

EVALUATING ESTROGEN RECEPTOR SIGNALING IN PANCREATIC β -CELLS

BY

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DEDICATION

Dedicated to everyone that has encouraged me to pursue my dreams.

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LIST OF ABBREVIATIONS

°C	Degrees Celsius
AF-1	Activation Function 1
AF-2	Activation Function 2
BPA	Bisphenol A
BSA	Bovine Serum Albumin
CMV	Cytomegalovirus
Cys	Cysteine
DBD	DNA Binding Domain
DMSO	Dimethyl Sulfoxide
DNA	Deoxyribonucleic Acid
E2	17 β -estradiol
EDC	Endocrine Disrupting Chemicals
ELISA	Enzyme Linked Immunoabsorbance Assay
ERE	Estrogen Response Element
ER α	Estrogen Receptor Alpha
ER β	Estrogen Receptor Beta

EV	Empty Vector
FBS	Fetal Bovine Serum
GFP	Green Florescent Protein
GLUT2	Glucose Transporter 2
GLUT4	Glucose Transporter 4
GPER	G protein-coupled estrogen receptor 1
HNF1a	Hepatic Nuclear Factor 1 Alpha
IF	Immunofluorescence
IGF1	Insulin Like Growth Factor 1
IIDP	Integrated Islet Distribution Program
IRF4	Interferon Regulatory Factor 4
IRS2	Insulin Receptor Substrate 2
LBD	Ligand Binding Domain
MAPK	Mitogen Activated Protein Kinase
mg	Milligrams
mL	Milliliters
MODY	Maturity Onset Diabetes of the Young

MPP	MPP Dihydrochloride
mRNA	Messenger Ribonucleic Acid
NCoA5	Nuclear Coactivator 5
NCoR	Nuclear Receptor Corepressor 1
ng	Nanogram
nM	Nanomolar
NPY	Neuropeptide Y
nRFP	Nuclear Red Fluorescent Protein
PBS	Phosphate Buffered Saline
PCB	Polychlorinated Biphenyl
PCR	Polymerase Chain Reaction
PHTPP	4-[2-Phenyl-5,7- <i>bis</i> (trifluoromethyl)pyrazolo[1,5- <i>a</i>]pyrimidin-3-yl]phenol
PI3K	Phosphoinositide 3 Kinase
POMC	Pro-opiomelanocortin
PPT	4,4',4''-(4-Propyl-[1 <i>H</i>]-pyrazole-1,3,5-triyl) <i>tris</i> phenol
PUMA	p53 Upregulated Modulator of Apoptosis
PVDF	Polyvinyl Difluoride

qPCR	Quantitative Polymerase Chain Reaction
Ral	Raloxifene Hydrochloride
RFP	Red Fluorescent Protein
RNA	Ribonucleic Acid
ROI	Region of Interest
RPMI	Roswell Park Memorial Institute Medium
SERM	Selective Estrogen Receptor Modulator
SNP	Single Nucleotide Polymorphism
SRC1	Steroid Receptor Coactivator 1
T1DM	Type 1 Diabetes Mellitus
T2DM	Type 2 Diabetes Mellitus
TBS	Tris-buffered Saline
TF	Transcription Factor
TRITC	Tetramethylrhodamine Isothiocyanate
μM	Micromolar
μL	Microliter
μg	Microgram

WB	Western Blot
Y134	[6-Hydroxy-2-(4-hydroxyphenyl)benzo[<i>b</i>]thien-3-yl]-[4-[4-(1-methylethyl)-1-piperazinyl]phenyl]methanone
ZDF	Zucker Diabetic Fatty
ZF	Zucker Fatty

ABSTRACT

Dover, Ellen Nicole

EVALUATING ESTROGEN RECEPTOR SIGNALING IN PANCREATIC β -CELLS

Dissertation under the direction of Peter Antinozzi, PhD, Assistant Professor

Estrogen receptor signaling is involved in the etiology of multiple diseases including the development and progression of Type 2 Diabetes Mellitus, T2DM. The role of estrogen receptor signaling in peripheral insulin resistance has been shown through studies in post-menopausal women; however, estrogen receptor signaling in the β -cell is less understood. Conflicting data have arisen that demonstrate estrogen receptor signaling in the β -cell has either no effect, a protective effect against induced stress, or deleterious effects after long term activation of signaling. The present study aimed to elucidate the role of estrogen receptor isoform and variant signaling on the health and function of pancreatic β -cells. The variants of ER α differ in the presence of two activation function (AF) domains, which regulate transcriptional activities. ER α 66 possesses both domains, ER α 46 lacks the AF-1 domain, and ER α 36 lacks both the AF-1 and AF-2 domains. Expression of ER α 66 induced constitutive ERE-dependent transcriptional activity, which was correlated to an increase in pancreatic β -cell death as observed through high throughput imaging analysis and apoptotic endpoint assays (caspase 3/7). ER α 46 expression resulted in increased transcriptional and caspase 3/7 activity similar to ER α 66. Interestingly, in all assays ER α 46 was more sensitive to ligand treatment than ER α 66, suggesting an inherent difference in the ligand responsiveness between these two variants in the INS-1E cell line. ER α 36 did not significantly alter transcriptional or caspase 3/7

activity. Expression of the estrogen receptor isoform, ER β , increased ERE-dependent transcriptional activity and caspase 3/7 activity, however the magnitude of activation was less than ER α . Expanding these assays into human islets revealed that raloxifene treatment results in a significant increase in insulin mRNA expression as well as differentially regulating genes that may be involved in β -cell health. In addition to testing isoforms and variants, coregulator interaction with the receptors was also assessed. The coregulator, NCoA5, significantly potentiated ER α 66 activity, but had no significant effect on ER α 46 or ER β implying a dependence on the AF-1 domain. Overall, this study elucidates that genomic estrogen receptor signaling is deleterious to β -cell health and function.

Chapter 1 Introduction

Diabetes

Diabetes is a metabolic disorder characterized by increased blood glucose levels, severe health complications, and high cost of care. The occurrence of diabetes is becoming more prevalent in the United States and throughout the world with the most dramatic increases in the last 20 years. The number of individuals with diabetes has been steadily rising since 1980, increasing from 5.6 million individuals to 29.1 million individuals, according to the latest Centers for Disease Control record¹⁻³. This staggering increase can be attributed to a rapid rise in the prevalence of type 2 diabetes mellitus (T2DM).

Diabetes is typically classified into one of two broad categories: type 1 diabetes mellitus (T1DM) or type 2 diabetes mellitus. While both of these conditions produce increased blood glucose levels, the etiology of the disease is vastly different. In type 1 diabetes, the increase in blood glucose levels is initiated by autoimmune-based destruction of pancreatic β -cells⁴. β -cells are the insulin producing cells of the pancreas, which release the hormone to mediate a blood glucose lowering effect. Due to the destruction of β -cells, individuals with T1DM become dependent on insulin injection⁵. T2DM is characterized by insulin resistance in peripheral tissues resulting in increased blood glucose levels and can also advance to pancreatic β -cell death through glucotoxicity or lipotoxicity^{4,6}. Additionally, there are sub-categories of diabetes including gestational diabetes and maturity onset diabetes of the young (MODY)⁵. Gestational diabetes arises much like T2DM and results from peripheral insulin resistance due to hormonal changes during pregnancy. Typically this type of diabetes

resolves itself following delivery of the offspring, but these women remain susceptible to T2DM development later in life⁷. While diabetes is usually a complex trait disease with multiple contributing factors, MODY develops from autosomal dominant mutations in genes involved in insulin production and secretion, including HNF1a and glucokinase, among others⁸. These examples illustrate the complexity of diabetes development and the variability in the etiology of the disease.

Blood Glucose Homeostasis

There are multiple tissues involved in regulating blood glucose homeostasis including skeletal muscle, liver, pancreas, adipose, and brain. As mentioned above, the pancreas is where the blood glucose lowering hormone, insulin, is produced in a specialized cell type called the β -cell. The other important endocrine cell type in the pancreas is the α -cell, which produces the blood glucose raising hormone, glucagon. These hormones are released and bind to insulin or glucagon receptors in peripheral tissues. When insulin binds to its receptor in the muscle it stimulates the translocation of glucose transporters (i.e. GLUT4) to the membrane to initiate glucose uptake⁹. In the liver, insulin binding stimulates the uptake of glucose, which is then converted into the storage molecule glycogen⁹. Glucagon signaling produces the opposite effect of insulin by binding to receptors in the liver to initiate the breakdown of glycogen into glucose to raise blood glucose levels⁹. An imbalance in this signaling pathway can lead to increased blood glucose levels and development of T2DM.

The brain is also important in regulating the development of obesity and T2DM through multiple mechanisms. The brain responds to signaling from insulin to reduce food intake. If components of the insulin sensing pathway are depleted (i.e. IRS2), then

the brain can no longer respond to insulin and obesity and diabetes can develop¹⁰. There are also specific neurons, which secrete hormones to regulate food intake. Neuropeptide Y (NPY) producing neurons are one of many types of neurons that stimulate food intake and reduce energy expenditure^{10,11}. Signaling from insulin inhibits NPY-producing neurons, thereby reducing food intake and increasing energy expenditure. These tissue interactions demonstrate the complexity involved in the development of diabetes, and highlight the importance of each player in contributing to disease progression. Interestingly, all of these systems are affected by signaling from the hormone, estrogen, through its interaction with nuclear hormone receptors, ER α and ER β .

As the incidence of the metabolic syndrome has risen in the past several decades, association between gender specific differences in the frequency of the disease has strengthened, demonstrating that estrogen signaling is associated with multiple traits of the metabolic syndrome including T2DM and obesity¹²⁻¹⁴. T2DM incidence increases in both men and women as they age, however post-menopausal women have a five-fold increase, whereas there is only a two-fold increase for males as they progress through aging¹⁵. The higher incidence of metabolic syndrome in post-menopausal women may be due to a decrease in the concentration of the hormone, estrogen, as women enter and progress through menopause. Clinical evidence has shown that women given estrogen and progesterone replacement therapy have a decreased prevalence of T2DM, further supporting estrogen's role in the prevention of metabolic dysfunction¹⁶. The ability of estrogen to decrease hyperglycemia and reduce insulin resistance arises mostly from estrogen action in the peripheral systems including adipose tissue, skeletal muscle, and

the brain, whereas the direct impact of estrogen on the pancreatic β -cell is less understood¹⁷.

Estrogen Effect on Insulin Sensitive Tissues

In the liver, inhibition of ER α signaling can lead to increased glucose production and lipid synthesis worsening the diabetic phenotype^{14,18}. As mentioned previously, GLUT4 is a glucose transporter critical to maintaining glucose homeostasis. In mouse skeletal muscle, ER α and ER β were shown to be important for both the transcription and translocation of GLUT4 to the plasma membrane¹⁹. In the brain, ER α inhibition can lead to obesity from hyperphagia and hypometabolism. This effect is mediated from distinct neurons in the brain including those responsible for hypometabolism, SF1 neurons, and hyperphagia, POMC (pro-opiomelanocortin) neurons²⁰. In adipose tissue, estrogen signaling has a direct effect on inhibiting lipogenesis^{21,22}. Additionally, reduced ER α signaling in adipose tissue can lead to increased adipocyte size, inflammation, and fibrosis, which leads to obesity (a phenotype associated with T2DM)²³. Estrogen receptor signaling involvement with diabetes is discussed in more detail below; however, before discussing the role of estrogen receptor signaling in diabetes, it is important to understand the basic factors that contribute to estrogen receptor action in cells.

Estrogen Receptor Signaling

Estrogen receptors belong to a category of transcription factors known as nuclear hormone receptors, and initiate cellular action in response to estrogenic ligand stimulation. There are two isoforms of estrogen receptor and multiple variants, which can signal through various pathways to initiate estrogen signaling.

Genomic Estrogen Signaling Pathway

The canonical estrogen receptor pathway involves the binding of ligand to the receptor in the cytoplasm, dimerization, and translocation to the nucleus to initiate transcription from estrogen response element (ERE) promoters (Figure 1.1). The

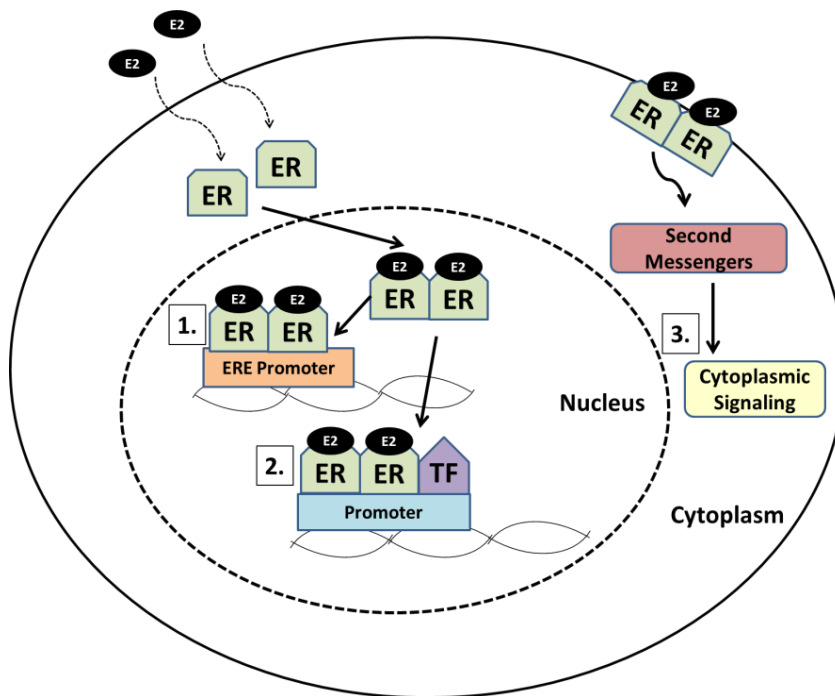


Figure 1.1 Estrogen Receptor Signaling Pathways.

The canonical estrogen receptor signaling pathway involves the binding of 17 β -estradiol (E2) to estrogen receptor isoforms in the nucleus, which induces dimerization, and translocation to the nucleus where estrogen receptors binding to estrogen response elements (ERE) in the promoter (1). Estrogen receptors have also been shown to associate with other transcription factors (SP1, c-FOS, Jun, etc.) to initiate transcription from Non-ERE promoter elements (2). A non-genomic signaling pathway involves the binding of E2 to cytoplasmic estrogen receptors to initiate second messenger cascades (i.e. PI3K, Src kinase, etc.) to mediate cytoplasmic signaling (3).

three factors governs much of the transcriptional activity of estrogen receptor, there are

canonical estrogen receptor signaling pathway is governed by the presence of three factors known as the tripartite pharmacology of estrogen receptors^{24,25}. Components of tripartite estrogen receptor signaling include: the estrogen receptor variant expressed, efficacy and type of ligand bound, and the recruitment of cofactors including coregulators^{25,26}. While the presence of these

many subcategories within each category. For example, ligands can include both natural (E2) and pharmacological agents like SERMs; the receptors can include the splice variants, isoforms, and post-translation modifications of each; and effectors can include transcription factors, corepressors, coactivators, and other transcriptional machinery. Although the ERE-dependent pathway is the traditional pathway for estrogen receptor transcriptional response, estrogen receptors may regulate the transcription of target genes with half ERE sites or no ERE site at all²⁷. Alternatively, estrogen receptors have been shown to participate in transcriptional cross-talk with transcription factors, such as c-Jun, FOS, or SP1, to regulate transcription from non-ERE promoter elements (Figure 1.1)^{28–30}. One gene that is regulated in this manner is cyclin d1, an important mediator of the cell cycle³¹. This signaling pathway opens up a new category of estrogen regulated target genes, and adds a layer of complexity to estrogen receptor signaling. Additionally, there are instances where estrogen receptors are transcriptionally active in the absence of estrogenic ligand leading to apo-estrogen receptor activity, once again highlighting the versatility in estrogen receptor signaling³².

Non-Genomic Estrogen Receptor Signaling

In addition to genomic signaling, estrogenic ligands can bind to extranuclear estrogen receptors to mediate a rapid non-genomic effect (Figure 1.1). While there is some debate on how estrogen receptors may associate with the plasma membrane to mediate non-genomic effects, the palmitoylation site that both ER α (Cys452) and ER β (Cys354) contain may facilitate their association with the plasma membrane^{33–35}. In addition to the palmitoylation sites found on both of these receptors, ER α 36 has three myristoylation sites, not found in the other receptors, which could enhance its association

with the plasma membrane³⁶. Other hypotheses for how estrogen receptors regulate plasma membrane signaling include the interaction of estrogen receptors with plasma membrane associated molecules, such as G-proteins or the p85 α subunit of PI3K, or through association with caveolae³⁷. Regardless of the mechanism, cytoplasmic estrogen signaling can initiate phosphorylation cascades including MAPK, tyrosine phosphorylation, Src kinase, and many others to induce its non-genomic effects^{38,39}. This additional signaling pathway leads to a rapid response of estrogenic ligand binding and bypasses the transcription and translation required for traditional estrogen signaling.

Estrogen Receptor Isoforms and Splice Variants

There are two isoforms of estrogen receptor, alpha (α) and beta (β), which are



Figure 1.2 Human Exon Structure of Estrogen Receptors.

ER α is located on chromosome 6 and contains 10 exons and a downstream exon (U), which is used in the ER α 36 variant. ER α 46 and ER α 36 skip the first coding exon of ER α , and thus lose the A/B domain of the protein (indicated by color). ER β is located on chromosome 14 and contains 9 exons and a downstream exon (cx), which is used in ER β 2 and ER β 5. ER β 2 and ER β 5 lack exon 9, which eliminates part of the E/F domain of the protein (indicated by color). Translation start sites indicated by arrow.

located on different chromosomes and function independently of one another (Figure 1.2)^{40,41}. In addition to the two isoforms of the receptor, there are multiple splice variants of each isoform, which perform diverse cellular activities

according to their structural differences^{40,42}. The three primary splice variants of ER α

include ER α 66, ER α 46, and ER α 36 (Figure 1.3)^{40,42,43}. The variants of ER α differ in the presence of two activation function (AF) domains, which recruit coregulators and other transcriptional machinery to drive nuclear activity⁴⁴. The AF-1 domain is responsible for transactivation of the receptor in a ligand independent manner, whereas the AF-2 domain is responsible for ligand dependent transactivation⁴⁴. ER α 66 possesses both of these domains and is the classical full length wild type estrogen receptor (Figure 1.3)⁴³.

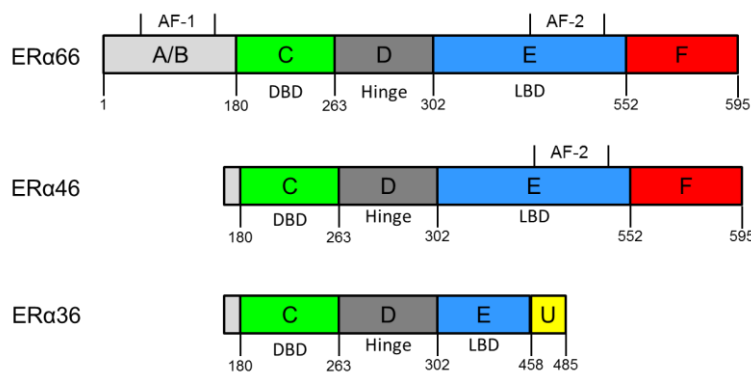


Figure 1.3 Estrogen Receptor Alpha Splice Variants.

ER α 46 and ER α 36 lack part of the A/B domain eliminating the AF-1 functionality. ER α 36 also lacks part of the E domain and the entire F domain, eliminating its AF-2 functionality as well. Additionally, ER α 36 contains 27 unique amino acids encoding an altered C-terminus.

Alternative promoter usage

can cause skipping of the first coding exon producing

the AF-1 deficient variant,

ER α 46 (Figure 1.2 and

Figure 1.3)⁴³. The lack of

an AF-1 domain prevents

the ligand independent

transactivation by ER α 46,

and has been shown to

inhibit the AF-1 dependent transcriptional activity of ER α 66 through heterodimerization

with this receptor⁴⁵. The third splice variant of ER α , ER α 36, has both an altered N-terminus and an altered C-terminus, making this receptor the shortest of the three⁴³.

ER α 36 lacks the first coding exon, similar to ER α 46, leading to a lack of an AF-1

domain, but also has a C-terminal truncation which eliminates the last 138 amino acids

thus removing the AF-2 domain as well (Figure 1.2 and Figure 1.3)⁴⁶. The C-terminus of

ER α 36 is further altered by the presence of a 27 amino acid sequence transcribed from a

downstream exon (U), not present in ER α 66 or ER α 46, which confers three myristoylation sites to this receptor (Figure 1.2 and Figure 1.3)⁴⁶. The presence of the three myristoylation sites increases the likelihood of ER α 36 insertion into the plasma membrane; thus it is postulated that ER α 36 is the predominant membrane associated variant^{36,46,47}.

The isoforms of ER β differ in the last exon, which affects the C-terminus (Figure 1.2 and Figure 1.4)^{40,42,43,48}. ER β 1 is the full length wild type receptor and possesses an active ligand binding domain as well as a functional AF-2 domain (Figure 1.4)⁴⁸. ER β 1 is structurally similar to ER α 66 in the DNA binding domain as well as the ligand binding domain/AF-2 domain, however varies significantly at the AF-1 domain⁴⁰. ER β 2

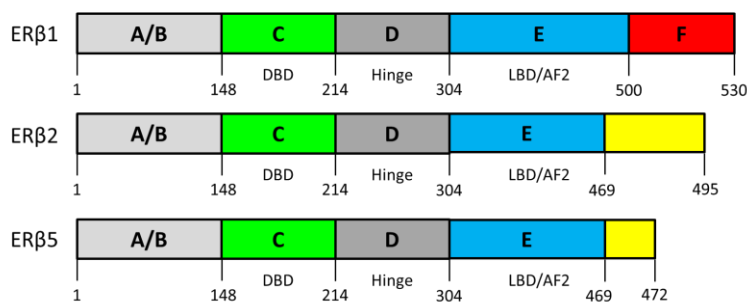


Figure 1.4 Estrogen Receptor Beta Splice Variant Domains.

ER β splice variants differ in the C-terminal region. ER β 1 is the wild-type ER β receptor containing all 530 amino acids. ER β 2 and ER β 5 contain C-terminal truncations and replacement of C-terminal amino acids with downstream sequence (shown by yellow boxes).

possesses a C-terminal truncation of 61 amino acids that are found in the wild-type receptor, ER β 1.

ER β 2 also has a unique C-terminal addition of 26 amino acids from a 3' exon, which blocks the

ligand binding domain making it incapable of ligand binding (Figure 1.2 and Figure 1.4)⁴⁹⁻⁵¹. ER β 5 possesses the same 61 amino acid C-terminal truncation as ER β 2, but contains a smaller 4 amino acid substitution encoded by a 3' exon (Figure 1.2 and Figure 1.4). Thus ER β 5 is capable of binding ligand, however only at supraphysiological concentrations⁴⁹⁻⁵¹. Additionally, ER β 1 is the only isoform capable of homodimerization,

as the deletions and substitutions in the C-terminus of ER β 2 and ER β 5 only allows for heterodimerization⁴⁸. While there are more splice variants of ER β that are not discussed here, ER β 1, ER β 2, and ER β 5 are the most well characterized variants of ER β . ER β 1 was the variant discussed in the present study, because it is the only isoform capable of both ligand binding and production of activating homodimers.

Effectors and Ligands

As mentioned previously, the tripartite pharmacology of estrogen receptors involves a bound ligand, estrogen receptor variant expression, and the presence of coregulators. There are two general categories of estrogen receptor coregulators, which includes corepressors and coactivators^{52,53}. Corepressors work to inhibit estrogen receptor mediated transcriptional activation by functioning as histone deacetylases or interfering with coactivator binding^{52,54,55}. Coactivators exert their transcriptional activation effects through multiple mechanisms including: histone acetylation, histone methylation, and tethering for other cofactors^{24,44}. In some cases of genomic estrogen receptor signaling, ligand binding to estrogen receptors induces a conformational change, which promotes either corepressor binding (antagonist ligand) or coactivator binding (agonist ligand)^{41,56}. The expression of coregulators influences the tissue specificity of a category of estrogenic ligands known as selective estrogen receptor modulators (SERMs), which can have either activating or repressing effects on estrogen receptor activity^{41,56}. Therefore, in examination of estrogen receptor involvement in cellular activity, one must take into account the expression of estrogen receptor variants and coregulators as well as estrogen ligand presence.

Estrogen Receptor Regulation of Cell Fate

Estrogen receptors are known to participate in a wide array of cellular activities, and can be major regulators of cell fate. Both ER α and ER β are known to be involved in proliferation, survival, and differentiation in multiple models. The following examples highlight the variability of estrogen receptor signaling in different cell types.

Interestingly, estrogen receptors regulate proliferation in two directions, inducing downregulation or upregulation dependent on the cell type. In a mouse mammary epithelial cell line, HC11, evidence has shown that inhibition of ER β signaling enhances ER α signaling to induce proliferation⁵⁷. In other breast cancer tissues, estrogen receptors are known to stimulate growth through induction of genes including insulin like growth factor (IGF1), c-fos, TGF- α , and many others⁵⁸. In the breast cancer cell line, MCF7, upregulation of estrogen receptor activity recruits the corepressor, NCoR, and histone deacetylases to reduce the expression of cyclin G2, a negative regulator of cell cycle⁵⁹. In bone, 17 β -estradiol treatment activates ER α to stimulate proliferation thereby strengthening osteoblasts in mice⁶⁰. Conversely, estrogen receptors can inhibit proliferative events as well. In MCF-7 cells, expression of ER β induces cell cycle arrest thus inhibiting proliferation⁶¹. Additionally, in vascular smooth muscle cells ER α signaling upregulates miR-203, a micro-RNA that inhibits proliferative events⁶².

In a neuronal cell line, PC12, extranuclear estrogen receptor signaling increased pro-survival kinase pathways to upregulate the anti-apoptotic protein Bcl2, enhancing cell survival⁶³. Additionally, p53 upregulated modulator of apoptosis (PUMA) expression induces apoptosis in breast cancer cells; activation of ER α signaling inhibits PUMA expression stopping the apoptotic effect⁶⁴. Conversely, estrogen receptor signaling can

promote cellular death through both non-genomic and genomic mechanisms. For example, in bone osteoclasts ER α signaling induces the expression of Fas ligand (FasL) inducing apoptosis and protecting bone from re-absorption to promote bone health⁶⁵. In addition to FasL regulation, non-genomic estrogen receptor signaling can upregulate p38 MAPK signaling in HeLa cells, which leads to apoptosis⁶⁶. These events highlight the importance of estrogen receptor signaling in the regulation of cell fate, and once again point to the delicate balance of estrogen signaling that exists to maintain proper cell health.

In addition to the defined role of estrogen signaling in cell proliferation and apoptosis, estrogen receptors also influence differentiation pathways. In myeloid progenitor cells, ER α can promote upregulation of interferon regulatory factor 4 (IRF4) to induce differentiation⁶⁷. In mouse ventral prostates, ER β depletion caused a lack of differentiated epithelium, revealing a role for both ER α and ER β in the control of differentiation⁶⁸. Not only is estrogen signaling important in triggering differentiation, but variation in estrogen receptor signaling can influence the formation of a particular cell type. An illustration of this comes from neural stem cells where 17 β -estradiol treatment triggers preferential development into neurons over glial cells⁶⁹.

The data from these studies highlights the various roles estrogen receptor signaling can play, and aids in the explanation of estrogen receptor action in pancreatic β -cell fate.

Estrogen Receptor Action in Pancreatic β -cells

One of the primary goals of diabetes research is to prevent the dysfunction and apoptosis of β -cells in order to preserve glucose homeostasis. Estrogens have been

identified as mediators of cell fate, as discussed previously, and are known as modulators of lipogenesis through prevention of abdominal adiposity^{70,71}. These two factors make estrogen signaling a promising avenue for study in T2DM and β -cells⁶.

Estrogen Receptor Effect on β -cell Health

Early evidence demonstrated that estrogen was selectively taken up into the Islets of Langerhans in baboons, suggesting that estrogen signaling could mediate some aspect of β -cell health or function⁷². A 2011 study by Tiano et al., found that activation of estrogen receptors in Zucker Diabetic Fatty (ZDF) rat islets can result in a reduction of triglyceride (TG) accumulation, although no significant effect on β -cell health was reported⁷³. Reduction in TG accumulation was observed through treatment with agonists of ER α and ER β as well as the G-protein coupled receptor, GPER, and substantiated by using pancreas specific ER α ablation, which reduced the protective effects of estrogen agonism on TG accumulation and Fas expression^{22,73}. However, when ER α and GPER were ablated in mouse islets and analyzed *in vitro*, triglyceride accumulation was reduced to the same level as the agonist treated wild type animals, convoluting the findings of the study⁷³. The same study also utilized INS-1 cells treated with ER α , ER β , and GPER agonists, which initiate a reduction in fatty acid synthetase (FAS) expression and TG content similar to ZDF treated islets^{22,73}. The pitfall of this particular analysis is that the result is not shown to be dependent on one receptor isoform or splice variant, since all agonists behaved similarly. Additionally, experimentation in this dissertation shows no detectable expression of ER α or ER β in the INS-1E cell line, questioning the findings of this study. Further, it is possible that the effect could be mediated solely through the action of the agonists on GPER, since G1 (the GPER agonist) only binds to this receptor,

and mediated a similar effect to all other agonists analyzed^{22,73}. Another study substantiates the hypothesis that GPER may be involved, since there was a more significant effect of G1 treatment than E2 treatment on components of fatty acid synthesis²². An additional caveat of this study is that the effects were seen at supraphysiological concentrations of E2 (100 nM or more), which may not translate into physiological relevance in humans.

In mouse models of streptozotocin induced diabetes (which induces diabetes by DNA damage after uptake by GLUT2 transporters), mice lacking ER α or ER β have increased susceptibility to diabetes development, which is not dependent on the DNA binding domain⁷⁴. However, the protective effects of ER β are significantly lower than ER α . In that study, the use of whole animal ER α and ER β knockout convolutes the findings because some of the protective effects could actually be mediated by signaling in peripheral tissues. Additionally, simultaneous ablation of both ER α and ER β did not eliminate the protective effect of estrogen treatment, suggesting an alternate mechanism of action. GPER, as discussed above, can exhibit anti-apoptotic and anti-lipogenic properties similar to ER α ⁷⁵. Knockout of GPER weakens estrogen's protective effect against induced β -cell stress similar to ER α knockout, which suggests a compensatory mechanism for extranuclear estrogen action in β -cells^{74,75}. In mouse islets, treatment with estrogen dendrimer conjugates (estrogens incapable of nuclear translocation) or 17 α -estradiol (an estrogen incapable of producing transcriptional activity) yielded the same effects as 17 β -estradiol treatment, validating that the protective effect is through extranuclear mechanisms⁷⁴. Other studies have shown that in INS-1 cells, estrogen treatment works through cytoplasmic signaling pathways to protect cells against

glucotoxicity by reducing oxidative stress through changes in ATF6-p50, p-ERK, UXB1, and SXP1 expression, however this effect was only present at 40 mM glucose (the equivalent of a 720 mg/dL blood sugar level)⁷⁶.

While these studies suggest there may be an effect of exogenous estrogen administered at supraphysiological concentrations, the effect of endogenous estrogen synthesis has also been examined. Estrogen is synthesized through a pathway involving the enzyme aromatase, which converts androgens to estrogens⁷⁷. In mice, knockout of the aromatase enzyme resulted in increased susceptibility of pancreatic β -cells to apoptosis after streptozotocin challenge⁷⁸. In both the ER α and aromatase knockout models, β -cells had normal morphology and function prior to challenge with streptozotocin or oxidative stress, suggesting a lack of importance in normal pancreatic β -cell function. These findings substantiate the need for further investigation of estrogen receptor action in pancreatic β -cells.

Estrogen Receptor Effect on Insulin Secretion

Estrogen receptor signaling has also been examined in relation to changes in β -cell function. In Zucker Diabetic Fatty rats, treatment with 17 β -estradiol leads to an increase in insulin content and secretion, suggesting that estrogens may exert an effect on β -cell function⁷³. However, in examination of these data, one must consider the methodology. The islets were isolated after E2 treatment of the whole animal, thus the increase in β -cell mass and insulin concentration may be due to improved insulin resistance in peripheral tissues, which might lead to reduced β -cell stress and failure, thus producing the observed result.

In the analysis of insulin secretion, the contribution of ER α and ER β is debatable. In one study, treatment of mouse pancreatic islets with 17 β -estradiol induced closure of K_{ATP} channels, which led to enhanced insulin secretion⁷⁹. This effect was mediated through the ER β receptor, as ER α knockout mice had no significant effect on the K_{ATP} channel modulation after treatment with 17 β -estradiol⁸⁰. In another study, mouse islets treated with 17 β -estradiol stimulated ER α to interact with the tyrosine kinase, Src, to activate the transcription factor, NeuroD1, and promote insulin synthesis⁸¹. These findings are in direct opposition to one another and show that in two different studies using one cell type (mouse islets) that the effect is exclusively mediated through either ER α or ER β .

These studies highlight a need for understanding the role of ER α or ER β in the modulation of insulin secretion, and suggest that there are other factors at play that may mediate the response in addition to these two receptors. Altogether, a balance may exist in which too much or too little estrogen receptor signaling potentiates β -cell death and dysfunction. In fact, Nadal, et al., 2009, generated the hypothesis that an imbalance of estrogen signaling can lead to hyperinsulinemia, insulin resistance, and progression to the diabetic condition⁸².

Estrogenic Ligands Effect on Isolated Islet Health

In human islet isolation procedures, treatment with estrogenic ligands can reduce pro-inflammatory cytokine mediated apoptosis⁸³. After transplanting islets into mouse recipients, the cytokine stress induced islets treated with estrogenic ligands maintained higher functionality as shown through an increase in plasma insulin levels and reduced apoptosis⁸³. In addition to these studies, treatment of isolated human islets with estrogen for four days prior to transplantation resulted in a greater euglycemic state in mouse

recipients, up to 30 days after transplant⁸⁴. In these studies it is possible that the effect of estrogen may be mediated by increased revascularization since estrogen has a known positive impact on vascularization, which would improve islet viability post-transplant⁸⁵.

Interestingly, treating rat islet donors with 17 β -estradiol for 7 hours prior to islet isolation increases both islet yield and islet viability by reducing the production of inflammatory cytokines, an effect which once again may be mediated through estrogen action in the periphery⁸⁶. The possibility also exists to treat recipients with estrogenic ligands after the transplantation of islets. Treatment of recipient mice with 17 β -estradiol during the process of islet transplantation increased islet engraftment and acutely reduced β -cell apoptosis⁸⁷. However, the effect of estrogen in the recipient mice could be due to estrogen's effect in peripheral tissues to improve insulin sensitivity and reduce blood glucose levels. These examples illustrate the necessity to look further into the action of estrogen in the periphery versus the β -cell in islet models of transplantation.

Selective Estrogen Receptor Modulators

Selective estrogen receptor modulators (SERMs) have the ability to act as agonists or antagonists dependent on the tissue type due to the multiple factors including the recruitment of coregulators and the isoform of estrogen receptor expressed⁵⁶. SERMs are widely given to women in the treatment of breast cancer, and recently tamoxifen treatment has been associated with a higher incidence of diabetes⁸⁸. However, another SERM, raloxifene (Evista), was found to have no effect on glycemic control in post-menopausal women, thus showing differential responses to SERMs in diabetes progression⁸⁹. Previously, raloxifene treatment of INS-1 cells was shown to reduce triglyceride content through extranuclear actions and agonism of cytoplasmic ER α while

antagonizing nuclear estrogen receptor activity, thus illustrating the diversity of SERM action in β -cells⁹⁰.

In another study using both mouse and human islets, an ER β selective SERM, WAY200070, was shown to exert anti-diabetic actions through increased glucose-stimulated insulin secretion and improved plasma insulin levels after streptozotocin induced diabetes⁹¹. However, in the same study the ER β selective agonist, DPN, had no effect on human islets, suggesting the mouse model may not mimic human tissues⁹¹. Nonetheless, ER α and ER β SERM based treatment strategies may open up a wide avenue of possibilities for the prevention of β -cell stress and apoptosis both during transplantation procedures and daily diabetes management.

Other studies provide evidence that estrogenic ligand exposure under normal conditions can cause β -cell dysfunction and apoptosis. The studies discussed below highlight the importance of environmental estrogen exposure and reveal a delicate balance for the effect of estrogen on the pancreatic β -cell.

Other Estrogenic Ligands

In addition to approved pharmacological estrogen receptor modulators, there are also compounds found in the environment that act as estrogen receptor ligands. These compounds, known as xenoestrogens, include bisphenol A (BPA) and polychlorinated biphenyl (PCB).

The xenoestrogen compounds are more widely known as endocrine disrupting chemicals (EDCs) and can have a broad impact on health by disrupting thyroid function, brain development, and pancreatic function⁹². Interestingly, BPA and PCB have been shown to have direct interaction with estrogen receptors and can activate both genomic

and non-genomic signaling pathways^{93,94}. There have been multiple studies examining the effects of xenoestrogens on the development and progression of diabetes, and most have found a positive correlation between compound exposure and diabetes incidence^{95–101}. Studies of post-menopausal women revealed there was a significant correlation between the concentration of PCBs in blood and a confirmed diagnosis of T2DM⁹⁵. BPA, a common environmental contaminant found in the plastic lining of cans and other sources, has been shown to be harmful to pancreatic β -cells even at low concentrations. Long-term exposure to low-level BPA in mice, which might mimic the environmental risk, was capable of inducing hyperinsulinemia and insulin resistance, which led to increased risk of T2DM through β -cell failure⁹⁶. Additionally, it was shown that prenatal BPA exposure increases the risk of developing weakened glucose-stimulated insulin secretion and has effects in peripheral tissues, reducing the expression of genes necessary for fatty acid metabolism⁹⁷. Expanding upon this knowledge, in a mouse β -cell line, exposure to BPA for as little as 24 hours increases the expression of HSP70, which is normally upregulated in response to endoplasmic reticulum stress¹⁰⁰. The induction of endoplasmic reticulum stress from BPA exposure could lead to decreased insulin production and eventually to β -cell apoptosis if stress is not alleviated. In this same study, there was also a decrease in the expression of GRP94 and GRP78, which have been shown to precede β -cell apoptosis in other systems^{100,102}. Thus, the activation of estrogen receptors by xenoestrogens in endogenous tissue may lead to the development of diabetes through induction of insulin resistance or initiation of β -cell dysfunction.

Single Nucleotide Polymorphisms of ER α Associate with Diabetes

There is also an association of single nucleotide polymorphisms (SNPs) with T2DM and the metabolic syndrome. In a study evaluating African American individuals with T2DM and end-stage renal failure, SNPs located in intronic regions 1 and 2 of the ER α gene were significantly associated with T2DM¹⁰³. To expand upon the knowledge of these SNPs in African American populations, individuals from the insulin resistance and atherosclerosis study (IRAS) were examined for association of intron 1 and intron 2 SNPs with T2DM and the metabolic syndrome. Significant association was found for multiple intronic SNPs to conditions of insulin sensitivity, fasting insulin levels, and the metabolic syndrome, confirming the results of the previous study¹⁰⁴. Other studies have found SNPs in intronic regions 3 and 4 are associated with T2DM in Swedish and French populations¹⁰⁵. While intronic SNPs may seem insignificant to the structure and function of the ER α gene, SNPs in these regions could affect splicing of the receptor or alter expression levels through changes in enhancer regions.

Impact of Splice Variants and Coregulators in Diabetes

Recent studies have provided evidence of domain specific effects of the ER α receptor in the regulation of metabolism in peripheral tissues. In mice, the AF-2 domain of ER α was shown to be important for the mediation of energy homeostasis, glucose metabolism, lipogenesis, and lipid oxidation¹⁰⁶. ER α regulates this effect through transcriptional control of several genes including fatty acid synthetase, acetyl-CoA carboxylase, SREBP1c, and others¹⁰⁶. These data suggest that there may be domain specific effects of estrogen receptor activity in the progression and development of T2DM that need to be addressed in the pancreatic β -cell as well.

The presence of domain specific effects of ER α suggests that there may be an impact on the recruitment of coregulators as well. NCoA5, a coregulator of ER α , was first associated to T2DM development through SNPs in its genomic region¹⁰⁷. In addition to these findings, NCoA5 haploinsufficient mice were shown to have reduced islet mass and increased blood glucose levels, suggesting there is an important role for NCoA5 in the endocrine pancreas as well¹⁰⁸. In the same study, haploinsufficiency of NCoA5 disrupted its interaction with ER α at the IL-6 promoter and increased IL-6 expression, which could impact insulin resistance in peripheral tissues¹⁰⁸. Together these studies substantiate the necessity for exploring the role of estrogen receptor splice variants and their coregulators in pancreatic β -cell function and health.

The present study will address the contribution of individual estrogen receptor isoforms and splice variants on INS-1E cell health to resolve some of the disparity that exists within the literature. In order to assess the activity of the receptors in the cell, death, proliferation, genomic activity, coregulator interaction, estrogenic ligand sensitivity, and downstream target genes will be examined in an isolated β -cell model. Additionally, the study will address the independent contributions of ER β and ER α expression on insulin secretion, as well as examining the role of three ER α variants on insulin mRNA expression to try to resolve the mechanism of estrogen receptor's role on β -cell function. Finally, more physiologically relevant concentrations of E2 and other estrogen receptor agonists will be used to eliminate any non-specific effects of estrogen treatment. Together these findings will aid in the understanding of estrogen signaling in the β -cell and elucidate new pathways for the regulation of β -cell fate and function. With

the knowledge gained from these insights, novel ways of regulating β -cell health in humans and during islet isolation procedures may be uncovered.

Chapter 2 Estrogen Receptor Signaling Triggers β -cell Death

ABSTRACT

Estrogen receptor signaling has been shown to regulate cellular processes including proliferation, survival, and differentiation in multiple model systems. This study aimed to evaluate the role of ER α and ER β in pancreatic β -cell health and function using the INS-1E cell line. Time course imaging analysis revealed no significant effect of estrogenic compound treatment on β -cell fate. Western blotting and immunofluorescence uncovered that the lack of response to estrogenic compounds was due to absence of endogenous estrogen receptor expression in the INS-1E cells. Thus, a transient transfection strategy was developed using a GFP-fused ER α 66 (ER α 66-GFP) or ER β (ER β -GFP). Transcriptional assays demonstrated that ER α 66-GFP was constitutively active in the untreated condition, and transcriptional activity could be inhibited 97% by antagonist treatment. Time course imaging analysis showed that expression of ER α 66-GFP increased cell death in the untreated condition, which could be inhibited 4-fold by antagonist treatment. Expression of ER α 66 also increased caspase 3/7 activity, which revealed a strong correlation between transcriptional activity and cell death. Interestingly, ER α 66-GFP activity was unresponsive to 17 β -estradiol treatment in any assay. The other isoform of estrogen receptor, ER β , also had increased transcriptional activity, which correlated to an increase in caspase 3/7 activity. However, unlike ER α 66-GFP, ER β -GFP was responsive to treatment with 17 β -estradiol in a concentration dependent manner. Surprisingly, expression of ER β -GFP did not induce a change in insulin secretion regardless of treatment group. However, ER α 66-GFP induced a mild reduction in insulin secretion, which was alleviated by treatment with MPP. Extending the study of estrogen

receptor signaling to human islets revealed that raloxifene treatment results in a significant upregulation of insulin mRNA as revealed through RNASeq analysis. Other genes which may be involved in pancreatic β -cell health were also differentially regulated in response to raloxifene. Overall, these studies provide evidence that the genomic activity of ER α 66 and ER β lead to increased cell death, and that ligand responsiveness is variable between these two isoforms in the INS-1E cell line. Additionally, the study suggests that human islets are responsive to SERM treatment and inhibition of estrogen receptor nuclear signaling activities can lead to differential expression of genes involved in pancreatic β -cell health and function.

INTRODUCTION

Pancreatic β -cell death is late onset symptom of type 2 diabetes (T2DM) progression, and can lead to a patient's dependence on insulin injection. Cell death can occur through multiple mechanisms including necrosis, autophagy, and apoptosis¹⁰⁹. In T2DM progression, β -cell death typically occurs through apoptosis following prolonged glucotoxicity or lipotoxicity^{4,110}. These stressors can induce apoptosis by activating the JNK pathway or through induction of endoplasmic reticulum and mitochondrial stress^{4,111–113}. The initiation of β -cell stress leads to the activation of caspases, which carry out the apoptotic process¹¹⁴. The apoptosis of β -cells reduces β -cell mass and leads to reduced insulin secretion, thereby worsening the diabetic phenotype. Thus, novel ways to regulate pancreatic β -cell death and function is a major goal of T2DM research. In other cell types, estrogen receptors, ER α and ER β , have been implicated in the control of cell fate through the regulation of apoptosis, proliferation, and differentiation^{57,58,63,69}. The ability to regulate cell fate through estrogen receptor activity has led to the investigation of the nuclear hormone receptor's involvement in the development and progression of diabetes^{37,38,41,42}.

Indeed, estrogen signaling has been linked to the development of T2DM in multiple model systems. In humans, the reduction of estrogen hormone levels and single nucleotide polymorphisms in the ER α gene have both been associated with development of T2DM^{103,104,16,115,116}. Animal models have shown that extranuclear mediated ER α and ER β signaling is protective against induced stress and can enhance insulin secretion^{83,84,80,91,73,74,117}. However, these protective effects are debatable. Evidence from studies using environmental xenoestrogens, such as BPA and PCB, which behave

similarly to SERMs, show that they can promote β -cell dysfunction and induce β -cell apoptosis, countering the previous findings of beneficial estrogen receptor activity^{95,96,101}. These studies highlight the variability reported in the literature regarding estrogen receptor action in β -cells, and demonstrate the need for additional knowledge.

Differences in the response of β -cells to estrogen exposure may be due to the activation of different signaling pathways. Estrogen receptors can participate in cytoplasmic signaling leading to rapid non-genomic responses or they can activate genomic signaling pathways to enhance transcription of target genes. The involvement of estrogen receptor signaling in both apoptosis and survival suggests that the activation of different signaling pathways may have divergent repercussions on cell health¹¹⁸.

This study aimed to elucidate the role of estrogen receptor isoforms, ER α and ER β , on pancreatic β -cell health and function. In order to assess estrogen receptor mediated β -cell death, a high throughput imaging strategy was developed using a GFP-fused estrogen receptor protein (Figure 2.3). This live cell imaging methodology is more impactful than traditional endpoint assays since GFP tracking enables cell-by-cell analysis of proliferation and survival in ER α 66-GFP expressing cells¹¹⁹. By examining GFP expression, one can then determine whether the cell proliferates, dies, or survives and can examine if changes in ER α 66-GFP localization impact these measures of cell fate (Figure 2.3)¹¹⁹. Caspase 3/7 analysis performed on ER α and ER β expressing cells corroborated the findings of the time course imaging, thus validating the method. Additional analysis revealed that the transcriptional activity ER α and ER β correlated to the activation of caspase 3/7. Extending the study to human islets revealed differential regulation of genes involved in pancreatic β -cell function in response to raloxifene

treatment. Overall, these studies provide novel evidence that activation of estrogen receptor genomic activity induces pancreatic β -cell dysfunction and leads to β -cell apoptosis.

RESULTS

Estrogenic Compound Treatment Doesn't Affect β -cell Health

To assess the effect of estrogenic compound treatment on the health of INS-1E cells, nRFP INS-1E cells were treated with a series of estrogenic compounds for 22 hours in a time lapse imaging assay.

The compounds in the screen included estrogen receptor agonists: 17β -estradiol (E2), PPT, and Way200070; estrogen receptor antagonists: MPP and PHTPP; and selective estrogen receptor modulators (SERMs):

cyclofenil (Sexovid), raloxifene (Evista), and Y134, an analogue of raloxifene. The total number of cells was scored at 0 and 22 hours and percentage change in

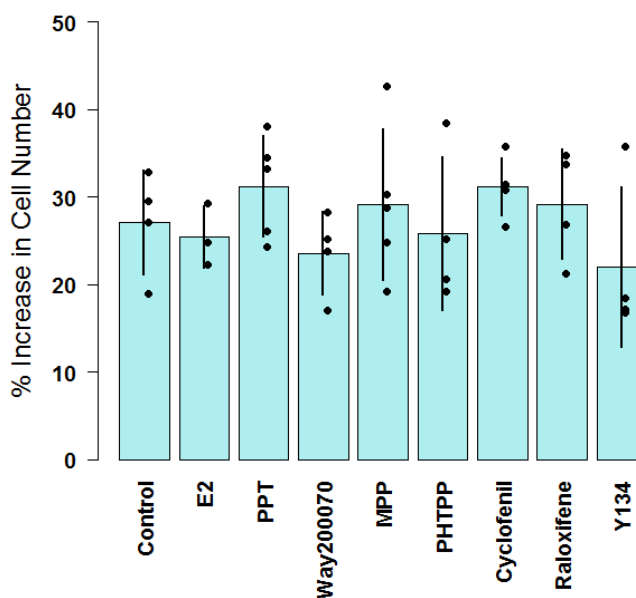


Figure 2.1 Estrogenic Compound Treatment Doesn't Affect β -cell Health.

nRFP INS-1E were treated with 10 nM estrogenic compounds (Cyclofenil, E2, MPP, PHTPP, PPT, raloxifene (Ral), Way200070 (Way), or Y134) during a 24 hour time course. RFP ROI were scored at time 0 and time 24 and the percentage increase in cell number was calculated. ($n \geq 3$)

cell number was calculated. If estrogenic compound treatment had an effect over the DMSO control, there would be a significant difference in total cell number between the control group and treatment groups. Significantly decreased cell number would be indicative of cellular death, whereas increased cell number would suggest increased proliferation. Examining all treatment groups, there was no significant increase or decrease in total cell number compared to the control (Figure 2.1). These data suggests

that simple addition of compounds to INS-1E cells had no significant effect on overall β -cell health.

Lack of Endogenous ER α Protein Expression was Confirmed

The lack of responsiveness in INS-1E cells after estrogenic compound treatment in the time course assay led to the hypothesis that ER α and ER β may not be active or

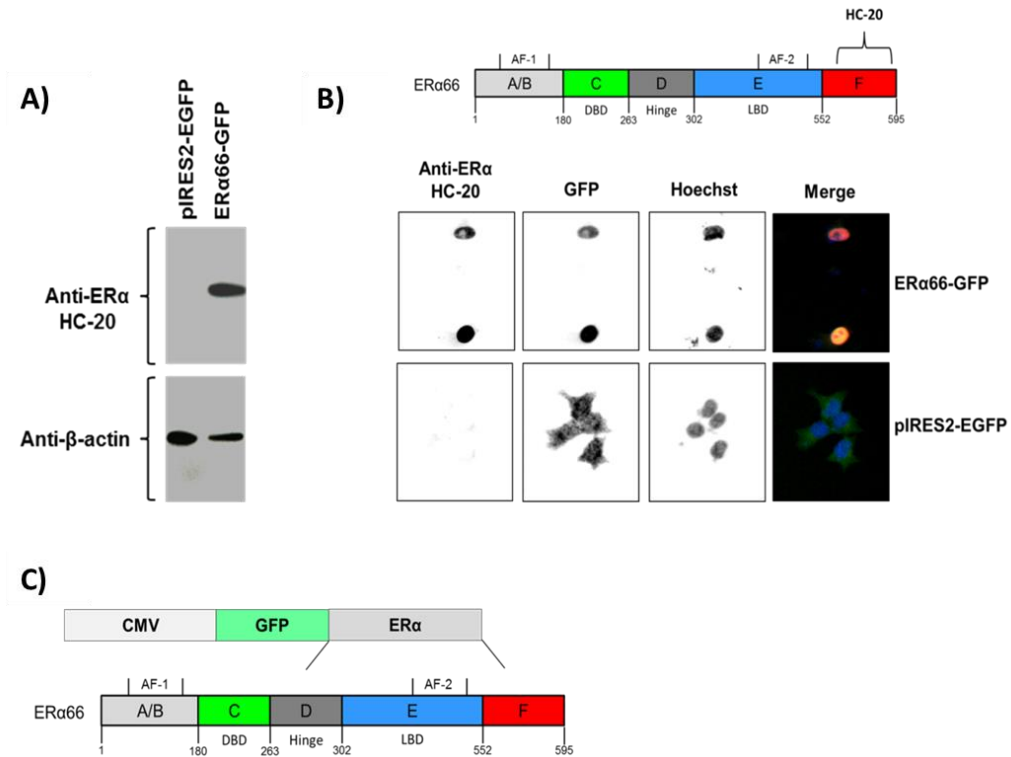


Figure 2.2 ER α is Not Endogenously Expressed in INS-1E cells.

INS-1E cells were transfected with either ER α 66-GFP or pIRES2-EGFP (an empty vector) and probed for protein expression using an HC-20 antibody, which binds to the C-terminal region of the protein. Immunoblotting confirmed lack of endogenous expression of ER α 66, whereas ER α 66-GFP (C) expression was detected (A). ER α 66-GFP nuclear localization was confirmed by GFP and probing with the anti ER α HC-20 antibody, which overlapped nuclear Hoechst staining (B).

expressed in this cell line. To address this hypothesis, ER α and ER β expression were analyzed through western blotting. Western blotting revealed a lack of endogenous ER α (Figure 2.2 A) and ER β (Figure 2.9 A) in this cell line. This finding explains the lack of responsiveness to estrogenic compound treatment observed in the previous assays. Thus, a transient expression strategy was designed that used GFP-fused ER α or ER β transfected into the INS-1E cells (Figure 2.2 C and Figure 2.9 A). Western blotting using a C-terminal anti-ER α antibody revealed that the expression vector produced protein in the cell (Figure 2.2 A). Additionally, immunofluorescence confirmed that GFP expression co-localized with ER α protein expression suggesting a successful fusion reporter (Figure 2.2 B). ER β -GFP expression was also confirmed in the INS-1E cells through western blotting (Figure 2.9 A).

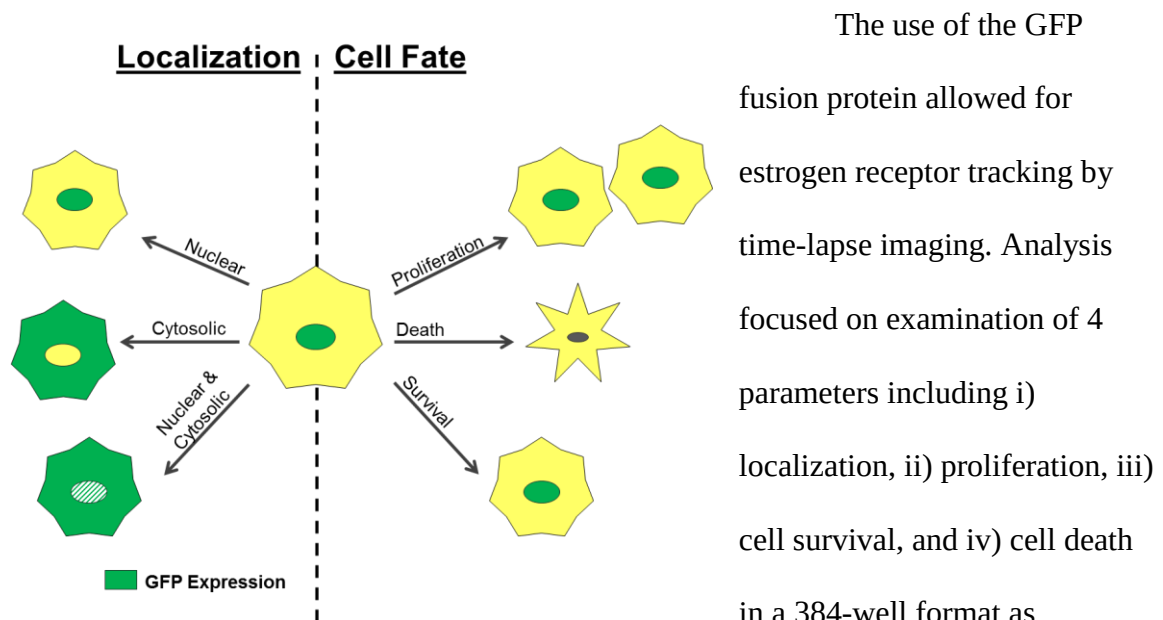


Figure 2.3 GFP Enabled Cell Tracking. The use of GFP-labeled ER α allowed for the examination of four parameters including localization (nuclear, cytosolic, or both nuclear and cytosolic), proliferation, death, and survival in a 384 well format.

ER α is Predominantly Nuclear Localized

Localization of ER α 66-GFP or EV in nuclear RFP expressing INS-1E (nRFP-INS1-E) was assessed at the beginning of the time course. In the no treatment group, ER α 66-GFP was nuclear localized in 74% of cells, while the remaining 26% of cells had both cytoplasmic and nuclear localized ER α 66-GFP (Figure 2.4 A and B). Cells were treated in three additional groups including agonist treatment (E2), antagonist treatment (MPP), and concurrent agonist and antagonist treatment (MPP + E2). Treatment with 10 nM E2 did not significantly alter the pattern of localization from the control treatment group in cells expressing ER α 66-GFP ($p=0.32$) (Figure 2.4 A and B). However, ER α 66-GFP cells treated with MPP or MPP + E2 had increased nuclear localization, 87% and 82% respectively ($p\leq 0.01$), with the remainder of protein in both the nucleus and cytoplasm (Figure 2.4 A and B). It is known that during apoptosis the nuclear envelope can be damaged due to the cleavage of lamins, thus increased nuclear localization could be due to increased integrity of the nuclear envelope, since MPP treatment dramatically increased β -cell health as discussed below (Figure 2.5 A and B)¹²⁰.

To test this hypothesis, RFP localization was assessed. RFP should only be present in the nucleus since it possesses a nuclear localization sequence, thus leakage of RFP into the cytoplasm suggests deterioration of the nuclear envelope which can occur during apoptosis or during prophase in mitosis. EV expressing cells retained RFP expression primarily in the nuclear compartment, 90-95%, with no significant difference between treatment groups (Figure 2.4 C and D). Upon measuring RFP localization in ER α 66-GFP expressing cells the control treatment group had 74% RFP localization in the nucleus, which was increased to 87% by MPP treatment (Figure 2.4 C and D). Thus,

it appears that MPP treatment improves cellular health resulting in reduced nuclear envelope deterioration and retention of both GFP and RFP in the nuclear compartment.

EV cells had GFP expression in both the cytoplasmic and nuclear compartments, with no significant difference between estrogenic compound treatment groups (Figure 2.4 C and D). These results demonstrate that ER α 66-GFP preferentially localizes to the nuclear compartment as seen previously³², and revealed a treatment specific effect on the localization of ER α 66.

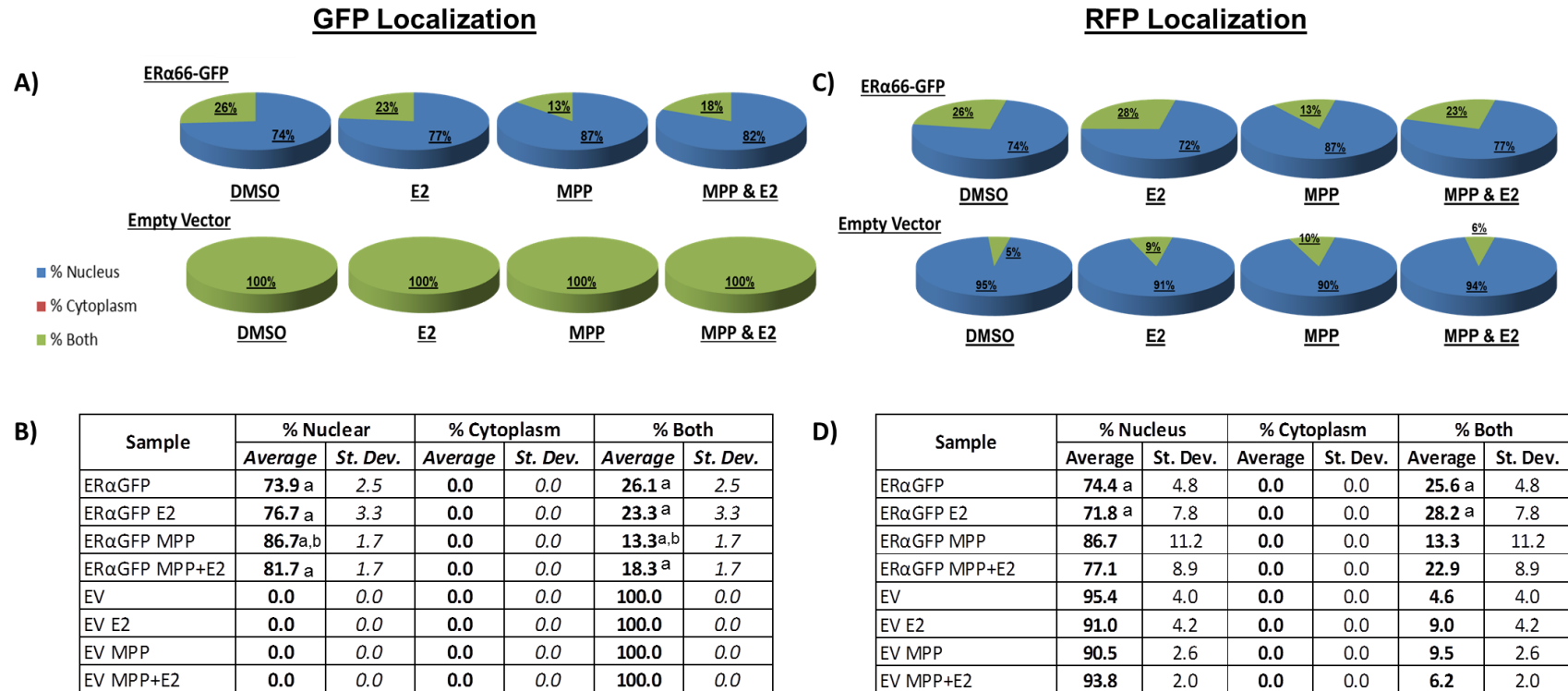


Figure 2.4 ERα is Predominantly Nuclear Localized.

nRFP INS-1E were transfected with either ERα66-GFP or EV, and treated with E2, MPP, or MPP + E2 for 24 hours. Localization of both GFP (representing protein localization) and RFP (representing nuclear membrane stability) were assessed. ERα66-GFP is primarily localized within the nucleus in all treatment groups with a significant increase in ERα66-GFP nuclear localization after MPP treatment (A & B). RFP localization was also assessed as a measure of nuclear membrane stability. EV expression has no effect on RFP expression in any treatment group. ERα66-GFP expressing cells had significantly increased RFP nuclear localization in the MPP treatment group (C & D). (a: ERα66-GFP treatment vs. EV control; b: ERα66-GFP treatment vs. ERα66-GFP control; $p \leq 0.01$). (n=180 ROI per treatment group).

ER α 66-GFP Expression Increases Cellular Death

In order to evaluate ER α 66-GFP effect on β -cell health in the INS-1E cell line, death, proliferation, and survival were examined during a 43 hour time course as outlined in the methods. Individual ROI were tracked for 43 hours and data was compiled at two time points, 8 hours and 43 hours, after the start of the time course.

At 8 hours into the time course, ER α 66-GFP expression did not significantly affect the percentage of proliferating cells compared to the EV control (Figure 2.5 A and B). Additionally, estrogenic compound treatment with E2, MPP, or MPP + E2 did not affect the percentage of proliferation in ER α 66-GFP or EV expressing cells (Figure 2.5 A and B). While proliferation was not affected by ER α 66-GFP expression, cellular death was significantly upregulated after expression of the receptor (Figure 2.5 A and B). ER α 66-GFP induced a 23 fold increase in the percentage of cellular death over the EV control in the no treatment group ($p \leq 0.01$) (Figure 2.5 A and B). The effect of ER α 66-GFP on cell death was not significantly altered by 10 nM E2 treatment (Figure 2.5 A and B). However, antagonism by MPP caused a significant 4-fold decrease in cellular death from 51.7% in the no treatment group to 13.3% in the MPP treatment group ($p \leq 0.01$) (Figure 2.5 A and B). Concurrent treatment with 10 nM MPP + 10 nM E2 fully reversed the MPP down-regulation of cellular death, which suggested that E2 can overcome MPP inhibition to restore ER α 66-GFP activity ($p \leq 0.01$) (Figure 2.5 A and B). As expected from the lack of endogenous ER α , cellular death was not altered in EV expressing cells in any estrogenic compound treatment group (Figure 2.5 A and B). Altogether, these analyses provided evidence for deterioration of β -cell health after expression of ER α 66-

GFP demonstrated by increased cell death. We next extended the analysis of cell fate data to include all data points through the 43 hour time course.

At 43 hours after the time course start, ER α 66-GFP expression results in a significant reduction in the percentage of proliferating cells in the control treatment group compared to EV expressing cells ($p \leq 0.01$) (Figure 2.5 C and D). This pattern was extended to all other treatment groups (E2, MPP, and MPP and E2) ($p \leq 0.01$) (Figure 2.5 C and D). Interestingly, MPP does not rescue reduced proliferation in ER α 66-GFP expressing cells. This may be due to the high percentage of death occurring in these cells, limiting the number of proliferative events and preventing any measurable difference.

As observed at the 8 hour time point, there was a significant increase in the percentage of dead cells in ER α 66-GFP expressing cells compared to EV expressing cells in the control treatment group at 43 hours ($p \leq 0.01$) (Figure 2.5 C and D). This pattern is extended to all other treatment groups (E2, MPP, and MPP and E2), where ER α 66-GFP expression leads to a significant increase in the percentage of dying cells compared to EV expression ($p \leq 0.01$) (Figure 2.5 C and D). The inhibition of cellular death seen after MPP treatment at 8 hours is still prevalent at 43 hours. MPP treatment of ER α 66-GFP expressing cells results in a significant inhibition, from 88% to 68%, of the percentage of dying cells compared to the ER α 66-GFP control group ($p \leq 0.01$) (Figure 2.5 C and D).

The evidence from the time course imaging assay combined with nuclear localization of the estrogen receptor led to the hypothesis that ER α mediated upregulation of cell death could be through its effect on transcription.

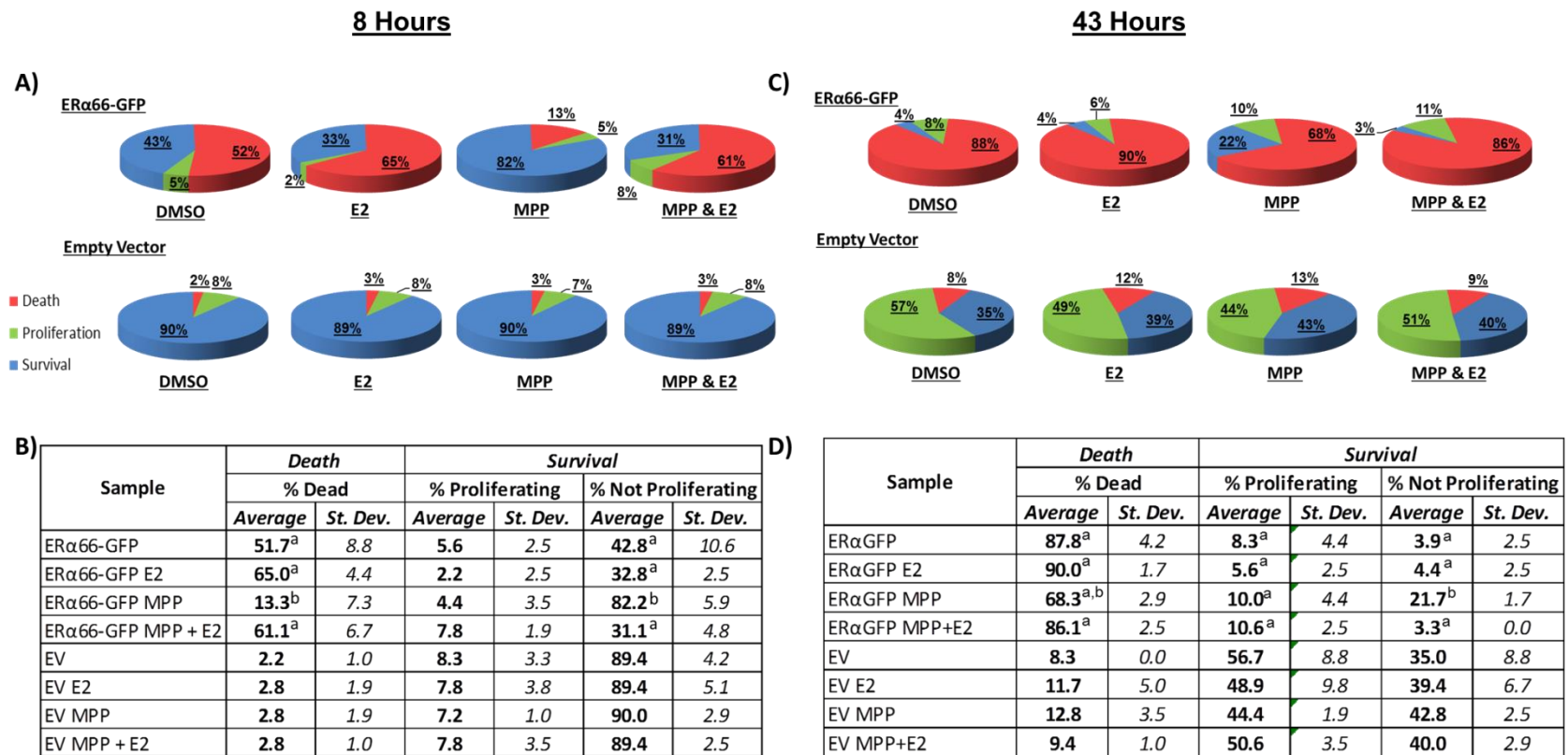


Figure 2.5 ERα66-GFP Increases β-cell Death.

nRFP INS-1E were transfected with ERα66-GFP or an EGFPN3 empty vector (EV) for visualization as described above. Proliferation was measured by examining duplication of GFP expression (ERα66-GFP) or duplication of RFP expression (EV). At 8 hours after treatment, no differences were observed in proliferation between ERα66-GFP and EV expressing cells (A & B). At 43 hours, fewer proliferation events were observed in ERα66-GFP expressing cells compared to EV expressing cells in all treatment groups (C & D). Cell death was dramatically increased at both 8 and 43 hours in ERα66-GFP expressing cells compared to EV cells, which was dramatically reduced by MPP treatment (A & C). (a: ERα66-GFP treatment vs. EV control; b: ERα66-GFP treatment vs. ERα66-GFP control; $p \leq 0.01$). (n=180 ROI per treatment group).

ER α 66-GFP is Transcriptionally Active in INS-1E cells

ER α 66 can regulate transcription from promoters containing ERE sites to mediate its genomic effect on cell apoptosis and survival. Thus, ER α -dependent transcription was measured using a dual luciferase reporter strategy consisting of a triplicate repeat of the canonical estrogen response element (ERE) luciferase reporter and a constitutively

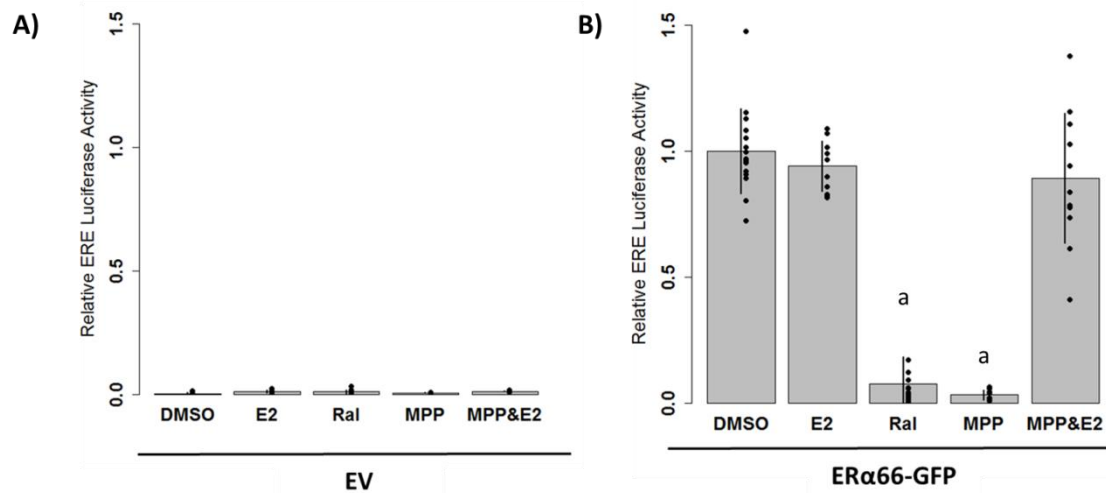


Figure 2.6 ER α Expression Increases Transcriptional Activation of ERE-Promoters. INS-1E cells were transfected with ER α 66-GFP or a pIRES2-EGFP empty vector (EV). No endogenous ERE-dependent transcriptional activity was observed in EV expressing cells (A). ER α expression results in a significant upregulation of ERE dependent transcriptional activation, which was inhibited by treatment with 10 nM raloxifene (Ral) or 10 nM MPP (B). Transcriptional activity was restored through concurrent treatment with 10 nM MPP and 10 nM E2, but was not significantly affected by 10 nM E2 treatment alone (B). Data is represented as normalized to the ER α 66-GFP DMSO control. (a: Treatment vs. ER α 66 DMSO $p \leq 0.01$) ($n \geq 11$)

expressed renilla luciferase reporter to control for transfection efficiency. ER α 66-GFP increased transcriptional activity 100-fold over the EV control in the DMSO treatment group, suggesting ER α 66 is activated in an unliganded state ($p \leq 0.01$) (Figure 2.6 A and B). The appearance of unliganded genomic activity has been previously reported after expression of ER α 66 in an ER α negative cell line³². 10 nM E2 treatment does not

potentiate ER α 66-GFP activation of the ERE-luciferase reporter, which corroborated the lack of E2 effect in the time-lapse imaging data ($p=0.30$). We hypothesized that MPP antagonism would result in decreased ER α 66-GFP transcriptional activity, since MPP is a known antagonist of ER α 66. Indeed, ER α 66-GFP transcriptional activity was inhibited 97% after treatment with 10 nM MPP compared to the DMSO control. This finding substantiates the 74% reduction in cellular death observed in the time course imaging assays ($p\leq 0.01$) (Figure 2.6 B). MPP inhibition of ER α 66-GFP mediated cell death was reversed by concurrent treatment with MPP + E2 in time-lapse imaging analysis. If transcriptional activity was correlated to cell death, MPP + E2 should restore transcriptional activation. As expected, concurrent treatment with MPP + E2 was capable of fully restoring ER α 66-GFP transcriptional activity to the level of the DMSO control (Figure 2.6 B). In addition to MPP treatment, ER α 66-GFP expressing cells were treated with the SERM, raloxifene. 10 nM raloxifene treatment inhibited ER α 66-GFP dependent transcriptional activation of the ERE-luciferase reporter by 92% ($p\leq 0.01$) (Figure 2.6 B), which is consistent with previous reports⁹⁰. Transcriptional activity in EV expressing cells was not affected by any estrogenic treatment, substantiating the lack of ER α protein expression in this cell line (Figure 2.6 A). Through the analysis of transcription, constitutive ER α 66-GFP activity was verified along with the inhibition of ER α 66-GFP activity by MPP, thereby supporting the results of the time lapse imaging analysis.

MPP Inhibition of ER α 66 Activity is Reversible

In the transcriptional assays discussed above, a full reversal of MPP inhibition

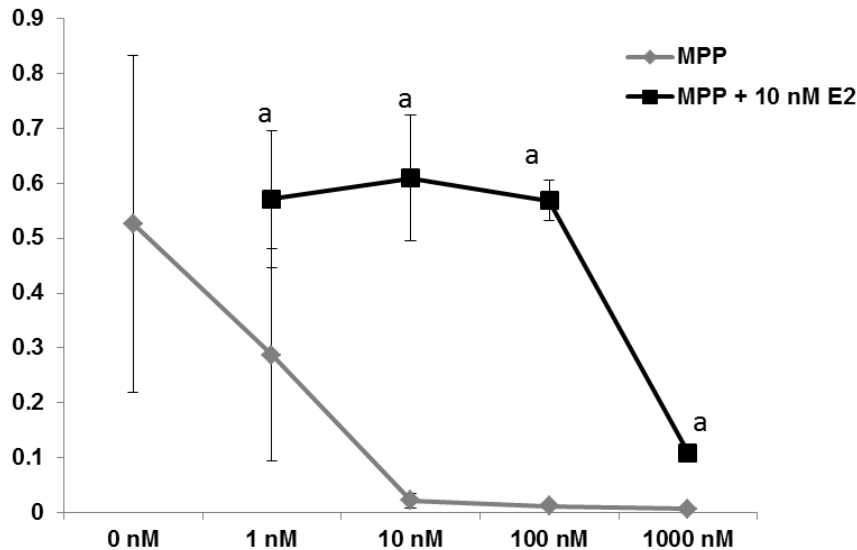


Figure 2.7 MPP inhibits ER α 66-GFP Activity in a Dose Dependent Manner.

INS-1E cells were transfected with ER α 66-GFP and assayed for transcriptional activity using a 3x-ERE luciferase reporter 24 hours after treatment with varying concentrations of MPP or concurrent MPP and 10 nM E2 treatment. MPP was found to inhibit ER α 66-GFP activity in a dose dependent manner, which was restored by treatment with 10 nM E2 at the 1, 10, and 100 nM concentrations of MPP. 10 nM E2 was unable to restore activity at the 1000 nM MPP concentration. (a: ER α 66 MPP vs. ER α 66 MPP + E2, $p \leq 0.01$) ($n \geq 3$ biological replicates)

after concurrent treatment with equivalent concentrations of E2 and MPP was observed.

To evaluate the competitive nature of MPP inhibition by E2, MPP was titrated in increasing amounts with or without 10 nM E2. Previously, it was shown that E2 reverses MPP inhibition of ER α activity at equivalent concentrations, which is diminished as the MPP concentration is increased¹²¹. As predicted, 10 nM E2 reversed MPP inhibition at low concentrations of the antagonist (Figure 2.7). Treatment with 10 nM E2 was unable to overcome 1 μ M MPP, suggesting that at this concentration MPP outcompetes E2 for

the ligand binding site of ER α (Figure 2.7). This experiment shows that the MPP inhibitory effect is partially reversible even at high concentrations of MPP.

Endogenous Estrogen Synthesis is Not Responsible for ER α 66 Activity

Since E2 was incapable of increasing ER α 66-GFP activity, the scenario that ER α 66 is activated through endogenous estrogen synthesis was addressed. INS-1E cells expressing ER α 66-GFP were treated with aromatase inhibitors, which block estrogen hormone synthesis¹²². Cells

were pretreated 48 hours with aromatase inhibitors, and then transfected with ER α 66-GFP or EV.

Treatment with aromatase inhibitors was continued 48 hours post-transfection, then transcriptional activity was

assayed. Treatment with aromatase inhibitors YM511, Exemestane, Letrozole, or Anastrozole showed no effect on ER α 66-GFP dependent transcriptional activation of the ERE-reporter (Figure 2.8). These

results suggest endogenous estrogen synthesis is not stimulating ER α activation in this

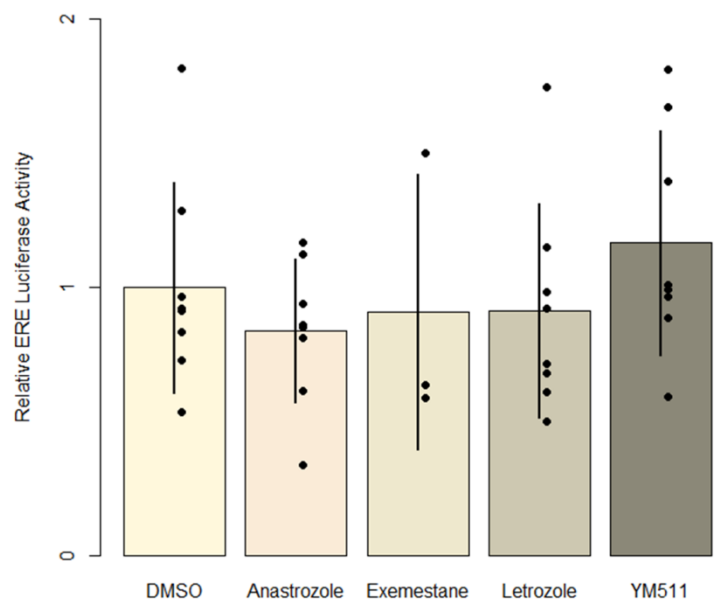


Figure 2.8 Depletion of Estrogen Synthesis does not Affect ER α Transcriptional Activity.

INS-1E cells were pretreated for 72 hours with aromatase inhibitor compounds Anastrozole, Exemestane, Letrozole, or YM511. Cells were then transfected with ER α 66-GFP, while remaining in aromatase inhibitor treatment. Transcriptional activity was measured 72 hours post-transfection using a 3x-ERE luciferase reporter. No inhibition of ER α 66-GFP dependent ERE transcriptional activation was observed. Data is represented as transcriptional activity normalized to the ER α 66-GFP DMSO control. (n $\geq$ 3)

system. Alternatively, the receptor may be stimulated due to activating post-translational modification or by binding to an unidentified endogenous ligand.

ER β Activity is E2 Inducible in INS-1E

From the previous analysis, ER α 66-GFP unliganded activity and lack of E2 responsiveness was seen (Figure 2.6). In order to determine if the lack of estrogen inducible activity was estrogen receptor isoform specific in the β -cell line, the activity of

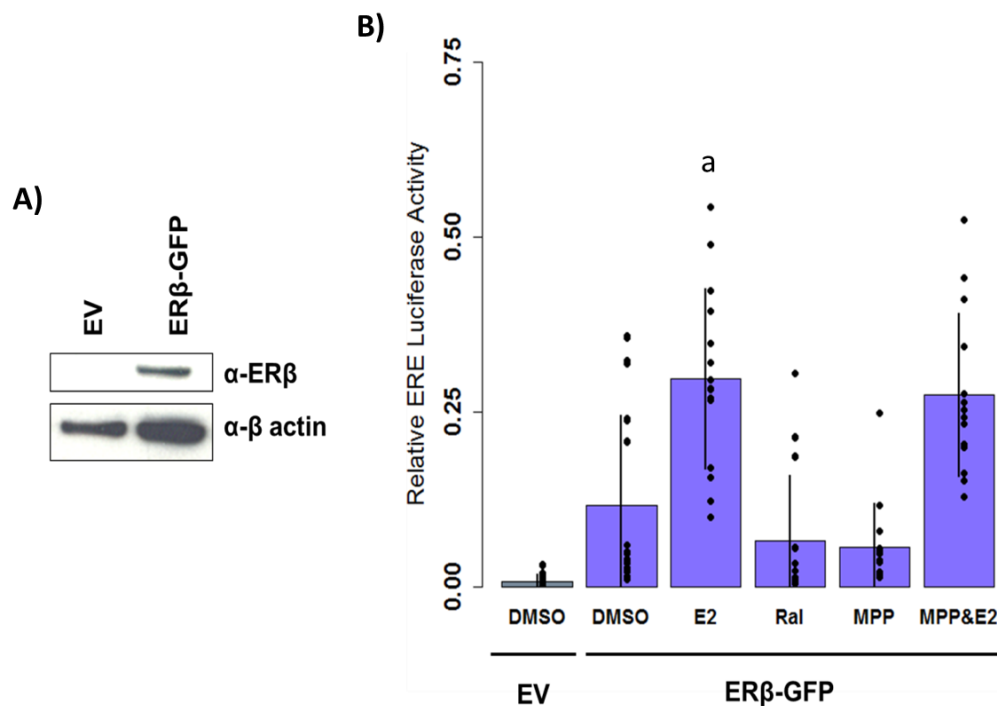


Figure 2.9 ER β Activity is E2 Inducible at ERE Promoters.

INS-1E cells were transfected with ER β -GFP or an EGFPN3 empty vector (EV). Expression was confirmed through western blotting (A). Transcriptional activity of ER β was assayed with a 3X-ERE luciferase reporter 24 hours after treatment with 10 nM 17 β -estradiol (E2), MPP, or raloxifene (Ral). E2 treatment significantly enhanced ER β transcriptional activity (B). Ral and MPP had no effect on ER β mediated transcription (B). (a: ER β Treatment vs. ER β DMSO, $p \leq 0.01$) ($n \geq 8$)

ER β was also evaluated. ER α and ER β do not significantly differ in their ligand binding affinity to E2, thus any differences observed in the action of the receptors in the cell line are likely to be due to the presence of coregulators, cofactors, or post-translational

modifications. Briefly, ER β -GFP was coexpressed with the ERE-luciferase dual reporter system and treated with estrogenic compounds (E2, Ral, MPP, and MPP + E2) to measure transcriptional activity. The transcriptional activity of ER β -GFP was not significantly increased over the EV control in the DMSO treatment group (Figure 2.9 B). However, unlike ER α 66-GFP activity, treatment with 10 nM E2 significantly increased ER β -GFP transcriptional activity ($p \leq 0.01$) (Figure 2.9 B). Treatment with raloxifene or MPP did not result in any significant change in ER β -GFP transcriptional activity from the DMSO control (Figure 2.9 B). Concurrent treatment with MPP and E2 increases ER β -GFP activity to the level of the E2 treatment group, suggesting that MPP has no effect on the affinity of E2 binding to ER β . (Figure 2.9 B). Overall, these data demonstrate that ER β -GFP is transcriptionally active in the INS-1E cell line after treatment with E2, revealing isoform specific inducible transcriptional activity in the β -cells. Additionally, the data substantiate the evidence from Figure 2.6 that the constitutive activity of ER α 66-GFP is due to unliganded transcriptional activity.

Titration of Estrogen Receptor Expressing Cells with E2 Increases ER β Activity, but not ER α Activity

To address E2 potentiation of ER α 66-GFP or ER β -GFP activity, cells were titrated with E2 in increasing amounts and activity from the ERE-luciferase reporter was assessed. Titration with E2 at concentrations up to 1 μ M did not increase ER α 66-GFP transcriptional activity, confirming the lack of an E2 stimulated response from this reporter (Figure 2.10 A). Unlike ER α 66-GFP activity, ER β -GFP transcriptional activity was increased in a stepwise manner up to 10 nM E2 treatment, and was significantly increased from 0.01 nM to 0.1 nM ($p \leq 0.05$) (Figure 2.10 B). Treatment with 100 nM E2 is not capable of further increasing ER β -GFP activity over the 10 nM treatment group (Figure 2.10 B). The data suggest that ER β -GFP

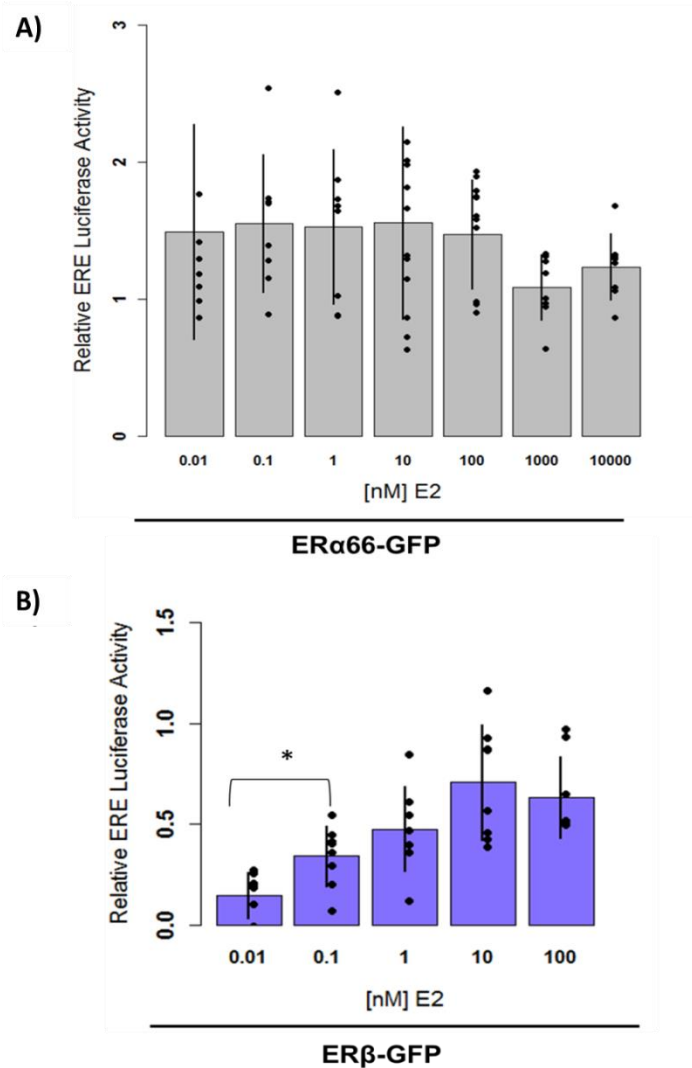


Figure 2.10 Isoform Specific Differences in Ligand Stimulation.

INS-1E cells were transfected with ER α 66-GFP or ER β -GFP and assayed for transcriptional activity using a 3x- ERE luciferase reporter 24 hours after 17 β -estradiol (E2) at the concentrations listed. E2 treatment did not potentiate ER α 66-GFP activity at any concentration (A). ER β transcriptional activity was increased in a stepwise manner up to 10 nM (B). (* $p \leq 0.05$) ($n \geq 5$)

transcriptional activity can be amplified in a step-wise manner by increasing concentrations of E2, whereas ER α 66-GFP activity cannot be potentiated by E2 treatment even in the micromolar range. These data validate the isoform specific response to E2 treatment in the INS-1E cells.

ER α 66-GFP Expression Increased Caspase 3/7 Activity

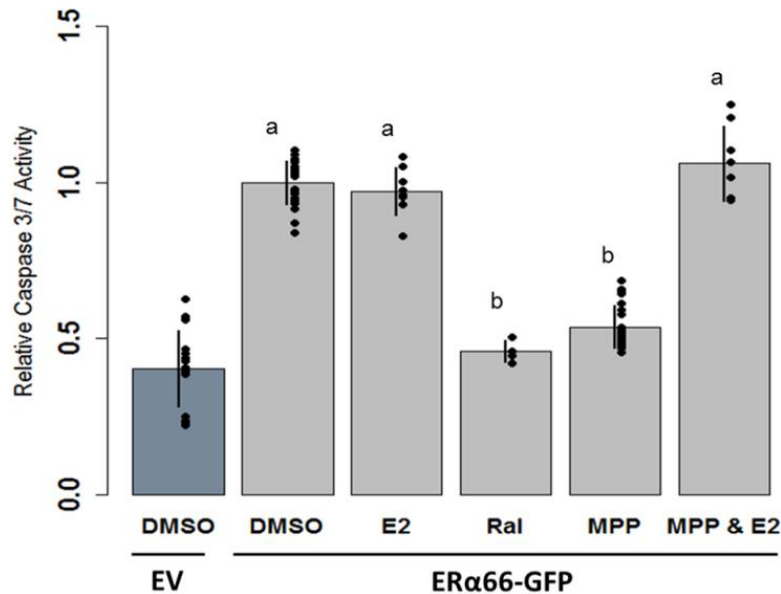


Figure 2.11 ER α 66 Expression Increases Caspase 3/7 Activity.

INS-1E cells were transfected with ER α 66-GFP or pIRES2-EGFP empty vector (EV) and selected by an antibiotic resistance marker for cells expressing the vectors. Selected cells were assayed for caspase 3/7 activity after chronic treatment with 10 nM compounds: MPP, E2, raloxifene (Ral), or MPP and E2. EV expression had no significant effect on caspase 3/7 activation. Expression of ER α 66-GFP resulted in a significant upregulation of caspase 3/7 activity, which was not further increased by treatment with 10 nM E2. Caspase 3/7 activity in ER α 66-GFP expressing cells was significantly inhibited by treatment with 10 nM MPP or 10 nM raloxifene and fully restored to DMSO control levels by concurrent 10 nM MPP and E2 treatment. Data are represented normalized to the ER α 66-GFP DMSO control. (a, Treatment vs. EV, $p \leq 0.01$) (b, Treatment vs. ER α 66 DMSO, $p \leq 0.01$) ($n \geq 4$ biological replicates)

Cell death can occur through three main pathways: apoptosis, necrosis, and autophagy¹⁰⁹. In cell lines, ER α has been shown to induce death by apoptosis through its regulation of transcriptional activity and through cytoplasmic signaling cascades^{66,123–125}. To assess the presence of cellular death in INS-1E cells, ER α

facilitated activation of caspase 3/7 activity was assessed. Briefly, INS-1E cells were transfected with ER α 66-GFP or EV. To evaluate caspase 3/7 activation in cells exclusively expressing ER α 66-GFP, cells were selected with an antibiotic. Cells were treated with estrogenic compounds in 5 treatment groups (DMSO, E2, Ral, MPP, or MPP + E2) for the duration of the experiment starting at selection. ER α 66-GFP increased caspase 3/7 activation 61% over the EV control in the DMSO treatment group, substantiating the cell death observed from time-lapse imaging analysis ($p \leq 0.01$) (Figure 2.11 and Figure 2.5). Treatment with E2 produced no significant change in ER α 66-GFP induced caspase 3/7 activity ($p = 0.36$) (Figure 2.11). Consistent with time-lapse imaging and transcriptional assays, MPP and raloxifene inhibited ER α 66-GFP mediated caspase 3/7 activation, 46% and 54%, respectively, from the ER α 66-GFP DMSO control ($p \leq 0.01$) (Figure 2.11). As observed with the transcriptional assays, concurrent treatment with MPP + E2 resulted in restoration of caspase 3/7 activity to the level of the ER α 66-GFP DMSO control (Figure 2.11). Altogether, these experiments aid in demonstrating that transcriptional activity is correlated to caspase 3/7 activation.

E2 Stimulates ER β Dependent Caspase 3/7 Activity

In order to determine if ER β -GFP activity also results in apoptosis, as seen with ER α 66-GFP, caspase 3/7 was assessed. ER β -GFP expression in the DMSO treatment

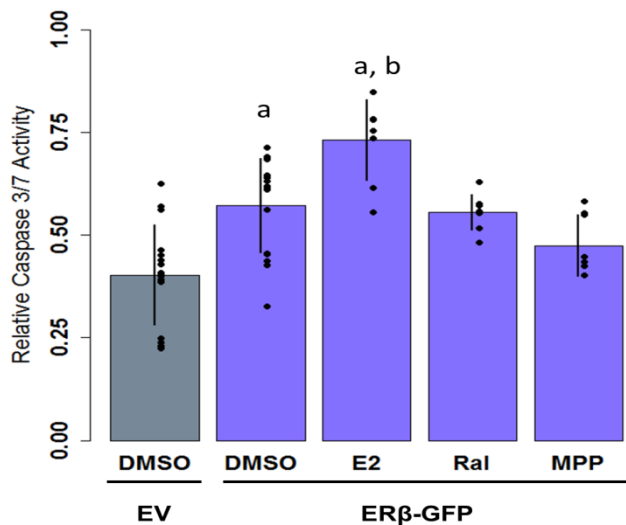


Figure 2.12 17 β -Estradiol Stimulates ER β Mediated Caspase 3/7 Activation.

INS-1E cells were transfected with either ER β -GFP or a pIRES2-EGFP empty vector (EV) and selected for cells expressing the vector of interest through an antibiotic resistance marker. Data were normalized to ER α 66-GFP DMSO control. ER β -GFP expression alone induces no upregulation of caspase 3/7 activity from the EV DMSO. Treatment with 10 nM E2 results in a significant upregulation in caspase 3/7 activity from the EV DMSO and ER β -GFP DMSO treatment groups. Data is represented as normalized to the ER α 66-GFP DMSO control. (a: $p \leq 0.01$ ER β vs. EV; b: $p \leq 0.01$ ER β treatment vs ER β DMSO) ($n \geq 5$)

group resulted in no significant increase in caspase 3/7 activity (Figure 2.12). In agreement with the transcriptional activity assays, treatment with E2 resulted in a significant increase of caspase 3/7 activity over the DMSO control ($p \leq 0.01$) (Figure 2.12). Treatment

with raloxifene had no significant effect on ER β -GFP mediated caspase 3/7 activation (Figure 2.12). MPP treatment also had no effect on caspase 3/7 activity in ER β -GFP expressing cells, which confirms the specificity of MPP for ER α 66-GFP (Figure 2.12). Overall, these results suggest that ER β -GFP

stimulates apoptotic cell death after treatment with E2 through activation of caspase 3/7.

Additionally, the data show that transcriptional activity correlates to increased apoptosis for both estrogen receptor isoforms.

Cyclofenil and Y134 Suppress ER α 66 Transcriptional Activity

As stated previously, SERMs are important estrogenic ligands because they can elicit tissue specific responses. If estrogen signaling proves to be important for the health and function of pancreatic β -cells, knowing how SERMs interact with each receptor isoform will be of critical importance. Therefore, in order to establish the sensitivity of ER α 66-GFP and ER β -GFP expressing cells to selective estrogen receptor modulation two SERMs were chosen. These two SERMs were chosen based on their known affinity for each receptor: Y134 has increased affinity for ER α , and Cyclofenil has increased affinity for ER β . Treatment with Y134 reduced ER α 66-GFP transcriptional activity significantly

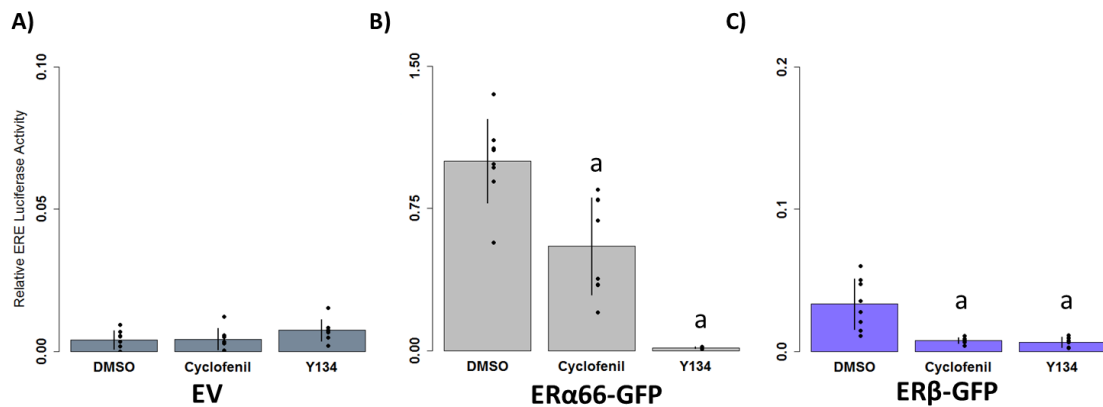


Figure 2.13 Cyclofenil and Y134 Significantly Reduce ER α Transcriptional Activity.

INS-1E cells were transfected with either an empty vector (EV), ER α 66-GFP, or ER β -GFP and assayed for transcriptional activity using a 3x-ERE luciferase reporter. Cells were treated 24 hours prior to transcriptional assays with DMSO, 10 nM Cyclofenil, or 10 nM Y134. Cyclofenil and Y134 had no significant effect on EV expressing cells (A). ER α 66-GFP transcriptional activity is significantly reduced by both Cyclofenil and Y134 (B). ER β -GFP transcriptional activity is also significantly downregulated by Cyclofenil or Y134 (C). (a: Treatment vs. DMSO in each transfection group, $p \leq 0.01$)

($p \leq 0.01$) (Figure 2.13 B). Interestingly, ER β -GFP transcriptional activity was further reduced from the low control levels after treatment with Y134 as well ($p \leq 0.01$) (Figure 2.13 C). Treatment with Cyclofenil also significantly reduced ER α 66-GFP and ER β -GFP

activity below the DMSO control ($p \leq 0.01$) (Figure 2.13 B and C). Treatment with either SERM had no significant effect in EV expressing cells (Figure 2.13 A). In both Cyclofenil and Y134 treatment groups, there was a greater suppression of ER α 66-GFP activity than ER β -GFP activity, which was expected since ER α 66-GFP is more active in the DMSO treatment group than ER β -GFP. These data show the ability of SERMs to inhibit the activity of ER α 66 and ER β in INS-1E cells. This suggests that SERMs could be a valuable tool in regulating endogenous estrogen receptor activity in pancreatic β -cells. The next measure examined tested estrogen receptor effect on insulin secretion as a measure of β -cell function.

Estrogen Receptor Effect on Insulin Secretion

The main role of the pancreas is to maintain blood glucose homeostasis, which is regulated through insulin

secretion and glucose

uptake¹²⁶. In order to test the

effect of estrogen receptor

isoforms on insulin secretion

ER α 66-GFP, ER β -GFP, or

an empty vector (EV) were

transfected into the INS-1E

cell line and treated with

either DMSO or MPP for the

duration of the experiment.

Cells expressing the vectors of

interest were selected for 48

hours in culture media

containing antibiotic to obtain a

pure population of estrogen

receptor expressing cells. Secretion was stimulated by incubation in high glucose (16

mM) or low glucose (2.5 mM) following a brief incubation in low glucose (2.5 mM). As

expected in EV expressing cells, treatment with high glucose results in significantly

increased insulin secretion compared to cells incubated in low glucose ($p \leq 0.05$) (Figure

2.14). ER α 66-GFP expression did not significantly increase insulin secretion compared to

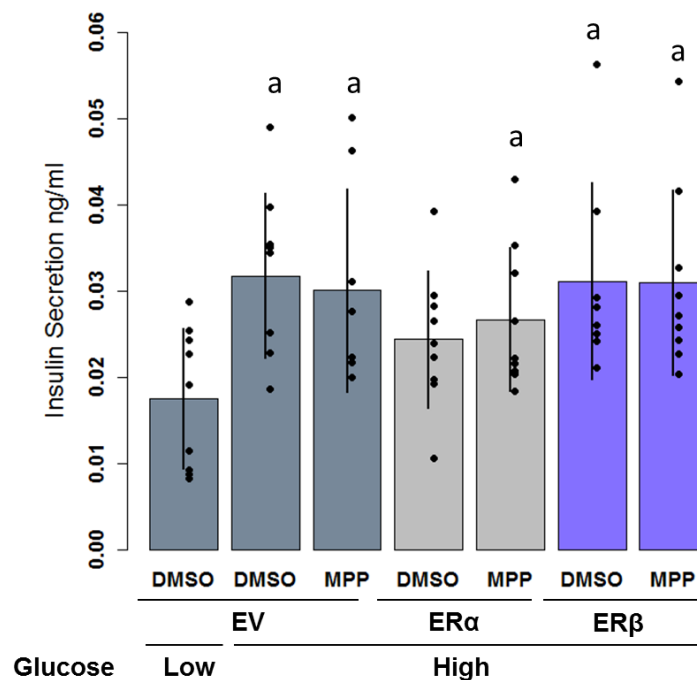


Figure 2.14 Estrogen Receptor Effect on Insulin Secretion.

INS-1E cells were transfected with ER α , ER β , or an EGFPN3 empty vector (EV). Cells were treated with 10 nM MPP or DMSO for the duration of the experiment. Cells were incubated in either high (16 mM) or low (2.5 mM) glucose for 3 hours prior to an insulin ELISA assay. Insulin secretion was normalized to total cell number. (a: Transfection/Treatment High Glucose vs. EV DMSO Low Glucose, $p \leq 0.05$) ($n \geq 8$ in 3 biological replicates)

the EV DMSO low glucose treatment group ($p=0.09$) (Figure 2.14). This suggests that ER α 66-GFP expression has a mild effect on insulin secretion by reducing insulin release in response to high glucose. Treatment with MPP alleviated the ER α 66-GFP mediated reduction in insulin secretion and restored a significant difference compared to EV DMSO low glucose ($p\leq 0.05$) (Figure 2.14). ER β -GFP expression did not result in a change of insulin secretion in either DMSO or MPP treatment groups compared to the EV DMSO high glucose control (Figure 2.14). None of the transfection conditions or treatments examined differed significantly from the EV DMSO high glucose treatment group. These data suggest that insulin secretion is not significantly affected by ER β expression in the INS-1E cells, but may be inhibited by ER α . The mechanism by which ER α 66-GFP decreases insulin secretion is currently not known, but may involve changes in insulin transcription or translation, changes to the insulin secretory machinery, or alterations in the responsiveness to high glucose.

Estrogen Receptor Inhibition Regulates Gene Expression in Human Islets

To verify that the observed responses in the pancreatic β -cell line, INS-1E, translated into human tissue types, estrogen receptor signaling was inhibited in isolated human islets. Briefly, two sets of pancreatic islets were obtained from the IIDP and incubated in 10 nM raloxifene or untreated for 24 hours. Following estrogen receptor inhibition, RNA was extracted, polyA selected, and processed through RNA Sequencing (RNASeq) as described below. Raloxifene was chosen because it is an FDA approved SERM, and previous analysis in the INS-1E cells revealed raloxifene treatment significantly downregulates ER α genomic activity (Figure 2.6). Differential expression analysis using DESeq revealed over 250 genes that were responsive to raloxifene

treatment compared to control in both of the islet samples. Surprisingly, in the analysis of overlapping hits only 19 genes were significantly altered in the same direction in both samples (i.e. up in both samples) (Figure 2.15 A). The implication of each of these genes in the regulation of β -cell health and function is unknown, however there were some genes identified that could significantly affect β -cell function.

The most promising find is the significant alteration of insulin gene expression between raloxifene and control treatment groups. When islet samples were treated with raloxifene, insulin expression was significantly upregulated compared to the control treatment group in both islet samples (Figure 2.15 B). The significant increase in insulin expression after estrogen receptor inhibition substantiates our previous findings in the INS-1E that activated estrogen receptor signaling impedes proper β -cell function.

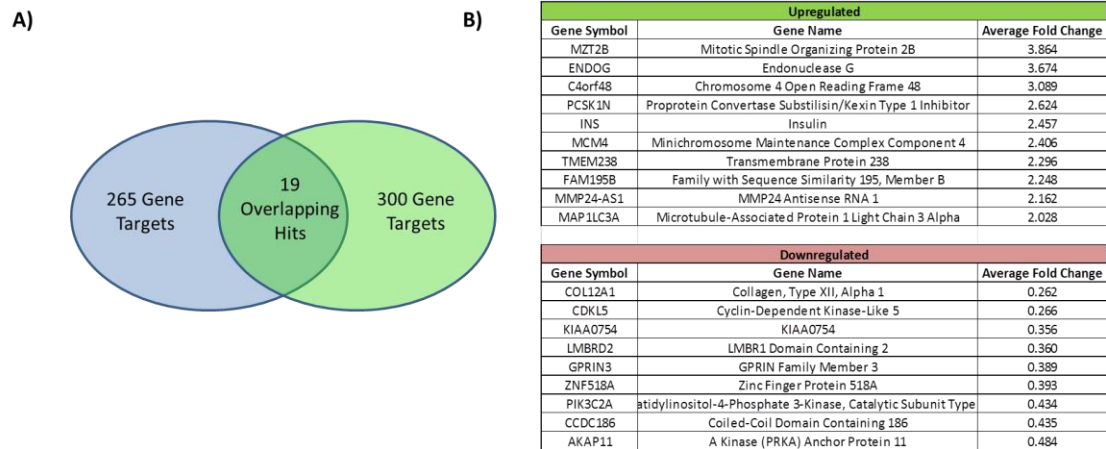


Figure 2.15 Human Islets Respond to SERM Treatment.

Human islets obtained from the IIDP were treated with raloxifene (10 nM) or untreated for 24 hours. Following raloxifene treatment, RNA was isolated and polyA selected. RNASeq was performed following cDNA library preparation. Two islet samples were analyzed for significant changes in raloxifene treatment versus control using DESeq. Of the hits obtained in the two islet samples, 19 genes were significantly altered in the same direction (upregulated or downregulated). Most significant is the upregulation of insulin in response to raloxifene treatment, suggesting an improvement of β -cell function.

In addition to this finding, several genes were identified that could be involved in mediating β -cell health including a significant upregulation in endonuclease G (ENDOG), minichromosome maintenance complex component 4 (MCM4), microtubule associated protein 1 light chain 3 alpha (MAP1LC3A), and mitotic spindle organizing protein 2B (MZT2B) (Figure 2.15). Additionally, a significant downregulation of genes

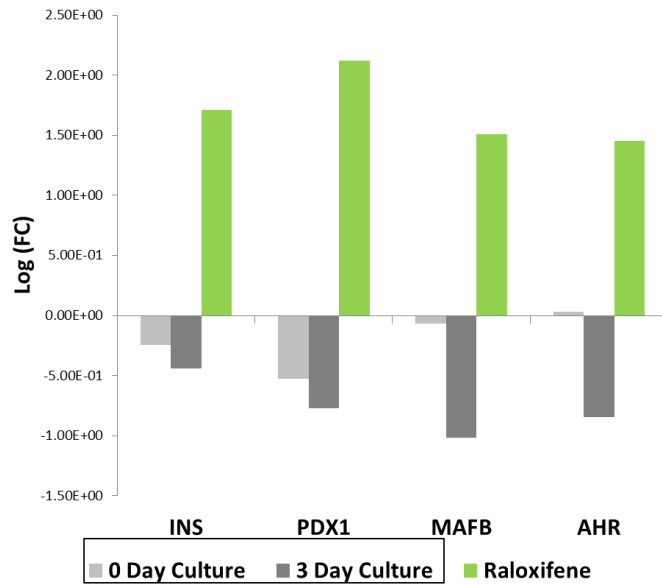


Figure 2.16 Raloxifene Treatment Improves Maintenance of β -cell Phenotype.

Human islets were obtained from the IIDP and cultured in either 10 nM raloxifene or untreated for 24 hours. Following raloxifene treatment, RNA was extracted and RNASeq was performed as described in the methods to generate RPKM values. Log(FC) of raloxifene treatment versus control is represented. 0 Day and 3 Day β -cell culture data was obtained from GSE29113 and Log(FC) values of 0 or 3 day culture versus intact pancreas are represented (Boxed Data). Raloxifene treatment resulted in a significant increase in genes involved in the maintenance of β -cell phenotype including INS, PDX1, MAFB, and AHR, whereas β -cell isolation and culture resulted in a decrease.

including collagen, type XII, alpha 1 (COL12A1), cyclin dependent kinase like 5 (CDKL5), and zinc finger protein 518A (ZNF518A), among others was observed (Figure 2.15). The function of some genes identified could mediate β -cell fate (ENDOG, MCM4, CDKL5), cellular function (MAP1LC3A, MZT2B, ZNF518A, AKAP11, COL12A1, PIK3C2A), or hormone processing and release (INS, PCSK1N). In addition, there are many differentially regulated genes

whose functions in the cell are not known. Thus, future studies are needed to determine the role of each of these genes in the modulation of pancreatic β -cell health and function.

In order to expand the analysis of the RNASeq data, changes in gene expression from raloxifene treated islets in the present study were compared to gene expression data from freshly isolated β -cells or β -cells which were cultured for three days as described in Negi, et al and found in the GEO data set GSE29113 (Figure 2.16)¹²⁷. Interestingly, culturing islets for 24 hours with raloxifene resulted in increased expression of genes involved in maintaining the pancreatic β -cell phenotype, which were decreased in β -cells from 0 day culture (freshly isolated) and islets cultured for 3 days (Figure 2.16). These genes included the blood glucose lowering hormone, insulin, as well as many transcription factors that are important for maintaining insulin secretion and the pancreatic β -cell phenotype, PDX1, AHR, and MAFB. This finding is significant in that

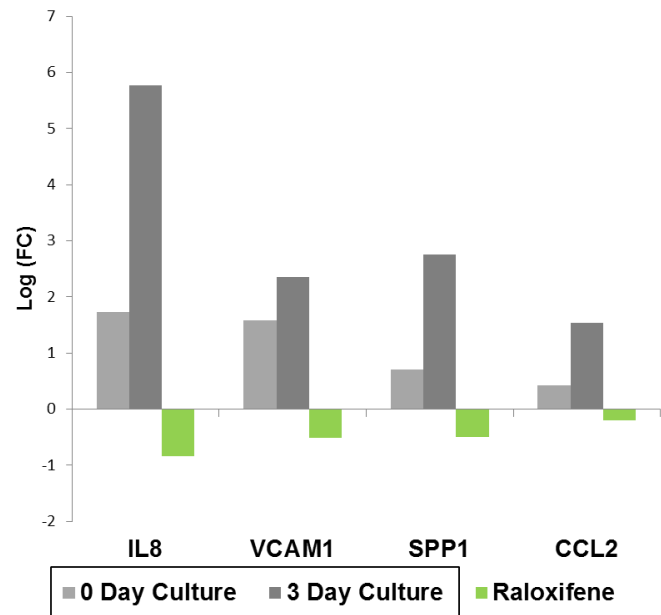


Figure 2.17 Raloxifene Treatment Reduces the Inflammatory Response in Isolated Islets.

Human islets were obtained from the IIDP and cultured in either 10 nM raloxifene or untreated for 24 hours. Following raloxifene treatment, RNA was extracted and RNASeq was performed as described in the methods to generate RPKM values. Log(FC) of raloxifene treatment versus control is represented. 0 Day and 3 Day β -cell culture data was obtained from GSE29113 and Log(FC) values of 0 or 3 day culture versus intact pancreas are represented (Boxed data). Raloxifene treatment of human islets resulted in a significant decrease in genes involved in the inflammatory response including IL8, VCAM1, SPP1 and CCL2, which are all significantly increased with 0 and 3 day islet culture.

it suggests raloxifene treatment of islets may reverse the negative effects of islet isolation and culture in order to maintain the β -cell phenotype. In addition to examining differences in the maintenance of insulin expression and β -cell phenotype, changes in the inflammatory response in isolated β -cells¹²⁷ versus raloxifene treated islets were also examined. Islet isolation significantly increases components of the inflammatory response in 0 day culture β -cells (freshly isolated) and worsens with 3 day culture β -cells as described previously (Figure 2.17)¹²⁷. Interestingly, 24 hour treatment of isolated islets with raloxifene significantly reduced components of the inflammatory response including reduced IL8, VCAM1, SPP1, and CCL2 expression (Figure 2.17). These findings are significant in that reduction in an inflammatory response and increase in β -cell phenotype genes could improve islet viability after isolation, which may increase success rates with processes such as islet transplantation.

DISCUSSION

One of the major hurdles of diabetes research is finding new ways to regulate β -cell fate and improve β -cell function. A novel way to mediate changes in β -cell health may be through the alteration of estrogen receptor signaling. Recently, modulation of estrogen receptor action has been associated with diabetes and β -cell health^{80,87,91,128–133}. Thus, this study aimed to characterize the function of estrogen receptor isoforms, ER α and ER β , in the INS-1E β -cell model.

In order to evaluate the impact of estrogen signaling on pancreatic β -cell fate, a high throughput imaging methodology was developed that allowed the screening of multiple estrogenic compounds for their effect on β -cell fate. The use of ER α and ER β agonists and antagonists had no significant effect on β -cell fate in the INS-1E cell line. These findings along with those of the estrogen receptor dependent transcriptional assay and western blotting revealed the lack of endogenous estrogen receptor expression in INS-1E cells. Previous studies have shown protein expression of ER α and ER β in mouse islets, MIN6 cells, and INS-1 cells; however, the expression level of these receptors may be very low^{74,87,129,134}. A 2008 study by Chuang, et al. found transcript abundance of ER α and ER β in mouse islets, MIN6 cells (β -cell line), α TC1 cells (α -cell line), and human islets fell below the level of detection when analyzed by qPCR¹³⁵. Nevertheless, the same study indicated that estrogen receptor expression could still be relevant because ER α expression levels rose in both mouse and human islets after incubation with high glucose^{135,136}. In another study, islets from young rats responded to hyperglycemia and oxidative stress by increasing ER α mRNA, a feature that is lost in the older rats, suggesting age and ER α expression may confer diabetes susceptibility¹³⁶.

In other cell types, estrogen receptors are known to regulate genes involved in apoptosis through the activation of consensus or partial ERE promoters, and induce genes like BCL2, DDIT3, CASP7, and many others¹³⁷. Also, it is important to remember that estrogen receptors can regulate transcription from non-canonical targets through their interaction with other transcription factors including AP1 and SP1 family members, expanding the list of potential estrogen receptor dependent gene targets^{28,29}. In INS-1E cells, ERE-dependent transcriptional activity of both ER α and ER β were examined, and data indicated that transcriptional activity and caspase 3/7 activation are tightly correlated. These data demonstrate that transcriptional activation of estrogen receptors influences downstream target gene expression to promote β -cell death. Furthermore, ER α was shown to be unresponsive to 17 β -estradiol treatment, whereas ER β was responsive and had titratable activation of transcriptional activity. These data suggest that the ligand response is different for each isoform. Other studies have found that estrogen signaling at the plasma membrane is beneficial for β -cell health in models of streptozotocin or H₂O₂ induced β -cell stress^{73,78,87,91,117,136}. However, in these studies the effects of estrogen could be due to estrogen signaling in peripheral tissues. Induction of hyperglycemia after streptozotocin administration is detrimental to β -cell health, thus increased insulin sensitivity and glucose uptake could mediate some of the observed beneficial effects of estrogen.

While at first this may seem to convolute the findings of previous studies showing the beneficial effects of estrogen signaling, we must separate estrogen receptor nuclear signaling from cytoplasmic signaling cascades. In the previous studies, estrogen treatment in islets appears to be mediated through the activation of cytoplasmic signaling,

as shown through the activation of extracellular components such as ERK1/2 and NeuroD1^{81,129}. The exclusion of nuclear signaling was further confirmed through the use of cytoplasmic restricted estrogens and estrogen receptors with mutated DNA binding domains, which reduced β -cell apoptosis as efficiently as 17 β -estradiol and wild type receptors, respectively^{74,117}. Thus, we must separate these two divergent signaling pathways when debating the role of estrogen receptors in β -cell health and function, focusing on the role of the cytoplasmic and nuclear estrogen receptor separately.

In addition to examining the role of estrogen receptors on β -cell health, it was also important to assess their impact on β -cell function, specifically insulin secretion. Estrogen receptor action has been linked to altered insulin expression and secretion in previous studies^{81,91,129}. However, as with estrogen receptor effects on β -cell health, insulin secretion appears to be mediated through changes at the plasma membrane and initiation of cytoplasmic signaling cascades. In this study, we found that expression of ER β did not result in a significant change in insulin secretion after stimulation with high glucose. However, expression of ER α results in a mild decrease in the responsiveness to high glucose, which is alleviated by treatment with MPP. This finding suggests that ER α nuclear activity in INS-1E cells exerts a mild effect to reduce insulin release from the β -cells, whereas ER β mediates no significant effect. While the data demonstrate a small change in insulin secretion, the mechanism by which ER α acts to reduce insulin release is not known and may be due to changes in insulin transcription or translation, inhibition of the secretory machinery, or changes in the response to high glucose.

Finally, the role of SERMs on estrogen receptor α and β action in the INS-1E cell model were examined. SERMs are estrogen receptor modulators that exert either

antagonistic or agonistic effects on estrogen receptor signaling dependent on the tissue type in which they are present^{41,56,138}. An example of the importance of SERM action lies in raloxifene's ability to be an antagonist in the breast and an agonist in the bone, preventing breast cancer while not influencing osteoporosis progression¹³⁹. In this study, three SERMs were examined for their effects on estrogen receptor transcriptional activity, since transcription is directly correlated to β -cell health in our model. The compounds used included two FDA approved SERMs, raloxifene (Evista) and cyclofenil (Sexovid) as well as Y134, a novel SERM derived from raloxifene. All three SERMs worked to inhibit both ER α and ER β transcriptional activity, and raloxifene was also shown to inhibit caspase 3/7 activity. Since we have evidence demonstrating that genomic estrogen signaling negatively regulates β -cell health, FDA approved SERMs could be used in islet transplantation to inhibit estrogen receptor signaling and increase transplant effectiveness as a treatment for diabetes. Currently, the outcome of islet transplantation is inadequate as it requires a large volume of islets and only 60% of patients achieve insulin independence four years after transplant¹⁴⁰. Thus, the use of FDA approved SERMs during islet transplantation procedures or during isolation and culture could reduce apoptosis, and improve islet function¹⁴¹. To test the effect of SERM treatment on human islet health, RNASeq analysis was performed on human islets following 24 hour treatment with raloxifene.

Raloxifene is a SERM widely used for inhibition of breast cancer cell proliferation, and in the INS-1E and INS-1 cells was shown to inhibit ER α genomic activity (Figure 2.6)^{90,142}. Treatment of isolated human islets with raloxifene for 24 hours resulted in differential regulation of over 250 genes in the analysis of two islet samples.

Interestingly, only 19 of these genes overlapped between the two samples. This suggests that there is an inherent heterogeneity between islet samples, which should be addressed in future studies. The heterogeneity could arise from factors including estrogen receptor splice variant and isoform expression, coregulator expression, or previous stress, health, age, and sex of the individual they were isolated from. Analysis of differentially expressed genes revealed a significant upregulation of insulin expression after estrogen receptor inhibition, which agrees with the model based on ER α signaling in the INS-1E cells. Additionally, it points to the mechanism of reduced insulin secretion from ER α expressing INS-1E cells to be mediated through a reduction in insulin gene expression. Furthermore, the modulation of genes involved in cell fate regulation including CDKL5, MCM4, and ENDOG by raloxifene treatment could have a significant impact on the health of isolated islets. MCM4 is known to be important for the replication of eukaryotic DNA, and increased expression (as observed in this study) is an indicator of increased proliferation¹⁴³. CDKL5 is a regulator of cell cycle and increased expression has been shown to cause cell cycle arrest, thus the decrease observed in this study may again allude to increased proliferation¹⁴⁴. Expanding the analysis of SERM treatment on human islets, we observed a significant reduction in genes involved in the inflammatory response and an increase in genes involved in maintenance of a pancreatic β -cell phenotype following treatment with raloxifene. These data were compared to data from isolated β -cells that were either freshly isolated from intact pancreas or cultured for 3 days, which showed a significant increase in the inflammatory response and a decrease in β -cell specific genes like insulin¹²⁷. Thus, if raloxifene treatment can protect the β -cell phenotype from inflammation while increasing insulin, it may become a useful tool to

increase islet viability in methods of islet isolation. Future studies should determine the function of each of these genes in relation to β -cell health and assess the reproducibility of the results between islet samples.

Overall, this study provides evidence that transient expression of ER α or ER β in the INS-1E cells results in a significant deleterious effect on β -cell health. Additionally, the study provides evidence that transcriptional activity of ER α and ER β is correlated to increased caspase 3/7 activity and β -cell death. We provide evidence that the deleterious effects of nuclear estrogen receptor signaling can be modulated with estrogen receptor antagonists and by FDA approved SERMs. Analysis of human islet samples revealed a significant effect of raloxifene treatment to differentially regulate gene expression, including a significant increase of insulin expression and decrease in the inflammatory response. Future studies should evaluate the significance of the genes identified by RNASeq analysis to determine the role in the mediation of islet health and function since many genes identified have a role in cell cycle and proliferation.

MATERIALS AND METHODS

INS-1E Cell Culture and Transfection

Rat Insulinoma (INS-1E) cells³⁷ or nuclear cherry red (nRFP) INS-1E were cultured in RPMI1640 (Gibco, 11875-093) supplemented with 6% FBS. For experiments, cells were plated on either 6- or 384- multi-well formats as previously described. Briefly, cells were harvested by trypsinization (0.5% trypsin, 0.05% EDTA) and washed once with RPMI supplemented with 6% FBS. Cells were resuspended in Optimem (Gibco, 31985-070) and plated at a density of approximately 225,000 cells for 6 well plates and 8,125 cells for 384 well plates. The cells were transfected with pEGFP-C1-ER α (addgene, 28230), pEGFP-C1-ER β (addgene, 28237), or empty vectors, EGFPN3 (Clontech, 6080-1) and pIRES2-EGFP (Clontech, 6029-1), using the cationic lipid DMRIE (Invitrogen, 10459014) with a $\mu\text{g}:\mu\text{l}$ DMRIE ratio of 0.62 (0.116:0.187) (384 well plate) and 0.40 (2.25:5.61) (6 well plate).

Compound Treatment

Estrogen modulating compounds included: 1,3,5 (10) Estratrien-3, 17 B-diol (Steraloids, E0950-000), raloxifene hydrochloride (Tocris, 2280), MPP dihydrochloride (Tocris, 1991), Cyclofenil (Tocris, 3999), PPT (Tocris, 1426), PHTPP (Tocris, 2662), Y134 (Tocris, 2676), or Way20070 (Tocris, 3366). Aromatase inhibitor compounds included: Anastrozole (Tocris, 3388), Exemestane (Tocris, 3759), Letrozole (Tocris, 4382), or YM511 (Tocris, 3278). Stock compounds were prepared in DMSO (Sigma 276855), and DMSO concentration did not exceed 0.3% in the described experiments.

Time Lapse Imaging Analysis

Untransfected nRFP-INS-1E were treated with 10 nM estrogen modulating compounds and imaged for 22 hours using the BD Pathway 855 Bioimager. Following data acquisition, Regions of Interest, ROI, representing the cells were segmented on the first and last frame of the experiment based on RFP expression, which highlights the nucleus. The percentage difference in cell number was calculated.

For imaging involving transfected cells, nRFP-INS-1E were transfected with the vectors designed to express ER α 66-GFP or a GFP-only empty vector (EV) in a 384 well plate format. Forty-eight hours post-transfection, cells were treated with 10 nM E2, MPP, or MPP + E2 and immediately recorded for 43 hours using fully automated time-lapse imaging at conditions of 37°C and 5% CO₂. Images were taken at 1 hour and 45 minute intervals on a BD Pathway 855 Bioimager. Following data acquisition, cells were segmented on GFP expression, in order to identify the protein of interest, and RFP expression, which allows for identification of the nucleus. Segmentation defines many aspects of the cell including the area occupied by the cell and location within the well, which allows for measures of cell size and cell tracking, respectively.

Localization of the GFP-only EV or ER α 66-GFP as well as RFP were analyzed on the first frame of the time course. GFP segmentation identified localization of the protein of interest in the nRFP-INS-1E cells. RFP segmentation allowed for identification of the nucleus, since the nRFP-INS-1E cells contain RFP in the nuclear compartment. Segmentation of the cells generates a segmentation mask, which can be used to determine the location of the fluorescence. The RFP segmentation mask was then overlaid onto the GFP fluorescence image in order to assess protein localization by visual

inspection. Localization of the GFP-only EV or ER α 66-GFP were scored as follows: nuclear, cytoplasmic, or both nuclear and cytoplasmic localization. Nuclear localization was defined as expression of GFP overlapping RFP expression and excluding the surrounding cytoplasmic compartment. Cytoplasmic localization was defined as GFP expression that excludes the area of RFP expression. Both cytoplasmic and nuclear expression was defined as GFP expression that both overlaps the nuclear RFP expression and is localized in the cytoplasmic compartment surrounding RFP expression.

Tracking of GFP in ER α 66-GFP and GFP-only EV expressing cells as well as nuclear RFP expression during the time lapse imaging allows for the identification of cellular fate events including proliferation, survival, and death. Once again, cells were segmented on both GFP, representing the protein of interest, and RFP, representing the nuclear compartment. Cell fate was analyzed by examining any changes in GFP or RFP expression in the ROI as described below over a period of 43 hours on a cell-by-cell basis. Death was classified by one of two measures: 1) Loss of GFP or RFP expression or 2) rapid expansion of the area occupied by an ROI, defined by leakage of either GFP or RFP into the area surrounding the cell. Proliferation was measured through a duplication of GFP or RFP expression from one ROI resulting in the appearance of two distinct ROI. Survival was measured as no change in GFP or RFP expression in the ROI being monitored.

Western Blotting

INS-1E cells were transfected in a 6 well plate format with ER α 66-GFP, ER β -GFP, or an EV. Cells were harvested using 1% NP40/TBS lysis buffer following a wash with PBS. Protein was loaded, electrophoresed on a 10% Bis-Tris Gel, and transferred to

a 0.2 μ m PVDF membrane. Blots were probed with 1:1000 ER α (SC-543) or 1:1000 ER β (AbCam, 3576) in 1% milk TBST or 5% milk TBST respectively overnight at 4°C. Anti- β -actin antibody 1:1000 (Sigma, A5316) in 1% milk PBST for 2 hours was used at a loading control. Secondary antibodies, HRP-linked anti-mouse 1:3000 (Bio-Rad, 172-1011) or anti-rabbit 1:2500 (Cell Signaling, 7074S), were added and incubated at room temperature for 2 hours. Blots were developed using the chemiluminescent substrate SuperSignal West Pico (Thermo Scientific, 34080).

Immunofluorescence

INS-1E cells transfected with ER α 66-GFP or EGFPN3 empty vector (EV) were fixed in 3.7% formaldehyde for 30 minutes at room temperature. Nuclei were stained with 2.025 μ M Hoechst 33342 (Invitrogen, H3570). Cells were permeabilized with 0.2% Triton X-100 and incubated with 1:200 ER α (sc-543)³⁸ in 1% BSA/PBS for 2 hours at room temperature. Cells were then incubated in an anti-rabbit TRITC 1:200 for 1 hour (Jackson ImmunoResearch, 711-025-152) in 2% BSA/PBS. Fluorescence was viewed with a BD Pathway 855 BioImager.

Luciferase Assay

For analysis of ERE transcriptional activity, the INS-1E cells were transfected with either ER α 66-GFP, ER β -GFP, or EV as well as a triple repeat estrogen response element (ERE) firefly luciferase reporter¹⁴⁵ and a transfection control, CMV-renilla luciferase (rLuc) (Promega, E2261) in a 384 well format. Cells were allowed to attain optimal protein expression for approximately 40 hours, and then treated twenty-four hours prior to the luciferase assay with estrogenic compounds. After 24 hours of treatment the cells were lysed using 10 μ l of passive lysis buffer (Promega) and

incubated at room temperature for 15 minutes. Following lysis a Promega Dual Luciferase Reporter Assay System was used (Promega, E1910). Briefly, 25 μ l of luciferase assay reagent II was added to the 10 μ l of lysates and ERE-dependent luminescence was observed. Next, 25 μ l of Stop and Glo reagent was added to determine renilla luminescence. Luminescence was detected in a BMG omega plate reader with a gain of 4000. ERE driven firefly luciferase activity was normalized to renilla luciferase activity to account for transfection efficiency, and further normalized to the ER α 66-GFP DMSO control.

Insulin Secretion

Insulin secretion was examined in INS-1E cells transfected with ER α , ER β , or empty vector in a 6 well plate as described above. DMSO or 10 nM MPP was used during the duration of the experiment starting at refeed. 40-42 hours after transfection cells were selected by 0.4 mg/ml geneticin treatment for 48 hours. Selected cells were then split to a 384 well plate where they were seeded at a density of 8,125 cells/well. The plate was allowed to incubate for 48 hours, and then insulin secretion was stimulated by either 2.5 mM glucose or 16 mM glucose Krebs Buffer for 3 hours. Insulin secretion was measured using a rat/mouse insulin ELISA kit (Millipore, EZRMI-13K). Data were analyzed by measuring insulin secreted in ng/ml and normalizing to total cell number.

Caspase 3/7 Assay

For analysis of caspase 3/7 activity, INS-1E cells were transfected with ER α 66-GFP, ER β -GFP, or an EV in a 6 well format as described above. Cells were treated with 10 nM compound for the duration of the experiment starting at the refeed of 6% FBS RPMI media 17 hours post-transfection. 40-42 hours after transfection cells were selected

by 0.4 mg/ml geneticin treatment for 48 hours. Selected cells were then split to a 384 well plate and seeded at a density of 6,675 cells/ well. The following day, 1.62 μ M Hoechst 33342 (Invitrogen, H3570) was added to the wells for 30 minutes and cells were imaged for total cell number. Following imaging analysis, 42 μ l of Caspase 3/7 Glo Reagent (Promega, G8091) was added to the wells and incubated for 30 minutes at room temperature in the dark. The luminescence was observed by a BMG omega plate reader with a gain of 3600. Caspase 3/7 activity was standardized by total cell number, counted by Hoechst positive cells, and normalized to the ER α DMSO control.

RNASeq

Human islet samples were obtained from the Integrated Islet Distribution Program (IIDP). Following arrival, islets were spun down at 1200 rpm for 5 minutes in 3, 50 ml conical tubes. Following the spin, supernatant was extracted and islets were combined in a 1 ml volume. Islets were plated on 96 well plates at a concentration of 500 islets per well. Following plating, islets were treated with 10 nM raloxifene or control treated. Islets were incubated for 24 hours at 37°C and 5% CO₂. After a 24 hour incubation 3 wells of islets were combined for 1500 total islets, and RNA was extracted following the RNeasy protocol (Qiagen, 74104). RNA was stored at 80°C until used for RNASeq. Prior to RNASeq, the RNA samples were polyA selected using Dynabeads mRNA DIRECT kit (Life Technologies, 61011). Following mRNA isolation, a cDNA library was built and RNASeq was performed. Counts were obtained using summarizeOverlaps function IntersectionNotEmpty in the GenomicRanges R package. Data were analyzed with the DESeq package in R using default settings as outlined in the workflow¹⁴⁶. Additional analysis included analyzing RPKM expression levels for β -cell phenotype genes and

inflammatory genes from the RNASeq data. Additional analyses included analyzed expression data for inflammatory and β -cell phenotype specific genes from an established pancreatic β -cell phenotype microarray data set GSE29113¹²⁷. Log(FC) of treatment (Day 0/Day 3 Culture or raloxifene treatment) versus control (intact pancreas or untreated islets) are represented.

Statistics

Data analysis was performed using a t-test. Where significant, p-values are reported within the text and on the figure.

Chapter 3 Estrogen Receptor Alpha Variants Have Differential Effects on β -cell Health and Function

ABSTRACT

Estrogen receptor alpha (ER α) signaling has been shown to both prevent and induce apoptosis in many cell types, including pancreatic β -cells. The duality of ER α signaling is due in part to the expression of three isoforms, ER α 66, ER α 46, and ER α 36¹⁴⁷. The structural difference in these isoforms lies in the presence or absence of two transactivation domains, AF-1 and AF-2, which are responsible for the ligand independent and ligand dependent activation of the receptor, respectively. ER α 66, the full length variant, possesses both domains, ER α 46 lacks the AF-1 domain, and ER α 36 lacks both transactivation domains. These differences lead to changes in the recruitment of transcriptional machinery, including coregulators, and produce differential ER α mediated target gene effects. In the present study, expression of ER α 66 or ER α 46 in INS-1E cells resulted in increased transcriptional activity from an estrogen response element (ERE) luciferase reporter. Transcriptional activity was tightly correlated to an increase in caspase 3/7 activation, suggesting that ER α 66 and ER α 46 activate genomic gene targets to induce apoptosis. Indeed, in examination of ER α target genes, ATF4 and DDIT3, components of the endoplasmic reticulum stress pathways, FOS and Gadd45 β , involved in apoptosis and DNA damage repair, and Bcl2, an anti-apoptotic gene, were all significantly affected by ER α 66 or ER α 46 expression. Interestingly, there was also a dramatic decrease in insulin gene expression indicating that ER α 66 and ER α 46 modulate pancreatic β -cell function as well. ER α 36 had no significant impact on either

transcriptional or caspase 3/7 activity, indicating there is no genomic activity of this receptor in the INS-1E cells.

INTRODUCTION

Multiple studies have linked estrogen receptor signaling to the development and progression of type 2 diabetes mellitus (T2DM) in both peripheral tissues and pancreatic β -cells^{16,74,84,103,104,115}. In pancreatic β -cells, extranuclear estrogen receptor signaling was shown to be beneficial through reduced apoptosis following streptozotocin or H_2O_2 induced stress, and reduced lipogenesis and triglyceride accumulation in islets to preserve β -cell health^{73,74,87,148}. However, there are also studies showing that estrogen signaling in β -cells can act to elicit a negative effect on cell health. Multiple studies have found that long-term exposure to xenoestrogens, environmental contaminants that mimic estrogen, induces β -cell dysfunction and apoptosis^{96,98,130}. These studies highlight the complexity of estrogen receptor signaling in β -cells, and suggest that the understanding of nuclear receptors on β -cell health is still poorly understood.

In order to understand if splice variants may be involved in mediating the differential effects of estrogen receptor signaling events in β -cells, this study examined three splice variants of ER α (ER α 66, ER α 46, and ER α 36). While ER α 66 signaling is thought to mediate the estrogenic effect in β -cells, the contribution of other variants cannot be dismissed⁷⁸. Previously, Tiano et al. theorized that ER α 36 could work in combination with an estrogen responsive G-protein coupled receptor (GPER) to mediate the extranuclear effect of estrogen in β -cells⁷³. Thus, the individual contribution of multiple ER α splice variants may work to facilitate the estrogenic effect in β -cells.

As stated above, the three primary splice variants of ER α are ER α 66, ER α 46, and ER α 36. Structurally, ER α consists of two activation function (AF-1 and AF-2) domains, a DNA binding domain, a hinge region, and a ligand binding domain^{40,42,43}. The variants

of ER α differ in the presence of the AF domains, which are responsible for the transcriptional activity of the receptors and work through ligand dependent, AF-2, and ligand independent, AF-1, mechanisms. ER α 66 has both AF-1 and AF-2 domains, ER α 46 lacks the AF-1 domain; and ER α 36 lacks both the AF-1 and AF-2 domains^{43,45,46}. The AF domains are responsible for the recruitment of transcriptional machinery and coregulators, thus the target genes affected by ER α variants differ as a result of the presence or absence of the AF-1 or AF-2 domains^{106,149,150}. Additionally, found within the AF-1 domain are regulatory phosphorylation sites, including Ser118, which can mediate the unliganded activation of the estrogen receptor¹⁵¹. Thus, ER α 46 and ER α 36, which does not have this site, may possess different patterns of transcriptional activity than ER α 66. Additionally, the domain specific recruitment of coregulators can lead to differential regulation of the same gene target producing ER α variant specific effects³².

In addition to a difference in the regulation of genes through the recruitment of coregulators, estrogen receptors also have two distinct signaling pathways, nuclear or cytoplasmic^{42,43}. The variants of ER α are known to participate in both pathways; however, their structural characteristics can influence the receptor to participate in predominantly cytoplasmic or nuclear signaling. For example, ER α 36 contains three myristoylation sites not contained in other ER α variants, which target it to be the predominant splice variant in the cytoplasm^{46,47}. ER α 46 and ER α 66 primarily initiate transcriptional activities, but may also have cytoplasmic related function due to a palmitoylation site (Cys452) found in the protein³³. Thus, the expression of each of these variants could drive diverse outcomes on β -cell health.

While the AF-1 and AF-2 domains of the estrogen receptor can work synergistically to regulate pancreatic β -cell function, there are instances in which one domain is necessary for ER α function. For example, in mice the AF-2 domain of ER α is necessary to protect against atherosclerosis, however its function is not necessary to speed endothelial healing^{152,153}. The necessity for ER α 's AF-2 domain was shown through changes in aortic gene expression of VCAM-1, GaIntl2, and Gremlin2, which are important in mediating atherosclerosis. Additionally, E2 has a well-defined role in the regulation of bone growth that was shown to be mediated through the ER α AF-2 dependent transactivation domain¹⁵⁴. In relation to diabetes, the independent effect of the AF-1 and AF-2 domains has been evaluated in peripheral tissues including skeletal muscle^{152,153}. In mice with a specific deletion of one or both of the domains, it was shown that the AF-2 domain is indispensable for ER α mediated insulin sensitivity through the regulation of downstream target genes¹⁰⁶. This finding highlights the potential importance of ER α variants in diabetes progression in peripheral tissues. However, there remains a lack of knowledge for the respective contributions of the AF-1 and AF-2 domains in the endocrine pancreas.

Overall, the present study aims to address the function of ER α splice variants in pancreatic β -cells using an estrogen receptor negative β -cell line, INS-1E. The results illustrate that AF-2 dependent activity of ER α triggers a significant deterioration of β -cell health, which was alleviated through inhibition of ER α activity with pharmacological agents. Additionally, the results suggest a distinction in ligand binding sensitivity between ER α 46 and ER α 66. This difference could affect future pharmacological treatment strategies for modulating ER α activity in β -cells that is dependent on the

expression level of each receptor. ER α 36 did not influence either transcriptional activity or cell death in this system. Altogether, the study provides a model for the potential use of therapeutic ligand treatment to modulate β -cell health, which is dependent on the expression of ER α variants.

RESULTS

ER α Variant Expression in INS-1E

In order to assess the effects of ER α variants in INS-1E cells, expression vectors were designed. ER α 66, ER α 46, and ER α 36 were constructed in a non-fusion pIRES2-EGFP expression vector to evaluate transfection efficiency without the interference of a fused GFP protein (Figure 3.1 A). ER α 46 was designed to eliminate the first 173 amino

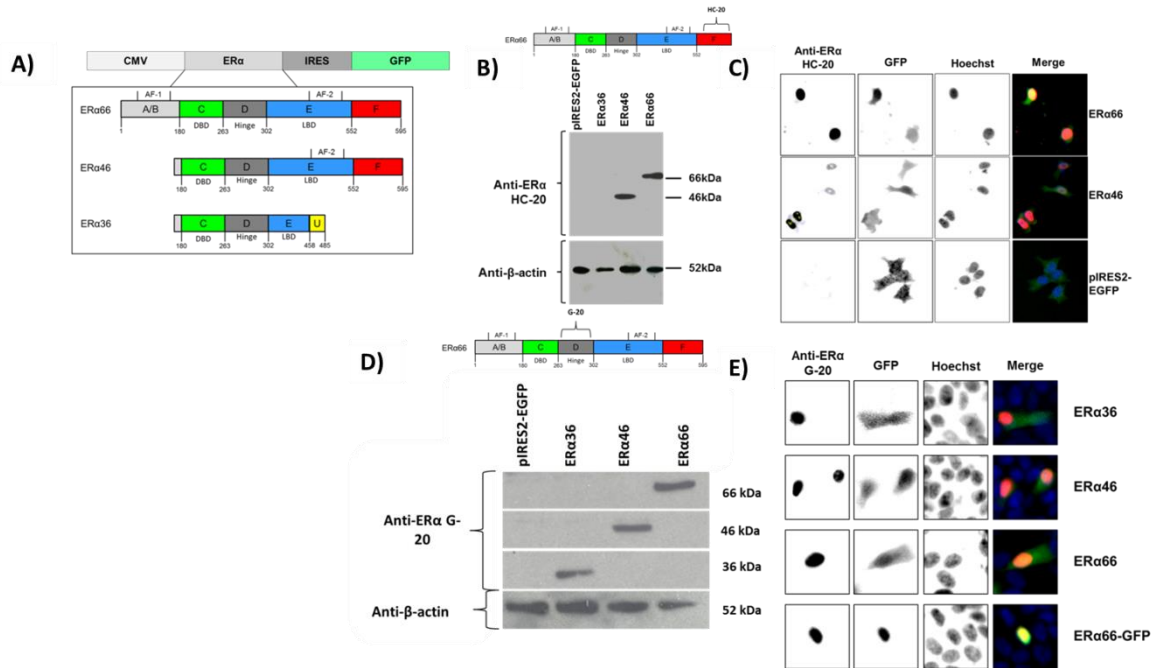


Figure 3.1 ER α Variant Expression in INS-1E.

ER α variants were constructed in a pIRES2-EGFP expression vector (A). Immunoblotting confirmed expression of ER α 46 and ER α 66 plasmids using the C-terminal HC-20 antibody with β -actin as the loading control (B). Immunofluorescence with the HC-20 antibody validates expression of the variants and demonstrates nuclear localization of ER α 66, and ER α 46 (C). Immunoblotting confirms the expression of ER α 66, ER α 46, and ER α 36 using the hinge region antibody G-20 with β -actin as the loading control, and confirms lack of endogenous expression of all three variants (D). Immunofluorescence confirms the nuclear expression of ER α 66, ER α 46, and ER α 36 (E).

acids that comprise the AF-1 domain, which is responsible for the recruitment of coregulators in a ligand independent manner (Figure 3.1 A). ER α 36 was designed to eliminate the first 173 amino acids of the AF-1 domain, as well as replace amino acids 495-598 with a 27 amino acid C-terminal end transcribed from a downstream exon (Figure 3.1 A). The deletion of the last 103 amino acids of ER α 36 eliminates the ligand dependent recruitment of coregulators from the AF-2 domain and partially affects the ligand binding domain. Expression of ER α 66 and ER α 46 was confirmed by western blotting using both a C-terminal antibody (HC-20) and a hinge region antibody (G-20) (Figure 3.1 B and D). Immunofluorescence with both the C-terminal and hinge region antibodies revealed that ER α 66 and ER α 46 are nuclear localized (Figure 3.1 C and E). ER α 36 expression was confirmed using the hinge region antibody (G-20) by western blotting, and immunofluorescence revealed its localization was also nuclear (Figure 3.1 D and E). The analysis of ER α protein expression by western blotting and immunofluorescence confirmed the lack of endogenous expression of all ER α variants in the INS-1E cell line (Figure 3.1 B-E). After confirming expression of the transfected proteins, the first functional measurement assessed was transcriptional activity.

ER α 46 and ER α 66 are Transcriptionally Active in INS-1E

To assess the transcriptional activity of ER α 66, ER α 46, and ER α 36, a dual luciferase assay was utilized. ER α dependent transcriptional activity was measured using a triplicate repeat of the canonical estrogen response element (ERE) fused to a firefly luciferase reporter, whose activity was normalized to renilla luciferase activity. ER α 66, the full length variant, increased transcriptional activity in the DMSO treatment group with no effect of E2 (Figure 3.2 A). This finding substantiates our previous studies with

an ER α 66-GFP fusion protein that showed constitutive activation in the absence of treatment (Figure 2.6 B). Antagonism with MPP decreased ER α 66 transcriptional activity by 67% from the DMSO treatment group ($p \leq 0.01$), which was reversed by concurrent treatment with MPP + E2 (Figure 3.2 A). The AF-1 deficient variant, ER α 46, also

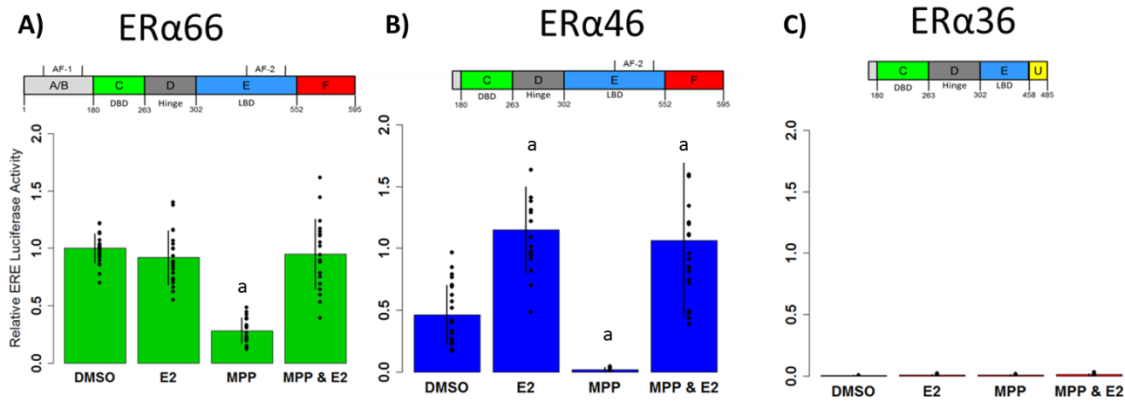


Figure 3.2 ER α Variant Expression Increases Transcriptional Activation.

INS-1E cells were transfected with ER α 66, ER α 46, or ER α 36 and assayed for transcriptional activity from a 3x-ERE luciferase reporter 24 hours after 10 nM ligand treatment (E2, MPP, or MPP and E2). Data was normalized to ER α 66 DMSO control. ER α 66 and ER α 46 expression induced an upregulation of luciferase activity, which was inhibited by 10 nM MPP in both cases (A & B). 10 nM E2 treatment caused a significant upregulation of ER α 46 dependent transcription on ERE promoters (B), but caused no significant change in ER α 66 expressing cells (A). ER α 36 expression induced no change in transcriptional activity on ERE-promoters (C). (a: Treatment vs. DMSO for each transfection group, $p \leq 0.01$) ($n \geq 5$ biological replicates)

increased transcriptional activity from the ERE-luciferase reporter in the absence of estrogenic treatment, although to a lesser extent than ER α 66 (Figure 3.2 B). Furthermore, contrary to ER α 66, ER α 46 transcriptional activity was significantly upregulated after E2 treatment, 59%, over the DMSO control, suggesting a differential response between the variants to this agonist ($p \leq 0.01$) (Figure 3.2 B). Similarly to ER α 66, ER α 46 transcriptional activity was inhibited 94% by MPP ($p \leq 0.01$) (Figure 3.2 B). However, the intensity of inhibition is substantially more than the 67% downregulation observed after

MPP treatment of ER α 66 expressing cells, once again suggesting a difference in the responsiveness to compound treatment between the two variants (Figure 3.2 A and B). The inhibitory effect elicited by treatment with MPP on ER α 46 activity was reversed by concurrent treatment with MPP + E2, which restored transcriptional activity to the E2 treatment level ($p \leq 0.01$) (Figure 3.2 B). ER α 36, the AF-1 and AF-2 deficient variant, did not activate transcriptional activity in any treatment group (Figure 3.2 C). These data show that ER α 66 and ER α 46 are transcriptionally active in INS-1E cells, and demonstrate that their activity can be modulated through estrogenic compound treatment. The data also establish a difference in the ligand responsiveness between ER α 66 and ER α 46, where ER α 46 is more responsive to both agonist and antagonist treatment.

ER α 66 and ER α 46 Increase β -cell Death

As described in chapter 2, ER α 66-GFP transcriptional activity is correlated to β -cell death (Figure 2.7). To evaluate if the transcriptional activity observed from ER α variants increased β -cell death in the same manner, caspase 3/7 activity was examined. INS-1E cells expressing ER α 66, ER α 46, or ER α 36 were selected to obtain a pure population of ER α variant expressing cells. Following selection, cells were assayed for caspase 3/7 activity in four treatment groups (DMSO, E2, MPP, or MPP + E2). Cells expressing ER α 66 or ER α 46 had elevated caspase 3/7 activity in the DMSO treatment group, with no effect of E2 treatment in ER α 66 expressing cells ($p=0.44$) (Figure 3.3 A and B). However, E2 treatment of ER α 46 expressing cells elicited a 19% increase in caspase 3/7 activity over the DMSO treatment group, confirming the E2 responsiveness in the transcriptional assays. As expected, the E2 stimulated caspase 3/7 activity is slightly less than the magnitude of transcriptional activation initiated by E2 due to the

necessity of FBS in the media used for the caspase 3/7 experiments ($p \leq 0.01$) (Figure 3.3 B). Treatment with MPP decreased caspase 3/7 activity in ER α 66 or ER α 46 expressing cells by 39% ($p \leq 0.01$) and 70% ($p \leq 0.01$) respectively, which is consistent with the level of decreased activity from the transcriptional assays (Figure 3.3 A and B). The inhibition of decreased activity from the transcriptional assays (Figure 3.3 A and B). The inhibition of ER α 46 mediated caspase 3/7 activation is more dramatic than observed for ER α 66; further suggesting enhanced ER α 46 ligand responsiveness in the INS-1E cells. Concurrent treatment with MPP + E2 overcame the inhibitory MPP effect in both ER α 66 and ER α 46 expressing cells to restore caspase 3/7 to the DMSO and E2 treatment levels,

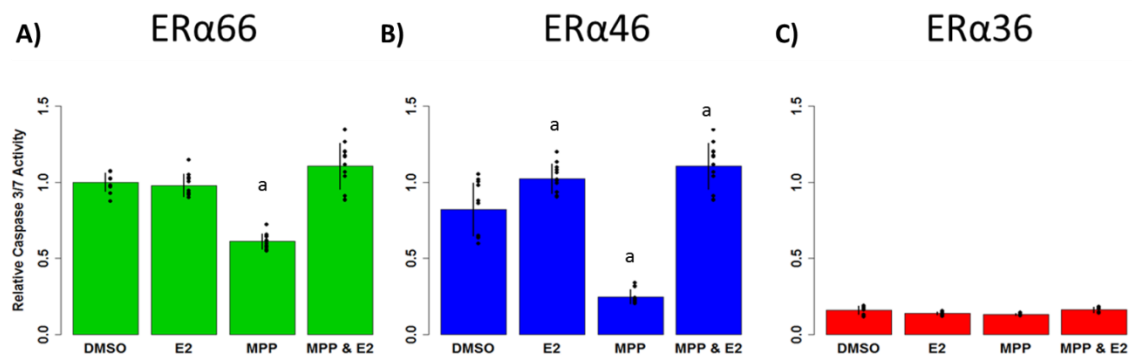


Figure 3.3 ER α Variant Expression Increases Caspase 3/7 Activity.

INS-1E cells were transfected with ER α 66, ER α 46, or ER α 36 and assayed for caspase 3/7 activity after chronic 10 nM estrogenic compound (E2, MPP, or MPP and E2) treatment. Data was normalized to ER α 66 DMSO control. Expression of ER α 66 and ER α 46 increased caspase 3/7 activity, which is again inhibited by 10 nM MPP treatment (A & B). In ER α 46 expressing cells, 10 nM E2 treatment increased caspase 3/7 activity (B), but was ineffective at changing ER α 66 activity (A). ER α 36 expression had no significant effect to increase caspase 3/7 activity (C). (a: Treatment vs. DMSO in each transfection group, $p \leq 0.01$) ($n \geq 8$)

respectively (Figure 3.3 A and B). ER α 36 expression initiated no change in caspase 3/7 activity in any treatment group, which substantiated the transcriptional assay results

(Figure 3.3 C).

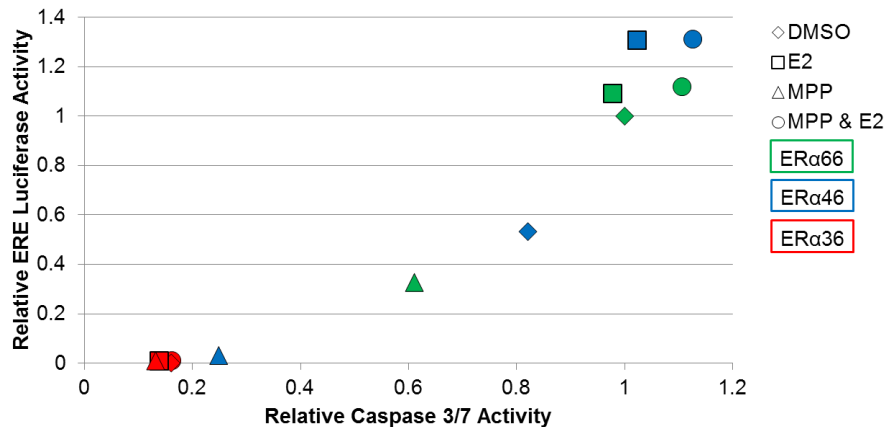


Figure 3.4 ER α Mediated Transcriptional Activation and Caspase Activity are Correlated.

Data points from luciferase and caspase 3/7 activity from **Figure 3.2** and **Figure 3.3** were averaged and plotted above. Estrogen receptor variant expression led to an increase in transcriptional activation, which was tightly correlated to an increase in caspase 3/7 activation. Treatment with 10 nM E2 inhibited both transcriptional activation and caspase 3/7 activation in ER α 66 and in ER α 46 expressing cells. 10 nM E2 treatment had significant effects only on ER α 46 expressing cells increasing both transcriptional activity and caspase 3/7 activation. ER α 36 displayed no significant effect on transcriptional activity or caspase activation in any treatment group.

As the data presented demonstrates, transcriptional activity of ER α variants was tightly correlated to the activation of caspase 3/7 (Figure 3.4). Further, the action of the receptor is likely

to be mediated by the AF-2 dependent activity of ER α , since ER α 46 lacks the AF-1 domain. This conclusion is substantiated by the lack of transcriptional and caspase 3/7 activation in cells that express the AF-1 and AF-2 deficient variant, ER α 36.

ER α Variants Respond Differentially to SERMs

To assess the variance in ligand responsiveness, transcriptional activity of ER α 66 or ER α 46 was measured after treatment with two selective estrogen receptor modulators (SERMs). SERMs have the unique property of being either an antagonist or agonist

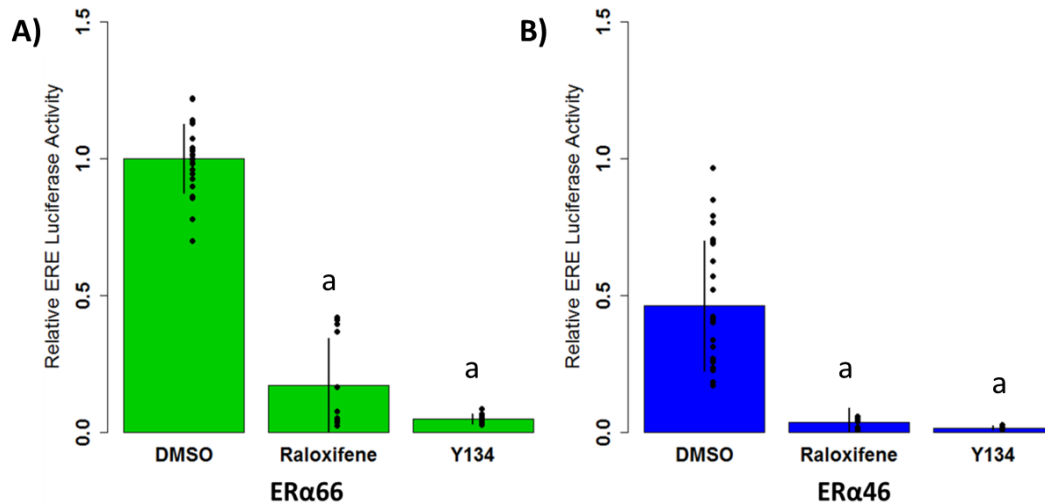


Figure 3.5 Raloxifene and Y134 Inhibit ER α 66 and ER α 46 Transcriptional Activity.

ER α 66 and ER α 46 were transfected in INS-1E cells, and transcriptional activity was assessed using an ERE-luciferase reporter normalized to renilla luciferase values. 24 hours prior to the luciferase assay cells were treated with DMSO, 10 nM raloxifene (Ral) or Y134. Transcriptional activity was reduced by raloxifene or Y134 treatment in cells expressing either ER α 66 (A) or ER α 46 (B). (a: Treatment vs. DMSO in each transfection group, $p \leq 0.01$) ($n \geq 10$)

depending on the tissue type in which they are present⁵⁶. Raloxifene is a widely used SERM, and previously was shown to be an antagonist of ER α transcriptional activity in INS-1 cells⁹⁰. In the current model, raloxifene significantly suppressed the activity of both ER α 66 and ER α 46 (Figure 3.5 A and B) ($p \leq 0.01$). Once again, ER α 46 transcriptional activity was more significantly inhibited, 91%, compared to ER α 66 activity, 82%. The second compound analyzed, Y134, is an analogue of raloxifene and exhibits high affinity for binding to ER α . Y134 was a potent antagonist of ER α 66 and

ER α 46 ERE-mediated transcriptional activity, exhibiting a greater inhibition of ER α 46 activity ($p \leq 0.01$) (Figure 3.5 A and B). These data advance the hypothesis that ER α 46 is more sensitive to compound treatment than ER α 66. Additionally, the results point to the importance of understanding the expression levels of each variant in the cell to tailor future *in vivo* modulation of estrogen receptor activity by pharmacological treatment.

However, we must take into account that in cells more than one variant may be expressed at once.

ER α Variant Coexpression Results in Repression of Transcriptional Activity

Previous literature has shown that heterodimerization of ER α 66 and ER α 46 in the same cell can cause inhibition of ER α 66 AF-1 dependent transcriptional activation⁴⁵. To assess the interaction of the two variants in the INS-1E β -cell model, ER α 66 and ER α 46 were co-expressed in one of 3 combination groups. With a 1:3 ratio of ER α 66:ER α 46, the transcriptional activation of the

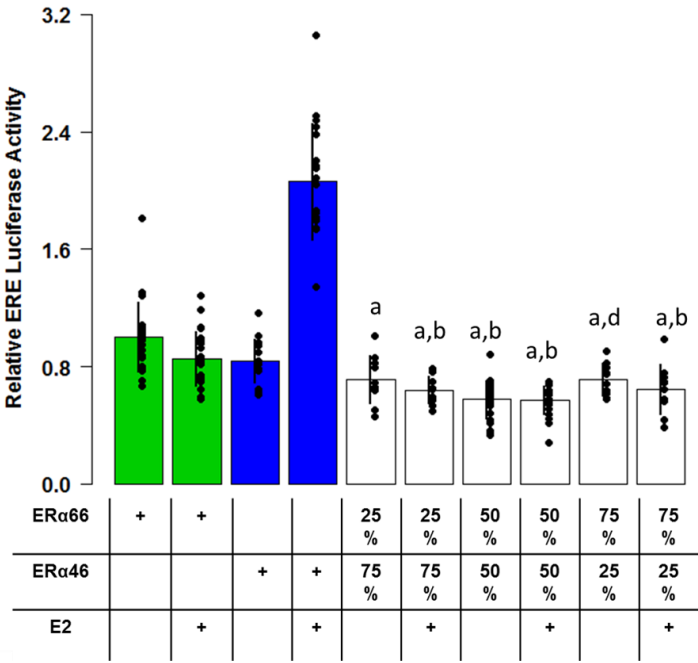


Figure 3.6 ER α 66/ER α 46 Co-expression Represses Transcriptional Activity.

INS-1E/Wnt cells were transfected with ER α 66 and ER α 46 at varying amounts including (25% ER α 66 / 75% ER α 46, 75% ER α 46 / 25% ER α 66, or 50% ER α 66 / 50% ER α 46). Transcriptional activity was assessed using a 3X ERE-luciferase reporter and values were normalized to renilla luciferase. 24 hours prior to the luciferase assay, cells were treated with either DMSO or 10 nM E2. Expression of ER α 46/ER α 66 at any combination reduced transcriptional activity from the ERE reporter. Treatment with E2 had no effect in any combination group. (a: $p \leq 0.01$ ER α 66 vs treatment) (b: $p \leq 0.01$ ER α 46 vs. treatment) (c: $p \leq 0.05$ ER α 66 vs treatment) (d: $p \leq 0.05$ ER α 46 vs. treatment) ($n \geq 10$)

ERE-luciferase reporter was significantly reduced, 28%, from ER α 66 expression alone ($p \leq 0.01$) (Figure 3.6). Combinations of 3:1 ratio of ER α 66:ER α 46 had a strikingly similar pattern of behavior as the 1:3 ER α 66:ER α 46 ratio causing a 28% reduction in transcriptional activity from the ERE-luciferase reporter ($p \leq 0.01$) (Figure 3.6). A 1:1 combination of ER α 66 and ER α 46 had a greater reduction, 46%, of ERE-transcription than variable concentrations of the receptors (i.e. 1:3 or 3:1 ratios) ($p \leq 0.01$), which could be due to increased formation of the inhibitory heterodimer at equivalent concentrations of each receptor (Figure 3.6). Treatment with 10 nM E2 had no effect on the transcriptional activity of the heterodimer in any combination group (Figure 3.6). Overall, these data indicate that any combination of ER α 66 and ER α 46 will have an inhibitory effect on ER α transcriptional activity at ERE promoters, with a more significant effect at equivalent receptor concentrations in INS-1E cells. Further, the data suggests that ER α 46 may suppress the AF-1-dependent activity of ER α 66 in β -cells.

ER α 46 and ER α 66 Influence β -cell Apoptosis and β -cell Function Target Gene Expression

While the readout of transcriptional activity is useful to elucidate the mechanism of ER α function in INS-1E cells, an identifiable gene target that would function to initiate the dramatic increase in apoptosis after ER α activation was not yet identified. Thus, in order to generate a list of potential ER α gene targets, we looked to the long-term estrogen deprived MCF7 cells as a model system to identify genes mediating estrogen induced apoptosis¹⁵⁵. The genes chosen included those involved in apoptosis and DNA damage repair (FOS, BCL2, Gadd45 β)^{156–161}, mediation of lipoapoptosis (ACLY)¹⁶², and genes involved in the unfolded protein response (ATF4, DDIT3)^{163,164}. ER α variants or an EV

were expressed in INS-1E cells, and then selected in order to obtain a pure population of ER α or EV expressing cells. mRNA levels were assessed following 24 hours of DMSO or E2 treatment. In this system, FOS expression was significantly upregulated over the EV control in cells expressing ER α 66 or ER α 46, with no significant effect of E2 treatment in either group ($p \leq 0.01$) (Table 1). Interestingly, during islet isolation procedures, increased FOS expression is indicative of apoptosis and leads to increased islet graft failure¹⁶⁵. Thus, inhibiting ER α activity in islets during isolation may reduce these negative effects. Further, ATF4 and DDIT3 were significantly upregulated in response to ER α 66 or ER α 46 expression compared to the EV control in the INS-1E model, again with no responsiveness to E2 treatment ($p \leq 0.05$) (Table 1). Interestingly, there is a greater increase in expression of ATF4 and DDIT3 in response to ER α 46 expression compared to ER α 66 expression, suggesting differential regulation of these genes by each receptor. The upregulation of ATF4 and DDIT3 could point to an increase in endoplasmic reticulum stress and activation of the unfolded protein response pathway. It is known that ATF4 is involved in mediating the upregulation of genes involved in the UPR, including DDIT3, which leads to apoptosis of cells¹⁶³. DDIT3, more commonly known as CHOP, is upregulated in response to ATF4 activity, and its increase can lead to apoptosis partly mediated through a decrease in BCL2 expression. Interestingly, we also observed a dramatic decrease in BCL2 expression in cells expressing ER α 66 compared to the EV control ($p \leq 0.05$) (Table 1), suggesting that DDIT3 upregulation may mediate a decrease in BCL2^{166,167}. In ER α 46 expressing cells, there was a trend towards decreased BCL2 expression compared to the EV control, however this was not significant (Table 1). The differential regulation of the BCL2 gene suggests that there may be domain specific

differences in the regulation of some genes we examined. Indeed, Gadd45 β also exhibits a variant specific regulation of its transcription. In ER α 46 expressing cells, a significant upregulation of Gadd45 β expression was observed compared to the EV control, with no effect of E2 treatment ($p \leq 0.01$) (Table 1). ER α 66 expression induced a slight upregulation of Gadd45 β expression over the EV control; however this was not significant (Table 1). Gadd45 β expression is known to be upregulated in response to genotoxic stress and regulates the DNA damage and repair response, substantiating the hypothesis that ER α transcriptional activity leads to β -cell apoptosis^{167,168}. In addition to the usual apoptotic gene targets, decreased expression of ACLY relative to the EV control was observed in cells expressing ER α 66 or ER α 46 ($p \leq 0.01$) (Table 1). This substantiates the hypothesis of an increased likelihood of apoptotic cell death after ER α 66 and ER α 6 expression since decreased expression of ACLY has been shown to induce β -cell death in the INS-1E model¹⁶². The expression of ER α 36 in the INS-1E cells did not elicit a significant alteration of expression in any of the genes in this assay, corroborating the transcriptional and caspase 3/7 assays (Table 1). The increase in pro-apoptotic and decrease in anti-apoptotic gene expression, as well as increased gene expression in the UPR pathway, agree with the caspase 3/7 data to validate that ER α activation of cell death is mediated by genomic signaling in INS-1E cells. In addition to measuring apoptotic cell death gene targets, we also analyzed a readout of β -cell function, insulin gene expression.

ER α Expression Decreases Insulin Production in INS-1E Cells

ER α 66 and ER α 46 expression stimulates a significant induction of β -cell death, however we wanted to evaluate if ER α expression also leads to changes in β -cell

function. To address the hypothesis that ER α variant expression would reduce β -cell function, insulin mRNA levels were measured in INS-1E cells selected for ER α variant expression as described previously. Expression of ER α 66 significantly reduced insulin mRNA levels compared to EV expressing cells ($p \leq 0.01$), with no effect of E2 treatment (Table 1). ER α 46 also reduced insulin mRNA levels 0.44 fold compared to the EV control ($p \leq 0.01$) (Table 1). The reduction of insulin expression was more significant in ER α 66 expressing cells, once again suggesting differential regulation of gene targets by each receptor in this cell line. Surprisingly, E2 treatment did not have a significant effect on insulin expression in the ER α 46 expressing cells (Table 1). The ligand independent regulation of non-ERE ER α target genes (i.e. insulin) has been reported previously, and may account for the lack of E2 responsiveness observed for insulin transcription¹⁶⁹. In agreement with previous luciferase assays, ER α 36 had no significant effect on the expression of insulin mRNA levels and was not affected by E2 treatment (Table 1). In order to confirm the reduced insulin mRNA expression, we set out to determine total insulin content in the INS-1E cells following expression of ER α 66 or an EV.

Table 1 ERα66 and ERα46 Regulate the Expression of Apoptotic and β-cell Function Genes

Gene	Treatment	ERα66				ERα46				ERα36			
		Average	-95% CI	+95% CI	p-value	Average	-95% CI	+95% CI	p-value	Average	-95% CI	+95% CI	p-value
Up-regulated													
FOS	DMSO	4.37	2.07	9.21	9E-04	4.87	0.97	24.37	0.01	0.97	0.81	1.16	0.80
FOS	E2	5.31	1.77	15.89	9E-04	5.99	5.22	6.88	3E-06	0.90	0.56	1.44	0.50
ATF4	DMSO	1.59	0.89	2.84	0.04	2.29	1.06	4.96	0.008	1.32	0.94	1.84	0.10
ATF4	E2	1.51	0.66	3.44	0.12	2.38	1.72	3.31	9E-04				
DDIT3	DMSO	2.50	0.79	7.92	0.04	6.34	2.98	13.48	7E-04	1.72	1.23	2.41	0.07
DDIT3	E2	2.05	1.13	3.72	0.04	7.97	4.69	13.53	2E-04				
Gadd45β	DMSO	2.46	0.70	8.61	0.13	8.46	2.92	24.51	9E-04	2.07	1.08	3.95	0.57
Gadd45β	E2	1.68	1.07	2.63	0.26	6.29	1.93	20.56	0.02				
Down-regulated													
INS	DMSO	0.14	0.12	0.17	6E-08	0.44	0.29	0.67	3E-04	1.17	0.94	1.47	0.15
INS	E2	0.10	0.08	0.14	5E-08	0.34	0.32	0.36	7E-05	1.11	0.74	1.66	0.29
ACLY	DMSO	0.19	0.08	0.47	2E-04	0.18	0.08	0.39	9E-05	0.84	0.41	1.71	0.34
ACLY	E2	0.11	0.05	0.26	4E-05	0.11	0.05	0.23	2E-05				
BCL2	DMSO	0.21	0.07	0.66	0.03	0.57	0.41	0.79	0.15	0.82	0.50	1.35	0.77
BCL2	E2	0.28	0.07	1.21	0.07	0.63	0.54	0.75	0.47				

INS-1E cells were transfected with vectors for expressing ERα66, ERα46, ERα36, or an empty vector (EV) and selected for cells expressing the vector of interest for 48 hours. Following selection, cells were treated with DMSO or 10 nM E2 for 24 hours and RNA was harvested. Increased expression for FOS was observed in cells expressing ERα66 and ERα46 with no effect of ERα36 expression. ATF4 and DDIT3 expression was significantly upregulated in cells expressing ERα46 and ERα66, but not ERα36 expressing cells. Treatment with 10 nM E2 had no significant effect on any gene. Insulin was significantly reduced in cells expressing ERα66 and ERα46, with no effect in ERα36 expressing cells. Data are presented as relative fold change to EV DMSO after data normalization. (n=3 biological replicates)

ER α 66 Expression Decreases Insulin Content

In order to assess if decreased insulin transcription in ER α expressing cells was correlated to a decrease in insulin content, insulin protein levels were assessed by ELISA. INS-1E cells expressing ER α 66 or EV were selected for expression of the plasmid to

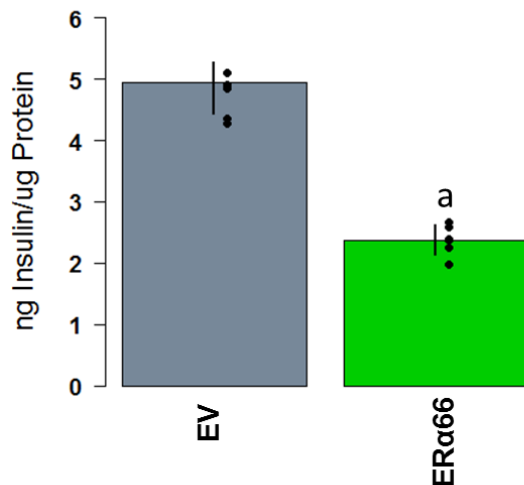


Figure 3.7 ER α Expression Decreases Insulin Protein.

INS-1E cells were transfected with ER α 66 or pIRES2 empty vector (EV), and cells were selected for those expressing the vector of interest. Following total protein extraction, insulin ELISA was performed and insulin content (ng/ml) was normalized to total protein content (μ g/ml). Insulin ELISA reveals that ER α 66 expression reduced total insulin protein levels. (a: ER α 66 vs. EV, $p \leq 0.01$) ($n \geq 3$)

obtain a pure population of ER α expressing cells. Following protein extraction, insulin protein content was assessed and normalized to total protein level. Not surprisingly, ER α 66 expression resulted in a significant 2-fold reduction of insulin content from EV expressing cells ($p \leq 0.01$) (Figure 3.7). These data confirm that the reduction in insulin gene expression translates to a decrease in protein synthesis. This indicates that long-term estrogen receptor activation will result in decreased insulin content possibly affecting downstream insulin secretion.

DISCUSSION

The present study aimed to address the role of ER α variants in the mediation of β -cell health and function. ER α variants differ in the presence of activation function (AF) domains, AF-1 and AF-2, which mediate the transactivation of the receptor⁴⁴. ER α 66, the classical full-length estrogen receptor, contains both domains, whereas ER α 46 only contains one domain (AF-2), and ER α 36 lacks both domains. The AF-1 and AF-2 domains are responsible for the recruitment of coactivators and corepressors; therefore the presence of one or both of the domains can lead to differential regulation of ER α target genes^{106,149,150}. Until now, the independent contributions of each estrogen receptor variant on β -cell health and function have not been assessed.

In the INS-1E cells, exogenous expression of ER α 66 or ER α 46 resulted in nuclear localization of the receptor and increased ER α dependent transcriptional activity from an ERE-reporter. This occurred even in the absence of estrogenic compound treatment, an effect which is consistent with a study of exogenously expressed ER α 66 and ER α 46 in an estrogen receptor negative breast cancer line¹⁷⁰. The activation of estrogen receptor dependent transcription has been shown to elicit a wide variety of responses ranging from apoptosis to proliferation^{57,64,69,123}.

The current study aimed to address whether the beneficial effect of estrogen signaling might be mediated through expression of different variants of ER α . Expression of either ER α 66 or ER α 46 resulted in increased caspase 3/7 activity, an indicator of apoptotic cell death. However, ER α 36, the traditionally cytoplasmically localized ER α variant, produced no genomic activity and no deleterious effect on β -cell health. Previously, it was shown that ER α activity resulted in reduced apoptosis after

streptozotocin or H₂O₂ challenge, suppressed lipogenesis, and reduced cytokine expression after islet isolation^{78,84,86,87}. The beneficial activity of ER α was shown to be through the activation of an extranuclear estrogen receptor, using both ER α DNA binding domain knockouts and nuclear excluded analogues of estradiol. Thus, it is possible that in the previous studies the beneficial effects are mediated through ER α 36 action rather than genomically active ER α 66 and ER α 46, since ER α 36 produced no detrimental effects on β -cell health in the present study.

The divergent responses observed between this and previous studies can be resolved by dissecting the role of the nuclear and the cytoplasmic estrogen receptors. Our study shows a strong correlation between increased ERE responsive transcriptional activity and death; whereas the previous studies show that the beneficial effects of estrogen signaling are initiated in the cytoplasm. The opposition of nuclear and cytoplasmic signaling effects, mediated through the presence of splice variants such as ER α 36 and ER α 66, is a theory proposed by Thomas and Gustafsson¹¹⁸. Thus, it is possible that in β -cells extranuclear signaling by cytoplasmically localized estrogen receptors is beneficial, whereas nuclear activity is detrimental to β -cell health.

To evaluate the effect of ER α 66 and ER α 46 expression on β -cell health, downstream target gene activation was assessed. ER α genomic activity has been linked to many apoptotic pathways through the regulation of genes including FasL, Bcl2, PUMA, NOXA, and many others^{64,124,160,161,171}. In this study, a panel of estrogen receptor responsive candidate genes were evaluated for responsiveness after ER α 66, ER α 46, or ER α 36 expression in the INS-1E cell line. From this list, seven genes were differentially regulated by either ER α 66 or ER α 46 expression including, FOS, ATF4, DDIT3,

Gadd45 β , INS, ACLY, and BCL2. The genes differentially regulated by estrogen receptor expression are involved in the regulation of proliferation and apoptosis (FOS, Gadd45 β , and BCL2), mediation of the endoplasmic reticulum stress pathway (ATF4 and DDIT3), lipoapoptosis (ACLY), and β -cell function (INS). Indeed, increased expression of Gadd45 β , which can induce apoptosis through p38 activation, and FOS, whose induction is correlated to islet graft failure, was observed^{141,155,165,172}. There appears to be independent effects of the AF-1 and AF-2 domains in the regulation of some genes, Gadd45 β and BCL2, whereas most genes appear to follow the same pattern of regulation after expression of both variants. Further, there was substantial upregulation of genes involved in the unfolded protein response including ATF4 and DDIT3. The UPR pathway is triggered in response to increased endoplasmic reticulum stress, and upregulation of these genes is associated with increased apoptosis¹⁶³. ER α 46 or ER α 66 expression induced upregulation of these genes in the INS-1E cells, suggesting nuclear ER α activity may mediate an endoplasmic reticulum stress response. In addition to these genes, significantly downregulated expression of ACLY, a gene involved in protection against lipid-induced apoptosis was observed in both ER α 66 and ER α 46 expressing cells¹⁶². In β -cells reduced expression of this gene can lead to an increase in endoplasmic reticulum stress and eventual apoptosis^{162,173}. Overall, these data suggest that estrogen receptor signaling from ER α 66 and ER α 46 in the nucleus leads to the alteration of genes involved in the regulation of apoptosis to initiate β -cell death. There was no significant effect of ER α 36 expression on any gene set suggesting that this AF-1 and AF-2 deficient variant has no effect on β -cell apoptosis in the INS-1E cell line.

In addition to the examination of apoptosis related genes, we were interested to see how ER α expression affected β -cell function. Thus, insulin gene expression was examined. In previous studies, extranuclear mediated estrogen receptor signaling has been shown to increase insulin gene expression^{81,129,174}. In the present study, expression of ER α 66 or ER α 46 resulted in a significant downregulation of insulin gene expression in INS-1E cells. The reduction in insulin observed in the present study may be due to ER α inhibition of transcriptional machinery necessary for insulin transcription, such as STAT3 or STAT5^{175,176}. Alternatively, the increase in endoplasmic reticulum stress as observed by the upregulation of DDIT3 and ATF4 and could cause reduced insulin mRNA levels. This effect has been observed previously in an induced endoplasmic reticulum stress model where both INS1/INS2 mRNA levels were reduced due to degradation of the transcripts¹⁷⁷.

In order to confirm the results of the insulin repression observed in these cells, protein was isolated to measure insulin protein levels. Substantiating the mRNA evidence, ER α 66 expression results in a significant decrease in total insulin content. Thus, in these cells a decrease in insulin mRNA translates to a decrease in insulin protein levels, which could produce a significant effect on downstream insulin secretion. This once again confirms our hypothesis that nuclear estrogen receptor activity is detrimental to β -cell health and function.

The negative effects elicited by nuclear ER α 46 and ER α 66 activity suggest that inhibiting nuclear estrogen receptor action in β -cells may provide a novel way to combat β -cell death. During islet isolation procedures, a dramatic increase in apoptosis is observed, and one gene associated with this is FOS, which was upregulated by ER α in

this study^{140,141,178,179}. If ER α signaling inhibition could mediate an increase in islet survival or improve islet outcome after transplant, then islet transplantation could become an effective means for diabetic therapy. The quickest way to implement anti-estrogen therapy to islets during transplant would be to use a compound already approved by the FDA. To this effect, we determined that raloxifene and Y134 reduced ER α 66 and ER α 46 transcriptional activity. Since transcriptional activity is tightly correlated to apoptotic cell death in the INS-1E β -cell line, we hypothesize that treatment with these inhibitors would lead to decreased β -cell death following nuclear ER α activation.

Realistically, the expression of ER α variants might not be limited to one variant per cell type. Co-expression of ER α 66 and ER α 46 resulted in decreased activity from the single transfections when co-expressed in any concentration group. It has been previously reported that ER α 46 can work to inhibit the AF-1 dependent transcriptional activity of ER α 66⁴⁵. Thus, in this system ER α 46 may be exerting this same inhibitory effect. Future studies should investigate the interaction between ER α 66 and ER α 46 to determine the mechanism of inhibited transcriptional activity.

In addition to ER α effect on β -cell health and function, differences in ligand sensitivity between ER α 66 and ER α 46 were detected. Cells expressing ER α 46 were more sensitive to antagonism by MPP and agonism by E2 as demonstrated by more significant changes in transcriptional and caspase 3/7 activity than observed for ER α 66. The different ligand sensitivities between the two estrogen receptors could be due to the absence of the AF-1 domain in ER α 46. There are many mechanisms which could mediate this effect including: post-translational modification of ER α 66 in the AF-1 domain, increased affinity of ER α 46 for ERE promoters, or altered folding of ER α 46 leading to

greater exposure of the ligand binding domain^{180,181}. As mentioned previously, the lack of an AF-1 domain in ER α 46 may affect its activity by eliminating activating phosphorylation sites such as Ser104/106, Ser118, Ser167, among others¹⁸². Another mechanism for differences in ligand sensitivity is the recruitment of coregulators.

Overall, this study provides evidence for the role of ER α 46 and ER α 66 in mediating pancreatic β -cell health. We provide stunning evidence that the transcriptional activity of both receptors is tightly correlated to the initiation of apoptotic cell death through caspase 3/7 activation. Additionally, we provide the first evidence of a difference in ligand responsiveness between ER α 46 and ER α 66 in β -cells, which could impact pharmacological modulation of the receptors *in vivo*. Further, we show that activation of ER α 66 and ER α 46 nuclear signaling impacts the expression of target genes associated with apoptosis, endoplasmic reticulum stress, and β -cell function. The reduction observed in insulin gene expression translates to reduced insulin content and could influence insulin secretion as well. In conclusion, future studies should examine the function of estrogen receptor signaling inhibition *in vivo* and in isolated islet samples to evaluate the role of estrogen receptor signaling in human cells.

MATERIALS AND METHODS

Cell Culture and Transfection

Rat Insulinoma (INS-1E) cells or constitutive Wnt expressing INS-1E (INS-1E/Wnt) were cultured in RPMI 1640 (Gibco, 11875-093) media supplemented with 6% FBS at 37°C in 5% CO₂. Cells were harvested by washing once with 1X PBS, trypsinized (0.5% trypsin, 0.05% EDTA), and collected in 10 ml 6% FBS RPMI. Following harvest, cells were spun at 1200 rpm for 5 minutes to form a pellet. The cell pellet was resuspended in Optimem (Gibco, 31985-070), and cells were plated at a density of approximately 225,000 cells for 6 well plates and 8,125 cells for 384 well plates. Cells were transfected using the cationic lipid DMRIE (Invitrogen, 10459014) with a µg:µl DNA:DMRIE ratio of 0.62 (0.116: 0.187) in 384 well plates and 0.40 (2.25:5.61) in 6 well plates.

Vector Generation

Primers used to generate ERα66-pIRES2-EGFP and ERα46-pIRES2-EGFP were designed according to the cDNA sequence BC128573.1. The 5' primer for ERα66 is 5'-ATAAGAATTCATCGCCACCATGACCATGACCCTCCACACCAA-3' and for ERα46 5'-ATAAGAATTCATCGCCACCATGGCTATGGAATCTGCCAAGGA-3' both possessing an *EcoRI* site at the 5' end. The 3' primer for both ERα66 and ERα46 is 5'-ATAAGGATCCTCAGACCGTGGCAGGGAAAC-3' possessing a *BamHI* site at the 3' end. The fragments produced were cloned into the pIRES2-EGFP vector to generate ERα66-pIRES2-EGFP (ERα66) and ERα46-pIRES2-EGFP (ERα46). The ERα36-pIRES2-EGFP (ERα36) vector was produced by extracting ERα36 from the PCB6+-ERα36 expression vector³⁰ using an *EcoRI*, *BamHI* digest and placed into the *EcoRI*,

*Bam*HI sites of the pIRES2-EGFP vector. Additional vectors used in experimentation included the empty vectors pIRES2-EGFP (Clontech, 6029-1) or EGFPN3 (Clontech, 6080-1), a triplicate repeat estrogen response element fused luciferase reporter¹⁴⁵, and a constitutive pRL-CMV renilla luciferase reporter (Promega, E2261).

Compound Treatment

Estrogen modulating compounds included: 1,3,5 (10) Estratrien-3, 17 β -diol (Steraloids, E0950-000), raloxifene hydrochloride (Tocris, 2280), MPP dihydrochloride (Tocris, 1991), or Y134 (Tocris, 2676). Stock compounds were prepared in DMSO (Sigma, 276855). The final concentration of DMSO in media for treatment of cells did not exceed 0.3% in the described experiments.

Western Blotting

INS-1E cells were transfected in a 6 well plate format as described above using the ER α 66, ER α 46, ER α 36, or pIRES2-EGFP vector (empty vector, EV). Cells were selected for ER α variant or EV expression for 48 hours by treatment with 0.4 mg/ml geneticin (G418) suspended in 6% FBS RPMI. Following selection, cells were washed 2x with ice cold 1X PBS and harvested with 60 μ l of lysis buffer containing 1 % NP40/1X TBS and a protease inhibitor tablet (Roche, 040693124001). Protein was loaded, electrophoresed on a 10% Bis-Tris Gel, and transferred to a 0.2 μ m PVDF membrane. Blots were probed with a C-terminal anti-ER α antibody 1:1000 (SC-543) or a hinge region anti-ER α antibody 1:1000 (SC-544) in 1% milk TBST overnight at 4°C. β -actin 1:1000 (Sigma, A5316) in 1% milk PBST for 2 hours was used as a loading control. Secondary antibodies included HRP-linked anti-mouse 1:3000 (Bio-Rad, 172-1011) or anti-rabbit 1:2500 (Cell Signaling, 7074S) incubated in either 1% milk PBST or 1% milk

TBST dependent on the first antibody buffer for a duration of 2 hours at room temperature. Blots were developed using SuperSignal West Pico chemiluminescent substrate (Thermo Scientific, 34080), and then exposed to x-ray film.

Immunofluorescence

INS-1E cells transfected with vectors designed to express ER α 66, ER α 46, ER α 36, or pIRES2-EGFP (empty vector, EV) were fixed in 3.7% formaldehyde for 30 minutes at room temperature. Nuclei were stained with 2.025 μ M Hoechst 33342 (Invitrogen, H3570). Cells were permeabilized with 0.2% Triton X-100 and incubated with 1:200 ER α (sc-543)³⁸ or ER α (sc-544) in 1% BSA/1X PBS for 2 hours at room temperature. Cells were then incubated in an anti-rabbit TRITC 1:200 secondary antibody for 1 hour (Jackson ImmunoResearch, 711-025-152) in 2% BSA/1X PBS. Fluorescence was viewed with a BD Pathway 855 BioImager.

Luciferase Assay

For analysis of ER α dependent transcriptional activity, INS-1E cells were transfected with vectors designed to express ER α 66, ER α 46, ER α 36, or pIRES2-EGFP (empty vector, EV) as well as a triple repeat estrogen response element (ERE) firefly luciferase reporter¹⁴⁵, and a transfection control, pRL CMV renilla luciferase (rLuc) (Promega, E2261) in a 384 well plate as indicated above. Seventeen hours post-transfection, cells were supplemented with 6% FBS RPMI. Twenty-four hours prior to the luciferase assay cells were washed once with 0% FBS RPMI, and treated with 10 nM estrogenic compounds in 0% FBS RPMI. After 24 hours of treatment, cells were lysed using 10 μ l of passive lysis buffer (Promega) and incubated at room temperature for 15 minutes. Following lysis, a Promega Dual Luciferase Reporter Assay System was used

(Promega, E1910). Briefly, 25 μ l of luciferase assay reagent II was added to 10 μ l of lysates and ERE-reporter luminescence was observed. Next, 25 μ l of Stop and Glo reagent was added to determine renilla luminescence. Luminescence was detected in a BMG omega plate reader with a gain of 4000. ERE driven firefly luciferase activity was normalized to renilla luciferase activity to account for transfection efficiency and further normalized to the ER α 66 DMSO control.

Caspase assay

For analysis of caspase 3/7 activity, INS-1E cells were transfected with vectors designed to express ER α 66, ER α 46, ER α 36 or pIRES2-EGFP (empty vector, EV) in a 6 well format as described above. Cells were refed 17 hours post-transfection with 6% FBS RPMI supplemented with 10 nM (E2, MPP, MPP + E2) or DMSO. Approximately forty hours after transfection, cells were selected by 0.4 mg/ml G418 suspended in 6% FBS RPMI supplemented with 10 nM estrogenic compounds or DMSO for 48 hours. Selected cells were split to a 384 well plate and seeded at a density of 6,675 cells/ well. The following day, 1.62 μ M Hoechst 33342 (Invitrogen, H3570) was added to the wells for 30 minutes, and cells were imaged for total cell number using the BD Pathway 855 BioImager. Following imaging analysis, 42 μ l of Caspase 3/7 Glo Reagent (Promega, G8091) was added to the wells, and incubated for 30 minutes at room temperature in the dark. Luminescence was observed on a BMG omega plate reader with a gain of 3600. Data were represented as caspase 3/7 activity relative to Hoechst estimated cell count and further normalized to the ER α 66 DMSO control.

RNA Extraction and qPCR

INS-1E cells were transfected with vectors designed to express ER α 66, ER α 46, ER α 36, or pIRES2-EGFP (empty vector, EV) as described above in a 6-well format. Seventeen hours post-transfection cells were refed with 6% FBS RPMI. After 40 hours of vector expression, cells were treated with 0.4 mg/ml G418 suspended in 6% FBS RPMI for 48 hours to select for cells expressing the transfected plasmids. Following selection, cells were washed once with 0% FBS RPMI, and treated with either 10 nM E2 or DMSO in 0% FBS RPMI for 24 hours. Following compound treatment, total RNA was harvested using an RNeasy kit (Qiagen, 74104). RNA was converted to cDNA using TaqMan reverse transcription reagents containing an Oligo_{dt} primer (Applied Biosystems, N808-0234). Genes were amplified according to the primers in Table 2 using Power Sybr Green (Applied Biosystems, 4367659) under the following cycling conditions: 1 cycle of 95°C-10 minutes; 40 cycles of 95°C-30 seconds, 60°C-1 minute. Amplified products were analyzed for size using dissociation curve analysis. Fold change was calculated compared to the EV DMSO control using an autoscaling technique¹⁸³.

Table 2 qPCR Primers

Gene	Primer 5'->3'
BCL2 Forward	GCGTCAACAGGGAGATGTCA
BCL2 Reverse	GTTCCACAAAGGCATCCCAG
INS1 Forward	CCCTAAGTGACCAGCTACAATCA
INS1 Reverse	CAGGTGAGGACCACAAAGGTG
ATF4 Forward	CCAAGCACTTCAAACCTCATG
ATF4 Reverse	GTCCATTTTCTCCAACATCCAATC
DDIT3 Forward	CTCATCCCCAGGAAACGAAG
DDIT3 Reverse	GAACTCTGACTGGAATCTGGAG
Gadd45 β Forward	ATCCAATCGTTCTGCTGCGA
Gadd45 β Reverse	GACAGTTCGTGACCAGGAGG
FOS Forward	GATACGCTCCAAGCGGAGAC
FOS Reverse	TCGGTGGGCTGCCAAAATAA
ACLY Forward	TTACGAGTGATGGGAGAAGTTG
ACLY Reverse	AGGAAGTTGGCAGTGTGAG
PPIA Forward	ATCTGCACTGCCAAGACTGA
PPIA Reverse	CATGCCTTCTTTACCTTCC

Insulin Content Assay

To analyze insulin content, INS-1E cells were transfected with vectors designed to express ER α 66 or pIRES2-EGFP (empty vector, EV) in a 6 well format as described above. Starting at refeed, cells were selected with 0.4 mg/ml G418 suspended in 6% FBS

RPMI for 48 hours. Following selection, cells were harvested using water or 1% NP40/1X TBS and a protease inhibitor cocktail (Roche, 040693124001) cell lysis buffer. Total protein content was measured using a Bradford assay. Insulin protein was measured using a mouse/rat insulin ELISA kit (Millipore, EZRMI-13K). Insulin content was calculated by dividing insulin protein by total protein.

Statistics

Data were analyzed using a t-test. Significance is noted in the text, figure legends, and on graphs.

Chapter 4 Coregulators Mediate the Activity of Estrogen Receptors

ABSTRACT

Estrogen receptors do not function alone, but require the recruitment of coregulators and other transcriptional machinery. In the present study, coregulator interaction with estrogen receptors was evaluated in the pancreatic β -cell line, INS-1E. NCoA5, a nuclear coactivator of estrogen signaling, was examined because of its known association with T2DM. Transcriptional analysis revealed that ER α 66 activity at ERE promoters was significantly enhanced by NCoA5 cotransfection. In order to determine if NCoA5 behaved differently in the presence of other ER α variants, the activity of ER α 46, the AF-1 deficient variant, was also examined. NCoA5 did not enhance ER α 46 transcriptional activity, regardless of estrogenic ligand treatment. These data suggest that the AF-1 domain of ER α is responsible for NCoA5 interaction with the receptor. Isoform specific NCoA5 potentiation was also assessed by examining the interaction of NCoA5 and ER β . NCoA5 had no effect on ER β transcriptional activity in any estrogenic ligand treatment group. These data demonstrate the specificity of NCoA5 to activate the estrogen receptor isoform, ER α 66, in the INS-1E cell line. However, when NCoA5 was expressed alone, caspase 3/7 activity was significantly upregulated independent of NCoA5 interaction with ER α . This suggests that NCoA5 may interact with other nuclear receptors to negatively impact β -cell health. Overall, this study highlights the importance of nuclear coregulators in the function of estrogen receptors and opens a new avenue of possibilities in modulating estrogen receptor activity in pancreatic β -cells.

INTRODUCTION

Type 2 diabetes mellitus (T2DM) is a complex disease that involves multiple signaling pathways and tissues¹⁸⁴. One pathway that has been identified to contribute to diabetes development and progression is the estrogen receptor signaling pathway. Estrogen receptor signaling has been linked to T2DM in peripheral tissues (skeletal muscle, liver, brain, etc.), and has been shown to directly affect pancreatic β -cells. For example, in post-menopausal women, where the concentration of estrogen is dramatically reduced, there is an increased incidence of T2DM due to insulin resistance in peripheral tissue^{16,115,116}. In pancreatic β -cells, estrogen administration has been shown to have protective effects against streptozotocin and H₂O₂ induced stress^{73,74,78,117,136}. However, there are also studies demonstrating that long term exposure to environmental estrogens can lead to reduced β -cell function, T2DM, and insulin resistance^{95,97,101,130}. These conflicting findings led to the hypothesis that the presence of other factors may regulate estrogen receptor's effect on diabetes progression. Understandably, estrogen receptor signaling does not occur in isolation, but requires the presence of multiple cofactors, including nuclear coregulators.

Coregulators function to mediate the transcriptional activities of nuclear hormone receptors, including estrogen receptors, after the binding of ligand⁵⁵. Coregulators work by binding to one of two activation function (AF) domains of ER α and ER β in a ligand dependent, AF-2, and ligand independent, AF-1, manner^{54,55}. The binding of estrogen receptor agonist recruits coactivators and antagonist binding recruits corepressors. Interestingly, there are certain estrogenic ligands known as selective estrogen receptor modulators (SERMs), which have tissue specific agonist or antagonist effects on estrogen

receptor signaling⁴¹. The tissue specific regulation of estrogen receptor activity is hypothesized to be due to the recruitment of coregulators, which leads to changes in estrogen receptor transcriptional activity^{41,56,185}. These findings highlight the importance of coregulators in maintaining proper estrogen receptor signaling, and demonstrate a way in which estrogen signaling can have a diverse tissue specific effect with one estrogenic ligand.

Interestingly, coregulators have also been implicated in the development of T2DM. Early genome wide association studies found that single nucleotide polymorphisms present in the location of the NCoA5 gene were linked with a higher incidence of T2DM^{107,186}. Functional evidence has shown that haploinsufficiency of NCoA5 reduced islet mass and increased blood glucose levels^{108,187}. This phenotype may be due to increased expression of IL-6, an inflammatory cytokine, induced by the disruption of an inhibitory ER α /NCoA5 interaction at its promoter^{108,187}. These data suggest that the presence of coregulators and ER α are important for maintaining glucose homeostasis. Further suggesting the importance of AF domains and coregulator recruitment is a study conducted in mice that showed an ER α AF-1 and AF-2 specific effect in the maintenance of blood glucose homeostasis in peripheral tissues¹⁰⁶. Data demonstrated that the AF-2 domain is necessary for the prevention of insulin resistance and for the transcription of genes involved in fatty acid metabolism. These domain specific differences may be influenced by the recruitment of certain coregulators necessary for gene transcription in these cell types, once again asserting the importance of coregulators in the maintenance of estrogen receptor signaling. Therefore, ER α 66, the

wild type receptor, ER α 46, the AF-1 deficient receptor, and ER β are likely to behave differently in the presence of coregulators.

This study addressed the contribution of NCoA5 to the transcriptional activity of ER α 66, ER α 46, or ER β in INS-1E pancreatic β -cells. Overall, we found that NCoA5 potentiates the transcriptional activity of the AF-1 and AF-2 containing estrogen receptor isoform, ER α 66, while not affecting the activity of ER α 46 or ER β . Additionally, the reactivity of SERMs on ER α 66 signaling was unaffected by the presence of NCoA5. NCoA5 expression alone increased caspase 3/7 activity, suggesting that NCoA5 may interact with other nuclear receptors in the INS-1E cell line to mediate an effect.

RESULTS

ERα66 Activity is Potentiated by NCoA5 Coexpression

ERα transcriptional activity is not only dependent on the expression of ERα, but also on the complement of coregulators present in the cell²⁵. NCoA5, a coregulator of

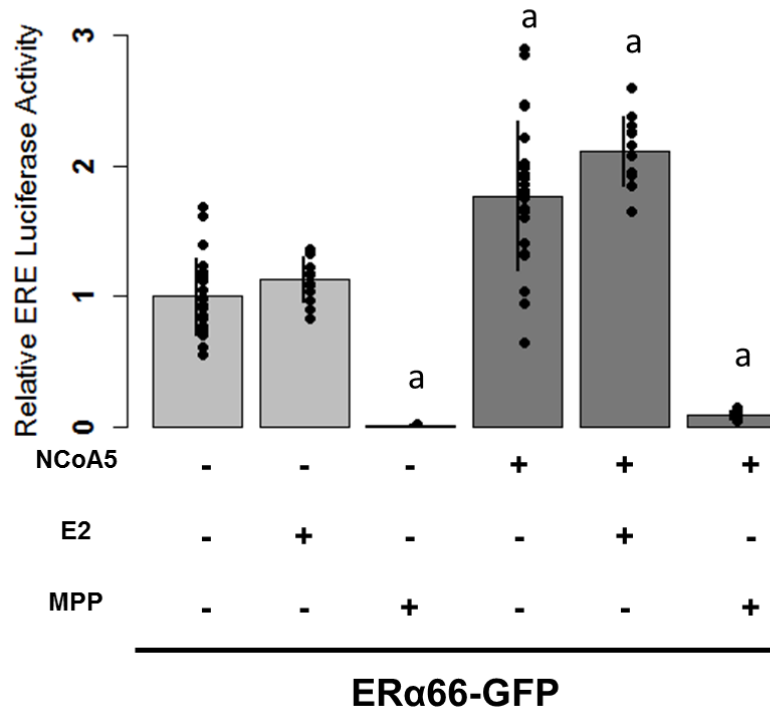


Figure 4.1 NCoA5 Potentiates ERα66 Transcriptional Activity.

INS-1E cells were co-transfected with ERα66-GFP and either a pIRES2-EGFP empty vector (EV) or NCoA5. Data are normalized to ERα66/EV DMSO control. Coexpression of ERα66-GFP and NCoA5 initiated a significant upregulation of transcriptional activity compared to the ERα/EV transfection alone. Treatment with 10 nM E2 had no significant effect on ERα/NCoA5 or ERα/EV transcriptional activity. 10 nM MPP treatment significantly reduced both ERα/NCoA5 and ERα/EV transcriptional activity. (a: Treatment vs. ERα66/EV DMSO, $p \leq 0.01$) ($n \geq 12$)

ERα, has been shown

to be a

transcriptional

activator of ERα66

in other systems¹⁸⁸.

Thus, we

hypothesized that

NCoA5 would

enhance ERα66

transcriptional

activity in β-cells. To

test this hypothesis,

ERα66-GFP and

either NCoA5 or an

EV were

cotransfected in INS-

1E cells, and

transcriptional

activity was assessed using a dual luciferase ERE-reporter assay. ERα66-GFP/NCoA5

coexpression significantly increased ER α transcriptional activity, 43%, over ER α 66-GFP/EV coexpression ($p \leq 0.01$) (Figure 4.1). Transcriptional activity was further potentiated 16% by treatment with E2 compared to DMSO in the ER α 66-GFP/NCoA5 coexpression group, however this did not reach significance ($p = 0.057$) (Figure 4.1). Treatment with the ER α antagonist, MPP, decreased ER α 66-GFP transcriptional activity in the EV and NCoA5 cotransfection conditions ($p \leq 0.01$) (Figure 4.1). The percentage of MPP inhibition was not significantly different between ER α 66-GFP and NCoA5 or EV cotransfection with 98% and 96% inhibition, respectively (Figure 4.1). This finding suggests that the coexpression of NCoA5 does not affect ER α responsiveness to antagonistic ligands. This hypothesis was further tested using a series of selective estrogen receptor modulators (SERMs) as discussed below.

NCoA5 Expression Doesn't Affect ER α Response to SERMs

In order to assess if other estrogenic ligands affect NCoA5 interaction with ER α , we assessed three SERMs including cyclofenil, raloxifene, and Y134. To test the impact of SERMs on ER α activity, ER α 66-GFP was cotransfected with either EV or NCoA5 and

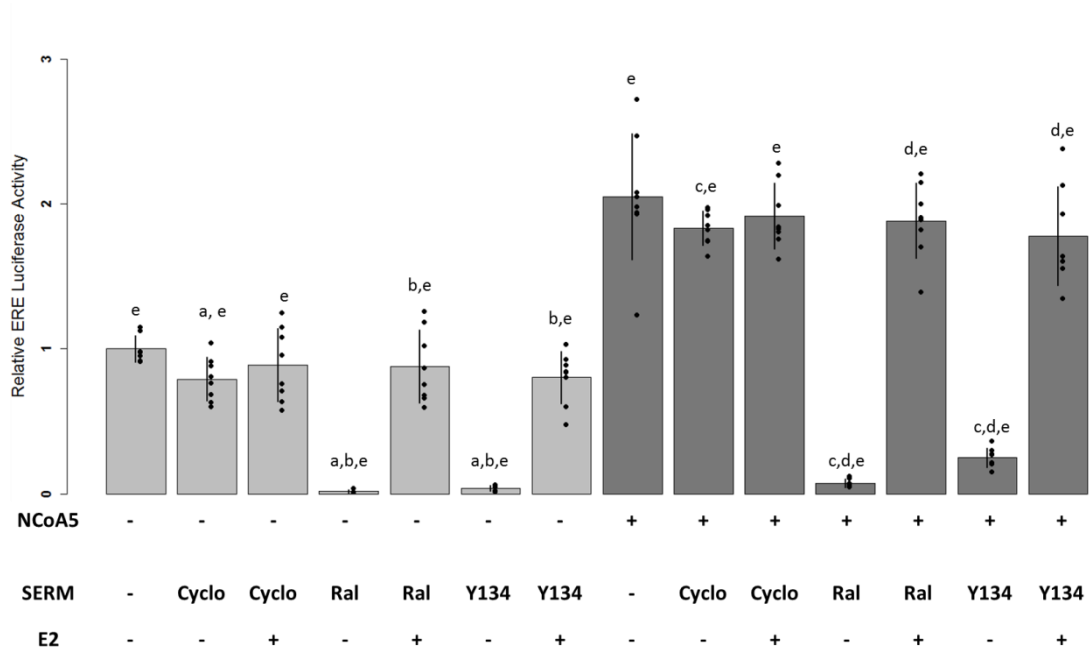


Figure 4.2 NCoA5 Doesn't Influence SERM Response.

INS-1E cells were co-transfected with ER α 66-GFP and either a pIRES2-EGFP empty vector (EV) or NCoA5. Transcriptional activity was assessed using a 3X-ERE Luciferase assay following 24 hour treatment with 10 nM cyclofenil, raloxifene, or Y134 alone or in combination with 10 nM E2. Cyclofenil had no significant effect on ER α 66-GFP activity. Both raloxifene and Y134 decreased ER α 66-GFP activity, in both EV and NCoA5 coexpression groups. (a: $p < 0.01$ ER α /EV DMSO vs. ER α /EV SERM; b: $p < 0.01$ ER α /EV SERM vs ER α /EV SERM E2; c: $p < 0.01$ ER α /NCoA5 DMSO vs. ER α /NCoA5 SERM; d: $p < 0.01$ ER α /NCoA5 SERM vs. ER α /NCoA5 SERM E2; e: $p < 0.01$ ER α /EV vs. ER α /NCoA5) ($n \geq 8$)

transcriptional activity was assessed after 24 hours of SERM treatment. Cyclofenil

inhibited ER α 66-GFP activity in both the EV and NCoA5 cotransfection groups

($p \leq 0.01$), however was more effective at inhibiting ER α 66-GFP/EV, 21%, versus ER α 66-

GFP/NCoA5, 11% (Figure 4.2). Raloxifene also inhibited ER α 66-GFP activity in both

the EV and NCoA5 cotransfection groups at a similar percentage of inhibition, 96-98% ($p \leq 0.01$) (Figure 4.2). Y134, a derivative compound of raloxifene, also inhibits ER α 66-GFP activity in the EV and NCoA5 cotransfection groups ($p \leq 0.01$), however is more effective at inhibiting ER α 66-GFP/EV activity, 96%, versus ER α 66-GFP/NCoA5, 88% (Figure 4.2). Treatment with E2 overcame the inhibitory effect of cyclofenil, raloxifene, and Y134 in both the ER α 66-GFP/EV and ER α 66-GFP/NCoA5 cotransfection groups (Figure 4.2). The ability of E2 to reverse the inhibitory effects of these compounds with the NCoA5 cotransfection demonstrates that NCoA5 does not affect the reversibility of SERM binding to ER α 66-GFP. The results suggest there is not a NCoA5 dependent effect on SERM response for any of the SERMs presented in this study.

NCoA5 Potentiation of ER α Transcriptional Activity Requires the AF-1 Domain

Since the possibility exists that the GFP fusion protein could affect inherent ER α and coregulator interaction, we investigated the interaction of NCoA5 with ER α 66 in a non-fusion vector as well. ER α 66/NCoA5 coexpression increased transcriptional activity 49% over ER α 66 expression alone in the DMSO treatment group ($p \leq 0.01$) (Figure 4.3 A). Treatment with E2 did not significantly increase transcriptional activity with either ER α 66/EV or ER α 66/NCoA5 coexpression (Figure 4.3 A). These data establish NCoA5 as a coactivator of ER α 66 activity in INS-1E cells and confirm the results obtained with the ER α 66-GFP fusion protein.

Coregulators function by binding to either the AF-1 or AF-2 domain of ER α to mediate its genomic activity. In order to determine if NCoA5 had domain dependent effects on ER α activity, we examined NCoA5 interaction with ER α 46, the AF-1 deficient variant of ER α . To evaluate the interaction between NCoA5 and ER α 46, they were coexpressed in INS-1E cells and transcriptional activity was assessed from the ERE-luciferase reporter. ER α 46/NCoA5 coexpression decreased transcriptional activity 13% over ER α 46/EV expression in the DMSO treatment group, although this was not significant ($p=0.30$) (Figure 4.3 B). Treatment with E2 induced a 94% increase in ER α 46 transcriptional activity in the ER α 46/EV coexpression group ($p\leq 0.01$) (Figure 4.3 B).

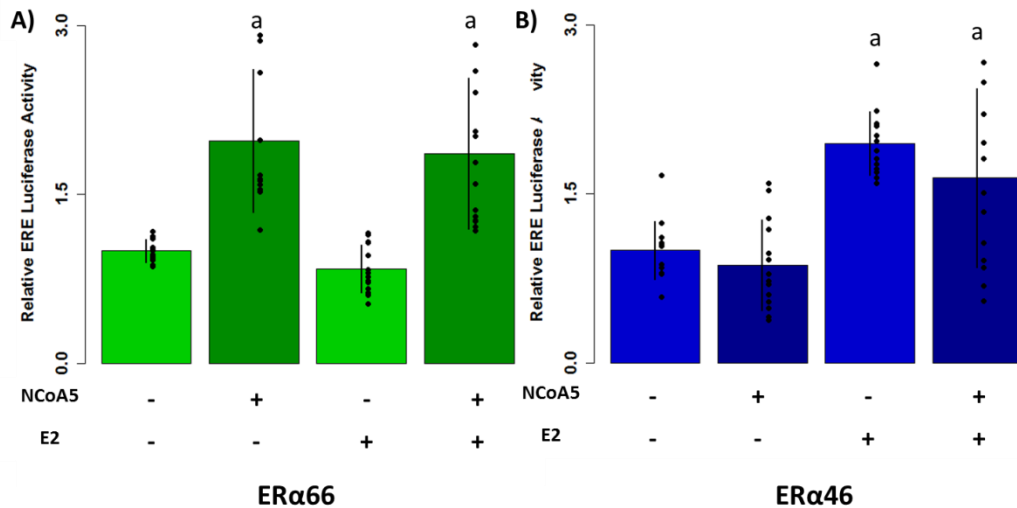


Figure 4.3 NCoA5 has Domain Dependent Effects on ER α Variants.

INS-1E cells were co-transfected with ER α 66 or ER α 46 and a pIRES2-EGFP empty vector (EV) or NCoA5. Transcriptional activity was assessed using a 3X-ERE luciferase reporter. Data were normalized to the solitary expression of each variant in the DMSO treatment group. ER α 66 coexpression with NCoA5 resulted in increased transcriptional activity, not enhanced by E2 treatment (A). Coexpression of ER α 46 and NCoA5 resulted in a downregulation of transcriptional activity, although not significant (B). Treatment with 10 nM E2 in cells coexpressing ER α 46 and NCoA5 resulted in an increase of transcriptional activity as observed with cells expression ER α 46/EV. (a: ER α Variant/NCoA5 or EV Treatment vs. ER α Variant/EV DMSO) $p \leq 0.01$ vs. DMSO ($n \geq 14$)

Treatment with E2 induced a more moderate increase, 89%, of transcriptional activity in the ER α 46/NcoA5 expressing cells, demonstrating that E2 may not be as effective in the ER α 46/NCoA5 coexpression group (Figure 4.3 B). Overall, NCoA5 had no significant effect on ER α 46 activity in any treatment group.

These data demonstrate that NCoA5 has domain dependent effects on ER α

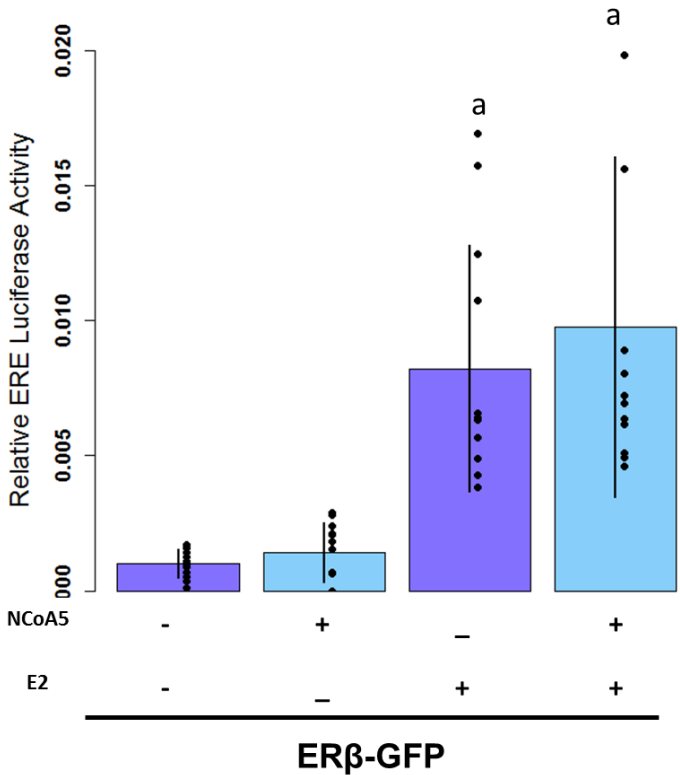


Figure 4.4 NCoA5 Does Not Enhance ER β Transcriptional Activity.

INS-1E cells were co-transfected with ER β -GFP and either a pIRES2-EGFP empty vector (EV) or NCoA5. Coexpression of ER β -GFP and NCoA5 induced no further transcriptional activation than the ER α /EV transfection alone. Treatment with 10 nM E2 increased ER β /NCoA5 transcriptional activity and ER β /EV transfection to equivalent amounts. (a: Treatment vs. ER β /EV DMSO, $p \leq 0.01$) ($n \geq 12$)

activity. The results from the luciferase assay reveal that the AF-1 domain is essential for NCoA5 mediated transcriptional potentiation, as ER α 46 activity was unaffected by NCoA5 cotransfection.

ER β Transcription is Unaffected by NcoA5 Coexpression

ER β , the other isoform of estrogen receptor, does not possess a functional AF-1 domain. Thus ER β may behave similarly to ER α 46, the AF-1 deficient ER α variant

(Figure 4.3). Indeed, ER β -GFP transcriptional activity was unaffected by coexpression with NCoA5 in the DMSO treatment group ($p=0.26$) (Figure 4.4). Treatment with E2 produced a significant increase in activity in both ER β /EV and ER β /NCoA5 coexpression groups ($p\leq 0.01$), with no appreciable difference between the two coexpression groups ($p=0.50$) (Figure 4.4). These findings substantiate the hypothesis that the NCoA5 coregulator activity requires a functional AF-1 domain.

NCoA5 Expression

Increases Caspase 3/7

Activity

RNASeq data showed that NCoA5 was expressed in the INS-1E cells in the absence of estrogen receptor expression. Therefore, it may have some effect unrelated to its action on estrogen receptor transcriptional activity. It has been shown that in addition to its role in estrogen receptor signaling, NCoA5 is involved in regulating the activity of orphan nuclear receptors, Rev-

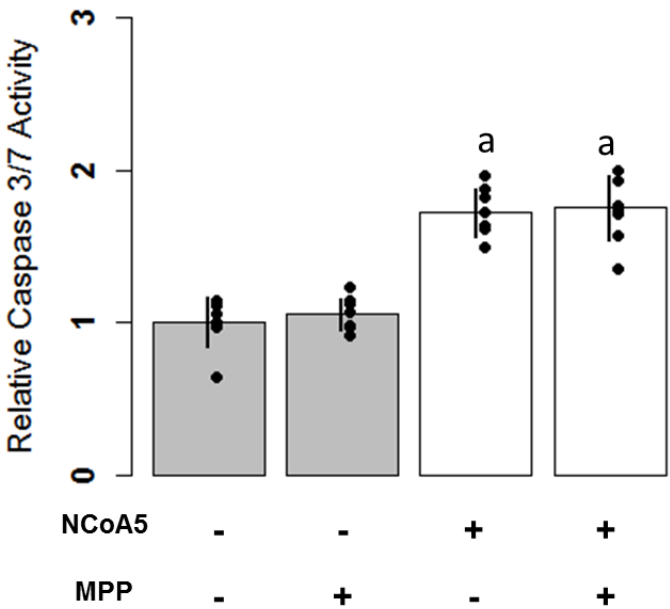


Figure 4.5 NCoA5 Increases Caspase 3/7 Activity Independently of Estrogen Receptors.

INS-1E cells were transfected with either NCoA5 or pIRES2-GFP empty vector (EV), and treated with DMSO or MPP for the duration of the experiment. Cells expressing the vector of interest were selected for 48 hours prior to plating on a 384 well plate. Cells were allowed to adhere for 24 hours prior to a caspase 3/7 assay. Data were normalized to the DMSO EV control. NCoA5 expression increased caspase 3/7 activity, and treatment with MPP has no effect. (a: Treatment vs. EV DMSO, $p\leq 0.01$) ($n\geq 8$)

erb α and Rev-erb β ^{188,189}. To assess the effect of NCoA5 on β -cell health, independent of its interaction with estrogen receptor, caspase 3/7 activity was assessed after NCoA5 expression in the ER α and ER β negative INS-1E cells. Caspase 3/7 activity was significantly increased after expression of NCoA5 ($p \leq 0.01$) (Figure 4.5). The activation of caspase 3/7 was unaffected by treatment with the ER α antagonist, MPP, verifying that this effect is independent of ER α activity (Figure 4.5). These data suggest that NCoA5 may interact with another endogenously expressed nuclear hormone receptor to modulate an effect on pancreatic β -cell health.

DISCUSSION

Estrogen signaling has been shown to regulate many aspects of human health including breast cancer development, reproductive health, bone maintenance, and more recently diabetes. Estrogenic ligands work through estrogen receptors to relay signals to the nucleus thereby inducing the transcription of target genes⁴². This signaling pathway requires the coordination of the multiple splice variants of both isoforms, ER α and ER β , recruitment of coregulators, and the type of estrogenic ligand that is bound. This study aimed to characterize the interaction between these three factors in a pancreatic β -cell line, INS-1E, as a model for understanding their interaction in human islets.

There are multiple coregulators of estrogen receptor activity, however NCoA5 was chosen for analysis in this study because of its association with T2DM in genome wide association studies and in functional analysis of glucose homeostasis^{107,108,186,187}. NCoA5 is a known coregulator for ER α and was first identified to interact with ER α in a non-AF-2 dependent manner¹⁸⁸. Therefore, we first examined NCoA5 and wild-type ER α , ER α 66-GFP, interaction in the INS-1E cells by assessing the transcriptional activation of an ERE-luciferase reporter. NCoA5 coexpression with ER α 66-GFP resulted in a significant potentiation of ER α 66 activity. This is meaningful because in previous analysis (Figure 2.6) ER α 66-GFP transcriptional activity is unaffected by treatment with estrogen receptor agonists. Thus, it appears that NCoA5 binds to ER α 66-GFP to enhance transcriptional activity showing the first inducible ER α 66 response on the ERE reporter in INS-1E cells. These results are substantiated by previous studies showing that NCoA5 can enhance ER α activity at ERE promoter elements¹⁸⁸. However, as observed in Figure 2.6, there is no significant increase in activity when ER α 66-GFP/EV or ER α 66-

GFP/NCoA5 are treated with 17 β -estradiol, suggesting that NCoA5 binding to ER α 66-GFP is not dependent on the presence of 17 β -estradiol. Previous studies have found that NCoA5 can associate with ER α in the absence of E2, substantiating our finding that ER α 66 and NCoA5 interact in the unliganded ER α conformation¹⁸⁸. Therefore, the ability of SERMs to disrupt NCoA5 and unliganded ER α 66-GFP interaction was assessed.

Previous studies have shown that ER α coregulators are imperative in mediating the tissue selectivity of SERMs^{41,52,54,56,185}. For example, the SERM, tamoxifen, can induce the recruitment of NCoR in the breast to antagonize ER α activity, but recruits SRC1 in the bone to enhance ER α activity^{185,190}. All SERMs examined in the present study inhibited transcriptional activity of ER α 66-GFP and NCoA5 or EV cotransfection, with raloxifene and Y134 being the most potent SERMs examined. This suggests that NCoA5 does not alter the responsiveness to the SERMs examined in the INS-1E cell line. However, there still exists the potential for differential regulation of ER α 66 activity when examining a larger panel of SERMs as shown in other studies^{55,188}. This may be important if SERMs become used for the mediation of estrogen receptor activity in islet isolation procedures.

To determine if NCoA5 exhibited domain dependent differences in the activation of estrogen receptor, NCoA5 was coexpressed with either ER α 46 or ER α 66. While ER α 66 activity was assessed in the previous experiments, we wanted to verify that the N-terminal GFP fusion on ER α 66 did not interfere with NCoA5 potentiation, since the AF-1 domain is also located nearer to the N-terminus. NCoA5 potentiated ER α 66 activity as observed in previous studies, and was still unresponsive to E2 treatment. NCoA5 potentiation of ER α 46, the AF-1 deficient variant, was also assessed. NCoA5 had no

effect on ER α 46 transcriptional activity in either the DMSO or the E2 treatment group. These data suggest that the AF-1 domain of ER α is required for NCoA5's potentiation of transcriptional activity. It is not surprising that there may be a domain specific effect of NCoA5 signaling. A study in mice found that the AF-2 domain of ER α is indispensable for maintaining glucose homeostasis and proper transcription of genes involved in fatty acid metabolism¹⁰⁶. This response is most likely due to the recruitment of coregulators to the AF-2 domain thus mediating ER α transcriptional responses.

To test NCoA5 interaction with estrogen receptors even further, we evaluated NCoA5's ability to potentiate ER β transcriptional activity in the INS-1E cells. Previous studies have shown that NCoA5 can interact with ER β as well as ER α ¹⁸⁸. However, ER β does not contain a fully functional AF-1 domain⁴⁸. In the INS-1E cells, NCoA5 did not potentiate ER β transcriptional activity at ERE promoters in the DMSO or in the E2 treatment groups. These data further suggest that there may be a level of dependence on a functional AF-1 domain to facilitate NCoA5 potentiation of estrogen receptor activity.

In addition to testing NCoA5 interaction with estrogen receptors, NCoA5 expression alone was tested in regards to its effect on insulin secretion and caspase 3/7 activity. In INS-1E cells, NCoA5 is expressed even though endogenous estrogen receptors are not, therefore it may have a physiological role outside of ER α interaction. Indeed, NCoA5 is known to interact with orphan nuclear receptors, Rev-erb α and Rev-erb β , which are expressed in INS-1E cells¹⁸⁹. Interestingly, when NCoA5 is expressed alone caspase 3/7 activity is induced. Treatment with MPP had no ability to reduce the NCoA5 mediated increases in caspase 3/7 activity, verifying that the effect is independent of ER α . Therefore, it appears that NCoA5 interacts with some other partner

in INS-1E cells to mediate deleterious effects when overexpressed. Future studies should examine the role of NCoA5 in relation to other transcription factors in the INS-1E cells to determine its binding partners.

Overall, this study demonstrates the importance of splice variants, isoforms, coregulators, and ligand treatment in mediating the effects of estrogen receptor signaling. We have shown that NCoA5 potentiates ER α 66 activity, but has no effect on the AF-1 deficient ER α variant, ER α 46, or the ER β isoform, which has a nonfunctional AF-1 domain. This evidence verifies the necessity to examine all factors of estrogen receptor signaling before formulating a paradigm of estrogen signaling in pancreatic β -cells. Knowing the complement of coregulators modulating estrogen receptor signaling in human β -cells will be critical for the evaluation of compounds to moderate pancreatic β -cell health and function. The AF domain specific activity of NCoA5 observed in the present study is a good example of the complex regulatory network that exists for estrogen receptor signaling. If modulation of estrogen receptor activity in β -cells becomes a therapeutic method for diabetes prevention or is used in islet isolation measures the system used in this study for testing the interaction of splice variants, coregulators, and estrogenic ligands will be advantageous. Overall, the knowledge gained from this study and model system could aid in finding new compounds that work with a β -cell specific coregulator and estrogen receptor expression profile to treat pancreatic β -cell failure in the future.

MATERIALS AND METHODS

INS-1E Cell Culture and Transfection

Rat Insulinoma (INS-1E) cells were culture in RPMI 1640 (Gibco 11875-093) supplemented with 6% FBS at 37°C in 5% CO₂. For experimentation, cells were plated in 6 or 384 well formats as described below. Cells were harvested by trypsinization (0.5% trypsin, 0.05% EDTA) and collected with 10 ml 6% FBS RPMI. Cells were spun at 1200 rpm for 5 minutes, then resuspended in Optimem (Gibco 31985-070). Cells were plated at a density of 225,000 cells for 6 well plates and 8,125 cells for 384 well plates. The cells were transfected with pEGFP-C1-ER α (addgene 28230), pEGFP-C1-ER β (addgene 28237), ER α 66-pIRES2, ER α 46-pIRES2, NCoA5 (Open Biosystems), or empty vector, pIRES2-EGFP (Clontech 6029-1) using the cationic lipid DMRIE-C (Invitrogen 10459014) with a $\mu\text{g}:\mu\text{l}$ DNA:DMRIE ratio of 0.82 (0.153:0.187) (384 well plate) and 0.40 (2.25:5.61) (6 well plate).

Compound Treatment

Estrogen modulating compounds included: 1,3,5 (10) Estratrien-3, 17 B-diol (Steraloids, E0950-000), MPP dihydrochloride (Tocris, 1991), Cyclofenil (Tocris, 3999), raloxifene hydrochloride (Tocris, 2280), or Y134 (Tocris, 2676). Stock compounds were prepared in DMSO (Sigma, 276855), and DMSO concentration did not exceed 0.3% in all described experiments.

Luciferase Assay

To assess ERE dependent transcriptional activity, INS-1E cells were transfected as described above with additional transfection of a triplicate repeat ERE firefly luciferase reporter¹⁴⁵, and a transfection control, CMV-renilla luciferase (rLuc) (Promega,

E2261) in a 384 well format. Cells were incubated for approximately 40 hours, then were treated with estrogenic compounds 24 hour prior to the start of the luciferase assay. After 24 hours, cells were lysed using 10 μ l passive lysis buffer (Promega) and incubated at room temperature for 15 minutes. Following lysis, a Promega Dual Luciferase Reporter Assay System (Promega, E1910) was used. Briefly, 25 μ l of luciferase assay reagent II was added to the 10 μ l of lysates and ERE-dependent luminescence was observed. Next, 25 μ l of Stop and Glo reagent was added to determine renilla luminescence.

Luminescence was detected in a BMG omega plate reader with a gain of 4000. ERE driven firefly luciferase activity was normalized to renilla luciferase activity to account for transfection efficiency.

Caspase 3/7 Assay

INS-1E cells were transfected with vectors designed to express either NCoA5 or pIRES2-EGFP as described above and treated started at refeed with 10 nM estrogenic ligands. Forty to Forty-two hours post-transfection, cells were selected for 48 hours in 0.4 mg/ml geneticin 6% FBS RPMI. After selection, cells were harvested and seeded at a density of 6,675 cells per well in a 384 well format. The following day, 1.62 μ M Hoechst 33342 (Invitrogen, H3570) was added to the wells for 30 minutes and cells were imaged for total cell number. Following imaging analysis, 42 μ l of Caspase 3/7 Glo Reagent (Promega, G8091) was added to the wells and incubated for 30 minutes at room temperature in the dark. The luminescence was observed by a BMG omega plate reader with a gain of 3600. Caspase 3/7 activity was normalized by total cell number (Hoechst positive cells).

Statistics

Data were analyzed using a t-test. Significance is noted within the text and figures as necessary.

Chapter 5 Conclusions

The major findings of the study are three-fold. (1) ER α and ER β nuclear signaling activities increase apoptosis in INS-1E cells. (2) ER α variants, ER α 66 and ER α 46, upregulate β -cell death and reduce β -cell function through transcriptional activation, whereas the ER α 36 variant has no effect. (3) NCoA5 exhibits isoform and splice variant specific transcriptional activity changes enhancing ER α 66 activity with no effect on ER α 46 or ER β . These three findings represent a major step forward in understanding the intricacies of estrogen receptor signaling in pancreatic β -cells.

This study revealed the detrimental effects of constitutive nuclear estrogen receptor signaling in the INS-1E pancreatic β -cell line. The potentiation of ER α 66, ER α 46, and ER β signaling in INS-1E cells induced apoptotic cell death, which would lead to a diabetic phenotype in humans. Previous studies have shown positive effects of non-genomic estrogen receptor activity on β -cell health in rodent and cell-based models^{22,74,80,84,87,133,136}. On the other hand, the literature also demonstrates that long term exposure to environmentally found estrogen mimics, xenoestrogens, leads to β -cell dysfunction and apoptosis^{97,98,101,130}. The factors modulating these diverse responses are poorly understood and can include divergent signaling pathways (nuclear or cytoplasmic) and splice variant or coregulator expression as addressed in the present study. Interestingly, we observe no effect on cell health when the traditionally non-genomic ER α splice variant, ER α 36, is expressed, further indicating that genomic estrogen receptor signaling is detrimental to β -cell health. Future studies will need to assess the activity of these each estrogen receptor isoform and splice variant in diabetic patients to

determine the expression level of the receptor, activity of the receptor (including its main signaling pathway, nuclear or cytosolic), and functional readouts of receptor activity.

If estrogen signaling is an active pathway in diabetic patients, then the option for treatment of individuals with FDA approved SERMs becomes a distinct possibility.

While antagonistic activity in the β -cell could be beneficial for health, it would be important to retain estrogen receptor signaling agonistic activity in the peripheral tissues (bone, skeletal muscle, liver, adipose). Thus, multiple SERMs would need to be tested for the specificity to activate peripheral estrogen receptor signaling while antagonizing pancreatic nuclear estrogen receptor signaling.

In addition to the potential modulation of estrogen receptor activity in human patients, there exists the possibility to alter estrogen receptor signaling during islet isolation procedures. Islet allo-transplantation is a method of diabetes treatment in which islets are isolated from donors, then transplanted into the diabetic patient using minimally invasive techniques^{140,178}. However, this method is plagued by many problems including the need for large quantities of donor islets, increased apoptosis after transplant, and reduced long-term functionality¹⁹¹. Additionally, there is a method known as islet auto-transplantation in which the pancreas is removed (mostly due to severe pancreatitis), and then islets are isolated and transplanted back into the same patient¹⁹². While islet allo-transplantation is still considered an experimental procedure, islet auto-transplantation is not, thus the need for increased survival of islets post-transplantation is a present need. Since there was a reduction in oxidative stress-induced apoptosis observed in animal models following estrogenic ligand treatment in other studies and increased insulin expression following raloxifene treatment in the present study, the beneficial effect of

estrogen could translate into reduced β -cell apoptosis and stress during islet transplant procedures. However, long-term culture of islets prior to transplant would not be feasible in human models of transplantation because islets begin to undergo dedifferentiation when cultured for prolonged periods¹²⁷. Thus, novel strategies that alleviate the problems with these procedures (apoptosis, reduced function, etc.) could enhance the effectiveness and increase the use of allo-transplant in diabetic patients as well as increasing the effectiveness of auto-transplantation. The findings from the present study showed that treatment of isolated human islets with the SERM, raloxifene, enhanced insulin RNA expression, regulated the expression of genes that could be involved in mediating β -cell fate, and decreased the inflammatory response. Future research should evaluate the effect of SERM treatment on isolated islets to determine if estrogen receptor signaling modulation has lasting consequences on the health and function of islets post-transplantation.

While the modulation of estrogen receptor signaling seems like a promising avenue of diabetic therapy research, the data gained from the present study using the INS-1E cell model highlights the complexity of the signaling pathways involved (Figure 5.1). A key finding of this study is that the variants of ER α , ER α 66, ER α 46, and ER α 36, localize to the nucleus in INS-1E cells. The lack of ER α expression in the cytoplasm suggests that the receptors in this particular cell line are not participating in the non-genomic signaling pathway. Analysis of a panel of estrogen responsive genes involved in cell fate pathways led to the identification of six genes that were significantly altered by ER α 66 or ER α 46 expression in the INS-1E cells (Figure 5.1). These genes included those involved in endoplasmic reticulum stress modulation (ATF4 and DDIT3) and apoptosis

regulation (FOS, Gadd45 β , BCL2, ACLY). In addition to these genes, insulin gene expression was significantly downregulated after ER α 66 and ER α 46 expression. This suggests that long-term activation of these receptors could produce a significant deterioration of insulin secretion from pancreatic β -cells. The present study also set out to determine if there were any significant differences in the signaling pathways and activity of each of the isoforms and variants of ER α and ER β .

Indeed, there were dramatic differences observed in the transcriptional activities of the two estrogen receptor isoforms, ER α and ER β . In the INS-1E cells, ER α was constitutively active on the ERE reporter system in the absence of ligand, whereas ER β

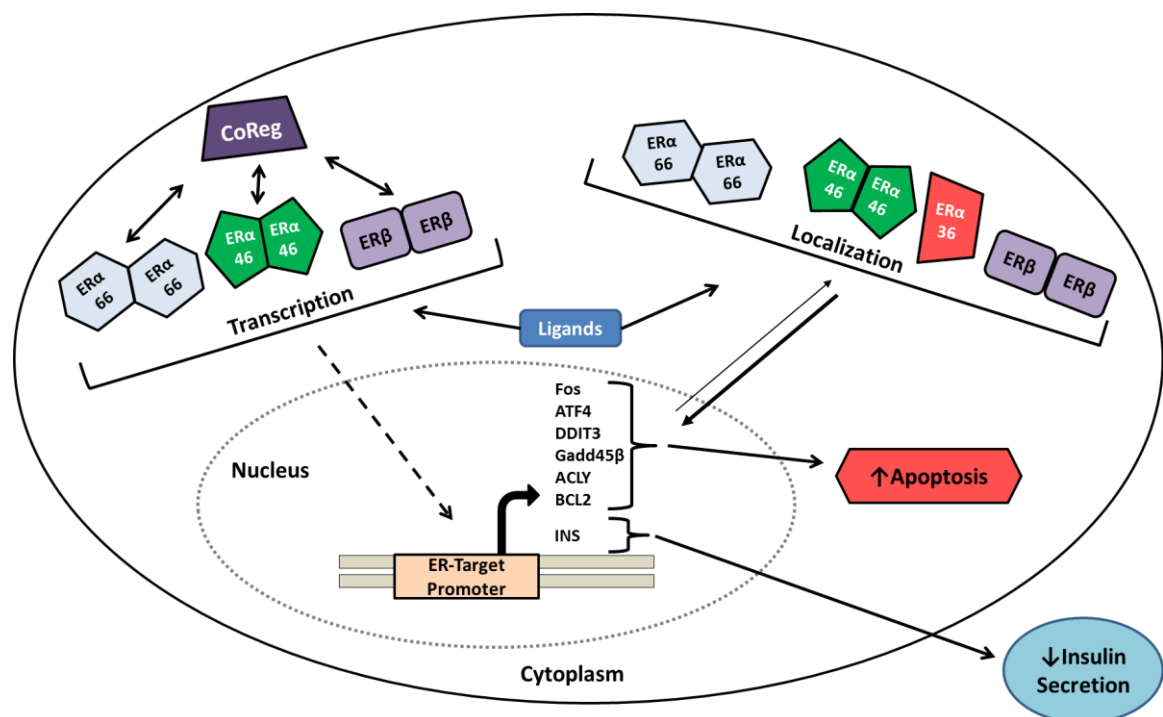


Figure 5.1 Proposed Estrogen Receptor Signaling Diagram.

Model of estrogen receptor signaling in the pancreatic β -cell. This study examined estrogen receptor localization, which was predominantly nuclear for all receptors examined, and could be modulated with ligand treatment. Additionally, estrogen receptor transcriptional activity was examined using an ERE-reporter and on downstream estrogen receptor target genes. Estrogen receptor signaling in the β -cells increased apoptosis and decreased insulin secretion through modulation of genes by transcriptional activity.

transcriptional activity was only observed following treatment with estrogen agonists. These data suggest an isoform specific response to transcriptional activation. In addition to the isoform specific responses, variation in the activity of ER α splice variants was also observed. ER α 66 and ER α 46 were both active in the no treatment groups, however the activity of ER α 46 was blunted compared to ER α 66. There was also a distinct difference in the action of bound ligand between these two receptors. Interestingly, this study revealed that ER α 46 is more responsive to ligand treatment than ER α 66 in this cell type. This was observed through a more vigorous inhibitory and activating response in response to agonist, antagonist, and SERM binding. Furthermore, there was no transcriptional response from ER α 36 in any of the experimental conditions, thus there was also no effect on apoptosis. The difference in the responsiveness of the isoforms and variants to ligand treatment and subsequent transcriptional activity changes could be due to many contributing factors including post-translational modifications, coregulator interaction, synergy among the AF-1 and AF-2 domains, among others^{40,193–195}. To address the interaction of coregulators, we also assessed whether coregulators influenced the transcriptional activity of estrogen receptors in the INS-1E cells (Figure 5.1).

NCoA5 was the coregulator chosen for this study since it has been linked to T2DM in both genomic and functional studies^{107,108,187}. NCoA5 is a known coregulator of ER α and can have both corepressor and coactivator activity dependent on the cell type¹⁸⁸. In the INS-1E cells, the coactivating potential of NCoA5 and ER α 66 interaction was confirmed. Interestingly, ER α 46 and ER β activity were not affected by NCoA5 coexpression. This finding leads to the belief that a functional AF-1 domain is required for NCoA5 potentiation of estrogen receptor transcriptional activity, since ER α 46 is

devoid of the domain and ER β shares only 20% similarity with ER α 66 in this domain⁴⁰.

The results of the NCoA5 and estrogen receptor interaction studies alludes to the importance of examining coregulator and estrogen receptor expression in determining the role of estrogen receptor signaling in the pancreatic β -cells (Figure 5.1).

Not only are all of these factors important in diabetes development and β -cell health, but there are multiple implications for these signaling pathways in other diseases and cell types. Divergent findings regarding estrogen receptor action has been observed in the study of both cardiovascular disease and breast cancer, similar to the findings in pancreatic β -cells¹⁹⁶. In cardiovascular disease, studies have shown both protective and null effects of estrogen signaling in the development of atherosclerosis, hypertrophy, and ischemia. It was proposed that estrogen signaling might only exert its protective effects in models of severe stress, such as during ischemia, much like the response in β -cells^{196,197}. The possibility also exists that the expression of different estrogen receptor isoforms in certain heart cell types may regulate the effect of estrogen signaling along with the interplay of coregulators, as observed in the present study. This same paradigm exists in breast cancer signaling¹⁹⁸. It is well established that anti-estrogen therapy in estrogen receptor positive breast cancers will inhibit proliferation and increase apoptosis, thus alleviating the cancer phenotype. However, there is a switch that can occur in these cells to adapt them to the anti-estrogen therapy and instead use it as an estrogen receptor agonist. This occurs through changes in coregulator usage. An example is tamoxifen, an anti-estrogenic SERM in breast tissue. When estrogen receptor positive breast cancer patients are given tamoxifen it inhibits cell proliferation, through the inhibition of estrogen receptor activity by binding to the corepressor, NCoR. However, some patients

develop resistance to this SERM treatment through coregulator switching¹⁹⁹. When this occurs tamoxifen switches and recruits coactivators, like SRC1, to potentiate estrogen receptor activity and worsen the prognosis for breast cancer patients¹⁹⁹. In addition to the role of coregulators in mediating estrogenic ligand response, the localization of the receptor is also very important. A 2014 study by Cook and Clarke showed that anti-estrogen resistance could also be dependent on localization of the receptor²⁰⁰.

Interestingly, anti-estrogen sensitive breast cancer cells respond to antagonist treatment by shifting the localization of ER α from the nucleus into the cytoplasm; however, anti-estrogen resistant breast cancer cells do not shift localization of the receptor and were thus unresponsive to antagonist treatment²⁰⁰. This study highlights the importance for examining receptor localization in the analysis of estrogen receptor signaling, as was performed in the present study.

Our results suggest that ER α is nuclear localized in INS-1E cells even in the absence of agonist ligand, and unresponsive to antagonist treatment. Previous studies of estrogen receptor signaling showed that extranuclear ER α and ER β signaling was beneficial in conditions of induced β -cell stress. Thus, the nuclear localization and lack of movement between the nucleus and cytoplasm may explain the negative impact of estrogen signaling on the health and function of β -cells. Therefore, the use of imaging techniques, coexpression experiments, transcriptional readouts, and quantitative gene assessment in this study may provide a model for studying estrogen receptor function in these other systems to gain a better understanding of the complexity of each estrogen receptor interacting component.

Overall Summary

This study provides evidence that estrogen receptor signaling is important in the regulation of pancreatic β -cell health and function. We provide the first results examining the independent contribution of each ER α splice variant and ER β isoform, and conclude that the transcriptional activity of ER α 66, ER α 46, and ER β is detrimental to β -cell health. Additionally, the results show that there are specific ligand bound activity differences between ER α splice variants and ER β . The importance of coregulator recruitment for transcriptional activity was also established using the coregulator, NCoA5, which demonstrated both isoform and splice variant specific activation differences. Overall, we provide a novel model to study the contributions of estrogen receptor signaling in an estrogen receptor negative β -cell line, and provide evidence that nuclear estrogen receptor signaling can be detrimental to both pancreatic β -cell health and function.

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200. Estrogen receptor- α signaling and localization regulates autophagy and unfolded protein response activation in ER⁺ breast cancer. *Recept. Clin. Investig.* (2014).
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CURRICULUM VITAE

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EDUCATION

Wake Forest University, Winston-Salem, NC

Department of Biochemistry and Molecular Biology

Ph.D. in Biochemistry and Molecular Biology, May 2015

Advisor: Peter Antinozzi, Ph.D.

Bowling Green State University, Bowling Green, OH

Department of Biology

M.S. in Biological Sciences, August 2011

Advisor: Lee Meserve, Ph.D.

Clemson University, Clemson, SC

B.S. *cum laude* in Genetics, May 2009

RESEARCH EXPERIENCE

Wake Forest School of Medicine, 2011-Present

Department of Biochemistry and Molecular Biology

Project: Doctoral research focuses on elucidating the effects of ER α variant signaling in pancreatic β -cells. Using high throughput imaging methods and the INS-1E β -cell model, I've found that ER α variants decrease β -cell survival. The initiation of cell death is dependent on the activation of caspase 3/7, and can be tightly controlled by pharmacological treatment. Additionally, analysis of differentially expressed genes by qPCR revealed increased expression of genes involved in the endoplasmic reticulum stress pathways in cells expressing ER α 66 or ER α 46. Expanding the knowledge gained by the cell model, we've shown through RNASeq analysis that treatment of isolated human islets with the selective estrogen receptor modulator, raloxifene, decreases expression of inflammatory factors and increases insulin expression, which could indicate improved islet health. Overall, this study provides evidence for ER α involvement in pancreatic β -cell health, and suggests that downregulation of ER α activity could improve β -cell survival.

Bowling Green State University, 2009-2011

Department of Biology

Project: Master's thesis research examined the effect of environmental toxins PCB 47 and PCB 77 on maternal care behavior and gene expression. Gestational exposure to

PCB 47/77 caused an increase in maternal nursing behaviors during the first 6 postnatal days. qPCR analysis of the oxytocin receptor in the hypothalamus of the dams showed a significant increase in expression on postnatal day 17. In addition to increased gene expression, preliminary bisulfite sequencing studies revealed a decrease in methylation of the promoter. Overall, this study provided evidence for a molecular link to changes in maternal care behavior.

Clemson University, 2008-2009

Project: Undergraduate research which examined a method for degrading bud scales from merino wool fibers using an environmentally friendly technique. Additionally, attaching a proprietary protein to the bud scale to reduce wool shrinkage was also explored. This research was performed in conjunction with an industry collaborator.

TECHNIQUES AND SKILLS

Molecular Biology and Biochemistry: expression vector design and construction, DNA sequencing analysis, RNA and DNA extraction, genomic PCR, qPCR, RNASeq, protein isolation, protein quantitation, western blotting, immunofluorescence, ELISA, luciferase assays, tissue culture, fluorescence microscopy

Animal Models: animal breeding, cross-fostering, animal dissection, behavior analysis

Data Analysis Software: R, Image J, Attovision Imaging Software, GraphPad, Access, Excel, Word, Powerpoint

ABSTRACTS AND PRESENTATIONS

American Diabetes Association

Dover E, Graham W, Antinozzi P. System Biology Informed by Islet Transcriptome Analyses Identifies a GSK3-beta Regulatory Node That Reciprocally Controls Estrogen Receptor and TCF7L2 Activities [abstract]. *Diabetes*. 2015. *Accepted*.

CMCS Fall Retreat, Advisor: Dr. Peter Antinozzi, October 2014

Poster Presentation: "ER α Variant Expression Upregulates Transcriptional and Caspase 3/7 Activity in INS-1E Cells"

Keystone Symposia, Advisor: Dr. Peter Antinozzi, April 2014

Poster Presentation: "ER α Variant Expression Affects Estrogenic Drug Responses in Pancreatic β -cells"

American Diabetes Association

Dover E, Graham W, Antinozzi P. Modulating Estrogen Receptor Activity and SERM Action in Pancreatic β -cells with Nuclear Coregulator Expression [abstract]. *Diabetes*. 2013; 62 (suppl 1).

American Diabetes Association

Antinozzi P, Graham W, **Dover E**. Lipid Signaling and Genotype Specific TCF7L2 Splice Variants Coordinate Transcriptional Activity of the Wnt Signaling Pathways in Pancreatic β -cells [abstract]. *Diabetes*. 2013; 62 (suppl 1).

Graduate Research Day, Advisor: Dr. Peter Antinozzi, March 2013

Poster Presentation: “Modulating Estrogen Receptor Activity and SERM Action in Pancreatic β -cells”

Keystone Symposia, P. Antinozzi, W. Graham, and **E. Dover**, January, 2013

Poster Presentation: “Lipid Signaling and Genotype Specific TCF7L2 Splice Variants Coordinate Transcriptional Activity of the Wnt Signaling Pathways in Pancreatic β -cells”

CMCS Fall Retreat, Advisor: Dr. Peter Antinozzi, October 2012

Oral Presentation: “Upregulation of Estrogen Signaling Modulates Pancreatic β -cell Survival Pathways”

JP Scott Brown Bag Lunch, Advisor: Dr. Lee Meserve, March 2011

Oral Presentation: “Effect of PCB 47/77 on Maternal Care Behavior and Associated Genes”

Annual Biology Department Retreat, Advisor: Dr. Lee Meserve, November 2010

Poster Presentation: “Effect of PCB 47/77 on Oxytocin Receptor and Arginine Vasopressin Receptor 1a and Associated Maternal Care Behaviors”

Undergraduate Research Day, Advisor: Dr. Albert Abbott, May 2009

Oral Presentation: “Enzymatic Digestion of Specific Amino Acids from the Surface of Wool Fibers”

Undergraduate Research Day, Advisor: Dr. Albert Abbott, December 2008

Poster Presentation: “Enzymatic Digestion of Specific Amino Acids from the Surface of Wool Fibers”

MANUSCRIPTS

Dover, E., Graham, W., and Antinozzi, P. ER α Variant Expression Induces INS-1E Cell Death in a Ligand Dependent Manner. *In Preparation*.

Dover, E., Mankin, D., Cromwell, H., Meserve, L., and Phuntumart, V. Polychlorinated biphenyl exposure alters oxytocin receptor gene expression and maternal behavior in a rat model. *Accepted in Endocrine Disruptors*.

PATENTS

Antinozzi, P., and **Dover, E.** 2013. Compositions and Methods for Maintaining and Improving Pancreatic Islet Cell Function. United States. U.S. Provisional Patent Application 61757071, filed January 2013. PCT filed January 2014.

TEACHING EXPERIENCE

Wake Forest School of Medicine, Spring 2014

Guest Lecturer in graduate level course, MCB 714. Invited to teach two lectures on Autoimmunity with a focus on Type 1 Diabetes.

Bowling Green State University, Fall 2009-Summer 2010

Teaching assistant for Anatomy and Physiology laboratory for undergraduates. Responsibilities included independently teaching and supervising two laboratory sections per semester, formulating laboratory exams, and grading.

HONORS AND AWARDS

Cowgill Fellowship, 2013-2014

Fellowship awarded to Ph.D. candidates based on academic merit, research experience, and written statement to enhance academic and career development.

JP Scott Fellowship, 2010-2011

Graduate student fellowship to cover tuition and stipend. Awarded based on academic merit, research experience, and Master's Thesis research project "Effect of PCB 47/77 on OXTR and AVPR1a Genes and Associated Maternal Care Behavior"

Palmetto Scholars Fellowship, 2005-2009

Undergraduate scholarship received in recognition of superior high school performance and maintenance of a college GPA above 3.5.

Trustee Scholarship, 2005-2009

Undergraduate scholarship received in recognition of academic merit.

Clemson Community Scholarship, 2005-2009

Undergraduate scholarship receive in recognition of outstanding community service record and commitment to perform 40+ hours of community service per college semester.