40,000 women will die from breast cancer in 2009 (2). It is thought that one million new cases of breast cancer are diagnosed each year worldwide and is correlated to a higher incidence in western countries (3). The US and Europe account for 16% of the world population but the account for approximately 60% of the worldwide incidence of breast cancer (42, 43). Today, breast cancer accounts for 22% of all female cancers (43). ACS suggests that approximately 1 in 8 will develop invasive breast cancer during her life and 1 in 35 cases will be fatal (2). Additionally, men are at risk for breast cancer in very rare cases (2).

Approximately, 40% of human primary cancer tissues express G-protein inwardly rectifying potassium channel 1 (GIRK1) mRNA and the expression of GIRK 1 mRNA is significantly correlated to lymph node metastasis and as a result tumors that express GIRK 1 tend to have a poor clinical prognosis (52). GIRK expression is normally restricted to the heart and brain, where they are known to form homo- and heterotetramers (52). However, GIRK1 requires one of the other subunits for formation of functional channels (22). Data from our lab has indicated that functional GIRK channels exist in breast cancer cell lines (12, 15) Additional data from our lab has demonstrates that a functional link exists between GIRK 1 expression and β_2 adrenergic receptor (ADRB2) function, which is part of the G-protein coupled receptor (GPCR) family (6, 7, 45, 46). Other investigators have shown that ovarian cancer invasiveness

can be mediated through ADRB2 activation of cell cyclic AMP (cAMP) protein kinase A (PKA) signaling pathway, which has been shown to stimulate angiogenesis and are associated tumor growth. (53). Our lab has demonstrated that estrogen receptor positive (ER (+)) cell lines MCF-7, MDA-MB-361, and ZR-75-1 and the ER negative (–) cell line MDA-MB-453 expressed mRNA for the GIRK1 channel while the ER (–) cell lines MDA-MB-468 and MDA-MB-435 did not express GIRK1 (45). Our lab also demonstrated GIRK 1, 2 and 4 protein expression in multiple breast cancer cell line types, which was the first report of GIRK protein expression outside of the tissues of origin (brain and heart) (12). Data from multiple laboratories suggest that GIRK is associated with proliferation and clinical aggressiveness, and as a result may be a valuable therapeutic target (5, 6, 7, 12, 15, 45, 47, 52). These reports indicate that GIRK1 is a potential biomarker of clinically aggressive breast cancer, but likely forms functional channels with another subunit, and may have therapeutic potential.

Estrogen (E2) is a well documented risk factor associated with breast cancer and is considered a carcinogen (61). The role of E2 in carcinogenesis is unclear, but it is thought to be the consequence of two complimentary pathways, specifically E2 metabolism and ER signaling (61). Estrogen receptors are a powerful predictor of breast cancer prognosis and ERα is a standard therapeutic target in treatment (2, 10, 19, 60). The carcinogenic action of E2 modulates signaling transduction pathways that are correlated with proliferation, inhibition of apoptosis, cell migration, and induction of angiogenic factors via both nongenomic actions and the estrogen receptor (13, 17, 32, 44, 46, 48, 50, 55, 61). Oxidative metabolites of estrogen have genotoxic, mutagenic, transforming, and carcinogenic effects on cells, suggesting that E2 may initiate and/or contribute to the progression of carcinogenesis (61). Previous studies have shown that estrogen metabolites can induce genetic mutations in MCF7 breast cancer cells and initiate

neoplastic transformation of non-turmorgenic MCF-10F breast cells treated with E2, which provides evidence of the mutagenic properties of E2 and E2 associated metabolites (48, 49).

Previous research from other labs has indicated that estrogen may have effects on potassium channels. Specifically, the activation of multiple potassium channels including GIRKs are responsive to the effects of E2 in the brain and heart (14, 21, 22, 25, 34, 54). E2 has been shown to induce rapid depolarization of inward rectifying potassium channels in hypothalamic neurons as part of the regulation of the female reproductive cycle (25). Additionally, it has been demonstrated that E2 can activate maxi-K potassium channels in breast cancer cells, and this activation was associated with increased cell proliferation (8). Evidence from our lab has demonstrated that GIRK protein and gene expression is decreased in cells cultured in serum free media possibly due to lack of estrogen in the media (12). We have also shown that treating MCF7 beast cancer cells with E2 resulted in increased the protein expression of GIRK1 and GIRK2, which was also correlated with increased intracellular potassium levels and increased cell proliferation (chapter 2). Taken together, these results suggest that GIRKs may be activated by E2 and could be functionally correlated with the ER expression and function breast cancer.

The primary focus of this study was to examine the effects of GIRK1 knockdown on ERα expression and function in ER (+) MCF7 breast cancer cells, specifically the functions that may be mediated through the GPCR, MAPK, and Akt signaling pathways. Our lab has previously shown that GIRK1 knockdown in the ER (-) MDA-MB453 breast cancer cell line resulted in decreased protein phosphorylation in these three signaling pathways. The GPCR, MAPK, and Akt signaling pathways have been shown to be responsive to the second messenger effects of E2-ERα signaling and possibly other non genomic actions associated with E2 signaling (chapter 2, 61). Our current results suggest that GIRK1 knockdown results in decreased ERα mRNA and

protein levels. These results suggest that GIRK expression is correlated to ERα function in MCF7 cells, which links GIRK function to a major breast cancer risk factor (estrogen) and suggests that GIRK may have relevance as a therapeutic target.

Materials and Methods

Cell Culture

The human estrogen responsive MCF7 breast cancer cell line was purchased from American Type Culture Collection (ATCC; Rockville, MD, USA) and was maintained RPMI 1640 media supplemented with fetal bovine serum (10% v/v), L-glutamine (2mM), 100 U/ml penicillin and 100 ug/ml streptomycin at 37 °C in an environment of 5% CO_2. Cells were treated with 200nM U50488H (trans-(±)3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]cyclohexyl-benzeneacetamide) dissolved in phosphate-buffered saline, a selective κ-opioid receptor agonist, to activate GIRK1/2 specific channels and as a positive control (57).

Real Time Polymerase Chain Reaction (PCR)

RNA was isolated by an Absolutely RNA kit (Stratagene, La Jolla, CA). After DNase treatment of RNA for 15 minutes at room temperature, reverse transcription was carried out using 2 μg RNA, 1 μl random decamer primers (Ambion, Foster City, CA) and nuclease-free water, which were heated to 70°C for 3 minutes for primer binding. This mixture was added to 0.5 mM of dNTPs, 10mM DTT, 40 U RNase ribonuclease inhibitor (Promega, Madison, WI), 200 U Moloney murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies, Carlsbad, CA), PCR buffer (10 mM Tris-HCl pH 8.3 50mM KCl, 1.5 mM $MgCl_2$) and nuclease-free water in a total volume of 20 μl. This mixture was incubated at 37 °C for 1h, and then the enzymes were heat inactivated for 10 min at 92°C. A negative control reaction was performed without the M-MLV to determine if there was genomic DNA contamination.

Real-time PCR was done using the Cepheid smart cycler. The GIRK1 primers for real-time PCR are forward 5´-ctctcggacctcttcaccac-3´ and reverse 5´-gccacggtgtaggtgagaat-3´ (Genbank Accession # NM_002239), and the internal TaqMan probe is 6-FAM-tcaagtggcgctggaacctc-TAMRA (Sigma-Genosys, Woodlands, TX). The real-time PCR conditions for GIRK1 were 95^{o} C for 120 seconds, followed by 45 cycles of 95^{o} C, 15 seconds; 62^{o} C, 10 seconds; and 72^{o} C, 15 seconds. The ERα primers for real-time PCR are forward 5´-aggccactggcttagagtac-3´ and reverse 5´-ctcccagtacccacagtccat-3´ (Genbank Accession # NM_000125), and the internal TaqMan probe is 6-FAM-tccttcccctgcatgacactga-BHQ1 (Biosearch Technologies, Novato, CA). The real-time PCR conditions for ERα were the same as GIRK1 except for the annealing temperature of 58^{o} C. 18S rRNA detection reagents (Eurogentec, San Diego, CA) were used for normalization of the data. Real time PCR data was analyzed using the $2^{-\Delta\Delta C}{}_{T}$ method (30).

siRNA Knockdown

Three stealth siRNA constructs (Invitrogen, Carlsbad, CA, U.S.A.) were made starting at bases 1104, 1315, and 1490 of the GIRK1 sequence (GenBank # NM_002239). These constructs are: 1104 sense—gga aac aac ugg gau gac uug uca a; 1104 antisense—uug aca agu cau ccc agu ugu uuc c (GC content 44%); 1315 sense—cca gcc aua acu aac agc aaa gaa a; 1315 antisense—uuu cuu ugc ugu uag uua ugg cug g (GC content 40%); 1490 sense—acu ugc cca uga aac uuc aac gaa u; 1490 antisense—auu cgu uga agu uuc aug ggc aag u (GC content 40%). In addition, the low GC RNAi negative control (35%–45% GC) was used as recommended by the manufacturer (Invitrogen). These constructs (200 pM) were transfected into the MCF7 breast cancer cell lines using lipofectamine 2000 (Invitrogen) (20 µl/60 mm dish), which was from our previously published protocol (15).

Analysis of Protein Expression by Western Blots

Cells were washed twice with phosphate buffered saline and lysed with cold RIPA lysis buffer containing Halt protease inhibitor (Thermo Scientific Pierce Protein Research Products, Rockford, IL) (50 mM Tris pH 7.4, 150 mM NaCl, 1% NP-40, 1% Triton X 100, 0.1% SDS, 1% sodium deoxycholate,1 mM EDTA, 50 mM NaF, 10 mM sodium pyrophosphate, 0.5 mM DTT). Cell lysates were collected from culture plates using a cell scraper, and protein collected by centrifugation. Protein concentrations determined by BCA protein assay (Pierce, Rockford, IL). Additional cell pellets were collected and membrane protein was isolated with the ReadyPrep protein extraction kit (signal) (Biorad, Hercules, CA). Protein levels of membrane proteins were determined using the RCDC kit (Biorad). Aliquots of 20 μg protein were boiled in 2x loading buffer (0.1 M Tris-Cl, pH 6.8, 4% SDS, 0.2% Bromophenyl blue, 20% glycerol) for 4 minutes, then resolved by electrophoresis on 12% SDS-PAGE, transferred to nitrocellulose membranes, blocked for 1 hour 1% (W/V) non-fat dry milk, and immunoblotted overnight with the specific primary antibodies. Primary antibodies were detected with horseradish peroxidase-coupled secondary antibody and enhanced chemiluminescence (Pierce, Rockford, IL). Antibodies for GIRK1, GIRK2 (Lifespan Biosciences, Seattle, WA), GIRK4 (Sigma, St. Louis, MO, USA), phospho-β2 (Ser 355/Ser 356) (Santa Cruz, Santa Cruz, CA), ERα (Upstate, Temecula, CA), GAPDH, phospho-CREB (Ser133), phospho-Akt (Ser473), phospho-c-Jun (Ser63), phospho-ERα (Ser104/106), phospho-ERK (Thr202/Tyr204) (Cell Signaling, Danvers, MA) were used in these studies. Membranes were probed with an antibody for GAPDH (Cell Signaling) to ensure equal loading of protein between samples. GAPDH has been previously used as a loading control (12, 15, 45). Actin was not used because GIRKs may affect actin expression (16, 35). Protein bands on the immunoblots were quantitated using Scion Image software, release Alpha 4.0.3.2

(http://www.scioncorp.com) using three separate measurements of each band on the gel, and each western blot was done at least twice (12, 15, 45). The protein bands were standardized by GAPDH loading control and converted to a percent based on the control.

Results

GIRK1 and ERα gene expression using real-time PCR

To further explore the relationship between GIRK activation and ER signaling in MCF7 cells, we performed RNA interference (RNAi) studies. As in previous studies (15), three stealth siRNA constructs (Invitrogen) were used starting at bases 1104, 1315, and 1490 of the GIRK1 sequence (GenBank # NM_002239) to knockdown GIRK1 levels in the MCF-7 breast cancer cell line. These constructs were transfected into MCF-7, and RNA was isolated 24 hours after transfection. GIRK1, ERα and 18S control levels were determined using real-time PCR. Unlike the previous results in ER(-) MDA-MB-453 cell line, only one of the three constructs(1315) noticeably decreased GIRK1 mRNA levels in the MCF7 cell line (figure 3.1). As a result only the results from the 1315 siRNA contruct have been included. These data were analyzed using the $2^{-\Delta\Delta C}{}_{T}$ method (30), the significant GIRK1 knockdown is 1.85 fold or 85% ($p<0.05$) (figure3.1). The 1315 construct also caused a 1.65 fold or 65% ($p<0.05$) knockdown of ERα mRNA (figure3.2).

GIRK membrane protein expression

Membrane proteins were isolated from additional MCF-7 cells transfected with the 1315 RNAi construct or cells treated with 200 nM U50488H (57) which activates GIRK1/GIRK2 channels at nanomolar doses. Enriched membrane proteins were isolated, western blotting was performed, and the membranes were probed with antibodies for GIRK1, 2, and 4. As in previous

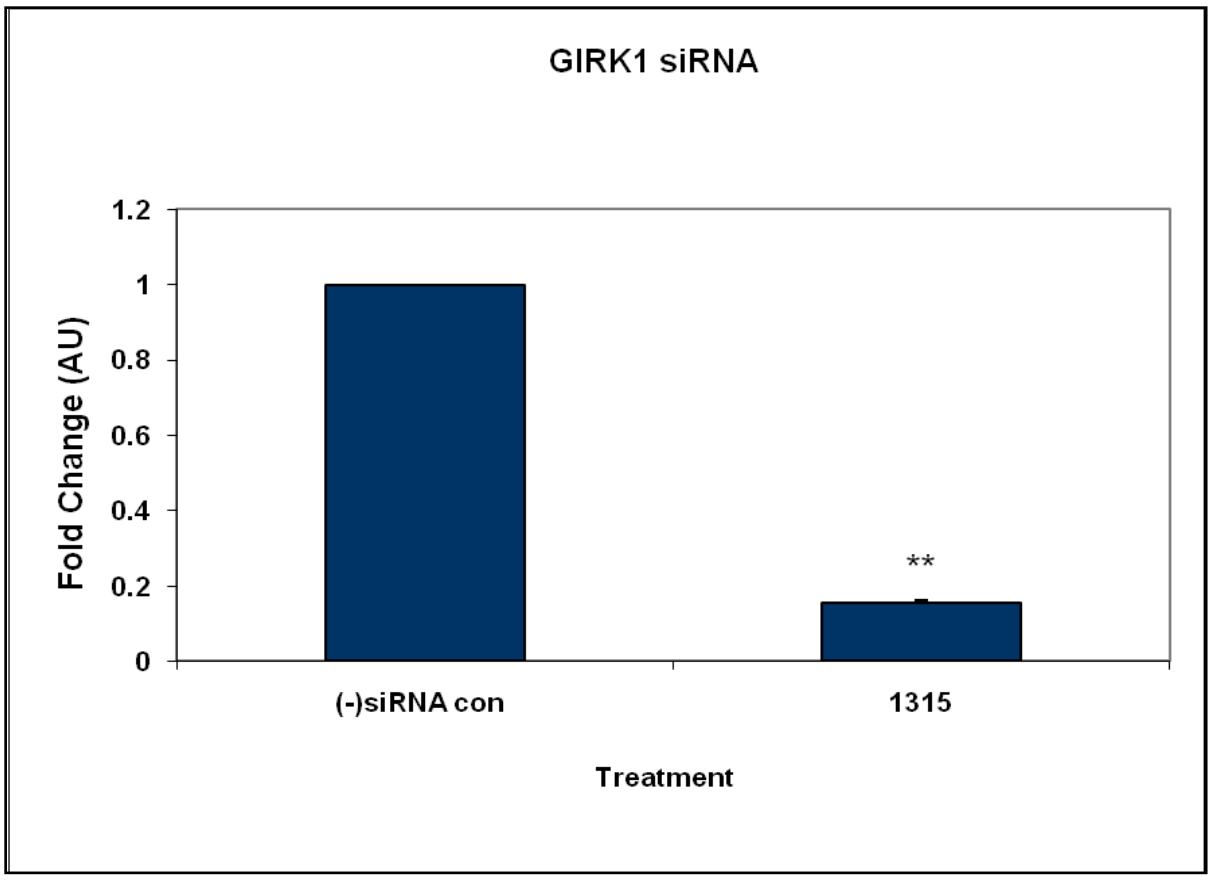

Figure 3.1 GIRK1 gene expression in MCF7 breast cancer cells using real time PCR. GIRK1 mRNA levels were reduced 1.85 fold or 85%. The low GC RNAi negative control (35-45% GC) was used as recommended by the manufacturer (Invitrogen). The stealth RNAi construct 1315 sense─cca gcc aua acu aac agc aaa gaa a; 1315 antisense─uuu cuu ugc ugu uag uua ugg cug g (GC content 40%). These data were analyzed analyzed using the $2^{-\Delta\Delta C}{}_{T}$ method (30). Asterisk (**) indicates a significant difference ($p<0.05$) in treatments. AU represents arbitrary units.

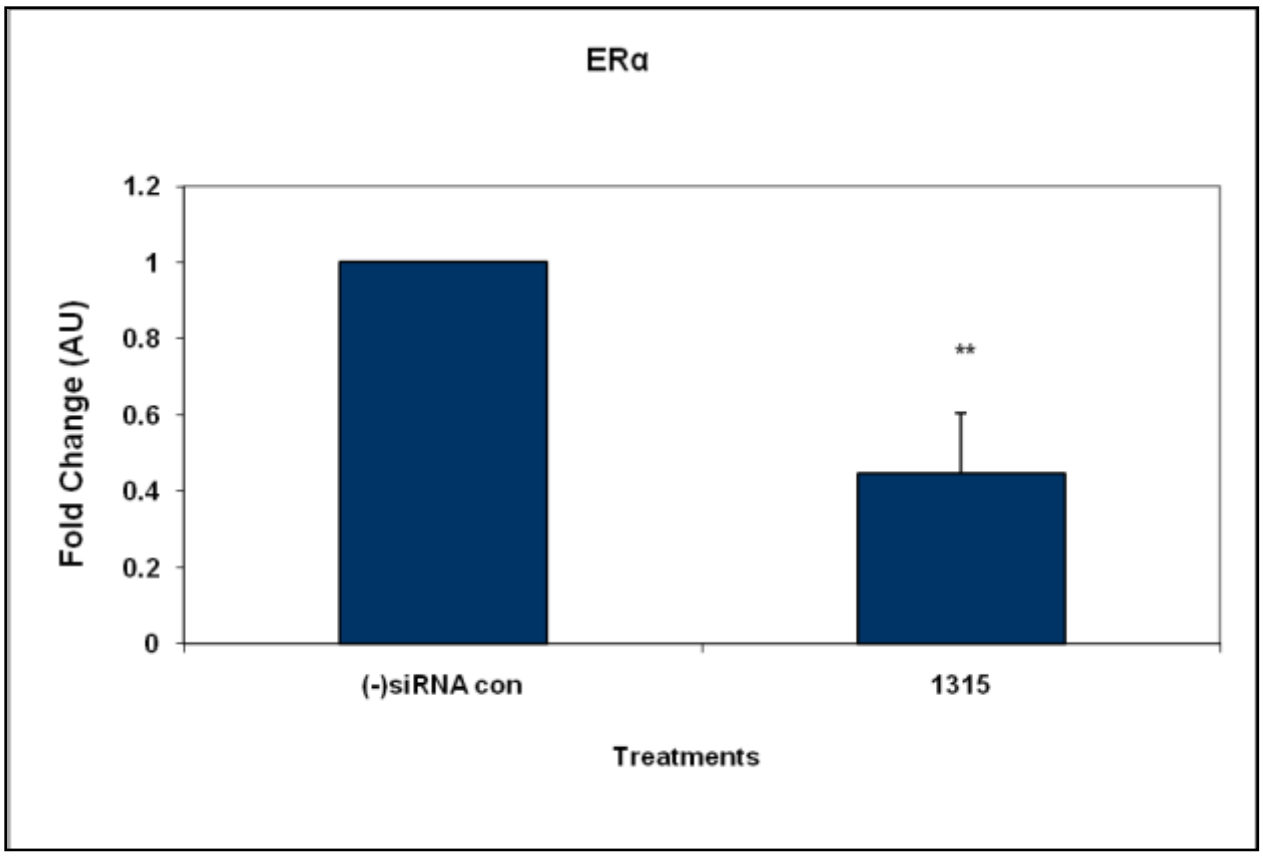

Figure 3.2 ERα gene expression in MCF7 breast cancer cells using real time PCR. ERα mRNA levels were reduced 1.65 fold or 65%. The low GC RNAi negative control (35-45% GC) was used as recommended by the manufacturer (Invitrogen). The stealth RNAi construct 1315 sense─cca gcc aua acu aac agc aaa gaa a; 1315 antisense─uuu cuu ugc ugu uag uua ugg cug g (GC content 40%). These data were analyzed analyzed using the $2^{-\Delta\Delta C}{}_{T}$ method (30). Asterisk (**) indicates a significant difference ($p<0.05$) in treatments. AU represents arbitrary units.

research, we only determined GIRK1, GIRK2 and GIRK4 protein expression (12, 15, 44) since the predominant GIRK heterotetramers are GIRK1/GIRK2 or GIRK1/GIRK4 (22). The 1315 construct reduced GIRK1, GIRK2 and GIRK4 membrane protein levels 24 hours after transfection (figure 3.3). Treatment with U5 increased GIRK1 and GIRK2 protein levels (figure 3.3). When the low GC RNAi negative control (Invitrogen) for the GIRK1 stealth siRNA was used, there were no effects on GIRK1 protein expression (data not shown).

Estrogen Receptor α expression and phosphorylation

Total cell lysates were isolated after 24 hours from additional MCF-7 cells transfected with the 1315 RNAi construct or cells treated with 200 nM U50488H. Western blotting was performed and the membranes were probed with antibodies to ERα. The 1315 construct decreased ERα protein and phosphorylation levels (figure 3.4). ERα protein levels were consistent in U5 treated cells and control (figure 3.4). Phosphorylation of ERα was reduced in U5 treated cells and the 1315 siRNA sample (figure 3.4).

G-Protein Couple Receptor Signaling

Previous data indicates a link between GIRK and the β-adrenergic GPCR signaling pathway including the downstream target cyclic AMP (cAMP) response element binding protein (CREB) (6, 15, 45). β_2-adrenergic and CREB activation was determined by western blot analysis of total cell lysates isolated after 24 hours from MCF-7 cells transfected with the 1315 RNAi construct or cells treated with 200 nM U50488H (figure 3.5). β_2 adrenergic receptor phosphorylation was unchanged in U5 treated cells but were significantly reduced in 1315 siRNA sample (figure 3.5). CREB phosphorylation was reduced in both U5 treated cells and the 1315 siRNA transfected cells (figure 3.5).

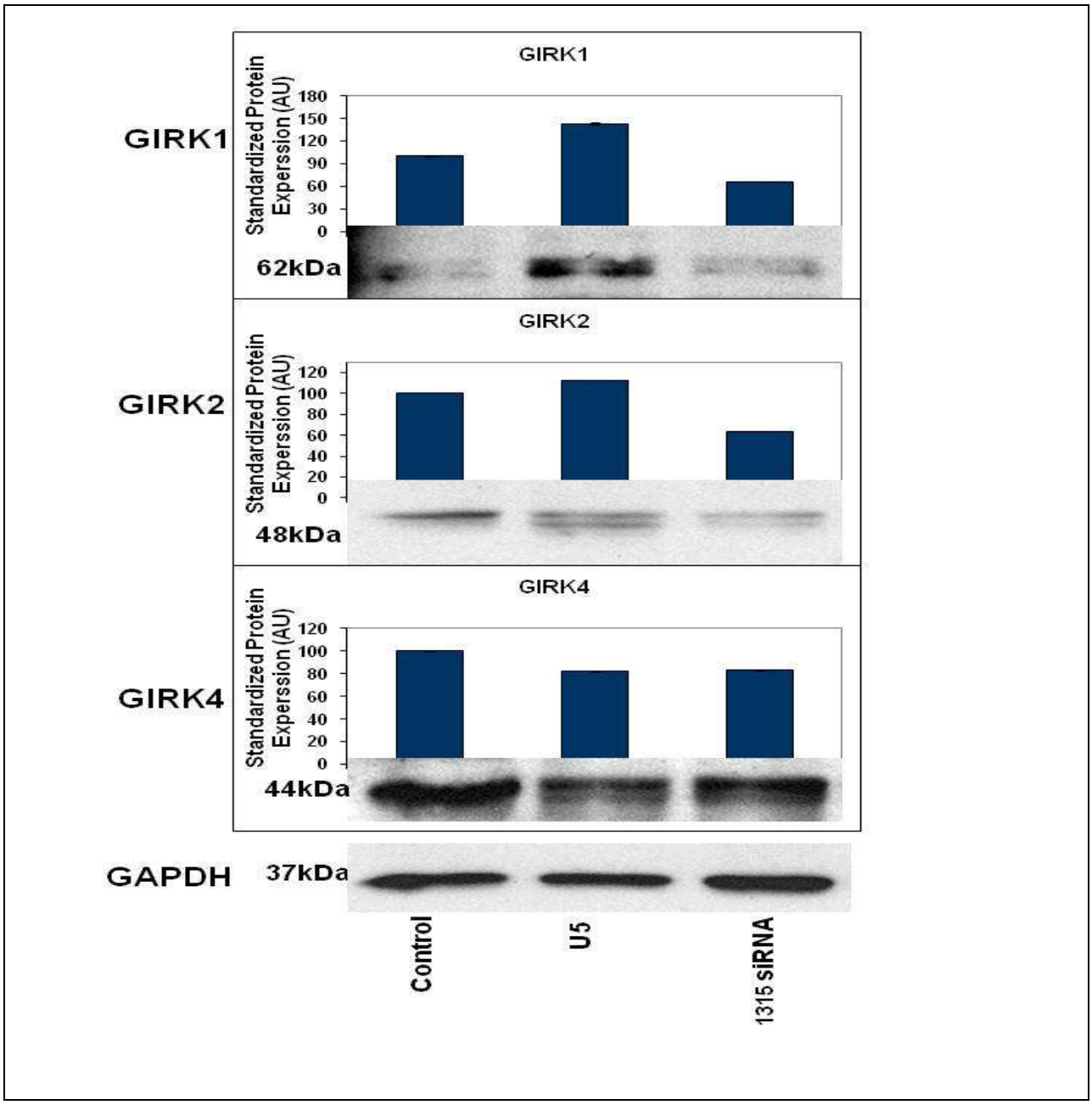

Figure 3.3 GIRK1 protein expression of MCF-7 cells transfected with GIRK1 stealth RNAi and treatment with U50488H. The bands are consistent with the expected size: GIRK1 (62 kDa); GIRK2 (48 kDa); GIRK4 (44 kDa); GAPDH (37 kDa). These are gels representative of two separate experiments. Graphs indicate densitometry of the Western blots. The densitometry of GIRK was divided by the densitometry of the control GAPDH (N=1). Each western blot was done at least twice with representative blot used in the figure. Abbreviations: AU represents (arbitrary units), control represents untreated MCF7 cells, U5 represents treatment withU50488H (trans-(±)3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]cyclohexyl-benzeneacetamide), and 1315 siRNA represents cells transfected with 1315 siRNA duplex

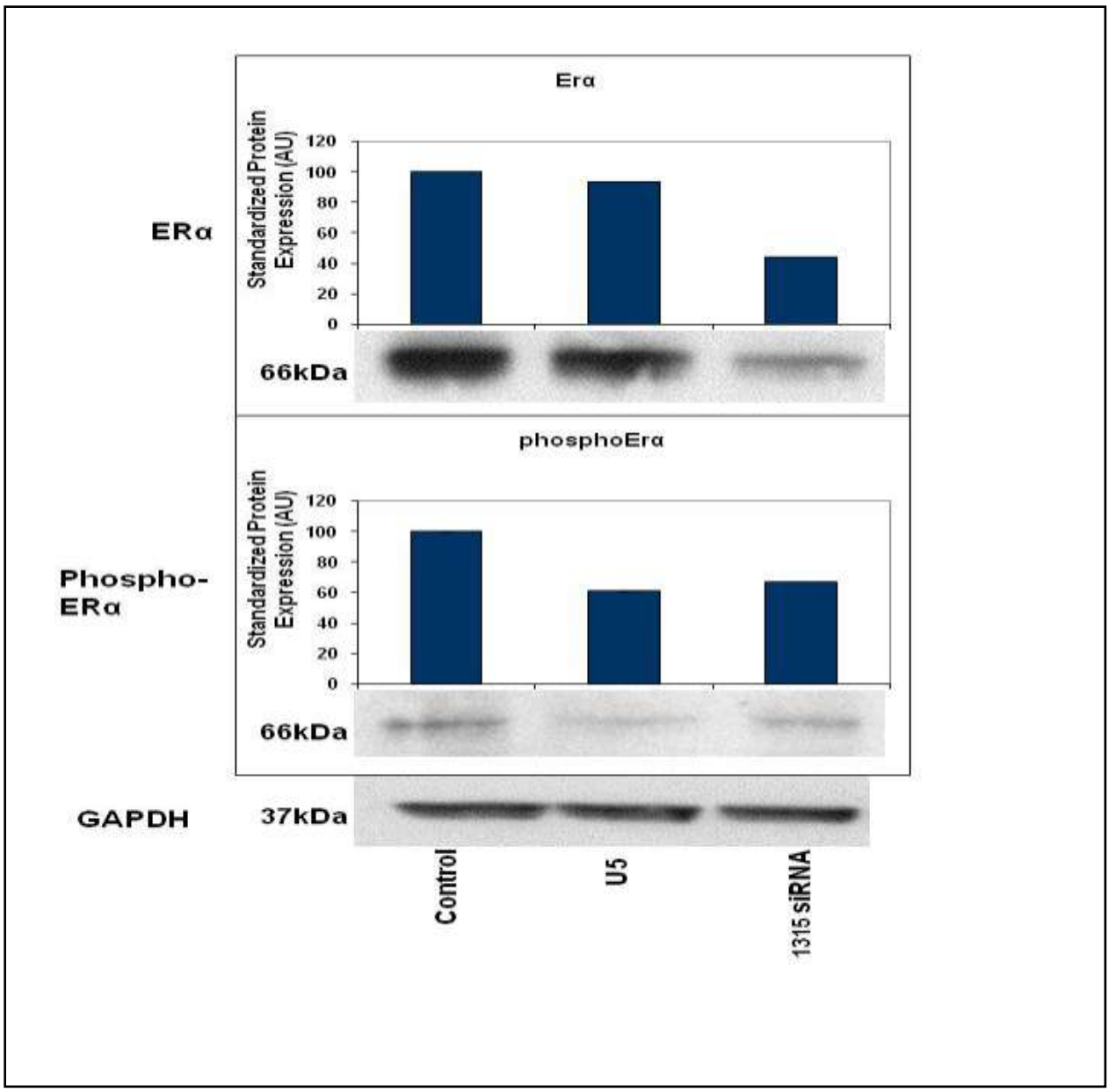

Figure 3.4 ERα protein expression and phosphorylation of MCF-7 cells transfected with GIRK1 stealth RNAi and treatment with U50488H. The bands are consistent with the expected size: GIRK1 (62 kDa); GIRK2 (48 kDa); GIRK4 (44 kDa); GAPDH (37 kDa). These are gels representative of two separate experiments. Graphs indicate densitometry of the Western blots. The densitometry of GIRK was divided by the densitometry of the control GAPDH (N=1). Each western blot was done at least twice with representative blot used in the figure. Abbreviations: AU represents (arbitrary units), control represents untreated MCF7 cells, U5 represents treatment withU50488H (trans-(±)3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]cyclohexyl-benzeneacetamide), and 1315 siRNA represents cells transfected with 1315 siRNA duplex

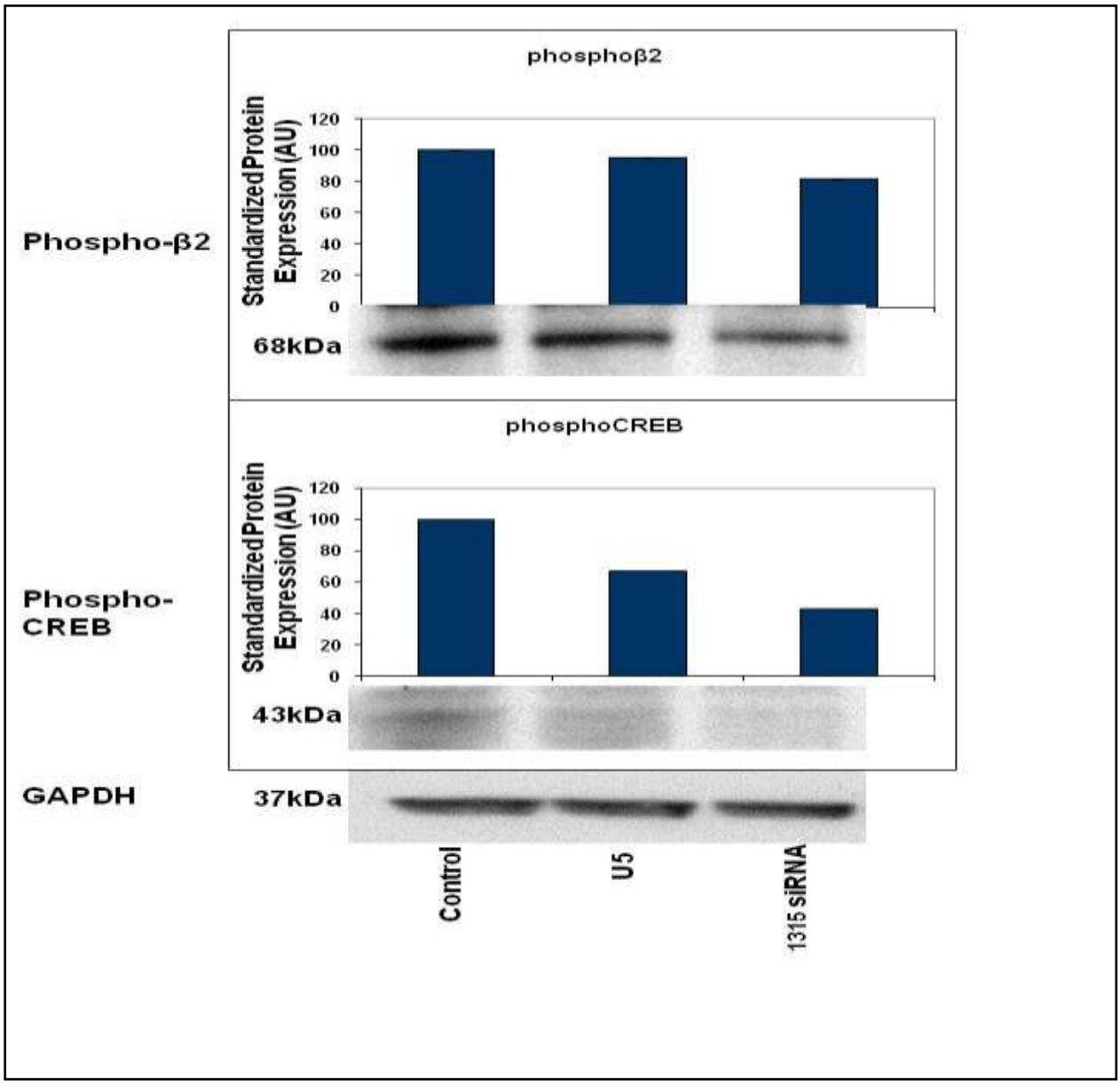

Figure 3.5 Effects of MCF-7 cells transfected with GIRK1 stealth RNAi and treatment withU50488H on G-Protein Couple Receptor Signaling. The bands are consistent with the expected size: phospho-β_2 (68 kDa); phospho-CREB (43 kDa); GAPDH (37 kDa). These are gels representative of two separate experiments. Graphs indicate densitometry of the Western blots. The densitometry of phospho-β_2 or phospho-CREB was divided by the densitometry of the control GAPDH (N=1). Each western blot was done at least twice with representative blot used in the figure. Abbreviations: AU represents (arbitrary units), control represents untreated MCF7 cells, U5 represents treatment with U50488H (trans-(±)3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]cyclohexyl-benzeneacetamide), and 1315siRNA represents cells transfected with 1315 siRNA duplex.

Akt and MAPK signaling

Our lab has previously shown that GIRK1 knockdown in the ER (-) MDA-MB453 breast cancer cell line resulted in decreased protein phosphorylation of Akt and MAPK (15), and these pathways have also been shown to be important in breast cancer progression (6, 12, 15, 31, 36, 45, 61). We used Western blot analysis to evaluated Akt, ERK1/2 and c-jun phosphorylation of total cell lysates isolated after 24 hours from MCF-7 cells transfected with the 1315 RNAi construct or cells treated with200 nM U50488H (figure 3.6). The 1315 siRNA transfection had reduced levels of Akt phosphorylation, ERK1/2 phosphorylation and c-jun phosphorylation (figure 3.6). The phosphorylation levels for all three proteins were unchanged in MCF7 cells treated with 200nM U50488H (figure 3.6).

Discussion

The present study demonstrates that knockdown of GIRK 1 mRNA and protein in ER (+) MCF7 breast cancer cells reduces the expression of ERα. This study supports previous published reports from our lab that identified a correlation between reduced GIRK expression and reduced phosphoylation of signaling intermediates in GPCR, Akt, and MAPK signaling pathways in ER(-) MDA MB453 breast cancer cells (15). Additionally, in the present study we used 200 nM U50488H, a selective κ opioid receptor agonist known to activate GIRK1/2 channels at nanomolar concentrations (57), to further confirm our previous results that functional GIRK channels are found in breast cancer cell lines (6, 12, 15, 45).

We transfected MCF7 cells with three siRNA constructs starting at base pairs 1104, 1315, and 1490, which we have used successfully in previously published investigations (15). Unlike the results we saw in MDA-MB453, only one of the three constructs resulted in GIRK1 reductions 24 hours after transfection, which was the 1315 construct which reduced GIRK

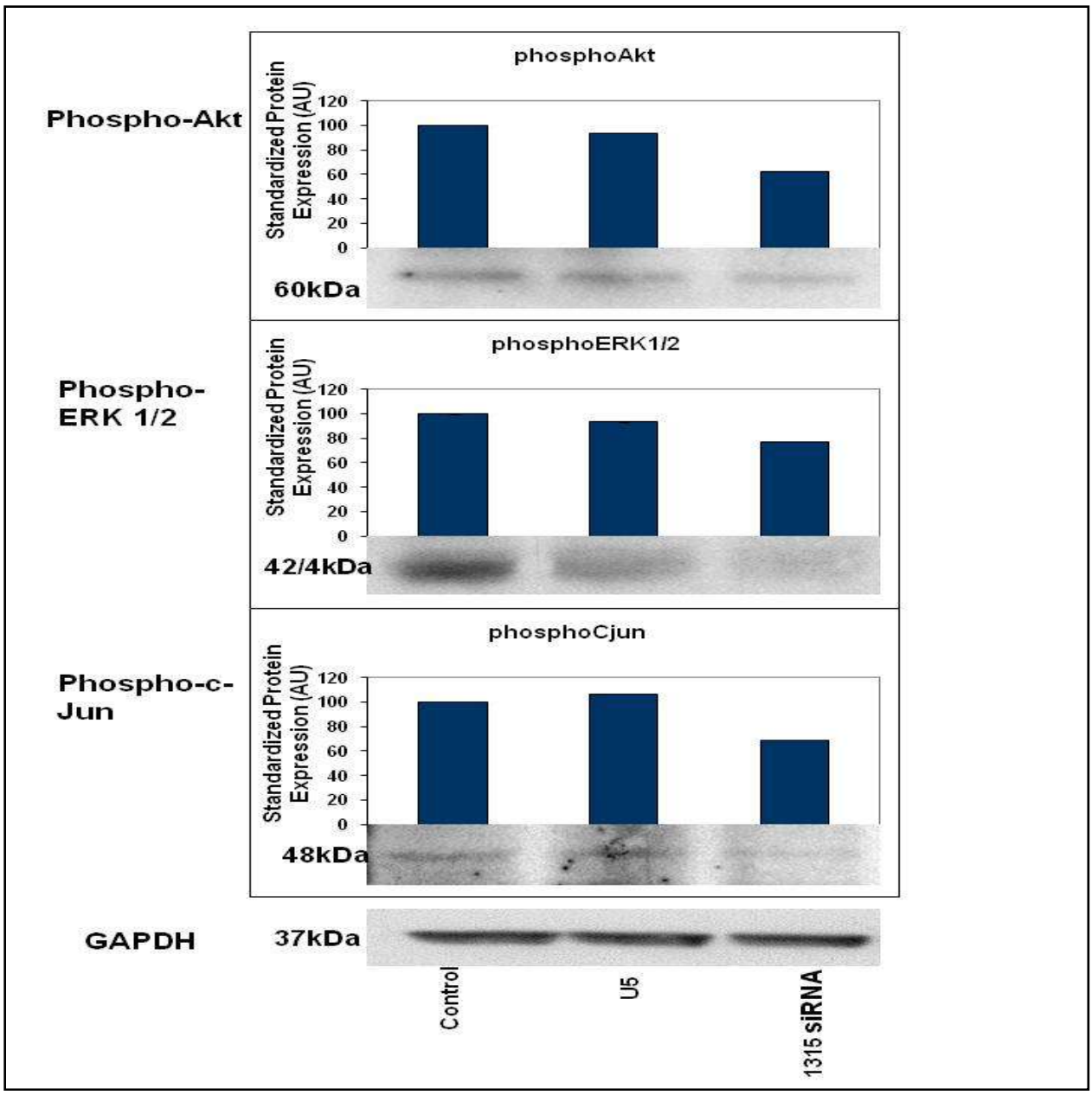

Figure 3.6 Effects of MCF-7 cells transfected with GIRK1 stealth RNAi and treatment withU50488H on Akt and MAPK signaling. The bands are consistent with the expected size: phospho-Akt (60 kDa); phospho-ERK (42/44 kDa); phospho-c-jun (48 kDa); GAPDH (37 kDa). These are gels representative of two separate experiments. Graphs indicate densitometry of the Western blots. The densitometry of phospho-Akt or phospho-ERK or phospho-c-jun was divided by the densitometry of the control GAPDH (N=1). Each western blot was done at least twice with representative blot used in the figure. Abbreviations: AU represents (arbitrary units), control represents untreated MCF7 cells, U5 represents treatment withU50488H (trans-(±)3,4-dichloro-N-methyl-N-(2-[1-pyrrolidinyl]cyclohexyl-benzeneacetamide), and 1315siRNA represents cells tranfected with 1315 siRNA duplex.

mRNA levels 1.85 fold (85%) (figure 3.1). There were no observable differences between the RNAi negative control (Invitrogen) for the GIRK1 stealth siRNA and the other two constructs (1109 and 1490). These differences between ER(-) MDA-MB453 and ER(+) MCF7 cells are likely the result of the variation between cell types, ER status and non genomic actions of E2 signaling. The 1315 siRNA construct was retained for the remaining experiments in this project and the other two duplexes were not used because they had no effect on GIRK1 mRNA levels.

GIRK protein expression was studied after additional MCF7 cells were transfected with the 1315 siRNA construct or treated with U50488H. U5 activates GIRK1/2 channels at nanomolar concentrations in a κ-opioid receptor mediated pathway (57). Data from our lab has shown the GIRK 1 and GIRK 2 protein expression dramatically increases after 24 hour and 48 hour estrogen treatment (chapter 2), which may suggest that channels composed of GIRK 1/2 are associated with proliferation and may be associated with the clinical aggressiveness that Stringer previously found in primary breast samples (52). Treatment with 200nM U50488H resulted in increases in GIRK 1 and GIRK2 membrane protein expression, which may suggest that there are functional GIRK channels in MCF7 breast cancer cells (figure 3.3). Transfection with the 1315 siRNA construct resulted in decreased GIRK1, 2 and 4 levels (figure 3.3). GIRK1 mRNA levels were reduced by 1.85 fold (85%) but GIRK1 protein levels (reduced approximately 34%) were not reduced as much possibly because of protein stability of GIRKs. GIRK1 can be glycosylated at Asn 119 without changing function but this glycosylation may add to protein stability (41). Other studies suggest that glycosylation of GIRK1 occurs during channel heteroassembly and is associated with plasma membrane localization (23). Another plausible explanation is our established laboratory protocol for transfection requires cells to be supplemented with serum 6 hours after transfection and serum contains endogenous levels of estrogen. Previous data from

our lab has demonstrated that estrogen increases protein expression of GIRK1 and GIRK2 in estrogen responsive MCF7 breast cancer cells but not in estrogen non resonsive MDA-MB453 breast cancer cells (Chap 2).

The membrane protein levels of GIRK2 and GIRK4 were reduced by GIRK1 knockdown. We determined that our 1315 siRNA construct was specific to only the GIRK1 subunit using the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/) nucleotide Blast Search. We did not evaluate the protein expression of GIRK2 and GIRK4 in our previously published siRNA study. However, Koyrakh et al. found that GIRK1 knockout mouse hippocampi resulted in reduced levels of other GIRK subunits using immunohistochemistry (23). Additionally, previous studies from our lab have demonstrated increased GIRK1 membrane protein expression in MDA-MB453 breast cancer cells 48 hours after transfection with plasmids containing GIRK1 or GIRK4 (12). These studies support our current findings and may suggest that reductions in GIRK mRNA or over expression of GIRK subunits may play a role in trafficking mechanism of GIRK channels to the cell membrane. These findings are poorly understood at present and will require further investigation.

Knockdown of GIRK 1 resulted in decreased estrogen receptor α (ERα) gene expression (approximately 55%) (figure 3.2), reduced ERα protein expression (approximately 55%) and phosphorylation (figure 3.4). This is the first report of GIRK1 knockdown resulting in reduced ERα and the mechanism of action is poorly understood at present. However, this data may suggest that targeting GIRK channel activity may interfere with estrogen mediated proliferation in ER(+) positive breast cancer. Experimental and clinical results have established that the role of estrogen exposure is a major risk factor that contributes to the rise of sporadic breast cancers (8, 13, 26, 27, 32, 48, 61). Multiple studies have identified a supportive role of ion channels,

specifically potassium channels, in proliferation and cancer progression (8, 24, 58). Additionally, multiple labs have demonstrated that pharmocological blockage of potassium channels exerted an inhibitory action on cellular proliferation, which suggests a significant role of potassium current across the cell membrane in maintaining cellular homeostasis and cell proliferation (8, 24, 37, 38, 39, 40, 58). Taken together, the reduction in ERα may be due to a loss of cellular homeostasis rather than a direct effect of GIRK function on ERα, but more work will be required to investigate homeostatic regulation and/or downstream funtions of GIRKs in estrogen mediated proliferation.

Previous data from our laboratory identified a functional correlation between β-adrenergic signaling and GIRK channels in breast cancer cells (6, 12, 15, 45). It has been reported that β-adrenergic stimulation resulted in increased GIRK currents in rat cardiomyocytes and *Xenopus laevis* oocytes coexpressiong β_2-adrenergic receptors, and GIRK 1/4 subunits (33). Additionally, rat atrial myocytes transfected with β-adrenergic receptors and treated with isoproterenol (β-adrenergic agonist) induced GIRK currents when compared to non-transfected rat atrial myocytes (59). Phosphorylation of β_2-adrenergic receptors (a member of the G-protein coupled receptor pathway) can lead to phosphorylation of CREB and/or ERK1/2 (11, 51, 60). In the present study, GIRK1 knockdown resulted in reduced phosphorylation of β_2-adrenergic receptors. Additionally, we saw a reduction in CREB phosphorylation. These results support and expand our previous findings from our laboratory (6, 12, 15, 45). Further research is needed to determine the mechanism of reduced β_2-adrenergic phosphorylation by GIRK1 knockdown.

We examined other pathways significant in cancer progression and estrogen signaling to determine if GIRK1 knockdown in ER(+) MCF7 cancer cells was similar to the GIRK1 knockdown results seen in ER(-) MDA-MB453 breast cancer cells (15). Akt signaling has been

suggested to mediate multiple transforming events in breast cancer including proliferation and metastasis (31, 56, 61). In the present study, Akt phosphorylation was reduced by GIRK1 knockdown at 24 hours after treatment and these results support similar results in ER(-) MDA-MB453 cells (15). These results demonstrates a functional correlation between GIRK activity and Akt signaling in breast cancer cells, indicating that GIRK function may be a contributing factor in supporting progression of normal tissue to metastatic tissue. Results from other investigators have shown that insulin-like growth factor (associated with GPCR and MAPK signaling) can increase the activity of *ether-a-go-go* potassium channels by stimulating Akt; these findings are significant because *ether-a-go-go* potassium channels play a significant role in mediating cellular proliferation (4, 26, 61, 62). The U5 treatment had no effect on Akt, which could be interpreted to mean that activation of GIRK1/2 channels are not necessarily a causative agent in cancer progression but GIRK channel activation may play more of a supportive role. However, further research is required to confirm this hypothesis.

MAP kinase, another significant pathway in cancer progression that is associated with estrogen signaling, has been shown to be a mediator of breast cancer cell migration and proliferation (36, 61). High potassium levels, in cerebellar granule neurons, resulted in sustained activation of MAP kinase, insulin-like growth factor activated Akt, and CREB phosphorylation (63). These data are significant because all of these pathways are involved in proliferation, metastasis, suppression of apoptosis, and angiogenesis, which are important in cancer progression (6, 13, 26, 27, 32, 61, 62). Suppression of MAP kinase activity has been shown to reduce the effects of PGE(2) on ROMK-like small conductance potassium channels in the distal tubule segment cortical collecting duct cells (18). MAPK has been shown to affect many ion channels in breast cancer including GIRKs (4, 5, 8, 15, 18, 24, 28). In the present study, GIRK

knockdown reduced ERK1/2 and c-jun phosphorylation, which may signify that GIRK is involved in MAPK signaling. Kunzelmann 2005 states that K^+ channels are required for oncogenic transformation, cell migration, metastasis and malignancy (24). These data suggest that GIRK may have relevance as a therapeutic target because GIRK 1 expression has been shown in primary breast cancer tissue to be associated with clinical aggressiveness (52). Additionally, other investigators identified GIRK1 mRNA expression was associated with lymph node metastasis in tissue samples collected from non-small cell lung cancer patients. The significance of GIRK1 is well established, but GIRK1 requires other subunits to form functional channels, which suggests future work is required to clarify how channel composition and function contribute to malignancy. Additionally, more work is required to elucidate the supportive role GIRKs may play in cancer and the potential ways to manipulate these channels as a therapeutic target.

MCF7 breast cancer cells treated with 200nM U50488H increased GIRK1 and GIRK2 subunits, which may suggests that these channels are functional. More work is require to elucidate the functionality of GIRK channels in breast cancer and the potential differences in functionality in regards to channel composition. We have previously, shown that estrogen treatment significantly increased the protein expression of GIRK 1 and GIRK 2 dramatically, which was correlated with increased cell proliferation and increased intracellular potassium concentrations (chapter 2). However, MCF7 cells treated with U50488H for 24 hours had similar protein phosphorylation levels of signal transduction pathways as our untreated control. We hypothesize that estrogen increased expression in GIRK1/GIRK2 channels may be ERα responsive and my contribute to cancer progression via increased proliferation. However, based on the data in the present study activation of GIRK1 and GIRK2 by 200nM U50488H treatment

did not result in similar results as estrogen treatment suggesting that activation of these channels alone does not have a causative role in breast cancer development. Other effects of U50488H on the cell may also contribute to the differences in the expected results. Plasma membrane potassium channels are required for cell proliferation and are directly involved in apoptosis (24). These data taken together puts forward a unique prospective that activation of these channels may provide an essential supportive environment in the development of cancer in conjunction with additional contributing factors.

Multiple sources indicate that potassium channels may be valuable targets for cancer therapy (6, 9, 12, 15, 22, 24, 37, 38, 39, 40, 45, 58). It has also been suggested that potassium channel blockers in combination with current clinical treatment strategies may improve current therapies because potassium channels appear to be essential for cell proliferation and apoptosis (1, 22, 24, 37, 39, 40, 53, 58). More work is needed to further define the supportive role ion channels play in cancer and how to specifically target these channels in a highly localized fashion (24). Additionally, our knowledge is limited on the functional role these channels play in tumor development or if these channels are differentially expressed in malignant tissues compared to normal tissues. The results of this study suggest that knocking down the expression of GIRK1 may interfere with multiple signaling pathways important in cancer progression, including estrogen signaling. These findings and our hypothesized mechanism of GIRK involvement are summarized in chapter 1 figure 1.7. These results and previously published results from our lab suggest that disruption of GIRK 1 may offer the potential means to interrupt these multiple signaling pathways regardless of the presence of the estrogen receptor and may interfere with estrogen mediated proliferation in ER(+) MCF7 breast cancer cells.

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Identification of Potential Candidate Genes Associated with G-Protein Inwardly Rectifying Potassium channels in 17β-Estradiol Treated MCF7 Breast cancer cells

Chapter 4

Abstract

Breast cancer is the second leading cause of cancer death and in 2009, the American Cancer Society estimates that over 192,000 new cases of breast cancer will be diagnosed, and over 40,000 women will die from breast cancer. Estrogen (E2) is required for normal female development and reproduction, but long-term exposure is carcinogenic and considered a risk factor for breast cancer. E2 acts as a mitogen and has been shown to modulate the action of various ion channels throughout the body. Accumulating evidence has shown the many of these ion channels are essential for cell proliferation and cell survival, which are key events in cancer progression. Previous work from our laboratory demonstrates that treating estrogen responsive MCF7 breast cancer cells with E2 increases the expression of G-protein inwardly rectifying potassium channel 1 (GIRK1) and GIRK2, which were also associated with increased cell proliferation and intracellular potassium concentrations. Based on these findings, we suggest that GIRK 1 and 2 are associated with cell proliferation but the mechanisms are currently unknown. In the present study, we used global gene expression profiling by Affymetrix GeneChip microarray analysis of nontreated and E2 treated MCF7 Cells. We observed 489 differentially expressed genes (283 upregulated and 206 downregulated) that were comprised largely of transcription and cell cycle associated genes. This study identified several human cell cycle associated genes that are both responsive to E2 treatment and are functionally correlated with GIRKs. Five genes were selected for further analysis by real time PCR. These genes include: Cell division cycle homolog 6 (*CDC6*), Defective in sister chromatid cohesion 1 homolog (*DSCC1*), Forkhead box M1(*FOXM1*), Maternal embryonice leucine zipper kinase (*MELK1*), and PDZ binding kinase (*PBK*).

Introduction

Breast cancer is the one of the most common cancers in women and is reported as the second highest cause of cancer death after lung cancer (1). Multiple risk factors contribute to the onset of breast cancer such as lifestyle (including smoking, drinking, and diet), family history, age, race, geographical location, and hormone exposure (including age of menarche, age of parturition, and age of menopause) (1). It is thought that one million new cases of breast cancer are diagnosed each year worldwide and it is correlated to a higher incidence in western countries (2). It has been suggested that the US and Europe account for 16% of the world population and approximately 60% of the worldwide incidence of breast cancer (51, 52). The American Cancer Society (ACS) approximates that 192,000 women will be diagnosed with breast cancer in 2009 and estimates 40,000 women will die from breast cancer in 2009 (1). Today, breast cancer accounts for 22% of all female cancers (51). ACS suggests that approximates 1 in 8 women will develop invasive breast cancer during her life and 1 in 35 cases will be fatal (1). In addition, men are at risk in rare cases (1).

Estrogen (E2) is a well documented risk factor associated with breast cancer and is considered a carcinogen (81). The role of E2 in carcinogenesis is unclear, but it is thought to be the consequence of two complimentary pathways, specifically E2 metabolism and ER signaling (81). Yager et al. states that oxidative metabolites of estrogen have genotoxic, mutagenic, and transforming effects on cells; suggesting that E2 may initiate and/or contribute to the progression of carcinogenesis (81). Previous studies have shown that estrogen metabolites can induced genetic mutations in MCF-7 breast cancer cells and induced neoplastic transformation of non-turmorgenic MCF-10F breast cells treated with E2, which provides evidence of the mutagenic properties of E2 and E2 associated metabolites (59, 61). The carcinogenic action of E2

modulates signaling transduction pathways that are correlated with proliferation, inhibition of apoptosis, cell migration, and induction of angiogenic factors via either nongenomic actions or the estrogen receptor (12, 27, 37, 43, 53, 56, 59, 64, 71). Estrogen receptors are a powerful predictor of breast cancer prognosis and ERα is a standard therapeutic target in treatment (1, 8, 28, 81).

It has been suggested that approximately 40% of human primary cancer tissues express G-protein inwardly rectifying potassium channel 1 (GIRK1) mRNA and the expression of GIRK1 mRNA is significantly correlated to lymph node metastasis(67). As a result tumors that express GIRK1 tend to have a poor clinical prognosis (67). GIRK expression is normally restricted to the heart and brain (67). Our lab has shown that estrogen exposure increases the protein expression and membrane localization of GIRK1 and GIRK2 (chapter 2). Estrogen exposure also increased intracellular potassium concentrations and cell proliferation (chapter 2). GIRK channels are responsive to various neurotransmitters and hormones, which may have the ability to modulate various cellular activities (32). Estrogen has been shown to negatively regulate the secretion of gonadotropin-releasing hormone from the mediobasal hypothalamus thereby regulating the reproductive cycle which can be achieved by rapid hyperpolarizaion of GIRK channels (5, 23, 29, 30, 36, 42, 65). Evidence from our lab has demonstrated that GIRK protein and gene expression are decreased in cells treated in serum free media (9). Serum-free media also decreased GIRK protein expression, possibly due to lack of estrogen in the media (9). Additional research has indicated that estrogen may have effects on potassium channels. Specifically, activation of potassium channels may be involved in the effects of E2 in the brain (29, 32, 47, 70). Results from our lab have also shown that GIRK1 knockdown using siRNA, reduced ERα mRNA levels and protein expression although the mechanism is not entirely

understood at present (chapter 3). Therefore, GIRKs and many other ion channels are thought to be a potential biomarkers of cancer invasiveness and may be associated with cell proliferation, and as a result may have value as a potential therapeutic target (chapter2, 4, 6, 9, 14, 19, 22, 32, 34, 54, 57, 63, 67, 73, 76, 77).

Our lab has demonstrated that a functional link exists between GIRK 1 expression and β_2 adrenergic receptor (ADRB2) function, which is part of the G-protein couple receptor (GPCR) family (6, 7, 54, 58). Current research suggests that ADRB2 activation of cell cyclic AMP (cAMP) protein kinase A (PKA) signaling pathway and is thought to stimulate angiogenesis and tumor growth in multiple cancers including ovarian cancer (69). Our lab has also demonstrated that estrogen receptor positive (ER (+)) cell lines MCF-7, MDA-MB-361, and ZR-75-1 and the ER negative (-) cell line MDA-MB-453 expressed mRNA for the GIRK1 channel while the ER (-) cell lines MDA-MB-468 and MDA-MB-435S did not express GIRK1 (54). Our lab demonstrated GIRK 1, 2 and 4 protein expression in multiple breast cancer cell types, which was the first report of GIRK protein expression outside of the tissues of origin (9). Data from multiple laboratories suggest that GIRK is associated with proliferation and clinical aggressiveness, and as a result may be a valuable therapeutic target (chapter 2, 4, 6, 7, 9, 54, 58, 67).

In the present study, our aim was to identify potential candidate genes associated with GIRK1 and GIRK2 increases seen in 10 nM E2 treated MCF7 breast cells. Treatment of MCF7 cells with E2 increased cell proliferation and intracellular potassium concentrations (chapter 2). It has been suggested that high intracellular concentrations of potassium are seen in rapidly dividing cells (34, 68). Additionally, many growth factors and mitosis related enzymes require a minimum intracellular potassium concentration and levels below this point result in reduced

proliferation (34). Our results suggest that increases in GIRK1 and GIRK2 are associated with both increased cell proliferation and increased intracellular potassium concentrations, and the proliferation and increased potassium levels may be inter-related (chapter 2). In this study, the Affymetrix human 1.0 ST chip platform was employed to identify differentially expressed genes between untreated cells and cells treated with E2, which was associated with increased GIRK1 and GIRK2 protein expression. We identified 489 differentially expressed genes (283 upregulated and 206 downregulated). Of these differentially expressed genes, we identified several human cell cycle associated genes that are responsive to E2 treatment and are functionally correlated with GIRKs. Five of these potentially GIRK related genes that were upregulated after 24 hour E2 treatement were selected for further analysis. These were Cell division cycle homolog 6 (*CDC6*), Defective in sister chromatid cohesion 1 homolog (*DSCC1*), Forkhead box M1(*FOXM1*), Maternal embryonice leucine zipper kinase (*MELK1*), and PDZ binding kinase (*PBK*). These cell cycle associated genes were selected because potassium channels are suggested to have a role in cell cycle progression, and proliferation, especially in cancer (34).

Materials and Methods

Cell Culture

The human estrogen responsive MCF7 breast cancer cell line was purchased from American Type Culture Collection (ATCC; Rockville, MD, USA) and was maintained RPMI 1640 media supplemented with fetal bovine serum (10% v/v), L-glutamine (2 mM), 100 U/ml penicillin and 100 ug/ml streptomycin at 37°C in an environment of 5% CO_2. Cells were treated with 10 nM 17β-estradiol (Sigma St. Louis, MO) that was dissolved in 100% ethanol (AAPER

Shelbyville, KY) and diluted with water, and the E2 was added to charcoal stripped serum enriched media. The final concentration of ethanol in the media is 0.001 %.

Microarray Analysis

The Affymetrix GeneChip Human Gene 1.0ST array (Affymetrix, Santa Clara, CA) were used to define gene expression profiles of untreated and 10nM E2 treated MCF7 breast cancer cells in triplicate. The RNA from these samples was provided to the University of Tennessee, Knoxville Affymetrix core facility. Target labeling and hybridization were performed following Affymetrix's protocol for Whole Transcript Sense Target Labeling at the Affymetrix core facility. Raw Affymetrix intensity measurements of all probe sets were background corrected, normalized, and summarized into gene expression level measurements using GC RMA (GeneChip Robust Multiarray Averaging) method with a quantile normalization (80). All subsequent analysis was performed using Partek Genomics Suite version 6.4.6.09.129 (Partek, Inc, St. Louis, MO). An initial principle components analysis (PCA on global gene expression indicated that samples grouped according to preparation date, so a batch effects transformation was used to reduce the variation between the samples from this effect. PCA run on the resultant data showed the samples grouping according to treatment. A one way ANOVA was used to select target genes that were differentially expressed between treatments (24 hour non treated and 10 nM E2 treatment), based on a global fold change criteria of ±1.5-fold and a q-value of <0.05 (q-value is the positive false discovery rate analogue of the p-value that eliminates the need to set the error rate before analysis) (66). Hierarchical clustering using the average linkage with Pearson's correlation was applied to the raw values of the whole set of differential expressed genes and markers. Gene ontology and gene function were generated using Onto-express (Wayne State University, Detroit MI) (10, 31).

RNA Isolation, cDNA Synthesis, and Real Time Polymerase Chain Reaction (PCR)

RNA was isolated by an Absolutely RNA kit (Stratagene, La Jolla, CA). After DNase treatment of RNA for 15 minutes at room temperature, reverse transcription was carried out using 2 μg RNA, 1 μl random decamer primers (Ambion, Foster City, CA) and nuclease-free water, which were heated to 70°C for 3 minutes for primer binding. This mixture was added to 0.5mM of dNTPs, 10mM DTT, 40U RNase ribonuclease inhibitor (Promega, Madision, WI), 200U Moloney murine leukemia virus (M-MLV) reverse transcriptase (Life Technologies, Carlsbad, CA), PCR buffer (10mM Tris-HCl pH 8.3 50mM KCl, 1.5mM $MgCl_2$) and nuclease-free water in a total volume of 20μl. This mixture was incubated at 37°C for 1h, and then the enzymes were heat inactivated for 10 min at 92°C. A negative control reaction was performed without the M-MLV to determine if there was a genomic DNA contamination.

Real-time PCR was done using the Cepheid smart cycler. The real-time PCR primers, probes and real-time conditions are listed in table 4.1. 18S rRNA detection reagents (Eurogentec, San Diego, CA) were used for normalization of the data. Real time PCR data was analyzed using the $2^{-\Delta\Delta C}{}_T$ method (39).

siRNA Knockdown

Three stealth siRNA constructs (Invitrogen, Carlsbad, CA, U.S.A.) were made starting at bases 1104, 1315, and 1490 of the GIRK1 sequence (GenBank # NM_002239). These constructs are: 1104 sense─gga aac aac ugg gau gac uug uca a; 1104 antisense─uug aca agu cau ccc agu ugu uuc c (GC content 44%); 1315 sense─cca gcc aua acu aac agc aaa gaa a; 1315 antisense─uuu cuu ugc ugu uag uua ugg cug g (GC content 40%); 1490 sense─acu ugc cca uga aac uuc aac gaa u; 1490 antisense─auu cgu uga agu uuc aug ggc aag u (GC content 40%). In addition, the low GC RNAi negative control (35%–45% GC) was used as recommended by the

Gene; Genbank Accession #	Primers and Probes	Conditions
***GIRK1*; NM_002239**	**forward 5´-ctctcggacctcttcaccac-3´ reverse 5´-gccacggtgtaggtgagaat-3´ TaqMan probe 6-FAM-tcaagtggcgctggaacctc-TAMRA (Sigma-Genosys, Woodlands, TX).**	**The real-time PCR conditions for *GIRK1* 95^{o} C for 120 seconds, followed by 45 cycles of 95^{o} C, 15 seconds; 62^{o} C, 10 seconds; and 72^{o} C, 15 seconds**
***CDC6*; NM_001245**	**forward 5'-gcgcgttctctgctcactac-3' reverse 5'-tggtgggcacctgtaatcc-3' TaqMan probe 6-FAM-agcacccgcttcccaggttgaa-BHQ1 (Biosearch Technologies, Novato, CA).**	**The real-time PCR conditions for *CDC6* were the same as GIRK1 except for the annealing temperature of 56^{o} C**
***DSCC1*; NM_024094**	**forward 5'-cacccaaactggaagcctctag-3' reverse 5'-cgccaggcagaggagtaa-3' TaqMan probe 6-FAM-atccatcgtttacatccatgaaattggc-BHQ1 (Biosearch Technologies).**	**The real-time PCR conditions for *DSCC1* were the same as GIRK1 except for the annealing temperature of 58^{o} C**
***FOXM1*; NM_202002**	**forward 5'-cctgctgcctgattatgcaaa-3' reverse 5'-ggacagcagaggaatcagatactc-3' TaqMan probe 6-FAM-cagtcacaccctagccactgctgg-BHQ1 (Biosearch Technologies).**	**The real-time PCR conditions for *FOXM1* were the same as GIRK1 except for the annealing temperature of 58^{o} C**
***MELK*; NM_014791**	**forward 5'-gcgatgcctgggtttacaaa-3' reverse 5'-tccggcaggatggaagaatc-3' TaqMan probe 6-FAM-agtggaagacatcctatctagctgca-BHQ1 (Biosearch Technologies).**	**The real-time PCR conditions for *MELK* were the same as GIRK1 except for the annealing temperature of 56^{o} C**
***PBK*; NM_018492**	**forward 5'-tggaagctgaggagaatatgcct-3' reverse 5'-gaggagatccaagagatcaagacaa-3' TaqMan probe is 6-FAM-agctccttggatacttcagactctggt-BHQ1 (Biosearch Technologies).**	**The real-time PCR conditions for *PBK* were the same as GIRK1 except for the annealing temperature of 58^{o} C**

Table 4.1. Real-time PCR primers, probes and real-time PCR conditions.

manufacturer (Invitrogen). These constructs (200 pMol) were transfected into the MCF7 breast cancer cell lines using lipofectamine 2000 (20 µl/60 mm dish) (Invitrogen) (19).

Results

Distribution of the biological functions of differentially expressed genes

Previous studies from our lab have shown the 10 nM E2 treatment increased membrane protein expression of GIRK1 and GIRK2 in MCF7 breast cancer cells (chapter 2). Additionally, these increases in GIRK expression were correlated with increased intracellular potassium concentrations and increased cell proliferation (chapter 2). Previous studies from other labs have suggested that cell cycle progression and the regulation of cell cycle relevant proteins may be directly regulated by membrane voltage, and intracellular potassium concentrations (34, 68). Previous genome-wide studies of MCF7 breast cancer cells treated with E2 identified up-regulation of positive proliferation regulators and genes involved in cell cycle progression (16). In the present study, we identified 489 differentially expressed genes (283 unregulated and 206 downregulated). We evaluated our list of differentially expressed genes from the comparison of untreated MCF7 cells to 24 hour 10 nM E2 treated MCF7 cells using Onto-express software (Wayne State University, Detroit MI) (10, 31). A pie chart illustrating the biological processes of all of the identified differentially expressed genes is shown in figure 4.1. The largest portion of genes fell into an unknown functional group. The remaining genes were classified proportionally from the largest functional groups to the smallest functional groups: cell cycle, transcriptional regulation and transcription, mitosis, cell division, metabolic processes, DNA replication, biological processes, signal transduction, transport and DNA repair, which may be associated with GIRK expression.

Cluster analysis of untreated MC7 and 10 nM E2 treated MCF7 breast cancer cells, and identification of candidate genes

Cluster analysis of untreated and 10 nM MCF7 treated breast cancer cells expression pattern was performed (figure 4.2). Raw intensity values were background corrected and normalized using GC RMA (80). A one-way ANOVA with a positive false discovery rate was performed with a global fold change criteria of ±1.5-fold and a q-value of <0.05. Sample replicates are shown horizontally and significant genes are clustered vertically (figure 4.2). Red represents up-regulated genes and blue represents down-regulated genes. Our laboratory is interested in the functional correlation of potassium channels and cancer. Potassium channels are essential for proliferation because membrane charge and intracellular potassium concentrations are thought to be associated with cell cycle progression and the regulation of cell cycle relevant proteins (34,68). As a result, we selected 5 five up-regulated candidate genes that are associated with cell cycle, transcriptional regulation/ transcription, mitosis, and cell division (figure 4.2 and table 4.2) for further analysis by real-time PCR because membrane bound potassium channels, including GIRKs, are thought to have a role on cell proliferation (chapter2, 32, 34, 63, 67, 68, 73, 76, 77).

GIRK1 gene expression using Real-Time PCR

GIRK1 gene expression was not one of the differentially expressed genes using the criteria outlined above to select potential candidate genes. These criteria were a one-way ANOVA with a positive false discovery rate that was performed with a global fold change criteria of ±1.5-fold and a q-value of <0.05. We had previously seen an increase in membrane protein expression of GIRK 1 and GIRK2 when treated with 10nM E2 for 24 hours (chapter 2). We evaluated GIRK1 gene expression of our microarray samples and found a 1.37 mean fold

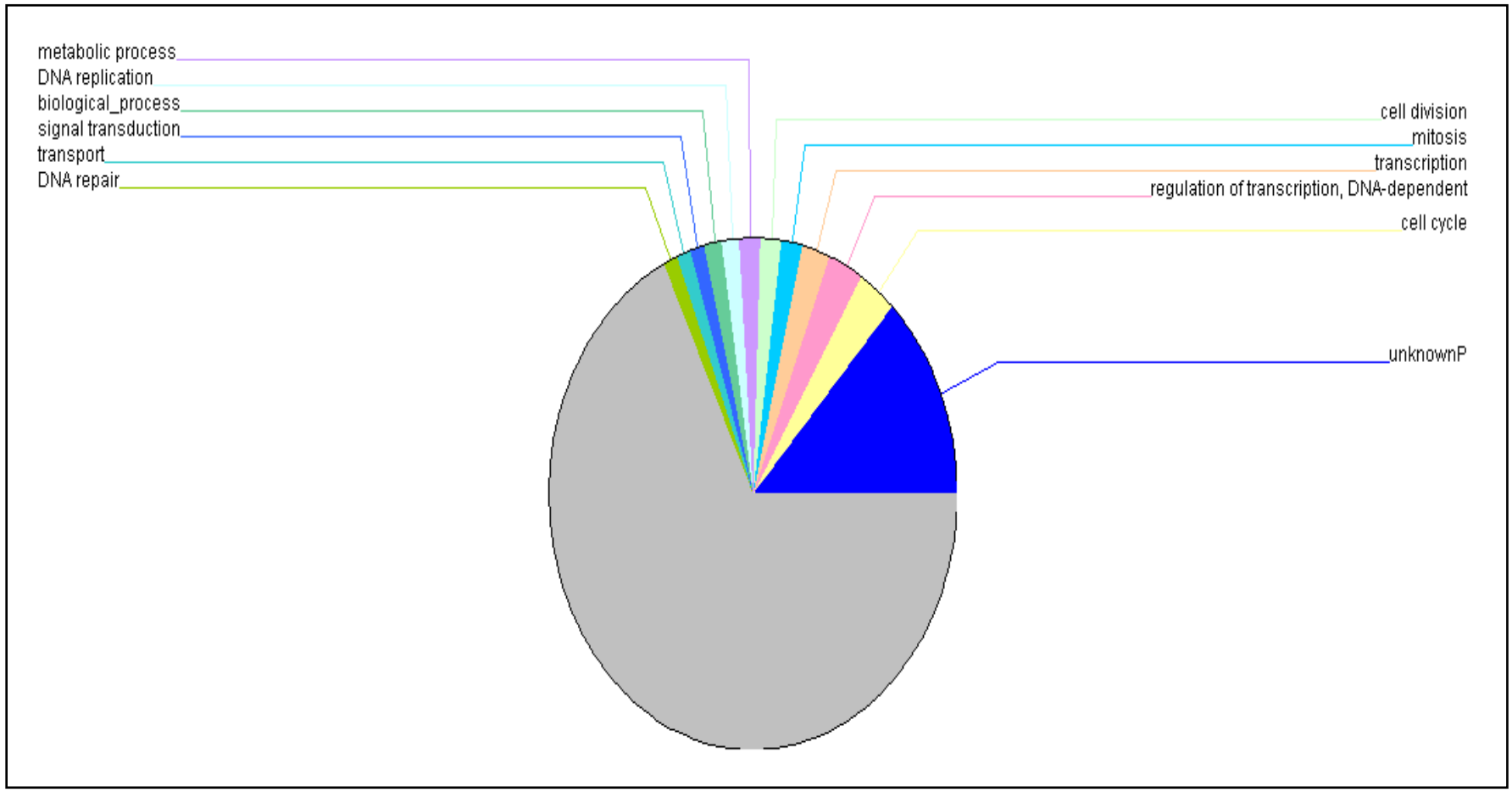

Figure 4.1 Biological functions of differentially expressed genes from estrogen treated MCF7 cells compared to untreated MCF7 cells. Biological processes pie chart generated using Onto-express (Wayne State University, Detroit MI) (10, 31).

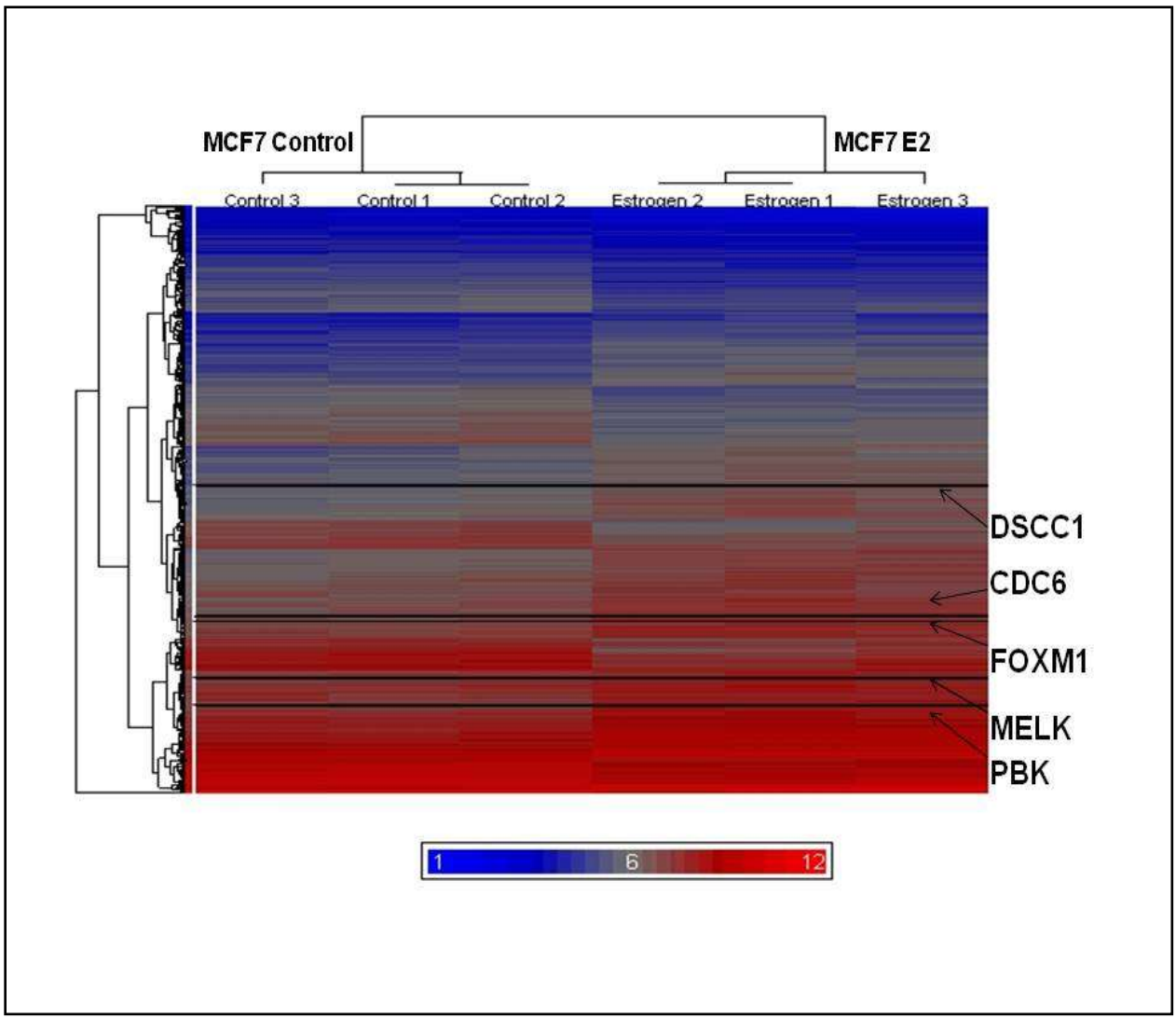

Figure 4.2 Summary of the 489 differential expressed genes and five selected genes by hierarchical clustering analysis. Raw intensity values were background corrected and normalized using GC RMA. One way ANOVA with a positive false discovery rate was performed with a global fold change criteria ±1.5-fold and a q-value of <0.05. Sample replicates are shown horizontally and significant genes are clustered vertically. Red represents up regulated genes and blue represents down regulated genes. Abbreviations Con represents (24hour non-treated control), E2 represents (24 hour 10 nM E2 treatment), DSCC1 represents (Defective in sister chromatid cohesion 1 homolog), CDC6 represents (Cell division cycle homolog 6), FOXM1 represents (Forkhead box M1), MELK represents (Maternal embryonice leucine zipper kinase), and PBK represents (PDZ binding Kinase).

RefSeq ID	Probe set ID	Gene symbol	Names	Fold change; q-value	Function	Chrom loc	Ref
NM_024094	8152582	*DSCC1*	Defective in sister chromatid cohesion 1 homolog	2.2; q=0.0002	DNA replication via involvement in replication factor complex; S phase of cell cycle	8q24.12	3, 11,45
NM_001254	8007071	*CDC6*	Cell division cycle homolog 6	2.0; q=0.0200	DNA replication; localized in the nucleus during G_1 of the cell cycle	17q21.3	11, 16, 80
NM_018492	8149955	*PBK*	PDZ binding Kinase	1.9; q=0.0019	Mitosis; serine/threonine kinase; mitogen-activated protein kinase kinase family	8p21.2	11, 13
NM_014791	8155214	*MELK*	Maternal embryonice leucine zipper kinase	1.8; q=0.0035	G_2-M transition and pre-mRNA splicing	9p13.2	11, 13
NM_202002	7960340	*FOXM1*	Forkhead box M1	1.7; q=0.0003	Regulation of S and M phase of cell cycle	12p13	11, 50

Table 4.2. List of 5 upregulated candidate genes selected from microarray results. A one way ANOVA was used to select target genes that were differentially expressed between treatments (24 hour non treated and 10 nM treatment), based on a global fold change criteria ±1.5-fold and a q-value of <0.05 (q-value, the positive false discovery rate analogue of the p-value that eliminates the need to set the error rate before analysis) (66).

(37%) increase in 24 hour 10 nM E2 treatment, but these results are not statistically significant due to variation between replicates (figure 4.3). Previous work had shown that GIRK1 gene expression ws decreased 1.85 fold or (85%) ($p<0.05$) by the 1315 stealth siRNA (figure 4.3). As a result we also analyzed these five candidate genes (table 4.2) by real-time PCR of MCF7 cells transfected with the 1315 stealth siRNA construct as an attempt to link these two sets of observations.

CDC6 gene expression using real-time PCR

Previous research has found the CDC6 gene expression, as well as the majority of cell cycle associated genes, are responsive to estrogen treatment in MCF7 breast cancer cells (16). Although time course studies have shown that most of these estrogen responsive cell cycle associated genes are regulated at 24 hours or later, Frasor et al. found that *CDC6* appears to be up-regulated in response to E2 treatment within the first 4 hours and remains up-regulated to 48 hours, and possibly longer (16). We selected *CDC6* as a target gene because it is involved with DNA replication and cell cycle progression (20, 80). Additionally, increased *CDC6* gene expression is correlated with loss of PTEN function and human prostate cancer metastasis (80). In the present study, MCF7 cells treated with 10nM E2 resulted in a significant 2.46 fold (146%) ($p<0.05$) increase in *CDC6* gene expression as assayed by real-time PCR (figure 4.4). Conversely, GIRK1 knockdown in non E2 treated cells resulted in 1.82 fold (82%) ($p<0.05$) reduction in *CDC6* gene expression (figure 4.4).

MELK gene expression using Real-Time PCR

MELK kinase is a regulator of the S-G_2, G_2-M transitions of the cell cycle and pre-mRNA splicing (13). Additionally, *MELK* is one of sixteen kinase genes used to predict luminal breast cancers that clinically demonstrate a poor prognosis (13). *MELK* was selected as a target gene

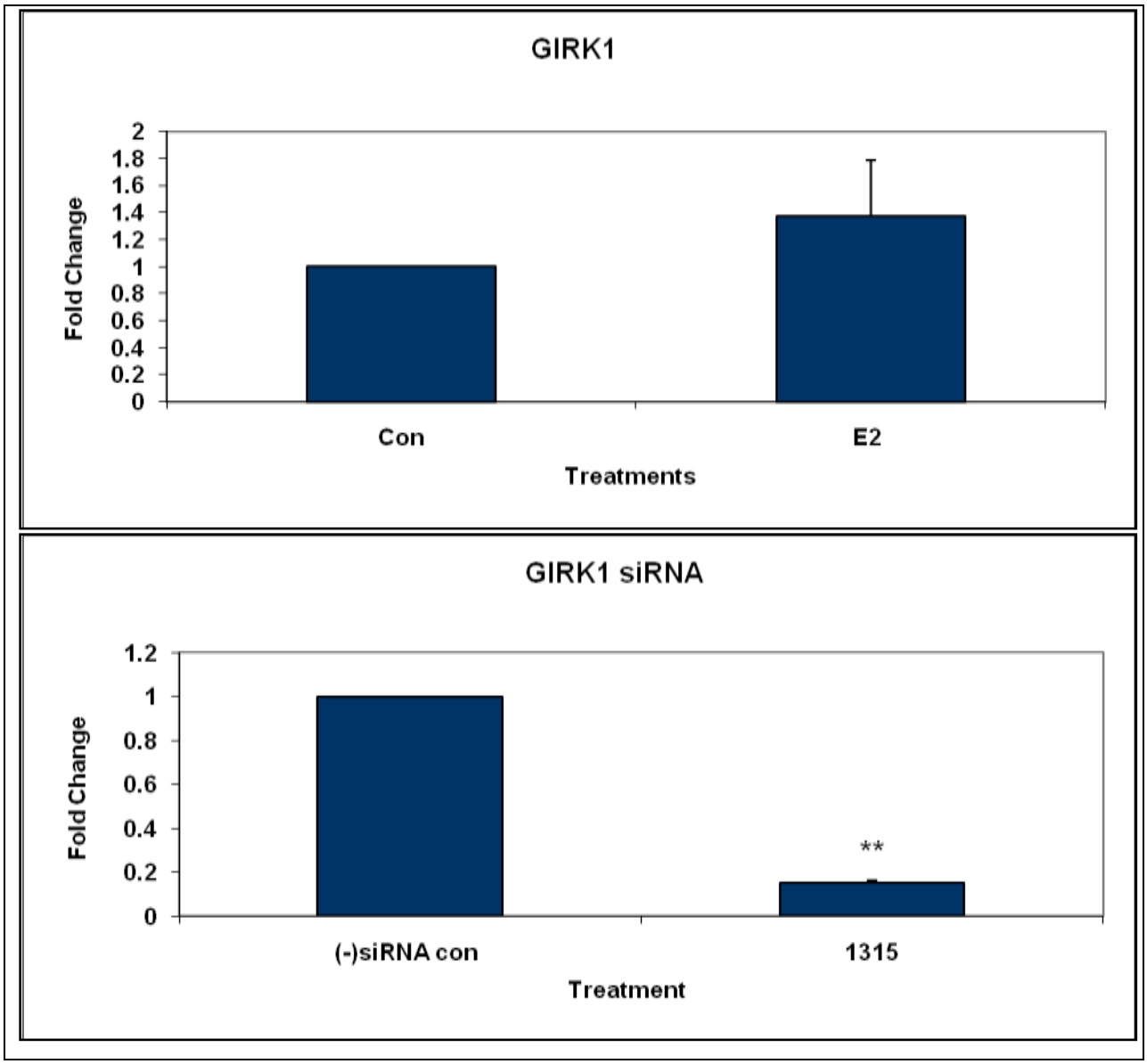

Figure 4.3 GIRK1 gene expression from estrogen treated MCF7 breast cancer cells and GIRK1 knockdown MCF7 cells using real-time PCR. RNA was isolated 24 hours after treatment of MCF-7 cells with 10 nM estradiol or transfection with 1315 siRNA construct. These data were analyzed analyzed using the $2^{-\Delta\Delta C}{}_{T}$ method (39). Asterisk (**) indicates a significant difference ($p<0.05$) in treatments. Abbreviations Con represents (24hour non-treated control), E2 represents (24 hour 10 nM E2 treatment), (-)siRNA con represents (MCF7 cells tranfected with the low GC RNAi negative control), and 1315 represents (MCF7 cells transfected with 1315 stealth RNAi construct).

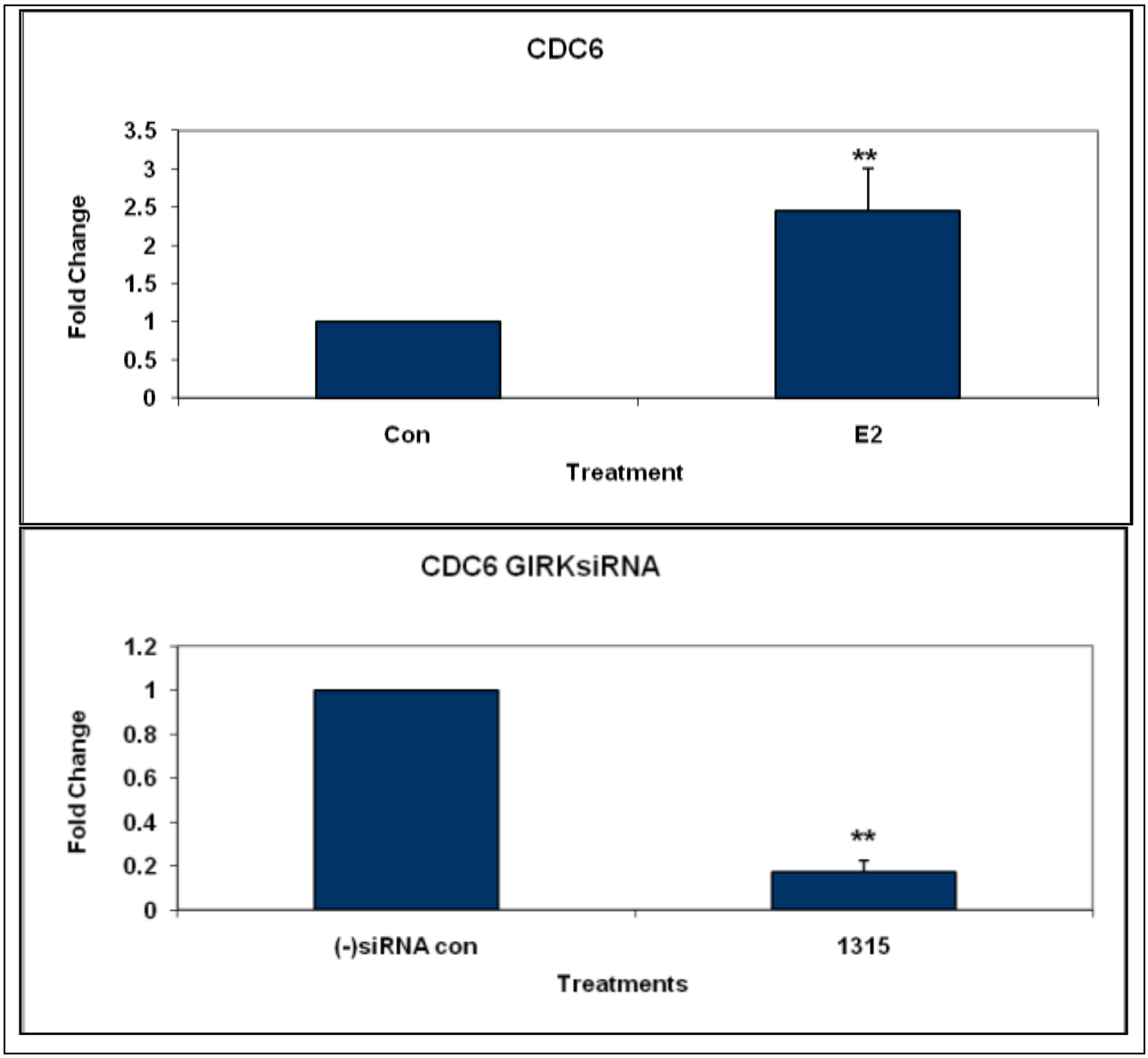

Figure 4.4 CDC6 gene expression from estrogen treated MCF7 breast cancer cells and GIRK1 knockdown MCF7 cells using real-time PCR. RNA was isolated 24 hours after treatment of MCF-7 cells with 10 nM estradiol or transfection with 1315 siRNA construct. These data were analyzed analyzed using the $2^{-\Delta\Delta C}{}_T$ method (39). Asterisk (**) indicates a significant difference ($p<0.05$) in treatments. Abbreviations Con represents (24hour non-treated control), E2 represents (24 hour 10 nM E2 treatment), (-)siRNA con represents (MCF7 cells tranfected with the low GC RNAi negative control), and 1315 represents (MCF7 cells transfected with 1315 stealth RNAi construct).

because it is usually upregulated in breast cancer and is thought to contribute to breast carcinogenesis by suppressing Bcl-G_L, a pro-apoptotic member of the Bcl-2 family (38). In the present study, MCF7 cells treated with 10 nM E2 resulted in a significant 2.17 fold (117%) ($p<0.05$) increase in *MELK* gene expression as assayed by real-time PCR (figure 4.5). GIRK1 knockdown in non E2 treated cells resulted in 1.77 fold (77%) ($p<0.05$) decrease in *MELK* gene expression (figure 4.5).

DSCC1 gene expression using real-time PCR

DSCC1 is involved in DNA replication via involvement in yeast replication factor C (RFC) complex and tied to sister chromatid cohesion, which is thought to occur during S phase of cell cycle (3, 45). It has been suggested that *DSCC1* may play a role in cancer because the gene product is involved chromosomal stability, which are likely early mutational targets in cancer (44, 45). Previous work from our lab, showed an increase in protein kinase A (PKA) activity in response to 10nM estrogen treatment for 24 hours (chapter 2). The human RFC complex, including *DSCC1*, is thought to interact with the RIα, PKA regulatory subunit in MCF7 breast cancer cells (18). We selected *DSCC1* as a target gene because the RFC and RIα are overexpressed in multiple cancers and thought to play a role in cell survival (18). MCF7 cells treated with 10 nM E2 resulted in a 1.68 fold (68%) increase in *DSCC1* gene expression (figure 4.6). These results were not significant due to variation between the replicates. However, GIRK1 knockdown in untreated cells resulted in 1.72 fold (72%) ($p<0.05$) decrease in *DSCC1* gene expression (figure 4.6).

PBK gene expression using Real-Time PCR

PBK is a member of the mitogen-activated protein kinase kinase family and is involved in early stage mitotic regulation (13). Knockdown of *PBK* gene expression using RNAi resulted

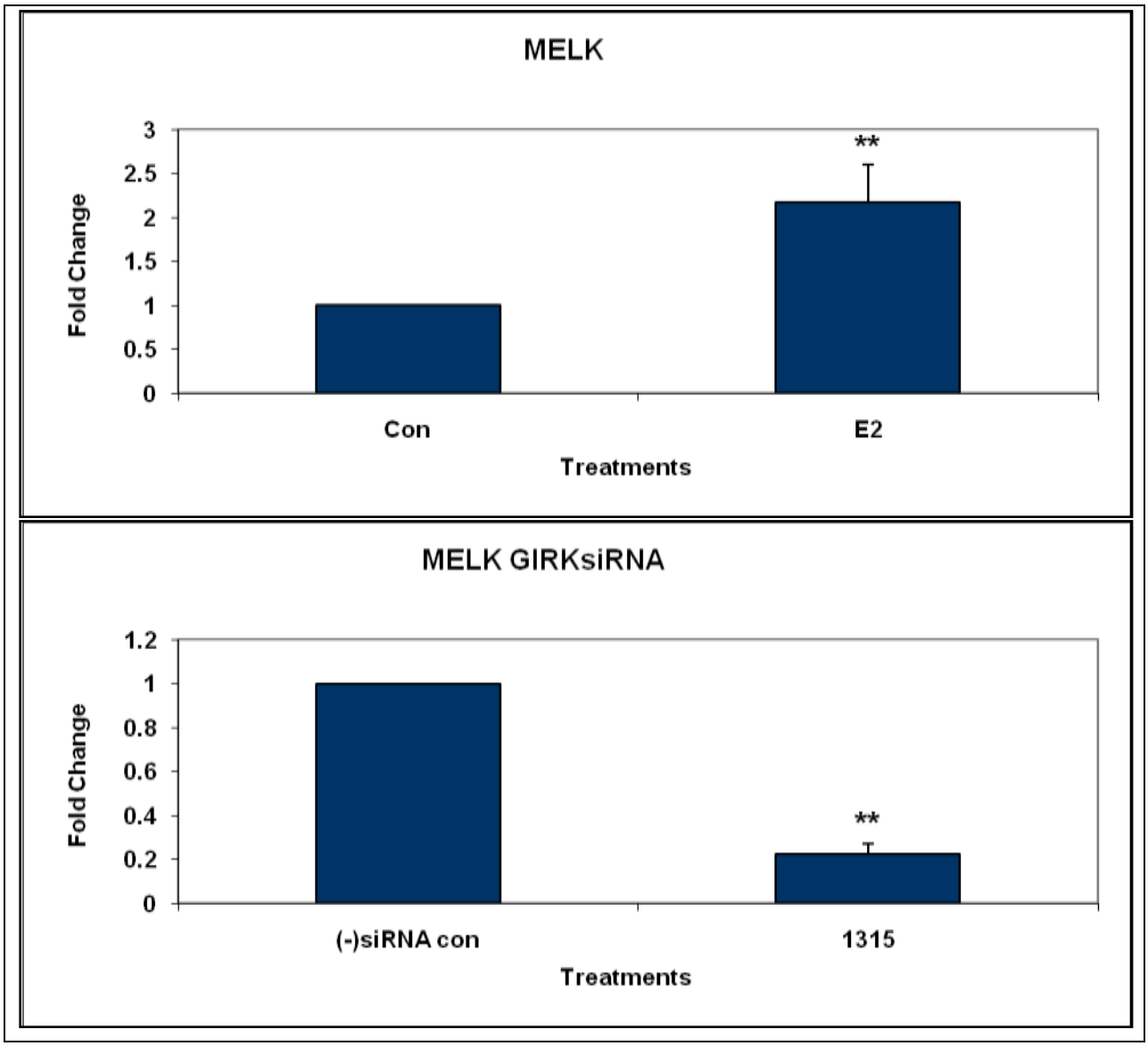

Figure 4.5 MELK gene expression from estrogen treated MCF7 breast cancer cells and GIRK1 knockdown MCF7 cells using real-time PCR. RNA was isolated 24 hours after treatment of MCF-7 cells with 10 nM estradiol or transfection with 1315 siRNA construct. These data were analyzed analyzed using the $2^{-\Delta\Delta C}{}_{T}$ method (39). Asterisk (**) indicates a significant difference ($p<0.05$) in treatments. Abbreviations Con represents (24hour non-treated control), E2 represents (24 hour 10 nM E2 treatment), (-)siRNA con represents (MCF7 cells tranfected with the low GC RNAi negative control), and 1315 represents (MCF7 cells transfected with 1315 stealth RNAi construct).

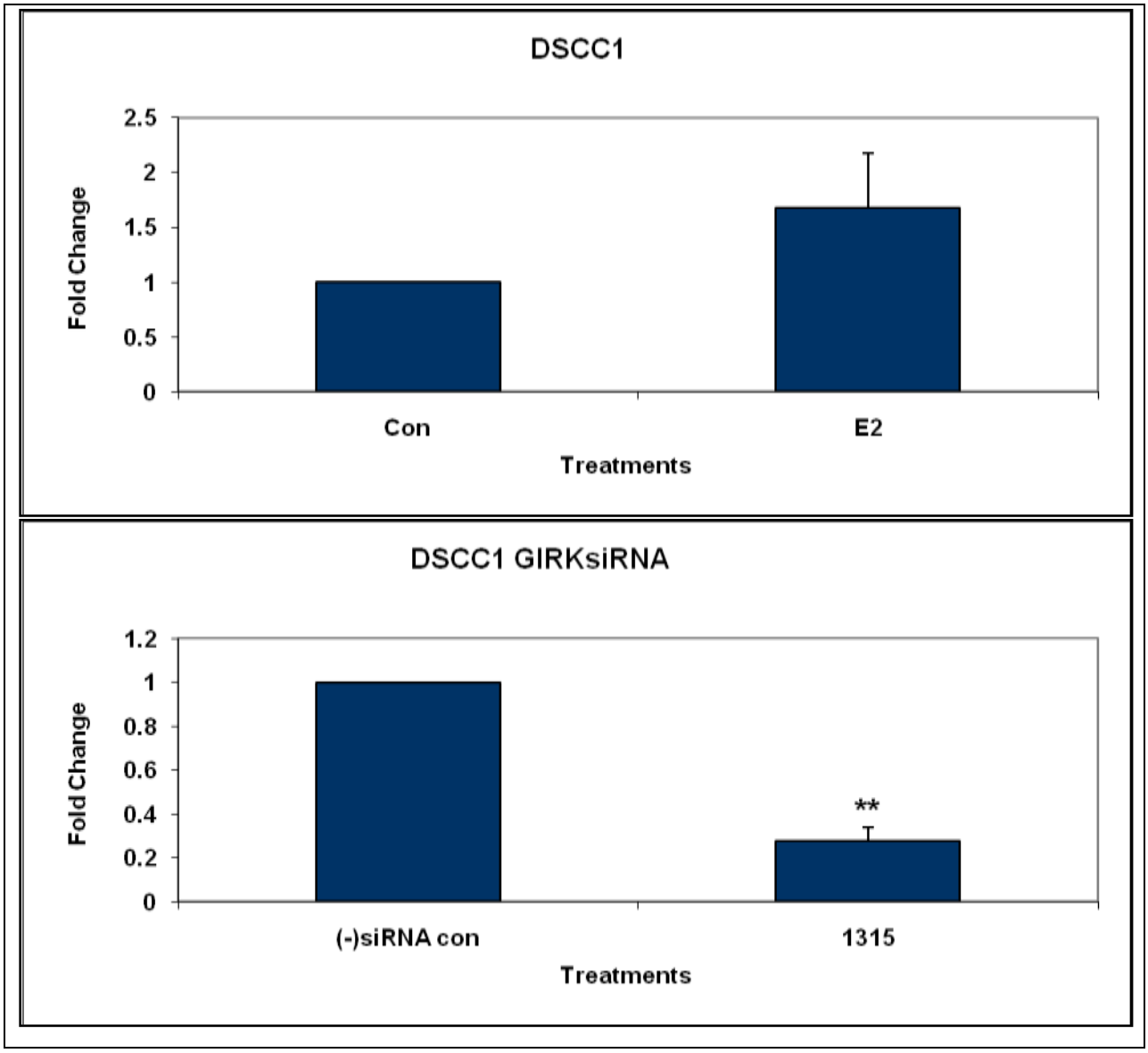

Figure 4.6 DSCC1 homolog gene expression from estrogen treated MCF7 breast cancer cells and GIRK1 knockdown MCF7 cells using real-time PCR. RNA was isolated 24 hours after treatment of MCF-7 cells with 10 nM estradiol or transfection with 1315 siRNA construct. These data were analyzed analyzed using the $2^{-\Delta\Delta C}{}_{T}$ method (39). Asterisk (**) indicates a significant difference ($p<0.05$) in treatments. Abbreviations Con represents (24hour non-treated control), E2 represents (24 hour 10 nM E2 treatment), (-)siRNA con represents (MCF7 cells tranfected with the low GC RNAi negative control), and 1315 represents (MCF7 cells transfected with 1315 stealth RNAi construct).

in reduced cell proliferation in multiple breast cancer cell lines (49). *PBK* is another one of sixteen kinase genes used to predict luminal breast cancers that clinically demonstrate a poor prognosis (13). MCF7 cells treated with 10 nM E2 resulted in a 1.83 fold (83%) increase in *PBK* gene expression (figure 4.7). These results were not significant due to variation between the replicates. However, GIRK1 knockdown in non treated cells resulted in 1.32 fold (32%) ($p<0.05$) decrease in *PBK* gene expression (figure 4.7).

FOXM1 gene expression using Real-Time PCR

FOXM1 is a transcriptional factor that activates the expression of cell cycle regulatory genes required for both S and M phase progression (50). It is normally expressed in multiple cancers types, cancer cell lines and has been shown to be required for tumorigenesis *in vivo* (33, 40, 50, 82, 83). The expression of *FOXM1* is only seen in a few proliferating cells types in adult tissues such as the thymus, testis and colon, but *FOXM1* expression is not normally seen in differentiated or resting cells in adult tissues (33, 50, 83). In the present study, MCF7 cells treated with 10 nM E2 resulted in a 1.3 fold (30%) increase in *FOXM1* gene expression as assayed by real-time PCR (figure 4.8). These results were not significant due to variation between the replicates. GIRK1 knockdown in non treated cells resulted in 1.36 fold (36%) increase in *FOXM1* gene expression (figure 4.8). These results were not significant and *FOXM1* gene expression does not appear to be affected by GIRK1 knockdown of MCF7 cells.

Discussion

The utility of ion channels is thought to be significant because of their appear to have a regulatory role in many diseases, which suggests they have potential in diagnosis, prognosis and therapeutic targeting (14, 15, 17, 22, 34, 57, 63, 73). Potassium channels are of significant interest because of their regulatory ability of essential cell functions, such as proliferation

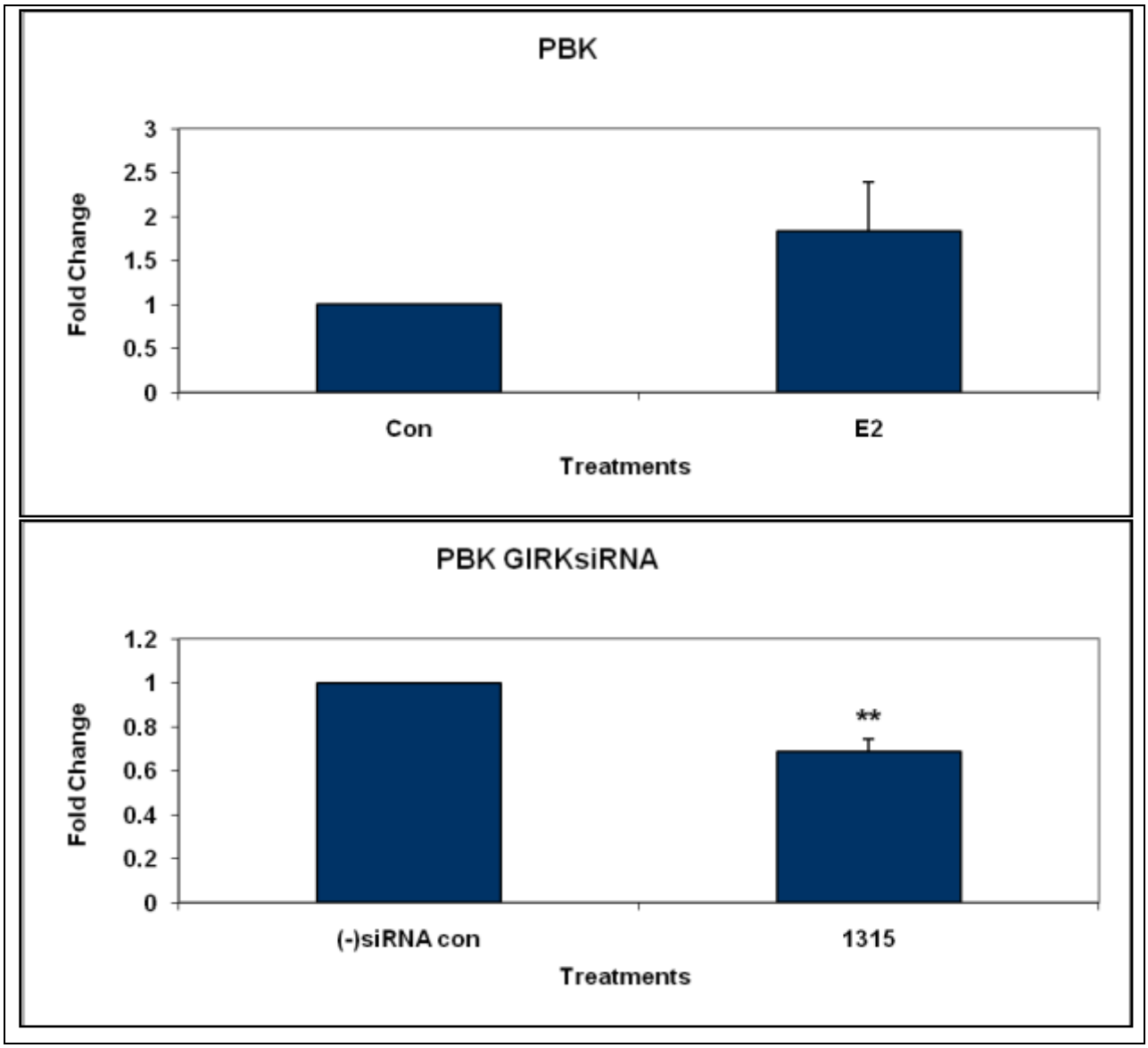

Figure 4.7 PBK gene expression from estrogen treated MCF7 breast cancer cells and GIRK1 knockdown MCF7 cells using real-time PCR. RNA was isolated 24 hours after treatment of MCF-7 cells with 10 nM estradiol or transfection with 1315 siRNA construct. These data were analyzed analyzed using the $2^{-\Delta\Delta C}{}_{T}$ method (39). Asterisk (**) indicates a significant difference ($p<0.05$) in treatments. Abbreviations Con represents (24hour non-treated control), E2 represents (24 hour 10 nM E2 treatment), (-)siRNA con represents (MCF7 cells tranfected with the low GC RNAi negative control), and 1315 represents (MCF7 cells transfected with 1315 stealth RNAi construct).

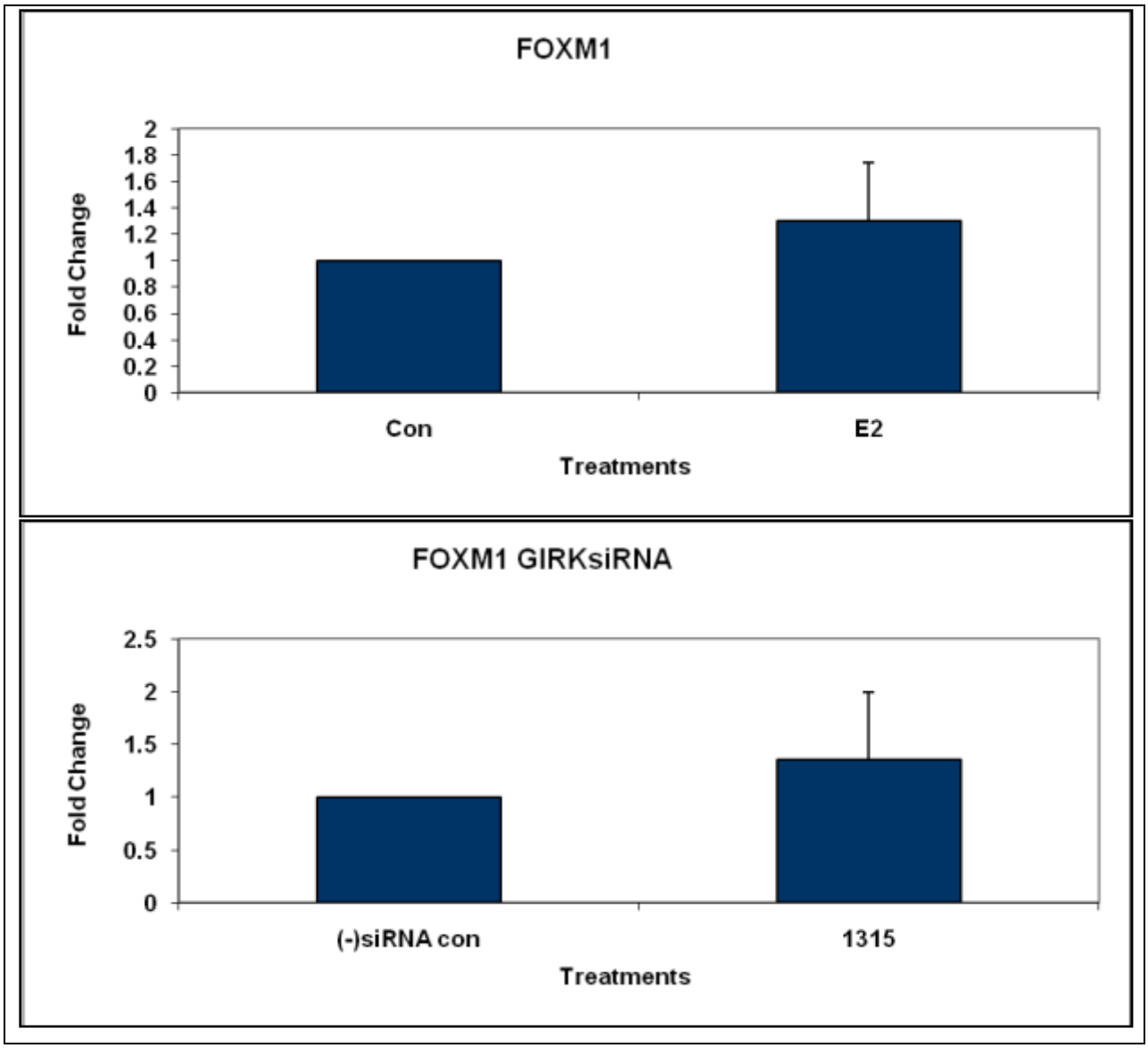

Figure 4.8 FOXM1 gene expression from estrogen treated MCF7 breast cancer cells and GIRK1 knockdown MCF7 cells using real-time PCR. RNA was isolated 24 hours after treatment of MCF-7 cells with 10 nM estradiol or transfection with 1315 siRNA construct. These data were analyzed analyzed using the $2^{-\Delta\Delta C}{}_{T}$ method (39). Asterisk (**) indicates a significant difference ($p<0.05$) in treatments. Abbreviations Con represents (24hour non-treated control), E2 represents (24 hour 10 nM E2 treatment), (-)siRNA con represents (MCF7 cells tranfected with the low GC RNAi negative control), and 1315 represents (MCF7 cells transfected with 1315 stealth RNAi construct).

and apoptosis, which may provide valuable contributions to furthering our understanding of cancer progression (15, 34). Additionally, there is growing support that targeting mitotic kinases and cell cycle regulatory proteins may provide novel treatment strategies in cancer management (62). The present study is the first report to identify potential candidate genes associated with increased GIRK1 and GIRK2 membrane protein expression. Previous results from our lab demonstrated that these increases in GIRK1 and GIRK2 membrane protein expression were correlated with increased intracellular potassium concentrations and increased cellular proliferation (chapter 2). Our results suggest that GIRK function may be associated with cell cycle regulatory genes thought to be involved in cancer progression, which may also tie GIRK function to cell cycle regulation.

Using a one way ANOVA to select target genes that were differentially expressed between treatments (24hour non treated and 10 nM E2 treatment) based on a global fold change criteria of ±1.5-fold and a q-value of <0.05 (66), we identified 489 differentially expressed genes, 283 unregulated and 206 downregulated. We used gene ontology software (Onto-Express; Wayne State University, Detroit, MI) to generate a categorized data set based on biological processes to aid in candidate gene selection (10, 31). Previous research from our lab as well as other labs, has demonstrated that membrane potassium channels have a role in cell proliferation and cell cycle progression (4, 9, 14, 19, 22, 32, 34, 63, 68, 73, 76, 77). Furthermore, it is also suggested that activation of cell cycle-relevant proteins may be directly regulated by intracellular potassium concentrations and membrane charge (34, 68). Another study indicated that global genome analysis of 10nM treated ER(+) MCF7 breast cancer cells resulted in increased expression of genes involved in proliferation and cell cycle regulaton (16). In the present study, the biological functions that represented the largest portion of differentially expressed genes

were unknown, but the largest remaining functional portions were identified as cell cycle, transcriptional regulation and transcription, mitosis and cell division (figure 4.1). We selected genes on the basis of involvement in different portions of cell cycle progression and mitosis, and a minimum global fold change up-regulation of 1.7 (figure 4.2 and table 4.2).

Neither GIRK1 nor GIRK2 were identified as upregulated by microarray analysis using our criteria. Although, Previous results from our lab have demonstrated that 10 nM E2 treatment increased GIRK1 and GIRK2 membrane protein expression, and increased cell proliferations (chapter 2). Analysis of GIRK1 gene expression of our microarray samples by real-time PCR indicated a 1.37 mean fold increase in the 24 hour 10 nM E2 treatment, but these results are not statistically significant (figure 4.3). One of the challenges in this study was variation between replicates, which suggests that we should have increased our replicates. Our initial PCA on global gene expression indicated that samples grouped according to preparation date, so a batch effects transformation was used to reduce the variation between the samples from this effect. Additionally, we opted to use a non-treated MCF7 control as opposed to a charcoal stripped control, which likely reduced the significance of some upregulated genes because of endogenous estrogen in serum used in the media. E2 treatment may also have a role in the regulatory mechanism that controls the translocation of ion channel proteins to the cell surface, which may explain the increases in membrane proteins levels of GIRK1 (chapter2) that were not seen in the mRNA levels (figure 4.3). As a means to compensate for these challenges, we included RNA isolated from MCF7 cells transfected with the 1315 stealth siRNA construct to further validate these candidate genes by real-time PCR. GIRK1 gene expression studies indicate that the candidate genes affected by estrogen treatment in the microarray studies were also affected by GIRK1 gene knockdown.

The *CDC6* gene is the target for transcriptional complexes, such as human E2F proteins, which are essential for cell growth and DNA replication (20). Blocking CDC6 protein expression, via microinjection of an anti-*CDC6* rabbit antiserum into T98G human glioblastoma cells blocks DNA sythesis, demonstrating that *CDC6* expression is required for the initiation of DNA replication (20). The *CDC6* gene has been shown to be overexpressed in human prostate tumors and induction of PTEN significantly reduced *CDC6* gene expression and protein levels in DU145 human prostate carcinoma cells (79). Loss of PTEN is seen in multiple types of cancer and is thought to upregulate *CDC6*, as well as other cell cycle regulated genes, and promote metastasis (79).Previous microarray studies of 10 nM E2 treated MCF7 cells, have identified increased gene expression of *CDC6*, which appears to be up-regulated within the first 4 hours (16). In the present study, *CDC6* was selected because it was increased 2 fold and it is associated with DNA replication and cell cycle progression (20, 79) (table 4.2). We confirmed the increase expression of *CDC6* using real-time PCR and we found that GIRK1 knockdown resulted in reduced *CDC6* gene expression (figure 4.4). These results suggest that *CDC6* expression is correlated with both GIRK1 expression and the effects of E2. Previous data (chapter 2) indicate a correlation between GIRK1 and E2, and this data further confirms this correlation.

Finetti et al. 2008, identified a set of sixteen serine/threonine mitosis-related kinases that are useful to identify an aggressive subform of luminal A breast cancer, luminal Ab breast cancer (13). Our list of up-regulated differentially expressed genes contained five of these mitosis-related kinase genes from this list including Maternal embryonice leucine zipper kinase (*MELK1*), and PDZ binding kinase (*PBK*). *MELK* and *PBK* were selected for further study (table 4.1). MELK kinase is a regulator of the S-G_2, G_2-M transitions of the cell cycle and pre-mRNA splicing (13). MELK is thought to contribute to breast carcinogenesis by suppressing Bcl-G_L, a

pro-apoptotic member of the Bcl-2 family (38). It is thought that *MELK* would be a valuable therapeutic target because is significantly overexpressed in a large majority of breast cancers based on results comparing expression profiles from human tumor samples against various normal tissue samples (38, 48 ,60). In the present study, MCF7 cells treated with E2 resulted in a significant 2.17 fold ($p<0.05$) increase in *MELK* gene expression as assayed by real-time PCR confirming the microarray results. Additionally, GIRK1 knockdown resulted in 1.77 fold ($p<0.05$) decrease in *MELK* gene expression (figure 4.5). *PBK* is a PDZ-binding Ser-Thr kinase related to the mitogen-activated protein kinase kinase family and is involved in early stage mitotic regulation, and at G2-M phase of the cell cycle (13, 49). Previous research has found that certain splice variants of GIRK2 contain a sequence at the carboxyl-terminus that is capable of interacting with members of the PDZ domain-containing family of proteins (21, 24, 25 , 26, 74). Reduced *PBK* gene expression using RNAi resulted in decreased cell proliferation in multiple breast cancer cell lines (49). It is thought that *PBK* could be a promising therapeutic target for breast cancer therapy because *PBK* is thought to mediate cell cycle progression though histone H3 phosphorylation at Ser^{10} , which is an essential event in mitosis (49, 55, 72). Additionally, *PBK* is significantly overexpressed in a large majority of breast cancers and breast cancer cells lines, including MCF7 cells, as well as in bladder cancer and non small cell lung cancer (49). We found that MCF7 cells treated with 10nM E2 resulted in a 1.83 fold increase in *PBK* gene expression by real-time PCR. These results were not significant due to variation between the replicates but the microarray study indicates an increase in *PBK* expression. In addition, we found that GIRK1 knockdown resulted in 1.22 fold ($p<0.05$) decrease in *PBK* gene expression (figure 4.7). Our data suggest that GIRK1 function and expression is associated with cell cycle

regulatory kinases that are also correlated to luminal Ab breast cancer, an aggressive subtype of luminal A breast cancer.

The gene expression of DSCC1 is tied to sister chromatid cohesion and involved in DNA replication via involvement in yeast replication factor C (RFC) complex during S phase of cell cycle (3, 45). There is very little published information about the potential roles DSCC1 may play in cancer. Although, DSCC1 is thought to have a role in cancer progression because the gene product is involved in chromosomal stability, which are likely early mutational targets in cancer (44, 45). Additionally, DSCC1 is part of human RFC complex, which has been shown to bind to with the RIα PKA regulatory subunit in MCF7 breast cancer cells and thought to contribute to cell survival (18). Previous work from our lab, showed an increase in PKA activity in response to 10nM estrogen treatment for 24 hours (chapter 2). Previous data also indicates a link between GIRK and the β-adrenergic GPCR signaling pathway (6, 19, 54). Both, cyclic AMP (cAMP) response element binding protein (CREB) and PKA are downstream targets of the β-adrenergic GPCR signaling pathway (69). Our microarray studies showed that DSCC1 had a 2.2 fold increase in 10 nM E2 treated MCF7 breast cancer cells (table 4.2). In the present study, MCF7 cells treated with 10nM E2 resulted in a 1.68 fold increase in DSCC1 gene expression assayed by real-time PCR (figure 4.6). These results were not significant due to variation between the replicates. Although, DSCC1 gene expression was significantly reduced by GIRK1 knockdown, indicating that reduced GIRK1 expression and function appears to negatively affect DSCC1 gene expression (figure 4.6).

Lastly, we evaluated FOXM1 gene expression, which was increased 1.7 fold by E2 treatment in the microarray studies but was not changed in the real-time studies (table 4.2 and figure 4.8). The FOXM1 gene encodes a transcriptional factor that activates the expression of

cell cycle regulatory genes required in mitotic timing of both S and M phase progression, and is associated with chromosomal stability (35, 50, 75). Deregulation of FOXM1 is thought to contribute to tumorigenesis and cancer progression and is overexpressed in multiple cancers including breast cancer (46, 78). FOXM1 has been suggested to be an attractive therapeutic target for breast cancer, because stable shRNA knockdown of FOXM1 in the BT-20 breast cancer cell line resulted decreased cell survivability and inhibited cell proliferation (78). GIRK1 knockdown resulted in a non-significant 1.36 fold (36%) increase in FOXM1 gene expression (figure 4.8). FOXM1 gene expression does not appear to be affected by GIRK1 knockdown of MCF7 cells. The expression of FOXM1 has been shown to be associated with ERα transcriptional regulation (41). Ectopic expression of FOXM1 in MCF7 and ZR75-30 breast cancer cells lead to increased expression of ERα at both the protein and transcript levels, but blocking FOXM1 suppressed the expression of ERα (41).

The present study is the first study to identify potential candidate genes associated with GIRK 1 and GIRK 2 protein expression and function in E2 treated MCF7 breast cancer cells. Furthermore, GIRK1 knockdown reduced the mRNA levels of *CDC6*, *MELK*, *PBK*, and *DSCC1*. At present, it is not understood how GIRK function is associated with these mitotic kinases and cell cycle regulatory proteins, which suggests more intense investigation is required. These findings are illustrated in "Potential mechanisms of GIRKs in MCF7 breast cancer cells" (chapter 1; figure 1.7). Estrogen treatment appears to increase the expression of multiple genes associated with cell cycle regulation, which are also associated with increasd GIRK1 and GIRK2 expression, and increased cellular proliferation. Conversely, non estrogen treated MCF7 breast cancer cells transfected with the 1315 siRNA construct reduced the expression of ERα, E2 responsive signal transduction pathways, and cell cycle regulatory genes, which may provide the

means to interupt E2 mediated proliferation. More extensive work is require to futher investigate the potential roles of GIRK involvement in cell cycle regulation and how these actions may contribute to breast cancer development. Taken together, the data from these microarray studies further suggest the potential value of therapeutically targeting GIRK1 in conjunction to current treatment stratigies.

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VITA

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To further his education, Michael enrolled at the University of Tennessee, Knoxville in August 1997 and finished his Bachelors of Science degree in biochemistry, cellular, and molecular biology in May 2001. In August 2002, Michael enrolled in the Comparative and Experimental Medicine graduate program at the University of Tennessee, Collage of Veterinary Medicine, which he completed in August 2005. During the course of his studies, Michael married his best friend, Emily Hance, because of the balance and joy she brought his life. Afterwards, he started his PhD in Comparative and Experimental Medicine graduate program investigating the role of ion channels in breast cancer.

Following the completion of his PhD in Comparative and Experimental medicine in August 2009, Michael is starting an NIH funded postdoctoral research position at the Hollings Cancer Center at the Medical University of South Carolina. His research project will focus on investigating the regulatory role of the tumor microenvironment on angiogenesis in glioblastoma and prostate cancer.