IDENTIFICATION OF UNIQUE GENE EXPRESSION PROFILES FOR ESTROGEN RECEPTOR ALPHA OR BETA

By

AMY GALADRIEL BIBY HURST

Bachelor of Science
Oklahoma State University
Stillwater, Oklahoma
2000

Submitted to the Faculty of the Graduate College of the
Oklahoma State University in partial fulfillment
of the requirements for the Degree of
DOCTOR OF PHILOSOPHY
May, 2005

IDENTIFICATION OF UNIQUE GENE EXPRESSION

PROFILES FOR ESTROGEN RECEPTOR

ALPHA OR BETA

Dissertation Approved:

Jerry R. Malayer
Dissertation Advisor

Rodney Geisert

Jerry Ritchey

Charlotte Ownby

Udaya DeSilva

A. Gordan Emslie
Dean of Graduate College

ACKNOWLEDGEMENTS

I would like to extend my deepest gratitude to Dr. Jerry R. Malayer, who has been more than a major advisor to me – he has been a mentor. He has been a driving force and an inspiration for my pursuit of a scientific career. He has offered guidance when needed, but also had the wisdom to allow me to explore research avenues on my own, which has prepared me for my future independent career. I would also like to thank the members of my committee; Dr. Rodney Geisert, Dr. Jerry Ritchey, Dr. Charlotte Ownby, and Dr. Udaya DeSilva. Each, in their own way, has contributed unique viewpoints which have been vital to my success as a student, and which I will take with me into my future career as a scientist. Additionally, I would like to extend my thanks to Dr. Larry Claypool in the Statistics Department who was vital in the statistical analysis of Chapters IV and V. I would also like to thank the Center for Veterinary Health Sciences and the Department of Physiological Sciences for granting me the opportunity to pursue this degree.

My thanks also goes out to all the members of our lab and the CVHS family who have provided such a tremendous support system since I started working with this group as an undergraduate in the spring of 1997. It goes without saying that it would take pages and pages to list all individuals who have helped me, but I would especially like to thank Vivienne Woods, and David Goad who provided great help at the beginning of my scientific career. I would also like to thank Leanne Weir who has been a great friend,

whom I will never forget, as well as Lori VanDeventer, Mahesh Mohan, Akhilesh Ramachandran, T.R. Nagaraja, James Flinchbaugh, and the 'guys' from Dr. Geisert's lab, Jason Ross and Morgan Ashworth, you have all made the time go so fast & the path enjoyable. Additionally, I would like to thank Ms. Betty Handlin for her expert technical assistance over the years.

Last, but by no means least, I would like to thank my family whose love and support has been immeasurable in my pursuit of this degree. Thank you to my mother, Judy A. Biby, and father, Dennis K. Biby, whom I hope that I have made proud. I love you both. I would especially like to thank my husband, Travis Hurst, for being so supportive (it is almost over dear). To my son Tanner; you have been the impetus for all that I have strived to become, never forget that you can achieve anything you set your heart to and that family will always help you to do this - I love you.

TABLE OF CONTENTS

LIST OF TABLES

Chapter III

Chapter IV

Appendix I

LIST OF FIGURES

Chapter II

Figures Page

Chapter III

Appendix I

NOMENCLATURE

AF-1	Activator function-1
AF-2	Activator function-2
ANOVA	Analysis of variance
AP-1	Activator protein 1
bER	Bovine ER
bERKO	ERβ knockout
BLAST	Basic local alignment search tool
CAT	Chloramphenicol Acetyltransferase
cDNA	Complementary deoxyribonucleic acid
CL	Corpus luteum
COL1A2	Pro – alpha – 2(I) collagen
CSFBS	Charcoal stripped fetal bovine serum
C_T	Cycle threshold
CtsL	Cathepsin L
DBD	DNA binding domain
DES	Diethylstilbestrol
DIG	Digoxigenin
DMEM	Dulbecco's modified eagle medium
DMSO	Dimethylsulphoxide
E_2	17β-Estradiol
EGF	Epidermal growth factor
ER	Estrogen receptor
ERE	Estrogen response element
ERKO	ERα knockout
EtOH	Ethanol
FAM	6-carboxyfluorescein/ Reporter dye
FSH	Follicle stimulating hormone
G-protein	Guanine nucleotide-binding protein
G_3PDH	Glyceraldehyde-3-phosphate dehydrogenase
GEN	Genistein
GH	Gonadotropin hormone
GSP	Gene Specific Primer
H12	Helix 12
HEPES	N-2-Hydroxyethylpiperazine-N′ -2-ethanesulfonic acid
hER	Human ER
HRE	Hormone response element
HRT	Hormone replacement therapy
HSP	Heat shock protein

IGF-1	Insulin-like growth factor 1
IL	Interleukin
K_d	Disassociation constant
LBD	Ligand binding domain
LH	Luteinizing hormone
MAPK	Mitogen-activated protein kinase
mRNA	Messenger ribonucleic acid
NCBI	National Center for Biotechnology Information
NCoR	Nuclear receptor co-repressor
NO	Nitric oxide
NR	Nuclear receptor
OHT	4-Hydroxytamoxifen
OVX	Ovariectomy
P_4	Progesterone
PBS	Phosphate buffered saline
P:C:I	Phenol: Chloroform: Isoamyl alcohol
PCOLCE	Procollagen C – proteinase enhancer protein
PCR	Polymerase Chain Reaction
$PGF_{2\alpha}$	Prostaglandin F2α
PKC	Protein kinase C
PL	Prolactin
PR	Progesterone receptor
RACE	Rapid elongation of cDNA ends
RACK1	Receptor for activated protein kinase C
RAL	Raloxifene HCl
RNAPII	RNA polymerase II
rRNA	Ribosomal RNA
RT	Reverse-transcription
RT-qPCR	Real time quantitative PCR
SAS	Statistical analysis system
Ser	Serine
SERM	Selective estrogen receptor modulator
SMRT	Silencing mediator for retinoid and thyroid hormone receptor
SRC-1	Steroid Receptor Coactivator-1
SSH	Suppression subtractive hybridization
TAF-Iβ	Template-activating factor Iβ
TAMRA	6-carboxytetramethylrhodamine/ Quencher
TF	Transcription factor
TGF-β	Transforming growth factor β
Thr	Threonine
TNF-α	Tumor necrosis factor α
Tyr	Tyrosine
U	Unit
V	Vehicle

Foreword

Chapter II of this dissertation is a peer-reviewed scientific publication, and is presented in the format required for publication in *Biology of Reproduction*.

Hurst AG, Goad DW, Mohan M, Malayer JR. Independent downstream gene expression profiles in the presence of estrogen receptor α or β. *Biology of Reproduction*. **2004**; 71: 1252-1261

Chapters IV and V are soon to be submitted for publication and have been formatted for the journal *Reproduction*.

CHAPTER I

INTRODUCTION

OVERVIEW OF PROJECT

Interest of Study. The study of estrogen receptors (ER)α and ERβ are of principal interest in relation to the critical role of the estrogen signaling system in the physiology of the reproductive organs, the cardiovascular, skeletal and central nervous systems, and in carcinogenesis. Estrogen receptor α and ERβ are structurally distinct, and phenotypic distribution and mouse ER gene knockout (KO) models demonstrate that the two subsets are differentially expressed in tissues. The two receptors also influence function of each other due to the ability of ERα and ERβ to heterodimerize. There is a need to better determine the involvement of each receptor in eliciting downstream gene expression in tissues where the receptors function autonomously and, especially, where both are present.

Model. Due to structural and functional differences and the complexity of ER biology, our interest lies in discovering unique independent gene expression profiles in the presence of ERα or ERβ. Therefore, it becomes important to be able to establish a model to study the two ER isoforms independent of each other. Research has yielded model cell lines that ectopically express the ER isoforms individually, in an undifferentiated rat

embryonic fibroblast cell line: Rat1 + ERα (Kaneko *et al.* 1993) and Rat1 + ERβ (Cheng & Malayer 1999). Estrogen receptors expressed in this model function in a physiologically relevant manner as evaluated in a chloramphenicol acetyltransferase reporter (CAT) assay and observed through their ability to up-regulate progesterone receptor (PR) gene expression following 17β-estradiol (E_2) treatment.

Hypothesis. These observations and the body of literature available on ER biology led us to make a two part hypothesis. The first part was that independent ERα and ERβ are able to impart unique downstream gene expression profiles within the same cell type. We further hypothesized that ligand and time of exposure could result in unique responses by target genes within any profiles identified.

Experimental design. Using an ER *in vitro* cell model, a treatment scheme involving E_2 treatment for 24h was used to identify unique gene expression profiles. Later K_i dependent single dose treatments of diethylstilbestrol (DES), 4-hydroxytamoxifen (OHT), raloxifene-HCl (RAL), or genistein (GEN) over a time-course were devised. Treatment was followed by extraction of total RNA. To evaluate roles of ER, cDNA were generated from Rat1+ERα, Rat1+ERβ and parental Rat1 cells following treatment with a single dose of E_2 [1nM] or an ethanol vehicle for 24 hours and subjected to suppression subtractive hybridization (SSH), followed by differential screening using dot blot hybridization. Genes pro – alpha – 2(I) collagen, procollagen C – proteinase enhancer protein, cathepsin L, and receptor for activated protein kinase C isolated through SSH, in addition to previously studied PR, were identified for real-time

quantitative polymerase chain reaction (RT-qPCR) analysis of profile changes in the presence of different ligands over time.

Results. Following SSH, in the presence of cellular ERα, gene products were identified that represent classic transcriptional responses to E_2, including markers for cell proliferation. In the presence of ERβ an alternate transcription profile was observed that included up-regulation of pro-alpha-2(I) collagen. These data support a model in which ERα and ERβ regulate unique subsets of downstream genes within a given cell type. When various ligands were applied to the *in vitro* cell culture over different time points, RT-qPCR indicated that gene expression profiles were affected in a differential manner which depended on an interaction between ER isotypes, ligand, and time. These results further expand our foundational understanding of the biological activity of ERs in the regulation of cell function.

Other work. Previously we have been unable to generate functional RT-qPCR primer probe sets that were sensitive enough to differentiate between ER isotypes in bovine samples. The bERβ cDNA was cloned and sequenced by Rosenfeld *et al*. (1999). However, due to a lack of comprehensive cDNA sequence for bovine ERα (bERα) at the commencement of this project, it became necessary to attempt 5′ rapid elongation of cDNA ends (RACE) to identify unique portions of bERα that could be utilized to develop probes sensitive enough to differentiate between ERα and ERβ. Endometrial samples were collected from non-bred Holstein cows at a local abattoir, and tissue homogenized for single step RNA extraction. The Invitrogen GeneRacer® protocol was then employed

to generate upstream cDNA from the previously identified ligand binding domain. Polymerase chain reaction products were then single pass sequenced using the CEQ 8000 (Beckman-Coulter, Fullerton CA) to verify product, cloned into the PCR4 vector (Invitrogen, Carlsbad CA), and re-sequenced. Two additional fragments of the bERα cDNA were obtained through this manner. From this sequence, it should be possible in the future to generate sensitive molecular tools for bERα from new sequence data obtained from bovine endometrial tissue.

CHAPTER II

LITERATURE REVIEW

Estrogen receptors

INTRODUCTION

Estrogen receptors are ligand-inducible transcription factors that bind a wide range of natural and environmental estrogenic compounds and through transcriptional modulation play a role in numerous physiological systems. Estrogen and its effects on tissue have long been examined, with the steroid first being isolated in the 1930's, and a receptor protein being discussed as early as 1962 (Jensen 1962). Toft and Gorski (1966) isolated and began characterizing a receptor protein for estrogen from the rat uterus in 1966 and through 1994 only one receptor protein had been identified. The cDNA sequence for human ER was first published in 1986 (Greene *et al.* 1986; Green *et al.* 1986), and for the rat ER in 1987 (Koike *et al.* 1987). In 1995 a second estrogen receptor, ERβ, was identified and cloned from rat prostate (Kuiper *et al.* 1996) and since that time ERβ has been characterized in the mouse (Tremblay *et al.* 1997; Pettersson *et al.* 1997), human (Mosselman *et al.* 1996), bovine (Rosenfeld *et al.* 1999), and numerous other species.

Estrogen receptors are type I members of the steroid receptor family and the nuclear receptor superfamily, which preferentially bind the active form of the steroid hormone estrogen, 17β-estradiol (Tsai & O'Malley 1994). Following binding of the cognate ligand, ER within the nucleus undergo phosphorylation (Arnold *et al.* 1997; Rogatsky *et al.* 1999), dimerization (Fawell *et al.* 1990) and commonly bind to DNA at specific palindromic *cis*-acting sites termed estrogen response elements (ERE) (Hall *et al.* 2002; Schultz *et al.* 2002). Through these interactions, in conjunction with differential receptor type and ligand affinity, ERs are able to affect differential transcriptional pathways that are the impetus for undertaking this research.

TRANSCRIPTIONAL REGULATION AND THE NUCLEAR RECEPTOR SUPERFAMILY

Transcriptional regulation. Cellular transcription factors regulate the efficient and proper transcription of mRNA. Transcription factors recognize and bind to DNA at specific *cis*-acting regulatory elements called hormone response elements or HREs. Complexes of general transcription factors, co-regulators, RNA polymerases and other mediators control the patterns of gene expression that ultimately manifests a physiological phenotype. These protein complexes work in tandem at the promoter regions of target genes with the end result of stabilizing or destabilizing the basal transcription machinery (Hager *et al.* 1998), which results in preinitiation complex recruitment of the RNA polymerase II (RNAPII) enzyme (Smith 1998; Roeder 1996). Thus, transcription is controlled by the interactions of general and site-specific transcription factors (Hartzog 2003).

Prior to activation transcription factors are often in a sequestered and complexed state with the 90 kd heat shock protein (HSP90) and other inhibitory proteins (Pratt & Toft 1997). As part of this sequestering, a template-activating factor (TAF-Iβ) is proposed to bind to the unbound receptor, maintain basal transcription rates through decreasing acetylation, masking the DNA-binding region of the ER, and provide a graded estrogen response after binding (Loven *et al.* 2003). Furthermore, acetylation acts as a regulatory mechanism for signaling through coordinated interactions with histone acetyltransferase that destabilize the inhibitory complex to allow binding to the promoter region of genes (Fu *et al.* 2004). Histones are DNA-binding proteins that form nucleosomes, which in turn make up chromatin. Histone acetylation leads then to a more open conformation for binding of the transcriptional machinery, resulting in increased rates of transcription.

The initiation point for transcription is the catalytic action of RNAPII that recognizes minimal DNA elements at the promoter region, the most common being the TATA box (Roeder, 1996). However, RNAPII lacks the ability to physically bind to the promoter on its own, and instead relies on general or basal (i.e. TFIIA, B, D, E, F, H), upstream, and inducible (i.e. nuclear receptors) transcriptional factors to create an initiation complex. These in turn regulate mRNA generation from the DNA template, and eventually result in protein expression (Voet & Voet 1995). Each of these classes of factors plays specific roles in creating the proper environment, including bending and conformational changes in the target DNA, for either activation or repression of gene expression that is dependent on other regulatory proteins present. For example, TFIIH has been shown to specifically

interact in nuclear transcription (Reese 2003) and to be recruited to ER target promoters (Wu *et al.* 2001). The transcriptional complexes formed at the promoter to support RNAPII function are not simply restricted to recruitment, and interaction with other proteins creates the proper environment for correct transcriptional regulation.

The nuclear receptors. The nuclear receptor superfamily is the largest family of transcription factors, and plays a pivotal role in the endocrine system by binding hormone ligands such as estrogen (E_2), progesterone (P_4), androgens, and glucocorticoids, as well as thyroid hormone, vitamin D, and retinoic acid. Nuclear receptors are commonly divided into three classes; type I, type II, and orphan receptors. Type I and II are classically ligand-inducible and undergo conformational changes upon binding their cognate ligand. Further conformational changes occur following interaction with a number of co-regulators resulting in transcriptional regulation. The regulatory ligands of orphan receptors have yet to be identified (Tsai & O'Malley 1994; Glass 1994), however, it is hypothesized that the orphan receptors may be an evolutionary ancestor of the type I and II nuclear receptors (Bertrand *et al.* 2004).

The type I subclass is comprised of the classical steroid hormone receptors for estrogen (ER), progesterone (PR), androgens (AR) and glucocorticoids (GR), while type II encompasses the thyroid hormone, vitamin D, and retinoic acids receptors. Type I and type II nuclear receptors further interact to form homodimers or heterodimers, respectively. During protein-DNA interactions unique estrogen response elements (ERE) are the classic *cis*-acting DNA targets of ER homo/heterodimers. Orphan receptors have

the ability to bind as monomers (Tsai & O'Malley 1994). Studies involving GR, which may be representative for the entire family, suggest that this interaction is not a static one, and instead involves the receptor complex rapidly moving on and off of these regulatory elements (Nagaich *et al.* 2004). There is also evidence for a C-terminal extension beyond the second zinc-finger of the two zinc finger motif within the structural DNA-binding domain of the NR, which acts distinctly in type I steroid receptors to increase DNA-binding affinity through recruitment of high mobility co-regulators (Melvin *et al.* 2002).

The understanding of nuclear receptor effects on gene expression patterns are of interest from several perspectives, as their role in endocrinology is diverse. Nuclear estrogen receptors mediate most of the actions of estrogenic compounds, resulting in transcriptional activation and repression, control of cell cycle progression, and integration of intracellular signaling pathways (Moggs & Orphanides 2001). This is accomplished through a cascade of complex interactions of repression and recruitment with a number of proteins, transcriptional regulators, and ligands that are not yet fully understood, but which are the basis for ongoing research.

LIGAND INDUCIBILITY OF ESTROGEN RECEPTORS

Natural physiological ligands. The estrogen signaling system plays a role in the physiology of the reproductive organs, as well as the cardiovascular, skeletal and central nervous systems, and in carcinogenesis. Estrogens are a group of C_{18} sterol compounds derived from cholesterol. Although ER preferentially bind the active metabolite E_2, there

are numerous other physiological estrogens that are recognized by the ligand binding domain, such as estrone, 17α-estradiol, and estriol (Kuiper *et al.* 1997). In addition to these physiological estrogens, ER also has the ability to bind to a number of synthetic estrogens such as diethylstilbestrol (DES) and selective estrogen receptor modulators (SERMs) as well as environmental estrogenic-like compounds such as those found in fungi, plants, and by-products of industrial processing such as moxesterol.

Synthetic and environmental ligands. The study of ERα and ERβ and their ligand specificities are of particular interest in regards to the selective activation of tissue-specific responses within the context of hormone replacement therapy (HRT) and treatment of disease (Pike *et al.* 1999; Sun *et al.* 2003). In the interest of capitalizing on this selective activation, pharmaceutical SERMs (such as tamoxifen and raloxifene) have been designed to make the most of the transcriptional activation differences when binding ERs (McDonnell 1999). Additionally, pharmacological means can be employed to elucidate specific receptor function when both isoforms are present. One such example of the versatility of synthetic compounds in expanding our understanding of ER function is the use of highly selective synthetic agonists for ERα that have linked the reliance of uterotrophic effects and bone protection to the induction of ERα over ERβ (Harris *et al.* 2002). This highlights how specific types of compounds can be used to complement our understanding of ER with physiological roles described through other models, such as knockout animal models.

Ligands designed to selectively activate only ERα or ERβ have been used to demonstrate differential effects of the two receptor subtypes (Harris *et al.* 2002; Waters *et al.* 2001), and have contributed significantly to understanding estrogen signaling. Limitations of these methodologies include the degree of selectivity by the ligand for one receptor over the other. Of significance to the present study, ERα and ERβ can respond to the same ligand with opposite effects on transcription (Paech *et al.* 1997). One such compound which elicits these kind of responses is the active metabolite of tamoxifen, 4-hydroxytamoxifen (OHT), a type I antiestrogen which inhibits cell growth in breast cancer, but promotes carcinogenic cell growth in uterine endometrium (Kedar *et al.* 1994). Furthermore, tamoxifen resistance that can arise in breast cancer treatment has been linked to levels of ERβ gene expression. However, whether those levels are increased (Speirs *et al.* 1999) or decreased (Murphy *et al.* 2002) is a point of contention, with the latter group reporting increased ERβ in tamoxifen sensitive cells.

This is important due to the ability for OHT to recruit regulatory elements in a manner different from the natural ligand E_2 (Fleming *et al.* 2004), and OHT to, following ER binding, as been demonstrated to disassociate from the ER/ERE site thereby leading to the weak transcriptional activation (Klinge *et al.* 1998).The ability of estrogens and antiestrogens to differentially alter ER function by such means has been linked back to possible allosteric interactions of the different functional domains of the ER protein (Tate *et al.* 1984). Understanding the different induction pathways that occur due to differential ligand selectivity will help us to explain the phenotypes observed as the endpoint of hormone action.

The SERM raloxifene (RAL), a benzothiophene, is also an antiestrogen yet it does not elicit the same of undesired endometrial proliferative effects as observed with OHT treatment and has been shown to have some positive skeletal effects (Turner *et al.* 1994; Black *et al.* 1994). Functional differences in cellular responses to raloxifene arise in part due to its ability to sterically restrict, due to its piperidine, the helical structure which comprises the ligand binding domain of the ER, namely helix 12 (H12). Within the binding cavity this is then recognized as an antagonistic position (Brzozowski *et al.* 1997). The difference between the activation of ERα and ERβ at an activator protein-1 (AP-1) recognition site within the target DNA is one explanation for these transcriptional regulatory differences (Paech *et al.* 1997). On a global level, microarray analysis has demonstrated that the two SERMs, OHT and RAL, have the ability to regulate different sets of genes within U2OS (human osteosarcoma) cell lines (Tee *et al.* 2004). The differential ability of SERMs to regulate gene expression is the drive behind research to discover not only the transcriptional pathways at the heart of SERM function, but also developing the next generation of therapeutic SERMs.

The selectivity of ligands by ERα and ERβ however is not limited to SERMs. Synthetic ligands such as non-steroidal based diethylstilbestrol (DES), and phytoestrogens such as the soy isoflavone genistein (GEN) have been shown to bind with varying affinities to the two receptors (Kuiper *et al.* 1997). Diethylstilbestrol was first developed in the 1940's and used through the 1970's in pregnant women for the prevention of miscarriage. However, reproductive abnormalities and increased risk of vaginal cancer in offspring caused its use as a therapeutic in humans to be terminated (Herbst *et al.* 1971; Newbold

2004). Due to the high binding affinity of DES for ER the development of therapeutic compounds based upon DES with different side chains is still of keen interest (Walter *et al.* 2004). There is an almost 2 fold difference between ERα and ERβ for affinity for DES (Kuiper *et al.* 1997) and this is of interest considering the physiological effects of the compound. Due to these properties DES may be used to elucidate certain aspects of ER function. Studies involving ER action in transgenic mice that act as indicators due to the inclusion of β-galactosidase genes that are expressed following ER activation have allowed for the further clarification of ER activity within tissue populations (Nagel *et al.* 2001). Work by Nagel (2001) with this animal model divided DES functional action into 5 tissue specific groups; 1) strongly induced (pituitary, uterus, and kidney), 2) moderate induction with high basal ER activity (hypothalamus liver, and adrenal gland), 3) moderate induction with low basal ER activity (thyroid, adipose, mammary and muscle), 4) significant basal ER activity not enhanced by DES (heart, thymus, intestine), and 5) no activity (spleen and lung). Classification of DES activity through this type of work has a two-fold usefulness, the first being a better understanding ER activity as a whole. Second, this is important in regards to development of new estrogenic compounds.

Phytoestrogens, such as GEN, are plant-derived estrogens found in dietary sources such as soybeans. They have been shown to have a weak affinity for ER, but an approximately 7-fold higher affinity for ERβ over ERα (Kuiper *et al.* 1997). The effects of plant-derived estrogens are not yet fully understood in terms of ER activation, but have long been a significant dietary source of estrogenic compounds in other cultures. These dietary proteins have been linked to decreases in breast cancer risks in premenopausal

women (Lee *et al.* 1991). A 3-year-study of non-human primates suggests that isoflavones such as GEN do not contribute to the negative proliferative effects on the myometrium or mammary tissue seen in other HRT and in high doses may result in a profile often linked with reduced breast cancer risks (Wood *et al.* 2004). Other recent studies have shown that GEN may be linked to increases in PR expression (Hughes *et al.* 2004), however the implications of this finding in regards to health management are not yet fully understood. Genistein has also been shown to have inhibitory effect on pro-collagen type COL1A2 synthesis in rat hepatic stellate cells (Kang *et al.* 2001), but the relation to ER in this system has not been explored.

Estrogen Receptor Distribution and Structure

Estrogen receptor distribution. Variations exist in the tissue distribution of ERα and ERβ. The two receptors appear to be coexpressed at similar levels in the testis, epididymis, bone, and adrenal gland (Couse & Korach 1999). Estrogen receptor α expression predominates in the proliferative cells of the mammary, pituitary, thyroid, uterus, theca cells of the ovary, skeletal muscle and the smooth muscle of the coronary arteries. The ERβ isoform is predominant in the prostate (Kuiper *et al.* 1996), granulosa cells of the ovary, lung, bladder, brain and hypothalamus (Kuiper *et al.* 1997). In tissues where both receptors are present it has been shown that the receptors may form either homodimers or heterodimers that will interact with the ERE (Pettersson *et al.* 1997; Cowley *et al.* 1997; Pace *et al.* 1997). Estrogen receptor β has been observed to function

as a partial antagonist to ERα activity in the human in part because of the ability to form heterodimers (Hall & McDonnell 1999).

In conjunction with these tissue distribution studies, it has been shown that the levels of ERα and ERβ are not equivalent and this inequity allows ERβ to have a regulatory effect on ERα by restricting the DNA-binding domain (Hall & McDonnell 1999). Furthermore, the distribution and relative ratio of ERα and ERβ to one another may play a role in differing cancer scenarios, such as switching from ERβ dominance to ERα dominance in ovarian cancer (Pujol *et al.* 1998). There is also an overall ERα/ERβ ratio decrease in post-menopausal endometrium due to ERβ expression decreasing with age, or an overall decrease in ERα as seen in some to adenocarcinomas (Jazaeri *et al.* 2001).

Organization of ERα and ERβ transcriptional domains. Estrogen receptor α and ERβ transcriptional activity is organized into 5 domains that show varying homology between the receptor types; a transcriptional regulation domain at the amino terminal end termed A/B, a DNA-binding domain (DBD) termed C, a hinge domain termed D, a ligand-binding domain (LBD) termed E, and a carboxyl terminal (F) that, depending on receptor, type plays a role in hormone-binding discrimination (Figure 1). While there is great similarity in their DNA- and ligand- binding domains, the two ER forms do exhibit significant structural differences. This is especially true in the NH_2-terminal A/B domain and the COOH-terminal F domain, and these characteristics account for some portion of differences seen in ligand affinity (Kuiper *et al.* 1996; Kuiper *et al.* 1997).

Within the A/B region is an activation function 1 (AF-1) domain which is located near the amino terminus (Lees *et al.* 1989). In this region, ERα is significantly longer than ERβ (Kuiper *et al.* 1996). Phosphorylation events in this region play an important part in transcriptional efficiency and ligand activation. There are conserved phosphorylation sites between ERα and β (Kuiper *et al.* 1996). In ERα, phosphorylation of serine^{167} aids in the AF-1 transcriptional activity in the presence of ligand through a Ser/Thr protein kinase reaction (Joel *et al.* 1998). Phosphorylation of Ser^{106} and Ser^{124} through the mitogen activated protein kinase (MAPK) pathway is the mechanism responsible for the recruitment of the regulatory protein SRC-1 to AF-1 in the ERβ subtype that can occur in a ligand-independent manner (Tremblay *et al.* 1999).

Studies with two-hybrid assays, mutations, and pull-down assays have shown direct binding of co-regulator proteins, such as p300 to AF-1 potentiate the synergism with AF-2 located in the LBD. This can occur in a ligand-inducible manner with no regard for ER isotype (Kobayashi *et al.* 2000), and is required for efficient transcriptional activity. In addition to synergistic cooperation between AF-1 and AF-2, physical bridges can be formed between the two by co-regulators and general transcription factors, such as the previously discussed TFIIH (Wu *et al.* 2001). It is also in this A/B domain, specifically the AF-1 region, that ligand-independent constitutive transactivation (Lees *et al.* 1989) is thought to be controlled. Another critical structural component within the ERα amino terminus is an α-helix core that is involved in the recruitment of co-activator proteins

(Metivier *et al.* 2001). Together these individual features create the first component of the machinery that is integral to transcriptional efficacy.

Several features of the DNA-binding region are responsible for contributing to differences in transcriptional regulation. The C domain or DBD is highly conserved (95%) between the two receptors and is in fact highly conserved within the entire steroid receptor family. This domain is composed of a two zinc-finger motif, where the first zinc finger has been identified by Green *et al*. (1986) to be the DNA discriminatory region, with the entire stretch having a vital role in dimerization and ERE recognition (Nilsson *et al.* 2001). The creation of this ER-ERE complex through binding of the DBD then induces structural changes not only in the target, but also to the full-length receptor through translated allosteric changes (Wood *et al.* 2001). The D domain lies between the C and E and is commonly referred to as the hinge domain. It functions primarily in conformational control of DBD and LBD interactions, as well as recognition of the co-regulators nuclear receptor co-repressor (NCor) and silencing mediator for retinoid and thyroid hormone receptor (SMRT) involved with repression (Ratajczak 2001). The D domain also contains motifs that, along with the C domain, are necessary for non-ERE promoter interactions (Teyssier *et al.* 2001).

The LBD is also known as the E domain and contains the activation function 2 (AF-2) region that is activated upon binding of ligand (Lees *et al.* 1989). In the ligand free receptor the conformational state of this domain is responsible for keeping the C domain from binding the ERE (Beekman *et al.* 1993). The structural change imparted is

proposed as the mechanism required for disassociation of HSPs and regulatory molecules such as TAF-Iβ which normally help to keep the receptors in a quiescent state (Loven *et al.* 2003), as well as unmasking elements necessary for dimerization, C domain interactions, and co-regulatory protein interactions (Beekman *et al.* 1993).

Ligands create differences in gene expression and transcriptional efficiency through the physical binding of ligand within the ER binding pocket and displacement of helical elements, especially H12 (Nichols *et al.* 1998). Work by Zajchowski et al. (1997) supports this model, showing that E_2 and OHT have distinctive structural requirements in regards to conformational changes affected in the ER protein. Molecular mechanisms of ER action involves promoters other than that used for direct DNA-binding to ERE (Zajchowski *et al.* 1997) determined by conformational variations. In this same manner ligand character can also affect the rate of dimer dissociation in the ER-ligand complex in a manner independent of binding affinity or dissociation kinetics (Tamrazi *et al.* 2002). Phosphorylation within the LBD helps lead to aforementioned conformational interactions required for ligand dependent transcriptional activity (Smith 1998).

The structure of the LBD is further organized into helices 3, 4, 5, and 12 where helix 12 functions as a lid over the ligand binding pocket (Brzozowski *et al.* 1997). In part, ligand selectivity depends upon the size of the ligand molecule. Hydrophobic interactions will also affect the positioning of H12 (Nichols *et al.* 1998). A difference in ERα and ERβ exists in this ligand binding pocket with ERβ having a smaller binding cavity than ERα (Nilsson *et al.* 2001). The F domain at the C- terminus is not conserved between the two

receptors, and is thought to play a role in discrimination of antagonist and agonist (Nichols *et al.* 1998). The F domain is not present in all members of the steroid receptor family, however, as in the N- terminus ERβ is significantly shorter than ERα and is thought that cell specific responses are regulated by this region (Kuiper *et al.* 1996). It has also been shown that this F domain is necessary for interactions with other DNA binding proteins, including Sp1and may be involved in some co-regulatory interactions (Kim *et al.* 2003).

ESTROGEN RECEPTOR TRANSCRIPTIONAL REGULATION

Phosphorylation events at the estrogen receptor. Proper and efficient transcriptional regulation requires specific protein complex formation at the promoter of the target gene. A primary step occurs once ER binds a ligand. Following binding rapid phosphorylation occurs in the 5' terminus which affects the subsequent binding to ERE at the DBD and transcriptional efficacy (Denton *et al.* 1992). Phosphorylation is observed predominately at serines [104, 106 , 118] of the ER in the A/B amino terminus (Le Goff *et al.* 1994), and complete ligand-induced transcriptional activation is facilitated by the cooperative phosphorylation of these sites.

Within the LBD, tyrosine^{537} acts as a basal phosphorylation site that is ligand dependent, and phosphorylation at tyr^{537} may be a requisite for ERE binding (Arnold *et al.* 1995). Phosphorylation is required for the recruitment of co-activators proteins, and when

phosphorylated helps lead to conformational interactions with the C domain required for transcriptional activation (Smith 1998). It has been suggested that mutation at this tyr^{537}, as well as at try^{443}, may have a role in hormone independent tumor progression. However, studies have shown that these types of mutations are anti-estrogen responsive, and do not lead to hormone resistance (Tremblay *et al.* 1998).

In some instances the capability exists for ligand independent activation (Power *et al.* 1991; Bunone *et al.* 1996). Cross-talk pathways with growth factors, can signal a cascade of events that allow for the phosphorylation of $serine^{118}$ through MAPK activation (Kato *et al.* 1995). One important cross-talk pathway has been demonstrated between epidermal growth factor (EGF)activation of the MAPK pathway, which can function in an ER-like manner in regards to phosphorylation of ER (Ignar-Trowbridge *et al.* 1992). It is unclear why this mechanism has evolved, but the result is that ER activity can be modulated when hormone concentrations are low. This requires the integration of multiple signaling pathways which appears to be critical.

Dimerization of ER. The type I subgroup of the nuclear receptor superfamily, such as androgen receptors and glucocorticoid receptors, typically form homodimers (Tsai & O'Malley 1994). Dimerization of the various NRs have been shown to be necessary for DNA binding and transcriptional activation (Glass 1994; Pettersson *et al.* 1997). Estrogen receptor α homodimers have been shown to be a necessary part of transcriptional regulation in the estrogen signaling pathway (Fawell *et al.* 1990). However, with the discovery of ERβ several studies have demonstrated that in addition to homodimers, ERs can form functional heterodimers.

Heterodimers of ERα/ERβ have been shown to form in the mouse (Pettersson *et al.* 1997) and human (Cowley *et al.* 1997; Pace *et al.* 1997). The formation of heterodimers has been shown to occur in part through interactions within the zinc-finger region of the DBD and within the LBD (Fawell *et al.* 1990; Glass 1994), and the dimer interface between ERα and ERβ appears to be distinct (Cowley *et al.* 1997). These observations have led to the idea that, in-part, reproductive abnormalities in ERKO mice may be linked to the loss of the cooperative formation of ERα/ERβ heterodimers (Pace *et al.* 1997). Due to this added complexity, it therefore becomes intriguing to be able to examine the two receptors independently of each other when examining expression profiles. In addition, the ERα/ ERβ heterodimer may have yet another role apart from the α/ α homodimer or β/ β homodimer in transcription.

Estrogen receptor target sites. Following ligand-binding, phosphorylation of the ER at the 5'-terminus region and dimerization, the DNA-binding region interacts with *cis*-acting DNA regulatory elements, EREs, in target DNA. Estrogen response elements are inverted palindromes where one ER monomer binds to each half-site (Tsai & O'Malley 1994). Estrogen response element recognition has been found to be primarily dependent on the AF-2 region of LBD domain (Saville *et al.* 2000). The ERE interaction is also affected by the receptor conformation assumed upon ligand (Krieg *et al.* 2004). However, ERs also have the ability to bind in a non-classical manner through protein complexes that do not require the presence of the ERE in the promoter region (Kim *et al.* 2003).

Involvement of the ER in indirect binding or non-ERE interactions. There are target genes where hormone regulation appears to rely on indirect binding of the receptor to promoter elements. These "non-classical" pathways occur because ERs also have the ability to bind in a protein-protein manner at AP-1 enhancer sites under the mediation of *c-fos* and *c-jun* transcription factors (Umayahara *et al.* 1994; Malayer *et al.* 1999), as well as through Sp1 protein and ERE half sites through the interaction of ER with GC-rich Sp1 sites (Porter *et al.* 1997). These protein-protein interactions are evident in the regulation of insulin-like growth factor (IGF-1), which lacks a traditional ERE site, but is sensitive to estrogen activity through post-translational modification through the AP-1 motif (Umayahara *et al.* 1994).

Even though these interactions with AP-1 rely on *c-fos*/*c-jun* regulation, it is important to note that the Jun protein physically binds to AP-1 DNA elements at this interface (Webb *et al.* 1995; Teyssier *et al.* 2001) which serves as another level of transcriptional control. Work by Webb *et al*. (1995) demonstrated that the AP-1 interaction with ER had varying sensitivity to activation by either E_2 or the antiestrogen OHT in a cell specific manner and that the OHT activation in uterine cell lines may be linked to the amino terminus of the ER. In addition, this work also suggested another pathway for OHT regulation at the AP-1 site that does not require direct ER binding to Jun, but instead activates a cascade that increases *c-jun* and *c-jun/c-fos* binding to the AP-1 site and this causes transcriptional alterations (Webb *et al.* 1995). Later work by Teyssier *et al*. (2001) further demonstrated direct Jun interaction with ER in pulldown assays and suggested

that in ovarian derived cell lines this interaction occurred as a result of elements in the C and D domains of the receptors. It is also of interest that the *c-fos* gene in this AP-1 complex is induced through a Sp1 site in the promoter and appears to be regulated by estrogen (Duan *et al.* 1998). The full implication of this level of control is not fully understood. Further complexity is added to the ER signaling pathway by the discovery that at AP-1 sites the two ER isotypes have opposing transcriptional effects (Paech *et al.* 1997).

Estrogen receptor and Sp1 interactions are further specific to the subtype of ER as well as the ligand used and the cell type (Saville *et al.* 2000). Studies involving the ERα promoter region have shown that transcription of the ERα gene relies heavily on interaction with the Sp1 factors (deGraffenried *et al.* 2002). Unlike the well recognized binding of ER at EREs, which are AF2 dependent, the Sp1 protein interaction with ERα, but not ERβ, appears to mediated by the AF-1 region of the A/B domain (Saville *et al.* 2000). The binding of Sp1 elements then occurs in conjunction with several of the structural domains including the C-terminus region, H12 of the ligand-binding domain as well as the F domain (Kim *et al.* 2003). A role for ERα/Sp1 interaction has been characterized for *cad* gene expression in MCF-7 cells which is a factor in cell proliferation (Khan *et al.* 2003). The mediation of PR gene expression following ligand activation of ERα is mediated through Sp1 binding and stabilization of Sp1-DNA interaction within the PR gene promoters (Schultz *et al.* 2003). The presence of an ERE half-site within the PR gene and the formation of DNA-bound Sp1 are thought to be the hormone activated pathway through ER, whereas Sp1 alone is thought to play a role in

endogenous, basal levels of PR expression observed in the absence of E_2 (Petz *et al.* 2004).

Estrogen receptor interactions with co-activators and co-repressors. Following ER binding at the promoter, the receptor protein is next involved in the recruitment of co-regulator proteins and other protein-protein interactions, resulting ultimately in modification of the rate of transcription from the target promoter (Hall *et al.* 2002; Xu *et al.* 1999; McKenna *et al.* 1999). The activator function 2 (AF-2) region of the LBD is one area that is critical to co-regulator interactions (Torchia *et al.* 1998; An *et al.* 1999), and is discussed in detail within the next section. One of the first steroid hormone related co-regulators was characterized and sequenced by Oñate et al. in 1995 via a yeast two-hybrid system and designated Steroid Receptor Coactivator-1 (SCR-1). This co-activator isolation was achieved through studies of interactions within portions of the hinge and LBD domains of hPR with cDNA from the SRC-1 protein and was shown to be a ligand-dependent event (Onate *et al.* 1995).

A number of co-regulators have been identified that interact at various sites within the DBD, hinge, and LBD regions of the ER protein (Figure 2) to either activate or repress the transcriptional machinery, often in a receptor and ligand dependent manner (Torchia *et al.* 1998; An *et al.* 2001; Ratajczak 2001). Co-activators are able to recognize and interact with the receptor through the NR box motif on the co-activators which have the capacity to be recruited by either ERα or β in a selective manner ligand (Bramlett *et al.* 2001). It has been demonstrated that some co-activators have the ability to bind ER

either a ligand-dependent or ligand-independent manner (Bramlett *et al.* 2001; Ozers *et al.* 2005). Like ERα and ERβ, co-regulators show tissue-specificity, and this is thought to account for the ability of selective agents to increase SRC-1 and SRC-3 in an ERα/AF-1 dependent manner leading to increased long-term stability (Lonard *et al.* 2004). Equally important in transcriptional activation is receptor and co-regulator turnover via proteosomes as shown through studies with SRC-1 and SRC-3 (Lonard *et al.* 2004).

Co-repressor proteins, which are of particular interest due to their role in regulation of ER/ERE complexes by SERMs (Fleming *et al.* 2004), include the NCoR and the SMRT proteins, which have been shown to interact with the unbound receptor to control transcriptional repression (Xu *et al.* 1999). The recruitment of these co-repressors by the receptor has been shown to be dependent on various ligand-affinity interactions, and occurs in a dose dependent manner (Ozers *et al.* 2005). For example, at the same promoter receptor bound OHT recruits the co-repressor SMRT, while receptor bound E_2 recruits the co-activator SRC-1 to the ER/ERE complex (Fleming *et al.* 2004).

Co-activator protein recruitment demonstrates tissue-specific localization, and is reliant on ligand selective activation. Tissue-specificity of the co-regulator protein population allows for specific ligands to recruit unique complexes to the promoter (Moras & Gronemeyer 1998), resulting in distinctive physiological responses. Additionally, SERMS can elevate steady state levels of co-regulators which inhibit ER turnover, (Lonard *et al.* 2004). Receptor bound GEN has also been shown to result in a distinct

transcriptional activation through selective recruitment of co-regulators to ERβ (An *et al.* 2001).

The SRC-1 knockouts where created to help to evaluate the interactions of the co-regulation machinery (Xu *et al.* 1998). Initial studies involving the disruption of this protein highlight the highly cooperative nature of the co-regulatory system and SRC family members, as null mutants had no observable changes in phenotype and showed only a decrease in efficiency of proliferation and differentiation in the mammary gland in response to E_2 and P_4 (Xu *et al.* 1998). Later studies involving this model have included OVX to compensate for the effects of E_2 resistance, and these studies have further shown the differential interaction of ERα and ERβ with SRC-1 to occur in a compartmentalized manner within specific tissues, such as bone (Mödder *et al.* 2004).

The known structural differences in ERs suggest unique independent downstream gene function. The amino and carboxyl terminal domains of the ERα protein are significantly longer than those of ERβ (Kuiper *et al.* 1996; Tremblay *et al.* 1997). These domains interact with the various co-regulator proteins crucial for transcriptional activation by the receptor to elicit cell specific responses (Tremblay *et al.* 1997). Furthermore, differences in amino acid composition and conformational differences within the binding pocket exist between ERα and ERβ (McDonnell 1999; Katzenellenbogen *et al.* 2001)and these likely account for differential recruitment and interactions with co-regulatory proteins which manifests as differences in affinity for various ligands. It is these differences that are at the heart of the action of SERMs.

ROLE OF ESTROGEN RECEPTOR IN HEALTH AND HEALTH MAINTENANCE

Estrogen receptors and reproductive physiology. Estrogen and ER are most commonly thought of in association with the female reproductive tract, however they are also found localized in the male reproductive tract, most notably ERβ in the prostate gland (Kuiper *et al.* 1996). Estrogen is necessary in spermatogenesis, affecting both quantity and quality of sperm (Eddy *et al.* 1996). Estrogen receptor α has been found to be located differentially with the male reproductive tract primarily in the Leydig cells, while ERβ is found within the Sertoli cells (Pelletier *et al.* 2000).

The major site of E_2 biosynthesis in females arises from the granulosa cell layer of the developing ovarian follicle (Hillier *et al.* 1981). Therefore, many animal models classically rely on ovariectomy (OVX) when it is desired to examine changes following treatment with E_2 or estrogenic-like compounds. Another site commonly associated with hormone precursor production is the adrenal gland that also serves as a regulator of the endocrine system through the secretion of steroid precursors (Weiss *et al.* 2004b).

The mammalian female reproductive system is comprised of two ovaries, two oviducts and a uterus that is either 1) duplex with two separate and independent tubes opening into two cervical canals joined at the cervix end, 2) bicornuate with two tubes joined at a common cervical canal, or 3) simplex with a single corpus and cervical canal (Mossmann 1987). Estrous or menstrual cycles, depending on species, are tightly controlled by hormonal fluctuations. Estrogen plays a major role in regulating these cycles by causing

endometrial proliferation in the uterus. This is orchestrated through levels and effects of follicle stimulating hormone (FSH), luteinizing hormone (LH), and progesterone (P_4) in ovulation that ultimately leads to either atresia or maintenance of the corpus luteum (CL) (Hillier *et al.* 1980; Hillier *et al.* 1981; Lessey *et al.* 1988; Snijders *et al.* 1992). The two main female reproductive organs associated with E_2 and ER function are the uterus and ovary, and a great deal of literature is devoted to this interplay.

The uterus is histologically divided into four layers; 1) the endometrium which is composed of luminal and glandular epithelium, and stroma, 2) the muscular myometrium, 3) the stratum vasculare, and 4) the perimetrium. The endometrium is of keen importance for implantation of the embryo, as well as the secretion of histotroph through the glandular surface secretions that are necessary for maintenance of pregnancy, and is proliferative in response to hormone action through ERα (Gray *et al.* 2001). The ovary consists of the total oocyte complement of a female, and thus is the major contributor of her reproductive potential. This follicle environment consists of two major cell types, the granulosa that surround and directly nurture the developing gamete, and theca cells that provide the necessary signaling for proper granulosa activation. The ERβ subtype is found preferentially in the granulosa cells with ERα localized to the theca cells (Byers *et al.* 1997; Rosenfeld *et al.* 1999). Hormonal signals from the pituitary gland in the form of gonadotropins signal gamete maturation (Hillier *et al.* 1980) and CL development. The CL then controls secretion of either P_4 under the correct conditions to maintain pregnancy or PGF2α to regress the CL to prepare the uterus for the next ovulation (Ganong 1991).

Estrogen receptors are localized in distinct patterns within the internal genitalia, which change during the maturation of the reproductive tract that occurs from the fetal stage until death. Estrogen receptors α and β play a role in the signaling pathway for estrogen to act in a proliferative manner upon the endometrium of the uterus (Bigsby 2002). In numerous studies the two ER have been shown to have distinct localization in the adult uterus and ovaries, such as those that have shown that ERα is preferentially expressed in the uterus (Kuiper *et al.* 1997; Shughrue *et al.* 1998). However, as with E_2 and P_4 signaling pathways, the expressions pattern within the ovine (Ing & Tornesi 1997) and rat (Wang *et al.* 1999) uterus is complex and highly compartmentalized. In the rat uterus, ERα is the predominant receptor gene in the glandular and luminal epithelium of the uterus (Wang *et al.* 1999), while ERα mRNA in the ovine uterus is up-regulated in all cell types except the luminal epithelium and myometrium following E_2 treatment (Ing & Tornesi 1997). The human and nonhuman primate endometrial vascular endothelium preferentially express ERβ. Since E_2 and P_4 are vital in the angiogenesis that occurs during the estrous or menstrual cycle it is hypothesized that the human endometrial vasculature may be partially regulated by ERβ (Critchley *et al.* 2001). Furthermore, even though ERα is the dominant receptor type, as previously mentioned, the ability to form heterodimers with available ERβ suggests that this receptor type may still play a vital role in the signaling pathways.

Ing and Tornesi, 1997 have provided evidence for a strong localized time-dependent expression of ER and PR within the uterus. This study demonstrated that even in steroid-deprived animals the receptor architecture remains intact to respond to a new influx of

hormones (Ing & Tornesi 1997), which is important when considering the physiological relevance of the existing pathways for E_2 signaling even in ER naïve cell line models. However, the reproductive system is affected by other systems, namely the hypothalamic-pituitary axis, as well as feedback loops by hormones from the reproductive system.

Estrogen and estrogen receptors in the neuroendocrine system. As mentioned previously, ERs are not solely "reproductive tissue" oriented, and also can play a number of roles in other physiological systems. The neuroendocrine system is another system that has systemic effects. The hypothalamus is classically seen as the boundary between the central nervous and endocrine systems (Couse & Korach 1999). As these systems age through a lifetime the related changes are of interest from a health perspective, and it has been shown that ERα levels increase, while ERβ decreases in an E_2 independent manner in the hypothalamus (Chakraborty *et al.* 2003). The implications of this finding have yet to be well described. Furthermore, maturation affects other regions of the neuroendocrine system, such as in rhesus monkeys, where it has been shown that ER is present in the adult adrenal gland, but is very low in the fetal organ (Hirst *et al.* 1992). It is observation such as these that further highlight the importance of temporal affects on the ER signaling pathway.

It is also through the neuroendocrine system that E_2 can stimulate the secretion of critical hormones for reproductive maintenance such as FSH, LH, gonadotropin hormone (GH), and prolactin from the pituitary gland (Nilsson & Gustafsson 2000). The hypothalamus-

pituitary axis is considered to be one part of the trigger to menopause in the human (Weiss *et al.* 2004b). Work with pituitary adenomas has linked ERα and β to distinct patterns within tumors arising from specialized cells types, such as ERα in prolactinomas (lactotropes) and gonadotrope tumors and ERβ in a majority of GH expressing tumors (Shupnik *et al.* 1998). Within the endocrine system differential ER isotype expression in both normal and abnormal tissues help to further understand how differential receptor profiles lead to variations in ER biology and signaling pathways.

ER in bone morphology. One high profile role of ER is in the physiology of bone through E_2 effects on bone resorption. The process of bone resorption is necessary for proper homeostasis and serves as a major pathway to the reservoir of minerals stored in bone that are needed by the body. This is especially noted in health maintenance and osteoporosis, as it is the loss of ovarian and pituitary hormone which is suggested to lead to the inverse relationship of increased bone resorption and decreased levels of new bone formation as a result of the respective system degradations (Yeh *et al.* 1997).

Bone density is mediated by the synergistic relationship between the breakdown and resorption of bone matrix by osteoclast cells, and the formation of new bone matrix by osteoblast cells (Riggs 2000). To understand the implication of this it is important to look at the roles that E_2 plays in bone morphogenesis. Estrogen is able to regulate the activity of osteoclast cells, which are responsible for bone degradation through the binding of ERα and subsequent down regulation of integrins and *c-jun/c-fos* (Saintier D *et al.* 2004). In conjunction, normal bone physiology relies on the E_2 down-regulation of

proinflammatory cytokines, which typically increase bone resorption. Estrogens also have a role in up-regulating inhibitors of resorption such as TGF-β, thereby effecting small, yet cumulative changes in the microenvironment of the bone (Riggs 2000). In light of these regulatory roles it is easy to see how the loss of E_2 would result in drastic changes in the normal balance of bone maintenance.

ER in the inflammatory response. Inflammation plays a role in immediate immune responses to injury, as well as in reproductive tissue as it relates to menstruation, estrus, or embryo implantation. There are well recognized roles for inflammation as it relates to health maintenance and dysfunction such as that seen in arthritis, cytokine impact on bone homeostasis and maintenance of the cardiovascular system. Steroid hormones, including estrogens have been shown to participate in these processes.

Estrogens and ER play a role in regulating cytokines involved in inflammation such as the interleukins 1 and 6 (IL) as well as tumor necrosis factor α (TNF-α), which in turn have roles in multiple physiological systems. Estrogen has a regulatory role in T-cell TNF-α secretion, B-cell population dynamics, as well as nitric oxide (NO) and cytokine inducible NO synthase (iNOS) levels (Pfeilschifter *et al.* 2002). Likewise, declines in E_2 levels lead ultimately to increases in release of free-radicals suggesting an antioxidant role for the ER system (Gurdol *et al.* 1997). The full implications for ER action through this system have yet to be realized. However, underlying interactions are of interest when considering the observation that inflammatory breast cancers show significantly decreased levels of ER (Van den Eynden *et al.* 2004). There is also a correlation between

increased ERβ over ERα immunoreactivity in the synovial fluid of individuals with rheumatoid arthritis which may play a role in the pathogenesis of this disease (Ishizuka *et al.* 2004). Together this demonstrates a role for ER in the inflammatory response that functions in health and disease.

Health and SERMS**.** In humans, the sudden decline of ovarian function and loss of LH during menopause serves as the classic trigger that accounts for the reduction in circulating E_2, and the resulting complications such as loss of reproductive function, hot flashes, bone density loss, and vaginal dryness (Cheung *et al.* 2004). There is also evidence that a loss of estrogen sensitivity in the hypothalamic-pituitary axis is involved in observable menopausal symptoms (Weiss *et al.* 2004). The exact trigger for human menopause, whether the loss of ovarian function or desensitization of the hypothalamic-pituitary axis, is not clear and may be a consequence of the decline of both systems (Wise *et al.* 1996).

The effects of aging and steroid hormone levels are not limited to females, as both male and female segments of the population suffer the effects of osteoporosis ,which is in part linked to E_2 levels (Wise *et al.* 1996). However, while men produce intragonadal E_2 in the cells of the testis it does not account for significant circulating amounts. Males rely on the metabolism of testosterone (Simpson *et al.* 1999), thus the dramatic and relatively sudden effects of estrogen loss in women is not observed in males. In additional to gonadal sources, non-gonadal sources in humans include the adrenal glands which supply the precursor dehydroepiandrosterone (Labrie *et al.* 1997), and tissues such as adipose

(Pedersen *et al.* 1996), brain and muscle where the conversion of precursors to E_2 appears to be site restricted (Simpson & Davis 2001; Labrie *et al.* 1997). These additional sites, which allow for localized E_2 production from available C_{19} precursors, may play significant roles as sources of E_2 in older individuals (Simpson *et al.* 1999). This in turn may affect those within the population which suffer from increased risk for certain cancers, such as the link between breast cancer and obesity (Huang *et al.* 1997).

Exogenous use of hormones in HRT is of interest in relieving not only the aforementioned uncomfortable symptoms that occur as a result of menopause, but also the detrimental effects in regards to bone density loss. However, the introduction of some hormone combinations that are used in HRT result in an unacceptable increased risk for certain cancers and cardiac dysfunction, which do not balance the positive bone effects. This was most notably seen in 2002, when the Women's Health Initiative ended a clinical trial involving the use of a combination of estrogen and progestin in women with a uterus, due to increased risk of breast cancer and to a lesser extent coronary artery disease (Writing group for the WHI Investigators 2002). However, clinical trials involving estrogen alone in women with a hysterectomy have been allowed to continue (Writing group for the WHI Investigators 2002). Understandably, an increased knowledge of the chief receptor type targeted by these treatments and their downstream effects is desired so that this data may lead to better health maintenance and therapeutics.

MODEL SYSTEMS FOR ESTROGEN RECEPTOR FUNCTION

Knockout models. Several complementary approaches have been used to verify and characterize physiological and genetic differences between the ER subtypes. The concept of functional knockouts is bolstered by the presence of natural knockouts found within the environment, such an ERα deficient male in humans (Smith *et al.* 1994). Engineered knockouts are created by targeting portions of genes, causing the receptor gene to become disrupted. The disrupted gene constructs are then cultured into embryonic stem cells, and added to blastocysts. Heterozygous offspring are then inbred to create ER null homozygotes, which are then studied for differences in phenotype (Couse & Korach 1999). These models have served as a cornerstone for understanding the physiological effects of ER in relation to phenotypic disparity. Phenotypic variation between mouse knockout models for ERα (Lubahn *et al.* 1993; Das *et al.* 1997) and ERβ (Krege *et al.* 1998) suggest that, in addition to differences in tissue distribution, the receptors exert different effects in the same tissue. These models have provided valuable insights into the differential roles of the estrogen receptors, as well as insight into cross-talk mechanisms such as the ablation of cross-talk between the membrane bound EGF and nuclear ER in the uterus of ERKO mice (Curtis *et al.* 1996).

The most notable phenotype of ERα knockouts (ERKO) is that both female (Lubahn *et al.* 1993) and male (Eddy *et al.* 1996) mice exhibit infertility. The ERβ knockout (bERKO) females exhibit only subfertility (Krege *et al.* 1998), highlighting the dominant nature of ERα. However, ERβ roles in the uterus have still been shown to be

developmentally important as bERKO mice have regulatory roles of ERα in the neonate (Weihua *et al.* 2000). Estrogen receptor α KO females display hypoplasia of the uterus and abnormal sexual behavior even following E_2 stimulation (Lubahn *et al.* 1993). The cause of female infertility stems mainly from the inability to ovulate, as seen by the dramatic differences in ovarian structure in ERKO versus wild-type (WT). Male infertility stems from disruption of the seminiferous tubules and a resulting decrease in the quantity and quality of sperm cells (Eddy *et al.* 1996).

In the ERKO mouse, the ovarian structure at postnatal day 10 is similar between ERKO and WT, but deteriorates by postnatal day20 to where an absence of corpora lutea, and a phenotype of hemorrhagic and cystic follicles is observed (Schomberg *et al.* 1999). This, in part, demonstrates the importance of temporal effects when considering physiological ER activation. In the male, the Sertoli cells are a main source of E_2 and also express ER. In ERKO males disruption of this process results in the lack of ability to successfully complete spermatogenesis (Couse & Korach 1999). In compound ER knockouts (ERαβKO) created by Dupont *et al*. (2000) ER null post-pubescent females displayed Sertoli cell-like structures within their ovaries. This has advanced ideas involving the nature of ER in female gonad developmental and differentiation, as well as suggesting the importance of time period when examining physiological effects.

Knockout models demonstrate the cooperative nature of ERα and ERβ, as well as the dose dependence of treatment, on the effects of the individual receptors. One example of this is the diversity in phenotypes with respect to of bone homeostasis seen between

females and males of the ERKO, βERKO, and double knockout αβERKO phenotypes. Defects in the skeletal system (although not overt) are seen in both female and male ERKO and βERKO models. The main effects tend to result from a decreased bone thickness and density in both sexes, while females show no effects in longitudinal bone and males demonstrate evidence of ER isoform compensatory effects (Sims *et al.* 2002). However, there is evidence that in males ERα is responsible for maintenance and cortical growth, and ERβ does not play a role in remodeling, while females require both receptors for remodeling but ERβ is ligand dependent (Sims *et al.* 2002). It is suggested that ERβ may mediate the negative regulatory effects of estrogenic compounds on bone (Couse & Korach 1999). This underscores the complexity of endocrine biology on multiple physiological systems and how ERα and ERβ may act differentially.

It is difficult, however, with knockout models to separate developmental effects from functional effects in adult tissue. There is evidence from ER knockouts that estrogenic compounds can retain effectiveness in uterine stimulation through non-nuclear ER pathways, splice-variants, or other receptor protein interactions (Das *et al.* 1997). In single ER knockouts compensation for each subtype is unavoidable. There also may be functional redundancy in the remaining members of the steroid receptor family (Dupont *et al.* 2000) and there may be effects of prenatal gene imprinting (Couse & Korach 1999). For these reasons other types of models are important in evaluating independent ERα and ERβ function as well as their interactions.

Cell line models. Cell line models provide valuable insight into the functional signaling pathways and gene expression profiles that may not be as easy to elucidate from knockout animal models. Numerous cell lines have been generated that give information on varying aspects of ER function, but for the purposes of this review the scope will be limited to a select few. In addition to breast tissue and breast cancer derived cell lines, osteoblast, pituitary, and embryonic fibroblast cell lines have been described for use in understanding the ER signaling pathway. The use of these models in understanding cell specific mechanisms are a valuable tools (Zhang *et al.* 2002), however it must also be kept in mind that they are a tool and a single cell line can not in and of itself imitate an intact tissue system.

In regard to ER function, one of the most widely used cell lines has been the metastatic breast cancer derived MCF-7 cell line (Soule *et al.* 1973) that expresses ERα preferentially and is growth inhibited by antiestrogens (Muller *et al.* 1998). The MCF-7 cells have been the classical cell culture model used to describe steroid function, including ER-PR interactions (Horwitz *et al.* 1975; Horwitz & McGuire 1978). Stable expression of the α and β receptors in a naïve cell line, such as fetal osteoblasts (Rickard *et al.* 2002; Harris *et al.* 1995; Monroe *et al.* 2003), has resulted in the demonstration of responses specific to ERα and ERβ mediated signaling. This osteoblast based cell culture model allows for characterization of the roles of the two receptors independent of one another in the presence of the same natural ligands, with a specific emphasis on how cellular signaling may function in context to bone morphology.

Work by Waters, *et al*. (2001) used human osteoblast cell lines (hFOB/ERα or ERβ) to demonstrate that ligand (E_2 or OHT) and ER isoforms affect responses of the cells in regards to cytokine expression in a "stage of differentiation" dependent manner. These researchers conclude that ER in this system may be modulated by co-regulators, cell matrix interactions, or cross-talk pathways along with other factors (Waters *et al.* 2001). However, it also highly likely that transcriptional regulation is dependent on the relationships between these interactions. This was more clearly seen in later studies by the same group which showed that ERα/ERβ co-expression decreased transcriptional response compared to the independent receptors, and that SRC family over expression favors one ER isotype over the other dependent on the SRC member present (Monroe *et al.* 2003).

Our lab has examined gene expression specifically regulated by either ERα or ERβ in an *in vitro* cell culture model based upon engineered rat embryonic fibroblast cell lines (Freeman *et al.* 1970; Kaneko *et al.* 1993; Cheng & Malayer 1999), although there are limitations with this approach as well. This cell line does not naturally express ER (Freeman *et al.* 1970), and permit the examination of changes in gene expression upon the retroviral addition of either ERα (Kaneko *et al.* 1993) or ERβ (Cheng & Malayer 1999) within the given cellular architecture. Ligand dependent activation of ER has been shown through estrogen responsiveness studies using chloramphenicol acetyltransferase (CAT) reporter assay (Kaneko *et al.* 1993; Cheng & Malayer 1999; Hurst *et al.* 2004), and the use of RT-PCR to determine the presence of PR (Kaneko *et al.* 1993; Cheng & Malayer 1999), a known downstream gene regulated by ER.

Due to the highly complex nature of estrogen signaling pathways, there is a need to determine with greater clarity the involvement of each independent receptor isotype in eliciting downstream gene expression both in tissues where the receptors function autonomously, and especially where both are present. Numerous methods, from knockouts to cell lines, have been used to study steroid hormones, their receptors and their roles in physiology. Due to the complexity of the steroid receptor paradigm it will take the investigation of multiple data sets to come to a more complete understanding of ER function and interactions. It is desired that this will ultimately lead to better understanding and management of the health and wellness issues with which we are currently faced. For these reasons we have examined the nature of unique gene regulation by ERα and ERβ in an undifferentiated *in vitro* cell model system designed to isolate each receptor protein from the influence of the other.

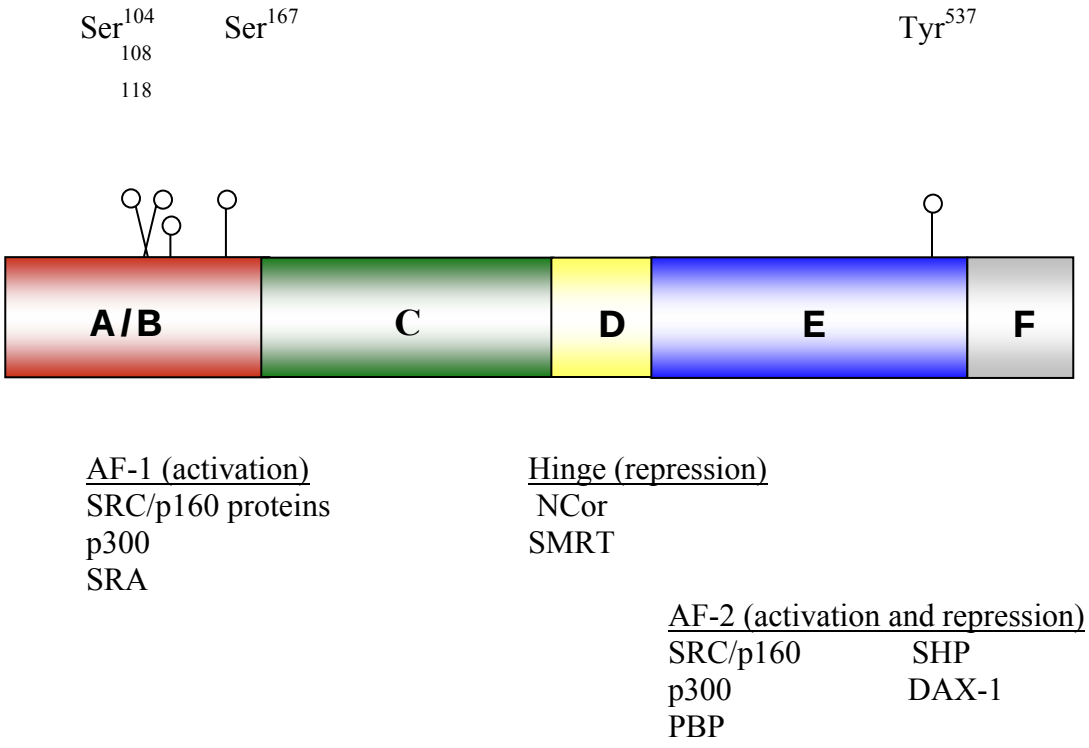


Figure 1: The nuclear receptor common domain structure. The estrogen receptor shares a common 5 domain structure with the NR family that displays specific serine and tyrosine phosphorylation sites, and interactions with co-regulatory proteins. Adapted from; Ratajczak, 2001 and Smith, 1998

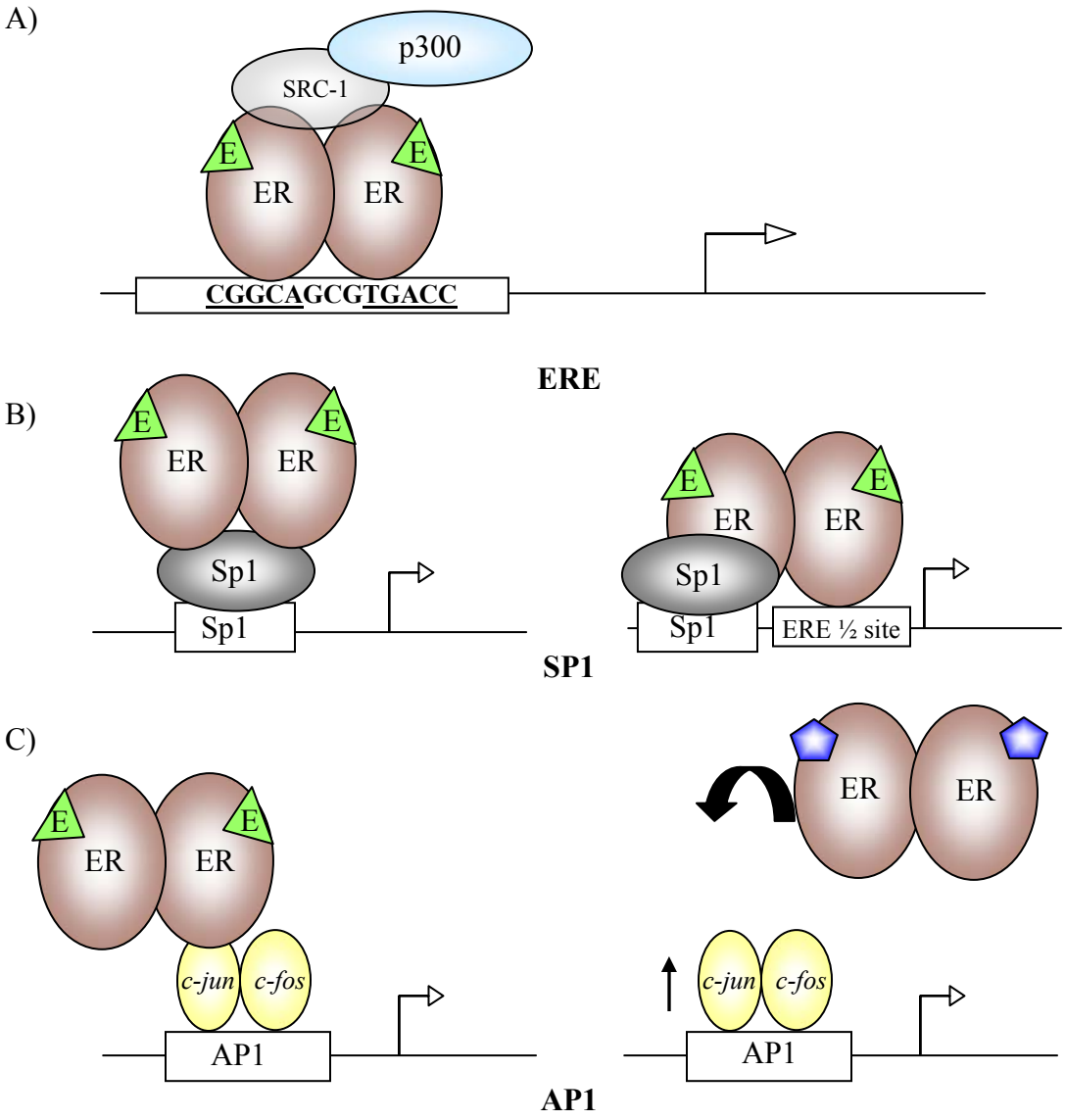


Figure 2: Multiple binding sites within the promoter region through which ER regulate gene expression. The most well recognized pathway is that which is mediated through DNA binding to ERE **(A)**. However, pathways arise from indirect binding through protein: protein interactions seen through Sp1 **(B)** and AP-1 **(C)**. This figure is a compilation from the following references; Webb et al., 1995 and DeNardo *et al.* 2005

REFERENCE LIST

An J, Ribeiro RCJ, Webb P, Gustafsson JA, Kushner PJ, Baxter JD & Leitman DC. 1999 Estradiol repression of tumor necrosis factor-alpha transcription requires estrogen receptor activation function-2 and is enhanced by coactivators. *Proceedings of the National Academy of Sciences* **96** 15161-15166.

An J, Tzagarakis-Foster C, Scharschmidt TC, Lomri N & Leitman DC. 2001 Estrogen receptor beta -selective transcriptional activity and recruitment of coregulators by phytoestrogens. *Journal of Biological Chemistry* **276** 17808-17814.

Arnold SF, Melamed M, Vorojeikina DP, Notides AC & Sasson S. 1997 Estradiol-binding mechanism and binding capacity of the human estrogen receptor is regulated by tyrosine phosphorylation. *Molecular Endocrinology* **11** 48-53.

Arnold SF, Vorojeikina DP & Notides AC. 1995 Phosphorylation of tyrosine 537 on the human estrogen receptor is required for binding to an estrogen response element. *Journal of Biological Chemistry* **270** 30205-30212.

Beekman JM, Allan GF, Tsai SY, Tsai MJ & O'Malley BW. 1993 Transcriptional activation by the estrogen receptor requires a conformational change in the ligand binding domain. *Molecular Endocrinology* **7** 1266-1274.

Bertrand S, Brunet FG, Escriva H, Parmentier G, Laudet V & Robinson-Rechavi M. 2004 Evolutionary genomics of nuclear receptors: from twenty-five ancestral genes to derived endocrine systems. *Molecular Biology and Evolution* **21** 1923-1937.

Bigsby RM. 2002 Control of growth and differentiation of the endometrium: the role of tissue interactions. *Annals of the New York Academy of Sciences* **955** 110-117.

Black LJ, Sato M, Rowley ER, Magee DE, Bekele A, Williams DC, Cullinan GJ, Bendele R, Kauffman RF, Bensch WR, Frolik CA, Termine JD & Bryant HU. 1994 Raloxifene (LY139481 HCI) prevents bone loss and reduces serum cholesterol without causing uterine hypertrophy in ovariectomized rats. *Journal of Clinical Invesigationt.* **93** 63-69.

Bramlett KS, Wu Y & Burris TP. 2001 Ligands specify coactivator nuclear receptor (NR) box affinity for estrogen receptor subtypes. *Molecular Endocrinology* **15** 909-922.

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA & Carlquist M. 1997 Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature.* **389** 753-758.

Bunone G, Briand PA, Miksicek RJ & Picard D. 1996 Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *EMBO J* **15** 2174-2183.

Byers M, Kuiper GGJM, Gustafsson JA & Park-Sarge OK. 1997 Estrogen receptor-β mRNA expression in rat ovary: down-regulation by gonadotropins. *Molecular Endocrinology* **11** 172-182.

Chakraborty TR, Ng L & Gore AC. 2003 Age-related changes in estrogen receptor β in rat hypothalamus: a quantitative analysis. *Endocrinology* **144** 4164-4171.

Cheng J & Malayer JR. 1999 Responses to stable ectopic estrogen receptor-beta expression in a rat fibroblast cell line. *Molecular and Cellular Endocrinology.* **156** 95-105.

Cheung A, Chaudhry R, Kapral M, Jackevicius C & Robinson G. 2004 Perimenopausal and postmenopausal health. *BMC Women's Health* **4** S23.

Couse JF, Hewitt SC, Bunch DO, Sar M, Walker VR, Davis BJ & Korach KS. 1999 Postnatal sex reversal of the ovaries in mice lacking estrogen receptors alpha and beta. *Science.* **286** 2328-2331.

Couse JF & Korach KS. 1999 Estrogen receptor null mice: what have we learned and where will they lead us? *Endocrine Reviews* **20** 358-417.

Cowley SM, Hoare S, Mosselman S & Parker MG. 1997 Estrogen receptors α and β form heterodimers on DNA. *Journal of Biological Chemistry* **272** 19858-19862.

Critchley HOD, Brenner RM, Henderson TA, Williams K, Nayak NR, Slayden OD, Millar MR & Saunders PTK. 2001 Estrogen receptor β, but not estrogen receptor α, is present in the vascular endothelium of the human and nonhuman primate endometrium. *Journal of Clinical Endocrinology Metabolism* **86** 1370-1378.

Curtis SW, Washburn T, Sewall C, DiAugustine R, Lindzey J, Couse JF & Korach KS. 1996 Physiological coupling of growth factor and steroid receptor signaling pathways: Estrogen receptor knockout mice lack estrogen-like response to epidermal growth factor. *Proceedings of the National Academy of Sciences* **93** 12626-12630.

Das SK, Taylor JA, Korach KS, Paria BC, Dey SK & Lubahn DB. 1997 Estrogenic responses in estrogen receptor-alpha deficient mice reveal a distinct estrogen signaling pathway. *Proceedings of the National Academy of Sciences* **94** 12786-12791.

deGraffenried LA, Hilsenbeck SG & Fuqua SAW. 2002 Sp1 is essential for estrogen receptor α gene transcription. *The Journal of Steroid Biochemistry and Molecular Biology* **82** 7-18.

DeNardo DG, Kim HT, Hilsenbeck S, Cuba V, Tsimelzon A & Brown PH. 2005 Global gene expression analysis of estrogen receptor transcription factor cross talk in breast cancer: identification of estrogen-induced/activator protein-1-dependent genes. *Molecular Endocrinology* **19** 362-378.

Denton RR, Koszewski NJ & Notides AC. 1992 Estrogen receptor phosphorylation. Hormonal dependence and consequence on specific DNA binding. *Journal of Biological Chemistry* **267** 7263-7268.

Duan R, Porter W & Safe S. 1998 Estrogen-induced *c-fos* protooncogene expression in MCF-7 human breast cancer cells: role of estrogen receptor Sp1 complex formation. *Endocrinology* **139** 1981-1990.

Dupont S, Krust A, Gansmuller A, Dierich A, Chambon P & Mark M. 2000 Effect of single and compound knockouts of estrogen receptors alpha (ERalpha) and beta (ERbeta) on mouse reproductive phenotypes. *Development* **127** 4277-4291.

Eddy EM, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB & Korach KS. 1996 Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinology* **137** 4796-4805.

Fawell SE, Lees JA, White R & Parker MG. 1990 Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor. *Cell.* **60** 953-962.

Fleming FJ, Hill ADK, McDermott EW, O'Higgins NJ & Young LS. 2004 Differential Recruitment of Coregulator Proteins Steroid Receptor Coactivator-1 and Silencing Mediator for Retinoid and Thyroid Receptors to the Estrogen Receptor-Estrogen Response Element by β-Estradiol and 4-Hydroxytamoxifen in Human Breast Cancer. *Journal of Clinical Endocrinology and Metabolism* **89** 375-383.

Freeman AE, Price PJ, Igel HJ, Young JC, Maryak JM & Huebner RJ. 1970 Morphological transformation of rat embryo cells induced by diethylnitrosamine and murine leukemia viruses. *Journal of the National Cancer Institutes.* **44** 65-78.

Fu M, Wang C, Zhang X & Pestell RG. 2004 Acetylation of nuclear receptors in cellular growth and apoptosis. *Biochemical Pharmacology* **68** 1199-1208.

Ganong WF. 1991 Role of the nervous system in reproduction. In *Reproduction in Domestic Animals*, pp. 1-24. Ed PT Cupps. New York: Harcourt Brace Jovanovich.

Glass CK. 1994 Differential recognition of target genes by nuclear receptor monomers, dimers, and heterodimers. *Endocrine Reviews* **15** 391-407.

Gray CA, Bartol FF, Tarleton BJ, Wiley AA, Johnson GA, Bazer FW & Spencer TE. 2001 Developmental biology of uterine glands. *Biology of Reproduction* **65** 1311-1323.

Green S, Walter P, Krust A, Bornert JM, Argos P & Chambon P. 1986 Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature.* **320** 134-139.

Greene GL, Gilna P, Waterfield M, Baker A & Shine J. 1986 Sequence and expression of human estrogen receptor complementary DNA. *Science.1986 Mar 7;231(4742):1150-4.* **231** 1150-1154.

Gurdol F, Oner-Yyidothan Y, Yalcyn O, Genc S & Buyru F. 1997 Changes in enzymatic antioxidant defense system in blood and endometrial tissues of women after menopause. *Research and Communications in Molecular Pathology and Pharmacology.* **97** 38-46.

Hager GL, Smith CL, Fragoso G, Wolford R, Walker D, Barsony J & Htun H. 1998 Intranuclear trafficking and gene targeting by members of the steroid/nuclear receptor superfamily. *Journal of Steroid Biochemistry and Molecular Biology.* **65** 125-132.

Hall JM & McDonnell DP. 1999 The estrogen receptor β-isoform (ERβ) of the human estrogen receptor modulates ERα transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology* **140** 5566-5578.

Hall JM, McDonnell DP & Korach KS. 2002 Allosteric regulation of estrogen receptor structure, function, and coactivator recruitment by different estrogen response elements. *Molecular Endocrinology* **16** 469-486.

Harris HA, Katzenellenbogen JA & Katzenellenbogen BS. 2002 Characterization of the biological roles of the estrogen receptors, ERα and ERβ, in estrogen target tissues in vivo through the use of an ERα-selective ligand. *Endocrinology* **143** 4172-4177.

Harris SA, Tau KR, Enger RJ, Toft DO, Riggs BL & Spelsberg TC. 1995 Estrogen response in the hFOB 1.19 human fetal osteoblastic cell line stably transfected with the human estrogen receptor gene. *Journal of Cellular Biochemistry.* **59** 193-201.

Hartzog GA. 2003 Transcription elongation by RNA polymerase II. *Current Opinion in Genetics & Development* **13** 119-126.

Herbst AL, Ulfelder H & Poskanzer DC. 1971 Adenocarcinoma of the vagina. Association of maternal stilbestrol therapy with tumor appearance in young women. *New England Journal of Medicine.* **284** 878-881.

Hillier S, Reichert LJ & Van Hall EV. 1981 Control of preovulatory follicular estrogen biosynthesis in the human ovary. *Journal of Clinical Endocrinology and Metabolism.* **52** 847-856.

Hillier S, Zeleznik A, Knazek R & Ross GT. 1980 Hormonal regulation of preovulatory follicle maturation in the rat. *Journal of Reproduction and Fertility* **60** 219-229.

Hirst JJ, West NB, Brenner RM & Novy MJ. 1992 Steroid hormone receptors in the adrenal glands of fetal and adult rhesus monkeys. *Journal of Clinical Endocrinology Metabolism* **75** 308-314.

Horwitz KB, Costlow ME & McGuire WL. 1975 MCF-7; a human breast cancer cell line with estrogen, androgen, progesterone, and glucocorticoid receptors. *Steroids.* **26** 758-795.

Horwitz KB & McGuire WL. 1978 Estrogen control of progesterone receptor in human breast cancer. Correlation with nuclear processing of estrogen receptor. *Journal of Biological Chemistry* **253** 2223-2228.

Huang Z, Hankinson SE, Colditz GA, Stampfer MJ, Hunter DJ, Manson JE, Hennekens CH, Rosner B, Speizer FE & Willett WC. 1997 Dual effects of weight and weight gain on breast cancer risk. *JAMA.* **278** 1407-1411.

Hughes CL, Liu G, Beall S, Foster WG & Davis V. 2004 Effects of genistein or soy milk during late gestation and lactation on adult uterine organization in the rat. *Experimental Biology and Medicine* **229** 108-117.

Hurst AGB, Goad DW, Mohan M & Malayer JR. 2004 Independent Downstream Gene Expression Profiles in the Presence of Estrogen Receptor α or β. *Biology of Reproduction* **71** 1252-1261.

Ignar-Trowbridge DM, Nelson KG, Bidwell MC, Curtis SW, Washburn TF, McLachlan JA & Korach KS. 1992 Coupling of dual signaling pathways: epidermal growth factor action involves the estrogen receptor. *Proceedings of the National Academy of Sciences* **89** 4658-4662.

Ing NH & Tornesi MB. 1997 Estradiol up-regulates estrogen receptor and progesterone receptor gene expression in specific ovine uterine cells. *Biology of Reproduction* **56** 1205-1215.

Ishizuka M, Hatori M, Suzuki T, Miki Y, Darnel AD, Tazawa C, Sawai T, Uzuki M, Tanaka Y, Kokubun S & Sasano H. 2004 Sex steroid receptors in rheumatoid arthritis. *Clinical Sciences (London).* **106** 293-300.

Jazaeri AA, Nunes KJ, Dalton MS, Xu M, Shupnik MA & Rice LW. 2001 Well-differentiated endometrial adenocarcinomas and poorly differentiated mixed mullerian tumors have altered ER and PR isoform expression. *Oncogene* **20** 6965-6969.

Jensen EV. 1962 On the mechanism of estrogen action. *Perspectives in Biology and Medicine.* **6** 47-59.

Joel PB, Smith J, Sturgill TW, Fisher TL, Blenis J & Lannigan DA. 1998 pp90rsk1 Regulates estrogen receptor-mediated transcription through phosphorylation of Ser-167. *Molecular and Cellular Biology* **18** 1978-1984.

Kaneko KJ, Gelinas C & Gorski J. 1993 Activation of the silent progesterone receptor gene by ectopic expression of estrogen receptors in a rat fibroblast cell line. *Biochemistry.* **32** 8348-8359.

Kang LP, Qi LH, Zhang JP, Shi N, Zhang M, Wu TM & Qi LH. 2001 Effect of genistein and quercetin on proliferation, collagen synthesis, and type I procollagen mRNA levels of rat hepatic stellate cells. *Acta Pharmacol Sin.* **22** 793-796.

Kato S, Endoh H, Masuhiro Y, Kitamoto T, Uchiyama S, Sasaki H, Masushige S, Gotoh Y, Nishida E, Kawashima H, Metzger D & Chambon P. 1995 Activation of the estrogen receptor through phosphorylation by mitogen-activated protein kinase. *Science* **270** 1491-1494.

Katzenellenbogen BS, Sun J, Harrington WR, Kraichely DM, Ganessunker DESH & Katzenellenbogen JA. 2001 Structure-function relationships in estrogen receptors and the characterization of novel selective estrogen receptor modulators with unique pharmacological profiles. *Annals of the New York Academy of Sciences* **949** 6-15.

Kedar RP, Bourne TH, Powles TJ, Collins WP, Ashley SE, Cosgrove DO & Campbell S. 1994 Effects of tamoxifen on uterus and ovaries of postmenopausal women in a randomised breast cancer prevention trial. *Lancet.* **343** 1318-1321.

Khan S, Abdelrahim M, Samudio I & Safe S. 2003 Estrogen Receptor/Sp1 complexes are required for induction of *cad* gene expression by 17β-estradiol in breast cancer cells. *Endocrinology* **144** 2325-2335.

Kim K, Thu N, Saville B & Safe S. 2003 Domains of estrogen receptor α (ERα) required for ERα/Sp1-mediated activation of GC-Rich promoters by estrogens and antiestrogens in breast cancer cells. *Molecular Endocrinology* **17** 804-817.

Klinge CM, Studinski-Jones AL, Kulakosky PC, Bambara RA & Hilf R. 1998 Comparison of tamoxifen ligands on estrogen receptor interaction with estrogen response elements. *Molecular and Cellular Endocrinology* **143** 79-90.

Kobayashi Y, Kitamoto T, Masuhiro Y, Watanabe M, Kase T, Metzger D, Yanagisawa J & Kato S. 2000 p300 Mediates functional synergism between AF-1 and AF-2 of estrogen receptor α and β by iteracting directly with the N-terminal A/B domains. *Journal of Biological Chemistry* **275** 15645-15651.

Koike S, Sakai M & Muramatsu M. 1987 Molecular cloning and characterization of rat estrogen receptor cDNA. *Nucleic Acids Research* **15** 2499-2513.

Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS, Gustafsson JA & Smithies O. 1998 Generation and reproductive phenotypes of mice lacking estrogen receptor beta. *Proceedings of the National Academy of Sciences* **95** 15677-15682.

Krieg AJ, Krieg SA, Ahn BS & Shapiro DJ. 2004 Interplay between estrogen response element sequence and ligands controls *in vivo* binding of estrogen receptor to regulated genes. *Journal of Biological Chemistry* **279** 5025-5034.

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S & Gustafsson JA. 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proceedings of the National Academy of Sciences* **93** 5925-5930.

Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S & Gustafsson JA. 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology* **138** 863-870.

Labrie F, Belanger A, Cusan L & Candas B. 1997 Physiological Changes in Dehydroepiandrosterone Are Not Reflected by Serum Levels of Active Androgens and Estrogens But of Their Metabolites: Intracrinology. *J Clin Endocrinology and Metabolism* **82** 2403-2409.

Le Goff P, Montano MM, Schodin DJ & Katzenellenbogen BS. 1994 Phosphorylation of the human estrogen receptor. Identification of hormone-regulated sites and examination of their influence on transcriptional activity. *Journal of Biological Chemistry* **269** 4458-4466.

Lee HP, Lee J, Gourley L, Duffy SW, Day NE & Esteve J. 1991 Dietary effects on breast-cancer risk in Singapore. *The Lancet* **337** 1197-1200.

Lees JA, Fawell SE & Parker MG. 1989 Identification of two transactivation domains in the mouse oestrogen receptor. *Nucleic Acids Research.* **17** 5477-5488.

Lessey BA, Killam AP, Metzger DA, Haney AF, Greene GL & McCarty KS Jr. 1988 Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle. *Journal of Clinical Endocrinology Metabolism* **67** 334-340.

Lonard DM, Tsai SY & O'Malley BW. 2004 Selective estrogen receptor modulators 4-hydroxytamoxifen and raloxifene impact the stability and function of SRC-1 and SRC-3 coactivator proteins. *Molecular and Cellular Biology* **24** 14-24.

Loven MA, Muster N, Yates JR & Nardulli AM. 2003 A novel estrogen receptor α-associated protein, template-activating factor Iβ, inhibits acetylation and transactivation. *Molecular Endocrinology* **17** 67-78.

Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS & Smithies O. 1993 Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proceedings of the National Academy of Sciences* **90** 11162-11166.

Malayer JR, Cheng J & Woods VM. 1999 Estrogen responses in bovine fetal uterine cells involve pathways directed by both estrogen response element and activator protein-1. *Biology of Reproduction* **60** 1204-1210.

McDonnell DP. 1999 The molecular pharmacology of SERMs. *Trends in Endocrinology and Metabolism* **10** 301-311.

McKenna NJ, Lanz RB & O'Malley BW. 1999 Nuclear receptor coregulators: cellular and molecular biology. *Endocrine Reviews* **20** 321-344.

Melvin VS, Roemer SC, Churchill MEA & Edwards DP. 2002 The C-terminal extension (CTE) of the nuclear hormone receptor DNA binding domain determines interactions and functional response to the HMGB-1/-2 co-regulatory proteins. *Journal of Biological Chemistry* **277** 25115-25124.

Metivier R, Penot G, Flouriot G & Pakdel F. 2001 Synergism between ERα transactivation function 1 (AF-1) and AF-2 mediated by steroid receptor coactivator protein-1: requirement for the AF-1 α-helical core and for a direct interaction between the N- and C-terminal domains. *Molecular Endocrinology* **15** 1953-1970.

Mödder UIL, Sanyal A, Kearns AE, Sibonga JD, Nishihara E, Xu J, O'Malley BW, Ritman EL, Riggs BL, Spelsberg TC & Khosla S. 2004 Effects of loss of steroid receptor coactivator-1 on the skeletal response to estrogen in mice. *Endocrinology* **145** 913-921.

Moggs JG & Orphanides G. 2001 Estrogen receptors: orchestrators of pleiotropic cellular responses. *EMBO Rep* **2** 775-781.

Monroe DG, Johnsen SA, Subramaniam M, Getz BJ, Khosla S, Riggs BL & Spelsberg TC. 2003 Mutual antagonism of estrogen receptors alpha and beta and their preferred interactions with steroid receptor coactivators in human osteoblastic cell lines. *J Endocrinol.* **176** 349-357.

Moras D & Gronemeyer H. 1998 The nuclear receptor ligand-binding domain: structure and function. *Current Opinion in Cell Biology* **10** 384-391.

Mosselman S, Polman J & Dijkema R. 1996 ERβ: Identification and characterization of a novel human estrogen receptor. *FEBS Letters* **392** 49-53.

Mossmann HW. 1987 Comparitive anatomy of the oviduct, uterus, and vagina. In *Vertebrate Fetal Membranes*, pp. 63-71. New Brunswick: Rutgers University Press.

Muller V, Jensen EV & Knabbe C. 1998 Partial antagonism between steroidal and nonsteroidal antiestrogens in human breast cancer cell lines. *Cancer Res.* **58** 263-267.

Murphy LC, Leygue E, Niu Y, Snell L, Ho S-M & Watson PH. 2002 Relationship of coregulator and oestrogen receptor isoform expression to *de novo* tamoxifen resistance in human breast cancer. *British Journal of Cancer;* **87** 1411-1416.

Nagaich AK, Rayasam GV, Martinez ED, Becker M, Qiu Y, Johnson TA, Elbi C, Fletcher TM, John S & Hager GL. 2004 Subnuclear trafficking and gene targeting by steroid receptors. *Annals of the New York Academy of Sciences* **1024** 213-220.

Nagel SC, Hagelbarger JL & McDonnell DP. 2001 Development of an ER action indicator mouse for the study of estrogens, selective ER modulators (SERMs), and xenobiotics. *Endocrinology* **142** 4721-4728.

Newbold RR. 2004 Lessons learned from perinatal exposure to diethylstilbestrol. *Toxicology and Applied Pharmacology* **199** 142-150.

Nichols M, Rientjes JM & Stewart AF. 1998 Different positioning of the ligand-binding domain helix 12 and the F domain of the estrogen receptor accounts for functional differences between agonists and antagonists. *EMBO J.* **17** 765-773.

Nilsson S & Gustafsson JÅ. 2000 Estrogen receptor transcription and transactivation: Basic aspects of estrogen action. *Breast Cancer Research* **2** 360-366.

Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, Andersson G, Enmark E, Pettersson K, Warner M & Gustafsson JA. 2001 Mechanisms of Estrogen Action. *Physiological Reviews* **81** 1535-1565.

Ozers MS, Ervin KM, Steffen CL, Fronczak JA, Lebakken CS, Carnahan KA, Lowery RG & Burke TJ. 2005 Analysis of ligand-dependent recruitment of coactivator peptides to estrogen receptor using fluorescence polarization. *Molecular Endocrinology* **19** 25-34.

Pace P, Taylor J, Suntharalingam S, Coombes RC & Ali S. 1997 Human Estrogen Receptor beta Binds DNA in a Manner Similar to and Dimerizes with Estrogen Receptor alpha. *Journal of Biological Chemistry* **272** 25832-25838.

Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ & Scanlan TS. 1997 Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* **277** 1508-1510.

Pedersen SB, Fuglsig S, Sjøgren P & Richelsen B. 1996 Identification of steroid receptors in human adipose tissue. *European Journal of Clinical Investigation* **26** 1051-1056.

Pelletier G, Labrie C & Labrie F. 2000 Localization of oestrogen receptor alpha, oestrogen receptor beta and androgen receptors in the rat reproductive organs. *Journal of Endocrinology* **165** 359-370.

Pettersson K, Grandien K, Kuiper GGJM & Gustafsson JA. 1997 Mouse estrogen receptor β forms estrogen response element-binding heterodimers with estrogen receptor α. *Molecular Endocrinology* **11** 1486-1496.

Petz LN, Ziegler YS, Schultz JR, Kim H, Kemper JK & Nardulli AM. 2004 Differential regulation of the human progesterone receptor gene through an estrogen response element half site and Sp1 sites. *The Journal of Steroid Biochemistry and Molecular Biology* **88** 113-122.

Pfeilschifter J, Koditz R, Pfohl M & Schatz H. 2002 Changes in proinflammatory cytokine activity after menopause. *Endocrine Reviews* **23** 90-119.

Pike AC, Brzozowski AM, Hubbard RE, Bonn T, Thorsell AG, Engstrom O, Ljunggren J, Gustafsson JA & Carlquist M. 1999 Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. *EMBO J.* **18** 4608-4618.

Porter W, Saville B, Hoivik D & Safe S. 1997 Functional synergy between the transcription factor Sp1 and the estrogen receptor. *Molecular Endocrinology* **11** 1569-1580.

Power RF, Mani SK, Codina J, Conneely OM & O'Malley BW. 1991 Dopaminergic and ligand-independent activation of steroid hormone receptors. *Science* **254** 1636-1639.

Pratt WB & Toft DO. 1997 Steroid receptor interactions with heat shock protein and immunophilin chaperones. *Endocrine Reviews* **18** 306-360.

Pujol P, Rey JM, Roger P, Gastaldi M, Laffargue F, Rochefort H & Maudelonde T. 1998 Differential expression of estrogen receptor-alpha and -beta messenger RNAs as a potential marker of ovarian carcinogenesis. *Cancer Research* **58** 5367-5373.

Ratajczak T. 2001 Protein coregulators that mediate estrogen receptor function. *Reproduction Fertility Development* **13** 229.

Reese JC. 2003 Basal transcription factors. *Current Opinion in Genetics & Development* **13** 114-118.

Rickard DJ, Waters KM, Ruesink TJ, Khosla S, Katzenellenbogen JA, Katzenellenbogen BS, Riggs BL & Spelsberg TC. 2002 Estrogen receptor isoform-specific induction of progesterone receptors in human osteoblasts. *Journal of Bone Mineral Research* **17** 580-592.

Riggs BL. 2000 The mechanisms of estrogen regulation of bone resorption. *Journal of Clinical Investigation* **106** 1203-1204.

Roeder RG. 1996 The role of general initiation factors in transcription by RNA polymerase II. *Trends in Biochemical Sciences* **21** 327-335.

Rogatsky I, Trowbridge JM & Garabedian MJ. 1999 Potentiation of human estrogen receptor alpha transcriptional activation through phosphorylation of serines 104 and 106 by the cyclin A-CDK2 complex. *Journal of Biological Chemistry* **274** 22296-22302.

Rosenfeld CS, Yuan X, Manikkam M, Calder MD, Garverick HA & Lubahn DB. 1999 Cloning, sequencing, and localization of bovine estrogen receptor-β within the ovarian follicle. *Biology of Reproduction* **60** 691-697.

Saintier D, Burde MA, Rey JM, Maudelonde T, de Vernejoul MC & Cohen-Solal ME. 2004 17beta-estradiol downregulates beta3-integrin expression in differentiating and mature human osteoclasts. *Journal Cellular Physiology* **198** 269-276.

Saville B, Wormke M, Wang F, Nguyen T, Enmark E, Kuiper G, Gustafsson JA & Safe S. 2000 Ligand-, cell-, and estrogen receptor subtype (α /β)-dependent activation at GC-rich (Sp1) promoter elements. *Journal of Biological Chemistry* **275** 5379-5387.

Schomberg DW, Couse JF, Mukherjee A, Lubahn DB, Sar M, Mayo KE & Korach KS. 1999 Targeted disruption of the estrogen receptor-α gene in female mice: characterization of ovarian responses and phenotype in the adult. *Endocrinology* **140** 2733-2744.

Schultz JR, Loven MA, Melvin VMS, Edwards DP & Nardulli AM. 2002 Differential modulation of DNA conformation by estrogen receptors alpha and beta. *Journal of Biological Chemistry* **277** 8702-8707.

Schultz JR, Petz LN & Nardulli AM. 2003 Estrogen receptor α and Sp1 regulate progesterone receptor gene expression. *Molecular and Cellular Endocrinology* **201** 165-175.

Sergio A.Onate, Sophia Y.Tsai, Ming-Jer Tsai & Bert W.O'Malley. 1995 Sequence and characterization of a coactivator for the steroid hormone receptor superfamily. *Science* **270** 1354-1357.

Shughrue PJ, Lane MV, Scrimo PJ & Merchenthaler I. 1998 Comparative distribution of estrogen receptor-[alpha] (ER-[alpha]) and [beta] (ER-[beta]) mRNA in the rat pituitary, gonad, and reproductive tract. *Steroids* **63** 498-504.

Shupnik MA, Pitt LK, Soh AY, Anderson A, Lopes MB & Laws ER, Jr. 1998 Selective expression of estrogen receptor α and β isoforms in human pituitary tumors. *Journal of Clinical Endocrinology Metabolism* **83** 3965-3972.

Simpson E, Rubin G, Clyne C, Robertson K, O'Donnell L, Davis S & Jones M. 1999 Local estrogen biosynthesis in males and females. *Endocrine-Related Cancer* **6** 131-137.

Simpson ER & Davis SR. 2001 Minireview: Aromatase and the Regulation of Estrogen Biosynthesis--Some New Perspectives. *Endocrinology* **142** 4589-4594.

Sims NA, Dupont S, Krust A, Clement-Lacroix P, Minet D, Resche-Rigon M, Gaillard-Kelly M & Baron R. 2002 Deletion of estrogen receptors reveals a regulatory role for estrogen receptors-β in bone remodeling in females but not in males. *Bone* **30** 18-25.

Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, Williams TC, Lubahn DB & Korach KS. 1994 Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. *New England Journal Medicine* **331** 1056-1061.

Smith CL. 1998 Cross-talk between peptide growth factor and estrogen receptor signaling pathways. *Biology of Reproduction* **58** 627-632.

Snijders MP, de Goeij AF, Debets-Te Baerts MJ, Rousch MJ, Koudstaal J & Bosman FT. 1992 Immunocytochemical analysis of oestrogen receptors and progesterone receptors in the human uterus throughout the menstrual cycle and after the menopause. *Reprodion and Fertility* **94** 363-371.

Soule HD, Vazguez J, Long A, Albert S & Brennan M. 1973 A human cell line from a pleural effusion derived from a breast carcinoma. *Journal of the Natoinal Cancer Institutes* **51** 1409-1416.

Speirs V, Malone C, Walton DS, Kerin MJ & Atkin SL. 1999 Increased expression of estrogen receptorβ mRNA in tamoxifen-resistant breast cancer patients. *Cancer Research* **59** 5421-5424.

Sun J, Baudry J, Katzenellenbogen JA & Katzenellenbogen BS. 2003 Molecular basis for the subtype discrimination of the estrogen receptor-β-selective ligand, diarylpropionitrile. *Molecular Endocrinology* **17** 247-258.

Tamrazi A, Carlson KE, Daniels JR, Hurth KM & Katzenellenbogen JA. 2002 Estrogen receptor dimerization: ligand binding regulates dimer affinity and dimer dissociation rate. *Molecular Endocrinology* **16** 2706-2719.

Tate AC, Greene GL, DeSombre ER, Jensen EV & Jordan VC. 1984 Differences between estrogen- and antiestrogen-estrogen receptor complexes from human breast tumors identified with an antibody raised against the estrogen receptor. *Cancer Research* **44** 1012-1018.

Tee MK, Rogatsky I, Tzagarakis-Foster C, Cvoro A, An J, Christy RJ, Yamamoto KR & Leitman DC. 2004 Estradiol and selective estrogen receptor modulators differentially regulate target genes with estrogen receptors α and β. *MolecularBiology of the Cell* **15** 1262-1272.

Teyssier C, Belguise K, Galtier F & Chalbos D. 2001 Characterization of the physical interaction between estrogen receptor α and JUN proteins. *Journal of Biological Chemistry* **276** 36361-36369.

Toft D & Gorski J. 1966 A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. *Proceedings of the National Academy Science U S A.* **55** 1574-1581.

Torchia J, Glass C & Rosenfeld MG. 1998 Co-activators and co-repressors in the integration of transcriptional responses. *Current Opinion in Cell Biology* **10** 373-383.

Tremblay A, Tremblay GB, Labrie F & Giguere V. 1999 Ligand-independent recruitment of SRC-1 to estrogen receptor beta through phosphorylation of activation function AF-1. *Molecular Cellular.* **3** 513-519.

Tremblay GB, Tremblay A, Labrie F & Giguere V. 1998 Ligand-independent activation of the estrogen receptors alpha and beta by mutations of a conserved tyrosine can be abolished by antiestrogens. *Cancer Research* **58** 877-881.

Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F & Giguere V. 1997 Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. *Molecular Endocrinology* **11** 353-365.

Tsai MJ & O'Malley BW. 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annual Reviews in Biochemistry.* **63** 451-486.

Turner CH, Sato M & Bryant HU. 1994 Raloxifene preserves bone strength and bone mass in ovariectomized rats. *Endocrinology* **135** 2001-2005.

Umayahara Y, Kawamori R, Watada H, Imano E, Iwama N, Morishima T, Yamasaki Y, Kajimoto Y & Kamada T. 1994 Estrogen regulation of the insulin-like growth factor I gene transcription involves an AP-1 enhancer. *Journal of Biological Chemistry* **269** 16433-16442.

Van den Eynden GG, Van der Auwera I, Van Laere S, Colpaert CG, van Dam P, Merajver S, Kleer CG, Harris AL, Van Marck EA, Dirix LY & Vermeulen PB. 2004 Validation of a tissue microarray to study differential protein expression in inflammatory and non-inflammatory breast cancer. *Breast Cancer Res Treat.* **85** 13-22.

Voet D & Voet J. 1995 *Biochemistry*. New York: Jon Wiley and Sons.

Walter G, Liebl R & von Angerer E. 2004 Synthesis and biological evaluation of stilbene-based pure estrogen antagonists. *Bioorganic & Medicinal Chemistry Letters* **14** 4659-4663.

Wang H, Masironi B, Eriksson H & Sahlin L. 1999 A comparative study of estrogen feceptors α and β in the rat uterus. *Biology of Reproduction* **61** 955-964.

Waters KM, Rickard DJ, Riggs BL, Khosla S, Katzenellenbogen JA, Katzenellenbogen BS, Moore J & Spelsberg TC. 2001 Estrogen regulation of human osteoblast function is determined by the stage of differentiation and the estrogen receptor isoform. *Journal of Cellular Biochemistry.* **83** 448-462.

Webb P, Lopez GN, Uht RM & Kushner PJ. 1995 Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. *Molecular Endocrinology* **9** 443-456.

Weihua Z, Saji S, Makinen S, Cheng G, Jensen EV, Warner M & Gustafsson JA. 2000 Estrogen receptor (ER) β, a modulator of ERα in the uterus. *Proceedings of the National Academy of Sciences* **97** 5936-5941.

Weiss G, Skurnick JH, Goldsmith LT, Santoro NF & Park SJ. 2004 Menopause and hypothalamic-pituitary sensitivity to estrogen. *JAMA: The Journal of the American Medical Association* **292** 2991-2996.

Wise PM, Krajnak KM & Kashon ML. 1996 Menopause: the aging of multiple pacemakers. *Science* **273** 67-70.

Wood CE, Register TC, Anthony MS, Kock ND & Cline JM. 2004 Breast and uterine effects of soy isoflavones and conjugated equine estrogens in postmenopausal female monkeys. *Journal of Clinical Endocrinology Metabolism* **89** 3462-3468.

Wood JR, Likhite VS, Loven MA & Nardulli AM. 2001 Allosteric modulation of estrogen receptor conformation by different estrogen response elements. *Molecular Endocrinology* **15** 1114-1126.

Writing group for the WHI Investigators. 2002 Risks and benefits of estrogen plus progesting in healthy postmenopausal women: principal results from teh Women's Health Initiative randomized controlled trial. *JAMA: The Journal of the American Medical Association* **288** 321-333.

Wu X, Li H & Chen JD. 2001 The human homologue of the yeast DNA repair and TFIIH regulator MMS19 is an AF-1-specific coactivator of estrogen receptor. *Journal of Biological Chemistry* **276** 23962-23968.

Xu J, Qiu Y, DeMayo FJ, Tsai SY & O'Malley BW. 1998 Partial hormone resistance in mice with disruption of the steroid receptor coactivator-1 (SRC-1) gene. *Science* **279** 1922-1925.

Xu L, Glass CK & Rosenfeld MG. 1999 Coactivator and corepressor complexes in nuclear receptor function. *Current Opinion in Genetics & Development* **9** 140-147.

Yeh JK, Chen MM & Aloia JF. 1997 Effects of estrogen and growth hormone on skeleton in the ovariectomized rat with hypophysectomy. *AJP - Endocrinology and Metabolism* **273** E734-E742.

Zajchowski DA, Webster L, Humm R, White III, Simmons SJ & Bartholdi M. 1997 Different estrogen receptor structural domains are required for estrogen- and tamoxifen-dependent anti-proliferative activity in human mammary epithelial cells expressing an exogenous estrogen receptor. *The Journal of Steroid Biochemistry and Molecular Biology* **62** 373-383.

Zhang HZ, Bennett JM, Smith KT, Sunil N & Haslam SZ. 2002 Estrogen mediates mammary epithelial cell proliferation in serum-free culture indirectly via mammary stroma-derived hepatocyte growth factor. *Endocrinology* **143** 3427-3434.

CHAPTER III.

Independent downstream gene expression profiles in the presence of estrogen receptor alpha or beta

ABSTRACT

The two known forms of estrogen receptor (ER), α and β, exhibit differences in structure, affinity for certain ligands, and tissue distribution, suggesting differential roles. It is of interest from several perspectives to determine whether the two receptors elicit similar or differing responses within the same cell type in the presence of the same ligand. To evaluate roles of ER we have examined responses to estrogen in a rat embryonic fibroblast cell line model, normally naive to ER, engineered to stably express ERα or ERβ. Rat1+ERα, Rat1+ERβ, and precursor Rat1 cell lines were treated with estradiol-17β (E_2; [1 nM]) or an ethanol vehicle for 24 hours. Total RNA was extracted, and cDNA generated and subjected to suppression subtractive hybridization (SSH), followed by differential screening using dot blot hybridization. In the presence of ERα products were identified that represent classic responses to E_2, including markers for cell proliferation. In the presence of ERβ an alternate transcription profile was observed, including up-regulation of pro-alpha-2(I) collagen. These data support a model in which ERα and ERβ regulate unique subsets of downstream genes within a given cell type.

INTRODUCTION

The estrogen signaling system plays a critical role in the physiology of the reproductive organs, as well as the cardiovascular, skeletal and central nervous systems, and in carcinogenesis. Estrogen receptors (ER) are members of the steroid receptor family and function as ligand-inducible transcription factors. Upon binding of the ligand, ER within the nucleus undergo phosphorylation (1, 2), dimerization (3), and binding to DNA at specific cis-acting sites termed estrogen response elements (ERE) (4, 5). The receptor protein is next involved in the recruitment of co-regulator proteins and other protein-protein interactions, resulting ultimately in modification of the rate of transcription from the target promoter (4, 6, 7). In some instances the capability exists for ligand independent activation through phosphorylation pathways (8,9), including MAP-kinases (10). Nuclear estrogen receptors mediate most of the actions of estrogens resulting in transcriptional activation and repression, control of cell cycle progression, and integration of intracellular signaling pathways (11).

Toft and Gorski (12) first isolated and began characterizing a receptor protein for estrogen in 1966 and through 1994 only one receptor isoform had been identified. In 1995 the second estrogen receptor, ERβ, was identified from rat prostate (13) and since that time ERβ has been characterized in the mouse (14), human (15), and numerous other species. Variations exist in the tissue distribution of ERα and ERβ, though they appear to have similar gene expression levels in the testis, epididymis, bone, and adrenal gland (16). ERα predominates in the proliferative cells of the mammary, pituitary and thyroid glands, as well as in uterus, skeletal muscle and the smooth muscle of the coronary

arteries, while ERβ is predominant in the prostate (13), granulosa cells of the ovary, and the lung, bladder, brain and hypothalamus (17). In tissues where both receptors are present it has been shown that the receptors may form either homodimers or heterodimers (18-20).

While there is great similarity in their DNA- and ligand- binding domains, the two ER forms exhibit significant structural differences, especially in the NH_2-terminal A/B domain and the COOH-terminal F domain, and there is some variation in affinity for certain ligands (13,17), suggesting differential physiological roles. Several complementary approaches have been used to verify and characterize these differences. Phenotypic variation between mouse knockout models for ERα (21, 22) and ERβ (23) suggest that, in addition to differences in tissue distribution, the receptors exert different effects in the same tissue. These models have provided valuable insights into the differential roles of the estrogen receptors. It is difficult, however, with knockout models to separate developmental effects from functional effects in adult tissue. Ligands designed to activate only ERα or ERβ have been used to show differential effects of the two receptors (24, 25), and contributed significantly to understanding estrogen signaling, although limitations include the degree of selectivity by the ligand for one receptor over the other. Stable expression of the receptors in a naïve cell line, such as fetal osteoblasts (26-28), in culture has resulted in the demonstration of responses specific to ERα and ERβ mediated signaling. This approach allows for characterization of the roles of the two receptors independent of one another in the presence of the same natural ligands. Although there are limitations with this approach as well, we have similarly examined

gene expression specifically regulated by ERα or ERβ in a set of engineered rat embryonic fibroblast cell lines (29-31). Utilizing the technique of suppression subtractive hybridization (SSH) we have attempted to identify examples of unique downstream genes autonomously activated by each receptor within the same cell type in response to the same ligand.

MATERIALS AND METHODS

CELL CULTURE AND REPORTER ASSAY

Cell Culture. Rat1 fibroblast cell lines (29) stably expressing either a mutant human HEG0 ERα (Rat1+ERα) (30) or rat ERβ (Rat1+ERβ) (31), were used as a model system for examining unique subsets of downstream genes regulated by each receptor type. Cells were grown in sterile filtered (0.22 μM), phenol red – free Dulbecco's Modified Eagle Medium (DMEM; Gibco-BRL, Grand Island NY), with $NaHCO_3$ (3.7 g/L). The cells were supplemented with bovine insulin (0.6 μg/ml) in HEPES (25 μM) (Sigma, St. Louis MO), 1X antibiotic – antimycotic (Sigma, St. Louis MO), and 10% charcoal – stripped/ dextran treated fetal bovine serum (CSFBS; Hyclone, Logan UT). Cells were maintained in approximately 0.133 ml/cm^2 medium at 37˚C with a humidified atmosphere of 5% CO_2 gas and 95% air, and media was replaced every 48 hours. Additionally, Rat1+ERα cells were supplemented with Hygromyocin B (100 μg/ml) in PBS (Gibco-BRL, Grand Island NY) beginning 24 h after plating (30). Rat1+ERβ cells were supplemented with Geneticin (50 μg/ml) (Gibco-BRL, Grand Island NY) (31).

CAT Reporter Assay. A chloramphenicol acetyltransferase (CAT) reporter assay was used to verify a functional response to E_2 by ER in each cell line and to verify that the precursor Rat1 cell line lacked ER expression and response to E_2. Cells were plated at 400,000 cells per well in a 6 well cluster (35 mm diameter) tissue culture plate (Costar, Cambridge MA), and transfected in triplicate with pERE15 (32) construct to determine E_2 responsiveness as described by Cheng and Malayer, 1999 (31). After a 24-hour recovery period in DMEM, transfected cells were treated with either E_2 [1nM] (98% 17-β estradiol; Sigma, St. Louis MO), or an equal volume of ethanol vehicle for 24 hours. Protein lysates were prepared and incubated with [^{3}H]chloramphenicol and n-butyryl-coenzymeA. Following extraction acetylated [^{3}H]chloramphenicol levels in the organic phase were measured via scintillation spectroscopy (31).

Treatments and RNA Extraction. Cells were grown in 225 cm^2 cell culture flasks (Corning, Corning NY) to 80% confluency and then treated with a single dose of E_2 [1 nM]. After 24 hours cells were washed 3 times in PBS and total RNA extracted as described by Chomczynski and Sacchi (33).

Detection of Unique Gene Expression

Suppression Subtractive Hybridization (SSH). RNA was subjected to SSH (34) modified to a kit available from BD Biosciences (BD Biosciences Clontech, Palo Alto CA). Total RNA (3-5 μg) extracted from cell culture, was used to generate cDNA from Rat1+ERα, Rat1+ERβ, and precursor Rat1. The cDNA was cut with *Rsa*I (BD Biosciences Clontech, Palo Alto CA), and 2 different adaptors (34) were ligated onto individual

aliquots of the tester population. Hybridizations were carried out as described by Mohan et al. (35) with modifications. The first hybridization was carried out on the tester with either Adaptor 1 or 2R, and these were heat denatured and hybridized in the presence of excess denatured driver. A second hybridization was then performed where the two tester populations were combined in the presence of fresh denatured driver. Due to the presence of the adaptors only those genes unique to the tester population were enriched and amplified in the PCR reaction (35). PCR products from SSH were then compared against unsubtracted products. To further enrich the products a secondary PCR was performed using nested primers to the adaptors.

Analysis of Unique Gene Expression

Cloning and Differential Analysis - Qualitative. Secondary PCR products were cloned into a pCRII Topo T/A vector (Invitrogen, Carlsbad, CA), and resulting colonies screened by differential analysis using DIG- labeled probes (Roche Molecular Biochemicals, Indianapolis IN) to verify subtractions. Due to the large number of inserts being transfected into the Topo vector, an extended incubation (1 hr) was used. Following chemical transfection, colonies were grown on selective agar plates at 37° C overnight. Colonies from the Rat1+ERβ tester population (96 clones) and from the Rat1+ERα tester population (120 clones) were then picked and grown in Terrific Broth (Fisher Biotech, Fair Lawn NJ) supplemented with carbanocillin (100 μg/ml; ICN, Costa Mesa, CA) in 96 well culture plates (Promega, Madison WI). DNA purification was carried out using Promega SV96 DNA purification system, and plasmid DNAs were spotted onto a series of 8.5 X 12 cm positively charged nylon membranes (Roche

Molecular Biochemicals, Indianapolis IN) using a BioDot apparatus (BioRad, Hercules, CA). Purified DNA was chemically denatured using denaturing solution [0.5 M NaOH, 1.5M NaCl] for 5 - 10 min in a 96 well plate and equally distributed to the BioDot apparatus followed by gentle vacuum for 1 min. Neutralization solution [0.7M Tris-HCl pH8.0, 1.5M NaCl] was then added to wells for 5 – 10 min. Membranes were rinsed in 2XSSC for 1 – 2 min, UV cross-linked, and stored at 4ºC.

Probes were prepared from the secondary PCR products generated by SSH. These were digested with *Rsa*I (Gibco-BRL, Grand Island NY) to remove adaptors, which cause background during hybridization. Excess inactivated enzyme, buffer, and adaptors were removed using Qiagen PCR purification columns (Qiagen, Valencia, CA). After denaturation (95˚C, 7 min) to generate ssDNA, SSH secondary PCR products [1 μg] were added to a pre-mixed DIG-HIGH Prime® containing; random primers, nucleotides, Klenow enzyme, buffers and the DIG-dUTP (Roche Molecular Biochemicals, Indianapolis IN), and incubated for 20 h at 37˚C. The reaction was stopped by heating to 65˚C for 10 min, and the labeled products were stored at -20˚C. Labeling efficiency was performed according to the manufactures instructions and compared to standard labeled product (Roche Molecular Biochemicals, Indianapolis IN) based on chemiluminescence (CSPD) detection. Labeled PCR probes were used at a concentration of 25 ng/ml in DIG EasyHyb hybridization solution (Roche Molecular Biochemicals, Indianapolis IN).

Membranes were pre-hybridized in DIG Easy-Hyb solution for 15 min (42˚C; 10 ml/100 cm^2 membrane) and probed in DIG EasyHyb solution with 25 ng/ml probe overnight (42˚C; 3.5 ml/100cm^2). Membranes were then washed as described by the manufacturer

to remove excess probe. Bound probe was detected using an anti-DIG conjugate for 30 min (75 mU/ml in 40 ml) at room temperature under constant agitation. Detection by CSPD was carried out via enzyme immunoassay (Roche Molecular Biochemicals, Indianapolis IN). Spots were qualitatively analyzed for signal intensity after exposure to Kodak X-OMAT LS 8 X 10 X-ray film (Kodak, Rochester NY). Comparison of subtracted and unsubtracted probes was conducted using criteria detailed in the Clontech Differential Screening manual (BD Biosciences Clontech, Palo Alto CA). Primary candidates for sequencing (Figure 2C; denoted +,+,-,-) hybridized to both subtracted and unsubtracted tester probes. Low abundance (Figure 2C; denoted +, -,-,-) products which hybridized only to subtracted tester probes were also sequenced. Colonies that were positive to the subtracted probe for the tester and driver and had 5-fold intensity in the tester were also picked and sequenced (Figure2C; denoted +>5, +, +, -). Colonies positive in the unsubtracted driver were sequenced (Figure 2C; denoted +, +, -, +). Though present in the driver population the products were in such low abundance as to not be detected through SSH, and therefore the differences between populations are still significant. Colonies positive on all four membranes were not considered.

Automated Sequencing. Once differential analysis of the subtracted products was verified, automated sequencing was carried out by the OSU Recombinant DNA/ Protein Resource Facility, and results analyzed using MacVector 7.0 in conjunction with NCBI Basic Local Alignment Search Tool (36) to identify homologous sequences in the GenBank database.

Real time PCR- Quantitative. Taqman® primers and probes were generated using Primer Express® software (PE Applied Biosystems, Foster City CA) to gene targets of interest defined by SSH, qualitative analysis, and sequence information. Quantitative PCR was then carried as described by Hettinger et al. (37) with modifications (38-40). ABI primer probe sets were generated from sequences deduced from SSH products. Probes contained a 3' fluorescent TAMRA quencher dye, and a 5' FAM reporter dye. Expression was examined for four individual targets in triplicate using total RNA (10 ng) from each of the treatment schemes for the cell lines (Rat1+ERα, Rat1+ERβ, Rat1 treated with E_2 or vehicle) by means of primer [300 nM] and probe [200 nM] sets shown in Table 1. Each population of total RNA (50 pg) was normalized in duplicate using 18S ribosomal RNA (Ribosomal RNA control kit, PE Biosystems) at a [200 nM] primer [100 nM] probe concentration, and the efficiency was checked via a standard curve of serial dilutions of Rat1+ERα E_2 treated. For individual targets 10 pg, 100 pg, 1 ng, 10 ng, 100 ng was used. For 18S ribosomal RNA 500 pg, 50 pg, 10 pg, 5 pg, and 1 pg was used. Real-time PCR was carried out in the ABI PRISM 7700 (PE Applied Biosystems, Foster City CA) under the following thermal cycler conditions; 48˚C for 30 min and 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min, in a 25 μL reaction. Analysis and fold differences were determined using the comparative C_T method as described in the ABI technical bulletin #2 for the ABI PRISM 7700 (38-40), where the Rat1 vehicle treated cell line was used as a calibrator.

Standard Reverse Transcription (RT) - PCR – Semi-quantitative. Reverse transcription followed by PCR was carried out for additional putative gene products identified through

SSH and differential screening, to further check for the presence of false positives in our data set. Annexin 1 (935 bp) identified in the Rat1 + ERα tester population, and Nuclear factor I/B (963 bp) identified in the Rat1+ERβ tester population were selected, and primers generated via MacVector® software (Table 2). Primers were synthesized by Integrated DNA Technologies (IDT: Coralville, Iowa), and G3PDH (500 bp) was run simultaneously as a PCR loading control. Conditions were carried out as described in Table 2.

Statistical Analysis. Results for CAT assay were analyzed with the Statistical Analysis System (SAS; Package 8.0) by constructing a 3 cell type by 2 treatment factorial Analysis of Variance (ANOVA) table and using least square differences in PROC-GLM to determine significant differences. Statistical comparison of RT-qPCR values with means ± S.D. were reported where n = 3 and results were tested using least square differences, reported as PROC-MIXED in a 3 cell type by 2 treatment factorial ANOVA table constructed using the SAS.

RESULTS

VERIFICATION OF E_2 STIMULATION

Similar to previously reported results, chloramphenicol acetyltransferase reporter assay confirmed an increase of pERE15 reporter gene activity in the presence of estradiol-17β (E_2) [1 nM] after 24 hours in cell lines expressing ERα and ERβ (31) (Figure 1). In the presence of ERα or ERβ, there was a significant increase in chloramphenicol

acetyltransferase activity ($P<0.01$), as determined by the amount of acetylated [^{3}H] chloramphenicol present following E_2 treatment, compared to either ethanol vehicle treatment or the parental cell line which does not express ERα or ERβ. There was no significant difference in chloramphenicol acetyltransferase activity between Rat1+ERα and Rat1+ERβ cells following E_2 treatment. This is in accordance with previously published data showing a 10X higher K_d of the HEG0 mutant (41) present in the Rat1+ERα cell line.

SUPPRESSION SUBTRACTIVE HYBRIDIZATION

Profiles of unique gene products were evaluated through comparisons of subtracted SSH products to unsubtracted controls (34). Four experiments were carried out, each consisting of a forward and reverse reaction: 1) comparison of Rat1+ERα and Rat1+ERβ following E_2 stimulation; 2) comparison of Rat1+ERα and Rat1+ERβ following vehicle treatment; 3) comparison of Rat1+ERα and Rat1 following E_2 stimulation; and 4) comparison of Rat1+ ERβ and Rat1 following E_2 stimulation. G3PDH (500 bp) efficiency controls were carried out to confirm that there was a decrease in levels of G3PDH in subtracted products. This was demonstrated by an increase in number of PCR cycles required to amplify G3PDH in comparison to the unsubtracted control.

QUALITATIVE ANALYSIS OF DIFFERENTIAL SCREENING FOLLOWING SSH

A total of 814 SSH products were screened in 4 forward and reverse experiments outlined above, and of these 208 were identified as differentially expressed through dot-blot

hybridization assays using digoxigenin (DIG)-labeled probes (Figure 2). These candidates were subjected to single-pass sequencing and BLAST analysis was performed (33). When clones from the four experiments were examined, 150/208 returned quality sequence data and 107/150 showed identity to known sequences with a 44% rate of redundancy, while 43/150 had no significant match to any known sequence with a 30% rate of redundancy (Tables 3,4,5). In the presence of E_2, Rat1+ERα appeared more robust in terms of gene activation and approximately 26 unique products exhibited identity to known genes (Table 3), while 6 showed no homology to GenBank sequences. Within the homologous putative genes, classic E_2 responsive gene products involved in cell growth, transcriptional activity and signal transduction were found. When Rat1+ERβ with E_2 stimulation was utilized as tester, 4 unique gene homologs were identified (Table 4), and 1 showed no homology. In vehicle treated experiments most results aligned with ribosomal RNA or mitochondrion products (Table 3 and 4). However, within the Rat1+ERα vehicle treated tester population a match occurred to procollagen C – proteinase enhancer protein (PCOLE) (Table 3). When the parental cell line was used as the tester the majority of products showed no homology to known sequences, or aligned to ribosomal or mitochondrion products (Table 5). These data support the concept that, in addition to differences in tissue distribution, ERα and ERβ regulate both overlapping and unique subsets of downstream genes in the same genetic background.

QUANTITATIVE RT-PCR ANALYSIS OF TARGET GENES OF INTEREST IDENTIFIED THROUGH SSH

Targets of particular interest, especially genes considered to be involved in inflammatory responses or MAP-kinase related pathways were selected for real-time quantitative RT-

PCR (RT-qPCR) (38-40) and normalized to 18S rRNA expression. Pro-alpha-2(I) collagen (COL1A2), procollagen C- proteinase enhancer protein (PCOLE), cathepsin L (CtsL), and receptor for activated protein kinase C (RACK1) were selected for RT-qPCR and analyzed using the comparative cycle threshold (C_T) method (Table 6). Validation of RT-qPCR efficiency was determined by the evaluation of the R^2-value of the standard curve for 18S ribosomal RNA which was 0.9818, with the R^2-values of the individual targets varying by less than $\pm$ 0.01 from this value. Efficiency of the PCR was further measured by the equation $((10^{1/-s})-1)$ and found to be $\geq$90% (40).

Pro-alpha-2(I) collagen was identified from the Rat1+ERβ E_2 tester/ Rat1+ERα E_2 driver comparison and increased 4-fold in the Rat1+ERβ cell line versus the Rat1+ERα following E_2 treatment ($p \leq 0.05$). An increase in COL1A2 of 14-fold was observed in the Rat1 + ERβ E_2 treated cell line over the predetermined calibrator Rat1 vehicle treated cells (Figure 3A).

Cathepsin L and RACK1 were identified from Rat1 + ERα E_2 tester/ Rat1+ERβ E_2 driver comparisons. The RACK1 target had a 2-fold increase in Rat1+ERα E_2 stimulated cells compared to Rat1 + ERβ E_2 cells ($p \leq 0.05$) (Figure 3B), and CtsL increased 2-fold in Rat1+ERα cells when compared to Rat1 + ERβ E_2 cells ($p \leq 0.05$) (Figure 3C).

Procollagen C – proteinase enhancer protein was identified in the Rat1+ERα vehicle treated tester subtracted from Rat1+ERβ vehicle treated driver comparison. However,

PCOLCE turned out to be a false positive in the population it was identified in as Rat1+ERα vehicle treated showed significantly lower expression levels than Rat1+ERβ vehicle treated ($p \leq 0.05$). Interestingly RT-qPCR detected a 3-fold increase in Rat1+ERα E_2 versus Rat1+ERβ E_2 ($p \leq 0.05$) (Figure 3D).

SEMI-QUANTITATIVE ANALYSIS OF ADDITIONAL GENES OF INTEREST USING RT-PCR

Semi-quantitative reverse transcription-PCR analysis of gene targets was also used to confirm the validity of targets detected through SSH. A 935 bp product for annexin 1 was detected in Rat1+ERα E_2 stimulated cDNA as expected (Figure 4A). A 963 bp product for nuclear factor I/B was detected in Rat1+ERβ E_2 stimulated, as well as in Rat1+ERα vehicle treated cells (Figure 4B). These data are congruent with the profiles predicted through SSH and differential screening.

DISCUSSION

The study of ERα and ERβ are of principal interest in relation to the critical role of the estrogen signaling system in the physiology of the reproductive organs, the cardiovascular, skeletal and central nervous systems, and in carcinogenesis. There is particular interest in the selective activation of tissue-specific responses within the context of hormone replacement therapy and treatment of disease (42, 43). There exists a need to better determine the involvement of each receptor in eliciting downstream gene expression both in tissues where the receptors function autonomously, and especially where both are present. For these reasons we have examined the nature of unique gene

regulation by ERα and ERβ in this undifferentiated model system designed to isolate each receptor protein from the influence of the other.

The known structural differences in ERs suggest unique independent downstream gene function. The amino and carboxyl terminal domains of the ERα protein are significantly longer than those of ERβ (13,14). These domains interact with the various co-regulator proteins crucial for transcriptional activation by the receptor to elicit cell specific responses (14). Furthermore, differences in amino acid composition and conformational differences within the binding pocket exist between ERα and ERβ (44, 45) and these likely account for differences in affinity for various ligands. It is these differences that are at the heart of such pharmaceuticals as selective estrogen response modulators (SERMs).

Using SSH, we have identified a profile of products differentially regulated by ERα and ERβ in response to exposure to a single dose of E_2 for 24 hours. This represents a small subset of potential target genes, as any response that would have occurred prior to 24 h, or at an alternative dose level, would not be recognized. Further, the experimental protocol has limited the number of products identified by limiting the number of colonies examined to 814 overall. Following sequencing of the cDNAs in the experimental set involving Rat1+ERβ tester population, only 5 gene products were found, and among these, redundancy was as high as 47% for human gastric associated differentially expressed protein. Conversely when Rat1+ERα was the tester population, there was low redundancy in the population of sequenced cDNA clones, no greater than 14%

redundancy for one unidentified product. Therefore it is expected that cloning and sequencing of more Rat1+ERα colonies would result in identification of an increased number of target gene products, including other known targets for E_2 modulation such as the progesterone receptor.

Among downstream genes identified in the present study, several were of particular interest due to potential involvement in processes associated with reproduction, diseases of aging, and carcinogenesis. Their quantitative expression following treatment was characterized to verify responses seen in the SSH analysis. In relation to bone and cartilage remodeling, type 1 collagen accounts for the majority of total collagens and is most abundant in bone (46). Additionally, COL1A2 is one of two alpha chains that comprise one-third of the type I collagen heterotrimer (47). PCOLCE is a glycoprotein enhancer element that binds the C-terminus of the type I procollagen propeptide and as such enhances the enzymatic ability of procollagen C-proteinase (48). It is present in high levels in the uterus (49), and plays a role in intracellular collagen formation and extracellular cell differentiation and proliferation, as well as possible stabilization of COL1A2 mRNA (50).

RACK1 binds the isozyme protein kinase C (PKC) and acts to stabilize the active conformation of PKC, which is necessary for subcellular translocation (51). RACK 1 is a homologue of guanine nucleotide-binding protein (G-protein) β subunit (51) and ERα studies have noted a relationship between E_2 and G - protein coupled receptors to affect PKC (52). Cathepsin L is a lysosomal cysteine protease implicated in human trophoblast

invasiveness (53), bone resorption (54), and degradation of extracellular matrix (55) to a name few cellular functions that tie into reproduction, inflammatory responses, and bone remodeling. It also has implications in oncogenesis and tumor invasiveness.

Additionally, to further verify our SSH profiles, annexin 1 and nuclear factor I/B (NFI-B) were selected for RT-PCR due to their role in the anti-inflammatory response and transcriptional regulation, respectively. Annexin 1 interacts with glucocorticoids to act in suppressing inflammation (56), which is an important component of implantation. Additionally, annexin 1 plays a role in cell growth and regulation (56), as well as DNA unwinding (57). Nuclear factor I/B is a member of the nuclear factor family and has a role in regulation of gene transcription through promoter interactions (58).

Due to the structural similarities in the ligand binding domains of ERα and ERβ, and the ability to form heterodimers *in vivo*, it becomes important to definitively separate the two ERs. An engineered cell line that truly expresses only one subtype offers numerous avenues for examining independent ER function. As an undifferentiated fibroblast cell line this model offers insight into the function of these ER in naïve developing systems as well as in a more global expression profile due to decreased complexity in comparison to highly differentiated cell lines. Previous studies have verified that the basic architecture is still present for the engineered cells to function in a physiologically relevant manner, such as the ability to activate progesterone receptor which is silent in the parental Rat1 cells (30-31). While not an alternative to *in vivo* studies, the Rat1, Rat1+ERα, and

Rat1+ERβ cell lines can indicate initial areas of interest at the gene level, which can in turn lead to directed, in-depth, focused physiological studies.

While there is overlap in the downstream targets for genomic effects in response to E_2, it is evident that ERα and ERβ are responsible for regulation of expression of unique subsets of downstream genes in response to the same ligand in the same cell type. This is in agreement with recent microarray studies at similar time-points that have examined the differing global profiles of ERα or ERβ in differentiated cell types (59). However, the genes identified by Monroe, et al. 2003 do not overlap with those profiles found in the present study. This is most likely due to the nature of the different levels of differentiation and complexity between models used, as well as the unique properties of SSH. In this study, ERα appeared to be responsible for regulating a larger number of products, of which a majority was involved in general cell house keeping and proliferation. ERβ activation resulted in fewer detectable products in comparison to ERα and most of these had specific cellular functions, although some general regulatory products were also detected. Differential activity of these two receptors in response to the same ligand has important implications for understanding cell regulatory functions and inflammatory responses, which are integral to reproductive processes, as well as oncogenesis, bone remodeling, and aging.

REFERENCE LIST:

1. Arnold SF, Melamed M, Vorojeikina DP, Notides AC, Sasson S. Estradiol-binding mechanism and binding capacity of the human estrogen receptor is regulated by tyrosine phosphorylation. Mol Endocrinol 1997; 11: 48-53
2. Rogatsky I, Trowbridge JM, Garabedian MJ. Potentiation of human estrogen receptor α transcriptional activation through phosphorylation of serines 104 and 106 by the cyclin A- CDK2 complex complex. J Biological Chemistry 1999; 274: 22296-22302
3. Fawell SE, Lees JA, White R, Parker MG. Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor. Cell 1990; 60: 953-62
4. Hall JM, McDonnell DP, Korach KS. Allosteric regulation of estrogen receptor structure, function, and coactivator recruitment by different estrogen response elements. Molecular Endocrinology 2002; 16: 469-486
5. Schultz JR, Loven MA, Melvin VMS, Edwards DP, Nardulli AM. Differential modulation of DNA conformation by estrogen receptors α and β. J Biol Chem 2002; 277: 8702-8707
6. Xu L, Glass CK, Rosenfeld MG. Coactivator and compressor complexes in nuclear receptor function. Current Opinion in Genetics and Development1999; 9: 140-147
7. McKenna NJ, Lanz RB, O'Malley BW. Nuclear receptor coregulators: cellular and molecular biology. Endocrine Reviews 1999; 20: 321-344

8. Power RF, Mani SK, Codina J, Conneely OM, O'Malley BW. Dopaminergic and ligand-independent activation of steroid hormone receptors. Science 1991; 254: 1636-1639
9. Bunone G, Briand PA, Miksicek RJ, Picard D. Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. EMBO J 1996; 15: 2174-2183
10. Kato S, Endoch H, Masuhiro Y, Kitamoto T, Uchiyama S, Haruna S, Masushige S, Gotch Y, Nishida E, Kawashima H, Metzger D, Chambon P. Activation of the estrogen receptor through phosphrylation by mitogen – activated protein kinase. Science 1995; 270: 1491 – 1494
11. Moggs JG, Orphanides G. Estrogen receptors: orchestrators of pleiotropic cellular responses. EMBO Reports 2001; 2: 775-781
12. Toft D, Gorski J. A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. Proc Natl Acad Sci 1966; 55: 1574 – 1581
13. Kuiper GGJM, Enmark E, Pelto-Huikko M, Nilsson S, Gustafsson J-A. Cloning of a novel estrogen receptor expressed in rat prostate and ovary. Proc. Natl Acad Sci USA 1996; 93: 5925-5930
14. Tremblay GB, Tremlay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, Giguere V. Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. Mol Endocrinol 1997; 11: 353-365
15. Mosselman S, Polman J, Dijkema R. ERβ: identification and characterization of a novel human estrogen receptor. FEBS Letters 1996; 392: 49-53

16. Couse JF, Korach KS. Estrogen receptor null mice: what have we learned and where will they lead us? Endocrine Reviews 1999; 20: 358-417

17. Kuiper GGJM, Carlsson B, Grandien KAJ, Enmark E, Haggblad J, Nilsson S, Gustafsson J-A. Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. Endocrinology 1997; 138: 863-870

18. Pettersson K, Grandien K, Kuiper GGJM, Gustafsson J-A. Mouse estrogen receptor β forms estrogen response element-binding heterodimers with estrogen receptor α. Mol Endocrinol 1997; 11: 1486-1496

19. Cowley SM, Hoare S, Mosselman S, Parker MG. Estrogen receptors α and β form heterodimers on DNA. J Biol Chem 1997; 272:19858-19862

20. Pace P, Taylor J, Suntharalingam S, Coombes RC, Ali S. Human estrogen receptor β binds DNA in a manner similar to and dimerizes with estrogen receptor α. J Biol Chem 1997; 41: 25832-25838

21. Lubahn DB, Moyer JS, Golding, TS, Couse JF, Korach KS, Smithies O. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. Proc Natl Acad. Sci USA 1993; 90: 11162-11166

22. Das SK, Taylor JA, Korach KS, Paria BC, Dey SK, Lubahn DB. Estrogenic responses in estrogen receptor-α deficient mice reveal a distinct estrogen sigaling pathway. Proc Natl Acad Sci USA 1997; 94: 12786-12791

23. Krege JH, Hodgin JB, Couse JF, Enmark E, Warner M, Mahler JF, Sar M, Korach KS. Gustafsson J-A, Smithies O. Generation and reproductive phenotypes of mice lacking estrogen receptor β. Proc Natl Acad Sci USA 1998; 95: 15677-15682

24. Harris HA, Katzenellenbogen JA Katzenellenbogen BS. Characterization of the biological roles of the estrogen receptors, ERalpha and ERbeta, in estrogen target tissues in vivo through the use of an ERalpha-selective ligand. Endocrinol 2002; 143:4172-7.

25. Waters KM, Rickard DJ, Riggs BL, Khosla S, Katzenellenbogen JA, Katzenellenbogen BS, Moore J, Spelsberg TC. Estrogen regulation of human osteoblast function is determined by the stage of differentiation and the estrogen receptor isoform. J Cell Biochem 2001; 83:448-62.

26. Rickard DJ, Waters KM, Ruesink TJ, Khosla S, Katzenellenbogen JA, Katzenellenbogen BS, Riggs BL, Spelsberg TC. Estrogen receptor isoform-specific induction of progesterone receptors in human osteoblasts. J Bone Miner Res 2002; 17:580-92.

27. Harris SA, Tau KR, Enger RJ, Toft DO, Riggs BL, Spelsberg TC. Estrogen response in the hFOB 1.19 human fetal osteoblastic cell line stably transfected with the human estrogen receptor gene. J Cell Biochem 1995; 59:193-201.

28. Monroe DG, Johnsen SA, Subramaniam M, Getz BJ, Khosla S, Riggs BL, Spelsberg TC. Mutual antagonism of estrogen receptors alpha and beta and their preferred interactions with steroid receptor coactivators in human osteoblastic cell lines. J Endocrinol 2003; 176: 349-57.

29. Freeman A, Price P, Igel H, Young J, Maryak J, Huebner R. Morphological transformation of rat embryo cells induced by diethylnitrosamine and murine leukemia viruses. J Natl Caner Inst 1970; 44: 64-78
30. Kaneko KJ, Gelinas C, Gorski J. Activation of the silent progesterone receptor gene by ectopic expression of estrogen receptors in a rat fibroblast cell line. Biochem 1993; 32: 8348-8359
31. Cheng J, Malayer JR. Responses to stable ectopic estrogen receptor-beta expression in a rat fibroblast cell line. Mol Cell Endocrinol 1999; 156: 95-105
32. Luckow B, Schutz G. CAT constructions with multiple unique restriction sites for the functional analysis of eukaryotic promoters and regulatory elements. Nucleic Acids Res 1987; 15: 5490
33. Chomczynski P, Sacchi N. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1987; 162: 156-159
34. Diatchenko L, Lau Y-F, Campbell AP, Chenchik A, Maqadam F, Huang B, Lukyanov S, Lukyanov K, Gurckaya N, Sverdlov ED, Siebert PD. Suppression subtractive hybridization: a method for generating differentially regulated or tissue-specific cDNA probes for libraries. Proc Natl Acad Sci *USA* 1996; 93: 6025-6030
35. Mohan M, Ryder S, Claypool PL, Geisert RD, Malayer JR. Analysis of gene expression in the bovine blastocyst produced in vitro using suppression-subtractive hybridization. Biol Reproduciton 2002; 67: 447-453

36. Altchul S, Gish W, Miller W, Myers E, Lipman D. Basic local alignment search tool. J Mol Biol 1990; 215: 403-410

37. Hettinger AM, Allen MR, Zhang BR, Goad DW, Malayer JR, Geisert RD. Presence of the acute phase protein, bikunin, in the endometrium of gilts during estrous cycle and early pregnancy. Biol Reproduction 2001; 65: 507-513

38. Gibson UE, Heid CA, Williams PM. A novel method for real time quantitative RT-PCR. Genome Res 1996; 6: 995-1001

39. Bustin SA. Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. J Mol Endcrinol 2002; 29: 23-39

40. Ginzinger DG. Gene quantification using real-time quantitative PCR: an emerging technology hits the mainstream. Experimental Hematology 2002; 30: 503-512

41. Tora L, Mullick A, Metzger D, Ponglikitmongkol M, Park I, Chmbon P. The cloned human oestrogen receptor contains a mutation which alters its hormone binding properties. EMBO J 1989b; 8: 1981-1986

42. Pike ACW, Brzozowski AM, Hubbard RE, Engström O, Ljunggren J, Gustafsson JÅ, Carlquist M. Structure of the ligand binding domain of oestrogen receptor beta in the presence of a partial agonist and a full agonist. The EMBO J 1999; 18: 4608-4618

43. Sun J, Baudry J, Katzenellenbogen JA, Katzenellenbogen BS. Molecular basis for the subtype discrimination of estrogen receptor-β-selective ligand, diarylpropionitrile. Mol Endocrinol 2003; 17: 247-258

44. McDonnell DP. The molecular pharmacology of SERMs. TEM 1999; 10: 301-310

45. Katzenellenbogen BS, Sun J, Harrington WR, Kraichely DM, Ganessunker D, Katzenellenbogen JA. Structure-function relationships in estrogen receptors and the characterization of novel selective estrogen receptor modulators with unique pharmacological profiles. Ann NY Acad Sci 2001; 946: 6-15
46. Nimni ME. Collagen: structure, function, and metabolism in normal and fibrotic tissues. Semin Arthritis Rheum 1983; 13: 1-86
47. Smith BD, Niles R. Characterization of collagen synthesized by normal and chemically transformed rat liver epithelial cell lines. Biochemistry 1980; 19: 1820-1825
48. Takahara K, Kessler E, Biniaminov L, Brusel M, Eddy RL, Jani-Sait S, Shows TB, Greenspan DS. Type I procollagen COOH-terminal proteinase enhancer protein identification, primary structure, and chromosomal localization of the cognate human gene (PCOLCE). J Biol Chem 1994; 269: 26280-26285
49. Scott IC, Clark TG, Takahara K, Hoffman GG, Greenspan DS. Structural organization and expression patterns of the human and mouse genes for the type I procollagen COOH-terminal proteinase enhancer protein. Genomics 1999; 55: 229-234
50. Matsui A, Yanase M, Tomiya T, Ikeda H, Fujiwara K, Ogata I. Stabilization of RNA strands in protein synthesis by type I procollagen C-proteinase enhancer protein, a potential RNA-binding protein, in hepatic stellate cells. Biochem and Biophys Res and Communication 2002; 290: 898-902

51. Ron D, Chen C-H, Caldwell J, Jamieson L, Orr E, Mochley-Rosen D. Cloning of an intracellular receptor for protein kinase C: a homolog of the β subunit of G proteins. Proc Natl Acad Sci *USA* 1994; 91: 839-843

52. Kelly MJ, Lagrange AH, Wagner EJ, Rønnekleiv OK. Rapid effects of estrogen to modulate G protein-coupled receptors via activation of protein kinase A and protein kinase C pathways. Steroids 1999; 64: 64 – 75

53. Divya , Chhikara P, Mahajan VS, Datta Gupta S, Chauhan SS. Differential activity of cathepsin L in human placenta at two different stages of gestation. Placenta 2002; 23: 59-64

54. Kakegawa H, Nikawa T, Tagami K, Kamioka H, Sumitani K, kawata T, Drobnič-Kosorok M, Lenarcic B, Turk V, Katunuma N. Participation of cathepsin L on bone resorption. FEBS 1993; 321: 247-250

55. Mason RW, Johnson DA, Barrett AJ, Chapman HA. Elastinolytic activity of human cathepsin L. Biochem J 1986; 233: 925-927

56. Roviezzo F, Getting SJ, Paul-Clark J, Ona S, Gavins FNE, Perretti M, Hannon R, Croxtall JD, Buckingham JC, Flower RJ. The annexin-1 knockout mouse: what it tells us about inflammatory responses. J Physiol Pharmacol 2002; 53: 541-553

57. Hirata A, Hirata F. DNA chain unwinding and annealing reactions of lipocortin (annexin) I heterotetramer: regulation by Ca(2+) and Mg(2+). Biochem Biophys Res Commun 2002; 291: 205-209

58. Gronostajski RM. Roles of the NFI/CTF gene family in transcription and development. Gene 2000; 249: 31-45

59. Monroe DG, Getz BJ, Johnsen SA, Riggs BL, Khosla S, Spelsbery TC. Estrogen receptor isoform-specific regulation of endogenous genes expression in human osteoblastic cell lines expressing either ERalpha or ERbeta. J Cell Biochem. 2003; 90: 315-326

Table 1 – Primers and probe sequences for Taqman® real-time quantitative PCR.

Target	**Primers [300 nM]**	**Probes [200 nM]**
Pro – alpha – 2(I) collagen (COL1A2)	**Forward** (398 – 415) 5' GGA CTT ATC TGG ATC ATA TTG CAC ACT 3' **Reverse** (475 – 451) 5' CCG TCT TTT CTA TGC ACC TAC ATC T 3'	(418 – 445) 5' 6FAMTCT GAC CAA TCC TTC TCT TTT GCC CAC – TAMRA 3'
Procollagen C – proteinase enhancer protein (PCOLE)	**Forward** (86 – 101) 5' CTG GCC TGA GTC GGA TTA CC 3' **Reverse** (152 – 133) 5' CTG GTT TGA GGG TGC AAT GA 3'	(107 – 129) 5' 6FAMCCC AGG CT CAG CTG TTC CTG GC – TAMRA 3'
Cathepsin L	**Forward** (174 – 197) 5' TCC ATC AAT TCA CGA TAG CAT AGC 3' **Reverse** (241 – 222) 5' CCA AAG ACC GGA ACA CC AC 3'	(199– 218) 5' 6FAMGGA CTT GCC ACC GCA GGC GA – TAMRA 3'
Receptor for activated protein kinase C	**Forward** (179 – 196) 5' CTG CGG ATG GGA CAA GCT 3' **Reverse** (249 – 230) 5' GGC CAA TGT GGT TGG TCT TT 3'	(199-227) 5' 6FAMCTT GCA GTT AGC CAG ATT CCA CAC CTT GA – TAMRA 3'

Table 2 – Primers for standard RT – PCR and conditions.

Target	Product size	Primers	Conditions
Annexin I	935 bp	**Forward** 5' AGC CCC TAC CCT TCC TTC AAT C 3' **Reverse** 5' GTT TAG TTT CCT CCA CAC AGA GCC 3'	50 pmol primers, 2.0 uM $MgCl^{2+}$, 38 cycles of : 94° C 10 sec., 56° C 1 min., 72° C 1 min.
Nuclear Factor I/B	963 bp	**Forward** 5' GAC TGG GTT TGT TGT GAA ATT GC 3' **Reverse** 5' TGC TTG GTG GAG AAG ACA GAG ACC 3'	50 pmol primers, 2.0 uM $MgCl^{2+}$, 40 cycles of: 94° C 10 sec., 60° C 1 min., 72° C 1 min.

Table 3 – GenBank dbEST submissions for putative cDNA clones identified through SSH with Rat1+ERα cell lines as tester.

Identity	Clone number	Accession Number	Base Pair Sequenced	Homology
Aldolase A	OKST ERA† -002	BQ703384	300	Rat 100% (251/251) Mouse 97% (239/246)
Annexin I	OKST ERA† -121	BQ703385	456	Rat 99% (444/445)
Bcl-2 related protein (Mcl-1)	OKST ERA† -067	BQ703386	254	Rat 94% (131/138)
c-HA-ras proto-oncogene-mechanism	OKST ERA† -044	BQ703387	578	Rat 96% (269/278)
Capping protein alpha 2	OKST ERA† -063	BQ703388	645	Mouse 98% (292/297)
Carboxypeptidase E	OKST ERA† -086	BQ703389	556	Rat 99% (552/556)
Cathepsin B	OKST ERA v Rat1‡ -005	CF269911	129	Rat 100% (129/129) Mouse 94% (115/122)
Cathepsin L	OKST ERA† -074	BQ703390	516	Rat 98% (509/516)
Cell division cycle 42 homolog mRNA (Cdc42)	OKST ERA† -094	BQ703391	639	Mouse 98% (627/639)
Cyclin G1	OKST ERA v Rat1‡ -012	CF269912	304	Rat 93% (285/304) Mouse 82% (186/226)
Chaperonin subunit 4 (delta)	OKST ERA† -079	BQ703392	474	Mouse 95% (449/470)
Dihydropyrimidinase-like 3	OKST ERA†-022	BQ703393	158	Human 93% (55/59)
DOC-2 p82 isoform (Dab2)	OKST ERA† -047	BQ703394	354	Rat 100% (164/164)
Elongation factor SIII p15 subunit (Tcbe1)	OKST ERA† -004	BQ703395	411	Rat 100% (390/390)
Epithelial membrane protein 1	OKST ERA† -014	BQ703406	490	Mouse 90% (311/342)
Heat shock protein, 70 kDa 4	OKST ERA v Rat1‡ -033	CF269914	366	Rat 99% (305/305)
Heat shock protein, 86 kDa 1	OKST ERA† -005 (2 clones)	BQ703396	537	Rat 99% (427/428) Mouse 96% (510/536)
Hypothetical protein, MGC: 7868	OKST ERA† -117	BQ703397	393	Mouse 89% (348/389)
Leucyl-specific aminopeptidase PILS	OKST ERA† -036	BQ703398	270	Rat 99% (267/269)
Nuclear autoantigen mRNA	OKST ERA† -097	BQ703399	527	Human 95% (209/220)
Nuclear phosphoprotein similar to PWP1	OKST ERA† -060	BQ703400	551	Human 90% (309/343)
Neural precursor cell	OKST ERA v Rat1‡ -013	CF269913	110	Rat 100% (90/90)
Procollagen C-proteinase enhancer protein	OKST ERAV§ -35	BQ783366	493	Rat 99% (447/448) Mouse 95% (419/440)
Proteasome z subunit mRNA	OKST ERA† -001	BQ703401	197	Rat 97% (338/354) Mouse 92% (315/339)

mRNA for receptor for activated C kinase	OKST ERA† -078	BQ703402	400	Bovine 100% (400/400) Mouse 99% (399/400)
Soc-2 homolog mRNA	OKST ERA† -120	BQ703403	351	Human 89% (257/286)
Suppressor of mif two 3 homolog 2 (SMT3)	OKST ERA† -018	BU551411		Rat 99% (418/421) Mouse 97% (410/422)
T – complex 1	OKST ERA† -103	BQ703404	507	Rat 100% (507/507)
Thioredoxin domain containing 5	OKST ERAV§ -001	CF269894	606	Mouse 85% (419/492
Similar to TAR DNA binding protein	OKST ERA† -064	BQ703405	459	Mouse 98% (446/453) Human 94% (433/457)
Unknown mRNA sequence	OKST ERA† -080	BQ703407	510	Rat 99% (509/510)
Vacuolar protein sorting 35	OKST ERA† -075	BQ703408	639	Mouse 97% (623/639)
Unknowns	OKST ERA† -006, 025, 054, 069, 082, 098	BU551405-10		
	OKST ERA v Rat1‡ -034, 062, 090	CF269915-19		
	OKST ERAV§ -072	CF269895		
Mitochondrion Genome and Products	OKST ERA v Rat1‡ -001 (14 clones)			
	OKST ERAV§ -049			
Ribosomal	OKST ERAV§ -002 (17 clones)			

† Putative products identified through Rat1+ERα E_2 stimulated tester and Rat1+ERβ E_2 stimulated driver SSH.

‡ Putative products identified through Rat1+ERα E_2 stimulated tester and Rat1 E_2 stimulated driver SSH

§ Putative products identified through Rat1+ERα vehicle stimulated tester and Rat1+ERβ vehicle stimulated driver SSH

Table 4 – GenBank dbEST submissions for putative cDNA clones identified through SSH with Rat1+ERβ cell lines as tester.

Identity	Clone Number	Accession Number	Base Pair Sequenced	Homology
Elongation factor 1-alpha 1	OKST ERBV[§] -037	CF269899	288	Human 99% (286/287)
Gastric associated differentially expressed protein	OKST ERB[†] -002 (14 clones)	BQ703710	490	Human 98% (442/490)
N-myc downstream regulated 1 mRNA	OKST ERB[†] -19	BQ703711	370	Mouse 90% (201/222) 88% (106/120)
Nuclear factor I/ B	OKST ERB[†] -043 (2 clones)	BQ703712	497	Human 96% (171/177)
Similar to splicing factor 3B, subunit 2	OKST ERB v Rat[‡] -035	CF269888	265	Rat 100% (245/245) Mouse 89% (212/236)
Pro – alpha – 2(I) collagen	OKST ERB[†] -067 (2 clones)	BQ703713	478	Rat 99% (202/203) 100% (19/19)
Unknown	OKST ERB[†] -032 (2 clones)	BQ783364		
	OKST ERB v Rat[‡] -012, 025, 101, 114 (4 clones), 120	CF269886-87 CF269891-93		
	OKST ERBV[§] -011, 020, 033, 060, 082, 085,	CF269896-98 CF269900-02		
Mitochondion genome	OKST ERB v Rat[‡] -023			
	OKST ERBV[§] -001 (3 clones)			
Ribosomal	OKST ERBV[§] -006 (5 clones)			

[†] Putative products identified through Rat1+ERβ E_2 tester and Rat1+ERα E_2 driver SSH.

[‡] Putative products identified through Rat1+ERβ E_2 stimulated tester and Rat1 E_2 stimulated driver SSH

[§] Putative products identified through Rat1+ERβ vehicle stimulated tester and Rat1+ERβ vehicle stimulated driver SSH

Table 5 – GenBank dbEST submissions for putative cDNA clones identified through SSH with Rat1 parental cell line as tester.

Identity	Clone Number	Accession Number	Base Pair Sequenced	Homology
Cathepsin L	OKST Rat vs ERB‡ -023 (2 clones)	CF269873	256	Rat 98% (253/256) Mouse 93% (240/256)
Cleavage and polyadenylation specific factor 2 (Cpsf2)	OKST Rat vs ERB‡ -055	CF269881	414	Mouse 94% (373/394) Bovine 87% (343/394) Human 86% (342/394)
Epithelial membrane protein 1	OKST Rat vs ERB‡ -033 (2 clones)	CF269876	438	Mouse 90% (311/342)
Methylthioadenosine phosphorylase	OKST Rat vs ERB‡ -088	CF269885	351	Mouse 94% (332/352)
Plastin 3 (T-isoform)	OKST Rat vs ERA† -116	CF269910	346	Mouse 93% (305/327) Hamster 86% (264/305)
Similar to BCS1-like	OKST Rat vs ERA† -112	CF269909	537	Rat 99% (442/444) Mouse 94% (345/367)
Unknowns	OKST Rat vs ERA† -009, 044, 085, 087, 096, 099	CF269903-08		
	OKST Rat vs ERB‡ -030, 032, 044, 046, 047, 050, 063, 081, 086,	CF269874-75, CF269877-80, CF269882-84		
Mitochondrial genome and products	OKST Rat vs ERB‡ -018 (2 clones)			
Ribosomal	OKST Rat vs ERA† -010 (3 clones)			
	OKST Rat vs ERB‡ -022 (4 clones)			

† Putative products identified through Rat1 E_2 stimulated tester and Rat1+ERα E_2 stimulated driver SSH.

‡ Putative products identified through Rat1 E_2 stimulated tester and Rat1+ERβ E_2 stimulated driver SSH.

Table 6 – Quantitative analysis of real-time PCR to targets of interest.

Transcript	Cell line and Treatment	Mean C_T[†]	18s rRNA Mean C_T[†]	ΔC_T[‡*]	$\Delta\Delta C_T$[§]	$2^{-\Delta\Delta CT}$¥
Pro – alpha – 2(I) collagen (COL1A2)	Rat1 + ERα E_2 [1nM]	21.70 ± 0.02	20.14 ± 0.04	1.56 ± 0.02 [a]	-1.58	2.99
	Rat1 + ERα V	23.90 ± 0.09	20.34 ± 0.31	3.56 ± 0.16 [b]	0.42	0.75
	Rat1 + ERβ E_2 [1nM]	21.10 ± 0.10	21.51 ± 0.07	-0.41 ± 0.02 [c]	-3.55	11.71
	Rat1 + ERβ V	21.07 ± 0.07	20.74 ± 0.13	0.34 ± 0.04 [d]	-2.80	6.96
	Rat1 E_2 [1nM]	24.24 ± 0.10	21.20 ± 0.21	3.30 ± 0.03[e]	-0.11	1.08
	Rat1 V	24.41 ± 0.09	21.26 ± 0.01	3.14 ± 0.06[e]	0.00	1.00
Procollagen C – proteinase enhancer protein (PCOLE)	Rat1 + ERα E_2 [1nM]	20.93 ± 0.07	20.73 ± 0.63	0.21 ± 0.39 [a]	-1.71	3.27
	Rat1 + ERα V	23.81 ± 0.06	20.14 ± 0.01	3.68 ± 0.04 [b]	1.76	0.30
	Rat1 + ERβ E_2 [1nM]	22.28 ± 0.03	20.42 ± 0.74	1.86 ± 0.50 [c]	-0.06	1.04
	Rat1 + ERβ V	22.25 ± 0.08	20.21 ± 0.13	2.04 ± 0.04 [c]	0.12	0.92
	Rat1 E_2 [1nM]	22.22 ± 0.13	20.14 ± 0.06	2.08 ± 0.05 [c]	0.16	0.90
	Rat1 V	22.11 ± 0.51	20.19 ± 0.05	1.92 ± 0.32 [c]	0.00	1.00
Cathepsin L	Rat1 + ERα E_2 [1nM]	21.97 ± 0.09	21.46 ± 0.13	0.50 ± 0.03 [a]	-4.99	31.78
	Rat1 + ERα V	24.14 ± 0.03	20.44 ± 0.13	3.71 ± 0.07 [b]	-1.78	3.43
	Rat1 + ERβ E_2 [1nM]	22.09 ± 0.09	20.81 ± 0.37	1.28 ± 0.19 [c]	-4.21	18.51
	Rat1 + ERβ V	21.83 ± 0.09	20.57 ± 0.01	1.26 ± 0.05 [c]	-4.23	18.77
	Rat1 E_2 [1nM]	25.38 ± 0.10	20.53 ± 0.31	4.85 ± 0.15 [d]	-0.65	1.57
	Rat1 V	25.47 ± 0.10	19.98 ± 0.13	5.49 ± 0.02 [e]	0.00	1.00
Receptor for Activated C Kinase (RACK1)	Rat1 + ERα E_2 [1nM]	21.66+ 0.16	21.46 + 0.13	0.19 + 0.02 [a]	-3.93	15.24
	Rat1 + ERα V	23.20 + 0.04	20.44 + 0.13	2.76 + 0.06[b]	-1.35	2.55
	Rat1 + ERβ E_2 [1nM]	22.09 + 0.07	20.81 + 0.37	1.28 + 0.21 [c]	-2.84	7.16
	Rat1 + ERβ V	22.03 + 0.10	20.57 + 0.01	1.46 + 0.06 [c]	-2.66	6.32
	Rat1 E_2 [1nM]	23.48 + 0.03	20.53 + 0.31	2.95 + 0.20[b]	-1.16	2.23
	Rat1 V	24.09 + 0.16	19.98 + 0.13	4.12 + 0.02 [d]	0.00	1.00

[†] Cycle threshold (C_T): The mean cycle number (target genes n=3, 18S rRNA n=2) at which the threshold crossed the geometric portion of the logarithmic amplification curve.

[‡] Normalized C_T values (ΔC_T): Mean C_T values for target genes were subtracted from the mean 18S rRNA gene C_T values to derive values for normalized expression.

[§] Calibrated value ($\Delta\Delta C_T$): Rat 1 V treated cells were set as a calibrator. Normalized C_T values were then subtracted from this value to derive the calibrated value used to determine fold differences ($2^{-\Delta\Delta CT}$).

¥ Fold differences ($2^{-\Delta\Delta CT}$): Target gene normalized to endogenous 18S reference, and relative to Rat1 parental cell line calibrator.

* Subscript number with different letters denote a significant difference ($P \leq 0.05$) between samples, while subscript letters that are the same denote no significant difference. Analysis was carried out using least square differences from a PROC-MIXED analysis in a 3 by 2 treatment factorial ANOVA arrangement analyzed by the Statistical Analysis System (SAS).

Figure 1. Chloramphenicol acetyltransferase reporter assay was carried out to test responsiveness of estrogen receptor to E_2 using a pERE15 construct. Responsiveness was determined by levels of acetylated [^{3}H]chloramphenicol levels in the organic phase determined by scintillation spectrometry. For CAT assay analysis of variance was carried out using PROC-GLM least square difference in SAS. E_2 treatment resulted in elevated reporter gene expression in ERα- and ERβ-expressing cell lines ($p \leq 0.05$) compared to vehicle control treatments, and E_2 or vehicle treatment of the parental Rat-1 cell line. There was no difference between ERα- and ERβ-expressing cell lines treated with E_2.

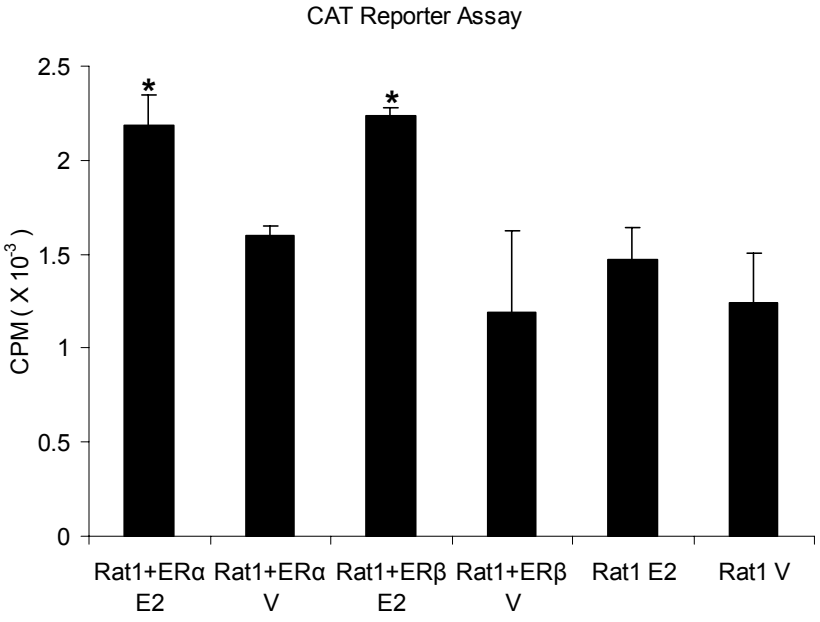
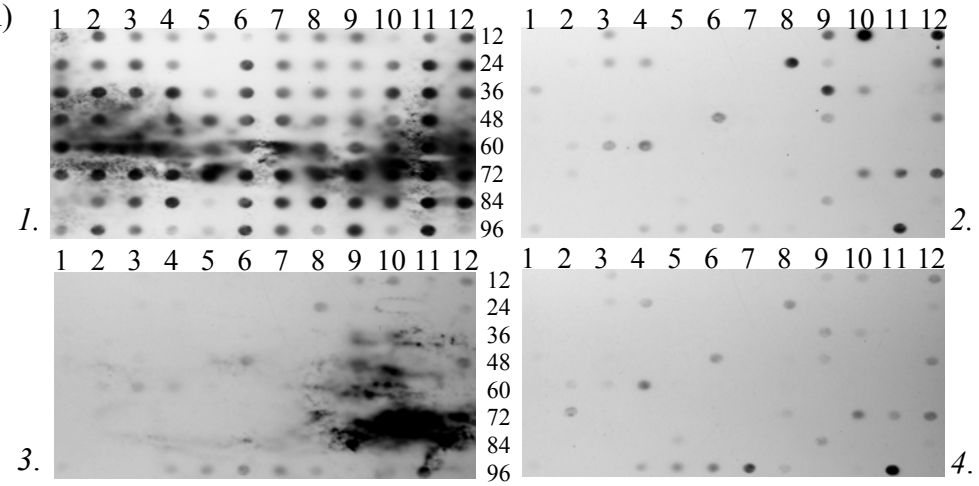
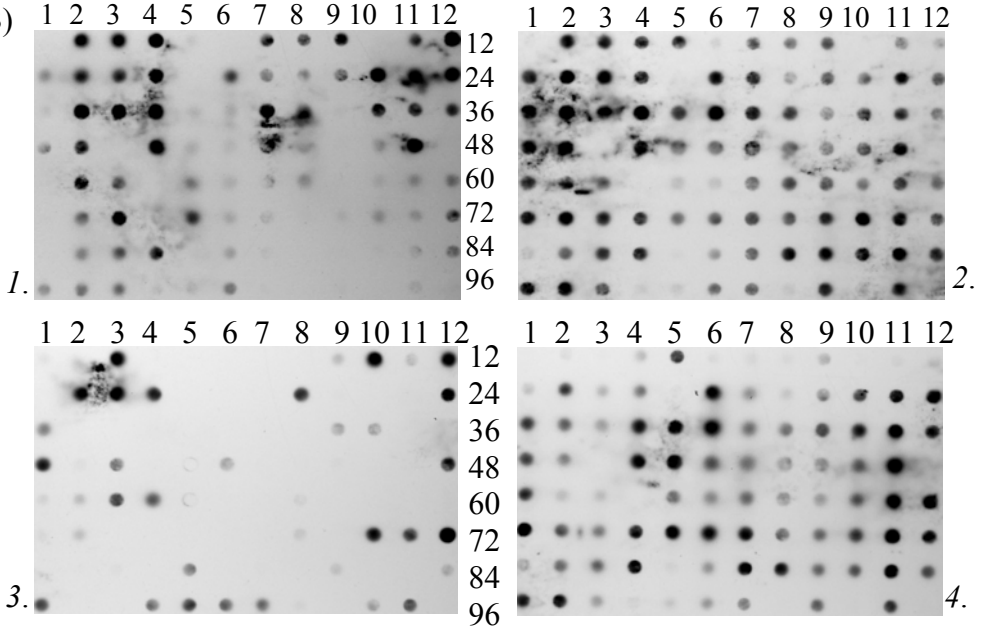

CAT Reporter Assay
CPM (X 10-3)
2.5
2
1.5
1
0.5
0
*
*
Rat1+ERα E2
Rat1+ERα V
Rat1+ERβ E2
Rat1+ERβ V
Rat1 E2
Rat1 V

Figure 2. Qualitative differential analysis of dot blot membranes probed using DIG – labeled probes: A) Differentially screened nylon membranes spotted with purified plasmid DNAs (96 of 120 shown) from subtracted population when Rat1+ERα E_2 was the tester and Rat1+ERβ E_2 was the driver. Membranes were probed with: *1*. Tester subtracted from driver (forward subtracted); *2*. Tester unsubtracted with both adaptors present but no driver present; *3*. Rat1+ERβ E_2 as tester and Rat1+ERα E_2 as driver (reverse subtracted); *4*. Reverse unsubtracted (absence of Rat1+ ERα E_2 driver added and both adaptors present). DIG labeled probes (25 ng/ mL) hybridized (42°C overnight), detected through CSPD, and exposed to X-OMAT blue film for approximately 30 seconds. B) Differentially screened nylon membranes spotted with purified plasmid DNAs (96) resulting from subtracted Rat1+ERβ E_2 tester with Rat1+ERα E_2 driver. Membranes were probed with: *1*. Rat1+ERβ E_2 tester subtracted from Rat1+ERα E_2 driver (forward subtraction); *2*. Rat1+ERβ E_2 unsubtracted (absence of ERα E_2 driver and both adaptors present); *3*. Rat1+ERα E_2 tester subtracted from Rat1+ERβ E_2 driver (reverse subtracted); *4*. Rat1+ERα E_2 unsubtracted (absence of Rat1+ ERβ E_2 driver added and both adaptors present). C) Definition of parameters considered for differential screening and sequencing of colonies.

A)



B)

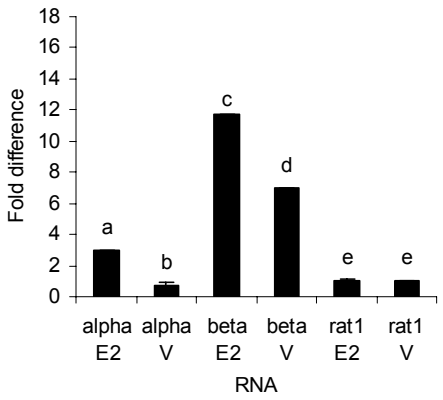


C)

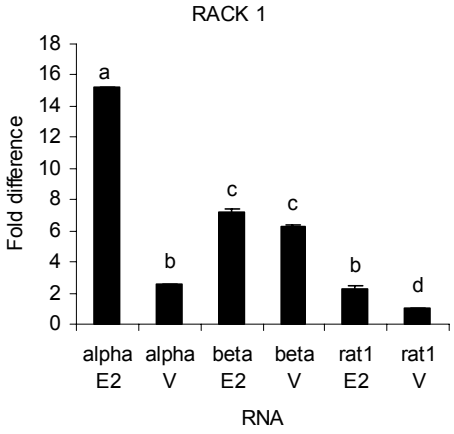
Probes				Analysis
1. Forward Subtracted	2. Forward Unsubtracted	3. Reverse Subtracted	4. Reverse Unsubtracted	
+	+	-	-	Differentially expressed products
+	-	-	-	Low abundance products
+ > 5	+	+	-	Differentially expressed products
+	+	-	+	Significantly different from reverse subtracted
+, -	+, -	+, -	+, -	No differential expression

Figure 3. Fold differences in target gene expression were determined by the comparative CT method. The difference between the C_T values of the target genes and the 18s rRNA (ΔC_T) were calibrated to an index value by subtracting all individual ΔC_T's from that of the Rat1 cell line receiving vehicle treatment to derive the $\Delta\Delta C_T$. Fold differences were then calculated by the equation $2^{-\Delta\Delta CT}$. Statistical analysis for fold differences are those determined for the $\Delta\Delta C_T$ using SAS PROC – MIXED. Fold differences shown for: A) COL1A2 detected in the Rat1+ERβ stimulated tester/ Rat1+ERα stimulated driver experiment; B) RACK1; and C) CtsL, both detected in Rat1+ERα stimulated tester/ Rat1+ERβ stimulated driver; and D) PCOLCE detected in the Rat1+ERα vehicle tester/ Rat1+ERβ vehicle driver SSH.

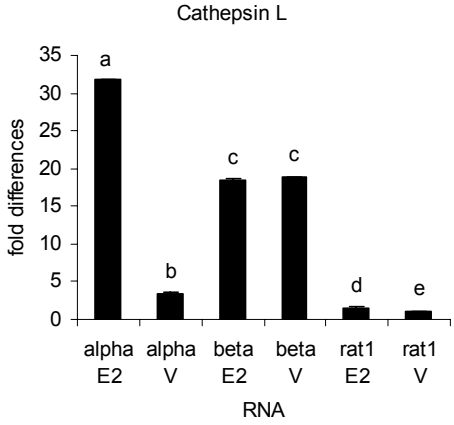
A)


COL1A2
Fold difference
18
16
14
12
10
8
6
4
2
0
a
b
c
d
e
e
alpha E2
alpha V
beta E2
beta V
rat1 E2
rat1 V
RNA

B)


RACK 1
Fold difference
18
16
14
12
10
8
6
4
2
0
a
b
c
c
b
d
alpha E2
alpha V
beta E2
beta V
rat1 E2
rat1 V
RNA

C)


Cathepsin L
fold differences
35
30
25
20
15
10
5
0
a
b
c
c
d
e
alpha E2
alpha V
beta E2
beta V
rat1 E2
rat1 V
RNA

D)

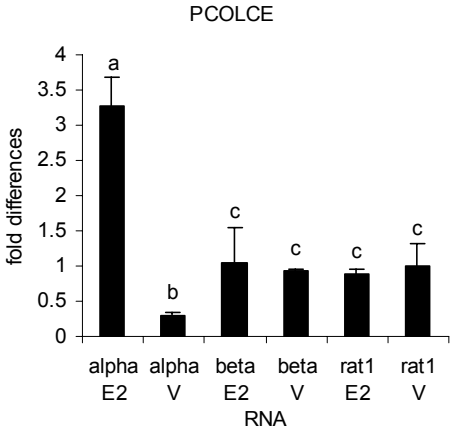
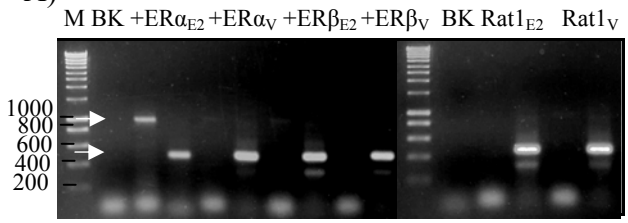

PCOLCE
fold differences
4
3.5
3
2.5
2
1.5
1
0.5
0
a
b
c
c
c
c
alpha E2
alpha V
beta E2
beta V
rat1 E2
rat1 V
RNA

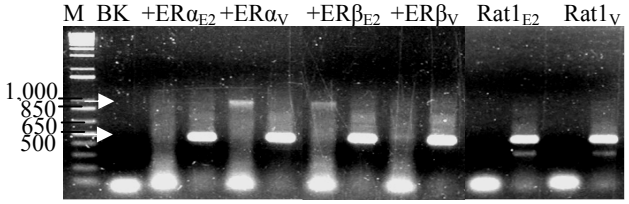
Figure 4. Amplification of target genes Annexin 1 and Nuclear Factor I/B identified through SSH: Gel electrophoresis in 2% agarose followed by ethidium bromide (5μg/ μL) staining was used to detect putative gene products identified through SSH. A) Annexin 1 (935 bp) and B) Nuclear Factor I/B (963 bp) amplification within the cell lines at 24 h E_2 [1nM] was analyzed beside G_3PDH (500 bp) positive controls, and bands extracted and sequenced to verify gene identification.

A)



Annexin 1 (935 bp), G_3PDH (500 bp)

B)



Nuclear Factor I/B (963 bp), G_3PDH (500 bp)

CHAPTER IV

Effect of ligand and exposure time on progesterone receptor gene expression in the presence of estrogen receptor alpha or beta

ABSTRACT

The estrogen signaling system plays a role in multiple physiological responses. The two known forms of estrogen receptor (ERα and β) exhibit differential affinity for estrogenic ligands in relation to structure and distribution. Estrogen receptors and progesterone receptor (PR) have a well recognized interaction with one another in the rat uterine model, in which ER activation results in increased expression of PR and progestins conversely down-regulate ER expression. We have measured PR up-regulation in response to estrogenic ligands in a rat embryonic fibroblast cell line model naive to ER, but engineered to stably express ERα or ERβ. Rat1+ERα, Rat1+ERβ, and precursor Rat1 lines were treated with estradiol-17β (E2), diethylstilbestrol (DES), 4-hydroxytamoxifen (OHT), genistein (GEN), raloxifene-HCl (RAL), or vehicle for 6, 9, 12, 18, 24 hours. Total RNA was extracted and subjected to DNAse treatment followed by quantitative real time PCR (RT-qPCR). Results were analyzed using the comparative C_T method and statistics carried out via a 3X5X5 factorial in a completely randomized design. Our data have demonstrated a statistically significant interaction of ligand, time, and ER isotype on PR expression. Specific examples include up-regulation of PR following E_2 administration in the ERα expressing cells at 9h. Alternatively in Rat1+ERβ cells GEN

was able to induce PR expression at 12h. These data support a model in which ERα and ERβ elicit unique downstream effects on target gene expression which are dependent on ligand and exposure time.

INTRODUCTION

Estrogens and estrogen receptors (ER) play pivotal roles in physiological systems, which vary with tissue and cell type. Members of the nuclear receptor (NR) superfamily, including ER, encompass an essential role in the endocrine system by binding hormone ligands such as estrogen, progestins, androgens, and glucocorticoids as well as thyroid hormone, vitamin D, and retinoic acids. In this role, the receptors act as ligand-inducible transcription factors. As a group, the NRs share a common structural organization of 5 domains responsible for DNA binding, ligand binding, and transcriptional regulation. Proper transcriptional activation relies on the cooperative effects of other protein-protein interactions, formation of dimers by the ER proteins (Fawell *et al.* 1990) acetylation and phosphorylation (Arnold *et al.* 1997; Rogatsky *et al.* 1999; Fu *et al.* 2004). Estrogen receptors can bind directly to estrogen response elements (ERE), or indirectly to AP-1 and Sp1 DNA binding sites through protein complexes (Tsai & O'Malley 1994; Umayahara *et al.* 1994; Porter *et al.* 1997).

The hypothesis of a receptor protein for estrogen was suggested as early as 1962 (Jensen 1962), followed by isolation of the receptor protein the rat uterus in 1966 (Toft & Gorski 1966). In 1995 a second estrogen receptor, ERβ, was identified from the rat prostate

gland (Kuiper *et al.* 1996), and the original ER was subsequently designated ERα. The two receptor isotypes exhibit both overlapping and divergent relationships in regards to tissue localization and ligand preferences (Kuiper *et al.* 1997). The distribution and ligand binding of ER ultimately leads to variations in gene expression by interactions at differing promoter sites (Saville *et al.* 2000; Webb *et al.* 1995; Paech *et al.* 1997), and co-regulatory protein recruitment (Hall *et al.* 2002; Xu *et al.* 1999; McKenna *et al.* 1999).

Estrogen receptors preferentially bind the active form of the steroid hormone estrogen, 17β-estradiol (E_2), and a range of physiological and environmental estrogenic like compounds (Pike *et al.* 1999; Sun *et al.* 2003). Selective estrogen receptor modulators (SERMs) such as tamoxifen (OHT) and raloxifene (RAL), have been designed to make use of differential transcriptional activation related to the two ER isotypes (McDonnell 1999). Synthetic ligands such as non-steroidal diethylstilbestrol (DES), and phytoestrogens such as the soy isoflavone genistein (GEN) have also been shown to bind with varying affinities to the two ER (Kuiper *et al.* 1997). Differences in gene expression and transcriptional efficiency stimulated by the various ER specific ligands occur through numerous pathway interactions, including steric interactions with the binding pocket (Nichols *et al.* 1998). There also exists an ability to recruit and regulate co-regulator proteins (Moras & Gronemeyer 1998; Lonard *et al.* 2004), effects on the dimerization reaction (Tamrazi *et al.* 2002) and effects on promoter binding (Klinge *et al.* 1998). These interfaces become of keen interest when considering the interplay of these effects on how the two receptors regulate specific gene expression profiles.

Progesterone receptor (PR) has a well recognized interaction with ER. Up-regulation of PR is induced following ER binding of a ligand. In the rat uterus this is followed by a negative feedback loop which acts to decrease ER levels (Horwitz & McGuire 1978). The up-regulation by estrogen stimulated ER is controlled through stimulation of PR mRNA and protein synthesis, even at basal levels, and estrogenic like compounds have been shown to function in a similar manner upon an interaction between ER and PR (Leavitt *et al.* 1977). Therefore, PR expression is often an indicator of ER responsiveness. To evaluate interplay between ER on downstream PR expression we have examined downstream effects to independent doses of different ligands in a rat embryonic fibroblast cell line model, normally naive to ER, engineered to stably express ERα or ERβ. Additionally, we have looked at these changes over a time period of 6, 9, 12, 18, 24 hours (h) in order to gain further insight into PR gene expression changes affected by ERα and ERβ.

MATERIALS AND METHODS

CHEMICALS

All estrogenic compounds used for treatment purposes were purchased through Sigma, St. Louis MO. Diethylstilbestrol, E_2, OHT, and GEN were reconstituted in 100% ethanol, while RAL was reconstituted in DMSO (Figure 1).

CELL CULTURE AND LIGAND TREATMENT

Cell culture. Cells were maintained as previously described by Hurst et al. 2004. Rat1 fibroblast cell lines (Freeman *et al.* 1970) stably expressing either a mutant human HEG0 ERα (Rat1+ERα) (Kaneko *et al.* 1993)or rat ERβ (Rat1+ERβ) (Cheng & Malayer 1999), were used as a model system for examining downstream PR expression regulated by each receptor type under differing ligand treatments. Cells were grown in sterile filtered (0.22 μM), phenol red – free Dulbecco's Modified Eagle Medium without glutamine, sodium pyruvate, or sodium bicarbonate (DMEM; Fisher, Plano TX), supplemented with $NaHCO_3$ (3.7 g/L) and 5mg/mL L-glutamine (Sigma, St. Louis MO). The cells were further supplemented with bovine insulin (0.6 μg/ml) in HEPES (25 μM) (Sigma, St. Louis MO), 1X antibiotic – antimycotic (Sigma, St. Louis MO), and 10% charcoal – stripped/ dextran treated fetal bovine serum (CSFBS; Hyclone, Logan UT). Cells were maintained at 37˚C with a humidified atmosphere of 5% CO_2 gas and 95% air, and media replaced every 48 hours. Additionally, Rat1+ERα cells were given the supplemental selective antibiotic Hygromyocin B (100 μg/ml) in PBS (Invitrogen, Carlsbad CA) beginning 24 h after plating (Kaneko *et al.* 1993). Rat1+ERβ cells were supplemented with Geneticin (50 μg/ml) in the same manner (Gibco, Grand Island NY) (Cheng & Malayer 1999).

Ligand treatment and RNA extraction. Cells were allowed to grow in triplicate subsets to 80% confluency in 75 cm^2 culture flasks and treated, in the absence of supplemental selective antibodies, with a single dose of E_2 [1nM] as previously described (Hurst *et al.* 2004) for 6, 9, 12, 18, 24 h. The mutant human HEG0 ERα used in the Rat1+ERα has a

10 fold higher K_d than native ER (Tora *et al.* 1989). Cells were therefore treated with a dose determined by established K_i values for hERα and rERβ (Kuiper *et al.* 1997), with a 10X amount of the published K_i used for ERα expressing cells. A single dose of DES (+ERα [0.4nM], +ERβ [0.05nM]), OHT (+ERα [1nM], +ERβ [0.04nM]), RAL (+ERα [1nM], +ERβ [0.4nM]), or GEN (+ERα [20nM], +ERβ [0.3nM) was applied for the stated time periods. An ethanol vehicle (V_{EtOH}) was used for E_2, DES, OHT and GEN, and a DMSO vehicle (V_{DMSO}) was used for RAL. For each vehicle treatment a volume equal to the highest ligand treatment was added to the cultures.

After the appropriate exposure time of 6, 9, 12, 18, 24 h had passed, the cells were washed 3X with 1X PBS (Gibco, Grand Island NY) and total RNA extracted in guanidinium thiocyanate (Promega, Madison WI) under the single step isolation protocol described by Chomczynski and Sacchi (Chomczynski & Sacchi 1987). Following extraction of RNA, samples were treated with RQ1 RNAse-free DNAse (Promega, Madison WI) at a concentration of 1U/10µl for 30 min at 37°C. This was followed by Phenol:Chloroform:Isoamyl alcohol (P:C:I) purification and ethanol precipitation. Amounts of RNA were determined by spectrophotometry.

Quantitative Analysis of Gene Expression

Real time-quantitative PCR. Taqman® primers and probe for PR were generated using Primer Express® software (PE Applied Biosystems, Foster City CA). Quantitative PCR was then carried and analyzed with modifications (Gibson *et al.* 1996; Bustin 2002;

Ginzinger 2002). The 5′ nuclease activity assay scheme was incorporated using probes that contained a 3′ fluorescent TAMRA quencher dye, a 5′ FAM reporter dye, and was carried out using the one-step RT-qPCR chemistry (Eurogentec North America Inc, San Diego, CA). Expression was examined for PR target using total RNA (10 ng) by means of primer [300 nM] and probe [200 nM] sets, with samples loaded in duplicate on each plate. Each population of total RNA (50 pg) was normalized in duplicate using 18S ribosomal RNA (Eurogentec North America Inc, San Diego, CA) at a [200 nM] primer and [100 nM] probe concentration. Efficiency was checked via a standard curve of serial dilutions of Rat1+ERα E_2 treated samples. For target PR a dilution series of 500, 100, 50, 10, and 5ng was used. For 18S ribosomal RNA a series of 5, 1, 0.5, 0.1, and 0.05 ng dilutions were used.

Real-time quantitative PCR was carried out in the ABI PRISM 7700 (PE Applied Biosystems, Foster City CA) under the following thermal cycler conditions; 48˚C for 30 min and 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min, in a 25 μL reaction with a 28 sec exposure time. Analysis and fold differences were determined using the comparative C_T method as described in the ABI technical bulletin #2 for the ABI PRISM 7700 (Gibson *et al.* 1996; Bustin 2002; Ginzinger 2002), where the Rat1 vehicle treated cell line was always used as the calibrator.

Statistical Analysis. Statistical comparison of RT-qPCR ΔC_T values, with means $\pm$ S.D., were reported where n=3 for cell cultures and n=2 for loading replication in the 5′ nuclease activity assay reaction. Results were tested using a 3X5X5 factorial ANOVA table generated through PROC-MIXED utilizing a complete randomized design

constructed using the Statistical Analysis System (Table 2) and p-values analyzed through least square differences. For cell line by compound by time comparisons degrees of freedom were equal to 150. Comparisons across these three factors were made in relation to the vehicle treatment for each experiment and statistical difference determined at $p \leq 0.05$.

RESULTS

PR EXPRESSION IN THE PRESENCE OF E_2

Previous studies from our lab demonstrated that PR expression could be detected by standard RT-PCR methods at 9 h post E_2 treatment while using 1ug of RNA template in Rat1+ERα and Rat1+ERβ cells (Cheng & Malayer 1999). In unpublished results we observed that this was highly time dependent as PR could not be detected at 6 h in either cell line and only Rat1+ERα at 12, 18, and 24 h, under the same conditions, although it was still detectable in Rat1+ERβ at the 9 h time point, albeit faintly and at a level similar to Rat1+ERα vehicle treated PR expression.

A statistically significant interaction was observed in a cell line by ligand by time manner ($p<0.0001$) in regards to target PR expression. Real-time quantitative PCR analysis of PR expression displayed an expression profile of induction following E_2 exposure in ERα expressing cells that varied over different time points (Figure 2a). A 90-fold increase of PR was seen in Rat1+ERα cells following treatment with E_2 for 9 h ($p<0.0001$) (Table 4;

Figure2a). There was no significant induction at 6 h (Table 3), while expression remained elevated in the ERα expression from 15 to 28-fold over the 12-24 h time period (Tables 4&5; Figure 2a).

Progesterone receptor gene expression following E_2 exposure was not detectable in Rat1+ERβ at any time point at the 10 ng amount of template (Figure 2a). Target PR expression was detected at 12 h in the vehicle treated ERβ cells ($p<0.0001$). This effect was observed in all ERβ vehicle treated samples at 12 h.

PR EXPRESSION IN THE PRESENCE OF SYNTHETIC DES

In ERα cells, treatment with DES for 24 h resulted in up-regulation ($p\leq0.0001$) of PR in a manner similar to E_2 treatment at the same time period in ERα ($p=0.1720$), with a 15-fold increase over Rat1 V (Table 6; Figure 2b). This increase was significantly less than that seen at 9 h E_2 with ERα, but did not differ from E_2 with ERα at 12 and 18h. There were no significant inductions of PR with DES at earlier time points. However, this led to a significant time by compound effect between 12-18 h DES and E_2 treatment.

Estrogen receptor β expressing cells showed no induction at 24 h and showed significant difference from vehicle at 9 ($p\leq0.0005$) and 12 h ($p\leq0.0191$) (Tables 3&4). The 12 h reduction from vehicle is significantly different from regulation events seen across treatments in ERβ at the same time. Likewise, while Rat1+ERβ is able to slightly activate PR expression at 18 h, but not in a statistically significant manner from its

vehicle ($p \leq 0.4188$) (Figure 5b). As with the other observations, there is a trend toward increased PR in the Rat1+ERβ vehicle at 12 h (Table 4; Figure 2b).

PR EXPRESSION FOLLOWING TREATMENT WITH THE SERMS OHT AND RAL

4-Hydroxytamoxifen is a partial selective antagonist, and as anticipated did not exhibit the ability to induce PR expression in ERα expressing cells with a significant reduction from vehicle treatment at 9 ($p \leq 0.0001$) and 12 h ($p \leq 0.0316$; Table 3 & 4). Raloxifene-HCL was able to weakly stimulate PR in ERα expressing cell lines at 9 h, but at a level similar to endogenous Rat1+ERα (Table 3; Figure 3b), which suggests that the effect may not be related to RAL.

Induction of PR at 12 h was observed in ERβ expressing cells, however this 34-fold was not at a level of significance over the vehicle treated cells ($p \leq 0.4639$). This highlighted an interesting observation that Rat1+ERβ also exhibits a profile for PR expression at 12 h that is not associated with cognate ligand activity. At 12 h post treatment OHT was also able to stimulate PR expression in naïve cells as well ($p \leq 0.001$) (Figure 3a). Overall there was no statistically significant induction of PR expression following RAL (Figure 3b); although the same trend toward increased expression at 12 h is observed.

PR EXPRESSION AFTER GEN TREATMENT

The phytoestrogen GEN, which has been shown to have a more favorable binding affinity for ERβ than ERα, was able to induce a significant 24-fold increase (Table 5; Figure 4) in PR expression in Rat1+ERβ cells at 12 h ($p \leq 0.0001$). Conversely, GEN was unable to regulate PR expression in the ERα expressing cells at any time point, but similar to OHT was able to stimulate PR expression in the parental line at the 12 h time point ($p < 0.0004$; Figure 4) and showed the same trend for increased ERβ vehicle treated expression.

DISCUSSION

This study has demonstrated that there are isotype by ligand by time dependent effects on regulation of PR gene expression by estrogen and estrogenic-like compounds (Figure 5a & 5b). To examine these interactions several decisions were made regarding experimental design. The PR protein has two functionally different subtypes, A and B, which are encoded by a single PR gene at two distinct translational start sites (Graham & Clarke 1997). In the interest of this study primer and probe sets were generated to the PR gene, instead of a particular subtype. Future studies elucidating promoter context in regard to PR activation would be of great interest with regards to the observation seen here. There was also a desire to normalize the ligand treatment so that affects seen were due to activation and not to higher dosage effects. Therefore, cells were treated with concentrations based on binding affinities observed for hERα and rERβ as reported by Kuiper *et al*. (1997), as these are the same species receptors used in our model. Concentrations were calibrated to take into account the 10X higher K_d value reported for

the HEG0 hERα mutant used in the Rat1+ERα cell lines (Tora *et al.* 1989; Kaneko *et al.* 1993).

One of the primary characteristics of the ER/PR relationship is the activation of PR in the rat uterus following binding of E_2 to ER (Clarke & Sutherland 1990), and therefore PR is often used as an indicator of ER function. Of importance when considering our results is research that has shown that in ERβ knock-out models induction of PR in the uterus appears to be an ERα mediated event, while repression of PR is ERβ mediated (Weihua *et al.* 2000). This falls into line with our observation of a strong induction of PR by ERα at 9 h, and makes it not wholly unexpected that ERβ does not have significant PR induction in response to E_2 within this data set, and actually has lower PR expression than vehicle at 9 and 12 h.

It is interesting that treatment with DES is able to have a delayed effect upon ERα expressing cells that is similar to E_2 at 24 h. The possible implications for DES ligand specificity in regards to early and late response of PR expression are intriguing. Future investigation into differential cellular pathway regulation involved with this observation could be valuable to understanding developmental phenotypes observed with DES exposure. It is also of interest that GEN is able to affect PR up-regulation in ERβ expressing cells, and this observation is corroborated by a recent study demonstrating that GEN may be linked to an increase in PR expression (Hughes *et al.* 2004). This fits with data that ERβ has a higher affinity for GEN, and that GEN is able to exert responses different than those seen by other ligands.

There are several examples of observed effects between ER and PR that have served as impetus for exploring how gene expression profiles change over time in the presence of different ligands. One such observation suggests a time sensitive component, with significant suppression of ERα by 12 h and ER levels returning to normal from 12-48 h (Okulicz 1989). Of interest in relation to ERα and ERβ variations and temporal differences, previous studies at delayed time-points of 24 and 48 h post E_2 treatment have shown no PR expression in ERβ expressing human osteoblast cell lines (Rickard *et al.* 2002). However, in Rat1+ERβ cell lines PR expression is reported as early as 9 h following E_2 treatment (Cheng & Malayer 1999). In our data set PR expression is not detected at 9 h with 10 ng of template total RNA. Previous standard PCR methods utilized 2 μg of template total RNA, and this may explain the ability to detect very low levels of PR in the ERβ expressing cells at 9 h. However, in human breast cancer cell lines it has been demonstrated that while may PR regulate both ERα and ERβ the relationship with ERβ is inversely associated with PR status (Dotzlaw *et al.* 1999).

Complexity of the PR interaction with ER following ligand binding is affected by the various protein complexes DNA binding can occur through, other than direct action of ER at EREs. Mediation of PR through E_2 activation of ERα has been shown to be mediated through Sp1 binding to recognition sites and stabilization of Sp1-DNA interaction within the PR gene (Schultz *et al.* 2003). The presence of an ERE half-site within the PR gene and the DNA-bound Sp1 are thought to be the hormone regulatory switch for ER, whereas Sp1 alone is thought to play a role in non-hormone PR expression

(Petz *et al.* 2004). Whereas the exact promoter context is not examined within the realm of this study, these influences help us to begin to understand some of the underlying mechanisms that may be involved in data reported here.

Several lines of research have demonstrated the ability of ERβ to regulate transcriptional activity in a ligand-independent manner as well as in response to the partial antagonist OHT. Work by Bramlett et al. (2001) has shown that ERβ has the capacity to exhibit significant ligand-independent binding to the SRC-1 NR box II peptide (Bramlett *et al.* 2001). In light of our results that ERβ expressing cells may have an ability to express PR at 12h with vehicle treatment it is tempting to speculate that cross-talk pathways and co-activator binding may be significantly impacting this portion of the pathway in a temporal manner. Since this is a time dependent profile it is likely that a component of the media is playing a role, and the most likely suspect is insulin since there are recognized insulin effects on PR expression (Katzenellenbogen & Norman 1990). Additionally, unpublished results by Jin Cheng in 1999 suggest that insulin in culture plays differential roles depending on ER isotype and responsiveness to ERE or AP-1 promoter binding sites. Therefore, we speculate that this effect is perhaps not related to an effect of the ethanol vehicle, but instead results from the influx of fresh media components that occurs with the concurrent feeding that occurs with treatment. Taken together, the 12h profile suggests that DES and E_2 may actually exerting a suppressive affect on PR through binding to ERβ which fits with research performed by Weihua et al. (2000). This opens up the possibility that this may be occurring through disruption of a

cross-talk pathway. At the same time OHT treatment may be rescuing this interaction, while GEN appears to be exerting a true induction event of PR through ERβ.

These data fit into a model in which ERα and ERβ play divergent roles in PR induction. Effects were highly dependent on time and ligand exposure. Data of this nature raise an interesting question as to the role of differential promoter context, co-regulator recruitment, and cross-talk pathways that are specific to events involving independent ERα and ERβ regulation. Examination of the promoters and co-regulatory molecules involved within these pathways could provide further information as to how the separate ERs are controlling transcription of PR, and this may lend insight into other gene expression interactions.

REFERENCE LIST

Arnold SF, Melamed M, Vorojeikina DP, Notides AC & Sasson S. 1997 Estradiol-binding mechanism and binding capacity of the human estrogen receptor is regulated by tyrosine phosphorylation. *Molecular Endocrinology* **11** 48-53.

Bramlett KS, Wu Y & Burris TP. 2001 Ligands specify coactivator nuclear receptor (NR) box affinity for estrogen receptor subtypes. *Molecular Endocrinology* **15** 909-922.

Bustin SA. 2002 Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *Journal of Molecular Endocrinology* **29** 23-39.

Cheng J & Malayer JR. 1999 Responses to stable ectopic estrogen receptor-beta expression in a rat fibroblast cell line. *Molecular Cellular Endocrinology.* **156** 95-105.

Chomczynski P & Sacchi N. 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry.* **162** 156-159.

Clarke CL & Sutherland RL. 1990 Progestin regulation of cellular proliferation. *Endocrinology Reviews.* **11** 266-301.

Dotzlaw H, Leygue E, Watson PH & Murphy LC. 1999 Estrogen receptor-β messenger RNA expression in human breast tumor biopsies: relationship to steroid receptor status and regulation by progestins. *Cancer Research* **59** 529-532.

Fawell SE, Lees JA, White R & Parker MG. 1990 Characterization and colocalization of steroid binding and dimerization activities in the mouse estrogen receptor. *Cell.* **60** 953-962.

Freeman AE, Price PJ, Igel HJ, Young JC, Maryak JM & Huebner RJ. 1970 Morphological transformation of rat embryo cells induced by diethylnitrosamine and murine leukemia viruses. *Journal of the National Cancer Institutes.* **44** 65-78.

Fu M, Wang C, Zhang X & Pestell RG. 2004 Acetylation of nuclear receptors in cellular growth and apoptosis. *Biochemical Pharmacology* **68** 1199-1208.

Gibson UE, Heid CA & Williams PM. 1996 A novel method for real time quantitative RT-PCR. *Genome Research.* **6** 995-1001.

Ginzinger DG. 2002 Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. *Experimental Hematology* **30** 503-512.

Graham JD & Clarke CL. 1997 Physiological Action of Progesterone in Target Tissues. *Endocrinology Reviews* **18** 502-519.

Hall JM, McDonnell DP & Korach KS. 2002 Allosteric regulation of estrogen receptor structure, function, and coactivator recruitment by different estrogen response elements. *Molecular Endocrinology* **16** 469-486.

Horwitz KB & McGuire WL. 1978 Estrogen control of progesterone receptor in human breast cancer. Correlation with nuclear processing of estrogen receptor. *Journal of Biological Chemistry* **253** 2223-2228.

Hughes CL, Liu G, Beall S, Foster WG & Davis V. 2004 Effects of genistein or soy milk during late gestation and lactation on adult uterine organization in the rat. *Experimental Biology and Medicine* **229** 108-117.

Hurst AGB, Goad DW, Mohan M & Malayer JR. 2004 Independent Downstream Gene Expression Profiles in the Presence of Estrogen Receptor α or β. *Biology of Reproduction* **71** 1252-1261.

Jensen EV. 1962 On the mechanism of estrogen action. *Perspectives in Biological Medicine* **6** 47-59.

Kaneko KJ, Gelinas C & Gorski J. 1993 Activation of the silent progesterone receptor gene by ectopic expression of estrogen receptors in a rat fibroblast cell line. *Biochemistry.* **32** 8348-8359.

Katzenellenbogen BS & Norman MJ. 1990 Multihormonal regulation of the progesterone receptor in MCF-7 human breast cancer cells: interrelationships among insulin/insulin-like growth factor-I, serum, and estrogen. *Endocrinology.* **126** 891-898.

Klinge CM, Studinski-Jones AL, Kulakosky PC, Bambara RA & Hilf R. 1998 Comparison of tamoxifen ligands on estrogen receptor interaction with estrogen response elements. *Molecular and Cellular Endocrinology* **143** 79-90.

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S & Gustafsson JA. 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proceedings of the National Academy of Sciences* **93** 5925-5930.

Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S & Gustafsson JA. 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology* **138** 863-870.

Leavitt W, Chen T & Allen T. 1977 Regulation of progesterone receptor formation by estrogen action. *Annals of the New York Academies of Science.* **286** 210-225.

Lonard DM, Tsai SY & O'Malley BW. 2004 Selective estrogen receptor modulators 4-hydroxytamoxifen and raloxifene impact the stability and function of SRC-1 and SRC-3 coactivator proteins. *Molecular and Cellular Biology* **24** 14-24.

McDonnell DP. 1999 The molecular pharmacology of SERMs. *Trends in Endocrinology and Metabolism* **10** 301-311.

McKenna NJ, Lanz RB & O'Malley BW. 1999 Nuclear receptor coregulators: cellular and molecular biology. *Endocrine Reviews* **20** 321-344.

Moras D & Gronemeyer H 1998. The nuclear receptor ligand-binding domain: structure and function. *Current Opinion in Cell Biology* **10** 384-391.

Nichols M, Rientjes JM & Stewart AF. 1998 Different positioning of the ligand-binding domain helix 12 and the F domain of the estrogen receptor accounts for functional differences between agonists and antagonists. *EMBO J.* **17** 765-773.

Okulicz WC. 1989 Temporal effects of progesterone inhibition of occupied nuclear oestrogen receptor retention in the rat uterus. *Journal Endocrinology.* **121** 101-107.

Paech K, Webb P, Kuiper GG, Nilsson S, Gustafsson J, Kushner PJ & Scanlan TS 9-5-1997 Differential ligand activation of estrogen receptors ERalpha and ERbeta at AP1 sites. *Science* **277** 1508-1510.

Petz LN, Ziegler YS, Schultz JR, Kim H, Kemper JK & Nardulli AM. 2004 Differential regulation of the human progesterone receptor gene through an estrogen response element half site and Sp1 sites. *The Journal of Steroid Biochemistry and Molecular Biology* **88** 113-122.

Pike AC, Brzozowski AM, Hubbard RE, Bonn T, Thorsell AG, Engstrom O, Ljunggren J, Gustafsson JA & Carlquist M. 1999 Structure of the ligand-binding domain of oestrogen receptor beta in the presence of a partial agonist and a full antagonist. *EMBO J.* **18** 4608-4618.

Porter W, Saville B, Hoivik D & Safe S. 1997 Functional synergy between the transcription factor Sp1 and the estrogen receptor. *Molecular Endocrinology* **11** 1569-1580.

Rickard DJ, Waters KM, Ruesink TJ, Khosla S, Katzenellenbogen JA, Katzenellenbogen BS, Riggs BL & Spelsberg TC. 2002 Estrogen receptor isoform-specific induction of progesterone receptors in human osteoblasts. *Journal of Bone Mineral Research* **17** 580-592.

Rogatsky I, Trowbridge JM & Garabedian MJ. 1999 Potentiation of human estrogen receptor alpha transcriptional activation through phosphorylation of serines 104 and 106 by the cyclin A-CDK2 complex. *Journal of Biological Chemistry* **274** 22296-22302.

Saville B, Wormke M, Wang F, Nguyen T, Enmark E, Kuiper G, Gustafsson JA & Safe S. 2000 Ligand-, cell-, and estrogen receptor subtype (α /β)-dependent activation at GC-rich (Sp1) promoter elements. *Journal of Biological Chemistry* **275** 5379-5387.

Schultz JR, Petz LN & Nardulli AM. 2003 Estrogen receptor α and Sp1 regulate progesterone receptor gene expression. *Molecular and Cellular Endocrinology* **201** 165-175.

Sun J, Baudry J, Katzenellenbogen JA & Katzenellenbogen BS. 2003 Molecular basis for the subtype discrimination of the estrogen receptor-β-selective ligand, diarylpropionitrile. *Molecular Endocrinology* **17** 247-258.

Tamrazi A, Carlson KE, Daniels JR, Hurth KM & Katzenellenbogen JA. 2002 Estrogen receptor dimerization: ligand binding regulates dimer affinity and dimer dissociation rate. *Molecular Endocrinology* **16** 2706-2719.

Toft D & Gorski J. 1966 A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. *Proceedings of the National Academy of Sciences.* **55** 1574-1581.

Tora L, Mullick A, Metzger D, Ponglikitmongkol M, Park I & Chambon P. 1989 The cloned human oestrogen receptor contains a mutation which alters its hormone binding properties. *EMBO J.* **8** 1981-1986.

Tsai MJ & O'Malley BW. 1994 Molecular mechanisms of action of steroid/thyroid receptor superfamily members. *Annual Reviews in Biochemistry* **63** 451-486.

Umayahara Y, Kawamori R, Watada H, Imano E, Iwama N, Morishima T, Yamasaki Y, Kajimoto Y & Kamada T. 1994 Estrogen regulation of the insulin-like growth factor I gene transcription involves an AP-1 enhancer. *Journal of Biological Chemistry* **269** 16433-16442.

Webb P, Lopez GN, Uht RM & Kushner PJ. 1995 Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. *Molecular Endocrinology* **9** 443-456.

Weihua Z, Saji S, Makinen S, Cheng G, Jensen EV, Warner M & Gustafsson JA. 2000 Estrogen receptor (ER) β, a modulator of ERα in the uterus. *Proceedings of the National Academy of Sciences* **97** 5936-5941.

Xu L, Glass CK & Rosenfeld MG. 1999 Coactivator and corepressor complexes in nuclear receptor function. *Current Opinion in Genetics & Development* **9** 140-147.

Table 1: Progesterone receptor primer/ probe set for 5′ nuclease activity RT-qPCR

Target	Primers [300 nM]	Probes [200 nM]
Progesterone Receptor gene (PR)	**Forward** (141-163) 5′ TGTAGTCTCGCCAATACCGATCT 3′ **Reverse** (190-207) 5′ CTCCTGAGCCTGGCAGGA 3′	(165-188) 5′ 6FAM/ CCTGGACCGGCTGCTCTTCTCTCG– TAMRA 3′

Table 2. ANOVA table for PR gene expression

Source	DF	Type I SS	Mean Square	F Value	Pr > F
CLINE	2	40.5764436	20.2882218	13.84	<.0001
COMP	4	59.6817004	14.9204251	10.18	<.0001
CLINE*COMP	8	186.0106142	23.2513268	15.86	<.0001
TIME	4	43.7926160	10.9481540	7.47	<.0001
CLINE*TIME	8	63.6060853	7.9507607	5.42	<.0001
COMP*TIME	16	181.1586462	11.3224154	7.72	<.0001
CLINE*COMP*TIME	32	145.2243458	4.5382608	3.10	<.0001

Table 3. Progesterone receptor expression at 6 h exposure to selected ligands

Cell line - **6 h**	Average C_T[†] Target	Average C_T[†] 18S	ΔC_T[‡]	$\Delta\Delta C_T$[§]	$2^{-\Delta\Delta CT}$[¥]
Rat1 + ERα DES[0.4nM]	39.78 ± 0.35	18.60 ± 1.43	21.18 ± 0.76[a]	0.06	0.96
Rat1 + ERα V_{EtOH}	40.00 ± 0.00	19.33 ± 0.34	20.67 ± 0.24[a]	-0.44	1.36
Rat1 + ERβ DES [0.05nM]	40.00 ± 0.00	18.62 ± 1.22	21.38 ± 0.86[a]	0.26	0.83
Rat1 + ERβ V_{EtOH}	40.00 ± 0.00	19.21 ± 0.34	20.79 ± 0.24[a]	-0.33	1.26
Rat1 DES[0.4nM]	40.00 ± 0.00	17.36 ± 0.38	22.64 ± 0.27[a]	1.52	0.34
Rat1 V_{EtOH}	40.00 ± 0.00	18.88 ± 0.24	21.12 ± 0.17[a]	0.00	1.00
Rat1 + ERα OHT [1nM]	39.40 ± 1.11	18.18 ± 0.33	21.21 ± 0.55[a]	-0.66	1.58
Rat1 + ERα V_{EtOH}	38.54 ± 1.24	18.32 ± 0.44	20.22 ± 0.56[a]	-1.65	3.15
Rat1 + ERβ OHT [0.04nM]	39.86 ± 0.34	18.01 ± 0.25	21.85 ± 0.07[a]	-0.28	1.02
Rat1 + ERβ V_{EtOH}	39.34 ± 1.04	18.13 ± 0.68	21.21 ± 0.25[a]	-0.66	1.58
Rat1 OHT [1nM]	40.00 ± 0.00	18.06 ± 0.34	21.94 ± 0.24[a]	0.07	0.95
Rat1 V_{EtOH}	40.00 ± 0.00	18.12 ± 0.64	21.87 ± 0.45[a]	0.00	1.00
Rat1 + ERα Gen [20nM]	39.44 ± 0.90	18.10 ± 0.98	20.71 ± 0.05[a]	2.16	0.22
Rat1 + ERα V_{EtOH}	39.45 ± 0.78	20.18 ± 0.50	19.32 ± 0.19[b]	0.77	0.59
Rat1 + ERβ Gen [0.3nM]	39.17 ± 1.03	18.20 ± 0.56	20.97 ± 0.33[a]	2.42	0.18
Rat1 + ERβ V_{EtOH}	39.05 ± 1.50	19.91 ± 0.70	19.14 ± 0.57[b]	0.59	0.66
Rat1 Gen [20nM]	39.00 ± 0.85	17.60 ± 0.55	21.40 ± 0.21[a]	2.85	0.14
Rat1 V_{EtOH}	38.39 ± 1.07	19.84 ± 0.97	18.55 ± 0.07[b]	0.00	1.00
Rat1 + ERα E_2 [1nM]	39.94 ± 0.10	19.88 ± 0.42	20.05 ± 0.22[a]	-0.66	1.58
Rat1 + ERα V_{EtOH}	40.00 ± 0.00	19.71 ± 0.39	20.29 ± 0.27[a]	-0.42	1.34
Rat1 + ERβ E_2 [1nM]	40.00 ± 0.00	19.47 ± 0.13	20.53 ± 0.09[a]	-0.19	1.14
Rat1 + ERβ V_{EtOH}	40.00 ± 0.00	19.74 ± 0.49	20.25 ± 0.35[a]	-0.46	1.38
Rat1 E_2 [1nM]	40.00 ± 0.00	19.35 ± 0.10	20.65 ± 0.07[a]	-0.06	1.05
Rat1 V_{EtOH}	40.00 ± 0.00	19.28 ± 0.27	20.72 ± 0.19[a]	0.00	1.00
Rat1 + ERα RAL [1nM]	39.26 ± 1.48	19.32 ± 0.62	19.93 ± 0.60[a]	-1.12	2.18
Rat1 + ERα V_{DMSO}	38.09 ± 1.04	18.82 ± 0.27	19.27 ± 0.55[a]	-1.79	3.46
Rat1 + ERβ RAL [0.04nM]	29.27 ± 0.88	19.44 ± 0.45	19.83 ± 0.31[a]	-1.23	2.36
Rat1 + ERβ V_{DMSO}	39.69 ± 0.75	19.36 ± 0.26	20.34 ± 0.34[a]	-0.72	1.65
Rat1 RAL [1nM]	39.75 ± 0.59	19.06 ± 0.33	20.70 ± 0.19[a]	-0.36	1.28
Rat1 V_{DMSO}	40.00 ± 0.00	18.93 ± 0.26	21.06 ± 0.18[a]	0.00	1.00

[†] Cycle threshold (C_T): The mean cycle number (target genes n=3, 18S rRNA n=2) at which the threshold crossed the geometric portion of the logarithmic amplification curve.

‡ Normalized C_T values (ΔC_T): Mean C_T values for target genes were subtracted from the mean 18S rRNA gene C_T values to derive values for normalized expression.

§ Calibrated value ($\Delta\Delta C_T$): Rat 1 V treated cells were set as a calibrator. Normalized C_T values were then subtracted from this value to derive the calibrated value used to determine fold differences ($2^{-\Delta\Delta CT}$).

¥ Fold differences ($2^{-\Delta\Delta CT}$): Target gene normalized to endogenous 18S reference, and relative to Rat1 parental cell line calibrator.

n Subscript number with different letters denote a significant difference ($P \leq 0.05$) in cell line interaction, while subscript letters that are the same denote no significant difference. Analysis was carried out using least square differences from a PROC-MIXED analysis in a 3X5X5 treatment factorial ANOVA arrangement analyzed by the Statistical Analysis System (SAS) and showing a significant cell line X ligand X time ($P \leq 0.0001$) effect.

Table 4. Progesterone receptor expression at 9 h exposure to selected ligands

Cell line - **9 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Rat1 + ERα DES[0.4nM]	38.50 ± 1.91	19.48 ± 1.98	19.02 ± 0.05[a]	-1.18	2.27
Rat1 + ERα V_{EtOH}	40.00 ± 0.00	19.88 ± 0.79	20.12 ± 0.56[a]	-0.08	1.05
Rat1 + ERβ DES [0.05nM]	40.00 ± 0.00	16.87 ± 0.82	23.13 ± 0.58[b]	2.93	0.13
Rat1 + ERβ V_{EtOH}	39.81 ± 0.46	19.17 ± 0.52	20.64 ± 0.04[a]	0.44	0.84
Rat1 DES[0.4nM]	40.00 ± 0.00	17.44 ± 0.76	22.56 ± 0.54[b]	2.36	0.19
Rat1 V_{EtOH}	40.00 ± 0.00	19.80 ± 0.71	20.20 ± 0.50[a]	0.00	1.00
Rat1 + ERα OHT [1nM]	38.61 ± 1.15	18.41 ± 0.29	20.19 ± 0.62[a]	2.82	0.14
Rat1 + ERα V_{EtOH}	37.90 ± 2.24	22.47 ± 0.78	15.42 ± 1.03[b]	-1.95	3.56
Rat1 + ERβ OHT [0.04nM]	39.35 ± 1.08	18.31 ± 0.33	21.03 ± 0.53[a]	3.65	0.07
Rat1 + ERβ V_{EtOH}	39.33 ± 0.98	21.77 ± 0.66	17.55 ± 0.22[c]	0.18	0.88
Rat1 OHT [1nM]	39.75 ± 0.41	18.16 ± 0.08	21.59 ± 0.23[a]	4.21	0.05
Rat1 V_{EtOH}	39.39 ± 0.74	22.02 ± 1.04	17.37 ± 0.21[c]	0.00	1.00
Rat1 + ERα Gen [20nM]	39.85 ± 0.37	19.41 ± 0.37	20.44 ± 0.01[a]	1.93	0.26
Rat1 + ERα V_{EtOH}	40.00 ± 0.00	19.44 ± 1.09	20.55 ± 0.77[a]	2.05	0.24
Rat1 + ERβ Gen [0.3nM]	40.00 ± 0.00	19.02 ± 0.31	20.97 ± 0.22[a]	2.48	0.17
Rat1 + ERβ V_{EtOH}	40.00 ± 0.00	20.67 ± 0.83	19.33 ± 0.59[b]	0.83	0.56
Rat1 Gen [20nM]	40.00 ± 0.00	19.22 ± 0.51	20.77 ± 0.36[a]	2.27	0.20
Rat1 V_{EtOH}	40.00 ± 0.00	21.49 ± 1.03	21.49 ± 0.72[b]	0.00	1.00
Rat1 + ERα E_2 [1nM]	34.40 ± 0.61	18.29 ± 0.92	16.12 ± 0.22[a]	-6.50	90.72
Rat1 + ERα V_{EtOH}	39.21 ± 1.52	17.93 ± 0.62	21.28 ± 0.64[b]	-1.34	2.53
Rat1 + ERβ E_2 [1nM]	40.00 ± 0.00	18.19 ± 0.46	21.81 ± 0.33[b]	-0.81	1.76
Rat1 + ERβ V_{EtOH}	39.75 ± 0.60	17.78 ± 0.89	21.97 ± 0.20[b]	-0.65	1.57
Rat1 E_2 [1nM]	39.69 ± 0.74	16.83 ± 0.56	22.86 ± 0.13[b]	0.24	0.84
Rat1 V_{EtOH}	40.00 ± 0.00	17.38 ± 0.73	22.62 ± 0.52[b]	0.00	1.00
Rat1 + ERα RAL [1nM]	39.03 ± 1.08	18.66 ± 0.63	20.37 ± 0.31[a]	-1.74	3.34
Rat1 + ERα V_{DMSO}	28.30 ± 0.90	18.61 ± 1.27	19.69 ± 0.27[a]	-2.43	5.37
Rat1 + ERβ RAL [0.04nM]	40.00 ± 0.00	18.98 ± 1.53	21.02 ± 1.08[a]	-1.09	2.14
Rat1 + ERβ V_{DMSO}	40.00 ± 0.00	18.54 ± 1.24	21.45 ± 0.88[a]	-0.65	1.58
Rat1 RAL [1nM]	40.00 ± 0.00	17.88 ± 0.24	22.12 ± 0.17[b]	0.01	0.99
Rat1 V_{DMSO}	40.00 ± 0.00	17.88 ± 0.18	22.11 ± 0.13[b]	0.00	1.00

Table 5. Progesterone receptor expression at 12 h exposure to selected ligands

Cell line - **12 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Rat1 + ERα DES[0.4nM]	40.00 ± 0.00	16.65 ± 0.19	23.35 ± 0.13[a]	0.55	0.68
Rat1 + ERα V_{EtOH}	40.00 ± 0.00	17.18 ± 0.19	22.82 ± 0.14[a]	0.01	0.99
Rat1 + ERβ DES [0.05nM]	40.00 ± 0.00	16.87 ± 0.40	23.13 ± 0.29[a]	0.32	0.80
Rat1 + ERβ V_{EtOH}	38.68 + 2.07	17.20 ± 0.18	21.47 ± 1.34[b]	-1.33	2.51
Rat1 DES[0.4nM]	40.00 ± 0.00	17.47 ± 0.99	22.53 ± 0.70[a]	-0.27	1.21
Rat1 V_{EtOH}	40.00 ± 0.00	17.19 ± 0.24	22.81 ± 0.17[a]	0.00	1.00
Rat1 + ERα OHT [1nM]	39.45 ± 0.88	19.87 ± 0.99	19.58 ± 0.08[a]	1.17	0.44
Rat1 + ERα V_{EtOH}	39.69 ± 0.53	21.62 ± 0.25	18.06 ± 0.20[a]	-0.34	1.27
Rat1 + ERβ OHT [0.04nM]	33.25 ± 0.47	19.93 ± 0.07	13.32 ± 0.28[b]	-5.09	33.98
Rat1 + ERβ V_{EtOH}	35.56 ± 1.97	21.73 ± 0.16	13.83 ± 1.28[b]	-4.57	23.81
Rat1 OHT [1nM]	35.46 ± 0.61	20.16 ± 0.22	15.29 ± 0.28[c]	-3.11	8.64
Rat1 V_{EtOH}	40.00 ± 0.00	21.59 ± 0.27	18.41 ± 0.19[a]	0.00	1.00
Rat1 + ERα Gen [20nM]	40.00 ± 0.00	23.97 ± 0.21	16.03 ± 0.15[a]	1.33	0.39
*Rat1 + ERα V_{EtOH}	40.00 ± 0.00	25.44 ± 1.10	14.56 ± 0.78[b]	-0.13	1.09
Rat1 + ERβ Gen [0.3nM]	35.06 ± 2.17	24.97 ± 1.13	10.09 ± 0.73[c]	-4.60	24.28
*Rat1 + ERβ V_{EtOH}	37.90 ± 1.69	24.88 ± 0.31	13.01 ± 0.98[d]	-1.68	3.20
Rat1 Gen [20nM]	36.71 ± 0.50	24.55 ± 0.35	12.16 ± 0.10[d]	-2.53	5.77
*Rat1 V_{EtOH}	40.00 ± 0.00	25.31 ± 0.61	14.69 ± 0.43[b]	0.00	1.00
Rat1 + ERα E_2 [1nM]	36.15 ± 0.75	16.00 ± 0.35	20.15 ± 0.29[a]	-3.95	15.44
Rat1 + ERα V_{EtOH}	40.00 ± 0.00	16.01 ± 0.10	23.99 ± 0.07[b]	-0.10	1.07
Rat1 + ERβ E_2 [1nM]	40.00 ± 0.00	15.90 ± 0.21	24.10 ± 0.15[b]	0.00	1.00
Rat1 + ERβ V_{EtOH}	34.49 ± 3.53	15.95 ± 0.19	19.55 ± 2.36[a]	-4.55	23.40
Rat1 E_2 [1nM]	40.00 ± 0.00	15.74 ± 0.39	24.25 ± 0.27[b]	0.16	0.89
Rat1 V_{EtOH}	40.00 ± 0.00	15.90 ± 0.21	24.10 ± 0.15[b]	0.00	1.00
Rat1 + ERα RAL [1nM]	39.32 ± 0.95	18.57 ± 1.06	20.76 ± 0.07[a]	-0.74	1.67
Rat1 + ERα V_{DMSO}	39.31 ± 0.86	18.22 ± 0.28	21.10 ± 0.41[a]	-0.40	1.32
Rat1 + ERβ RAL [0.04nM]	39.41 ± 1.08	18.67 ± 0.56	20.74 ± 0.37[a]	-0.76	1.69
Rat1 + ERβ V_{DMSO}	38.37 ± 1.92	18.80 ± 0.78	19.56 ± 0.81[a]	-1.94	3.83
Rat1 RAL [1nM]	39.92 ± 0.21	17.98 ± 0.30	21.93 ± 0.07[a]	1.43	0.74
Rat1 V_{DMSO}	40.00 ± 0.00	18.50 ± 0.28	21.50 ± 0.20[a]	0.00	1.00

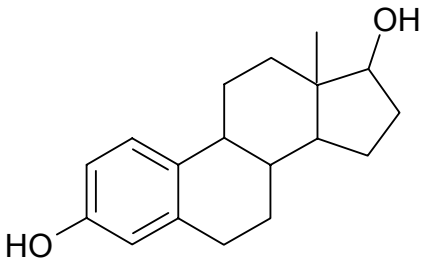
Table 6. Progesterone receptor expression at 18 h exposure to selected ligands

Cell line - **18 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Rat1 + ERα DES[0.4nM]	38.44 ± 1.79	15.32 ± 0.11	23.12 ± 1.18[a]	-1.43	2.70
Rat1 + ERα V_{EtOH}	39.47 ± 0.85	15.39 ± 0.33	24.08 ± 0.37[a]	-0.46	1.38
Rat1 + ERβ DES [0.05nM]	37.80 ± 1.03	15.54 ± 0.15	22.25 ± 0.62[a]	-2.30	4.91
Rat1 + ERβ V_{EtOH}	38.22 ± 1.03	15.40 ± 0.46	22.82 ± 0.49[a]	-1.73	3.32
Rat1 DES[0.4nM]	40.00 ± 0.00	15.48 ± 0.11	24.52 ± 0.08[a]	-0.03	1.02
Rat1 V_{EtOH}	40.00 ± 0.00	15.45 ± 0.35	24.55 ± 0.25[a]	0.00	1.00
Rat1 + ERα OHT [1nM]	40.00 ± 0.00	18.24 ± 0.66	21.76 ± 0.46[a]	0.47	0.72
Rat1 + ERα V_{EtOH}	40.00 ± 0.00	18.19 ± 0.47	21.81 ± 0.33[a]	0.52	0.69
Rat1 + ERβ OHT [0.04nM]	40.00 ± 0.00	18.56 ± 0.76	21.44 ± 0.53[a]	0.15	0.90
Rat1 + ERβ V_{EtOH}	40.00 ± 0.00	18.40 ± 0.39	21.60 ± 0.28[a]	0.31	0.80
Rat1 OHT [1nM]	40.00 ± 0.00	18.08 ± 0.40	21.92 ± 0.28[a]	0.63	0.64
Rat1 V_{EtOH}	40.00 ± 0.00	18.71 ± 0.47	21.29 ± 0.33[a]	0.00	1.00
Rat1 + ERα Gen [20nM]	40.00 ± 0.00	16.56 ± 0.29	23.44 ± 0.20[a]	1.11	0.46
Rat1 + ERα V_{EtOH}	40.00 ± 0.00	16.94 ± 0.38	23.06 ± 0.27[a]	0.73	0.60
Rat1 + ERβ Gen [0.3nM]	40.00 ± 0.00	16.59 ± 0.23	23.41 ± 0.17[a]	1.09	0.47
Rat1 + ERβ V_{EtOH}	40.00 ± 0.00	17.11 ± 0.41	22.89 ± 0.29[a]	0.57	0.67
Rat1 Gen [20nM]	40.00 ± 0.00	16.98 ± 1.10	23.02 ± 0.77[a]	0.69	0.62
Rat1 V_{EtOH}	40.00 ± 0.00	17.67 ± 0.49	22.33 ± 0.35[a]	0.00	1.00
Rat1 + ERα E_2 [1nM]	33.93 ± 1.71	16.48 ± 0.46	17.44 ± 0.89[a]	-4.84	28.57
Rat1 + ERα V_{EtOH}	40.00 ± 0.00	17.62 ± 0.35	22.38 ± 0.17[b]	0.10	0.93
Rat1 + ERβ E_2 [1nM]	40.00 ± 0.00	15.95 ± 0.28	24.05 ± 0.20[c]	1.77	0.29
Rat1 + ERβ V_{EtOH}	40.00 ± 0.00	17.50 ± 0.24	22.50 ± 0.17[b]	0.22	0.86
Rat1 E_2 [1nM]	40.00 ± 0.00	15.97 ± 0.14	24.03 ± 0.10[b]	1.75	0.30
Rat1 V_{EtOH}	40.00 ± 0.00	17.72 ± 0.53	22.28 ± 0.38[b]	0.00	1.00
Rat1 + ERα RAL [1nM]	40.00 ± 0.00	16.82 ± 0.42	23.18 ± 0.29[a]	0.11	0.92
Rat1 + ERα V_{DMSO}	40.00 ± 0.00	16.79 ± 0.26	23.21 ± 0.19[a]	0.14	0.91
Rat1 + ERβ RAL [0.04nM]	40.00 ± 0.00	16.98 ± 0.22	23.02 ± 0.15[a]	-0.05	1.03
Rat1 + ERβ V_{DMSO}	40.00 ± 0.00	16.87 ± 0.26	23.13 ± 0.18[a]	0.06	0.96
Rat1 RAL [1nM]	40.00 ± 0.00	16.86 ± 0.20	23.15 ± 0.14[a]	0.07	0.95
Rat1 V_{DMSO}	40.00 ± 0.00	16.93 ± 0.12	23.07 ± 0.08[a]	0.00	1.00

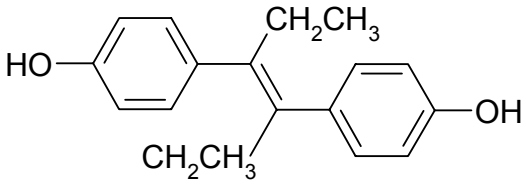
Table 7. Progesterone receptor expression at 24 h exposure to selected ligands

Cell line - **24 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Rat1 + ERα DES[0.4nM]	34.95 ± 1.86	16.48 ± 0.33	18.47 ± 1.08[a]	-3.92	15.12
Rat1 + ERα V_{EtOH}	39.71 ± 0.71	18.11 ± 0.56	21.59 ± 0.10[b]	-0.79	1.73
Rat1 + ERβ DES [0.05nM]	39.77 ± 0.56	16.30 ± 0.19	23.47 ± 0.26[b]	1.07	0.47
Rat1 + ERβ V_{EtOH}	40.00 ± 0.00	17.53 ± 1.71	22.47 ± 1.21[b]	0.08	0.94
Rat1 DES[0.4nM]	40.00 ± 0.00	16.33 ± 0.43	23.67 ± 0.30[b]	1.27	0.41
Rat1 V_{EtOH}	40.00 ± 0.00	17.61 ± 1.32	22.39 ± 0.93[b]	0.00	1.00
Rat1 + ERα OHT [1nM]	40.00 ± 0.00	17.18 ± 0.29	22.82 ± 0.21[a]	2.37	0.19
Rat1 + ERα V_{EtOH}	40.00 ± 0.00	18.96 ± 0.46	21.03 ± 0.33[b]	0.58	0.67
Rat1 + ERβ OHT [0.04nM]	40.00 ± 0.00	17.07 ± 0.15	22.93 ± 0.11[a]	2.47	0.18
Rat1 + ERβ V_{EtOH}	40.00 ± 0.00	17.35 ± 0.14	22.64 ± 0.10[a]	2.19	0.22
Rat1 OHT [1nM]	40.00 ± 0.00	17.21 ± 0.15	22.79 ± 0.11[a]	2.33	0.20
Rat1 V_{EtOH}	40.00 ± 0.00	19.54 ± 0.08	20.46 ± 0.06[b]	0.00	1.00
Rat1 + ERα Gen [20nM]	38.57 ± 2.22	17.52 ± 0.70	21.05 + 1.07[a]	0.05	0.97
Rat1 + ERα V_{EtOH}	40.00 ± 0.00	19.57 ± 0.34	20.43 + 0.24[a]	-0.57	1.48
Rat1 + ERβ Gen [0.3nM]	40.00 ± 0.00	18.05 ± 0.22	21.95 + 0.15[a]	0.95	0.51
Rat1 + ERβ V_{EtOH}	40.00 ± 0.00	17.35 ± 1.09	22.65 + 0.77[a]	1.65	0.32
Rat1 Gen [20nM]	40.00 ± 0.00	17.59 ± 0.35	22.41 + 0.25[b]	1.41	0.38
Rat1 V_{EtOH}	40.00 ± 0.00	19.00 ± 1.09	21.00 + 0.77[a]	0.00	1.00
Rat1 + ERα E_2 [1nM]	35.21 ± 0.79	19.94 ± 0.77	15.27 ± 0.01[a]	-4.31	19.90
Rat1 + ERα V_{EtOH}	39.84 ± 0.38	22.81 ± 0.87	17.03 ± 0.34[b]	-2.55	5.84
Rat1 + ERβ E_2 [1nM]	40.00 ± 0.00	20.30 ± 0.55	19.70 ± 0.39[c]	0.11	0.92
Rat1 + ERβ V_{EtOH}	40.00 ± 0.00	20.89 ± 0.70	19.11 ± 0.49[c]	-0.48	1.39
Rat1 E_2 [1nM]	40.00 ± 0.00	20.43 ± 0.83	19.57 ± 0.59[c]	-0.02	1.01
Rat1 V_{EtOH}	40.00 ± 0.00	20.42 ± 1.96	19.58 ± 1.39[c]	0.00	1.00
Rat1 + ERα RAL [1nM]	39.71 ± 0.71	14.09 ± 0.60	25.63 ± 0.46[a]	0.23	0.85
Rat1 + ERα V_{DMSO}	39.70 ± 0.70	14.43 ± 0.16	25.28 ± 0.40[a]	-0.11	1.09
Rat1 + ERβ RAL [0.04nM]	40.00 ± 0.00	14.27 ± 0.11	25.73 ± 0.08[a]	0.34	0.79
Rat1 + ERβ V_{DMSO}	40.00 ± 0.00	14.35 ± 0.31	25.65 ± 0.22[a]	0.26	0.83
Rat1 RAL [1nM]	40.00 ± 0.00	14.32 ± 0.15	25.68 ± 0.11[a]	0.28	0.81
Rat1 V_{DMSO}	40.00 ± 0.00	14.60 ± 0.19	25.39 ± 0.13[a]	0.00	1.00

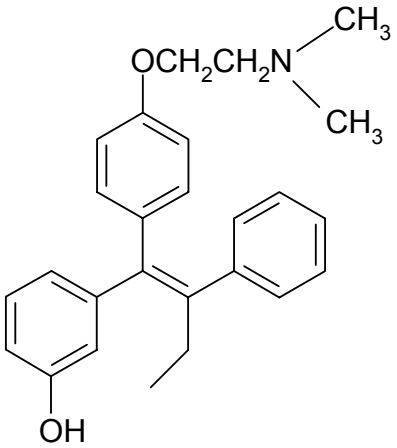
Figure 1. Chemical structures of estrogenic compounds used in treatment schemes. All chemicals were of pharmaceutical grade



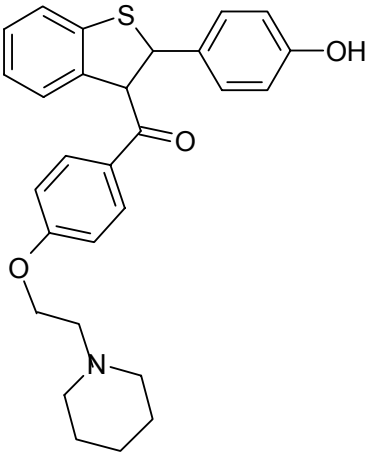
17β-estradiol (E_2)



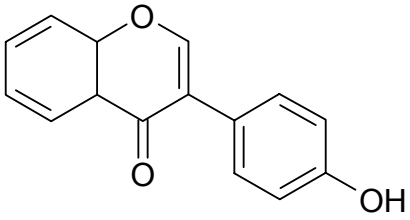
Diethylstilbestrol (DES)



4-hydroxytamoxifen (OHT)



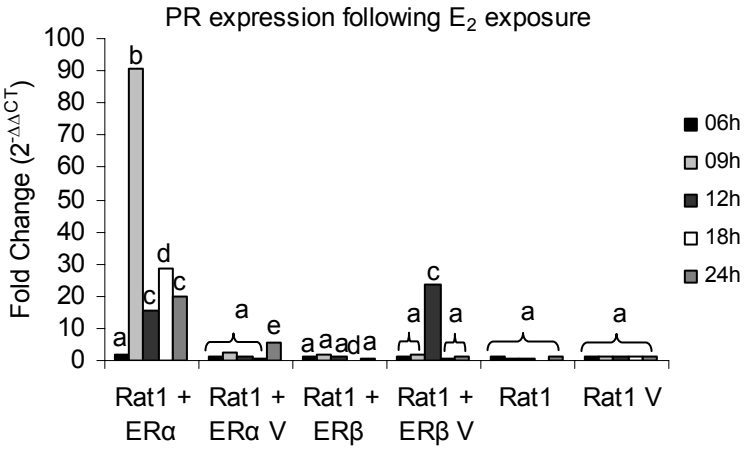
Raloxifene (RAL)



Genistein (GEN)

Figure 2. Estrogen and synthetic estrogen agonist DES effects on PR expression. Time by cell effects for each ligand exposure were analyzed by ANOVA ($P<0.0001$) performed on the ΔC_T values. Letters denote significant interactions between time and isotype, with significance set at $P \leq 0.05$. **a)** Progesterone receptor expression following E_2 exposure over time. Greatest expression occurred in the presence of ERα at 09h with an increase 90 fold over Rat1 V. This 9 h induction is nearly 6-fold greater than that seen at 12 h, 3-fold than that at 18 h, and 4.5-fold over 24h. This suggests a predominant role for E_2 bound ERα in mediation of PR induction that is time sensitive. An increase was also seen in the presence of ERβ following the control vehicle treatment, with of 23-fold increase that was similar to ERα E_2 exposed cells at 12 and 24 h. This may result from being fed with fresh media at the same time as vehicle treatment. **b)** Progesterone receptor expression following DES exposure over time. The only significant induction in expression of PR was seen in the presence of ERα at 24 h with an approximate 15-fold increase over Rat1 V. This suggests that DES bound ERα may function in a manner divergent from E_2 bound ERα in regards to PR mediation. There was also a significant decrease in PR expression at 12 h in the ERβ treated, when compared with that which was observed with control treatment of vehicle.

a)


PR expression following E_2 exposure
Fold Change ($2^{-\Delta\Delta CT}$)
100
90
80
70
60
50
40
30
20
10
0
06h
09h
12h
18h
24h
Rat1 + ERα
Rat1 + ERα V
Rat1 + ERβ
Rat1 + ERβ V
Rat1
Rat1 V

b)

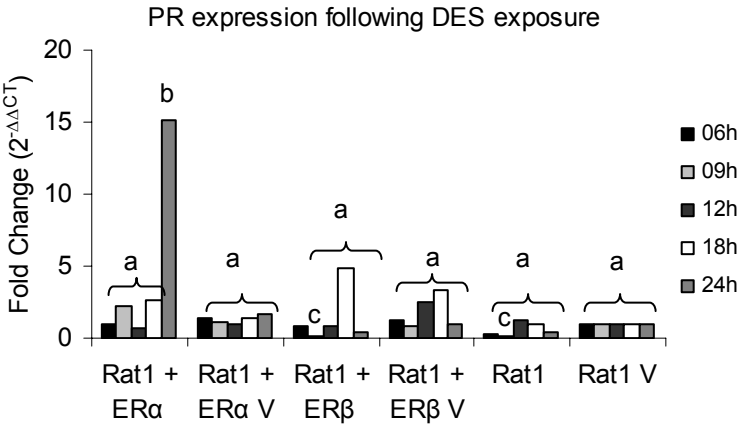
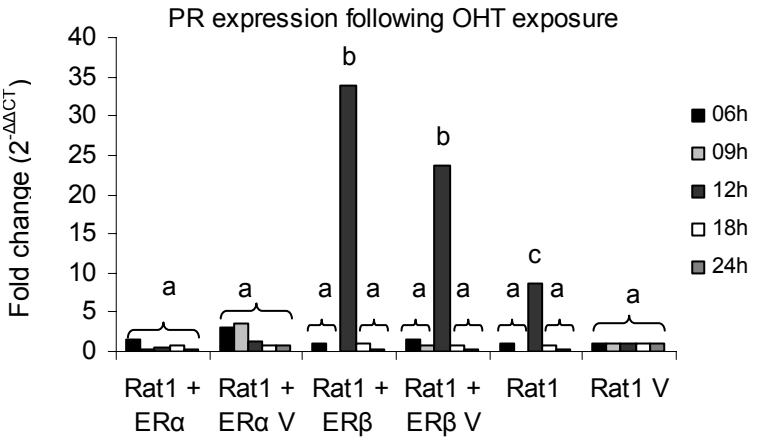

PR expression following DES exposure
Fold Change ($2^{-\Delta\Delta CT}$)
20
15
10
5
0
06h
09h
12h
18h
24h
Rat1 + ERα
Rat1 + ERα V
Rat1 + ERβ
Rat1 + ERβ V
Rat1
Rat1 V

Figure 3. Evaluation of PR expression following exposure to the SERMs OHT and RAL in cells expressing either ERα or ERβ. Time by cell effects for each ligand exposure were analyzed by ANOVA ($P<0.0001$) with Least Square Difference analysis being performed on the ΔC_T values. Letters denote significant interactions between time and cell line isotype, with significance set at $P \leq 0.05$. **a)** 4-Hydroxytamoxifene had no induction effect on PR with independent ERα. However, treatment did yield a significant increase in PR expression at 12 h in ERβ expressing cells following treatment that was statistically different across time, but did not differ from the control vehicle treated PR expression at 12 h. To an extent an increase was seen in the Rat1 treated cells. **b)** Raloxifene had no significant effect on PR expression, but a trend toward increased expression at 12h in ERβ vehicle treated cells.

a)


PR expression following OHT exposure
Fold change ($2^{-\Delta\Delta CT}$)
40
35
30
25
20
15
10
5
0
a
a
a
b
a
a
b
a
a
c
a
a
a
Rat1 + ERα
Rat1 + ERα V
Rat1 + ERβ
Rat1 + ERβ V
Rat1
Rat1 V
06h
09h
12h
18h
24h

b)

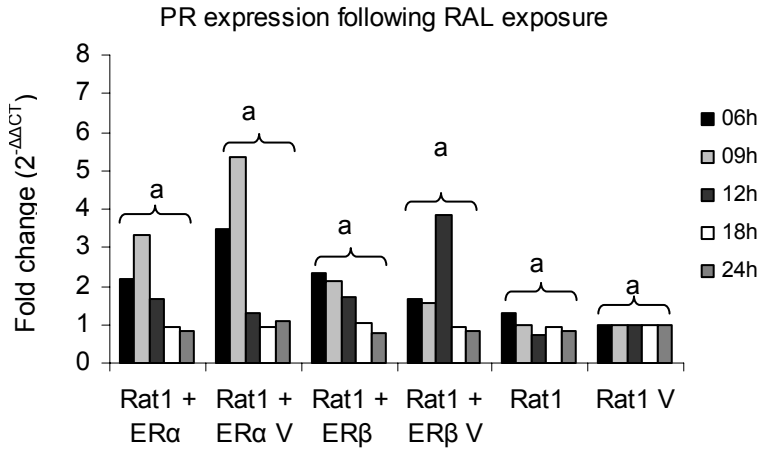

PR expression following RAL exposure
Fold change ($2^{-\Delta\Delta CT}$)
8
7
6
5
4
3
2
1
0
a
a
a
a
a
a
Rat1 + ERα
Rat1 + ERα V
Rat1 + ERβ
Rat1 + ERβ V
Rat1
Rat1 V
06h
09h
12h
18h
24h

Figure 4. Effects of phytoestrogen GEN treatment on PR expression over time. A significant effect with a 24 fold increase of PR expression is seen at 12 h in the presence of GEN in the Rat1+ERβ cell line ($P<0.0001$). Differences were also detected in the vehicle treated ERβ and Rat1 treated at the same time-point, though these were significantly less than the response observed following GEN treatment. This is consistent with observed higher binding affinities for ERβ and suggests a unique activation pathway through GEN binding of ERβ.

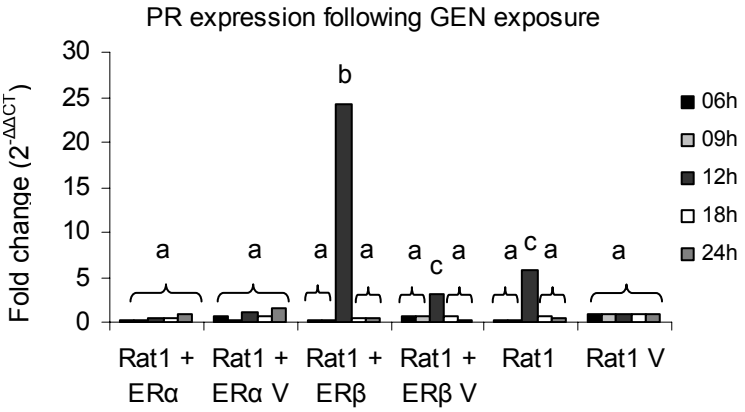
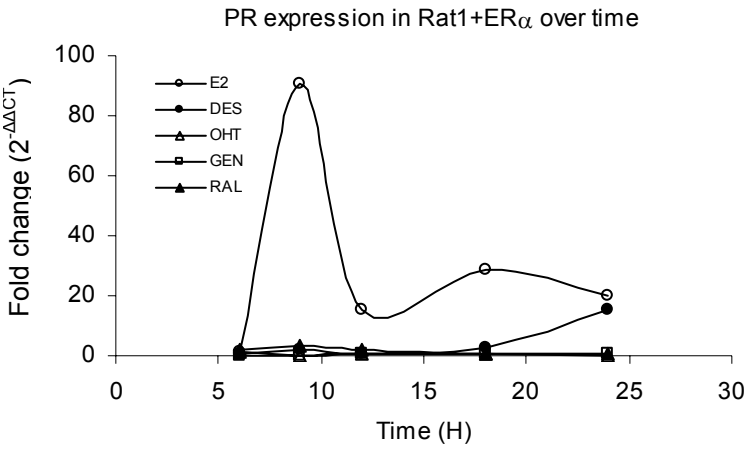
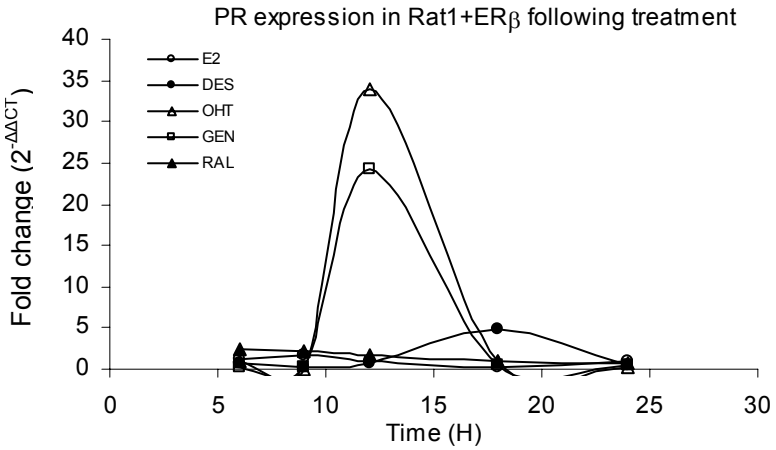

PR expression following GEN exposure
Fold change ($2^{-\Delta\Delta CT}$)
30
25
20
15
10
5
0
a
a
a
b
a
a
a
c
a
a
c
a
a
06h
09h
12h
18h
24h
Rat1 + ERα
Rat1 + ERα V
Rat1 + ERβ
Rat1 + ERβ V
Rat1
Rat1 V

Figure 5. PR expression over time for all treatments observed in either ERα or ERβ expressing cell lines. Cell line by compound by time effects were detected ($P<0.0001$) using an ANOVA generated in SAS. Summary expression profiles of PR are separated based on the presence of ERα or ERβ within the cells. **a)** Rat1+ERα following E_2 treatment is the main regulator for PR expression with peak expression reached by 9 h. It is also observed that DES exposure mirrors E_2 exposure, but only following a 24 h period. **b)** Rat1+ERβ cells were slightly, though not statistically affected by DES treatment at 18 h, while the highest statistical induction of PR is seen by GEN at 12 h. 4-Hydroxytamoxifen also shows a large regulation event at 12 h, however this effect is mirrored in the vehicle treated cells at this time.

a)


PR expression in Rat1+ERα over time
Fold change (2-ΔΔCT)
Time (H)
E2
DES
OHT
GEN
RAL
0
20
40
60
80
100
0
5
10
15
20
25
30

b)


PR expression in Rat1+ERβ following treatment
Fold change (2-ΔΔCT)
Time (H)
E2
DES
OHT
GEN
RAL
0
5
10
15
20
25
30
35
40
0
5
10
15
20
25
30

CHAPTER V

Ligand and time course effect on RACK1, COL1A2, PCOLCE, and CtsL gene expression in the presence of estrogen receptor alpha or beta

ABSTRACT

Estrogen receptors (ER) function in the endocrine system by binding estrogen and estrogenic like compounds, regulating transcription, and eliciting a myriad of physiological responses. Two distinct ER isotypes, ERα and ERβ, display differential localization patterns and ligand binding affinities. We have previously described unique gene expression profiles for the two ER subtypes, ERα and ERβ, in the context of the engineered Rat1 embryonic fibroblast cell lines Rat1+ERα and Rat1+ERβ following E_2 treatment and utilizing suppression subtractive hybridization. In the current study, we have evaluated expression of four genes; receptor for activated protein kinase C (RACK1), pro-α-2(I)-collagen (COL1A2), procollagen C – proteinase enhancer protein (PCOLCE), and cathepsin L (CtsL), identified as differentially regulated in the presence of ERα or ERβ to examine the effects of ligand treatment and temporal effects at a single dose exposure. The individual cell lines were treated for 6, 9, 12, 18, or 24 h with estradiol-17β (E_2), diethylstilbestrol (DES), 4-hydroxytamoxifen (OHT), genistein (GEN), raloxifene-HCl (RAL), or vehicle. Total RNA was extracted and subjected to DNAse treatment followed by quantitative PCR (RT-qPCR). Results were analyzed using the comparative C_T method and statistical analysis carried out using a 3X5X5

factorial in a completely randomized design. All four genes demonstrated unique responses over time in response to the various ligands. This data further describes a model in which ERα or ERβ are able to independently regulate transcription in a distinctive manner in regards to context of treatment.

INTRODUCTION

Estrogen receptors (ER) are ligand inducible transcription factors that can bind a wide range of natural and environmental estrogenic compounds and through transcriptional modulation play a role in multiple physiological systems. There are two recognized isotypes, ERα (Toft & Gorski 1966) and ERβ (Kuiper *et al.* 1996) that exhibit divergent and overlapping structure, tissue localization, affinity to ligands, and transcriptional activation. Since the relatively recent discovery of a second ER, there has been an impetus to understand the different levels of control that can be affected between receptor types. The two ERs appear to be co-expressed at similar levels in the testis, epididymis, bone, and adrenal gland (Couse & Korach 1999) while ERα expression predominates in the proliferative cells of the mammary, pituitary and thyroid glands, as well as uterus, theca cells of the ovary, skeletal muscle and the smooth muscle of the coronary arteries, and ERβ is predominate in the prostate (Kuiper *et al.* 1996), granulosa cells of the ovary, and the lung, bladder, brain and hypothalamus (Kuiper *et al.* 1997).

When both receptors are present they may form homo or heterodimers that will interact with the ERE (Pettersson *et al.* 1997; Cowley *et al.* 1997; Pace *et al.* 1997).

Alternatively, the ER dimers can bind at non-classical promoter sites through protein-protein interactions of primarily two differing complexes; *c-fos/c-jun* at an AP-1 binding site and the Sp1 protein (Webb *et al.* 1995; Saville *et al.* 2000). The ERβ has also been observed to antagonize ERα function through heterodimer formation (Hall & McDonnell 1999). For these reasons it is of interest to examine ERα and ERβ in an independent manner to determine differential patterns of gene regulation within a given cell type.

In addition to physiological estrogens such as 17β-estradiol (E_2), ER also has the ability to bind to a number of synthetic estrogens such as diethylstilbestrol (DES), selective estrogen receptor modulators (SERMS) like 4-hydroxytamoxifen (OHT) and raloxifene (RAL), as well as environmental estrogenic-like compounds such as those found in plants, for example the isoflavone genistein (GEN). These compounds have been shown to exhibit distinct binding affinities for ERα and ERβ (Kuiper *et al.* 1997), in addition to being able to effect differential transcription control at the indirect promoter sites AP-1 (Webb *et al.* 1995) and Sp1 (Saville *et al.* 2000). Additionally, there is evidence that differing ligands exert changes in conformation of the ER protein which in turn lead to the recruitment of distinct co-regulators to the transcriptional site (Torchia *et al.* 1998; An *et al.* 2001; Ratajczak T 2001).

The ERα and ERβ have distinct and differential transcriptional regulation. We have previously described unique independent expression profiles for the two ER subtypes, ERα and ERβ, in the context of engineered Rat1 embryonic fibroblast cell lines Rat1+ERα and Rat1+ERβ following E_2 treatment and utilizing suppression subtractive

hybridization (Hurst *et al.* 2004). Here we examine four of these previously identified genes to determine effects of ligand and time of exposure; receptor for activated protein kinase C (RACK1), pro-α-2(I)-collagen (COL1A2), procollagen C – proteinase enhancer protein (PCOLCE), and cathepsin L (CtsL), to examine the effects of ligand and time exposure.

MATERIALS AND METHODS

CHEMICALS

All chemicals were obtained through Sigma, St. Louis MO and were of pharmaceutical grade. The compounds E_2, DES, OHT, and GEN were dissolved in 100% ethanol. Raloxifene-HCl was dissolved in DMSO.

CULTURE CONDITIONS

Rat1, Rat1+ERα, and Rat1+ERβ cell lines were maintained as previously described (Cheng & Malayer 1999; Hurst *et al.* 2004). Cells were grown in sterile filtered (0.22 μM), phenol red–free Dulbecco's Modified Eagle Medium without glutamine, sodium pyruvate, or sodium bicarbonate (DMEM; Fisher, Plano TX), supplemented with $NaHCO_3$ (3.7 g/L) and 5mg/mL L-glutamine (Sigma, St. Louis MO). Further supplementation was provided with bovine insulin (0.6 μg/ml) in HEPES (25 μM) (Sigma, St. Louis MO), 1X antibiotic – antimycotic (Sigma, St. Louis MO), and 10% charcoal – stripped/ dextran treated fetal bovine serum (CSFBS; Hyclone, Logan UT). Cells were maintained at 37˚C in a humidified atmosphere of 5% CO_2 gas, 95% air, and

media replaced every 48 hours. Rat1+ERα cells were supplemented with the selective antibiotic Hygromyocin B (100 μg/ml) in PBS (Invitrogen, Carlsbad CA) beginning 24 h after plating (Kaneko *et al.* 1993). Rat1+ERβ cells were supplemented with 50 μg/ml Geneticin (Gibco, Grand Island NY) in the same manner (Cheng & Malayer 1999).

RNA EXTRACTION

Following the appropriate time period of 6, 9, 12, 18, or 24 h, cells were washed 3 times with 1X PBS (Gibco, Grand Island NY) and lysed in guanidinium thiocyanate (Promega, Madison WI) per the total RNA extraction method described by Chomczynski and Sacchi (Chomczynski & Sacchi 1987). Following extraction, DNA contamination was corrected for through treatment with RQ1 RNAse-free DNAse (Promega, Madison WI) at a concentration of 1U/10μl for 30 min at 37°C. This was followed by phenol:chloroform:isoamyl alcohol (P:C:I) purification, ethanol precipitation, and concentrations were determined by spectrophotometry.

REAL-TIME QUANTITATIVE PCR

Quantitative PCR was then carried and analyzed with modifications (Gibson *et al.* 1996; Bustin SA. 2002; Ginzinger 2002). The 5′ nuclease activity assay scheme was incorporated using probes that contained a 3′ fluorescent TAMRA quencher dye, and a 5′ FAM reporter dye, and carried out using the one-step RT-qPCR chemistry (Eurogentec North America Inc, San Diego, CA). Taqman® primers and probe for target genes were generated using Primer Express® software (PE Applied Biosystems, Foster City CA), as

previously reported (Hurst et al, 2004). Expression was examined using total RNA (10 ng) by means of primer [300 nM] and probe [200 nM] sets, with a loading duplicate. Each population of total RNA (50 pg) was normalized in duplicate using 18S ribosomal RNA (Eurogentec North America Inc, San Diego, CA) at a [200 nM] primer [100 nM] probe concentration, and the efficiency was checked via a standard curve of serial dilutions of Rat1+ERα E_2 treated samples. For target, a dilution series of 10, 5, 1, 0.5, and 0.1 ng was used. For 18S ribosomal RNA a series of 5, 1, 0.5, 0.1, and 0.05 ng dilutions were used. Real-time PCR was carried out in the ABI PRISM 7700 (PE Applied Biosystems, Foster City CA) under the following thermal cycler conditions; 48˚C for 30 min and 95˚C for 10 min, followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min, in a 25 μL reaction with a 28 sec exposure time. Analysis and fold differences were determined using the comparative C_T method as described in the ABI technical bulletin #2 for the ABI PRISM 7700 (Gibson *et al.* 1996; Bustin SA. 2002; Ginzinger 2002), where the Rat1 vehicle treated cell line was always used as the calibrator for determining the $2^{-\Delta\Delta CT}$ fold difference values.

Statistical Analysis

Statistical comparison of RT-qPCR ΔC_T values with means $\pm$ S.D. and reported for n = 3 for cell cultures and n=2 for loading replication in the 5′ nuclease assay reaction. Results were tested using 3X5X5 factorial ANOVA tables generated through PROC-GLM utilizing a complete randomized design constructed using the Statistical Analysis System (Table 1), and p-values analyzed through least square differences. For cell line by compound by time comparisons degrees of freedom were equal to 150. Comparisons

across these three factors were made in relation to the vehicle treatment for each experiment, and statistical difference determined at $p \leq 0.05$.

RESULTS

PRO-A-2(I)-COLLAGEN

A significant cell by compound by time effect was seen for COL1A2 ($p<0.0306$). In Rat1+ERα cells DES and E_2 treatments resulted in significant decreases from vehicle expression at 6 h (Table 2a; Figure 1a & b). Treatment with GEN resulted in decreased expression at 6 ($p<0.0420$) and 24 h ($p<0.0001$), and OHT treatment also resulted in a significant decrease at 24h ($p<0.0176$) (Figure 2a). Cells exposed to E_2 or DES for24 h had observed increases in expression to a level that was no longer significantly different from the vehicle treated parental cell line. When this 24 h DES exposure was compared to treatments of OHT ($p<0.0033$), RAL ($p<0.0393$), or GEN ($p<0.0001$) the DES treatment was statistically higher.

Significant induction over its own vehicle treatment occurred only in ERβ expressing cells with DES at 09h ($p<0.0319$), GEN at 18h ($p<0.0001$), and RAL at 06h ($p<0.0084$) (Figure 2b). Treatment with OHT resulted in expression that was higher than that seen in Rat1, ERα expressing, or ERβ E_2 or DES treated at the same 6 h time (Table 2a). Likewise, at 6 h ERβ expressing cells exposed to GEN differed from E_2 or DES treated cells, as well as any ERα expressing cell treated at the same time-point. Previous analysis of COL1A2 noted an increase in expression in ERβ expressing cells at 24h with

a single dose of E_2, and where an increase in the profile is observable it did not reach a level of significance ($p<0.4737$), which may be a result of difference in analysis and replication. However, ERβ E_2 treated cells at 24h had significantly higher COL1A2 compared to vehicle than the other four treatments at the same time-point and in the same cell type.

CATHEPSIN L

Cell by compound by time effects ($p<0.0062$) were observed for CtsL. As previously reported, CtsL expression had a significant increase in ERα expressing cells ($p<0.0080$) at 24h following a single exposure to E_2 with a 4-fold increase over ERβ (Table 3E). Additionally CtsL levels remained above vehicle at 18h with E_2 ($p<0.0079$), but fell below Rat1 ($p<0.0004$). Interestingly, at 6, 9, and 12 h a significant level of repression was observed for the same treatment parameters (Figure 3a). Genistein, though a weak ERα agonist, was able to significantly increase CtsL expression at 9 h ($p<0.0001$) in the ERα expressing cells in a manner greater than that seen by ERβ at the same time ($p<0.0058$).

Rat1+ERβ showed a pulse-like change in CtsL expression with up-regulation at 0 9 and 18h in response to GEN exposure, however only 18 h reached a level of significance over its own vehicle ($p<0.0001$), even though this did not exceed expression seen in the Rat1 ER naïve cells (Figure 4). Neither of the SERMs, OHT or RAL, were able to effect

significant increases in levels of CtsL, and OHT actually was able to significantly decrease CtsL in the ERβ expressing cells at 24 h ($p<0.0020$).

RECEPTOR FOR ACTIVATED PROTEIN KINASE C

Cell by time ($p<0.0034$) and compound by time ($p<0.0001$) interactions were observed for RACK1. Rat1+ERα had a significant regulation event occurring at 24h ($p<0.007$), which would appear to be in line with previous results with ERα expressing cells having a 5-fold higher expression of RACK1 than ERβ (Table 4E). Also at 24h DES treatment of ERα expressing cells resulted in a significant 6.5 fold increase over ERβ that was of a greater magnitude than that seen in response to E_2. Interestingly, OHT also was able to induce a significant increase in the amount of RACK1 at 06h following exposure in ERα expressing cells ($p<0.0001$; Table 4A). An increase, though smaller was also seen in RAL treated ERα cells at this time. This up-regulation was lost by 09h, but at this time point GEN showed an up-regulation of RACK1 which was greater in ERα, but also observed in ERβ (Figure5). Raloxifene also had an up-regulation affect on RACK1 at 09h that was specific to ERα (Figure 5 and 6a). Treatment with OHT resulted in an increase in RACK1 expression compared to control in ERβ at 06h ($p<0.0137$), however this was the only significant induction seen in the Rat1+ERβ cell line.

A compound by time affect was detected for PCOLCE expression ($p<0.0001$). Similar to results reported by Hurst *et al*. (2004) PCOLCE had a nearly 4-fold increase in ERα expressing cells over ERβ expressing cells at 24 h (Table 5E). This was mirrored in the DES treated ERα cells, however this increase was less than the E_2 effect ($p<0.0163$; Figure 7). At 9 h following OHT exposure a non-cell specific significant increase in PCOLCE expression was observed in all three cell lines ($p<0.0001$) (Figure 7). Additionally, GEN treatment at 09h was able to induce an increase in ERα expressing cells compared to 06, 18 and 24h (Figure 8). A trend towards increased PCOLCE was observed at 18 h in ERβ expressing cells following treatment when compared to controls, with RAL having the greatest effect (Figure 8).

DISCUSSION

It has been shown that ERα and ERβ exhibit unique control over transcriptional gene expression profiles in response to a single ligand at a single time-point. However, fixation on a single exposure narrows the breadth of the dynamics that may be involved in a ligand response. Additionally, it is well accepted that regulation pathways are differentially affected in relation to varying ligands. With these points in mind we have examined previously identified genes (Hurst *et al.* 2004) in response to different exposures of ligand over time to increase our understanding of the independent nature of gene regulation by ERα and ERβ. Concentrations of applied ligands were normalized to reported binding affinities for hERα and rERβ (Kuiper *et al.* 1997), which are present in

our cell line, with regard for the 10X lower bind affinity of the HEG0 hERα mutant (Tora *et al.* 1989). These data have demonstrated that ligand and time affect differential control upon these genes. This is not unexpected as it is well recognized that gene expression occurs in early and delayed waves of response, but what is of interest is that alternate ligands have the ability to alter the timing of the response depending on which receptor is present.

Type 1 collagen accounts for the majority of total collagens and is most abundant in bone, and is involved in bone and cartilage remodeling (Nimni 1983). These type I collagens are fibril-forming and are synthesized as larger precursors which are cleaved by proteinases to create the functional protein (Prockop 1995). Pro-alpha-2(I) collagen is one of two alpha chains that comprise one-third of the type I collagen heterotrimer (Smith & Niles 1980). The COL1A2 polypeptide chains are commonly synthesized by fibroblasts, and osteoblasts, together with COL1A1 which then aggregate to form collagen (Verrecchia & Mauviel 2004). Transcription of COL1A2 requires complex and cooperative protein-protein interactions that are not yet fully understood. Up-stream binding elements interact with proteins such as Sp1, AP-1 and *cis*-acting elements in a strongly tissue-specific manner (Tanaka *et al.* 2004).

The cytokines TGF-β and TNF-α play a pivotal role in the transduction of collagens, with TGF-β requiring Smad3 and Smad4 transcription factors for COL1A2 transcription (Verrecchia & Mauviel 2004). This is relevant to ER due to cross-talk pathways in which ER inhibit TGF-β signaling through Smad3 association and repression (Matsuda *et al.*

2001). The TNF-α promoter is also strongly inhibited by ERβ and slightly so by ERα through repression of transcription via the AF-2 region of the LBD (An *et al.* 1999). With the shifts in profiles related to time seen with our results, it is possible that factors such as TGF-β and TNF-α may be interacting in a cascade of events that begins with the selective binding of ERs to ligand. This would fit with the strong apparent inhibition by ERα at early time points, and suggest that due to levels returning close to baseline with a given compound in a temporal manner that repression events are ligand dependent.

The cathepsin gene family is composed of lysosomal proteases that play multiple roles in cellular maintenance and remodeling events. As such, they are often unregulated in cancers and therefore have major implications in oncogenesis and tumor invasiveness (Koblinski et al. 2000). As a lysosomal cysteine protease, CtsL is implicated in human trophoblast invasiveness (Divya *et al.* 2002), bone resorption (Kakegawa *et al.* 1993), and degradation of extracellular matrix (Mason *et al.* 1986) are an example of cellular functions that tie into reproduction, inflammatory responses, and bone remodeling. Proteases such as CtsL also play important roles in male fertility and spermatozoa production in the testis (Peloille *et al.* 1997). Estrogen receptors also have regulational control in the same tissues that are affected by cathepsins, and our results suggest that CtsL expression may be linked to ERα activation. The ability of the different ligand treatments to result in increased expression in ERβ at 18 h mirrors only RAL exposure in ERα, suggesting that perhaps specific ligands can create a response that is apparently similar to an isotype switch. It is also interesting that compounds like GEN (9 h) and

OHT (6 h) can function in the same manner in both ERα and ERβ, when other compounds fail to interact in a similar manner.

Receptor for activated kinase C binds the isozyme protein kinase C (PKC) and acts to stabilize the active conformation of PKC, which is necessary for subcellular translocation (Ron *et al.* 1994). The RACK 1 protein is a homologue of guanine nucleotide-binding protein (G-protein) β subunit (Ron *et al.* 1994) and ERα studies have noted a relationship between E_2 and G-protein coupled receptors to affect PKC (Kelly *et al.* 1999). The ability of ER to interact with this gene in a time and compound manner have implications for basic transcriptional machinery function. We have observed that ERα has the ability, in the presence of E_2, to increase RACK1 at 24 h in a manner greater than ERβ, and that this is amplified in the presence of the synthetic estrogen DES. Raloxifene and GEN at 06h increase RACK1 in the presence of ERα in a manner greater than E_2 treatment, while OHT with ERβ creates the only appreciable increase in RACK1 in that cell line. This suggests a role for partial agonist/antagonist SERMs with ERβ, and further defines the preferential binding and transcriptional activation role of GEN in cells expressing ERβ. In conjunction with an apparent time component this observation suggests significant roles for exogenous estrogenic compounds to interact with the ERβ isotype in the context of the RACK1 gene.

Procollagen C–proteinase enhancer protein is an elongated glycoprotein (Bernocco *et al.* 2003) enhancer element that binds the C-terminus of the type I procollagen propeptide, and as such enhances the enzymatic ability of procollagen C-proteinase (Takahara *et al.*

1994). Procollagen C-proteinases are involved with cleavage of the C-propeptide from the collagen precursor to create a mature collagen (Prockop 1995). Procollagen C – proteinase enhancer protein is present in high levels in bone, tendon (Shalitin *et al.* 2003), and the uterus (Scott *et al.* 1999), and plays a role in intracellular collagen formation and extracellular cell differentiation and proliferation, as well as possible stabilization of COL1A2 mRNA (Matsui *et al.* 2002).

The induction of PCOLCE expression has already been shown to be linked with TGF-β expression in a fibrogenic cell line (Lee *et al.* 1997), and correlates with expression of type I collagens in the culture media of cardiac fibroblast cell lines (Shalitin *et al.* 2003). Again the regulation events here, like with COL1A2, would suggest that ER can affect target genes such as cytokines like TGF-β in a manner that is manifest in the expression of downstream gene products such as PCOLCE. It is of interest that OHT at 9 h has an impact on ERα PCOLCE expression, and to a lesser extent ERβ and the naïve cell line, which suggests that ERα may only be serving to potentiate an otherwise non-receptor OHT mediated event in this target.

Ligands create differences in gene expression and transcriptional efficacy through pathway interactions, one being the physical binding of ligand within the ER binding pocket and displacement of H12 (Nichols *et al.* 1998). An example of this is the ability of raloxifene to sterically restrict H12 within the binding cavity in a recognized antagonistic position due to its piperidine ring (Brzozowski *et al.* 1997). The ligands E_2 and OHT have been shown to have distinctive structural requirements and utilizing

distinguishable regulatory elements (Zajchowski *et al.* 1997). Additionally, co-regulators have been identified that interact at various sites within the ER protein to either activate of repress the transcriptional machinery, often in a receptor and ligand dependent manner (Torchia *et al.* 1998; An *et al.* 2001; Ratajczak 2001). Using global techniques such as microarrays it has also been demonstrated that OHT and RAL have the ability to regulate different sets of genes within U2OS (human osteosarcoma) cell lines (Tee MK *et al.* 2004). These observations help to explain, in part, the divergent effects that compound exposure may impart upon the gene expression profiles examined here.

Taken together these data suggest unique regulation for genes in the presence of ligands of varying binding affinities, which in turn has a temporal component which may be modified through ligand involvement with promoters and co-regulator recruitment. Protein profiles and microarray analysis to examine global changes over time would be of great interest, as would determining the promoter site interactions and co-regulator recruitment profiles that are occurring within these cell lines at the given treatment regimes described.

REFERENCE LIST

An J, Ribeiro RCJ, Webb P, Gustafsson JA, Kushner PJ, Baxter JD & Leitman DC. 1999 Estradiol repression of tumor necrosis factor-alpha transcription requires estrogen receptor activation function-2 and is enhanced by coactivators. *Proceedings of the National Academy of Sciences* **96** 15161-15166.

An J, Tzagarakis-Foster C, Scharschmidt TC, Lomri N & Leitman DC. 2001 Estrogen receptor beta -selective transcriptional activity and recruitment of coregulators by phytoestrogens. *Journal of Biological Chemistry* **276** 17808-17814.

Bernocco S, Steiglitz BM, Svergun DI, Petoukhov MV, Ruggiero F, Ricard-Blum S, Ebel C, Geourjon C, Deleage G, Font B, Eichenberger D, Greenspan DS & Hulmes DJS. 2003 Low Resolution Structure Determination Shows Procollagen C-Proteinase Enhancer to be an Elongated Multidomain Glycoprotein. *J.Biol.Chem.* **278** 7199-7205.

Brzozowski AM, Pike AC, Dauter Z, Hubbard RE, Bonn T, Engstrom O, Ohman L, Greene GL, Gustafsson JA & Carlquist M. 1997 Molecular basis of agonism and antagonism in the oestrogen receptor. *Nature.* **389** 753-758.

Bustin SA. 2002 Quantification of mRNA using real-time reverse transcription PCR (RT-PCR): trends and problems. *J Mol Endocrinol.* **29** 23-39.

Cheng J & Malayer JR. 1999 Responses to stable ectopic estrogen receptor-beta expression in a rat fibroblast cell line. *Mol Cell Endocrinol.* **156** 95-105.

Chomczynski P & Sacchi N. 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem.* **162** 156-159.

Couse JF & Korach KS. 1999 Estrogen receptor null mice: what have we learned and where will they lead us? *Endocrine Reviews* **20** 358-417.

Cowley SM, Hoare S, Mosselman S & Parker MG. 1997 Estrogen receptors α and β form heterodimers on DNA. *Journal of Biological Chemistry* **272** 19858-19862.

Divya, Chhikara P, Mahajan VS, tta Gupta S & Chauhan SS. 2002 Differential activity of cathepsin L in human placenta at two different stages of gestation. *Placenta* **23** 59-64.

Gibson UE, Heid CA & Williams PM. 1996 A novel method for real time quantitative RT-PCR. *Genome Res.* **6** 995-1001.

Ginzinger DG. 2002 Gene quantification using real-time quantitative PCR: An emerging technology hits the mainstream. *Experimental Hematology* **30** 503-512.

Hall JM & McDonnell DP. 1999 The estrogen receptor β-isoform (ERβ) of the human estrogen receptor modulates ERα transcriptional activity and is a key regulator of the cellular response to estrogens and antiestrogens. *Endocrinology* **140** 5566-5578.

Hurst AGB, Goad DW, Mohan M & Malayer JR. 2004 Independent downstream gene expression profiles in the presence of estrogen receptor α or β. *Biology of Reproduction* **71** 1252-1261.

Kakegawa H, Nikawa T, Tagami K, Kamioka H, Sumitani K, Kawata T, Drobnic-Kosorok M, Lenarcic B, Turk V & Katunuma N. 1993 Participation of cathepsin L on bone resorption. *FEBS Letters* **321** 247-250.

Kaneko KJ, Gelinas C & Gorski J . 1993 Activation of the silent progesterone receptor gene by ectopic expression of estrogen receptors in a rat fibroblast cell line. *Biochemistry.* **32** 8348-8359.

Kelly MJ, Lagrange AH, Wagner EJ & Ronnekleiv OK. 1999 Rapid effects of estrogen to modulate G protein-coupled receptors via activation of protein kinase A and protein kinase C pathways. *Steroids.* **64** 64-75.

Koblinski JE, Ahram M & Sloane BF. 2000 Unraveling the role of proteases in cancer. *Clinica Chimica Acta* **291** 113-135.

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S & Gustafsson JA. 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proceedings of the National Academy of Sciences* **93** 5925-5930.

Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S & Gustafsson JA. 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology* **138** 863-870.

Lee S, Solow-Cordero DE, Kessler E, Takahara K & Greenspan DS. 1997 Transforming Growth Factor-beta Regulation of Bone Morphogenetic Protein-1/Procollagen C-proteinase and Related Proteins in Fibrogenic Cells and Keratinocytes. *J.Biol.Chem.* **272** 19059-19066.

Mason RW, Johnson DA, Barrett AJ & Chapman HA. 1986 Elastinolytic activity of human cathepsin L. *Biochem J.* **233** 925-927.

Matsuda T, Yamamoto T, Muraguchi A & Saatcioglu F. 2001 Cross-talk between Transforming Growth Factor-beta and Estrogen Receptor Signaling through Smad3. *J.Biol.Chem.* **276** 42908-42914.

Matsui A, Yanase M, Tomiya T, Ikeda H, Fujiwara K & Ogata I . 2002 Stabilization of RNA strands in protein synthesis by type I procollagen C-proteinase enhancer protein, a potential RNA-binding protein, in hepatic stellate cells. *Biochemical and Biophysical Research Communications* **290** 898-902.

Nichols M, Rientjes JM & Stewart AF. 1998 Different positioning of the ligand-binding domain helix 12 and the F domain of the estrogen receptor accounts for functional differences between agonists and antagonists. *EMBO J.* **17** 765-773.

Nimni ME. 1983 Collagen: structure, function, and metabolism in normal and fibrotic tissues. *Semin Arthritis Rheum.* **13** 1-86.

Pace P, Taylor J, Suntharalingam S, Coombes RC & Ali S. 1997 Human Estrogen Receptor beta Binds DNA in a Manner Similar to and Dimerizes with Estrogen Receptor alpha. *Journal of Biological Chemistry* **272** 25832-25838.

Peloille S, Esnard A, Dacheux JL, Guillou F, Gauthier F & Esnard F. 1997 Interactions between ovine cathepsin L, cystatin C and α 2-macroglobulin. Potential role in the genital tract. *Eur J Biochem.* **244** 140-146.

Pettersson K, Grandien K, Kuiper GGJM & Gustafsson JA. 1997 Mouse estrogen receptor β forms estrogen response element-binding heterodimers with estrogen receptor α. *Molecular Endocrinology* **11** 1486-1496.

Prockop JD. 1995 Collagens: Molecular Biology, Diseases, and Potentials for Therapy. *Annual Review of Biochemistry* **64** 403-434.

Ratajczak T. 2001 Protein coregulators that mediate estrogen receptor function. *Reprod.Fertil.Dev.* **13** 229.

Ron D, Chen C, Caldwell J, Jamieson L, Orr E & Mochly-Rosen D. 1994 Cloning of an intracellular receptor for protein kinase C: A homolog of the β subunit of G proteins. *Proceedings of the National Academy of Sciences* **91** 839-843.

Saville B, Wormke M, Wang F, Nguyen T, Enmark E, Kuiper G, Gustafsson JA & Safe S. 2000 Ligand-, cell-, and estrogen receptor subtype (α /β)-dependent activation at GC-rich (Sp1) promoter elements. *Journal of Biological Chemistry* **275** 5379-5387.

Scott IC, Clark TG, Takahara K, Hoffman GG & Greenspan DS. 1999 Structural organization and expression patterns of the human and mouse genes for the type I procollagen COOH-terminal proteinase enhancer protein. *Genomics* **55** 229-234.

Shalitin N, Schlesinger H, Levy MJ, Kessler E & Kessler-Icekson G. 2003 Expression of procollagen C-proteinase enhancer in cultured rat heart fibroblasts: evidence for co-regulation with type I collagen. *Journal of Cellular Biochemistry* **90** 397-407.

Smith BD & Niles R. 1980 Characterization of collagen synthesized by normal and chemically transformed rat liver epithelial cell lines. *Biochemistry* **19** 1820-1825.

Takahara K, Kessler E, Biniaminov L, Brusel M, Eddy RL, Jani-Sait S, Shows TB & Greenspan DS. 1994 Type I procollagen COOH-terminal proteinase enhancer protein: identification, primary structure, and chromosomal localization of the cognate human gene (PCOLCE). *Journal of Biological Chemistry* **269** 26280-26285.

Tanaka S, Antoniv TT, Lui K, Wang L, Wells DJ, Ramirez F & Bou-Gharios G. 2004 Cooperativity between far-upstream enhancer and proximal promoter elements of the human alpha2(I) collagen (COL1A2) gene instructs tissue specificity in transgenic mice. *Journal of Biological Chemistry* M411406200.

Tee MK, Rogatsky I, Tzagarakis-Foster C, Cvoro A, An J, Christy RJ, Yamamoto KR & Leitman DC. 2004 Estradiol and selective estrogen receptor modulators differentially regulate target genes with estrogen receptors α and β. *MolecularBiology of the Cell* **15** 1262-1272.

Toft D & Gorski J. 1966 A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. *Proc Natl Acad Sci U S A.* **55** 1574-1581.

Tora L, Mullick A, Metzger D, Ponglikitmongkol M, Park I & Chambon P. 1989 The cloned human oestrogen receptor contains a mutation which alters its hormone binding properties. *EMBO J.* **8** 1981-1986.

Torchia J, Glass C & Rosenfeld MG. 1998 Co-activators and co-repressors in the integration of transcriptional responses. *Current Opinion in Cell Biology* **10** 373-383.

Verrecchia F & Mauviel A. 2004 TGF-β and TNF-α: antagonistic cytokines controlling type I collagen gene expression. *Cellular Signalling* **16** 873-880.

Webb P, Lopez GN, Uht RM & Kushner PJ. 1995 Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. *Molecular Endocrinology* **9** 443-456.

Zajchowski DA, Webster L, Humm R, White III, Simmons SJ & Bartholdi M. 1997 Different estrogen receptor structural domains are required for estrogen- and tamoxifen-dependent anti-proliferative activity in human mammary epithelial cells expressing an exogenous estrogen receptor. *The Journal of Steroid Biochemistry and Molecular Biology* **62** 373-383.

Table 1. ANOVA output for target genes

COL1A2

Source	DF	Type I SS	Mean Square	F Value	Pr > F
CLINE	2	12.4380542	6.2190271	5.06	0.0075
COMP	4	5.9086996	1.4771749	1.20	0.3129
CLINE*COMP	8	24.6335324	3.0791916	2.50	0.0140
TIME	4	21.7113840	5.4278460	4.41	0.0021
CLINE*TIME	8	19.9391147	2.4923893	2.03	0.0470
COMP*TIME	16	113.6800293	7.1050018	5.78	<.0001
CLINE*COMP*TIME	32	63.3974987	1.9811718	1.61	0.0306

CtsL

Source	DF	Type I SS	Mean Square	F Value	Pr > F
CLINE	2	17.6798969	8.8399484	5.72	0.0040
COMP	4	49.9608116	12.4902029	8.09	<.0001
CLINE*COMP	8	7.7070098	0.9633762	0.62	0.7569
TIME	4	67.6761404	16.9190351	10.95	<.0001
CLINE*TIME	8	51.6693476	6.4586684	4.18	0.0002
COMP*TIME	16	205.2562196	12.8285137	8.31	<.0001
CLINE*COMP*TIME	32	93.0768124	2.9086504	1.88	0.0062

RACK1

Source	DF	Type I SS	Mean Square	F Value	Pr > F
CLINE	2	10.2210560	5.1105280	4.57	0.0118
COMP	4	18.9066373	4.7266593	4.23	0.0029
CLINE*COMP	8	15.6394240	1.9549280	1.75	0.0917
TIME	4	35.5803262	8.8950816	7.96	<.0001
CLINE*TIME	8	27.1468684	3.3933586	3.03	0.0034
COMP*TIME	16	259.9020249	16.2438766	14.53	<.0001
CLINE*COMP*TIME	32	45.7664738	1.4302023	1.28	0.1651

PCOLCE

Source	DF	Type I SS	Mean Square	F Value	Pr > F
CLINE	2	5.2710080	2.6355040	2.18	0.1171
COMP	4	37.6232596	9.4058149	7.77	<.0001
CLINE*COMP	8	6.5552498	0.8194062	0.68	0.7116
TIME	4	27.0912729	6.7728182	5.59	0.0003
CLINE*TIME	8	13.8971298	1.7371412	1.43	0.1866
COMP*TIME	16	140.8954871	8.8059679	7.27	<.0001
CLINE*COMP*TIME	32	39.3918569	1.2309955	1.02	0.4528

Table 2A. Expression of COL1A2 at 6 h exposure to selective ligand

Target	Cell line - **6 h**	Average C_T [†] Target	Average C_T 18S	ΔC_T [‡]	$\Delta\Delta C_T$ [§]	$2^{-\Delta\Delta CT}$ [¥]
Pro – alpha – 2(I) collagen (COL1A2)	Rat1 + ERα DES[0.4nM]	26.48 ± 0.31	19.59 ± 0.42	6.89 ± 0.07[a]	3.47	0.09
	Rat1 + ERα V_{EtOH}	25.58 ± 0.16	21.13 ± 0.59	4.45 ± 0.30[b]	1.03	0.49
	Rat1 + ERβ DES [0.05nM]	24.46 ± 0.51	19.76 ± 0.32	4.70 ± 0.14[b]	1.29	0.41
	Rat1 + ERβ V_{EtOH}	23.91 ± 0.42	21.02 ± 0.13	2.89 ± 0.20[c]	-0.53	1.43
	Rat1 DES[0.4nM]	25.85 ± 0.46	19.55 ± 0.53	6.30 ± 0.05[a]	2.89	0.13
	Rat1 V_{EtOH}	24.22 ± 0.59	20.80 ± 0.17	3.41 ± 0.30[c]	0.00	1.00
	Rat1 + ERα OHT [1nM]	27.39 ± 0.29	21.53 ± 0.72	5.85 ± 0.30[a]	0.14	0.91
	Rat1 + ERα V_{EtOH}	27.15 ± 0.30	20.44 ± 0.51	6.71 ± 0.15[a]	1.00	0.50
	Rat1 + ERβ OHT [0.04nM]	25.04 ± 0.96	20.45 ± 0.33	4.59 ± 0.45[a]	-1.13	2.18
	Rat1 + ERβ V_{EtOH}	26.18 ± 1.07	20.97 ± 0.25	5.21 ± 0.58[a]	-0.50	1.42
	Rat1 OHT [1nM]	26.05 ± 0.58	20.63 ± 0.23	5.42 ± 0.24[a]	-0.29	1.22
	Rat1 V_{EtOH}	26.43 ± 0.50	20.73 ± 0.48	5.71 ± 0.02[a]	0.00	1.00
	Rat1 + ERα Gen [20nM]	27.38 ± 0.76	19.15 ± 0.61	8.22 ± 0.11[a]	1.87	0.28
	Rat1 + ERα V_{EtOH}	27.22 ± 0.28	20.30 ± 0.61	6.91 ± 0.23[b]	0.56	0.68
	Rat1 + ERβ Gen [0.3nM]	24.82 ± 0.37	19.48 ± 1.88	5.34 ± 1.07[c]	-1.02	2.02
	Rat1 + ERβ V_{EtOH}	26.35 ± 0.82	20.54 ± 0.34	5.80 ± 0.34[c]	-0.55	1.46
	Rat1 Gen [20nM]	26.43 ± 0.60	18.84 ± 0.47	7.59 ± 0.09[b]	1.23	0.42
	Rat1 V_{EtOH}	26.71 ± 1.13	20.35 ± 0.61	6.35 ± 0.37[b]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	27.24 ± 0.45	20.10 ± 0.25	7.14 ± 0.13[a]	2.80	0.14
	Rat1 + ERα V_{EtOH}	25.28 ± 0.12	20.04 ± 0.68	5.24 ± 0.40[b]	0.90	0.53
	Rat1 + ERβ E_2 [1nM]	25.46 ± 0.57	19.86 ± 0.49	5.60 ± 0.05[b]	1.27	0.41
	Rat1 + ERβ V_{EtOH}	23.55 ± 0.55	19.75 ± 0.26	3.79 ± 0.21[c]	-0.54	1.45
	Rat1 E_2 [1nM]	24.41 ± 0.29	19.54 ± 0.09	4.86 ± 0.14[c]	0.54	0.69
	Rat1 V_{EtOH}	23.85 ± 0.70	19.51 ± 0.17	4.33 ± 0.37[c]	0.00	1.00
	Rat1 + ERα RAL [1nM]	25.11 ± 0.70	20.47 ± 1.21	4.63 ± 0.37[a]	0.51	0.70
	Rat1 + ERα V_{DMSO}	25.05 ± 0.48	19.97 ± 0.34	5.08 ± 0.09[a]	0.95	0.52
	Rat1 + ERβ RAL [0.04nM]	23.99 ± 0.50	20.29 ± 0.73	3.73 ± 0.16[b]	-0.39	1.31
	Rat1 + ERβ V_{DMSO}	24.52 ± 0.59	19.08 ± 0.64	5.45 ± 0.03[a]	1.32	0.40
	Rat1 RAL [1nM]	23.55 ± 0.11	19.95 ± 1.37	3.60 ± 0.89[b]	0.52	1.04
	Rat1 V_{DMSO}	23.61 ± 0.63	19.49 ± 0.17	4.12 ± 0.32[b]	0.00	1.00

[†] Cycle threshold (C_T): The mean cycle number (target genes n=3, 18S rRNA n=2) at which the threshold crossed the geometric portion of the logarithmic amplification curve.

‡ Normalized C_T values (ΔC_T): Mean C_T values for target genes were subtracted from the mean 18S rRNA gene C_T values to derive values for normalized expression.

§ Calibrated value ($\Delta\Delta C_T$): Rat 1 V treated cells were set as a calibrator. Normalized C_T values were then subtracted from this value to derive the calibrated value used to determine fold differences ($2^{-\Delta\Delta CT}$).

¥ Fold differences ($2^{-\Delta\Delta CT}$): Target gene normalized to endogenous 18S reference, and relative to Rat1 parental cell line calibrator.

n Subscript number with different letters denote a significant difference ($P \leq 0.05$) in cell line interaction, while subscript letters that are the same denote no significant difference. Analysis was carried out using least square differences from a PROC-MIXED analysis in a 3X5X5 treatment factorial ANOVA arrangement analyzed by the Statistical Analysis System (SAS)

Table 2B. Expression of COL1A2 at 9 h exposure to selective ligand

Target	Cell line - **9 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Pro – alpha – 2(I) collagen (COL1A2)	Rat1 + ERα DES[0.4nM]	27.51 ± 0.89	21.00 ± 1.15	6.51 ± 0.18[a]	2.32	0.20
	Rat1 + ERα V_{EtOH}	28.92 ± 0.57	21.72 ± 1.06	7.20 ± 0.35[a]	3.01	0.12
	Rat1 + ERβ DES [0.05nM]	25.86 ± 0.47	20.29 ± 1.12	5.57 ± 0.46[b]	1.38	0.38
	Rat1 + ERβ V_{EtOH}	27.77 ± 1.23	20.82 ± 1.06	6.95 ± 0.13[a]	2.76	0.15
	Rat1 DES[0.4nM]	26.45 ± 0.79	21.26 ± 1.71	5.19 ± 0.65[b]	1.00	0.50
	Rat1 V_{EtOH}	24.62 ± 0.81	20.42 ± 0.76	4.19 ± 0.04[b]	0.00	1.00
	Rat1 + ERα OHT [1nM]	25.79 ± 0.67	18.50 ± 0.55	7.29 ± 0.09[a]	3.03	0.12
	Rat1 + ERα V_{EtOH}	29.38 ± 0.62	22.31 ± 0.82	7.06 ± 0.17[a]	2.81	0.14
	Rat1 + ERβ OHT [0.04nM]	24.49 ± 0.59	18.42 ± 0.23	6.06 ± 0.25[a]	1.80	0.29
	Rat1 + ERβ V_{EtOH}	28.19 ± 0.81	22.14 ± 1.56	6.05 ± 0.53[a]	1.79	0.29
	Rat1 OHT [1nM]	24.80 ± 1.27	17.84 ± 0.72	6.93 ± 0.39[a]	2.70	0.15
	Rat1 V_{EtOH}	25.28 ± 0.75	21.02 ± 0.91	4.26 ± 0.11[b]	0.00	1.00
	Rat1 + ERα Gen [20nM]	26.56 ± 0.79	21.27 ± 0.58	5.29 ± 0.14[a]	1.03	0.49
	Rat1 + ERα V_{EtOH}	29.47 ± 0.94	22.38 ± 1.19	7.09 ± 0.18[b]	2.83	0.14
	Rat1 + ERβ Gen [0.3nM]	26.05 ± 0.20	21.19 ± 0.55	4.85 ± 0.25[a]	0.60	0.66
	Rat1 + ERβ V_{EtOH}	27.87 ± 1.09	21.85 ± 1.16	6.03 ± 0.05[b]	1.78	0.29
	Rat1 Gen [20nM]	25.79 ± 0.51	20.95 ± 0.90	4.84 ± 0.27[a]	0.59	0.66
	Rat1 V_{EtOH}	25.66 ± 0.87	21.41 ± 0.73	4.25 ± 0.09[a]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	28.03 ± 0.89	20.23 ± 0.94	7.80 ± 0.04[a]	3.16	0.11
	Rat1 + ERα V_{EtOH}	27.80 ± 0.49	20.33 ± 0.89	7.47 ± 0.28[a]	2.84	0.14
	Rat1 + ERβ E_2 [1nM]	24.53 ± 0.47	19.69 ± 0.98	4.84 ± 0.35[b]	0.21	0.87
	Rat1 + ERβ V_{EtOH}	26.20 ± 0.94	20.61 ± 1.65	5.59 ± 0.50[b]	0.95	0.52
	Rat1 E_2 [1nM]	24.09 ± 0.51	19.99 ± 1.70	4.10 ± 0.84[b]	-0.53	1.44
	Rat1 V_{EtOH}	23.67 ± 0.70	19.04 ± 1.10	4.64 ± 0.28[b]	0.00	1.00
	Rat1 + ERα RAL [1nM]	26.89 ± 0.55	20.14 ± 0.39	6.75 ± 0.11[a]	2.37	0.19
	Rat1 + ERα V_{DMSO}	26.71 ± 0.29	20.28 ± 0.29	6.43 ± 0.00[a]	2.05	0.24
	Rat1 + ERβ RAL [0.04nM]	25.51 ± 0.26	20.19 ± 0.20	5.33 ± 0.04[b]	0.95	0.52
	Rat1 + ERβ V_{DMSO}	25.58 ± 0.43	20.45 ± 0.13	5.13 ± 0.21[b]	0.75	0.59
	Rat1 RAL [1nM]	25.44 ± 0.48	20.20 ± 0.52	5.24 ± 0.03[b]	0.85	0.55
	Rat1 V_{DMSO}	24.31 ± 0.33	19.93 ± 0.25	4.39 ± 0.06[b]	0.00	1.00

Table 2C. Expression of COL1A2 at 12 h exposure to selective ligand

Target	Cell line - **12 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Pro – alpha – 2(I) collagen (COL1A2)	Rat1 + ERα DES[0.4nM]	24.69 ± 0.35	17.33 ± 0.16	7.35 ± 0.14[a]	1.62	0.32
	Rat1 + ERα V_{EtOH}	25.09 ± 0.76	17.81 ± 0.20	7.28 ± 0.39[a]	1.55	0.34
	Rat1 + ERβ DES [0.05nM]	23.86 ± 0.71	17.43 ± 0.26	6.43 ± 0.32[a]	0.70	0.62
	Rat1 + ERβ V_{EtOH}	24.66 ± 0.74	17.89 ± 0.19	6.77 ± 0.38[a]	1.04	0.49
	Rat1 DES[0.4nM]	24.24 ± 0.46	17.37 ± 0.08	6.87 ± 0.27[a]	1.14	0.45
	Rat1 V_{EtOH}	23.62 ± 0.80	17.89 ± 0.16	5.73 ± 0.45[b]	0.00	1.00
	Rat1 + ERα OHT [1nM]	25.83 ± 0.53	17.06 ± 0.30	8.77 ± 0.17[a]	0.98	0.51
	Rat1 + ERα V_{EtOH}	27.24 ± 1.33	19.68 ± 0.66	8.56 ± 0.48[a]	0.77	0.59
	Rat1 + ERβ OHT [0.04nM]	24.41 ± 0.36	16.97 ± 0.12	7.44 ± 0.17[a]	-0.35	1.27
	Rat1 + ERβ V_{EtOH}	25.31 ± 0.60	17.33 ± 0.37	7.98 ± 0.16[a]	0.19	0.88
	Rat1 OHT [1nM]	24.93 ± 0.50	17.06 ± 0.08	7.86 ± 0.30[a]	0.07	0.95
	Rat1 V_{EtOH}	25.02 ± 1.01	17.23 ± 0.32	7.79 ± 0.49[a]	0.00	1.00
	Rat1 + ERα Gen [20nM]	26.87 ± 0.39	19.85 ± 0.30	7.02 ± 0.06[a]	3.00	0.12
	Rat1 + ERα V_{EtOH}	28.13 ± 1.50	22.43 ± 2.32	5.69 ± 0.59[a]	1.68	0.31
	Rat1 + ERβ Gen [0.3nM]	25.71 ± 0.60	20.28 ± 0.67	5.43 ± 0.05[a]	1.41	0.38
	Rat1 + ERβ V_{EtOH}	26.49 ± 0.58	20.85 ± 0.57	5.64 ± 0.01[a]	1.62	0.32
	Rat1 Gen [20nM]	25.01 ± 1.00	19.95 ± 0.44	5.05 ± 0.40[a]	1.04	0.49
	Rat1 V_{EtOH}	25.01 ± 1.12	20.99 ± 0.67	4.01 ± 0.33[b]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	24.99 ± 1.17	17.37 ± 0.37	7.62 ± 0.57[a]	1.77	0.29
	Rat1 + ERα V_{EtOH}	25.07 ± 0.75	17.05 ± 0.18	8.03 ± 0.41[a]	2.17	0.22
	Rat1 + ERβ E_2 [1nM]	24.75 ± 0.91	17.23 ± 0.16	7.52 ± 0.53[a]	1.66	0.31
	Rat1 + ERβ V_{EtOH}	24.56 ± 0.46	17.14 ± 0.13	7.42 ± 0.23[a]	1.55	0.34
	Rat1 E_2 [1nM]	23.96 ± 1.25	17.18 ± 0.25	6.77 ± 0.70[b]	0.92	0.53
	Rat1 V_{EtOH}	23.00 ± 1.05	17.14 ± 0.19	5.86 ± 0.60[b]	0.00	1.00
	Rat1 + ERα RAL [1nM]	26.82 ± 0.77	20.13 ± 1.11	6.69 ± 0.23[a]	1.89	0.27
	Rat1 + ERα V_{DMSO}	26.25 ± 0.44	20.57 ± 1.78	5.67 ± 0.95[a]	0.86	0.55
	Rat1 + ERβ RAL [0.04nM]	25.41 ± 0.89	19.77 ± 1.34	5.63 ± 0.31[a]	0.83	0.56
	Rat1 + ERβ V_{DMSO}	24.47 ± 0.98	20.76 ± 1.64	3.71 ± 0.47[b]	-1.09	2.12
	Rat1 RAL [1nM]	24.03 ± 0.62	20.25 ± 1.87	3.77 ± 0.88[b]	-1.02	2.03
	Rat1 V_{DMSO}	23.65 ± 0.56	18.85 ± 0.53	4.80 ± 0.01[b]	0.00	1.00

Table 2D. Expression of COL1A2 at 18 h exposure to selective ligand

Target	Cell line - **18 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Pro – alpha – 2(I) collagen (COL1A2)	Rat1 + ERα DES[0.4nM]	27.00 ± 0.58	19.35 ± 0.15	7.65 ± 0.31[a]	2.44	0.18
	Rat1 + ERα V_{EtOH}	27.15 ± 0.43	19.22 ± 0.21	7.94 ± 0.16[a]	2.73	0.15
	Rat1 + ERβ DES [0.05nM]	24.91 ± 0.58	19.44 ± 0.14	5.47 ± 0.31[b]	0.26	0.83
	Rat1 + ERβ V_{EtOH}	25.03 ± 0.57	19.35 ± 0.27	5.68 ± 0.21[b]	0.47	0.72
	Rat1 DES[0.4nM]	23.89 ± 0.87	19.44 ± 0.09	4.45 ± 0.55[b]	-0.76	1.69
	Rat1 V_{EtOH}	24.57 ± 1.49	19.36 ± 0.44	5.21 ± 0.74[b]	0.00	1.00
	Rat1 + ERα OHT [1nM]	28.55 ± 1.00	19.81 ± 0.59	8.74 ± 0.30[a]	3.49	0.09
	Rat1 + ERα V_{EtOH}	27.65 ± 0.20	19.85 ± 0.21	7.80 ± 0.01[a]	2.55	0.17
	Rat1 + ERβ OHT [0.04nM]	25.81 ± 0.60	19.52 ± 0.14	6.29 ± 0.33[b]	1.04	0.49
	Rat1 + ERβ V_{EtOH}	25.41 ± 0.59	19.43 ± 0.27	5.98 ± 0.22[b]	0.73	0.60
	Rat1 OHT [1nM]	24.28 ± 0.82	19.48 ± 0.31	4.81 ± 0.36[b]	-0.44	1.35
	Rat1 V_{EtOH}	24.90 ± 1.97	19.65 ± 0.43	5.25 ± 1.09[b]	0.00	1.00
	Rat1 + ERα Gen [20nM]	25.44 ± 1.36	19.69 ± 0.91	5.75 ± 0.32[a]	0.95	0.51
	Rat1 + ERα V_{EtOH}	25.13 ± 1.20	20.19 ± 0.69	4.94 ± 0.36[a]	0.15	0.90
	Rat1 + ERβ Gen [0.3nM]	24.14 ± 1.69	19.67 ± 0.73	4.47 ± 0.67[a]	-0.33	1.25
	Rat1 + ERβ V_{EtOH}	27.86 ± 0.19	20.73 ± 0.73	7.13 ± 0.68[b]	2.33	0.20
	Rat1 Gen [20nM]	24.97 ± 0.68	20.55 ± 0.67	4.41 ± 0.01[a]	-0.38	1.30
	Rat1 V_{EtOH}	25.30 ± 1.84	20.50 ± 0.88	4.80 ± 0.68[a]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	23.68 ± 0.29	16.92 ± 0.40	6.75 ± 0.08[a]	1.81	0.28
	Rat1 + ERα V_{EtOH}	26.67 ± 0.34	18.77 ± 0.39	7.89 ± 0.03[a]	2.96	0.13
	Rat1 + ERβ E_2 [1nM]	23.20 ± 0.39	16.76 ± 0.24	6.44 ± 0.11[a]	1.50	0.35
	Rat1 + ERβ V_{EtOH}	23.87 ± 1.09	18.00 ± 0.59	5.86 ± 0.35[a]	0.93	0.53
	Rat1 E_2 [1nM]	22.01 ± 0.74	16.78 ± 0.39	5.23 ± 0.24[b]	0.30	0.81
	Rat1 V_{EtOH}	23.59 ± 1.69	18.66 ± 0.56	4.93 ± 0.79[b]	0.00	1.00
	Rat1 + ERα RAL [1nM]	25.15 ± 0.29	17.31 ± 0.51	7.83 ± 0.16[a]	1.46	0.36
	Rat1 + ERα V_{DMSO}	25.15 ± 0.41	17.38 ± 0.36	7.77 ± 0.03[a]	1.40	0.38
	Rat1 + ERβ RAL [0.04nM]	23.65 ± 1.29	17.56 ± 0.17	6.09 ± 0.80[b]	-0.27	1.21
	Rat1 + ERβ V_{DMSO}	23.73 ± 0.59	17.43 ± 0.20	6.30 ± 0.28[b]	-0.07	1.05
	Rat1 RAL [1nM]	24.15 ± 0.34	17.57 ± 0.09	6.58 ± 0.17[b]	0.21	0.86
	Rat1 V_{DMSO}	23.74 ± 0.63	17.37 ± 0.30	6.37 ± 0.23[b]	0.00	1.00

Table 2E. Expression of COL1A2 at 24 h exposure to selective ligand

Target	Cell line - **24 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Pro – alpha – 2(I) collagen (COL1A2)	Rat1 + ERα DES[0.4nM]	23.44 ± 0.09	18.49 ± 0.23	4.96 ± 0.09[a]	-0.24	1.17
	Rat1 + ERα V_{EtOH}	24.84 ± 0.43	18.71 ± 0.67	6.13 ± 0.16[a]	0.93	0.52
	Rat1 + ERβ DES [0.05nM]	25.20 ± 0.62	18.11 ± 0.29	7.09 ± 0.23[a]	1.89	0.27
	Rat1 + ERβ V_{EtOH}	24.86 ± 0.53	18.80 ± 0.21	6.07 ± 0.22[a]	0.87	0.54
	Rat1 DES[0.4nM]	23.87 ± .51	18.39 ± 0.18	5.48 ± 0.23[a]	0.28	0.82
	Rat1 V_{EtOH}	23.58 ± 0.93	18.39 ± 0.22	5.20 ± 0.50[a]	0.00	1.00
	Rat1 + ERα OHT [1nM]	26.29 ± 1.22	17.24 ± 0.13	9.05 ± 0.77[a]	2.45	0.18
	Rat1 + ERα V_{EtOH}	24.81 ± 0.58	17.30 ± 0.38	7.51 ± 0.14[b]	0.91	0.53
	Rat1 + ERβ OHT [0.04nM]	25.54 ± 0.65	17.36 ± 0.14	8.19 ± 0.36[b]	1.58	0.33
	Rat1 + ERβ V_{EtOH}	24.75 ± 0.61	17.24 ± 0.13	7.51 ± 0.34[b]	0.90	0.53
	Rat1 OHT [1nM]	23.30 ± 0.93	16.83 ± 0.65	6.47 ± 0.20[b]	-0.13	1.10
	Rat1 V_{EtOH}	23.62 ± 0.78	17.01 ± 0.14	6.61 ± 0.45[b]	0.00	1.00
	Rat1 + ERα Gen [20nM]	26.59 ± 0.44	17.33 ± 0.90	9.25 ± 0.33[a]	3.85	0.07
	Rat1 + ERα V_{EtOH}	25.75 ± 0.55	19.63 ± 0.64	6.12 ± 0.07[b]	0.72	0.61
	Rat1 + ERβ Gen [0.3nM]	24.90 ± 0.55	17.92 ± 0.22	6.98 ± 0.24[b]	1.58	0.33
	Rat1 + ERβ V_{EtOH}	25.42 ± 0.75	19.62 ± 0.19	5.80 ± 0.40[b]	0.40	0.76
	Rat1 Gen [20nM]	24.27 ± 0.64	16.73 ± 0.63	7.53 ± 0.01[a]	2.13	0.23
	Rat1 V_{EtOH}	24.16 ± 0.94	18.76 ± 0.13	5.40 ± 0.57[b]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	23.49 ± 0.51	20.98 ± 0.40	2.52 ± 0.08[a]	-0.36	1.29
	Rat1 + ERα V_{EtOH}	23.28 ± 0.67	21.22 ± 0.47	2.07 ± 0.14[a]	-0.81	1.76
	Rat1 + ERβ E_2 [1nM]	23.08 ± 0.74	21.09 ± 0.34	1.98 ± 0.28[a]	-0.90	1.86
	Rat1 + ERβ V_{EtOH}	23.82 ± 0.62	21.38 ± 0.28	2.45 ± 0.24[a]	-0.43	1.35
	Rat1 E_2 [1nM]	23.37 ± 0.34	20.76 ± 0.31	2.61 ± 0.03[a]	-0.27	1.21
	Rat1 V_{EtOH}	23.27 ± 0.30	20.39 ± 0.15	2.88 ± 0.10[a]	0.00	1.00
	Rat1 + ERα RAL [1nM]	23.36 ± 0.69	16.30 ± 0.28	7.07 ± 0.29[a]	2.99	0.13
	Rat1 + ERα V_{DMSO}	23.37 ± 0.73	17.01 ± 0.28	6.35 ± 0.32[a]	2.27	0.21
	Rat1 + ERβ RAL [0.04nM]	23.44 ± 0.75	16.84 ± 0.22	6.61 ± 0.37[a]	2.53	0.17
	Rat1 + ERβ V_{DMSO}	22.87 ± 0.85	16.85 ± 0.83	6.02 ± 0.01[a]	1.94	0.26
	Rat1 RAL [1nM]	21.51 ± 0.79	16.95 ± 0.39	4.56 ± 0.29[a]	0.48	0.71
	Rat1 V_{DMSO}	21.49 ± 0.67	17.41 ± 0.56	4.08 ± 0.07[a]	0.00	1.00

Table 3A. Expression of CtsL at 6 h exposure to selective ligand

Target	Cell line – **6 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Cathepsin L (CtsL)	Rat1 + ERα DES[0.4nM]	25.75 ± 0.23	19.31 ± 0.56	6.43 ± 0.24[a]	3.15	0.11
	Rat1 + ERα V_{EtOH}	25.13 ± 0.11	21.07 ± 0.54	4.05 ± 0.31[b]	0.77	0.59
	Rat1 + ERβ DES [0.05nM]	25.67 ± 0.44	19.38 ± 0.53	6.28 ± 0.07[a]	3.00	0.12
	Rat1 + ERβ V_{EtOH}	25.37 ± 0.35	20.91 ± 0.34	4.45 ± 0.01[b]	1.17	0.44
	Rat1 DES[0.4nM]	25.36 ± 0.62	19.47 ± 0.34	5.89 ± 0.20[a]	2.61	0.16
	Rat1 V_{EtOH}	24.02 ± 0.91	20.74 ± 0.22	3.28 ± 0.48[b]	0.00	1.00
	Rat1 + ERα OHT [1nM]	24.85 ± 0.39	19.67 ± 0.46	5.18 ± 0.05[a]	-0.69	1.61
	Rat1 + ERα V_{EtOH}	25.54 ± 0.14	19.28 ± 0.33	6.26 ± 0.14[a]	0.38	0.77
	Rat1 + ERβ OHT [0.04nM]	25.24 ± 0.76	19.35 ± 0.67	5.89 ± 0.06[a]	0.02	0.99
	Rat1 + ERβ V_{EtOH}	26.25 ± 0.33	19.87 ± 0.46	6.39 ± 0.09[a]	0.51	0.70
	Rat1 OHT [1nM]	24.73 ± 0.54	18.96 ± 0.82	5.77 ± 0.20[a]	-0.10	1.07
	Rat1 V_{EtOH}	24.53 ± 0.94	18.66 ± 0.42	5.87 ± 0.37[a]	0.00	1.00
	Rat1 + ERα Gen [20nM]	26.88 ± 1.32	19.43 ± 0.89	7.45 ± 0.31[a]	1.28	0.41
	Rat1 + ERα V_{EtOH}	26.31 ± 0.41	20.02 ± 0.56	6.29 ± 0.11[a]	0.12	0.92
	Rat1 + ERβ Gen [0.3nM]	26.19 ± 0.32	17.95 ± 0.94	8.24 ± 0.44[b]	2.07	0.24
	Rat1 + ERβ V_{EtOH}	27.71 ± 0.52	19.70 ± 0.53	8.01 ± 0.01[b]	1.84	0.28
	Rat1 Gen [20nM]	26.99 ± 1.63	18.57 ± 0.36	8.42 ± 0.89[b]	2.25	0.21
	Rat1 V_{EtOH}	26.12 ± 0.36	19.95 ± 0.55	6.17 ± 0.13[a]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	26.45 ± 0.74	18.09 ± 0.23	8.35 ± 0.36[a]	3.11	0.12
	Rat1 + ERα V_{EtOH}	23.79 ± 0.25	18.01 ± 0.36	5.78 ± 0.08[b]	0.54	0.69
	Rat1 + ERβ E_2 [1nM]	25.79 ± 0.79	17.61 ± 0.09	8.17 ± 0.49[a]	2.93	0.13
	Rat1 + ERβ V_{EtOH}	23.89 ± 0.38	17.60 ± 0.22	6.29 ± 0.11[b]	1.05	0.48
	Rat1 E_2 [1nM]	24.92 ± 0.43	17.36 ± 0.17	7.57 ± 0.19[a]	2.33	0.20
	Rat1 V_{EtOH}	22.77 ± 0.87	17.52 ± 0.33	5.24 ± 0.38[b]	0.00	1.00
	Rat1 + ERα RAL [1nM]	23.83 ± 0.81	20.24 ± 0.79	3.59 ± 0.02[a]	0.41	0.75
	Rat1 + ERα V_{DMSO}	24.08 ± 0.64	21.01 ± 1.11	3.07 ± 0.33[a]	-0.11	1.08
	Rat1 + ERβ RAL [0.04nM]	24.60 ± 0.34	20.40 ± 0.12	4.20 ± 0.15[b]	1.02	0.49
	Rat1 + ERβ V_{DMSO}	25.03 ± 0.54	20.31 ± 0.88	4.71 ± 0.24[b]	1.53	0.35
	Rat1 RAL [1nM]	23.57 ± 0.38	20.08 ± 0.68	3.49 ± 0.21[a]	0.32	0.81
	Rat1 V_{DMSO}	23.36 ± 0.57	20.18 ± 0.36	3.18 ± 0.14[a]	0.00	1.00

Table 3B. Expression of CtsL at 9 h exposure to selective ligand

Target	Cell line – **9 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Cathepsin L (CtsL)	Rat1 + ERα DES[0.4nM]	25.69 ± 0.78	18.60 ± 0.85	7.09 ± 0.05[a]	3.79	0.07
	Rat1 + ERα V_{EtOH}	26.03 ± 0.66	20.16 ± 0.84	5.86 ± 0.12[a]	2.57	0.17
	Rat1 + ERβ DES [0.05nM]	25.49 ± 0.53	17.57 ± 0.55	7.91 ± 0.02[a]	4.62	0.04
	Rat1 + ERβ V_{EtOH}	25.92 ± 0.44	19.51 ± 0.52	6.40 ± 0.05[b]	3.10	0.12
	Rat1 DES[0.4nM]	25.58 ± 0.48	18.46 ± 0.75	7.12 ± 0.19[a]	3.83	0.07
	Rat1 V_{EtOH}	23.13 ± 0.75	19.83 ± 0.57	3.30 ± 0.13[c]	0.00	1.00
	Rat1 + ERα OHT [1nM]	24.08 ± 0.55	18.27 ± 0.15	5.81 ± 0.28[a]	4.02	0.06
	Rat1 + ERα V_{EtOH}	26.55 ± 0.40	21.23 ± 0.74	5.31 ± 0.24[a]	3.53	0.09
	Rat1 + ERβ OHT [0.04nM]	24.74 ± 1.27	17.31 ± 0.31	6.43 ± 0.68[a]	4.64	0.04
	Rat1 + ERβ V_{EtOH}	26.56 ± 0.59	21.16 ± 0.87	5.41 ± 0.20[a]	3.62	0.08
	Rat1 OHT [1nM]	24.31 ± 0.81	18.15 ± 0.26	6.16 ± 0.38[a]	4.37	0.05
	Rat1 V_{EtOH}	23.50 ± 0.78	21.72 ± 0.31	1.78 ± 0.32[b]	0.00	1.00
	Rat1 + ERα Gen [20nM]	24.35 ± 0.65	18.89 ± 0.40	5.46 ± 0.18[a]	-0.85	1.80
	Rat1 + ERα V_{EtOH}	28.15 ± 0.75	19.44 ± 1.09	8.71 ± 0.24[b]	2.40	0.19
	Rat1 + ERβ Gen [0.3nM]	25.24 ± 0.30	19.67 ± 1.32	5.57 ± 0.72[a]	-0.73	1.67
	Rat1 + ERβ V_{EtOH}	27.80 ± 0.72	20.22 ± 1.52	7.58 ± 0.57[b]	1.27	0.41
	Rat1 Gen [20nM]	25.04 ± 1.15	18.34 ± 1.15	6.70 ± 0.01[a]	0.39	0.76
	Rat1 V_{EtOH}	24.78 ± 0.67	18.47 ± 1.09	6.31 ± 0.30[a]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	26.55 ± 0.77	19.15 ± 0.89	7.40 ± 0.09[a]	3.76	0.07
	Rat1 + ERα V_{EtOH}	24.30 ± 0.20	19.39 ± 0.81	4.92 ± 0.43[b]	1.27	0.41
	Rat1 + ERβ E_2 [1nM]	25.09 ± 0.35	18.97 ± 0.41	6.11 ± 0.05[a]	2.47	0.18
	Rat1 + ERβ V_{EtOH}	25.18 ± 0.46	18.95 ± 0.38	6.23 ± 0.05[a]	2.59	0.17
	Rat1 E_2 [1nM]	25.45 ± 0.64	18.42 ± 0.45	7.03 ± 0.13[a]	3.38	0.09
	Rat1 V_{EtOH}	22.77 ± 0.63	19.13 ± 0.39	3.64 ± 0.17[b]	0.00	1.00
	Rat1 + ERα RAL [1nM]	25.86 ± 0.49	19.33 ± 0.24	6.54 ± 0.17[a]	1.10	0.46
	Rat1 + ERα V_{DMSO}	26.07 ± 0.40	19.66 ± 0.29	6.41 ± 0.08[a]	0.97	0.51
	Rat1 + ERβ RAL [0.04nM]	25.96 ± 0.49	19.50 ± 0.17	6.46 ± 0.22[a]	1.02	0.49
	Rat1 + ERβ V_{DMSO}	26.08 ± 0.42	19.57 ± 0.18	6.51 ± 0.17[a]	1.07	0.47
	Rat1 RAL [1nM]	24.94 ± 0.18	19.37 ± 0.18	5.57 ± 0.01[a]	0.14	0.91
	Rat1 V_{DMSO}	24.70 ± 0.29	19.27 ± 0.15	5.44 ± 0.10[a]	0.00	1.00

Table 3C. Expression of CtsL at 12 h exposure to selective ligand

Target	Cell line - **12 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Cathepsin L (CtsL)	Rat1 + ERα DES[0.4nM]	21.85 ± 0.51	15.60 ± 0.22	6.25 ± 0.20[a]	-0.08	1.06
	Rat1 + ERα V_{EtOH}	22.61 ± 0.88	15.90 ± 0.40	6.70 ± 0.34[a]	0.37	0.77
	Rat1 + ERβ DES [0.05nM]	23.96 ± 1.21	15.84 ± 0.48	8.12 ± 0.51[b]	1.79	0.29
	Rat1 + ERβ V_{EtOH}	24.18 ± 0.71	16.17 ± 0.17	8.00 ± 0.38[b]	1.68	0.31
	Rat1 DES[0.4nM]	22.40 ± 0.60	15.34 ± 0.60	7.05 ± 0.01[a]	0.72	0.60
	Rat1 V_{EtOH}	22.52 ± 1.02	16.19 ± 0.20	6.33 ± 0.58[a]	0.00	1.00
	Rat1 + ERα OHT [1nM]	22.20 ± 0.74	14.36 ± 1.08	7.84 ± 0.24[a]	0.71	0.61
	Rat1 + ERα V_{EtOH}	22.92 ± 0.90	15.32 ± 0.17	7.60 ± 0.52[a]	0.47	0.72
	Rat1 + ERβ OHT [0.04nM]	23.25 ± 0.35	14.35 ± 0.56	8.90 ± 0.15[a]	1.78	0.29
	Rat1 + ERβ V_{EtOH}	24.91 ± 1.42	15.48 ± 0.23	9.43 ± 0.85[a]	2.31	0.20
	Rat1 OHT [1nM]	22.55 ± 0.77	14.19 ± 0.13	8.35 ± 0.45[a]	1.23	0.43
	Rat1 V_{EtOH}	22.54 ± 1.28	15.41 ± 0.16	7.13 ± 0.80[a]	0.00	1.00
	Rat1 + ERα Gen [20nM]	23.68 ± 0.49	17.62 ± 0.29	6.06 ± 0.14[a]	0.68	0.63
	Rat1 + ERα V_{EtOH}	25.14 ± 0.89	18.30 ± 0.77	6.85 ± 0.08[a]	1.47	0.36
	Rat1 + ERβ Gen [0.3nM]	25.10 ± 0.68	18.33 ± 0.49	6.77 ± 0.14[a]	1.39	0.38
	Rat1 + ERβ V_{EtOH}	26.27 ± 0.83	18.21 ± 0.65	8.06 ± 0.13[a]	2.68	0.16
	Rat1 Gen [20nM]	23.66 ± 1.07	17.97 ± 0.55	5.69 ± 0.37[a]	0.31	0.81
	Rat1 V_{EtOH}	24.61 ± 1.05	19.23 ± 0.47	5.38 ± 0.41[a]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	26.95 ± 1.03	17.12 ± 0.19	9.83 ± 0.60[a]	1.57	0.33
	Rat1 + ERα V_{EtOH}	24.17 ± 0.86	16.90 ± 0.19	7.27 ± 0.47[b]	-0.98	1.98
	Rat1 + ERβ E_2 [1nM]	30.36 ± 3.34	16.86 ± 0.21	13.50 ± 2.21[c]	5.24	0.03
	Rat1 + ERβ V_{EtOH}	26.68 ± 0.82	16.84 ± 0.18	9.84 ± 0.46[a]	1.59	0.33
	Rat1 E_2 [1nM]	27.28 ± 2.78	16.81 ± 0.30	10.47 ± 1.75[a]	2.21	0.22
	Rat1 V_{EtOH}	24.93 ± 1.29	16.68 ± 0.10	8.25 ± 0.84[b]	0.00	1.00
	Rat1 + ERα RAL [1nM]	25.27 ± 0.15	18.52 ± 0.33	6.75 ± 0.12[a]	2.03	0.24
	Rat1 + ERα V_{DMSO}	25.45 ± 0.20	18.89 ± 0.29	6.56 ± 0.27[a]	1.84	0.28
	Rat1 + ERβ RAL [0.04nM]	24.59 ± 0.20	18.89 ± 0.51	5.69 ± 0.22[a]	0.98	0.51
	Rat1 + ERβ V_{DMSO}	24.58 ± 0.66	19.09 ± 0.42	5.48 ± 0.17[a]	0.76	0.60
	Rat1 RAL [1nM]	23.44 ± 0.49	18.59 ± 0.37	4.85 ± 0.08[a]	0.13	0.91
	Rat1 V_{DMSO}	23.47 ± 0.62	18.74 ± 0.81	4.72 ± 0.14[a]	0.00	1.00

Table 3D. Expression of CtsL at 18 h exposure to selective ligand

Target	Cell line - **18 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Cathepsin L (CtsL)	Rat1 + ERα DES[0.4nM]	25.53 ± 0.66	14.85 ± 0.23	10.68 ± 0.31[a]	1.68	0.31
	Rat1 + ERα V_{EtOH}	25.64 ± 0.25	14.88 ± 0.59	10.77 ± 0.24[a]	1.77	0.29
	Rat1 + ERβ DES [0.05nM]	23.81 ± 0.76	14.81 ± 0.39	9.00 ± 0.26[b]	-0.01	1.00
	Rat1 + ERβ V_{EtOH}	24.04 ± 0.98	14.93 ± 0.61	9.10 ± 0.26[b]	0.10	0.93
	Rat1 DES[0.4nM]	22.45 ± 0.62	15.03 ± 0.59	7.43 ± 0.02[b]	-1.57	2.97
	Rat1 V_{EtOH}	23.53 ± 0.86	14.53 ± 0.67	9.00 ± 0.35[b]	0.00	1.00
	Rat1 + ERα OHT [1nM]	27.19 ± 1.04	16.07 ± 0.37	11.12 ± 0.47[a]	4.01	0.06
	Rat1 + ERα V_{EtOH}	25.87 ± 0.46	16.02 ± 0.12	9.84 ± 0.24[a]	2.74	0.15
	Rat1 + ERβ OHT [0.04nM]	23.18 ± 0.61	16.03 ± 0.17	7.16 ± 0.32[b]	0.05	0.96
	Rat1 + ERβ V_{EtOH}	24.25 ± 1.25	15.97 ± 0.30	8.27 ± 0.68[b]	1.17	0.44
	Rat1 OHT [1nM]	22.82 ± 1.17	16.00 ± 0.29	6.81 ± 0.62[b]	-0.29	1.22
	Rat1 V_{EtOH}	23.09 ± 0.86	15.98 ± 0.52	7.10 ± 0.24[b]	0.00	1.00
	Rat1 + ERα Gen [20nM]	27.27 ± 0.27	19.80 ± 0.60	7.46 ± 0.23[a]	2.88	0.13
	Rat1 + ERα V_{EtOH}	28.35 ± 0.45	20.60 ± 0.55	7.75 ± 0.07[a]	3.17	0.11
	Rat1 + ERβ Gen [0.3nM]	25.10 ± 0.44	20.03 ± 0.69	5.07 ± 0.10[b]	0.49	0.71
	Rat1 + ERβ V_{EtOH}	26.33 ± 1.46	19.73 ± 0.78	6.61 ± 0.48[a]	2.03	0.25
	Rat1 Gen [20nM]	24.05 ± 0.43	19.64 ± 0.50	4.41 ± 0.05[b]	-0.17	1.13
	Rat1 V_{EtOH}	25.41 ± 1.37	20.82 ± 0.33	4.58 ± 0.73[b]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	23.86 ± 0.33	16.32 ± 1.79	7.53 ± 1.03[a]	1.82	0.28
	Rat1 + ERα V_{EtOH}	26.25 ± 0.45	16.77 ± 0.33	9.47 ± 0.09[b]	3.76	0.07
	Rat1 + ERβ E_2 [1nM]	23.43 ± 0.51	15.24 ± 0.17	8.19 ± 0.25[a]	2.48	0.18
	Rat1 + ERβ V_{EtOH}	23.81 ± 1.61	16.88 ± 0.27	6.94 ± 0.94[a]	1.22	0.43
	Rat1 E_2 [1nM]	22.69 ± 0.75	15.22 ± 0.27	7.48 ± 0.34[a]	1.77	0.29
	Rat1 V_{EtOH}	22.87 ± 1.27	17.15 ± 0.37	5.71 ± 0.63[c]	0.00	1.00
	Rat1 + ERα RAL [1nM]	24.33 ± 0.38	16.47 ± 0.54	7.86 ± 0.11[a]	0.16	0.90
	Rat1 + ERα V_{DMSO}	24.36 ± 0.53	16.44 ± 0.33	7.92 ± 0.14[a]	0.21	0.86
	Rat1 + ERβ RAL [0.04nM]	23.45 ± 0.46	16.57 ± 0.22	6.89 ± 0.17[a]	-0.82	1.76
	Rat1 + ERβ V_{DMSO}	23.53 ± 0.51	16.47 ± 0.26	7.05 ± 0.18[a]	-0.65	1.57
	Rat1 RAL [1nM]	24.49 ± 0.18	16.55 ± 0.18	7.94 ± 0.01[a]	0.24	0.84
	Rat1 V_{DMSO}	24.26 ± 0.94	16.56 ± 0.19	7.70 ± 0.53[a]	0.00	1.00

Table 3E. Expression of CtsL at 24 h exposure to selective ligand

Target	Cell line - **24 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Cathepsin L (CtsL)	Rat1 + ERα DES[0.4nM]	23.00 ± 0.35	18.93 ± 0.28	4.08 ± 0.05[a]	-1.00	2.00
	Rat1 + ERα V_{EtOH}	24.90 ± 0.55	19.53 ± 0.39	5.37 ± 0.12[a]	0.29	0.82
	Rat1 + ERβ DES [0.05nM]	25.29 ± 0.69	18.02 ± 0.20	7.27 ± 0.35[b]	2.19	0.22
	Rat1 + ERβ V_{EtOH}	26.15 ± 1.24	19.09 ± 2.19	7.05 ± 0.6[b]7	1.97	0.25
	Rat1 DES[0.4nM]	22.60 ± 0.57	18.71 ± 0.45	3.90 ± 0.08[a]	-1.18	2.27
	Rat1 V_{EtOH}	23.71 ± 0.72	18.63 ± 0.79	5.08 ± 0.05[a]	0.00	1.00
	Rat1 + ERα OHT [1nM]	25.08 ± 1.22	18.33 ± 0.27	6.75 ± 0.68[a]	2.40	0.19
	Rat1 + ERα V_{EtOH}	25.05 ± 0.49	19.75 ± 0.83	5.29 ± 0.24[b]	0.94	0.52
	Rat1 + ERβ OHT [0.04nM]	25.95 ± 0.77	18.51 ± 0.22	7.44 ± 0.39[a]	3.09	0.12
	Rat1 + ERβ V_{EtOH}	26.34 ± 1.53	21.23 ± 1.01	5.11 ± 0.37[b]	0.76	0.59
	Rat1 OHT [1nM]	21.83 ± 0.78	18.10 ± 0.69	3.73 ± 0.06[c]	-0.62	1.53
	Rat1 V_{EtOH}	23.92 ± 0.54	19.57 ± 1.16	4.34 ± 0.44[c]	0.00	1.00
	Rat1 + ERα Gen [20nM]	24.95 ± 0.44	17.12 ± 0.33	7.83 ± 0.08[a]	1.41	0.37
	Rat1 + ERα V_{EtOH}	25.28 ± 0.58	18.66 ± 0.53	6.62 ± 0.03[a]	0.20	0.87
	Rat1 + ERβ Gen [0.3nM]	25.09 ± 0.09	17.15 ± 0.09	7.94 ± 0.01[a]	1.52	0.35
	Rat1 + ERβ V_{EtOH}	25.38 ± 0.61	17.51 ± 0.91	7.87 ± 0.21[a]	1.45	0.37
	Rat1 Gen [20nM]	22.32 ± 0.56	17.25 ± 0.35	5.07 ± 0.15[b]	-1.35	2.55
	Rat1 V_{EtOH}	24.22 ± 0.63	17.80 ± 0.50	6.42 ± 0.09[b]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	22.89 ± 0.86	19.65 ± 0.41	2.63 ± 0.32[a]	-1.01	2.02
	Rat1 + ERα V_{EtOH}	24.72 ± 0.48	20.49 ± 0.27	4.22 ± 0.15[b]	0.57	0.67
	Rat1 + ERβ E_2 [1nM]	24.41 ± 0.29	19.80 ± 0.25	4.61 ± 0.03[b]	0.96	0.51
	Rat1 + ERβ V_{EtOH}	26.02 ± 1.61	19.18 ± 1.79	6.84 ± 0.13[c]	3.19	0.11
	Rat1 E_2 [1nM]	21.73 ± 0.66	19.71 ± 0.38	2.03 ± 0.20[a]	-1.62	3.07
	Rat1 V_{EtOH}	23.53 ± 0.64	19.88 ± 0.85	3.64 ± 0.15[a]	0.00	1.00
	Rat1 + ERα RAL [1nM]	24.89 ± 0.49	17.26 ± 0.38	7.63 ± 0.07[a]	2.73	0.15
	Rat1 + ERα V_{DMSO}	25.52 ± 0.66	18.14 ± 0.28	7.38 ± 0.26[a]	2.49	0.18
	Rat1 + ERβ RAL [0.04nM]	24.58 ± 0.64	17.95 ± 0.30	6.63 ± 0.23[a]	1.73	0.30
	Rat1 + ERβ V_{DMSO}	24.51 ± 0.19	18.16 ± 1.06	6.35 ± 0.61	1.45	0.37
	Rat1 RAL [1nM]	23.32 ± 0.58	18.23 ± 0.44	5.09 ± 0.10[b]	0.20	0.87
	Rat1 V_{DMSO}	23.78 ± 0.50	18.88 ± 0.08	4.89 ± 0.07[b]	0.00	1.00

Table 4A. Expression of RACK1 at 6 h exposure to selective ligand

Target	Cell line - **6 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Receptor for Activated C Kinase (RACK1)	Rat1 + ERα DES[0.4nM]	23.34 ± 0.17	19.21 ± 0.37	4.12 ± 0.13[a]	1.34	0.39
	Rat1 + ERα V_{EtOH}	23.05 ± 0.29	21.44 ± 0.71	1.60 ± 0.30[c]	-1.17	2.24
	Rat1 + ERβ DES [0.05nM]	24.19 ± 0.35	19.62 ± 0.75	4.57 ± 0.28[a]	1.79	0.29
	Rat1 + ERβ V_{EtOH}	23.98 ± 0.26	21.18 ± 0.26	2.80 ± 0.01[b]	0.03	0.98
	Rat1 DES[0.4nM]	24.54 ± 0.37	19.25 ± 0.79	5.30 ± 0.30[a]	2.52	0.17
	Rat1 V_{EtOH}	23.76 ± 0.49	20.98 ± 0.30	2.77 ± 0.13[b]	0.00	1.00
	Rat1 + ERα OHT [1nM]	23.22 ± 0.44	19.75 ± 0.75	3.47 ± 0.22[a]	-2.66	6.32
	Rat1 + ERα V_{EtOH}	23.63 ± 0.15	18.28 ± 0.32	5.35 ± 0.11[b]	-0.78	1.72
	Rat1 + ERβ OHT [0.04nM]	23.79 ± 0.53	19.74 ± 0.41	4.04 ± 0.08[c]	-2.09	4.24
	Rat1 + ERβ V_{EtOH}	25.17 ± 0.55	19.30 ± 0.74	5.87 ± 0.13[b]	-0.26	1.20
	Rat1 OHT [1nM]	24.17 ± 0.29	19.12 ± 0.78	5.05 ± 0.35[b]	-1.08	2.11
	Rat1 V_{EtOH}	24.84 ± 0.41	18.70 ± 0.46	6.13 ± 0.04[b]	0.00	1.00
	Rat1 + ERα Gen [20nM]	24.56 ± 1.49	18.31 ± 0.50	6.25 ± 0.70[a]	0.13	0.91
	Rat1 + ERα V_{EtOH}	23.98 ± 0.32	19.72 ± 0.35	4.27 ± 0.02[b]	-1.85	3.60
	Rat1 + ERβ Gen [0.3nM]	24.37 ± 0.39	18.06 ± 0.50	6.30 ± 0.07[a]	0.19	0.88
	Rat1 + ERβ V_{EtOH}	25.64 ± 0.44	19.93 ± 0.92	5.71 ± 0.33[c]	-0.41	1.32
	Rat1 Gen [20nM]	25.44 ± 1.15	17.67 ± 0.53	7.76 ± 0.44[a]	1.65	0.32
	Rat1 V_{EtOH}	25.45 ± 0.36	19.34 ± 0.78	6.11 ± 0.30[c]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	25.62 ± 0.54	18.93 ± 0.17	6.70 ± 0.26[a]	1.31	0.40
	Rat1 + ERα V_{EtOH}	22.77 ± 0.15	18.90 ± 0.58	3.87 ± 0.30[b]	-1.52	2.87
	Rat1 + ERβ E_2 [1nM]	24.62 ± 0.62	18.75 ± 0.22	5.87 ± 0.28[a]	0.47	0.72
	Rat1 + ERβ V_{EtOH}	23.83 ± 0.34	18.74 ± 0.14	5.09 ± 0.14[b]	-0.29	1.23
	Rat1 E_2 [1nM]	24.61 ± 0.50	18.61 ± 0.21	5.99 ± 0.21[a]	0.60	0.66
	Rat1 V_{EtOH}	23.76 ± 0.49	18.37 ± 0.21	5.39 ± 0.19[b]	0.00	1.00
	Rat1 + ERα RAL [1nM]	22.79 ± 0.67	20.15 ± 0.90	2.64 ± 0.16[a]	-1.82	3.52
	Rat1 + ERα V_{DMSO}	22.97 ± 0.48	19.92 ± 0.67	3.04 ± 0.13[a]	-1.41	2.66
	Rat1 + ERβ RAL [0.04nM]	23.81 ± 0.16	20.13 ± 0.41	3.68 ± 0.18[b]	-0.78	1.71
	Rat1 + ERβ V_{DMSO}	25.30 ± 1.65	19.75 ± 0.46	5.54 ± 0.84[b]	1.09	0.47
	Rat1 RAL [1nM]	23.69 ± 0.17	19.60 ± 0.82	4.09 ± 0.45[b]	-0.37	1.29
	Rat1 V_{DMSO}	23.73 ± 0.42	19.27 ± 0.46	4.46 ± 0.03[b]	0.00	1.00

Table 4B. Expression of RACK1 at 9 h exposure to selective ligand

Target	Cell line - **9 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Receptor for Activated C Kinase (RACK1)	Rat1 + ERα DES[0.4nM]	26.24 + 0.30	21.52 + 1.31	4.72 + 0.71[a]	1.79	0.29
	Rat1 + ERα V_{EtOH}	25.88 + 0.55	23.01 + 0.70	2.86 + 0.11[b]	-0.06	1.04
	Rat1 + ERβ DES [0.05nM]	26.47 + 0.41	21.88 + 0.72	4.59 + 0.21[a]	1.67	0.31
	Rat1 + ERβ V_{EtOH}	26.59 + 0.30	22.37 + 0.42	4.21 + 0.09[a]	1.29	0.41
	Rat1 DES[0.4nM]	26.75 + 0.40	21.13 + 0.54	5.62 + 0.10[a]	2.70	0.15
	Rat1 V_{EtOH}	25.71 + 0.47	22.78 + 0.91	2.93 + 0.30[b]	0.00	1.00
	Rat1 + ERα OHT [1nM]	23.72 + 0.32	18.97 + 0.23	4.74 + 0.06[a]	1.96	0.25
	Rat1 + ERα V_{EtOH}	26.16 + 0.63	22.06 + 0.75	4.10 + 0.09[a]	1.32	0.40
	Rat1 + ERβ OHT [0.04nM]	24.34 + 0.27	18.85 + 0.41	5.50 + 0.10[a]	2.72	0.15
	Rat1 + ERβ V_{EtOH}	26.30 + 0.47	22.06 + 0.58	4.24 + 0.08[a]	1.46	0.37
	Rat1 OHT [1nM]	24.74 + 0.50	18.58 + 0.15	6.16 + 0.25[b]	3.38	0.09
	Rat1 V_{EtOH}	24.93 + 0.67	22.15 + 1.06	2.78 + 0.27[c]	0.00	1.00
	Rat1 + ERα Gen [20nM]	24.28 + 0.47	20.27 + 0.17	4.01 + 0.21[a]	-2.19	4.57
	Rat1 + ERα V_{EtOH}	27.35 + 0.45	20.74 + 1.40	6.61 + 0.67[b]	0.40	0.76
	Rat1 + ERβ Gen [0.3nM]	25.08 + 0.44	19.97 + 0.71	5.10 + 0.19[c]	-1.10	2.14
	Rat1 + ERβ V_{EtOH}	27.53 + 0.55	20.67 + 0.83	6.86 + 0.20[b]	0.65	0.63
	Rat1 Gen [20nM]	25.21 + 0.70	19.89 + 0.83	5.31 + 0.09[c]	-0.89	1.86
	Rat1 V_{EtOH}	26.47 + 0.24	20.27 + 1.43	6.21 + 0.84[b]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	25.29 + 0.69	19.10 + 0.65	6.19 + 0.03[a]	1.71	0.30
	Rat1 + ERα V_{EtOH}	24.10 + 0.28	19.87 + 0.66	4.23 + 0.27[a]	-0.25	1.19
	Rat1 + ERβ E_2 [1nM]	24.19 + 0.43	19.27 + 0.71	4.92 + 0.19[a]	0.44	0.74
	Rat1 + ERβ V_{EtOH}	24.79 + 0.30	19.13 + 0.27	5.66 + 0.02[a]	1.19	0.44
	Rat1 E_2 [1nM]	24.37 + 0.58	19.08 + 0.34	5.28 + 0.16[a]	0.80	0.57
	Rat1 V_{EtOH}	23.98 + 0.40	19.50 + 0.48	4.47 + 0.06[a]	0.00	1.00
	Rat1 + ERα RAL [1nM]	23.82 + 0.37	21.38 + 1.39	2.44 + 0.72[a]	-2.75	6.73
	Rat1 + ERα V_{DMSO}	23.87 + 0.27	20.01 + 0.29	3.87 + 0.29[b]	-1.32	2.51
	Rat1 + ERβ RAL [0.04nM]	24.87 + 0.28	19.93 + 0.40	4.94 + 0.09[b]	-0.26	1.19
	Rat1 + ERβ V_{DMSO}	24.84 + 0.29	19.83 + 0.21	5.01 + 0.06[b]	-0.18	1.13
	Rat1 RAL [1nM]	24.62 + 0.15	19.72 + 0.33	4.90 + 0.12[b]	-0.29	1.22
	Rat1 V_{DMSO}	24.38 + 0.27	19.19 + 0.27	5.19 + 0.01[b]	0.00	1.00

Table 4C. Expression of RACK1 at 12 h exposure to selective ligand

Target	Cell line - **12 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Receptor for Activated C Kinase (RACK1)	Rat1 + ERα DES[0.4nM]	22.79 ± 0.23	16.36 ± 0.19	6.43 ± 0.03[a]	-1.61	3.07
	Rat1 + ERα V_{EtOH}	23.80 ± 0.74	16.88 ± 0.25	6.62 ± 0.34[a]	-1.43	2.69
	Rat1 + ERβ DES [0.05nM]	24.46 ± 0.57	16.71 ± 0.24	7.75 ± 0.23[a]	-0.30	1.23
	Rat1 + ERβ V_{EtOH}	25.38 ± 0.30	16.84 ± 0.23	8.54 ± 0.05[a]	0.50	0.71
	Rat1 DES[0.4nM]	25.54 ± 0.50	16.56 ± 0.41	8.98 ± 0.07[a]	0.94	0.52
	Rat1 V_{EtOH}	24.79 ± 1.61	16.74 ± 0.18	8.05 ± 1.01[a]	0.00	1.00
	Rat1 + ERα OHT [1nM]	24.42 ± 0.44	16.77 ± 0.25	7.65 ± 0.13[a]	-0.71	1.63
	Rat1 + ERα V_{EtOH}	25.43 ± 0.66	18.29 ± 0.24	7.15 ± 0.30[a]	-1.21	2.32
	Rat1 + ERβ OHT [0.04nM]	26.51 ± 0.36	16.99 ± 0.12	9.53 ± 0.17[b]	1.17	0.44
	Rat1 + ERβ V_{EtOH}	26.76 ± 0.45	18.37 ± 0.11	8.39 ± 0.24[b]	0.03	0.98
	Rat1 OHT [1nM]	26.82 ± 0.40	17.13 ± 0.31	9.68 ± 0.07[b]	1.32	0.40
	Rat1 V_{EtOH}	26.71 ± 1.47	18.35 ± 0.22	8.36 ± 0.89[b]	0.00	1.00
	Rat1 + ERα Gen [20nM]	24.38 ± 0.29	24.57 ± 0.37	-0.19 + 0.01[a]	-1.40	2.64
	*Rat1 + ERα V_{EtOH}	26.09 ± 1.01	26.46 ± 1.08	-0.37 ± 0.04[a]	-1.58	2.98
	Rat1 + ERβ Gen [0.3nM]	26.16 ± 0.80	25.87 ± 1.17	0.29 ± 0.26[b]	-0.92	1.89
	*Rat1 + ERβ V_{EtOH}	27.11 ± 0.38	25.55 ± 0.61	1.56 ± 0.16[b]	0.35	0.78
	Rat1 Gen [20nM]	26.27 ± 1.19	25.01 ± 0.85	1.27 ± 0.25[b]	0.05	0.96
	*Rat1 V_{EtOH}	26.57 ± 1.51	25.36 ± 0.72	1.21 ± 0.56[b]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	24.79 ± 0.27	16.52 ± 0.15	8.27 ± 0.08[a]	-0.27	1.20
	Rat1 + ERα V_{EtOH}	23.25 ± 0.29	16.45 ± 0.14	6.81 ± 0.10[a]	-1.72	3.00
	Rat1 + ERβ E_2 [1nM]	24.72 ± 0.31	16.59 ± 0.28	8.13 ± 0.02[a]	-0.40	1.32
	Rat1 + ERβ V_{EtOH}	25.29 ± 0.52	16.52 ± 0.15	8.77 ± 0.26[a]	0.24	0.84
	Rat1 E_2 [1nM]	24.87 ± 1.23	16.42 ± 0.17	8.45 ± 0.75[a]	-0.07	1.05
	Rat1 V_{EtOH}	24.92 ± 1.54	16.39 ± 0.18	8.53 ± 0.96[a]	1.00	1.00
	Rat1 + ERα RAL [1nM]	24.67 ± 0.17	19.39 ± 0.64	4.98 ± 0.33[a]	-0.23	1.17
	Rat1 + ERα V_{DMSO}	24.57 ± 0.22	20.06 ± 0.61	4.50 ± 0.28[a]	-0.71	1.63
	Rat1 + ERβ RAL [0.04nM]	24.93 ± 0.20	19.45 ± 0.83	5.47 ± 0.45[a]	0.26	0.83
	Rat1 + ERβ V_{DMSO}	25.22 ± 0.65	19.94 ± 1.31	5.28 ± 0.47[a]	0.07	0.95
	Rat1 RAL [1nM]	24.86 ± 0.31	19.57 ± 0.42	5.29 ± 0.07[a]	0.08	0.95
	Rat1 V_{DMSO}	24.87 ± 0.34	19.66 ± 0.68	5.21 ± 0.24[a]	0.00	1.00

Table 4D. Expression of RACK1 at 18 h exposure to selective ligand

Target	Cell line - **18 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Receptor for Activated C Kinase (RACK1)	Rat1 + ERα DES[0.4nM]	27.76 ± 0.39	16.93 ± 0.31	10.82 ± 0.06[a]	0.73	0.60
	Rat1 + ERα V_{EtOH}	27.72 ± 0.30	16.89 ± 0.66	10.83 ± 0.26[a]	0.73	0.60
	Rat1 + ERβ DES [0.05nM]	26.90 ± 0.24	17.01 ± 0.70	9.90 ± 0.33[a]	-0.19	1.14
	Rat1 + ERβ V_{EtOH}	27.39 ± 0.82	16.70 ± 0.33	10.69 ± 0.35[a]	0.59	0.66
	Rat1 DES[0.4nM]	27.22 ± 0.34	16.60 ± 0.15	10.62 ± 0.14[a]	0.53	0.70
	Rat1 V_{EtOH}	27.61 ± 0.83	17.51 ± 1.50	10.09 ± 0.47[a]	0.00	1.00
	Rat1 + ERα OHT [1nM]	30.57 ± 0.72	17.67 ± 0.31	12.90 ± 0.29[a]	1.24	0.42
	Rat1 + ERα V_{EtOH}	29.64 ± 0.34	17.77 ± 0.21	11.86 ± 0.09[a]	0.20	0.87
	Rat1 + ERβ OHT [0.04nM]	28.17 ± 1.22	17.51 ± 0.16	10.67 ± 0.75[a]	-0.99	1.99
	Rat1 + ERβ V_{EtOH}	29.05 ± 0.90	17.77 ± 0.22	11.29 ± 0.48[a]	-0.37	1.30
	Rat1 OHT [1nM]	28.68 ± 0.73	17.43 ± 0.20	11.25 ± 0.37[a]	-0.41	1.32
	Rat1 V_{EtOH}	29.38 ± 0.37	17.72 ± 0.39	11.66 ± 0.02[a]	0.00	1.00
	Rat1 + ERα Gen [20nM]	28.70 ± 0.30	19.75 ± 0.56	8.94 ± 0.19[a]	0.68	0.62
	Rat1 + ERα V_{EtOH}	29.26 ± 0.23	19.94 ± 0.45	9.32 ± 0.15[a]	1.06	0.48
	Rat1 + ERβ Gen [0.3nM]	27.59 ± 0.42	19.49 ± 0.26	8.10 ± 0.11[a]	-0.16	1.12
	Rat1 + ERβ V_{EtOH}	28.42 ± 0.91	20.06 ± 0.57	8.36 ± 0.24[a]	0.10	0.93
	Rat1 Gen [20nM]	28.23 ± 0.38	19.69 ± 0.48	8.54 ± 0.07[a]	0.29	0.82
	Rat1 V_{EtOH}	29.17 ± 1.04	20.91 ± 0.52	8.26 ± 0.37[a]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	28.36 ± 0.35	16.79 ± 0.88	11.57 ± 0.37[a]	-0.10	1.07
	Rat1 + ERα V_{EtOH}	29.21 ± 0.26	17.92 ± 0.19	11.29 ± 0.05[a]	-0.38	1.30
	Rat1 + ERβ E_2 [1nM]	28.03 ± 0.38	16.87 ± 1.25	11.16 ± 0.62[a]	-0.51	1.42
	Rat1 + ERβ V_{EtOH}	28.81 ± 1.09	17.69 ± 0.27	11.11 ± 0.58[a]	-0.55	1.47
	Rat1 E_2 [1nM]	27.87 ± 0.81	16.76 ± 1.17	11.10 ± 0.25[a]	-0.57	1.48
	Rat1 V_{EtOH}	29.63 ± 0.71	17.96 ± 0.49	11.67 ± 0.16[a]	0.00	1.00
	Rat1 + ERα RAL [1nM]	23.25 ± 0.17	17.02 ± 0.52	6.22 ± 0.25[a]	-0.97	1.96
	Rat1 + ERα V_{DMSO}	23.54 ± 0.45	17.08 ± 0.39	6.46 ± 0.04[a]	-0.73	1.66
	Rat1 + ERβ RAL [0.04nM]	23.35 ± 0.16	17.23 ± 0.17	6.12 ± 0.01[a]	-1.07	2.10
	Rat1 + ERβ V_{DMSO}	23.58 ± 0.36	17.15 ± 0.40	6.43 ± 0.03[a]	-0.76	1.69
	Rat1 RAL [1nM]	24.39 ± 0.13	17.15 ± 0.17	7.24 ± 0.03[a]	0.05	0.96
	Rat1 V_{DMSO}	24.40 ± 0.64	17.21 ± 0.10	7.19 ± 0.38[a]	0.00	1.00

Table 4E. Expression of RACK1 at 24 h exposure to selective ligand

Target	Cell line - **24 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Receptor for Activated C Kinase (RACK1)	Rat1 + ERα DES[0.4nM]	24.03 ± 0.17	18.49 ± 0.23	5.55 ± 0.04[a]	-2.60	6.06
	Rat1 + ERα V_{EtOH}	24.93 ± 0.30	17.81 ± 0.18	7.13 ± 0.08[b]	-1.02	2.03
	Rat1 + ERβ DES [0.05nM]	26.17 ± 0.41	17.90 ± 0.16	8.27 ± 0.17[c]	0.12	0.92
	Rat1 + ERβ V_{EtOH}	26.16 ± 0.44	17.99 ± 0.28	8.17 ± 0.11[c]	0.02	0.98
	Rat1 DES[0.4nM]	25.18 ± 0.45	18.18 ± 0.29	7.00 ± 0.11[b]	-1.14	2.21
	Rat1 V_{EtOH}	25.81 ± 0.38	17.66 ± 0.18	8.15 ± 0.14[c]	0.00	1.00
	Rat1 + ERα OHT [1nM]	23.69 ± 0.81	17.63 ± 0.33	6.07 ± 0.34[a]	1.85	0.27
	Rat1 + ERα V_{EtOH}	23.59 ± 0.42	20.45 ± 0.51	3.14 ± 0.06[b]	-1.07	2.11
	Rat1 + ERβ OHT [0.04nM]	25.33 ± 0.65	17.75 ± 0.25	7.59 ± 0.28[a]	3.37	0.10
	Rat1 + ERβ V_{EtOH}	24.92 ± 0.41	20.25 ± 0.12	4.68 ± 0.20[c]	0.47	0.72
	Rat1 OHT [1nM]	23.42 ± 0.61	17.33 ± 0.84	6.09 ± 0.16[a]	1.87	0.27
	Rat1 V_{EtOH}	24.47 ± 0.38	20.25 ± 0.41	4.21 ± 0.02[c]	0.00	1.00
	Rat1 + ERα Gen [20nM]	23.92 ± 0.18	17.51 ± 0.72	6.42 ± 0.29[a]	2.36	0.19
	Rat1 + ERα V_{EtOH}	23.70 ± 0.42	20.57 ± 0.63	3.14 ± 0.15[b]	-0.92	1.89
	Rat1 + ERβ Gen [0.3nM]	25.13 ± 0.31	18.14 ± 0.20	6.99 ± 0.08[a]	2.93	0.13
	Rat1 + ERβ V_{EtOH}	24.88 ± 0.50	20.52 ± 0.43	4.36 ± 0.05[b]	0.31	0.81
	Rat1 Gen [20nM]	23.92 ± 0.47	17.33 ± 0.90	6.59 ± 0.30[a]	2.17	0.17
	Rat1 V_{EtOH}	24.39 ± 0.39	20.33 ± 0.31	4.06 ± 0.05[b]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	23.45 ± 0.52	17.40 ± 0.32	6.06 ± 0.14[a]	-0.92	1.89
	Rat1 + ERα V_{EtOH}	24.80 ± 0.35	18.47 ± 0.19	6.33 ± 0.11[a]	-0.65	1.57
	Rat1 + ERβ E_2 [1nM]	25.82 ± 0.32	17.37 ± 0.20	8.45 ± 0.08[b]	1.47	0.36
	Rat1 + ERβ V_{EtOH}	25.98 ± 0.42	18.91 ± 0.37	7.07 ± 0.04[b]	0.09	0.94
	Rat1 E_2 [1nM]	24.37 ± 0.46	17.60 ± 0.23	6.77 ± 0.16[a]	-0.22	1.16
	Rat1 V_{EtOH}	25.58 ± 0.26	18.60 ± 0.29	6.99 ± 0.02[a]	0.00	1.00
	Rat1 + ERα RAL [1nM]	28.01 ± 0.29	16.00 ± 0.16	12.00 ± 0.30[a]	1.47	0.36
	Rat1 + ERα V_{DMSO}	28.21 ± 0.43	16.76 ± 0.24	11.45 ± 0.31[b]	0.91	0.53
	Rat1 + ERβ RAL [0.04nM]	28.51 ± 0.65	16.42 ± 0.28	12.09 ± 0.25[a]	1.55	0.34
	Rat1 + ERβ V_{DMSO}	27.61 ± 0.26	16.45 ± 0.89	11.16 ± 0.45[b]	0.62	0.65
	Rat1 RAL [1nM]	28.25 ± 0.51	16.63 ± 0.39	11.61 ± 0.08[a]	1.08	0.47
	Rat1 V_{DMSO}	27.91 ± 0.63	17.37 ± 0.55	10.53 ± 0.06[b]	0.00	1.00

Table 5A. Expression of PCOLCE at 6 h exposure to selective ligand

Target	Cell line - **6 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Procollagen C – proteinase enhancer protein (PCOLCE)	Rat1 + ERα DES[0.4nM]	23.88 ± 0.35	21.61 ± 0.39	2.27 ± 0.02[a]	1.98	0.25
	Rat1 + ERα V_{EtOH}	23.50 ± 0.11	23.04 ± 0.85	0.46 ± 0.52[b]	0.17	0.89
	Rat1 + ERβ DES [0.05nM]	23.39 ± 0.43	21.85 ± 1.00	1.54 ± 0.41[a]	1.25	0.42
	Rat1 + ERβ V_{EtOH}	23.13 ± 0.48	23.02 ± 0.69	0.11 ± 0.15[b]	-0.18	1.13
	Rat1 DES[0.4nM]	24.50 ± 1.01	22.63 ± 0.71	1.87 ± 0.21[a]	1.59	0.33
	Rat1 V_{EtOH}	23.09 ± 0.54	22.81 ± 0.45	0.29 ± 0.07[b]	0.00	1.00
	Rat1 + ERα OHT [1nM]	22.98 ± 0.04	20.33 ± 0.77	2.65 ± 0.52[a]	-1.39	2.62
	Rat1 + ERα V_{EtOH}	23.24 ± 0.21	19.73 ± 0.59	3.05 ± 0.27[a]	-0.54	1.45
	Rat1 + ERβ OHT [0.04nM]	22.29 ± 0.71	19.61 ± 0.27	2.78 ± 0.31[a]	-1.27	2.40
	Rat1 + ERβ V_{EtOH}	23.70 ± 0.33	20.21 ± 0.37	3.49 ± 0.02[a]	-0.55	1.47
	Rat1 OHT [1nM]	23.57 ± 0.77	19.80 ± 0.19	3.78 ± 0.41[a]	-0.27	1.20
	Rat1 V_{EtOH}	23.32 ± 0.43	19.28 ± 0.58	4.04 ± 0.10[a]	0.00	1.00
	Rat1 + ERα Gen [20nM]	24.41 ± 1.69	20.22 ± 1.87	4.18 ± 0.13[a]	0.94	0.52
	Rat1 + ERα V_{EtOH}	24.15 ± 0.24	21.05 ± 0.85	3.10 ± 0.42[b]	-0.15	1.10
	Rat1 + ERβ Gen [0.3nM]	22.92 ± 0.14	18.95 ± 0.44	3.95 ± 0.21[b]	0.71	0.61
	Rat1 + ERβ V_{EtOH}	24.21 ± 0.32	20.43 ± 1.33	3.79 ± 0.72[b]	0.54	0.69
	Rat1 Gen [20nM]	24.10 ± 1.02	19.32 ± 1.33	4.77 ± 0.21[a]	1.53	0.35
	Rat1 V_{EtOH}	23.99 ± 0.66	20.75 ± 0.73	3.25 ± 0.05[b]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	24.49 ± 0.48	20.03 ± 1.03	4.46 + 0.39[a]	1.55	0.34
	Rat1 + ERα V_{EtOH}	23.01 ± 0.05	19.64 ± 0.20	3.37 + 0.11[b]	0.47	0.72
	Rat1 + ERβ E_2 [1nM]	23.42 ± 0.42	19.61 ± 0.48	3.80 + 0.04[a]	0.90	0.53
	Rat1 + ERβ V_{EtOH}	22.32 ± 0.25	19.59 ± 0.45	2.72 + 0.15[b]	-0.18	1.13
	Rat1 E_2 [1nM]	23.20 ± 0.24	19.36 ± 0.54	3.85 + 0.21[a]	0.94	0.52
	Rat1 V_{EtOH}	22.43 ± 0.58	19.52 ± 0.24	2.90 + 0.24[b]	0.00	1.00
	Rat1 + ERα RAL [1nM]	23.29 ± 0.48	21.31 ± 0.30	1.99 ± 0.13[a]	0.21	0.86
	Rat1 + ERα V_{DMSO}	23.39 ± 0.37	21.46 ± 0.38	1.83 ± 0.01[a]	0.05	0.96
	Rat1 + ERβ RAL [0.04nM]	23.03 ± 0.09	21.41 ± 0.25	1.61 ± 0.11[a]	-0.16	1.11
	Rat1 + ERβ V_{DMSO}	23.70 ± 0.85	21.26 ± 0.38	2.44 ± 0.33[a]	0.67	0.63
	Rat1 RAL [1nM]	23.19 ± 0.11	21.17 ± 0.14	2.03 ± 0.01[a]	0.25	0.84
	Rat1 V_{DMSO}	23.20 ± 0.44	21.43 ± 0.49	1.77 ± 0.04[a]	0.00	1.00

Table 5B. Expression of PCOLCE at 9 h exposure to selective ligand

Target	Cell line – **9 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Procollagen C – proteinase enhancer protein (PCOLCE)	Rat1 + ERα DES[0.4nM]	24.29 ± 0.70	20.99 ± 1.12	3.29 ± 0.29[a]	1.99	0.25
	Rat1 + ERα V_{EtOH}	24.53 ± 0.92	22.29 ± 0.94	2.23 ± 0.01[a]	0.94	0.52
	Rat1 + ERβ DES [0.05nM]	24.07 ± 0.72	20.09 ± 0.84	3.98 ± 0.09[a]	2.69	0.15
	Rat1 + ERβ V_{EtOH}	24.31 ± 0.61	20.77 ± 0.37	3.54 ± 0.17[a]	2.25	0.21
	Rat1 DES[0.4nM]	24.84 ± 0.38	20.21 ± 1.01	4.63 ± 0.44[a]	3.34	0.10
	Rat1 V_{EtOH}	22.89 ± 0.50	21.60 ± 0.63	1.29 ± 0.09[b]	0.00	1.00
	Rat1 + ERα OHT [1nM]	21.75 ± 0.34	25.65 ± 2.02	-3.89 ± 1.19[a]	-5.01	32.30
	Rat1 + ERα V_{EtOH}	24.75 ± 0.87	24.97 ± 2.79	-0.22 ± 1.36[b]	-1.34	2.52
	Rat1 + ERβ OHT [0.04nM]	22.47 ± 0.74	24.63 ± 1.02	-2.16 ± 0.20[a]	-3.27	9.69
	Rat1 + ERβ V_{EtOH}	24.48 ± 0.50	23.33 ± 2.09	1.15 ± 1.22[b]	0.03	0.98
	Rat1 OHT [1nM]	23.04 ± 0.58	26.04 ± 3.00	-3.00 ± 1.71[a]	-4.11	17.32
	Rat1 V_{EtOH}	22.96 ± 0.61	21.86 ± 2.16	1.11 ± 1.09[b]	0.00	1.00
	Rat1 + ERα Gen [20nM]	22.20 ± 0.35	21.11 ± 0.50	1.09 ± 0.11[a]	-1.20	2.30
	Rat1 + ERα V_{EtOH}	24.46 ± 1.20	22.39 ± 1.01	2.07 ± 0.13[a]	-0.22	1.17
	Rat1 + ERβ Gen [0.3nM]	23.19 ± 0.53	20.73 ± 0.43	2.47 ± 0.07[a]	0.18	0.88
	Rat1 + ERβ V_{EtOH}	24.60 ± 0.60	21.65 ± 0.72	2.95 ± 0.08[a]	0.66	0.63
	Rat1 Gen [20nM]	23.46 ± 0.45	20.96 ± 0.36	2.50 ± 0.07[a]	0.21	0.86
	Rat1 V_{EtOH}	23.47 ± 0.58	21.19 ± 0.67	2.29 ± 0.06[a]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	23.89 ± 0.77	19.76 ± 0.49	4.13 ± 0.20[a]	1.29	0.40
	Rat1 + ERα V_{EtOH}	22.37 ± 0.26	19.93 ± 0.72	2.44 ± 0.32[a]	-0.40	1.32
	Rat1 + ERβ E_2 [1nM]	22.47 ± 0.64	19.41 ± 0.39	3.06 ± 0.18[a]	0.22	0.95
	Rat1 + ERβ V_{EtOH}	22.93 ± 0.36	19.33 ± 0.71	3.60 ± 0.25[a]	0.77	0.58
	Rat1 E_2 [1nM]	22.86 ± 0.62	19.22 ± 0.24	3.65 ± 0.27[a]	0.81	0.57
	Rat1 V_{EtOH}	21.72 ± 0.31	18.88 ± 0.37	2.84 ± 0.04[a]	0.00	1.00
	Rat1 + ERα RAL [1nM]	23.45 ± 0.43	19.34 ± 0.20	4.11 ± 0.16[a]	0.63	0.65
	Rat1 + ERα V_{DMSO}	23.38 ± 0.26	19.30 ± 0.39	4.08 ± 0.09[a]	0.59	0.66
	Rat1 + ERβ RAL [0.04nM]	22.90 ± 0.29	19.65 ± 0.13	3.26 ± 0.11[a]	-0.22	1.17
	Rat1 + ERβ V_{DMSO}	22.84 ± 0.48	19.47 ± 0.27	3.38 ± 0.15[a]	-0.11	1.08
	Rat1 RAL [1nM]	23.26 ± 0.32	19.42 ± 0.19	3.84 ± 0.09[a]	0.35	0.78
	Rat1 V_{DMSO}	22.76 ± 0.39	19.27 ± 0.30	3.48 ± 0.06[a]	0.00	1.00

Table 5C. Expression of PCOLCE at 12 h exposure to selective ligand

Target	Cell line - **12 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Procollagen C – proteinase enhancer protein (PCOLE)	Rat1 + ERα DES[0.4nM]	20.87 ± 0.37	16.35 ± 0.23	4.51 ± 0.10[a]	-0.42	1.34
	Rat1 + ERα V_{EtOH}	21.13 ± 0.44	16.62 ± 0.15	4.50 ± 0.21[a]	-0.43	1.35
	Rat1 + ERβ DES [0.05nM]	21.88 ± 0.69	16.51 ± 0.36	5.37 ± 0.23[a]	0.44	0.74
	Rat1 + ERβ V_{EtOH}	22.11 ± 0.25	16.85 ± 0.09	5.27 ± 0.11[a]	0.33	0.79
	Rat1 DES[0.4nM]	22.54 ± 0.25	16.25 ± 0.08	6.29 ± 0.12[a]	1.36	0.39
	Rat1 V_{EtOH}	21.77 ± 0.84	16.83 ± 0.28	4.93 ± 0.40[a]	1.00	1.00
	Rat1 + ERα OHT [1nM]	21.98 ± 0.30	16.73 ± 0.23	5.24 ± 0.05[a]	-0.37	1.29
	Rat1 + ERα V_{EtOH}	23.54 ± 1.12	18.38 ± 1.33	5.17 ± 0.15[a]	-0.45	1.36
	Rat1 + ERβ OHT [0.04nM]	22.87 ± 0.35	16.90 ± 0.19	5.97 ± 0.11[a]	0.36	0.78
	Rat1 + ERβ V_{EtOH}	23.18 ± 0.20	16.99 ± 0.38	6.19 ± 0.13[a]	0.57	0.67
	Rat1 OHT [1nM]	23.28 ± 0.23	16.91 ± 0.24	6.37 ± 0.01[a]	0.75	0.59
	Rat1 V_{EtOH}	22.85 ± 1.06	17.24 ± 0.41	5.61 ± 0.46[a]	0.00	1.00
	Rat1 + ERα Gen [20nM]	22.06 ± 0.51	21.12 ± 0.57	0.93 ± 0.04[a]	-0.10	1.07
	Rat1 + ERα V_{EtOH}	23.63 ± 1.26	22.77 ± 1.72	0.86 ± 0.32[a]	-0.18	1.13
	Rat1 + ERβ Gen [0.3nM]	22.70 ± 0.58	21.56 ± 0.40	1.15 ± 0.12[a]	0.11	0.92
	Rat1 + ERβ V_{EtOH}	23.92 ± 0.67	21.95 ± 0.48	1.97 ± 0.13[a]	0.94	0.52
	Rat1 Gen [20nM]	22.97 ± 1.12	21.07 ± 0.44	1.90 ± 0.48[a]	0.87	0.55
	Rat1 V_{EtOH}	22.73 ± 1.26	22.44 ± 0.57	1.03 ± 0.49	0.00	1.00
	Rat1 + ERα E_2 [1nM]	21.95 ± 0.32	16.42 ± 0.40	5.54 ± 0.06[a]	0.63	0.65
	Rat1 + ERα V_{EtOH}	20.59 ± 0.46	16.42 ± 0.39	4.17 ± 0.05[a]	-0.74	1.67
	Rat1 + ERβ E_2 [1nM]	21.78 ± 0.28	16.49 ± 0.19	5.30 ± 0.07[a]	0.39	0.76
	Rat1 + ERβ V_{EtOH}	21.69 ± 0.22	16.50 ± 0.58	5.19 ± 0.25[a]	0.28	0.82
	Rat1 E_2 [1nM]	21.57 ± 0.89	16.34 ± 0.46	5.23 ± 0.30[a]	0.32	0.80
	Rat1 V_{EtOH}	21.19 ± 0.95	16.29 ± 0.23	4.91 ± 0.51[a]	0.00	1.00
	Rat1 + ERα RAL [1nM]	23.52 ± 0.25	20.21 ± 0.90	3.31 ± 0.46[a]	0.62	0.65
	Rat1 + ERα V_{DMSO}	23.48 ± 0.16	19.93 ± 0.53	3.55 ± 0.26[a]	0.87	0.55
	Rat1 + ERβ RAL [0.04nM]	22.49 ± 0.21	19.61 ± 0.64	2.87 ± 0.30[a]	0.19	0.88
	Rat1 + ERβ V_{DMSO}	22.72 ± 0.58	20.53 ± 0.51	2.19 ± 0.05[a]	-0.51	1.42
	Rat1 RAL [1nM]	22.75 ± 0.38	19.94 ± 0.44	2.81 ± 0.05[a]	0.13	0.92
	Rat1 V_{DMSO}	22.54 ± 0.41	19.85 ± 0.51	2.69 ± 0.07[a]	0.00	1.00

Table 5D. Expression of PCOLCE at 18 h exposure to selective ligand

Target	Cell line - **18 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Procollagen C – proteinase enhancer protein (PCOLCE)	Rat1 + ERα DES[0.4nM]	22.54 ± 0.23	16.99 ± 0.15	5.55 ± 0.06[a]	0.08	0.57
	Rat1 + ERα V_{EtOH}	22.65 ± 0.27	16.78 ± 0.26	5.87 ± 0.01[a]	1.14	0.45
	Rat1 + ERβ DES [0.05nM]	20.97 ± 0.33	17.01 ± 0.15	3.96 ± 0.13[a]	-0.77	1.70
	Rat1 + ERβ V_{EtOH}	21.30 ± 0.51	16.97 ± 0.39	4.33 ± 0.09[a]	-0.39	1.31
	Rat1 DES[0.4nM]	21.15 ± 0.75	17.17 ± 0.32	3.97 ± 0.30[a]	-0.75	1.68
	Rat1 V_{EtOH}	21.56 ± 0.96	16.83 ± 0.42	4.72 ± 0.39[a]	0.00	1.00
	Rat1 + ERα OHT [1nM]	24.55 ± 0.60	18.75 ± 0.88	5.81 ± 0.19[a]	1.39	0.38
	Rat1 + ERα V_{EtOH}	23.77 ± 0.21	18.68 ± 0.21	5.09 ± 0.01[a]	0.66	0.63
	Rat1 + ERβ OHT [0.04nM]	22.11 ± 0.20	18.49 ± 0.11	3.63 ± 0.06[a]	-0.80	1.74
	Rat1 + ERβ V_{EtOH}	22.33 ± 0.60	18.37 ± 0.21	3.97 ± 0.28[a]	-0.46	1.37
	Rat1 OHT [1nM]	22.43 ± 0.61	18.41 ± 0.25	4.01 ± 0.25[a]	-0.41	1.33
	Rat1 V_{EtOH}	23.13 ± 0.82	18.71 ± 0.83	4.42 ± 0.01[a]	0.00	1.00
	Rat1 + ERα Gen [20nM]	22.88 ± 0.29	17.43 ± 0.38	5.46 + 0.06[a]	0.21	0.86
	Rat1 + ERα V_{EtOH}	24.0+ ± 0.23	17.81 ± 0.42	6.27 + 0.14[a]	1.03	0.49
	Rat1 + ERβ Gen [0.3nM]	21.73 ± 0.42	17.38 ± 0.18	4.35 + 0.17[a]	-0.90	1.86
	Rat1 + ERβ V_{EtOH}	22.43 ± 0.61	17.78 ± 0.38	4.65 + 0.16[a]	-0.60	1.51
	Rat1 Gen [20nM]	22.18 ± 0.62	17.37 ± 0.27	4.81 + 0.25[a]	-0.43	1.35
	Rat1 V_{EtOH}	23.71 ± 1.22	18.47 ± 0.74	5.25 + 0.34[a]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	21.74 ± 0.23	16.84 ± 0.21	4.90 + 0.01[a]	-0.61	1.52
	Rat1 + ERα V_{EtOH}	23.84 ± 0.13	17.21 ± 0.30	6.63 + 0.12[b]	1.13	0.46
	Rat1 + ERβ E_2 [1nM]	21.38 ± 0.21	16.85 ± 0.14	4.52 + 0.05[a]	-0.98	1.97
	Rat1 + ERβ V_{EtOH}	22.24 ± 0.56	17.10 ± 0.60	5.14 + 0.03[a]	-0.36	1.29
	Rat1 E_2 [1nM]	21.70 ± 0.58	16.71 ± 0.16	4.99 + 0.30[a]	-0.51	1.43
	Rat1 V_{EtOH}	23.15 ± 1.00	17.65 ± 0.27	5.50 + 0.52[a]	0.00	1.00
	Rat1 + ERα RAL [1nM]	21.25 ± 0.10	16.73 ± 0.50	4.51 ± 0.28[a]	-0.53	1.44
	Rat1 + ERα V_{DMSO}	21.26 ± 0.39	16.83 ± 0.30	4.43 ± 0.06[a]	-0.61	1.53
	Rat1 + ERβ RAL [0.04nM]	20.72 ± 0.30	17.07 ± 0.23	3.65 ± 0.04[a]	-1.39	2.63
	Rat1 + ERβ V_{DMSO}	20.81 ± 0.36	17.00 ± 0.15	3.81 ± 0.14[a]	-1.23	2.35
	Rat1 RAL [1nM]	22.47 ± 0.36	17.05 ± 0.11	5.42 ± 0.01[a]	0.38	0.77
	Rat1 V_{DMSO}	22.11 ± 0.56	17.08 ± 0.12	5.04 ± 0.31[a]	0.00	1.00

Table 5E. Expression of PCOLCE at 24 h exposure to selective ligand

Target	Cell line - **24 h**	Average C_T Target	Average C_T 18S	ΔC_T	$\Delta\Delta C_T$	$2^{-\Delta\Delta CT}$
Procollagen C – proteinase enhancer protein (PCOLE)	Rat1 + ERα DES[0.4nM]	20.88 ± 0.30	18.86 ± 0.19	2.02 ± 0.07[a]	-1.10	2.15
	Rat1 + ERα V_{EtOH}	22.51 ± 0.41	19.28 ± 0.43	3.24 ± 0.01[a]	0.11	0.92
	Rat1 + ERβ DES [0.05nM]	22.79 ± 0.44	17.82 ± 0.24	4.97 ± 0.14[a]	1.85	0.28
	Rat1 + ERβ V_{EtOH}	23.39 ± 0.54	19.45 ± 0.42	3.84 ± 0.09[a]	0.72	0.61
	Rat1 DES[0.4nM]	21.58 ± 0.51	18.68 ± 0.34	2.90 ± 0.11[a]	-0.22	1.16
	Rat1 V_{EtOH}	22.06 ± 0.52	18.94 ± 0.23	3.12 ± 0.21[a]	0.00	1.00
	Rat1 + ERα OHT [1nM]	22.41 ± 0.78	18.03 ± 0.26	4.38 ± 0.37[a]	0.87	0.55
	Rat1 + ERα V_{EtOH}	22.61 ± 0.45	18.82 ± 0.27	3.80 ± 0.13[a]	0.29	0.82
	Rat1 + ERβ OHT [0.04nM]	23.03 ± 0.59	18.16 ± 0.29	4.87 ± 0.20[a]	1.35	0.39
	Rat1 + ERβ V_{EtOH}	23.37 ± 0.63	19.17 ± 0.69	4.19 ± 0.04[a]	0.69	0.62
	Rat1 OHT [1nM]	20.97 ± 0.65	17.64 ± 0.69	3.33 ± 0.03[a]	-0.18	1.13
	Rat1 V_{EtOH}	22.40 ± 0.48	18.89 ± 0.50	3.50 ± 0.01[a]	0.00	1.00
	Rat1 + ERα Gen [20nM]	22.29 ± 0.24	17.83 ± 0.83	4.46 ± 0.42[a]	1.18	0.44
	Rat1 + ERα V_{EtOH}	22.64 ± 0.40	19.42 ± 0.29	3.22 ± 0.07[a]	-0.07	1.05
	Rat1 + ERβ Gen [0.3nM]	22.54 ± 0.19	18.50 ± 0.22	4.03 ± 0.02[a]	0.75	0.59
	Rat1 + ERβ V_{EtOH}	23.08 ± 1.07	19.21 ± 2.21	3.87 ± 0.80[a]	0.59	0.67
	Rat1 Gen [20nM]	21.35 ± 0.42	19.62 ± 0.77	3.73 ± 0.24[a]	0.45	0.73
	Rat1 V_{EtOH}	22.67 ± 0.51	19.39 ± 0.30	3.29 ± 0.15[a]	0.00	1.00
	Rat1 + ERα E_2 [1nM]	20.69 ± 0.60	19.41 ± 0.18	1.28 ± 0.30[a]	-2.23	4.70
	Rat1 + ERα V_{EtOH}	22.64 ± 0.52	19.82 ± 0.33	2.81 ± 0.14[b]	-0.70	1.63
	Rat1 + ERβ E_2 [1nM]	22.57 ± 0.16	19.32 ± 0.22	3.25 ± 0.04[a]	-0.27	1.21
	Rat1 + ERβ V_{EtOH}	23.02 ± 1.16	19.22 ± 2.16	3.81 ± 0.71[a]	0.29	0.82
	Rat1 E_2 [1nM]	20.96 ± 0.57	19.37 ± 0.45	1.59 ± 0.09[a]	-1.93	3.81
	Rat1 V_{EtOH}	22.22 ± 0.52	18.70 ± 1.25	3.52 ± 0.52[b]	0.00	1.00
	Rat1 + ERα RAL [1nM]	20.81 ± 0.35	17.50 ± 0.33	3.31 ± 0.02[a]	1.40	0.38
	Rat1 + ERα V_{DMSO}	21.11 ± 0.57	18.16 ± 0.30	2.95 ± 0.19[a]	1.04	0.49
	Rat1 + ERβ RAL [0.04nM]	20.79 ± 0.46	18.12 ± 0.53	2.60 ± 0.05[a]	0.69	0.62
	Rat1 + ERβ V_{DMSO}	20.39 ± 0.20	18.09 ± 0.90	2.30 ± 0.50[a]	0.38	0.77
	Rat1 RAL [1nM]	20.79 ± 0.57	18.15 ± 0.34	2.64 ± 0.16[a]	0.72	0.61
	Rat1 V_{DMSO}	20.92 ± 0.59	19.00 ± 0.59	1.92 ± 0.00[a]	0.00	1.00

Figure 1. Fold changes in COL1A2 over time and ligand exposure were determined by the comparative C_T method. The difference between the C_T values of the target genes and the 18s rRNA (ΔC_T) were calibrated to an index value by subtracting all individual ΔC_T's from Rat1 cell line ΔC_T receiving vehicle treatment to derive the $\Delta\Delta C_T$. Fold differences were then calculated by the equation $2^{-\Delta\Delta CT}$. Statistical analysis for fold differences are those determined for the ΔC_T through factorial ANOVA using SAS PROC-MIXED. A significant cell by compound by time effect was detected ($p<0.0306$). Fold differences are shown for: **a)** COL1A2 detected when treated with E_2; **b)** COL1A2 detected when treated with DES; **c)** COL1A2 detected when treated with OHT; **d)** COL1A2 detected when treated with RAL; **e)** COL1A2 detected when treated with GEN.

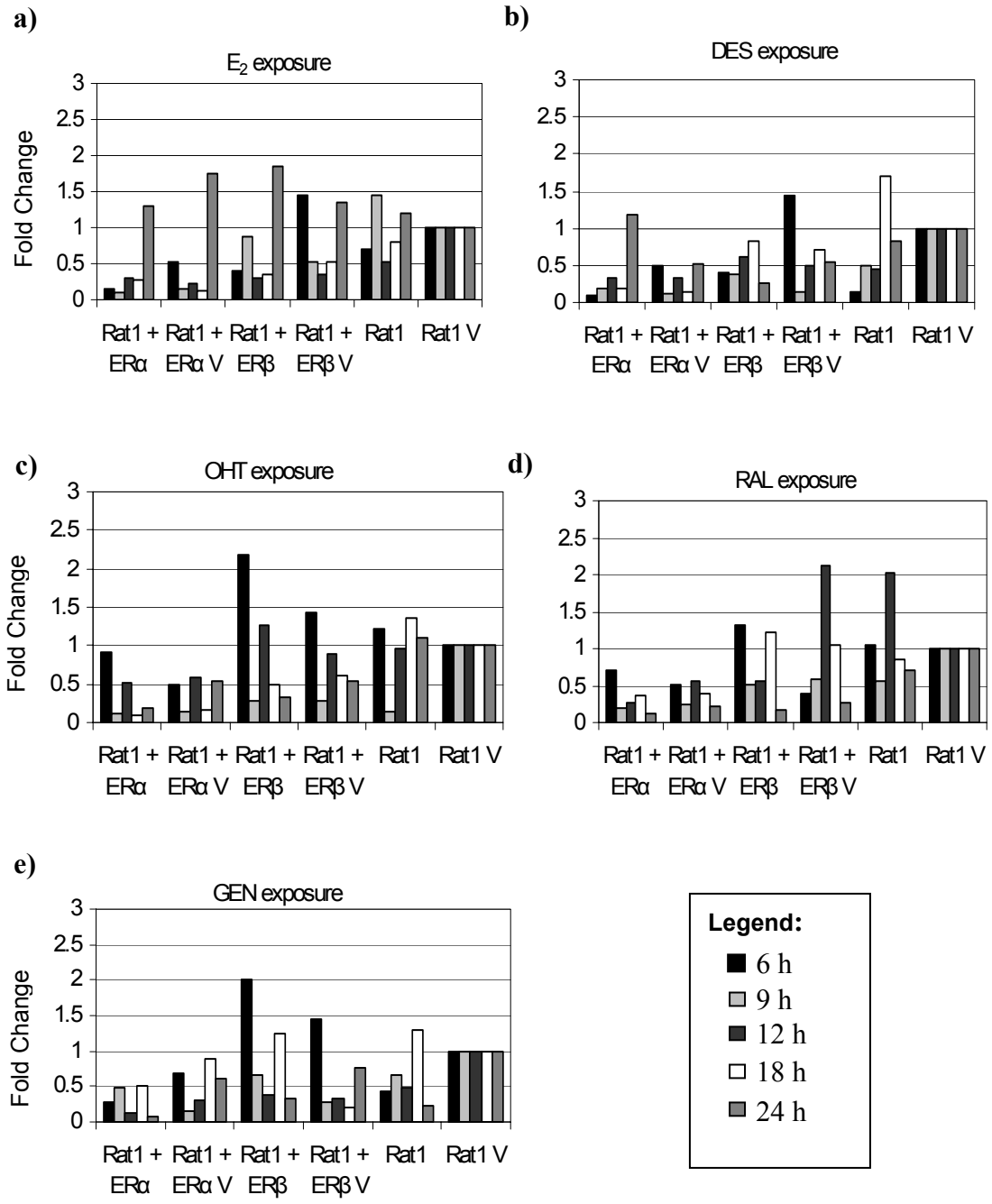
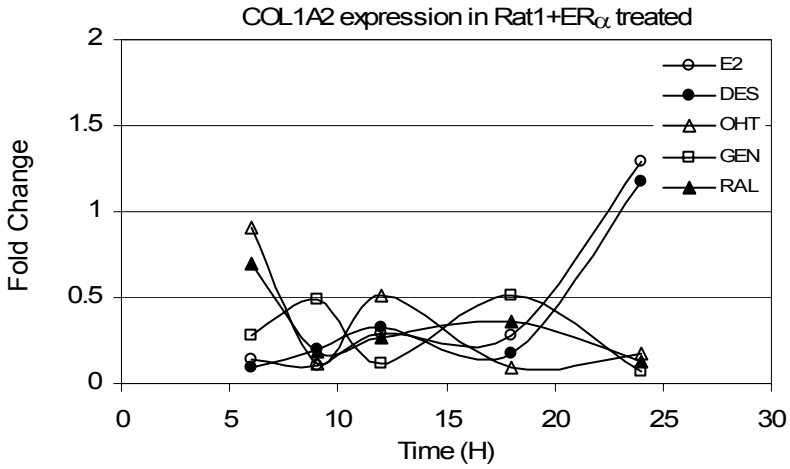

a)
E2 exposure
Fold Change
3
2.5
2
1.5
1
0.5
0
Rat1 + ERα
Rat1 + ERα V
Rat1 + ERβ
Rat1 + ERβ V
Rat1
Rat1 V
b)
DES exposure
c)
OHT exposure
d)
RAL exposure
e)
GEN exposure
Legend:
6 h
9 h
12 h
18 h
24 h

Figure 2. Independent expression trends of COL1A2 in either ERα or ERβ expressing cell culture lines over time. **a)** Rat1+ERα changes in profile for COL1A2 expression demonstrate that E_2 or DES treatment at 24h is able to reach a level of expression at or above baseline expression seen in Rat1 cells. These treatments result in levels of expression that are significantly higher than the COL1A2 detected with the other three treatments at 24 h and higher than expression seen following 9, 12, and 18 h of RAL, E_2 or DES treatment. The SERMs OHT and RAL were also significantly higher than DES, E_2 or GEN at 6 h. **b)** Rat1+ERβ had increased expression of COL1A2 in OHT and RAL similar to that observed in ERα exposed cells at 6 h. An increase observed following 6 h GEN treatment was significantly higher than E_2 or DES treatments. Though not different from its own vehicle, this effect did not appear to be as repressive as E_2 and DES treatment at the time point. Though not different from levels detected in ERα expressing cells at 24 h, E_2 treatment did affect a trend toward increased expression that was significantly higher than all other treatments at this time. These data suggest a trend toward possible gene repression in receptor expressing cells that OHT and RAL treatment may negate at 6 h in ERα and ERβ as well as following GEN treatment in ERβ. A reversal of this trend is demonstrated following treatment at 24 h with E_2 in both receptors and DES treatment in ERβ expressing cells.

a)


COL1A2 expression in Rat1+ERα treated
Fold Change
Time (H)
E2
DES
OHT
GEN
RAL
0
0.5
1
1.5
2
0
5
10
15
20
25
30

b)

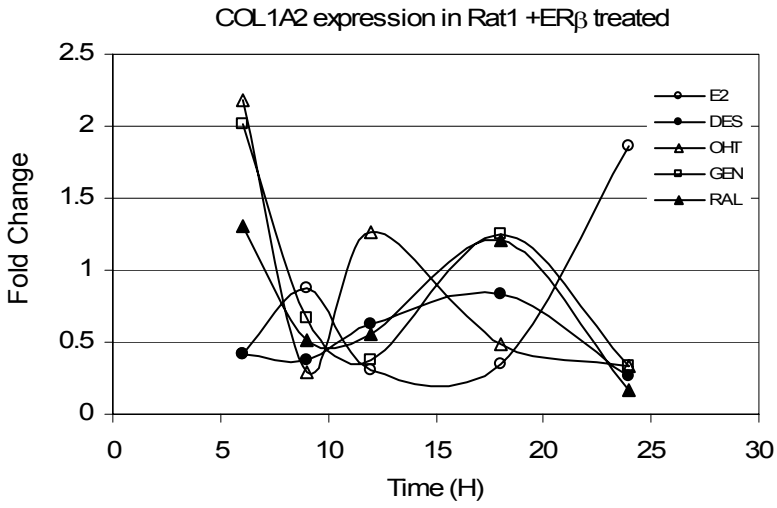

COL1A2 expression in Rat1 +ERβ treated
Fold Change
Time (H)
E2
DES
OHT
GEN
RAL
0
0.5
1
1.5
2
2.5
0
5
10
15
20
25
30

Figure 3. Fold changes in CtsL over time and ligand exposure were determined by the comparative C_T method. Statistical analysis determined a significant cell by compound by time interaction ($p<0.0062$). Fold differences shown for: **a)** CtsL detected when treated with E_2; **b)** CtsL detected when treated with DES; **c)** CtsL detected when treated with OHT; **d)** CtsL detected when treated with RAL; **e)** CtsL detected when treated with GEN.

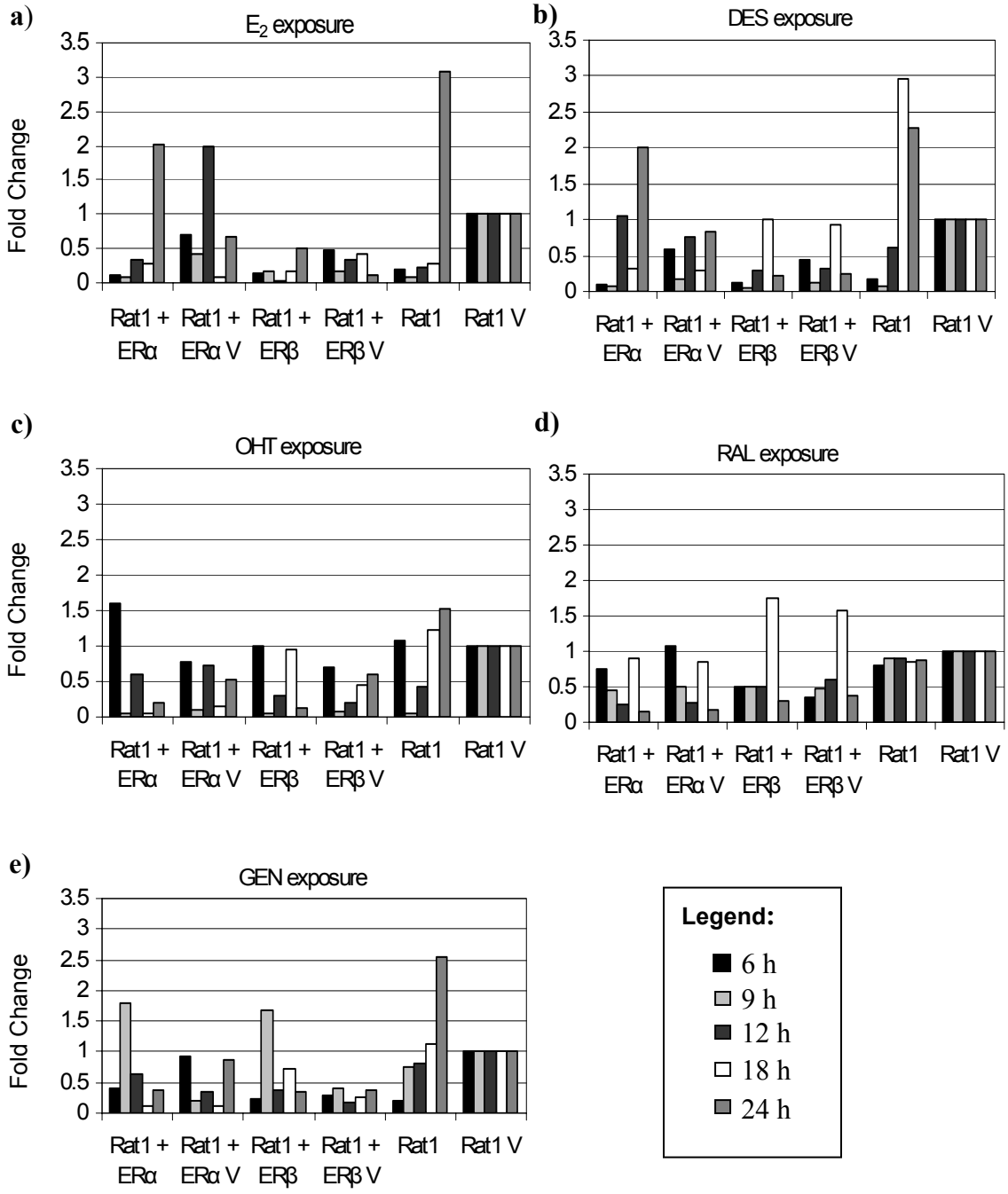
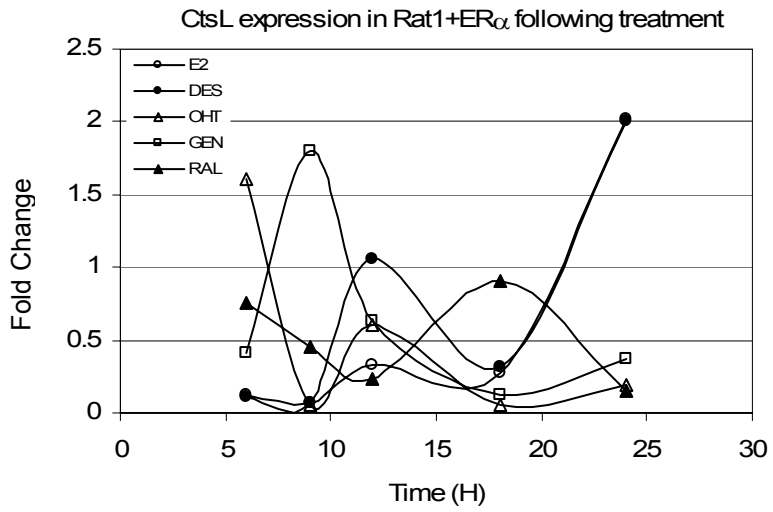

a)
E2 exposure
Fold Change
3.5
3
2.5
2
1.5
1
0.5
0
Rat1 + ERα
Rat1 + ERα V
Rat1 + ERβ
Rat1 + ERβ V
Rat1
Rat1 V
b)
DES exposure
c)
OHT exposure
d)
RAL exposure
e)
GEN exposure
Legend:
6 h
9 h
12 h
18 h
24 h

Figure 4. Independent expression trends of CtsL in either ERα or ERβ expressing cell lines over time. **a)** Rat1+ERα changes in profile for CtsL expression show that treatment with either E_2 or DES closely mirror each other following 24 h exposure, yet are significantly different from any other interaction at 18 and 12 h in the presence of ERα.. Expression of CtsL following 24 h exposure to either E_2 or DES is also greater than expression detected in the ERβ expressing cells following the same treatment regime. These data suggest complementary roles for CtsL gene expression effects following treatment with E_2 or DES following 24 h. **b)** Rat1+ERβ changes in profile for CtsL expression show a similar effect following a 9 h exposure to GEN in comparison to the ERα expressing cells ($p<0.0001$). There is also a trend toward increased target expression with RAL exposure following 18 h, however, this induction is not significant over its own vehicle ($p<0.1572$). This suggests that ERβ may have a minor role in up-regulation of CtsL at these observed time points, but treatment with compounds such as E_2 or DES can create differing transcriptional scenarios between the two receptor isotypes.

a)


CtsL expression in Rat1+ERα following treatment
E2
DES
OHT
GEN
RAL
Fold Change
Time (H)
0
0.5
1
1.5
2
2.5
0
5
10
15
20
25
30

b)

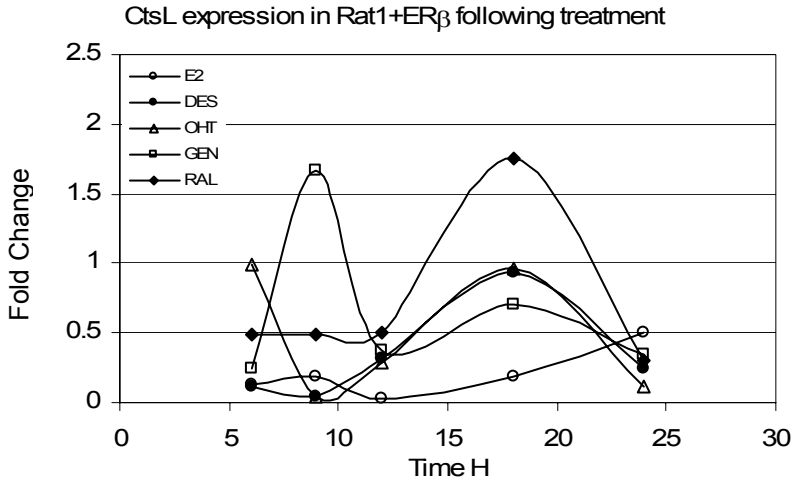

CtsL expression in Rat1+ERβ following treatment
E2
DES
OHT
GEN
RAL
Fold Change
Time H
0
0.5
1
1.5
2
2.5
0
5
10
15
20
25
30

Figure 5. Fold changes in RACK1 over time and ligand exposure were determined by the comparative C_T method. Cell by time ($p<0.0034$) and compound by time ($p<0.0001$) interactions were statistically determined using a 3X5X5 factorial in the SAS package. Fold differences are shown for: **a**) RACK1 detected when treated with E2; **b)** RACK1 detected when treated with DES; **c)** RACK1 detected when treated with OHT; **d)** RACK1 detected when treated with RAL; **e)** RACK1 detected when treated with GEN.

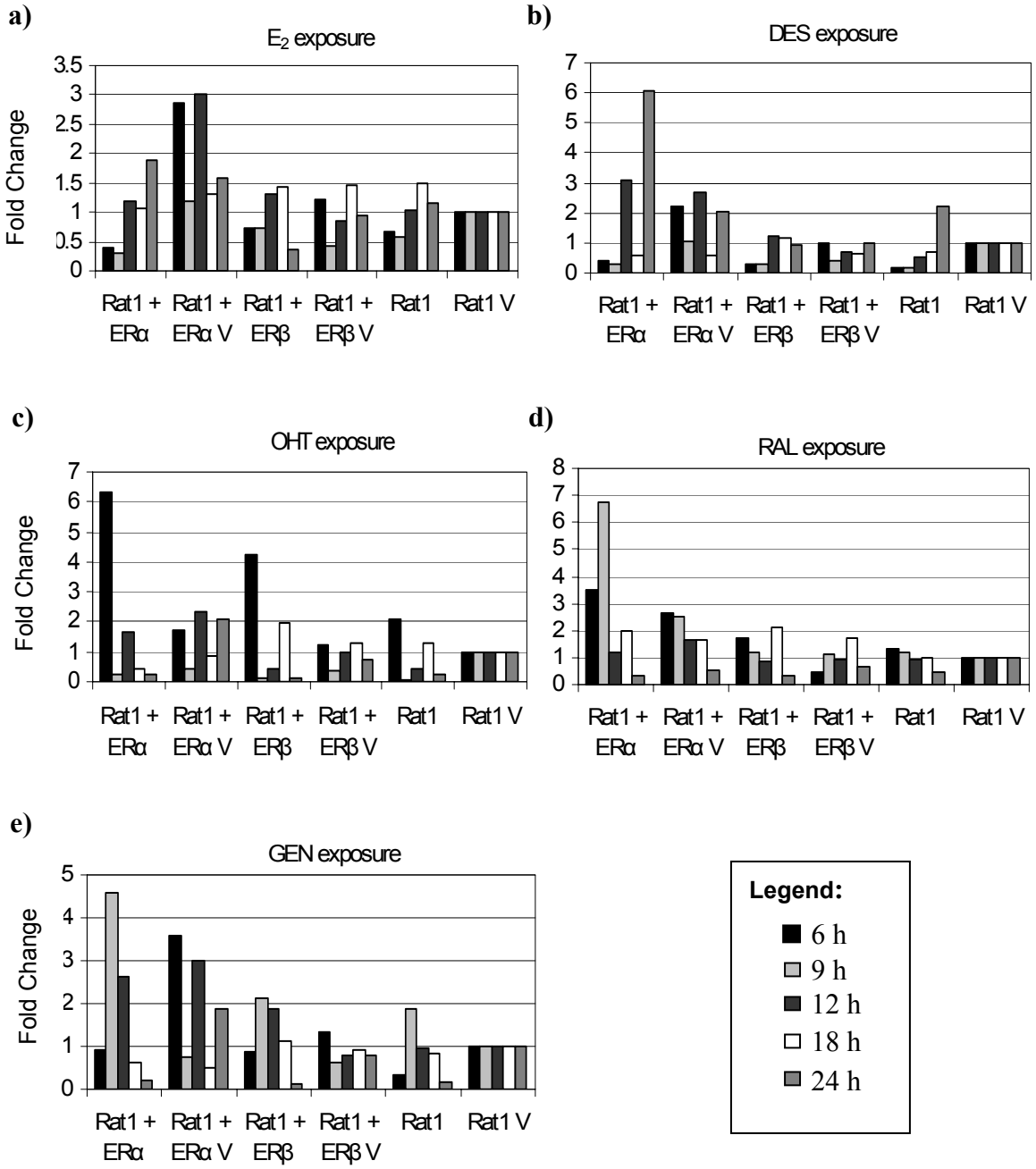
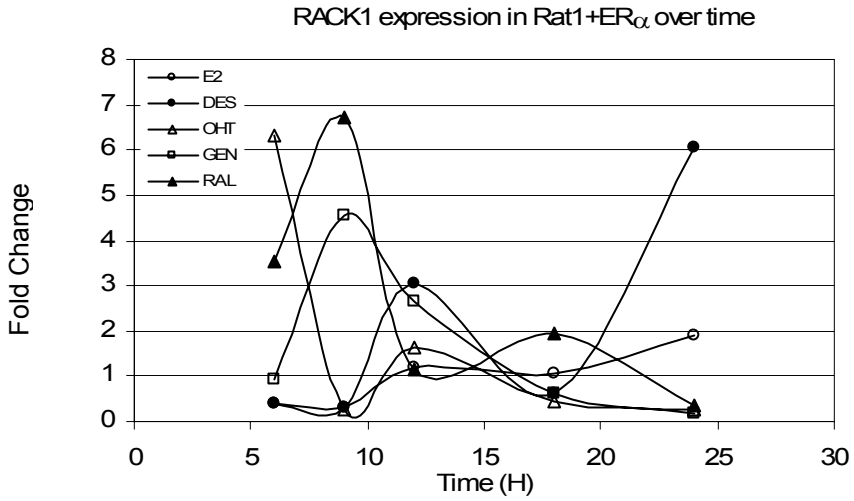

a)
E2 exposure
Fold Change
3.5
3
2.5
2
1.5
1
0.5
0
Rat1 + ERα
Rat1 + ERα V
Rat1 + ERβ
Rat1 + ERβ V
Rat1
Rat1 V
b)
DES exposure
7
6
5
4
3
2
1
0
c)
OHT exposure
d)
RAL exposure
8
e)
GEN exposure
Legend:
6 h
9 h
12 h
18 h
24 h

Figure 6. Independent expression trends of RACK1 in either ERα or ERβ expressing cell lines over time. **a)** The Rat1+ERα profile for RACK1 expression demonstrate that treatment with OHT for 6 h is similar to RAL treatment for 9 h, and that these SERMs may illicit an early RACK1 response in a manner similar to the later 24 h exposure to DES. Treatment with either E_2 or DES for a 24 h period is demonstrates an increase in RACK1 gene expression, however the presence of DES appears to have a more profound effect. **b)** In Rat1+ERβ an increase in the detectable expression of RACK1 following 6 h with OHT was demonstrated that is similar to expression observed in the ERα expressing cells under the same conditions. The detection of RACK1 expression following this early time-point is considerably decreased in the ERβ expressing cells suggesting unique ligand to ER isotype interactions.

a)


RACK1 expression in Rat1+ERα over time
E2
DES
OHT
GEN
RAL
Fold Change
Time (H)
0
1
2
3
4
5
6
7
8
0
5
10
15
20
25
30

b)

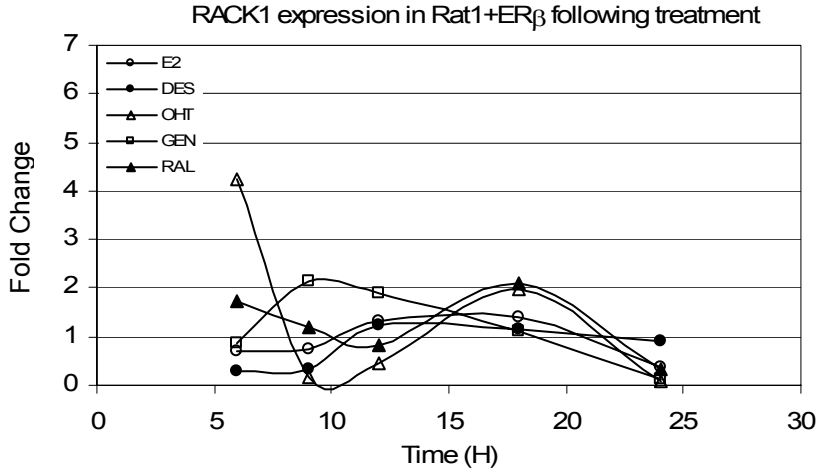

RACK1 expression in Rat1+ERβ following treatment
E2
DES
OHT
GEN
RAL
Fold Change
Time (H)
0
1
2
3
4
5
6
7
0
5
10
15
20
25
30

Figure 7. Fold changes in PCOLCE expression over time and ligand exposure were determined by the comparative C_T method. Compound by time effects ($p<0.0001$) were statistically determined for the interaction using a 3X5X5 factorial in the SAS package. Fold differences are shown for: **a)** PCOLCE detected when treated with E_2; **b)** PCOLCE detected when treated with DES; **c)** PCOLCE detected when treated with OHT; **d)** RACK1 detected when treated with RAL; **e)** PCOLE detected when treated with GEN.

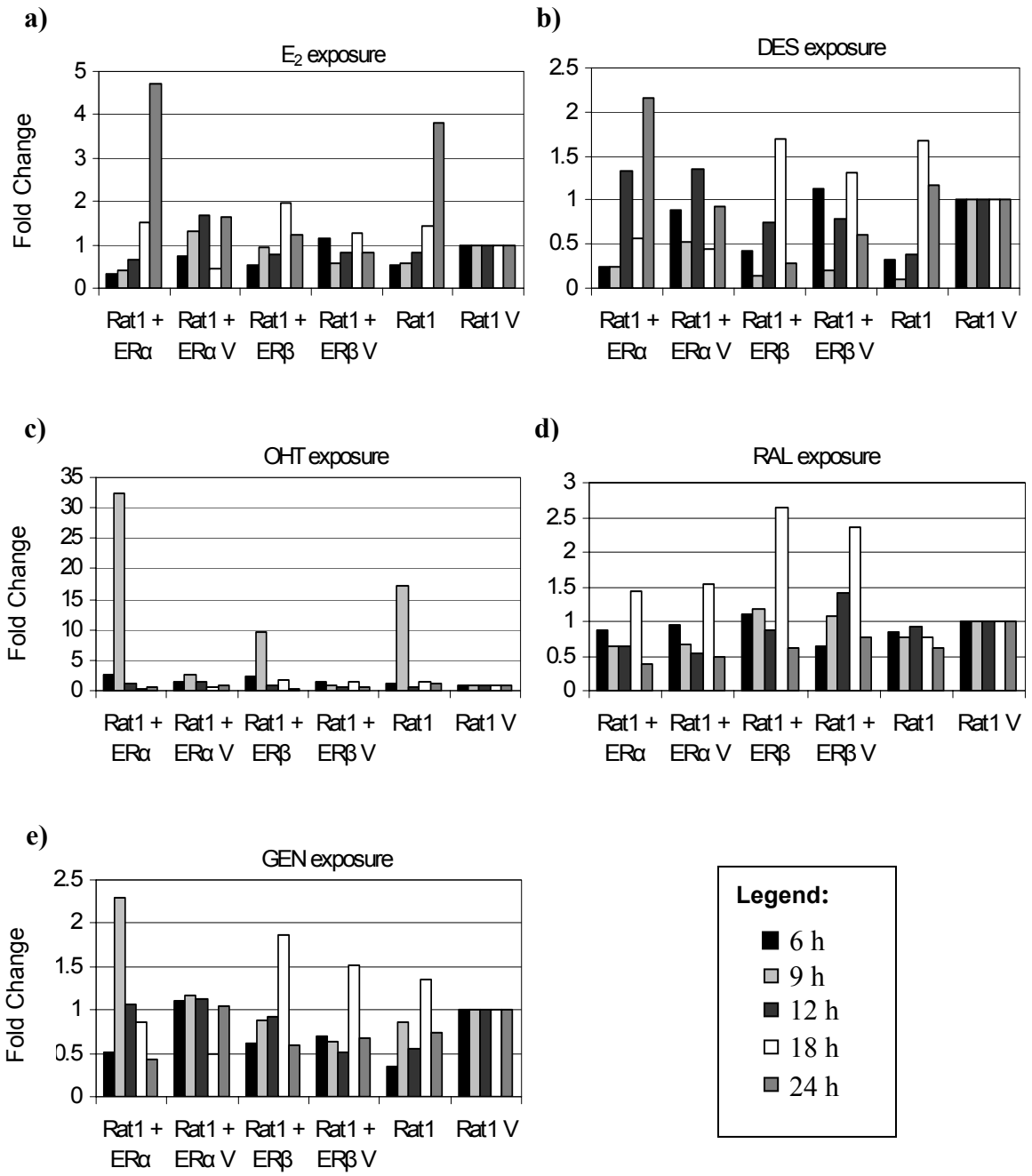
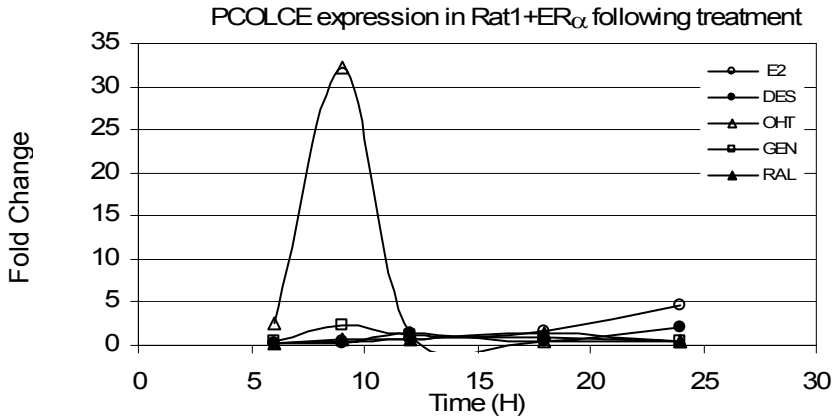
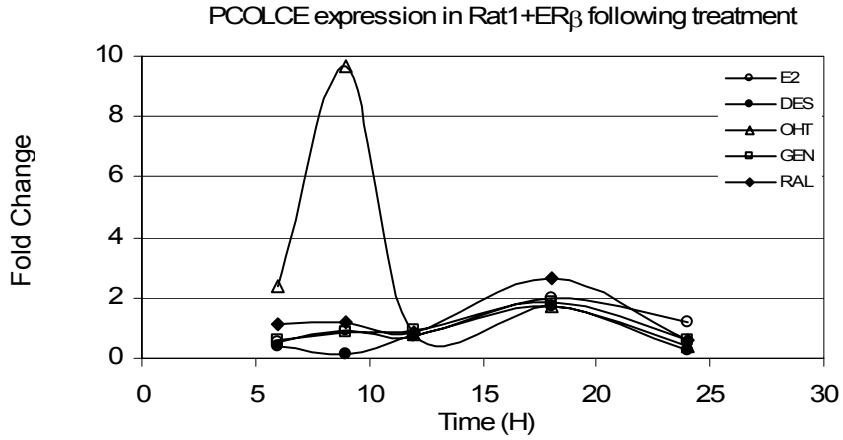

a)
E2 exposure
Fold Change
5
4
3
2
1
0
Rat1 + ERα
Rat1 + ERα V
Rat1 + ERβ
Rat1 + ERβ V
Rat1
Rat1 V
b)
DES exposure
2.5
2
1.5
1
0.5
0
c)
OHT exposure
35
30
25
20
15
10
5
0
d)
RAL exposure
3
2.5
2
1.5
1
0.5
0
e)
GEN exposure
Legend:
6 h
9 h
12 h
18 h
24 h

Figure 8. Independent expression trends for PCOLCE in either ERα or ERβ expressing cell lines over time. **a)** Rat1+ERα changes in profile for PCOLCE expression show an increase following E_2 treatment at 24 h. Notably a large change in PCOLCE is observed at 9 h following treatment with the tissue selective partial agonist/antagonist OHT. **b)** Little significant effects are seen in Rat1+ERβ for PCOLCE expression, except for a large increase at 9 h, which mirrors what is seen in Rat1+ERα under the same conditions. Slight, yet non-significant increases are seen following 18 h across all treatments.

a)


PCOLCE expression in Rat1+ERα following treatment
Fold Change
Time (H)
E2
DES
OHT
GEN
RAL
0
5
10
15
20
25
30
35

b)


PCOLCE expression in Rat1+ERβ following treatment
Fold Change
Time (H)
E2
DES
OHT
GEN
RAL
0
2
4
6
8
10
0
5
10
15
20
25
30

CHAPTER VI

Discussion and Conclusions

SUMMARY

We have examined the nature of gene regulation by ERα and ERβ within an undifferentiated model system designed to examine effects of each receptor protein isolated from the influence of the other. These data have demonstrated that unique global gene expression patterns exist within cells of the same genetic background, and that these profiles are specific in response to ERα or ERβ activation for ligand and time. Suppression subtractive hybridization following a 24h exposure to E_2 demonstrated that ERα and ERβ can result in differential gene expression. The effects of the presence of specific ER isotypes as it relates to PR expression suggests that ERα in the presence of physiological E_2 is a major factor in up-regulation, as well as suggesting possible non-ligand associated interactions through ERβ. The effects upon genes involved in extracellular matrix formation and general cell processes in the presence of independent ERα and ERβ helps to understand the breadth of effects that ligand and time can induce and supplies impetus for future work to further understand the complex pathway regulation that the ER biology paradigm represents.

PROGESTERONE RECEPTOR

Progesterone plays a vital role in reproductive physiology, and PR function is intertwined with estrogen treatment and ER activation. Therefore, the relationship of ER to PR is of extreme interest in regards to reproductive health as well as breast, endometrial and ovarian cancer prognostics. The PR protein has two functionally different subtypes, A and B, which are encoded by a single PR gene at two distinct translational start sites (Graham & Clarke 1997). Within the female reproductive tract it plays a major role in the endometrial and uterine stroma, where it is responsible for proliferation, differentiation, and maintenance (Clarke CL & Sutherland RL 1990; Li *et al.* 2004), and the mammary tissue where ER-PR pathways and interactions have a role in breast cancer (Horwitz & McGuire 1978; Dotzlaw *et al.* 1999).

One of the primary characteristics of the ER/PR relationship is the activation of PR by ER and the negative feedback ability of PR to inhibit ER expression levels (Clarke & Sutherland 1990). This relationship has been shown to be controlled through different mechanisms in different tissues. This is exemplified through 17β-hydroxysteroid dehydrogenase, an enzyme in the metabolic pathway of steroids, performing a role in the PR down-regulation of ER in the anterior pituitary gland, but not the uterus (Fuentes *et al.* 1990), suggesting that the down regulation of ER in this region may be due to steroid availability more than mRNA and protein synthesis effects seen in the uterus.

Other studies have demonstrated a temporal component of this interaction with a significant suppression of ERα by 12 h and ER levels returning to normal from 12-48 h

(Okulicz 1989). Of interest in relation to ERα and ERβ variations and temporal differences, previous studies at delayed time-points of 24 and 48 h post E_2 treatment have shown no PR expression in ERβ expressing human osteoblast cell lines (Rickard *et al.* 2002). In the Rat1+ERβ cell line, PR expression is reported as early as 9 h following E_2 treatment (Cheng & Malayer 1999). In human breast cancer cell lines, PR regulates both ERα and ERβ; however, the relationship with ERβ is inversely associated with PR status (Dotzlaw *et al.* 1999). These data fit into a model in which ERα and ERβ play pivotal, if not divergent, functions in PR induction, which is furthermore highly dependent on time and ligand exposure. Our present model supports the role of ERα as the main regulator of PR, with ERβ possibly functioning as a repressor. However, the ability of ERβ to up-regulate PR in a non-estrogenic manner at 12 h, possibly as a response to insulin in the media that is refreshed upon feeding, also raises interesting questions as to the role of differential promoter context, co-regulator recruitment, and cross-talk pathways that are specific to events involving independent ERα and ERβ regulation.

EXTRACELLULAR MATRIX AND CELL MAINTENANCE

The COL1A2 polypeptide chains are commonly synthesized by fibroblasts and osteoblasts together with COL1A1, which then aggregate to form collagen (Verrecchia & Mauviel 2004). Transcription of COL1A2 requires complex and cooperative protein-protein interactions that are not yet fully understood. Up-stream binding elements interact with proteins such as Sp1, AP-1 and *cis*-acting elements in a strongly tissue-specific manner (Tanaka *et al.* 2004). This is exciting in the context of what we know about the ability of various ligands to function through Sp1 and AP-1 elements (Webb *et*

al. 1995; Saville *et al.* 2000), and the effects we see here, with significant induction events in ERβ 06h compared to vehicle following treatment with OHT, GEN, and RAL, even while ERα is exhibiting repressive effects with E_2 and DES treatment at the same time-point.

The cathepsin gene family is composed of lysosomal proteases that play multiple roles in cellular maintenance and remodeling events. Cathepsin L has multiple roles in reproduction, inflammatory responses, and bone remodeling (Divya *et al.* 2002; Kakegawa *et al.* 1993; Mason *et al.* 1986). Estrogen receptors also have regulational control in the same tissues that are affected by cathepsins, and our results suggest that CtsL expression may be linked to ERα activation. A non-ligand differentiating response to treatments at ERβ at 18h mirrors RAL exposure in ERα in comparison to vehicle, and this suggests that perhaps specific ligands, like RAL, may be responsible for an effect that is similar to an isotype switch. It is also interesting that compounds like GEN (09h) and OHT (06h) can behave the same in both ERα and ERβ, when other compounds fail to behave similarly.

The RACK1 protein functions in subcellular translocation and protein kinase C (PKC) stabilization (Ron *et al.* 1994), and there is a known relationship for ERα with E_2 in affecting PKC (Kelly *et al.* 1999). This is in agreement with our observed ability of E_2 to increase RACK1 gene expression at 24 h in a manner greater than ERβ. It is therefore of interest, though not surprising, that DES treatment can stimulate induction at a greater

magnitude at the same time point. What was not expected was the ability of RAL and GEN at 6 h to increase RACK1 in the presence of ERα in a manner greater than E_2 treatment, or that OHT with ERβ would create the only appreciable increase in RACK1 in that cell line.

The induction of PCOLCE, a protein involved in extracellular matrix formation, expression has already been shown to be linked with TGF-β expression in a fibrogenic cell line (Lee *et al.* 1997), and correlates with expression of type I collagens in the culture media of cardiac fibroblast cell lines (Shalitin *et al.* 2003). Here, PCOLCE appears to be only slightly effected by ligand binding events upon ER, except at 9 h when OHT has an impact on ERα PCOLCE expression, ERβ and the naïve cell line. This suggests that ERα may only be serving to potentiate an otherwise non-receptor OHT mediated event in this target.

CONCLUSIONS

Through the use of a physiological relevant embryonic fibroblast cell line we have shown that there are ERα and ERβ independent gene profiles that are further regulated by ligand and time of exposure. The allosteric changes that are imposed upon the various domains of the ER upon ligand binding and promoter interactions, lead to conformational changes upon the target DNA that control efficient transcriptional events. Within the context of these interactions multiple signaling events, including phosphorylation and cross-talk can offer additional levels of control.

Implications for cross-talk pathways through ERβ, possibly as it relates to insulin and insulin-like growth factor I induction of the AP-1 promoter interaction through the MAPK pathway, are hinted through ligand independent PR expression at 12h post treatment. The complex changes induced over time in the presence of varying ligands on the four genes; COL1A2, CtsL, RACK1, and PCOLCE, highlights that what may be thought of as early and late gene responses have the ability to be shifted and changed in regards to ligand interaction over time, and this may in turn be related ligand affects on co-regulator recruitment and binding events at promoter regions. Taken together this furthers our understanding of a dynamic and complex system that has implications in the health and health maintenance of not only humans, but multiple species. The physiological system is in no way static, and strives constantly for a state of homeostasis which may be greatly affected by the steroid hormones to which we are exposed, not only through the environment but by our own response to a range of stimuli.

The future directions that could be investigated as a direct extension of these data are numerous. Determining if the protein profiles for the genes examined herein would be of paramount need, to verify if these gene expression profiles are indeed having direct effects on physiological end-points. Protein identification can be approached from several directions, the main two being Edman degradation or mass spectrometry. Protein microarrays utilizing antibodies are also becoming available which would allow for examination of differential protein profiles. Another avenue of research that should be examined is the promoter context with which each target gene is associated with; ERE, AP-1 or Sp1. The CAT assay could be employed utilizing plasmids that are specific to

the given binding sites, to determine levels of responsiveness to AP-1 and Sp1. However, DNA footprinting would be the main experiment that would need to be performed to determine the effects of treatment on ERα and ERβ promoter interactions that may go towards explaining data reported with this project. In regards to PR, specifically, it should be interesting to determine which promoters, PRA or PRB, are being activated in the specific treatments examined within.

The interactions of co-regulatory elements and other protein-protein interactions should be studied as well to help us to elucidate the pathways that various the treatments effect, and to determine how recruitment of accessory proteins affects these profiles. Coimmunoprecipitation studies could begin to provide insight into these interactions. Expanding on general gene expression profile differences in the presence of ERα or ERβ, it would be important to analyze the cell, time, and ligand treatments using rat microarrays as a platform. Finally, expanding on previous work performed in our lab involving insulin effects on gene expression between the two receptors in the culture system may provide answers as to why an increase in PR expression was observed in the ERβ cells following treatment with vehicle at 12h.

By better understanding the underlying differential control which is occurring at the transcriptional level in response to ER isotypes over time and in the presence of various ligands we can begin to be prepared for reactions that occur in a physiological relevant manner. The inducible transcriptional activity of these two receptors in response to the same ligand, as well as the effects imparted by different ligands has important

implications for understanding cell regulatory functions and inflammatory responses, which are integral to reproduction, as well as oncogenesis, bone remodeling, and the aging process.

REFERENCE LIST

Cheng J & Malayer JR. 1999 Responses to stable ectopic estrogen receptor-beta expression in a rat fibroblast cell line. *Molecular Cellular Endocrinology.* **156** 95-105.

Clarke CL & Sutherland RL. 1990 Progestin regulation of cellular proliferation. *Endocrinology Review.* **11** 266-301.

Divya, Chhikara P, Mahajan VS, tta Gupta S & Chauhan SS. 2002 Differential activity of cathepsin L in human placenta at two different stages of gestation. *Placenta* **23** 59-64.

Dotzlaw H, Leygue E, Watson PH & Murphy LC. 1999 Estrogen receptor-β messenger RNA expression in human breast tumor biopsies: relationship to steroid receptor status and regulation by progestins. *Cancer Research* **59** 529-532.

Fuentes MA, Muldoon TG & Mahesh VB. 1990 Role of 17β-hydroxysteroid dehydrogenase in the modulation of nuclear estradiol receptor binding by progesterone in the rat anterior pituitary gland and the uterus. *The Journal of Steroid Biochemistry and Molecular Biology* **37** 57-63.

Graham JD & Clarke CL. 1997 Physiological Action of Progesterone in Target Tissues. *Endocrinology Review* **18** 502-519.

Horwitz KB & McGuire WL. 1978 Estrogen control of progesterone receptor in human breast cancer. Correlation with nuclear processing of estrogen receptor. *Journal of Biological Chemistry* **253** 2223-2228.

Kakegawa H, Nikawa T, Tagami K, Kamioka H, Sumitani K, Kawata T, Drobnic-Kosorok M, Lenarcic B, Turk V & Katunuma N. 1993 Participation of cathepsin L on bone resorption. *FEBS Letters* **321** 247-250.

Kelly MJ, Lagrange AH, Wagner EJ & Ronnekleiv OK. 1999 Rapid effects of estrogen to modulate G protein-coupled receptors via activation of protein kinase A and protein kinase C pathways. *Steroids* **64** 64-75.

Lee S, Solow-Cordero DE, Kessler E, Takahara K & Greenspan DS. 1997 Transforming Growth Factor-beta Regulation of Bone Morphogenetic Protein-1/Procollagen C-proteinase and Related Proteins in Fibrogenic Cells and Keratinocytes. *Journal of Biology and Chemistry* **272** 19059-19066.

Li X, Lonard DM & O'Malley BW. 2004 A contemporary understanding of progesterone receptor function. *Mechanisms of Ageing and Development* **125** 669-678.

Mason RW, Johnson DA, Barrett AJ & Chapman HA. 1986 Elastinolytic activity of human cathepsin L. *Biochemistry Journal* **233** 925-927.

Okulicz WC. 1989 Temporal effects of progesterone inhibition of occupied nuclear oestrogen receptor retention in the rat uterus. *Journal of Endocrinology* **121** 101-107.

Rickard DJ, Waters KM, Ruesink TJ, Khosla S, Katzenellenbogen JA, Katzenellenbogen BS, Riggs BL & Spelsberg TC. 2002 Estrogen receptor isoform-specific induction of progesterone receptors in human osteoblasts. *Journal of Bone and Mineral Research* **17** 580-592.

Ron D, Chen C, Caldwell J, Jamieson L, Orr E & Mochly-Rosen D. 1994 Cloning of an intracellular receptor for protein kinase C: A homolog of the β subunit of G proteins. *Proceedings of the National Academy of Sciences* **91** 839-843.

Saville B, Wormke M, Wang F, Nguyen T, Enmark E, Kuiper G, Gustafsson JA & Safe S. 2000 Ligand-, cell-, and estrogen receptor subtype (α /β)-dependent activation at GC-rich (Sp1) promoter elements. *Journal of Biological Chemistry* **275** 5379-5387.

Shalitin N, Schlesinger H, Levy MJ, Kessler E & Kessler-Icekson G. 2003 Expression of procollagen C-proteinase enhancer in cultured rat heart fibroblasts: evidence for co-regulation with type I collagen. *Journal of Cellular Biochemistry* **90** 397-407.

Tanaka S, Antoniv TT, Lui K, Wang L, Wells DJ, Ramirez F & Bou-Gharios G. 2004 Cooperativity between far-upstream enhancer and proximal promoter elements of the human alpha2(I) collagen (COL1A2) gene instructs tissue specificity in transgenic mice. *Journal of Biological Chemistry* M411406200.

Verrecchia F & Mauviel A. 2004 TGF-[beta] and TNF-[alpha]: antagonistic cytokines controlling type I collagen gene expression. *Cellular Signalling* **16** 873-880.

Webb P, Lopez GN, Uht RM & Kushner PJ. 1995 Tamoxifen activation of the estrogen receptor/AP-1 pathway: potential origin for the cell-specific estrogen-like effects of antiestrogens. *Molecular Endocrinology* **9** 443-456.

Appendix I

Determination of portions of the cDNA sequence of the bovine estrogen receptor alpha from bovine endometrium using 5′ RACE

Other work

ABSTRACT

Estrogen receptors (ER) are ligand-induced transcription factors that have numerous physiological roles. Since the discovery of a second ER type (ERβ) much effort has been directed at characterization of the localization and actions of ERα and ERβ. The ERs are organized into 5 functional domains which are highly conserved in the DNA- and ligand-binding domains. Estrogen receptors are least homologous in their A/B domain, with ERβ being significantly truncated in comparison to ERα. In the study of bovine ER (bER) the cloning and sequencing of the 5′ region of bERα has proven difficult, limiting the ability to construct ER specific molecular tools. We have applied 5′ GeneRacer® to total RNA extracted from bovine endometrium to isolate regions 5′ of the known hormone binding domain. Thermoscript® RT and deazo-GTPs were used for synthesis of the first strand template, followed by GC-Melt® and a 2 polymerase PCR system for generating the amplified cDNA template. The GeneRacer® PCR product was directly sequenced using the 5′GeneRacer® forward primer and a gene specific (GSP) reverse primer designed from the *Bos taurus* hormone-binding domain. To date we have sequenced the hinge domain and a portion of the DNA binding domain. There was a 96% homology to *Ovis aries* ERα, a 91% homology to *Sus scrofa* ERα and 88% homology to *Homo sapiens*

ERα. The full length bERα cDNA sequence will enable us to develop probes and strategies to better differentiate between bER isotypes, assisting in future studies into ER expression, localization and roles in gene regulation.

INTRODUCTION

Estrogen receptors (ER) are members of the steroid receptor family and act as ligand-inducible transcription factors. From 1966, when Toft and Gorski (Toft & Gorski 1966) first isolated and began characterizing a protein receptor for estrogen, through 1994, only one receptor form was known. In 1995 the second receptor type, ERβ, was identified from the prostate gland of the rat (Kuiper *et al.* 1996). Subsequently, clones for human ER were first published in 1986 (Greene *et al.* 1986; Green *et al.* 1986), and for the rat ER in 1987 (Koike *et al.* 1987). In 1995 a second estrogen receptor, ERβ, was identified and cloned from rat prostate (Kuiper *et al.* 1996) and since that time ERβ has been characterized in the mouse (Tremblay *et al.* 1997; Pettersson *et al.* 1997), human (Mosselman *et al.* 1996), bovine (Rosenfeld *et al.* 1999), and numerous other species.

The ERα and ERβ proteins are organized into 5 domains that show varying homology between the receptor types, with the central DNA binding domain (or C domain) and ligand binding domain (E domain) being reasonably conserved (95% - 55% respectively) between the two receptors (Kuiper *et al.* 1996). The amino terminal A/B domain is less conserved and in rat ERα (rERα) is significantly longer, by approximately 87 amino acid residues, than rERβ (Kuiper *et al.* 1996). In comparison to rERα, the carboxyl terminal F

domain of rERβ is also shorter by 15 amino acid residues. Interactions of the receptor with co-activator and co-repressor proteins occur across these A/B, E, and F domains and it is likely that receptor-specific responses in the context of different promoters and cell types occur as a result of these variable interactions (Kuiper *et al.* 1997). We have previously generated probes for bERα based on the E domain. The lack of specific sequence information about other, more variable regions of the bovine ER (bER) limits our ability to develop molecular probes capable of discriminating between mRNAs of the alpha and beta forms of the receptor when both are present.

To date, the cloning and sequencing of the 5′ portion of the bERα cDNA has proven difficult. Only recently has a nearly full-length cDNA encoding bERα been placed into the GenBank database (accession number: AY538775.1). In addition, the first 39 bp of cDNA sequence immediately 3' of the bERα promoter has been reported (accession number: AY332655.1). Through the use of 5′ GeneRacer® (Invitrogen, Carlsbad CA) in conjunction with the GC-Melt® and Advantage 2® PCR system (BD Biosciences, Palo Alto CA) we have cloned and sequenced portions of the previously undefined 5′ region of bERα.

MATERIALS AND METHODS

RNA EXTRACTION

Endometrium was collected from non-bred Holstein cows at a local abattoir, and transported in RNAlater® (Invitrogen, Carlsbad CA). Samples were stored at 4°C until

RNA extraction. Tissues (200 mg) were mechanically homogenized and total RNA extracted per the single-step Chomczynski and Sacchi method (Chomczynski & Sacchi 1987). Concentrations were determined by spectrophotometry and total RNA was stored at -80°C until GeneRacer® was performed.

5′ Rapid Elongation of cDNA Ends

An RNA oligo was ligated onto the 5′ region of decapped mRNA. Reverse transcription was performed on 2 μg of template total RNA, with random hexamers, performed as described in the Invitrogen protocol, with modifications of using the thermal stable reverse transcriptase ThermalAce (Invitrogen, Carlsbad CA) at 60°C and dNTPs with deazo-GTPs. GeneRacer PCR was then performed in the presence of GC-Melt® (BD Biosciences Clontech, Palo Alto CA) and DMSO (Sigma, St. Louis MO) under the conditions described in Table 1. Products were resolved on 1.5% agarose gels and band extracted. Bands were then purified through S.N.A.P. columns (Invitrogen, Carlsbad CA), PCR performed 4 times, and this was pooled and PCR purified (Quiagen, Valencia CA) for sequencing.

Sequencing and Cloning

Single pass sequencing using the CEQ 8000 and well-read dyes (Beckman-Coulter, Fullerton CA) was performed on PCR products (200 fmol) to confirm homology. Sequence was screened through the Basic Local Alignment Search Tool (Altschul *et al.* 1990) in the NCBI database. Confirmed fragments were cloned into the Topo PCR4

vector (Invitrogen, Carlsbad CA) for preservation. These clones were then subjected to sequencing. Gene specific primers (GSP) were then designed and used to perform primer walking in order to obtain additional sequence information in the bERα 5' region (Table 1).

RESULTS

An approximately 900 bp band (Figure 1A) was obtained after the first round of GeneRacer® PCR with homologies of 96% to *Ovis aries*, 91% to *Sus scrofa*, and 88% to *Homo sapiens*. This PCR product was then cloned and sequence verified. A second primer was then designed from the 5′ region of this fragment and a second 493 bp band was obtained (Figure 1B), which showed similar homologies to those above. Together these new sequences in conjunction with the previously known ligand binding domain have given us 1108 bp (Figure 2) which showed 95% homology to *O. aries*, 92% homology to *S. scrofa*, and 89% homology to *H. sapiens*.

DISCUSSION

Sequence information for cDNA of ERα has been reported in human (1986 (Greene *et al.* 1986; Green *et al.* 1986), rat (1987 (Koike *et al.* 1987), as well as ovine and porcine. Following identification and cloning of ERβ in the rat (Kuiper *et al.* 1996), ERβ has also been cloned from the mouse (Tremblay *et al.* 1997; Pettersson *et al.* 1997), human (Mosselman *et al.* 1996), and bovine (Rosenfeld *et al.* 1999). However, even with cloning of a bERβ in 1999, until recently full length bERα has been unavailable.

Recently full length clones have been generated and placed into the NCBI database (accession number: AY538775.1)), yet these do not have consensus 5′ sequence with available mRNA sequence available downstream of promoter data (accession number: AY332655.1). Our data, in conjunction with the time taken for full-length sequence to be reported in a database, suggest that a molecular characteristic of bERα may create difficulties in the amplification process of this gene. With the available sequence that we have created, with the newly available sequence, should provide data to create new molecular tools for detecting bERα. This in turn may lead to new exploratory research into how this complex gene functions.

REFERENCE LIST

Altschul SF, Gish W, Miller W, Meyers EW & Lipman DJ. 1990 Basic local alignment search tool. *Journal of Molecular Biology* **215** 403-410.

Chomczynski P & Sacchi N. 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Annals of Biochemistry.* **162** 156-159.

Green S, Walter P, Krust A, Bornert JM, Argos P & Chambon P. 1986 Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature.* **320** 134-139.

Greene GL, Gilna P, Waterfield M, Baker A & Shine J. 1986 Sequence and expression of human estrogen receptor complementary DNA. *Science* **231** 1150-1154.

Koike S, Sakai M & Muramatsu M. 1987 Molecular cloning and characterization of rat estrogen receptor cDNA. *Nucleic Acids Research* **15** 2499-513.

Kuiper GG, Enmark E, Pelto-Huikko M, Nilsson S & Gustafsson JA. 1996 Cloning of a novel estrogen receptor expressed in rat prostate and ovary. *Proceedings of the National Academy of Sciences* **93** 5925-5930.

Kuiper GGJM, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S & Gustafsson JA. 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors α and β. *Endocrinology* **138** 863-870.

Mosselman S, Polman J & Dijkema R. 1996 ERβ: Identification and characterization of a novel human estrogen receptor. *FEBS Letters* **392** 49-53.

Pettersson K, Grandien K, Kuiper GGJM & Gustafsson JA. 1997 Mouse estrogen receptor β forms estrogen response element-binding heterodimers with estrogen receptor α. *Molecular Endocrinology* **11** 1486-1496.

Rosenfeld CS, Yuan X, Manikkam M, Calder MD, Garverick HA & Lubahn DB. 1999 Cloning, sequencing, and localization of bovine estrogen receptor-β within the ovarian follicle. *Biology of Reproduction* **60** 691-697.

Toft D & Gorski J. 1966 A receptor molecule for estrogens: isolation from the rat uterus and preliminary characterization. *Proc Natl Acad Sci U S A.* **55** 1574-1581.

Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F & Giguere V. 1997 Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor β. *Molecular Endocrinology* **11** 353-365.

Table 1. Gene specific and GeneRacer® primers and PCR conditions

	Primers	Conditions
GeneRacer® 5' *Forward*	5' CGACTGGAGCACGAGGACACTGA 3'	100 pmol primers, 1.25µl DMSO, 2.5 µl GC-Melt® 35cycles; 95° 5min, 58°C 1 min, 72° 1 min
Gene Specific 1 *Reverse*	5' GCAGAGTCAGGCCTGCTTTGGCCATCA 3'	
Gene Specific 2 *Reverse*	5' CCTCACTGAAAGGTGGTAGGGTCATACT 3'	

Figure 1. Electrophoresis analysis of PCR products following 5' RACE. A) The 3' GSP1 primer was used with the 5' primer specific to the GeneRacer oligonucleotide at 58°C annealing temperature, to amplify a 900 bp band. The band was PCR amplified, purified and sequenced. Once homology was verified the product was cloned into the PCR4® vector (Invitrogen), and the plasmid sequenced. B) A 3' GSP2 was generated from the newly identified sequence. Polymerase chain reaction was carried out with the 5' GeneRacer primer at 60°C annealing temperature. The 497 bp amplified product was extracted and subjected to PCR amplification and purification prior to sequencing. The verified product was cloned and sequenced again.

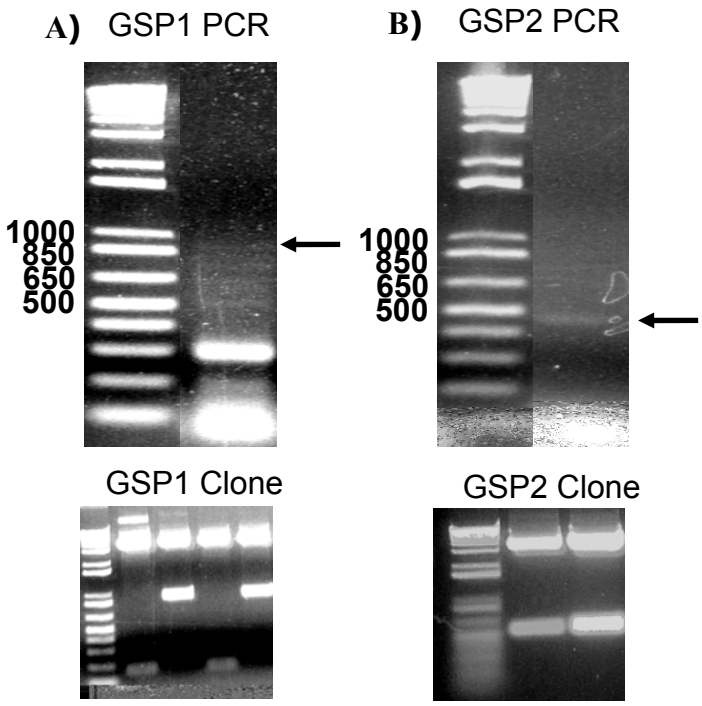

A)
GSP1 PCR
1000
850
650
500
B)
GSP2 PCR
1000
850
650
500
GSP1 Clone
GSP2 Clone

Figure 2. Alignment and proposed protein analysis. Base pair alignment of proposed sequence to AY538775.1, with the top line representing sequence obtained through the GeneRacer® protocol. The two reverse GSP used to obtain sequence are in red font, with the proposed DBD and zinc finger region highlighted in blue and the proposed LBD highlighted in green.

```
1    aaggagactcgctactgtgcaggggcaangactaagcctcaggctaccaatacggaaga 60
     ||||||||||||||||||||| | |||| ||||| |||||||||||||| |||||| |
693  aaggagactcgctactgtgcagtgtgcaatgactatgcctcaggctaccattacgga-gt 751

61   taggtcttgcgagggctgtaaggccttcctcaagagaagtatttcaaggacataaagact 120
     | |||||||||||||||||||||||||| |||||||||||||| |||||||||| ||||
752  ttggtcttgcgagggctgtaaggccttcttcaagagaagtatt-caaggacataatgact 810

121  tacaagagtccggccaccaaccagggtacgattgataaaaacaggaggaagaggctgtca 180
      ||| | |||||||||||||||| | |||||||||||||||||||||||| ||||||
811  -acatgtgtccggccaccaaccagtgcacgattgataaaaacaggaggaagag-ctgtca 868

181  ggcctgccggctaagcaagtggctatggaagtgggcatgatgaaaggcggaatacggaaa 240
     |||||||||||||| ||||||| ||||| ||||||||||||||||||||||||||||||
869  ggcctgccggctacgcaagtg-ctatg-aagtgggcatgatgaaaggcggaatacggaaa 926

241  gaccgaagaggagggagaatgttgaaacacaagcgccagagagatgatggggagggcagg 300
     |||||||||||||||||||||| ||||||||||||||||||||||||||||||||||||
927  gaccgaagaggagggagaatgttgaagcacaagcgccagagagatgatggggagggcagg 986

301  aacggaagcggggccctccggagacatgagagctgccaacctttggccaagccccattca 360
     |||| |||||| ||||||||||||||||||||||||||||||||||||||||||||||| ||
987  aacg-aagcggtgccctccggagacatgagagctgccaacctttggccaagccccat-ca 1044

361  tgatttaaacacactaagaagaacagtccggtgttgtccctgacagctgaccagaatgat 420
     ||||| |||||||||||||||||| |||||||||||||||||||||||||||| ||||
1045 tgatt-aaacacactaagaagaacagcccggtgttgtccctgacagctgaccaga-tgat 1102

421  ctagtgccttgctggaggctgagccccccataatttattcttgagtatgaccctaccag 480
     | |||||||||||||||||||||||||||||||| | ||||| ||||||||||||||||
1103 c-agtgccttgctggaggctgagcccccccataatct-attct-gagtatgaccctaccag 1159

481  acctttcagtgaggcttcaatgatgggcttgctgaccaaccttgcagacagggagctggt 540
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1160 acctttcagtgaggcttcaatgatgggcttgctgaccaaccttgcagacagggagctggt 1219

541  acacatgatcaactgggcaaagagggtgccaggatttgtggatctggccctccatgatca 600
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1220 acacatgatcaactgggcaaagagggtgccaggatttgtggatctggccctccatgatca 1279

601  agtccacctttggaatgtgcctggctagagatcctcatgattggtcttgtctggcgctc 660
     |||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1280 agtccacctttggaatgtgcctggctagagatcctcatgattggtcttgtctggcgctc 1339
```

```
661  catggagcatccagggaagctcctatttgctcctaaccttctcctggacaggaaccaggg 720
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1340 catggagcatccagggaagctcctatttgctcctaaccttctcctggacaggaaccaggg 1399

721  aaaatgtgtggaaggcatggtggagatctttgacatgttgctggctacttcgtctcggtt 780
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1400 aaaatgtgtggaaggcatggtggagatctttgacatgttgctggctacttcgtctcggtt 1459

781  ccgtatgatgaatctccagggagaagagtttgtgtgcctcaaatccatcatcttgcttaa 840
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1460 ccgtatgatgaatctccagggagaagagtttgtgtgcctcaaatccatcatcttgcttaa 1519

841  tttctggagtgtacacatttctgtccagcacttctgaggtcttctggaagagaaggacca 900
      |||||||||||||||||||||||||||||| ||||||||| ||||||||||||||||||
1520 -ttctggagtgtacacatttctgtccagcac-tctgaggtc-tctggaagagaaggacca 1576

901  catccaccgcgtcctggacaagatcacagacacctgatccatctgatggccaaagcagg 960
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1577 catccaccgcgtcctggacaagatcacagacaccttgatccatctgatggccaaagcagg 1636

961  cctgactctgcagcagcagcaccggcgtctgggccaactcctcctcatcctctctcactt 1020
     |||||||||||||||||||||||||||||| ||||||||||||||||||||||||||||
1637 cctgactctgcagcagcagcaccggcgtctggcccaactcctcctcatcctctctcactt 1696

1021 caggcacatgagcaacaaaggcatggagcatctatacagcatgaagtgcaagaacgtggt 1080
     ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
1697 caggcacatgagcaacaaaggcatggagcatctatacagcatgaagtgcaagaacgtggt 1756

1081 gcctctctatgacctgctgctggagatg 1108
     ||||||||| ||||||||||||||||||
1757 gcctctctacgacctgctgctggagatg 1784
```

Legend:

- DNA Binding Domain
- Ligand Binding Domain
- Reverse GSPs

VITA

Amy Galadriel Biby Hurst

Candidate for the Degree of

Doctor of Philosophy

Thesis: IDENTIFICATION OF UNIQUE GENE EXPRESSION PROFILES FOR ESTROGEN RECEPOTR ALPHA OR BETA

Major Field: Veterinary Biomedical Sciences

Biographical:

Personal Information: Born in Fort Ord, California, USA, June 25, 1978, the daughter of Judy A. Biby and Dennis K. Biby.

Education: Graduated from Waurika High School, Waurika, Oklahoma, in May 1996; received Bachelor of Science in Biochemistry and Molecular Biology from Oklahoma State University, Stillwater, Oklahoma, in May 2000. Completed the requirements for the Doctor of Philosophy degree at Oklahoma State University, Stillwater, Oklahoma, in May 2005.

Experience: Undergraduate Research Assistant, Oklahoma State University, Stillwater, Oklahoma, 1998-2000. Graduate Research Assistant, Oklahoma State University, Stillwater, Oklahoma, 2000-2005. Teaching Assistant, Howard Hughes Critical Thinking in Biological Sciences Program, Oklahoma State University, Stillwater, Oklahoma, 2003-2005.

Professional Organizations: Society for the Study of Reproduction, Sigma Xi Scientific Research Society, Oklahoma Microscopy Society, College of Veterinary Medicine Graduate Student Association.

Name: Amy Galadriel Biby Hurst Date of Degree: May 2005

Institution: Oklahoma State University Location: Stillwater, OK

Title of Study: IDENTIFICATION OF UNIQUE GENE EXPRESSION PROFILES FOR ESTROGEN RECEPTOR ALPHA OR BETA

Pages in study: 218 Candidate for the degree of Doctor of Philosophy

The study of estrogen receptors are of interest due to the critical role of the estrogen signaling system in the physiology of multiple tissue systems. There are two distinct receptor types, ERα or ERβ. Due to structural and functional differences, our interest lies in discovering unique independent gene expression profiles in the presence of ERα or ERβ. We have examined gene regulation of the two ER isotypes within a rat embryonic fibroblast cell culture model designed to evaluate effects of each receptor protein isolated from the influence of the other; Rat1+ERα, Rat1+ERβ, and precursor Rat1. Using this *in vitro* cell model, a treatment scheme involving 17β-estradiol (E_2) treatment for 24h was used to identify unique gene expression profiles. This was later followed by single dose treatments of diethylstilbestrol, 4-hydroxytamoxifen, raloxifene-HCl, or genistein for 6, 9, 12, 18, or 24 h. Treatment was followed by extraction of total RNA. To evaluate independent roles of ER, cDNA were generated from Rat1+ERα, Rat1+ERβ and parental Rat1 cells following treatment with a single dose of E_2 or an ethanol vehicle for 24 hours and subjected to suppression subtractive hybridization (SSH). The SSH technique demonstrated that ERα and ERβ can result in differential gene expression. Genes pro–alpha–2(I) collagen, procollagen C–proteinase enhancer protein, cathepsin L, and receptor for activated protein kinase C isolated through SSH, in addition to previously studied progesterone receptor (PR), were identified for real-time quantitative polymerase chain reaction analysis to determine profile changes in the presence of different ligands over time. These data demonstrate that unique gene expression patterns exist within cells of the same genetic background, and that profile interactions are specific to ERα or ERβ expression. The effects of ER isotypes as it relates to PR expression suggest that ERα in the presence of E_2 has a major regulatory role, and that a possible cross-talk pathway exists through ERβ mediation. The effects upon genes involved in extracellular matrix formation and general cell processes in the presence of ERα or ERβ aids in understanding treatment effects and supplies impetus to better elucidate the pathway regulation involved in ER biology.

Advisor's Approval: Jerry R. Malayer