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Ligand- and phosphorylation-dependent modulation of estrogen receptor target gene expression

Kristen L. Koterba
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Ligand- and Phosphorylation-dependent Modulation of Estrogen Receptor Target Gene Expression

Submitted by
Kristen L. Koterba

In partial fulfillment of the requirements for the degree of Master of Science in Biomedical Sciences

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acknowledgments</td>
<td>ii</td>
</tr>
<tr>
<td>Table of Contents</td>
<td>iii</td>
</tr>
<tr>
<td>Abbreviations</td>
<td>iv</td>
</tr>
<tr>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>Literature Review</td>
<td>6</td>
</tr>
<tr>
<td>Materials and Methods</td>
<td>16</td>
</tr>
<tr>
<td>Protocols</td>
<td>19</td>
</tr>
<tr>
<td>Results</td>
<td>26</td>
</tr>
<tr>
<td>Discussion</td>
<td>43</td>
</tr>
<tr>
<td>Summary</td>
<td>48</td>
</tr>
<tr>
<td>References</td>
<td>49</td>
</tr>
<tr>
<td>Abstract</td>
<td>57</td>
</tr>
</tbody>
</table>
ABBREVIATIONS

Bioluminescence resonance energy transfer: BRET$^2$

Estrogen Receptor: ER ($\alpha$, $\beta$)

$17\beta$-Estradiol: E2

Estrogen response element: ERE

Fluorescence resonance energy transfer: FRET

Green Fluorescent Protein: GFP

Nuclear receptor corepressor: NCoR

*Renilla* luciferase: Rluc

Selective Estrogen Receptor modulator: SERM

Steroid Receptor Coactivator 1: SRC-1
INTRODUCTION

17β-estradiol (E$_2$) has a central role in the proliferation of estrogen responsive cells through the modulation of target gene expression within estrogen responsive tissues. The effects of E$_2$ are mediated through binding to the Estrogen Receptor α (ERα) and Estrogen Receptor β (ERβ) nuclear receptor transcription factors. Upon ligand binding, the receptor dimerizes and interacts with coregulator proteins important for histone modification. The receptor-coregulator complex binds to specific DNA sequences termed estrogen response element (ERE) within the promoter regions of Estrogen Receptor genes (for review see (Evans 1988)).

Coactivators are proteins that interact with nuclear receptors and potentiate receptor dependent transcriptional activity. Coactivators of the p160 family such as Steroid Receptor Coactivator -1/Nuclear Coactivator -1 possess intrinsic histone acetyltransferase activity that relaxes chromatin nucleosome structure allowing the DNA to become more accessible to other transcription factors. Steroid Receptor Coactivator -1 (SRC-1) functions synergistically with other coactivators to enhance transcription (Chen et al. 2000). SRC-1 is of particular importance because high expressions levels of SRC-1 in Ishikawa cells (an estrogen receptor positive endometrial carcinoma cell line) is required for tamoxifen agonist action (Shang et al. 2000).

Tamoxifen is a selective estrogen receptor modulator (SERM) that binds to the estrogen receptor to modulate ER-mediated gene transcription. Tamoxifen is used as an effective treatment and chemopreventative agent for hormone-responsive breast cancer (Demissie et al. 2001; Dutertre and Smith 2000a). Although tamoxifen is an antiestrogen
in breast tissue, it also contains partial estrogenic activity in the uterus and is associated with an increased risk of uterine cancer (Friedrich et al. 1999). The molecular mechanism underlying tamoxifen action is unclear. Tamoxifen can act as an ER antagonist by competing with E2 for receptor binding thus inducing a conformational change in the receptor that blocks its interaction with coactivators and subsequent histone acetylation (Dutertre and Smith 2000b). However, this does not explain tamoxifen’s agonist activity in the uterus.

The importance of ERα in human physiology and breast cancer has been clearly demonstrated. However, the role of ERβ in breast cancer has only recently become more evident. For example, the ERα/ERβ ratio may be altered during carcinogenesis such that ERα expression proportionally increases as cells progress to malignancy (Clarke 2003; Gustafsson and Warner 2000). Co-expression of ERα and ERβ and treatment with tamoxifen increases the antagonistic effect in an ERβ dose-dependent manner suggesting that ERβ is a negative regulator of ERα action (Gustafsson and Warner 2000; Pettersson et al. 2000b). ERα and ERβ may bind as hetero- or homo-dimers at ERE-containing promoters (Chen et al. 1999; Ogawa et al. 1998; Tamrazi et al. 2002; Tremblay et al. 1999). The preferential dimerization between ERs may be key in understanding the mechanisms regulating either tamoxifen agonist or antagonist activity. The consequences of heterodimerization on tamoxifen regulated expression of ER target genes have not been determined. ERβ may decrease the overall cellular sensitivity to E2 in ERα mediated gene transcription and may have a protective role against hyper-proliferation and carcinogenesis.

Estrogen receptor α (ERα) and Estrogen receptor β (ERβ) can be phosphorylated
on multiple sites, and stimulation by a number of growth factor receptors and/or protein
kinases leads to ligand-independent and/or an increase in ligand-dependent
transcriptional activation (Fowler et al. 2004; Kurebayashi et al. 2004; Osborne and Schiff
2003; Rowan et al. 2000b). It is increasingly recognized that the pathways involved in
growth factor receptor signaling are involved in the non-genomic transcriptional activity
of estrogen responsive genes. Tyrosine phosphorylation of the EGF family of receptors
(specifically, epidermal growth factor receptors 1 and 2) can activate downstream
signaling pathways and cross-talk between kinases and estrogen receptor, thereby
regulating the expression and activity of genes involved in cell growth and survival
(Kurokawa and Arteaga 2003). Antiestrogens can also activate the transcription of EGFR
and ErbB2 genes (Newman et al. 2000; Salvatori et al. 2003; Yarden et al. 2001);
conversely, EGFR and other signaling pathways can stimulate ER-dependent
transcription (El Tanani and Green 1997; Wang et al. 2000). Activation of the EGFR
pathway may switch tamoxifen activity through phosphorylation of ERα and coactivators
(Kurokawa and Arteaga 2001; Kurokawa and Arteaga 2003).

We hypothesize that structural modification of the estrogen receptor induced by
ligand binding, as well as site specific phosphorylation of ERα, ERβ, and coregulators
resulting in changes in recruitment of the coregulators SRC-1 and NCoR, thereby altering
interaction with ER dependent promoters. To test this hypothesis, we examined the role
of selective estrogen receptor modulator ligands such as tamoxifen and its analogs in
modifying the protein-protein interaction between estrogen receptor and coregulator and
the protein:DNA interaction between ER and promoter regions of ER-target genes.

Most of these types of studies have been performed using artificial reporter assays
and co-immunoprecipitation. To our knowledge, we are the first to examine the role of ER ligands in regulating the interaction between full-length ER isoforms, coregulators, and promoters in a real-time, in-vivo environment using a combination of bioluminescence resonance energy transfer (BRET²), confirmed by co-immunoprecipitations and ER-dependent reporter assays. Resonance energy transfer has become an invaluable mechanism for the quantitative analysis of the transient protein-protein interactions of hormone receptors. Eidne et. al (Eidne et al. 2002) has written an excellent review of applying the various methods of resonance energy transfer as techniques for the use of studying dynamic hormone receptor interactions within living cells. BRET² has been recently used extensively in the study of dimerization and subsequent signaling pathways of G-protein coupled receptors (Berthouze et al. 2005; Harikumar et al. 2005; Huttenrauch et al. 2005; Roy et al. 2006; Urizar et al. 2005) as well as growth factor receptors. In this study, we have extensively explored this technology and its use as a novel approach to study steroid receptor protein-protein interactions. Understanding the role of ERα, ERβ, and SRC-1 phosphorylation in the initial steps of tamoxifen-regulated gene transcription will identify specific phosphorylation sites that may be targeted to promote tamoxifen antagonist action on ER-dependent gene transcription.
Estrogen Receptor Structure and Function

17β-estradiol (E₂) plays a crucial role in the normal functional development of female gynecologic characteristics and reproductive behavior. However, the E₂-Estrogen Receptor complex is also involved in the development and growth of a variety of cancers, most prominently in breast tissue. There are two forms of the Estrogen Receptor that have been identified, Estrogen Receptor α (ERα) and Estrogen Receptor β (ERβ). They are members of the type I nuclear receptor superfamily that includes the progesterone receptor (PR), androgen receptor (AR), glucocorticoid receptor (GR), and mineralcorticoid receptor (MR). These transcription factors are defined relative to other steroid receptors in the absence of ligand by their sequestration in 9S heterocomplexes that contain heat-shock proteins, immunophilins, and other chaperones. When ERα or ERβ binds to estrogen, it undergoes a conformational change that renders it protease resistant. Estrogen binding also triggers the release of heat shock protein-90 (HSP-90) from the complex thereby promoting formation of dimers. Upon dimerization, ER translocates to the nucleus where the receptor then binds to specific DNA elements, called an estrogen response element, 5’-GGTCAnnnTGACC-3’, which is a palindromic inverted repeat located within the promoters of estrogen responsive genes (Klein-Hitpass et al. 1988). The estrogen receptor complex then recruits the steroid receptor coactivator family of coactivators (SRCs), p300/CBP (a histone acetyltransferase), and the TRAP/DRIP complex. The acetyltransferase activity relaxes nucleosome structure, which in turn facilitates the binding of basal transcription machinery, such as RNA polymerase...
II (for review see (Evans 1988; Rollerova and Urbancikova 2000)). The intrinsic acetylation of p160 coactivators leads to the release and disassembly of the protein complex from the DNA, and the cycle is repeated (Shang et al. 2000).

ERα and ERβ are homologous, particularly in the DNA binding domain (DBD) and the C-terminal ligand binding domain (LBD; Schematic 1). The N-terminal A/B domain of ERα contains the ligand-independent but promoter and cell-type dependent transactivation function 1 (AF-1) which activates target genes by interacting with the components of the core transcription machinery. The C domain contains the DNA binding domain (DBD) and is the most strongly conserved region within nuclear receptors. This domain contains two zinc fingers, the first of which contains amino acids termed proximal box or P-box which is involved in the sequence specificity to DNA binding. The second zinc finger contains residues called the distal box or D-box, which is responsible for the selectivity of binding to classical palindromic half-site estrogen response elements (EREs), or by binding indirectly through tethering to other transcription factors such as Sp1 and AP-1. The hinge D region is hypervariable and allows the receptor to alter conformation to allow maximum ligand binding affinity. The C-terminal domain (designated E/F domain) contains a ligand-binding domain (LBD), the second activation function domain (AF-2), and has regions allowing receptor dimerization by a leucine-zipper mechanism and interaction with transcription coactivators and corepressors (for review see (Klinge 2001)).
While the homology between ERα and ERβ are similar, they differ in their ligand binding affinity, recruitment of coregulators, and subsequent transcriptional response of ER-target genes (McInerney et al. 1998). For example, tamoxifen is a cell- and tissue-specific mixed agonist-antagonist for ERα but is a pure antagonist on ERβ (Tremblay et al. 1997). This is due to the fact that, contrary to ERα, the AF-2 domain of ERβ is independent within the receptor, whereas the AF-1 region functions as a repressor domain, and when removed, increases overall transcriptional activity (Hall and McDonnell 1999). In addition, while both ERα and ERβ produce similar effects on promoter activity at EREs, they have opposite effects on transcription at other sites, such as AP-1 and SP-1 (Castro-Rivera et al. 2001; Paech et al. 1997).

Selective Estrogen Receptor Modulators (SERMs)

The ER activation process depends on the ligand and its mechanism of action. ER ligand agonist and antagonist complexes differ in receptor conformation, DNA binding, protein stability, and recruitment of cofactors required in activation of gene transcription. Estrogen promotes tumorigenesis by the dysregulation of cellular growth. Selective
Estrogen Receptor Modulators (SERMs), such as the antiestrogen tamoxifen, are used as a treatment for breast cancer by competitive binding to ER thereby blocking estrogen-stimulated cellular proliferation (Dutertre and Smith 2000b). Tamoxifen was introduced as hormonal therapy and approved for chemoprevention and is widely used for ER-positive breast cancer, but the mechanism of action and resistance is still unclear (1992). The absence of ER is a common de novo resistance to tamoxifen therapy, whereas acquired resistance is more complex. For instance, tamoxifen is a cell-type and tissue-specific mixed agonist/antagonist for ER\(\alpha\), but a pure antagonist for ER\(\beta\). Whether antiestrogen resistance is caused by a switch from predominantly ER\(\alpha\) to ER\(\beta\) expression and/or altered nuclear receptor coregulator expression remains unknown.

As tamoxifen is a useful therapeutic agent in invasive breast carcinoma, it also has estrogen-like effects in the uterus, resulting in a higher incidence of uterine cancer (Assikis et al. 1996; Friedrich et al. 1999). With the advent of new SERMs, it is important to characterize the estrogen or antiestrogen activity in a tissue-specific manner, to minimize the risk of endometrial cancer development. Like tamoxifen therapy resistance, the agonist/antagonist tissue selectivity of SERMs is unclear. Variations in protein-protein interactions due to altered ER\(\alpha\), ER\(\beta\), and coregulator expression in the breast versus the uterus has been implicated in this tissue selectivity. Defining the roles of ER\(\alpha\), ER\(\beta\), and coregulators in ligand-independent and dependent effects may help elucidate the mechanisms of tissue selectivity of tamoxifen that has not yet been determined.

**Nuclear Receptor Coregulators**

Nuclear receptor coactivators and corepressors are necessary for the transcription
initiation or repression, respectively, of estrogen-responsive target genes. Coregulators interact with nuclear receptors through a Leu-X-X-Leu-Leu amino acid motif termed the receptor interaction domain (RID). These coregulators interact with the AF-2 region of the ER hormone dependently and function in chromatin remodeling through histone acetylation and deacetylation to mediate opposing effects of hormones through varying intracellular levels. Histones are positively charged proteins that tightly bind negatively charged DNA. Coactivators contain an intrinsic histone acetyltransferase that loosens the chromatin nucleosome structure coiled around the DNA, allowing the DNA to become more accessible to other transcription factors. Corepressors repress basal transcription by recruiting histone deacetylase complexes which remove acetyl groups from histones to confer a tight chromatin formation (for review see (McKenna et al. 1999)).

Coactivators are proteins that interact with steroid receptors and potentiate transcriptional activity. Numerous steroid coactivators have been identified, but for our study we will focus on the steroid receptor coactivator (SRC-1), which is one of the p160 family of coactivators, and the c-AMP response element-binding protein (CBP) which together have been shown to function synergistically to enhance transcription (Chen et al. 2000). SRC-1 is of particular importance because of its required high expression in tamoxifen stimulated uterine cells (Shang and Brown 2002). The functional domain structure of SRC-1 includes an amino terminal basic helix-loop-helix (bHLH) domain and a PAS domain. The PAS region is an approximately 300 amino-acid conserved region which was first isolated in the Drosophila protein period clock (PER), the Ah receptor nuclear translocater (ARNT) and the Drosophila single-minded (SIM). It is composed of two or more imperfect repeats. Within the bHLH/PAS proteins the PAS
region is involved in protein dimerization with another protein of the same family. SRC-1 also contains two nuclear receptor-interacting domains (NR1, NR2) and two activation domains (AD1, AD2; Schematic 2). The central nuclear receptor box (NR1) contains three LXXLL consensus sequence motifs.


Corepressors interact with antagonist bound nuclear receptors to repress transcriptional activity. Nuclear receptor corepressor (NCoR) is one of the well characterized nuclear receptor corepressors. The nuclear receptor interaction domains of NCoR contain two I/LXXI/V-I motifs (Schematic 3). Once the receptor is bound by an antagonist, NCoR mediates transcriptional repression by recruiting histone deacetylase complexes which remove acetyl groups from histone proteins, therefore conferring a tight conformation repressing basal transcription. Smith et al. (Smith et al. 1997) have demonstrated that NCoR interacts with antagonist bound ER and blocks the partial agonist activity of the mixed antagonist tamoxifen, indicating that the relative expression of NCoR and SRC-1 can modulate tamoxifen’s ability to enhance or repress ER-dependent gene transcription in a cell and tissue-specific manner.

Schematic 3. Domain structure of Nuclear Receptor Corepressor. Light grey areas indicate repressor
Ligand-dependent Estrogen Receptor Activation

In the classical model of receptor activation, estradiol crosses the cell membrane by passive diffusion and binds to the ligand-binding domain of the receptor. This stimulates the release of heat shock proteins and immunophilins. A conformational change of the receptor signals homo-dimerization of ERα or ERβ, or hetero-dimerization of ERα and ERβ. The complex translocates to the nucleus, and binds to specific DNA elements, called estrogen response elements (ERE) in the promoters of target genes. The binding of ligand to the ER stabilizes helix 12 in the AF-2 domain region. This conformation allows interactions with coactivator proteins to potentiate transcription. In a recent study published by Shang et al. on cofactor dynamics, DNA-bound ER recruits p160 (SRC family), p300/CBP/pCAF (histone acetyltransferases), and the TRAP/DRIP complex. The HAT activity then relaxes chromatin structure which facilitates RNA polymerase II binding and release of the p160 coactivators along with ER (Shang et al. 2000).

ERs not only activates transcription at a consensus ERE, but can also regulate gene transcription by binding indirectly to non-ERE dependent sites within the promoter. In contrast to the classical mechanism of estrogen action, ER does not bind directly to DNA, but tethers to DNA through jun/fos proteins (Cheung et al. 2005). These alternate pathways result from the binding of liganded ER through tethering to other transcription factors such as AP-1 (Kushner et al. 2000) and Sp1 (Kim et al. 2003; Salvatori et al. 2003). In respect to tamoxifen agonist activity, it is hypothesized that the recruitment of
coactivators to ER through jun/fos increases the coactivation property of coactivators (Paech et al. 1997; Webb et al. 1995). It has been found that a GC-rich oligonucleotide region containing an ERE half-site independent to direct DNA binding by ER, forms a complex with the Sp1 and ER proteins (Porter et al. 1997).

Ligand-independent ER Activation by Crosstalk with Signaling Pathways

Nuclear receptor phosphorylation by growth factor receptors and/or protein kinases has been shown to regulate the ligand-independent transcriptional activity of the ER (Arnold et al. 1997; Bunone et al. 1996; Dayani et al. 1990; Denton et al. 1992). This phosphorylation indicates a crucial role for development of breast and uterine cancers (Gullick et al. 1991). In many of these tumors, the MAPK (mitogen-activated protein kinase) and Akt (protein kinase b) kinase pathways amplify the cell growth and survival properties of these cancers due to upregulation of receptor protein tyrosine kinases such as epidermal growth factor receptors 1 and 2 (EGFR and ErbB2) (Amin et al. 2004; Harari and Yarden 2000; Shou et al. 2004). Cross-talk between nuclear receptors and signal-transduction pathways involves not only the phosphorylation of the nuclear receptor, but also of their coregulators. The phosphorylation of ER and coregulators can modulate the transcriptional activity of the receptor (Ali et al. 1993; Arnold et al. 1995; Aronica and Katzenellenbogen 1993; Cerillo et al. 1998; Stoica et al. 2000; Stoica et al. 2003; Yudt et al. 1999). The major phosphorylation sites on the ER are within the N-terminal domain at serine 104 (Le Goff et al. 1994), serine 106 (Le Goff et al. 1994), serine 118 (Joel et al. 1995; Le Goff et al. 1994), and serine 167 (Arnold et al. 1994). Serine 236 is located within the DNA-binding domain (Chen et al. 1999; Lannigan 2003),
and threonine 311 (Lee and Bai 2002) and tyrosine 537 (Arnold et al. 1995; Arnold et al. 1997) within the ligand-binding domain.

Nuclear receptor coregulators are phosphorylated indirectly through growth factor stimulation (Font de Mora and Brown 2000; Rowan et al. 2000a; Rowan et al. 2000b; Wu et al. 2002). There are seven phosphorylation sites have been identified in SRC-1: serine 372, serine 395, serine 517, serine 569, serine 1033, threonine 1179, and serine 1185 (Rowan et al. 2000b). All the sites contained consensus sequences for proline-directed protein kinases. In the same study, Erk-2 phosphorylated threonine 1179 and serine 1185 in vitro, suggesting the importance of this pathway for SRC-1 regulation (Rowan et al. 2000b). In-vitro studies for other family members of the p160 family (SRC-2 and SRC-3) were phosphorylated by the Erk-2 in the map kinase pathway (Font de Mora and Brown 2000; Lopez et al. 2001). Although the nuclear corepressors NCoR and SMRT are also phosphoproteins and have significant sequence homology, SMRT is subject to negative regulation by MAPK signaling pathways operating downstream of growth factor and stress signaling pathways, whereas NCoR is not (Hong and Privalsky 2000; Jonas and Privalsky 2004; Zhou et al. 2001).

**Estrogen Receptor and Physiological Importance in Breast Cancer**

Breast cancer is a leading cause of death in women in the United States (Love et al. 1996; Stewart et al. 2004). The importance of ERα in human physiology and its biological significance in human breast cancer and antiestrogen resistance has been clearly implicated. (Clarke et al. 2003; Friedrich et al. 1999; Katzenellenbogen and Katzenellenbogen 2000; Shou et al. 2004). However only recently has the role of ERβ
during normal development and hyperplasia become more evident. For example, the
ERα/ERβ ratio may be altered during carcinogenesis such that ERα expression
proportionally increases as cells progress to malignancy (Clarke 2003; Gustafsson and
Warner 2000). Homozygous estrogen receptor knockout mice (ERα−/−) are infertile,
females have a 30–40% increase in ovarian tumors by 18 months, while ERβ−/− exhibit
normal development, though females have reduced fertility. Males develop prostatic
hyperplasia (Couse and Korach 1999; Krege et al. 1998). Co-expression of ERα and ERβ
and subsequent treatment with tamoxifen increases the antagonistic effect in an ERβ
dose-dependent manner suggesting that ERβ is a negative regulator of ERα action
(Pettersson et al. 2000a). The ER isoforms form heterodimeric complexes in vitro, but the
consequences of the heterodimerization on tamoxifen regulated expression of ER target
genes has not been determined. Taken together, ERβ may decrease the overall cellular
sensitivity to E2 in ERα mediated gene transcription, thus may have a protective role
against hyper-proliferation and carcinogenesis.

Hormone independent growth and/or resistance to antiestrogens have been
proposed to result from a number of phenomena observed with estrogen receptors. These
include 1) alterations in the ERα and ERβ ratio, 2) preferential homo-dimerization of
ERα rather than hetero-dimerization between ERα and ERβ, and 3) steroid/antisteroid–
induced phosphorylation of ERs and/or coregulators. In this study, we examined how
these relationships affect the tamoxifen-dependent transcriptional activity of estrogen
positive carcinoma through direct protein interactions.
MATERIALS AND METHODS

Plasmid Construction - The human pGFP-C, pGFP-N, pRluc(h)-C, and pRluc(h)-N terminal expression vectors were purchased from Perkin-Elmer Life Sciences. The GFP² vector encodes a mutant of GFP which absorbs and emits light at a longer wavelength than conventional GFP. The GFP-N1-ERα, Rluc-N3-SRC-1, and Rluc-C1-SRC-1 fusion proteins were constructed by amplifying the estrogen receptor and SRC-1 fragments from the pCR3.1 vector by PCR and digesting the KpnI and ApaI sites of both the PCR products and the expression vectors. The GFP-C2-ERα fusion protein was constructed by amplifying the estrogen receptor by PCR and digesting the BamHI and BspEI sites of the insert as well as the expression vector. All constructs were verified by direct DNA sequencing.

Western blotting - HEK-293 cells (ATCC) were cultured and maintained in DMEM supplemented with 10% FBS, l-glutamine, and antibiotics at 37°C, 95% O₂, and 5% CO₂. Cells (3 x 10⁶) were seeded in 10-cm dishes and transfected with 10µg each of the above expression vectors using Lipofectamine 2000 transfection reagent (Invitrogen). 24 hours post-transfection, cells were rinsed with cold PBS, scraped from the plates and the cell pellet was lysed in 400µl of lysis buffer containing 50mM Tris-HCl (pH 7.5), 420mM NaCl, 1% NP-40, and supplemented with a protease inhibitor cocktail (Sigma). The cell lysate was centrifuged at 15,000 x g at 4°C for 20 minutes. The protein concentration in the supernatant was determined and 50µg of protein lysate was boiled in Laemmli loading buffer and subjected to a 10% SDS-PAGE.
Co-immunoprecipitations- HEK-293 cells were cultured and transfected as described above. Cells (3 x 10^6) were transfected with 10ug of Rluc-C-SRC-1 and 10ug GFP-C-ERa or vector. 48 hours post-transfection, cells were rinsed in cold PBS, scraped from the dishes and the cell pellet was lysed in 400ul of hypotonic lysis buffer (20mM HEPES pH 7.5, 10% glycerol, 1mM PMSF) using five strokes of a dounce homogenizer. The lysate was centrifuged at 1000x g at 4°C for 20 minutes to pellet the nuclei. The cytosolic supernatant was removed and set aside at 4°C. The volume of the packed nuclei (Packed Cell Volume, or PCV) was determined and low-salt NP-40 lysis buffer (20mM HEPES pH7.5, 150mM NaCl, 1% NP-40, 50mM Tris-HCl pH 7.5, 1mM PMSF) was added at twice the PCV. The nuclear pellet was vortexed at 5 minute intervals for 30 minutes at 4°C, then centrifuged at 15,000 x g for 10 minutes at 4°C. The nuclear extract was combined with the cytosolic extract and the protein concentration was determined. Separating the cytosolic and nuclear extracts in this manner was found to preserve the protein-protein interactions and increase the yield. 500ug of combined cell lysate was pre-cleared by incubation with Protein A agarose beads for 30 minutes at 4°C. The pre-cleared supernatant was then incubated with SRC-1 antibody (0.5µg/100µg lysate; M-341, Santa Cruz) rotating overnight at 4°C. 20µl of Protein A agarose beads were added and the solution and incubated for 1 hour at 4°C while rotating. The resulting protein A/immunocomplex was then centrifuged at 15,000 x g for 1 minute at 4°C, washed in low-salt NP-40 lysis buffer, and repeated 3x. The beads were boiled in Laemmli loading buffer for 10 minutes and followed by electrophoresis by 10% SDS-PAGE.
Luciferase Assays- HeLa cells were cultured in phenol-red free DMEM with 5% fetal bovine serum and plated in 6-well dishes at a confluency of $2 \times 10^5$ cells/well. The medium was replaced with medium containing 2% dextran-coated charcoal-stripped serum and the cells were incubated for 48 hours. Cells were then transfected with 500ng of EREelb-luciferase reporter using Fugene6 transfection reagent. 24 hours post-transfection, cells were incubated with vehicle or $10^{-8}$ M estradiol for 24 hours. The cells were lysed with luciferase lysis buffer and centrifuged at 20000 x g at room temperature for 5 min. to remove cellular debris. The supernatant was used for a standard luciferase assay using a luminometer and luciferase assay values were normalized to protein measured by Bradford assay.

Bioluminescence Resonance Energy Transfer (BRET$^2$)- HEK-293 cells were plated in 6-well plates (200,000 cells/well) and incubated in medium containing 5% charcoal-stripped serum overnight. Cells were transfected with varying amounts of the BRET$^2$ expression vectors using Lipofetamine 2000 (Invitrogen) transfection reagent. 48 hours posttransfection, the cells were harvested and washed 2x in BRET Buffer (PBS plus 2ug/ml aprotinin). 50,000 cells in 40ul buffer were incubated with vehicle or $10^{-8}$M estradiol for 30 minutes at room temperature in 96-well white Optiplates (PerkinElmer). Following addition of 5uM DeepBlueC substrate, luminescence and fluorescence emissions were measured immediately on the Packard Fusion instrument at 410nm and 515nm, respectively. An extensively detailed protocol for BRET$^2$ follows.
PROTOCOLS

BRET\textsuperscript{2} requires transfection of fusion proteins, therefore receptor negative cell lines that exhibit high transfection efficiency should be used. We have found HEK-293 cells yield the most robust BRET\textsuperscript{2} signal. Standardization experiments must be performed before assaying fusion proteins of interest. We suggest using known interacting proteins and their respective agonists as a positive control when first applying BRET\textsuperscript{2} methodology. The protocol detailed below is adapted from the PerkinElmer technical data sheet for catalog number 6310556 (www.perkinelmer.com) and designed specifically for quantitating resonance energy transfer in steroid receptors.

A. Preliminary Experiments:

1. Create a minimum of four fusion protein vectors for each protein to test whether differences in protein folding and post-translational modifications may contribute to the interaction. The orientation of the BRET\textsuperscript{2} tag can also effect protein expression and function. GFP\textsuperscript{2} and Rluc fusion construct combinations are shown below.

<table>
<thead>
<tr>
<th>Combination</th>
<th>C-terminal GFP\textsuperscript{2} fusion protein</th>
<th>N-terminal GFP\textsuperscript{2} Fusion protein</th>
<th>C-terminal Rluc fusion protein</th>
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</table>


2. Verify the functionality and expression of all combinations of fusion proteins.

Expression can be assayed by immunoblot, and the functionality of each nuclear receptor fusion protein can be confirmed with a standard reporter gene assay in which the reporter contains the hormone responsive element for the nuclear receptor. Quantitative expression of each fusion protein can also be tested individually by the Fusion instrument. Testing the expression of each bioluminescent/fluorescent fusion protein signal separately will provide the user with the maximum level of fluorescence/luminescence output without resonance energy transfer.

- For the GFP signal: In a 96-well black Optiplate, aliquot 50,000 transfected cells/well (25ul) and add 175ul BRET$^2$ buffer. Read on the Fusion instrument (Fluorescence mode). (PMT:1000 volts, Gain:1, read time 0.5 sec, excitation 425/20, emission 515/30).

- For the Rluc signal: In a 96-well white Optiplate, aliquot 50,000 cells/well (25ul) and add 150ul BRET$^2$ buffer. Add 50ul of 20uM coelenterazine h (5uM final). Incubate covered with foil 18 minutes. Read on the Fusion instrument (Luminescence mode). (PMT:900, Gain:1, read time 1 sec, emission 410).

B. BRET$^2$ Assay:

The optimal amount of each fusion protein construct used in co-transfection experiments depends on factors such as the affinity of the two interacting domains and the levels at which each construct is expressed. For this reason, a simple 1:1 ratio of construct DNA may not yield the best results. Therefore, a transfection matrix experiment
must be performed with various ratios of each construct and used for BRET\(^2\) measurements. Below is an example of a BRET\(^2\) transfection matrix.

<table>
<thead>
<tr>
<th>Rluc-Protein B</th>
<th>0ug</th>
<th>1ug</th>
<th>3ug</th>
<th>10ug</th>
</tr>
</thead>
<tbody>
<tr>
<td>0ug</td>
<td>Condition 1</td>
<td>Condition 2</td>
<td>Condition 7</td>
<td>Condition 12</td>
</tr>
<tr>
<td>3ug</td>
<td>-</td>
<td>Condition 3</td>
<td>Condition 8</td>
<td>Condition 13</td>
</tr>
<tr>
<td>10ug</td>
<td>-</td>
<td>Condition 4</td>
<td>Condition 9</td>
<td>Condition 14</td>
</tr>
<tr>
<td>20ug</td>
<td>-</td>
<td>Condition 5</td>
<td>Condition 10</td>
<td>Condition 15</td>
</tr>
<tr>
<td>40ug</td>
<td>-</td>
<td>Condition 6</td>
<td>Condition 11</td>
<td>Condition 16</td>
</tr>
</tbody>
</table>

3. Transfect HEK-293 cells in 100mm dishes with combinations of DNA listed above using Lipofectamine 2000 according to the manufacturer’s instructions. Use GFP\(^2\) vector (without insert) when transfecting the Rluc-Protein B alone to keep the DNA concentration constant (Conditions 2, 7, and 12).

4. BRET\(^2\) assays can be performed 24-72 hours post-transfection depending on protein turnover and cell doubling-time. At the time of the assay, wash cells in BRET\(^2\) buffer (Dulbecco’s Phosphate Buffered Saline (D-PBS) containing CaCl\(_2\) (0.1g/l), MgCl\(_2\) (0.1g/l), and D-Glucose (1g/l) supplemented with Aprotinin (2µg/ml)) twice, and harvest in BRET\(^2\) buffer.

5. Count the cells and resuspend 1x10\(^6\)/ml in BRET\(^2\) buffer. Aliquot 50µl (50,000) cells in triplicate into a 96-well white Optiplate. This constitutes the non-treated
cells. Aliquot another 50µl cells in triplicate for each ligand. An example is described below:

6. Add ligand (diluted in BRET² buffer) or buffer alone to each well. Incubate at room temperature for 30 minutes. For example, add 6µl of a 1µM dilution of Estradiol for a final concentration of 10nM (56µl total).

7. Start BRET² reaction by adding 14µl of 25µM (5µM final) DeepBlueC substrate one column at a time (70µl total volume). Read each column immediately on the Fusion instrument before addition of substrate to the next column.

8. Immediately following addition of DeepBlueC, read the plate on a Fusion microplate analyzer with the following settings: one second per well, PMT: 1100 volts and gain: 100, using the following 410/515 nm filter pairs:
   a) RLuc emission: 410 nm bandpass, 80 nm
   b) GFP2 emission: 515 nm bandpass, 30 nm
BRET$^2$ can be further optimized by altering the number of cells assayed. After determining the greatest difference in signal to background from the transfection matrix (see data analysis), titrate the number of cells (50,000 to 200,000) from that particular ratio. The number of cells per well depends on protein expression levels and transfection efficiencies (i.e. the lower the protein expression, the higher number of cells required). For each co-transfection, it is also necessary to assay Rluc-Protein B alone and untransfected cells alone for calculation purposes (see below). For example, if Condition 3 (a co-transfection of both GFP-Protein A and Rluc-Protein B) yield the highest BRET$^2$ signal, one must simultaneously assay the same amount of Rluc-Protein B expressed alone (Condition 2) as well as assay untransfected cells (Condition 1).

9. Resuspend cells in varying concentrations ($1\times10^6$/ml to $4\times10^6$/ml) and aliquot 50µl of cells in triplicate per concentration. Below is an example on a 96-well microplate:

<table>
<thead>
<tr>
<th>Condition 1 Non-treated</th>
<th>50,000 cells</th>
<th>100,000 cells</th>
<th>200,000 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Condition 1 + Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Condition 2 Non-treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Condition 2 + Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Condition 3 Non-treated</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Condition 3 + Treatment</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>


10. Follow with steps 6-8 for BRET² analysis.

C. Data Analysis:

The average counts obtained between the triplicate wells with non-transfected cells (treated or untreated with ligand) are subtracted from the reading of transfected cells in each well (with or without ligand, respectively) to obtain the corrected counts at both wavelengths. Using the corrected counts, the BRET² signal is then determined as the ratio between GFP² emission (515 nm) and Rluc emission (410 nm). This is illustrated in Calculation #1 below. [When subtraction of the background counts yields negative values, a BRET² ratio of zero is assigned.]

There are two calculations to analyze the BRET² ratio. Both are independent and derived from the basic principle of resonance energy transfer; GFP emission/Rluc emission. Both are correct and either can be used depending on presentation of data. Calculation #1 is best for presenting ligand-dependent versus ligand-independent interactions because it simply subtracts raw background emission from itself. In the assay above, subtract the fluorescence signal of Condition 1 (non-transfected) from the fluorescence signal of Condition 3 (GFP-Protein A and Rluc-Protein B co-transfection) divided by the luminescence signal of Condition 1 subtracted from the luminescence signal of Condition 3.

**Calculation #1:**

\[
\frac{\text{GFP}^2 \text{ emission (515nm)} - \text{GFP}^2 \text{ emission of non-transfected cells (515nm)}}{\text{Rluc emission (410 nm)} - \text{Rluc emission of non-transfected cells (410 nm)}} = \text{Corrected BRET}^2 \text{ Ratio}
\]
Calculation #2 is the corrected BRET$^2$ ratio (Calculation #1) and subtracts the correction factor. The correction factor is the background subtracted fluorescent/luminescent signal for the Rluc-Protein B expressed alone. Instead of visualizing basal interactions as with Calculation #1, it incorporates possible false positive resonance energy transfer signals due to forced interactions between over-expressed Rluc or GFP fusion proteins. This calculation is best for comparing time-course or multiple ligands in one graph. In the assay described above, Calculation #1 is used to obtain the corrected BRET$^2$ ratio of both the Rluc-Protein B expressed alone (Condition 2, or Cf) and the GFP-Protein A:Rluc-Protein B co-transfection (Condition 3). Then subtract corrected Condition 2 from the corrected Condition 3.

**Calculation #2:**

\[
\text{Corrected BRET}^2 \text{ Ratio} - \text{Correction Factor (Cf)}
\]

Where \( \text{Cf} = \)

\[
\frac{\text{GFP}^2 \text{ emission (515nm)} - \text{GFP}^2 \text{ emission of non-transfected cells (515nm)}}{\text{Rluc emission (410nm)} - \text{Rluc emission of non-transfected cells (410)}}
\]

for the Rluc vector expressed alone
RESULTS

Expression of GFP-C and –N Terminal Estrogen Receptor and Rluc-C-SRC-1

*Fusion Proteins*- BRET\(^2\) uses the transient transfection of a bioluminescent fusion protein and a fluorescent fusion protein of the interacting proteins of interest. N- and C-terminal fusion proteins must be constructed to determine whether differences in protein folding and post-translational modifications may play a role in the interaction. To establish BRET\(^2\) in a ligand inducible system, we chose to use the nuclear coactivator SRC-1 as the donor moieity, and the estrogen receptor as the acceptor moiety. We have found that the C- terminal NCoR fusion protein is not functional by luciferase assay, however, we have had success with the coactivator SRC-1 and the following reflects those preliminary results. Figure 1 demonstrates the expression of the GFP-C and -N terminal estrogen receptor and Rluc-C-SRC-1 fusion proteins by Western analysis in the ER negative human cervical carcinoma cell line, HeLa.

Figure 1
Figure 1: Expression of the GFP-C and -N terminal estrogen receptor and Rluc-C1-SRC-1 fusion proteins by Western analysis. HeLa cells were plated in 10-cm dishes and transfected with 5ug each of the above expression vectors. 100ug of total cell extract was prepared for 8% SDS-PAGE and Western blotting with antibody to hERα (A and B), or Rluc (C).

*Testing for the function of N- and C- Terminal Fusion Proteins of ERα and SRC-1*

Functionality of the fusion proteins must be confirmed prior to assessing protein interactions by BRET\(^2\). Once the function of the fusion proteins is confirmed, titrations of the transfected construct must also be performed to exclude background and non-specific BRET signals. Background BRET\(^2\) signal is defined as the fluorescent/luminescent quantitative number given by the Fusion for untransfected cells. A non-significant BRET\(^2\) signal is a signal that can occur by overexpression of the fusion proteins. Figure 2 demonstrates the functionality of the fusion proteins as shown by a luciferase reporter assay using the ERE\(_{e1b}\)-luciferase reporter plasmid transiently co-transfected in ER-negative HeLa cells. Nuclear receptor function may also be assessed by other common approaches such as ligand binding, DNA binding, subcellular localization, and endogenous receptor-regulated genes expression. Coactivators are commonly assessed by their ability to enhance the transcriptional activity of steroid receptors. Other functional assays specific for the protein of interest may also be employed. Coactivator proteins interact with the estrogen receptor to potentiate its transcriptional activity. All combinations of N- and C- terminal SRC-1 fusion proteins potentiate the effect of estradiol on the receptor except for the combination of GFP-N-ERα and Rluc-C-SRC-1. The non-functional combination of GFP-N-ERα and Rluc-C-SRC-1 may be caused by interference of the BRET\(^2\) fluorescent tags thereby allowing an unfavorable orientation of
the fusion proteins for coactivator interaction. In data not shown, these results are comparable to the effects of WT-SRC-1 on GFP-ERα as well as SRC-1 fusion proteins on WT-ERα.

Figure 2

**Figure 2:** The different combinations of N- and C-terminal constructs has no effect on functionality. HeLa cells were transfected with 100ng of GFP-C-ER (A and B) or GFP-N-ER (C and D) expression vector with increasing amounts either Rluc-C1-SRC-1 (A and C) or Rluc-N-SRC-1 (B and D) expression vector, an 50ng of ERE-luciferase reporter, and the remaining amount of DNA with a vector containing a CMV promoter. The DNA levels were equivalent, with 1600ng of total transfected DNA. The cells were incubated with either vehicle or 10^{-8} M estradiol for 24 hours. The cells were lysed and luciferase expression was measured by a luminometer.

*Interaction of ERα Homodimers Demonstrated by the BRET^2 assay* - The ERα negative human embryonic kidney cell line HEK-293 was chosen as a model system to measure interactions by BRET^2 because the cells are ERα negative, easily transfectable,
and especially amenable to BRET$^2$ analysis (www.perkinelmer.com). There have been previous reports that show dimerization is estradiol dose-dependent by resonance energy transfer (Michelini et al. 2004). To determine the optimum dose of estrogen needed in HEK-293 cells to stimulate homodimerization by BRET$^2$, we examined an agonist dose response curve and found that estradiol-dependent ER homodimerization and ER:SRC-1 interaction was dose dependent with $10^{-8}$M estradiol as the optimal dose for dimerization (Figure 3).

**Figure 3**

**GFP-C-ERa, Rluc-C-ERa BRET E2 Dose Titration**

**Figure 3: Agonist Dose-Response Curves for Estrogen Receptor using the BRET assay:** HEK-293 cells were transiently co-expressed with the GFP-C-ERa (40ug) and Rluc-C-ERa (10ug) fusion proteins to generate a dose-response curve with estradiol treatment. 48 hours post-transfection, cells were harvested and plated in a 96-well Optiplate. The cells were treated increasing concentration of estradiol agonist ranging from $10^{-6}$ M to $10^{-12}$ M for 30 minutes. Dose titration is shown as the molar log of estradiol.
Figure 4 demonstrates the transfection efficiency (~80%) of the GFP-Rluc positive control vector at 24 hours post-transfection in HEK-293 cells. We assessed estradiol-dependent homodimerization by BRET\(^2\) using different combinations of GFP and Rluc, C- and N- terminal ER\(\alpha\) fusions. By titration of GFP-ER\(\alpha\) and Rluc-ER\(\alpha\) expression vectors it was determined that 10\(\mu\)g transfection of both the GFP-ER\(\alpha\) and the Rluc-ER\(\alpha\) fusion vectors resulted in the strongest estradiol-dependent dimerization by BRET\(^2\).

![Figure 4](image)

**Figure 4:** Transfection efficiency of the GFP-Rluc positive control vector in HEK-293 cells. HEK-293 cells were transfected with 10\(\mu\)g of the GFP-Rluc positive control vector (PerkinElmer) for 24 hours. Panel A represents the GFP filter, and Panel A overlay of the GFP filter onto the phase contrast.

We show that all combinations of C- and N-terminal ER\(\alpha\) fusion proteins respond to estradiol by interaction (presumably homodimerization), where the maximal BRET\(^2\) ratio occurred between the GFP-N-ER\(\alpha\), and Rluc-C-ER\(\alpha\) fusion proteins (Figure 5).
Figure 5: The estradiol-induced interaction by BRET\(^2\) occurs between all combinations of ER\(\alpha\) fusion proteins, regardless of differences in tertiary structure. HEK-293 cells were transfected with 10μg each of a GFP-ER\(\alpha\) and Rluc-ER\(\alpha\) fusion protein and treated with vehicle or 10\(^{-8}\) M estradiol for 30 minutes. Luminescence and fluorescence emissions were then read immediately following addition of DeepBlueC substrate.

In Figure 6 we assessed the BRET\(^2\) signal between GFP and Rluc, C- and N-terminal ER fusion proteins and the ability to homodimerize in the presence of estrogen. The dashed line represents background BRET\(^2\) signal activity as assessed by cells co-transfected with Rluc-C-ER\(\alpha\) and the GFP vector. This negative control accounts for any false-positive BRET\(^2\) signals due to protein over-expression. In addition to estradiol induced homodimerization, we observed ligand-independent homodimerization (Figure 6, indicated by untreated co-transfected GFP-N-ER\(\alpha\) and Rluc-C-ER\(\alpha\)). This experiment was repeated five times.
**Figure 6:** Ligand-dependent and –independent homodimerization of ERα. HEK-293 were transfected with 10µg of Rluc-C-ERα and 10µg of either GFP-C-ERα or the GFP-C vector alone and treated for 30 minutes with 10^{-8}M estradiol. The ligand-dependent and –independent interaction of GFP-C-ERα and Rluc-C-ERα with and without estradiol treatment is indicated by bars above the dashed line. The dashed line represents basal activity. This figure is representative of five experiments.

Dimerization was confirmed by GFP-C-ERα and RlucC-ERα co-transfected, estradiol treated HEK-293 cells, and co-immunoprecipitated with an antibody to GFP of the fusion proteins and probed with antibody to Rluc (Figure 7).
**Figure 7:** Co-immunoprecipitation confirms estradiol-induced homodimerization between GFP-C-ERα and Rluc-C-ERα. HEK-293 cells were untransfected (Lane 1) or transfected with 10µg each of Rluc-C-ERα and 10µg of GFP-C vector (Lane 3), and treated with $10^{-8}$M estradiol. Immunoprecipitated Protein A agarose beads without antibody was used as a control (Lane 2). Whole cell lysate was immunoprecipitated with ERα antibody followed by western blot for ERα. The molecular weight of Rluc-C-ERα is 101kD.

**Interaction of ERα and the Nuclear Coactivator SRC-1 by the BRET² assay**

Figure 8 demonstrates the estradiol-dependent interaction of ERα and SRC-1 by titration of the GFP-N-ERα and Rluc-C-SRC-1 fusion proteins by BRET². The maximum BRET² signal occurred at 10µg of both GFP-N-ERα and Rluc-C-SRC-1, approximately two-fold compared to basal interaction. All combinations of GFP-ERα and Rluc-SRC-1 resulted in ligand-dependent interaction (as shown by the basal BRET² signal (dashed line)). The basal BRET² signal is represented by the Rluc-C-SRC-1 fusion protein expressed without
the estrogen receptor. This experiment was repeated five times.

Figure 8

![Graph showing the ligand-dependent interaction between GFP-C-ERα and Rluc-C-SRC-1.](image)

**Figure 8: Ligand-dependent interaction between GFP-C-ERα and Rluc-C-SRC-1.** HEK-293 were transfected with 10µg of Rluc-C-SRC-1 and 10µg of either GFP-C-ERα or the GFP-C vector alone and treated for 30 minutes with 10-8M estradiol. The ligand-dependent interaction of GFP-C-ERα and Rluc-C-SRC-1 in the presence of estradiol treatment is indicated the bar above the dashed line. The dashed line represents basal activity. This figure is a representative graph of five experiments.

To confirm the interaction is specific, the Rluc-C-SRC-1 control was co-expressed with a non-interacting protein (GFP). The interaction was confirmed by *in vitro* co-immunoprecipitation (Figure 9). HEK-293 cells were co-transfected with Rluc-C-SRC-1 and/or Rluc vector alone with GFP-C-ERα with or without estradiol treatment.
**Figure 9: Co-immunoprecipitation confirms estradiol-induced interaction between GFP-C-ERα and Rluc-C-SRC-1.** HEK-293 cells were untransfected (Lane 1) or transfected with 10µg of Rluc-C-ERα and 10µg of GFP-C vector (Lanes 2 and 3) or 10µg GFP-C-ERα (Lanes 4 and 5), and treated with 10⁻⁸M estradiol (Lanes 1, 3, 5, and 6) or without (lanes 2 and 4). Non-immunoprecipitated whole cell lysate from GFP-C-ERα and Rluc-C-SRC-1 co-transfection was used as a positive control (Lane 6). Cells were immunoprecipitated with SRC-1 antibody followed by western blot for ERα. The molecular weight of GFP-C-ERα is 93kD.

*BRET² Detection of Ligand-induced ERα Homodimerization and ERα/SRC-1 interaction in a Time Course* - It has been shown in previous studies that estradiol upregulates and ICI182,780 reduces ERα protein levels in MCF-7 cells (Wang et al. 2004; Welshons et al. 1993). To determine that the lagands used in BRET² are functional, HEK-293 cells were treated with vehicle (DMSO), 10⁻⁸M estradiol (E2), 10⁻⁷M 4-hydroxytamoxifen (4-OHT), 10⁻⁷M EM-652, 100nM ICI182,780, and 50ng/ml EGF in a
time course ranging from 1 to 12 hours. EM-652 is a partial potent anti-estrogen exhibiting a higher affinity for the estrogen receptor, including estradiol. ICI182-780 is a pure anti-estrogen with anti-estrogenic activity in all tissues. All ligands except for EGF induced GFP-C-ERα and Rluc-C-ERα homodimerization within 30 minutes (data not shown), and ERα/SRC-1 interaction by 1 hour (Figure 11) and continued to stimulate this interaction up to 12 hours (Figure 10 and 11).

Figure 10: GFP-C-ERα and Rluc-C-ERα Ligand-Induced Homodimerization

![Figure 10](image)

**Figure 10: BRET² detection of ligand-induced ERα homodimerization.** HEK-293 cells were co-transfected with Rluc-C-ERα and either GFP-C-ERα or the GFP vector alone. The cells were treated with vehicle (DMSO), 10⁻⁸ M estradiol (E2), 10⁻⁷ M 4-hydroxytamoxifen (4-OHT), 10⁻⁷ M EM-652, 100nM ICI182-780, or 50ng/ml EGF. The BRET² assay was performed at a time course of 1, 4, 8, and 12 hours. The BRET² signal was performed in triplicate and repeated three times.
**Figure 11: BRET$^2$ detection of ligand-induced ER$\alpha$ and SRC-1 interaction.** HEK-293 cells co-transfected with Rluc-C-SRC-1 and either GFP-C-ER$\alpha$ or the GFP vector as a control. The Rluc-C-SRC-1/GFP vector co-transfected cells The cells were treated with vehicle (DMSO), $10^{-8}$M estradiol (E2), $10^{-7}$M 4-hydroxytamoxifen (4-OHT), $10^{-7}$M EM-652, 100nM ICI182-780, or 50ng/ml EGF. The BRET$^2$ assay was performed at a time course of 1, 4, 8, and 12 hours. The BRET$^2$ signal was performed in triplicate and repeated three times.

It is important to remember that the BRET$^2$ signal is not a quantitative measurement within live cells, due to variable distances of fluorescent proteins within tertiary structures and chemical concentration, and can only describe whether there is simply an interaction or not. Therefore, we can assess that all ligands (except EGF) induce a BRET$^2$ interaction, but that not one of them is greater than another. This experiment has been repeated in triplicate.

*ER$\alpha$ protein detection in BRET$^2$ ligand treated HEK-293 cells-* It has been shown in previous studies that estradiol upregulates and ICI182,780 degrades ER$\alpha$ protein levels
in MCF-7 cells (Fawell et al. 1990; Preisler-Mashek et al. 2002). To determine that the ligands used in the BRET\(^2\) experiments are functional, HEK-293 cells were treated with vehicle (DMSO), \(10^{-8}\)M estradiol (E2), \(10^{-7}\)M 4-hydroxytamoxifen (4-OHT), \(10^{-7}\)M EM-652, 100nM ICI182-780, and 50ng/ml EGF for 2 hours and probed for total ER protein levels (Figure 12, top panel). An antibody to \(\beta\)-actin was used as a loading control (Figure 12, bottom panel). Western blotting shows that ICI182,780 is indeed functional as it reduces total ER\(\alpha\) protein levels in HEK-293 cells.

**Figure 12**

![Western Blot](image)

**Figure 12: The effect of ligand on ER\(\alpha\) protein expression in HEK-293 cells.** HEK-293 cells were treated for 2 hours with vehicle (DMSO), \(10^{-8}\)M estradiol (E2), \(10^{-7}\)M 4-hydroxytamoxifen (4-OHT), \(10^{-7}\)M EM-652, 100nM ICI182,780, or 50ng/ml EGF. Cells were lysed and total ER\(\alpha\) protein (Figure 12, top panel) was assayed by western blot. An antibody to \(\beta\)-actin was used as a loading control (Figure 12, bottom panel).

*The effect of tamoxifen activity on the activation of an ERE-luciferase reporter in MCF-7 and MCF-7-LCC2 cells*- In order to minimize the risk of endometrial cancer
associated with prolonged tamoxifen use, there is a clinical need to develop an improved and more efficacious antiestrogenic drug. In collaboration with Ralph Bernacki at the Roswell Park Cancer Institute, we have obtained a tamoxifen analog (99149) which is a structurally different chiral enantiomer that is two-fold more potent than monohydroxytamoxifen in the ER positive breast cancer cell line MCF-7 as shown by the IC50 of a growth inhibition assay; its potency becomes decreased as cells become more resistant, i.e. MCF-7-LCC2 cells. To characterize the chiral tamoxifen analog 99149, we will be able to increase our understanding of antagonist-ER interactions that may lead to the development of new agents with more specific antitumor activity. MCF-7 (Figure 13A) and its tamoxifen-resistant subline MCF-7-LCC2 cells (Figure 13B) were treated with varying doses of mono-hydroxytamoxifen and/or estradiol for 24 hours, and luciferase expression quantitated. Compared to parental MCF-7, MCF-7-LCC2 cells exhibits a greater resistance to combined treatment with 10nM estradiol and 1µM of tamoxifen.

![Figure 13A](image-url)
Figure 13: The effect of estrogen and tamoxifen on the activation of an ERE-luciferase promoter in tamoxifen sensitive MCF-7 cells and tamoxifen resistant MCF-7-LCC2 cells. The induction of luciferase expression in A. MCF-7 cells and B. MCF-7-LCC2 cells after 24 incubation with varying doses of estrogen and tamoxifen.

To determine the extent of the effect of 99149 and the more potent 4-hydroxytamoxifen on tamoxifen sensitive and resistant breast cancer cells MCF-7 (Figure 14A) and MCF-7-LCC2 (Figure 14B), respectively, were treated with $10^{-7}$M of 99149 and 4-OHT, with or without $10^{-8}$M estradiol, and quantitated by luciferase assay.
Figure 14: The effect of the tamoxifen analog 99149 on the activation of an ERE-luciferase promoter in tamoxifen sensitive MCF-7 cells and tamoxifen resistant MCF-7-LCC2 cells. The induction of luciferase expression in A. MCF-7 cells and B. MCF-7-LCC2 cells after 24 incubation with $10^{-6}$M estradiol, $10^{-7}$M 99149, $10^{-7}$M 4-hydroxytamoxifen, or 50ng/ml EGF.
Although MCF-7-LCC2 cells showed a greater resistance to combined treatment with estradiol and monohydroxytamoxifen compared to parental MCF-7 (Figure 13A and 13B), co-treatment with estradiol and 4-hydroxytamoxifen MCF-7-LCC2 was less resistant. Noteworthy is the difference in effect of 99149 or 4-hydroxytamoxifen and EGF in MCF-7 versus MCF-7-LCC2. The tamoxifen analogs both appear to reduce luciferase activity in combination with EGF in compared to EGF alone in LCC2 cells whereas there is not a significant difference in parental cells.
DISCUSSION

Estrogen receptor (ER) α and β may hetero- or homo-dimerize and recruit coregulators in a ligand dependent and independent manner, initiating transcriptional activity. Dimerization is the first step in initiating the transcriptional activity of the estrogen receptor. The preferential dimerization between ERs may be key in understanding the mechanisms regulating either tamoxifen agonist or antagonist activity. The consequences of heterodimerization on tamoxifen regulated expression of ER target genes has not been determined. We demonstrate here for the first time the use of bioluminescence resonance energy transfer (BRET<sup>2</sup>) as a model system for real-time, in-vivo protein interactions between nuclear receptors and coregulators.

Bioluminescent resonance energy transfer (BRET<sup>2</sup>, PerkinElmer) is a novel technique used to measure protein-protein interactions in a live, cell-based system. It is characterized by the efficient transfer of excited energy between a bioluminescent donor molecule (Renilla luciferase) and a fluorescent acceptor molecule (a mutant of Green Fluorescent Protein). The BRET<sup>2</sup> assay is advantageous to FRET because it does not require an external light source thereby eliminating the problems of photobleaching and autofluorescence. The absence of contamination by light results in a low background that allows detection of very small changes in the BRET signal. BRET is dependent on the orientation and distance between two fusion proteins and therefore requires preliminary standardization experiments to conclude a positive BRET signal, independent of variations in protein titrations and folding arrangement in tertiary structures.

To establish BRET in a ligand inducible system, we chose to use the nuclear

43
coactivator SRC-1 as the donor moiety, and the estrogen receptor as the acceptor moiety. Functionality of these fusion proteins was assessed by transient transfection in HeLa cells, followed by Western blot analysis for protein expression and reporter gene activity. Our preliminary results reveal that titration of ER and SRC-1 protein levels is necessary to obtain an accurate molar ratio of the Rluc and GFP fusion proteins needed for BRET. It has previously been shown that estrogen receptor signaling is modulated by the nuclear coactivator SRC-1 and corepressor NCoR. Elucidation of the subcellular localization of estrogen receptors and determination of interactions between the receptors as well as coregulators will provide important information about ligand stimulated transcriptional activity of ER target genes.

Studies on truncated estrogen receptor and/or coregulator protein-protein interactions has been shown by several in vitro mechanisms (Chen et al. 1999; Cowley et al. 1997; Resnick et al. 2000; Valentine et al. 2000), as well as in vivo assays using fluorescently labeled fusion proteins such as fluorescence resonance energy transfer (FRET) and fluorescence recovery after photobleaching (FRAP) (Bai and Giguere 2003; Smith et al. 1997; Tamrazi et al. 2002). However, previous literature has not been able to identify the role of site specific phosphorylation of ERα, ERβ, and coregulators in regulation of tamoxifen-dependent transcription of ER-target genes in a real time, in-vivo assay. Published work with BRET² does not contain standardization experiments that we have found imperative to conclude a positive BRET² signal in interpreting the data. The BRET² assay is a sensitive assay for determining ligand-dependent and –independent interactions. However, a major limitation of BRET² is that it is not quantitative, and can only describe simply whether there is an interaction or not. This is because ligand-
induced conformational changes of the receptor can render the GFP moiety too far from
the Rluc moiety for efficient resonance energy transfer. Therefore, because there may not
be a significant BRET$^2$ signal, this does not necessarily indicate the proteins of interest
do not interact. Conversely, a false positive resonance energy transfer signal may occur
from over-expressed proteins. This BRET2 protocol has been developed to incorporate
and eliminate these false positives, thereby increasing specificity.

Tamoxifen induced proliferation of endometrial tissue can cause an increased risk
of uterine cancer in patients using the drug for breast cancer therapy. Tamoxifen works
by interfering with the growth activity of estrogen, reducing the mortality and recurrence
of estrogen receptor positive breast cancer. The benefits of tamoxifen for treatment of
breast cancer are greater than the risk of developing endometrial carcinoma. However,
because tamoxifen is the most widely used therapy for hormone responsive breast cancer,
there is an urgent need to further understand the molecular basis of the tissue specificity
of tamoxifen. In addition to an increase in the rate of incidence of uterine cancer,
estrogen receptor positive breast cancer, over time, can become unresponsive to
tamoxifen therapy. It is not known how this resistant phenotype occurs, but several
mechanisms have been proposed. These include the loss of estrogen receptor, alterations
in the binding of tamoxifen, and modulation in the interaction between estrogen receptor
and transcription coregulators. This acquired tamoxifen resistance often occurs
concurrently with the over-expression of the epidermal growth factor family of receptors.

Two of the receptors within the epidermal growth factor receptor family (EGFR
and ErbB2) are involved in the poor prognosis of breast cancer. When these receptors are
activated by ligand they enhance the growth and metastasis of cancers by signaling to
downstream kinases involved in cell growth. Both receptors have been shown to phosphorylate the estrogen receptor α (ERα), estrogen receptor β (ERβ), and the steroid receptor coactivator SRC-1. This phosphorylation can lead to the secondary modulation of estrogen receptor action such as dimerization, coactivator recruitment, and promoter interaction. ER-promoter interaction subsequently leads to transcription of ER-dependent genes involved in cell growth. Therefore, knowledge of the molecular mechanism of ligand-independent and dependent activation of the estrogen receptor is a crucial step in understanding the pathogenesis of breast cancer. It will also contribute to the development of improved anti-hormonal therapies that do not exhibit estrogen-like activity or promote anti-hormonal resistance.

The BRET\textsuperscript{2} assay confirmed previous reports of an estradiol dependent dimerization of ERα. Remarkably, there SERMs of varying ERα antagonist properties also induced a robust dimerization within 1 hour. 4-hydroxytamoxifen is a mixed antiestrogen with estrogen agonist activity in the uterus. EM-652 is a pure antiestrogen in the uterus and breast and ICI 182,780 is a pure antagonist that induces degradation of ERα. While 4-hydroxytamoxifen and EM-652 permit DNA binding by ERα, ICI 182,780 prevents this association. Future studies will examine the relative importance of these SERM induced dimerization for antagonist action and ERα proteolysis. A similar remarkable finding was that all SERM ligands induced recruitment of the coactivator SRC-1 to ERα within 1 hour. Although this may seem contradictory, especially for the pure antagonist ligands, a recent paper revealed a novel mechanism linking recruitment of coactivator proteins to nuclear receptors bound to antagonist ligands. Recruitment of SRC-1 to ERα complexed with ICI 182,780 was required for recruitment of the proteosomal machinery to the ERα
complex that in turn downregulated ERα protein (Shao et al. 2004). The present data suggest that the coactivator SRC-1 may also serve this role as a platform for recruitment of proteosomal proteins.

EGF did not induce ERα dimerization or recruitment of the coactivator SRC-1 to ERα within 12 hours. EGF induces ERα ligand independent activity (Stoica et al. 2000; Stoica et al. 2003). These data would suggest that the ligand independent activity of ERα is not dependent upon dimerization or recruitment of SRC-1 although EGF recruitment of other related coactivators was not tested.
SUMMARY

We demonstrate the use of BRET\textsuperscript{2} as a model system for real-time, \textit{in vivo} protein interactions between nuclear receptors and coregulators. To establish BRET in a ligand inducible system, we chose to use the nuclear coactivator SRC-1 as the donor moiety, and the Estrogen Receptor as the acceptor moiety. Functionality of these fusion proteins was assessed by transient transfection in HeLa cells, followed by Western blot analysis for protein expression and reporter gene activity. Titration of ER and SRC-1 protein levels was needed to obtain an accurate molar ratio of the Rluc and GFP fusion proteins needed for BRET.

Published work with BRET\textsuperscript{2} does not contain standardization experiments that we have found imperative to conclude a positive BRET\textsuperscript{2} signal in interpreting the data. Although BRET\textsuperscript{2} is a sensitive assay for measuring ligand and time dependent changes in nuclear receptor interaction in live cells, one major limitation of BRET\textsuperscript{2} is that it is not quantitative but rather simply determines whether or not an interaction has occurred. This is because ligand-induced conformational changes of the receptor may place the GFP moiety too distant from the Rluc moiety for efficient resonance energy transfer. Consequently, a large increase in the BRET\textsuperscript{2} ratio may not necessarily indicate a quantitatively greater interaction between proteins than smaller increases in the BRET\textsuperscript{2} ratio.

Elucidation of the key mechanisms involved in promoting tamoxifen agonist activity will contribute to the development of novel therapeutics that circumvents tamoxifen resistance.


ABSTRACT

$17\beta$-estradiol ($E_2$) has a central role in the proliferation of estrogen responsive cells through the modulation of target gene expression within estrogen responsive tissues. The effects of $E_2$ are mediated through binding to the Estrogen Receptor $\alpha$ nuclear receptor transcription factor. Tamoxifen also binds to the estrogen receptor to modulate ER-mediated gene transcription. Although tamoxifen is an antiestrogen in breast tissue, it also contains partial estrogenic activity in the uterus and is associated with an increased risk of developing uterine cancer. Tamoxifen can act as an ER antagonist, inducing a conformational change in the receptor that blocks its interaction with coactivators. This study has employed a novel methodology specifically called bioluminescent resonance energy transfer (BRET$^2$) to accurately measure protein-protein interactions. Understanding the roles of ER$\alpha$ and coregulators in a ligand-independent and dependent manner may elucidate a mechanism in the tissue selectivity of tamoxifen that has not yet been determined.