

University of Louisville

## ThinkIR: The University of Louisville's Institutional Repository

---

Electronic Theses and Dissertations

---

5-2007

# Regulation of nuclear respiratory factor-1 expression by 17beta-estradiol : a new mechanism for coordinating mitochondrial gene expression.

Kathleen AnnMarie Mattingly 1979-  
*University of Louisville*

Follow this and additional works at: <https://ir.library.louisville.edu/etd>

---

### Recommended Citation

Mattingly, Kathleen AnnMarie 1979-, "Regulation of nuclear respiratory factor-1 expression by 17beta-estradiol : a new mechanism for coordinating mitochondrial gene expression." (2007). *Electronic Theses and Dissertations*. Paper 924.  
<https://doi.org/10.18297/etd/924>

This Doctoral Dissertation is brought to you for free and open access by ThinkIR: The University of Louisville's Institutional Repository. It has been accepted for inclusion in Electronic Theses and Dissertations by an authorized administrator of ThinkIR: The University of Louisville's Institutional Repository. This title appears here courtesy of the author, who has retained all other copyrights. For more information, please contact [thinkir@louisville.edu](mailto:thinkir@louisville.edu).

REGULATION OF NUCLEAR RESPIRATORY FACTOR-1 EXPRESSION BY 17 $\beta$ -  
ESTRADIOL: A NEW MECHANISM FOR COORDINATING MITOCHONDRIAL  
GENE EXPRESSION

By

Kathleen AnnMarie Mattingly  
B.A., Bellarmine University, 2000  
M.S., University of Louisville, 2004

A Dissertation  
Submitted to the Faculty of the  
Graduate School of the University of Louisville  
in Partial Fulfillment of the Requirements  
for the Degree of

Doctor of Philosophy

Department of Biochemistry and Molecular Biology  
University of Louisville  
Louisville, Kentucky

May 2007

REGULATION OF NUCLEAR RESPIRATORY FACTOR-1 EXPRESSION BY 17 $\beta$ -  
ESTRADIOL: A NEW MECHANISM FOR COORDINATING MITOCHONDRIAL  
GENE EXPRESSION

By

Kathleen AnnMarie Mattingly  
B.A., Bellarmine University, 2000  
M.S., University of Louisville, 2004

A Dissertation Approved on

March 22, 2007

By the following Dissertation Committee:

---

Dissertation Director

## ACKNOWLEDGMENTS

I would like to thank my mentor, Dr. Carolyn M. Klinge for her invaluable support and the opportunities that she has afforded me in her lab. I would also like to thank my committee members Dr. Barbara J. Clark, Dr. Brian Wattenberg, Dr. Russell A. Prough, and Dr. William M. Pierce, Jr. for their support and patience over the course of my studies. Finally, I would like to thank my colleagues in Dr. Klinge's lab, my husband, Robert, and my children Rose and Jacob.

## ABSTRACT

### REGULATION OF NUCLEAR RESPIRATORY FACTOR-1 EXPRESSION BY 17 $\beta$ -ESTRADIOL: A NEW MECHANISM FOR COORDINATING MITOCHONDRIAL GENE EXPRESSION

Kathleen A. Mattingly

March 22, 2007

The mechanisms by which estrogens regulate mitochondrial activity are not completely understood. Chronic treatment of ovariectomized rats with estradiol (E<sub>2</sub>) increased the amount of Nuclear Respiratory Factor-1 (NRF-1) protein in cerebral blood vessels. NRF-1 is a transcription factor that regulates the expression of nuclear-encoded mitochondrial genes including mitochondrial transcription factor A (*TFAM*), which, in turn, controls transcription of the mitochondrial genome. Here, I tested the hypothesis that E<sub>2</sub> increases NRF-1 transcription through a genomic activation of estrogen receptor (ER) resulting in a coordinate increase in nuclear- and mitochondrial- encoded genes, mitochondrial respiratory activity, and mitochondrial biogenesis. E<sub>2</sub> increased NRF-1 mRNA and protein in MCF-7 breast and H1793 lung adenocarcinoma cells in a time-, concentration-, and ER-dependent manner. E<sub>2</sub>-induced NRF-1 expression was inhibited by Actinomycin D, but not by inhibitors of the PI3K or MAPK pathways, indicating a genomic mechanism of E<sub>2</sub> action. An estrogen response element in the NRF-1 promoter bound ER $\alpha$  and ER $\beta$  *in vitro* and in chromatin immunoprecipitation assays in MCF-7 cells and activated reporter gene expression in transfected cells. The E<sub>2</sub>-induced increase in NRF-1 was followed in time by increased *TFAM*; Tfam-regulated, mtDNA-encoded

*COI* and *NDI*; and mitochondrial biogenesis. The selective ER modulators (SERMs) 4-hydroxytamoxifen (4-OHT) and raloxifene (RAL) also increased NRF-1 expression by a mechanism involving ER nongenomic and genomic activities. E<sub>2</sub>, 4-OHT, and RAL also increased NRF-1 expression in Human Umbilical Vein Endothelial Cells (HUVEC) by a genomic ER mechanism. Exposure to Diesel Exhaust Particle Extracts (DEPE) may promote vascular disease and DEPE are antagonists of genomic estrogen responses. DEPE suppressed the basal expression of NRF-1 in HUVEC and ablated the stimulatory effect of E<sub>2</sub>, 4-OHT, and RAL on NRF-1 transcription. Lastly, a known cardioprotective phytoestrogen, resveratrol, stimulated NRF-1 expression in HUVEC and inhibited the ability of DEPE to suppress basal NRF-1. In summary, the research presented here characterizes a possibly important ER-mediated pathway to account for the observed beneficial effects of E<sub>2</sub> on mitochondrial function. These results suggest that administration of E<sub>2</sub> or SERMs may be beneficial in treating pathological conditions involving mitochondrial dysfunction including heart disease, neurodegenerative disorders, and cancer.

## TABLE OF CONTENTS

	PAGE
ACKNOWLEDGEMENTS.....	iii
ABSTRACT.....	iv
LIST OF TABLES.....	x
LIST OF FIGURES.....	xi
LIST OF ABBREVIATIONS.....	xv
CHAPTER	
I.    GENERAL INTRODUCTION	
A. Estrogen Receptor Structure, Classical Function, and Tissue Distribution.....	1
B. Breast Cancer, Lung Cancer, and Estrogen.....	4
C. ERE and Coactivator Interactions with ER.....	7
D. Selective Estrogen Receptor Modulators.....	8
E. Mitochondrial Function and Maintenance.....	10
F. Estrogen Activity in Mitochondria.....	11
G. Lessons from Knockout and Ovariectomized Mice.....	13
H. Nuclear Respiratory Factor-1 (NRF-1) and Mitochondrial Regulation.....	14
I. NRF-1 and Breast Cancer.....	16
J. E <sub>2</sub> Induced Changes in Mitochondria in Normal Tissues.....	21
K. Goals of the Project.....	27
II.  REGULATION OF NUCLEAR RESPIRATORY FACTOR-1 EXPRESSION BY 17 $\beta$ -ESTRADIOL	

A. Introduction.....	29
B. Materials and Methods.....	31
i. Cell Culture and Treatment Description.....	31
ii. RNA Isolation, RT-PCR and Quantitative Real Time RT-PCR (QRT-PCR).....	32
iii. Transient Transfection of MCF-10A Cells.....	33
iv. Protein Isolation and Western Blot Analysis.....	33
v. Mitochondrial Biogenesis.....	36
vi. siRNA Knockdown of ER $\alpha$ , ER $\beta$ , and NRF-1.....	38
vii. Statistics.....	39
C. Results.....	40
i. E <sub>2</sub> -Induced NRF-1 Transcription is Mediated by Genomic ER.....	40
ii. NRF-1 is a Primary Estrogen-Responsive Gene Mediated by Genomic ER.....	42
iii. Both ER $\alpha$ and ER $\beta$ Increase NRF-1 Transcription.....	45
iv. siRNA Knockdown of ER $\alpha$ and ER $\beta$ Inhibits the E <sub>2</sub> -induced Increase in NRF-1.....	50
v. NRF-1 Protein Expression is Increased by E <sub>2</sub> .....	55
vi. The Expression of Tfam, COI, and NDI mRNAs are Increased by E <sub>2</sub> .....	55
vii. Mitochondrial Biogenesis is Increased by E <sub>2</sub> .....	59
viii. siRNA Knockdown of NRF-1 in MCF-7 Cells.....	64
D. Discussion.....	70

III. ANALYSIS OF THE NUCLEAR RESPIRATORY FACTOR-1 PROMOTER REGION AND CONTRIBUTION OF THE PUTATIVE



ESTROGEN RESPONSE ELEMENT	
A. Introduction.....	74
B. Materials and Methods.....	80
i. Cloning of the NRF-1 Promoter.....	80
ii. Dual Luciferase Assays in MCF-7 and HEK-293 Cells with NRF-1 Promoter Constructs.....	82
iii. Chromatin Immunoprecipitation Assays.....	83
iv. ER $\alpha$ and ER $\beta$ Proteins.....	85
v. EMSA and ER-NRF-1-ERE $K_i$ Determination.....	85
C. Results.....	89
i. Luciferase Reporter Assays with E <sub>2</sub> and ICI 182,780.....	89
ii. ER Binds to the Putative NRF-1 ERE <i>in vivo</i> and <i>in vitro</i> .....	99
D. Discussion.....	103
IV. REGULATION OF NUCLEAR RESPIRATORY FACTOR-1 IN MCF-7 AND H1793 CELLS BY 4-HYDROXYTAMOXIFEN AND RALOXIFENE	
A. Introduction.....	105
B. Materials and Methods.....	107
C. Results.....	108
i. 4-OHT and RAL Increase NRF-1 mRNA in a Concentration- and Time- Dependent Manner in MCF-7 and H1793 Cells...108	
ii. Dual Luciferase Assays in MCF-7 and HEK-293 Cells with NRF-1 Promoter Constructs in the Presence of SERMs.....114	
iii. ChIP Assays Examining the ER Occupancy of the NRF-1 Promoter in the Presence of SERMs.....118	
D. Discussion.....	120

V.	REGULATION OF NUCLEAR RESPIRATORY FACTOR-1 EXPRESSION BY ESTRADIOL, 4-HYDROXYTAMOXIFEN, AND RALOXIFENE IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS	
	A. Introduction.....	121
	B. Materials and Methods.....	128
	i. Cell Culture and Treatments.....	128
	ii. Bromodeoxyuridine (BrdU) Proliferation Enzyme Linked Immunosorbant Assay (ELISA).....	128
	iii. Complex IV Activity Assay.....	129
	C. Results.....	132
	i. E <sub>2</sub> , 4-OHT and RAL Increase NRF-1 Transcription in HUVEC.....	132
	ii. Secondary Gene Expression is Increased in Treated HUVEC.....	135
	iii. Complex IV Activity in Treated HUVEC.....	136
	iv. DEPE Inhibit NRF-1 mRNA Expression.....	140
	v. DEPE Do Not Stimulate Cell Proliferation in HUVEC as Assessed in BrdU Assays.....	141
	vi. Resveratrol Induces an Increase in NRF-1 Through Activation of Genomic ER.....	147
	D. Discussion.....	150
VI.	CONCLUDING DISCUSSION.....	154
	REFERENCES.....	159
	CURRICULUM VITAE.....	180

## LIST OF TABLES

TABLE	PAGE
1. SYBR Green Primer Sequences for QRT-PCR.....	34
2. PCR Primers Used for Mitochondrial Biogenesis Experiments.....	37
3. Primer Sequences for Cloning and Mutagenesis of the NRF-1 Promoter Constructs.	81
4. Primers Used for ChIP Experiments.....	87
5. Concentrations of PAHs Having Four or More Rings in the DEPE samples.....	131

## LIST OF FIGURES

FIGURES	PAGE
1. Illustration of the domains and percent homology between ER $\alpha$ and ER $\beta$ .....	2
2. NRF-1 expression is increased in breast cancer cell lines compared to normal mammary epithelium.....	18
3. NRF-1 expression is increased in response to E <sub>2</sub> in some ER positive breast cancer cell lines.....	19
4. NRF-1 is increased in preneoplastic mammary tissue and mammary tumors in a MMTV-neu mouse model.....	20
5. NRF-1 mRNA expression is increased in higher tumor grades and in ER positive human breast tumors. ....	22
6. NRF-1 expression in normal tissues.....	25
7. NRF-1 expression in uterine tissue in response to E <sub>2</sub> .....	26
8. E <sub>2</sub> treatment induces an increase in NRF-1 mRNA expression in MCF-7 and H1793 cells.....	41
9. ICI 182,780 blocks the E <sub>2</sub> -induced increase in NRF-1 mRNA in MCF-7 and H1793 cells.....	43
10. EPC blocks the E <sub>2</sub> -induced increase in NRF-1 mRNA in MCF-7 cells.....	44
11. E <sub>2</sub> -induced NRF-1 transcription is mediated by genomic ER.....	46
12. Both ER $\alpha$ and ER $\beta$ upregulate NRF-1 gene transcription.....	47
13. Both ER $\alpha$ and ER $\beta$ upregulate NRF-1 gene transcription in MCF-10A cells.....	49
14. siRNA knockdown of ER $\alpha$ .....	51
15. siRNA knockdown of ER $\alpha$ inhibits the E <sub>2</sub> -induced increase in NRF-1 mRNA.....	52

16. siRNA knockdown of ER $\beta$ .....	53
17. siRNA knockdown of ER $\beta$ inhibits the E <sub>2</sub> -induced increase in NRF-1 mRNA.....	54
18. NRF-1 protein levels are increased by E <sub>2</sub> treatment.....	56
19. Secondary gene expression is increased in E <sub>2</sub> treated MCF-7 cells.....	57
20. Protein expression of secondary genes is increased in E <sub>2</sub> -treated MCF-7 cells .....	58
21. E <sub>2</sub> increases mtDNA copy number.....	61
22. EtOH does not affect O <sub>2</sub> consumption rates.....	62
23. E <sub>2</sub> increases O <sub>2</sub> consumption in MCF-7 cells.....	63
24. Transfection with siRNA targeting NRF-1 decreases NRF-1 protein expression.....	66
25. Secondary gene expression is not increased in E <sub>2</sub> -treated MCF-7 cells transfected with siRNA targeting NRF-1.....	67
26. Protein expression of secondary genes is not increased in E <sub>2</sub> -treated MCF-7 cells transfected with siRNA targeting NRF-1.....	68
27. The E <sub>2</sub> -induced increase in mtDNA copy number is inhibited in MCF-7 cells transfected with siRNA targeting NRF-1.....	69
28. A model of the regulation of NRF-1 by E <sub>2</sub> and the subsequent downstream effects of this stimulation.....	73
29. 5' promoter region of the hNRF-1 gene.....	77
30. Promoter deletion and ERE mutant constructs of the hNRF-1 promoter.....	78
31. Comparison of the nucleotide sequence of consensus ERE with the putative NRF-1 ERE and mutated NRF-1 ERE.....	79
32. Flow chart describing the CHIP method.....	86
33. Activity of the NRF-1 promoter constructs in MCF-7 cells.....	91
34. Effect of E <sub>2</sub> on luciferase activity from NRF-1 promoter constructs in transiently transfected MCF-7 cells.....	92
35. Effect of E <sub>2</sub> on luciferase activity from pGL3-pro-NRF-1-(1007-1807) in transiently transfected MCF-7 cells.....	93

36. Basal activity of the NRF-1 promoter constructs in HEK-293 cells.....	96
37. Effect of E <sub>2</sub> on luciferase activity from NRF-1 promoter constructs in transiently transfected HEK-293 cells.....	97
38. Effect of E <sub>2</sub> on luciferase activity from pGL3-pro-NRF-1-(1007-807) in transient transfections in HEK-293 cells.....	98
39. ER $\alpha$ and ER $\beta$ bind to the putative NRF-1 ERE <i>in vivo</i> .....	100
40. ER $\alpha$ and ER $\beta$ bind to the putative NRF-1 ERE <i>in vitro</i> .....	101
41. ER $\alpha$ and ER $\beta$ bind the NRF-1 ERE with reduced affinity in comparison to a palindromic ERE.....	102
42. 4-OHT and RAL increase NRF-1 mRNA in a concentration- and time-dependent manner in MCF-7 cells.....	109
43. 4-OHT and RAL increase NRF-1 mRNA in a concentration- and time- dependent manner in H793 cells.....	110
44. 4-OHT- and RAL- induced NRF-1 transcription is mediated by both genomic and nongenomic ER in MCF-7 cells.....	112
45. 4-OHT- and RAL- induced NRF-1 transcription is mediated by both genomic and nongenomic ER action in H1793 cells.....	113
46. Effect of SERMs on luciferase activity from non-ERE containing NRF-1 promoter constructs.....	115
47. Effect of SERMs on luciferase activity from ERE-containing NRF-1 promoter constructs .....	116
48. Effect of SERMs on luciferase activity from the pGL3-pro-NRF-1-(1007-807) construct.....	117
49. ER $\alpha$ and ER $\beta$ bind to the putative NRF-1 ERE <i>in vivo</i> in the presence of 4-OHT and RAL.....	119
50. E <sub>2</sub> , 4-OHT, and RAL activate NRF-1 mRNA expression in HUVEC.....	133
51. ICI 182,780 blocks the E <sub>2</sub> , 4-OHT, and RAL stimulated NRF-1 mRNA induction in HUVEC.....	134
52. Genomic ER is responsible for the increase in NRF-1 mRNA induced by E <sub>2</sub> ,	

4-OHT, and RAL in HUVEC.....	137
53. Secondary gene mRNA expression is increased in response to treatment with E <sub>2</sub> , 4-OHT, and RAL in HUVEC.....	138
54. Effect of E <sub>2</sub> , 4-OHT, and RAL on Complex IV activity in HUVEC.....	139
55. L50, L75, and S80 inhibit basal transcription of NRF-1 in HUVEC.....	142
56. L50 and L75 inhibit the E <sub>2</sub> -induced increase in NRF-1 at all concentrations tested in HUVEC.....	143
57. L0 and L75 inhibit the E <sub>2</sub> -induced increase in NRF-1 at all concentrations tested in HUVEC.....	144
58. S80 inhibits the E <sub>2</sub> -, 4-OHT-, and RAL-induced increase in NRF-1 mRNA in HUVEC.....	145
59. DEPE do not stimulate cell proliferation in HUVEC.....	146
60. Resveratrol increases NRF-1 mRNA expression in a concentration-dependent manner through genomic ER activity in HUVEC.....	148
61. Resveratrol prevents basal suppression of NRF-1 transcription by L75 and S80...	149

## LIST OF ABBREVIATIONS

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)

4-hydroxytramoxifen (4-OHT)

Actinomycin D (Act D)

activation function-1 (AF-1)

activation function-2 (AF-2)

amino acid (aa)

Aromatase knockout mice (ArKO)

blood pressure (BP)

Bromodeoxyuridine (BrdU)

charcoal stripped (CCS)

Coactivator-associated Arginine Methyltransferase (CARM1)

conjugated equine estrogens (CEE)

coronary heart disease (CHD)

cycloheximide (CHX)

cytochrome *c* oxidase subunit I (COI)

cytochrome *c* oxidase subunit II (COII)

cytochrome *c* oxidase subunit III (COIII)

cytochrome *c* oxidase subunit IV (COIV)

cytochrome *c* oxidase subunit VII (COVII)



Dulbecco's Modified Eagle's Medium (DMEM)

Diesel Exhaust Particles (DEP)

Diesel Exhaust Particle Extracts (DEPE)

DNA binding domain (DBD)

diarylpropionitrile (DPN)

E<sub>2</sub>-Penta Compound (EPC)

Endothelial Cell Basal Medium-2 (EBM-2)

Enzyme Linked Immunosorbant Assay (ELISA)

estrogen receptor (ER)

ER alpha (ER $\alpha$ )

ER $\alpha$  knockout mice (ERKO)

ER beta (ER $\beta$ )

Estradiol (E<sub>2</sub>)

estrogen response elements (EREs)

Ethanol (EtOH)

fetal bovine serum (FBS)

Heart and Estrogen/Progestin Replacement Study (HERS)

heart rate (HR)

histone acetyltransferase (HAT)

hormone replacement thereby (HRT)

Human Umbilical Vein Endothelial Cells (HUVEC)

ICI 182,780 (ICI)

integrated optical density (IOD)

Iscove's Modified Eagle's Medium (IMEM)

ligand binding domain (LBD)

manganese superoxide dismutase (MnSOD)

MAP kinase kinase (MEK)

medoxyprogesterone acetate (MPA)

mitochondrial EREs (mtEREs)

mitochondrial respiratory chain function (MRC)

mitochondrial RNA (mtRNA)

mitochondrial targeting protein sequences (mTPS)

mitochondrial transcription factor A (Tfam)

mitochondrial transcription factor B1 (TFB1)

mitochondrial transcription factor B2 (TFB2)

Mitogen Activated Protein Kinase (MAP Kinase)

NADH dehydrogenase subunit 1 (NDI)

Nuclear Respiratory Factor-1 (NRF-1)

non-small cell lung carcinoma (NSCLC)

ovariectomized (Ovx)

oxygen (O<sub>2</sub>)

PD98059 (PD)

Pertussis Toxin (Ptx)

phosphoinositide-3 (PI3)

polycyclic aromatic hydrocarbons (PAHs)

propyl pyrazole triol (PPT)

Protein Arginine Methyltransferase 1 (PRMT1)

Protein Disulfide Isomerase (PDI)

Quantitative Real Time RT-PCR (QRT-PCR)

R,R-enantiomer of tetrahydrochrysene (R,R-THC)

raloxifene (RAL)

reactive oxygen species (ROS)

relative luciferase units (RLU)

Raloxifene Use for the Heart (RUTH)

resveratrol (Res)

selective ER modulators (SERMs)

small cell lung carcinoma (SCLC)

standard error of the mean (SEM)

Study of Tamoxifen and Raloxifene (STAR)

tamoxifen (TAM)

Thyroid hormone (T<sub>3</sub>)

Women's Health Initiative (WHI)

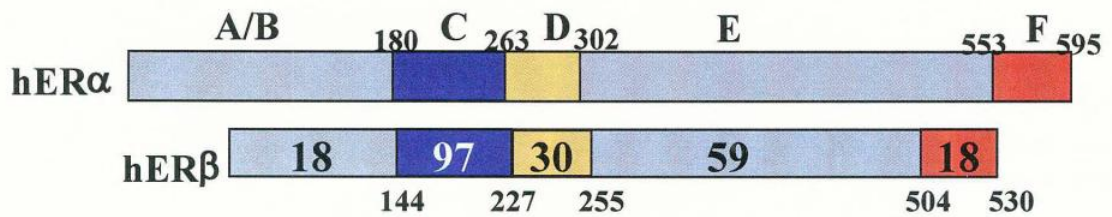
Wortmannin (Wort)

## CHAPTER I

### GENERAL INTRODUCTION

#### **Estrogen Receptor Structure, Classical Function, and Tissue Distribution**

Estrogen action in the cell is mediated by binding to estrogen receptors (ER) in the cell nucleus. ER, like other steroid receptors, is a member of the class I steroid/nuclear receptor superfamily of transcriptional enhancers. There are two subtypes of ER, ER alpha (ER $\alpha$ ) and ER beta (ER $\beta$ ). The ER protein is divided into six domains termed A-F (Figure 1) (1). A/B is the variable N-terminal domain, which regulates transcription through its activating factor 1 (AF-1) in a ligand-independent and cell-specific manner. This region contains low amino acid (aa) homology between ER $\alpha$  and ER $\beta$ . In contrast, the DNA binding domain (DBD), located in domain C, has the highest aa homology when the two ER subtypes are compared. The ligand binding domain (LBD) and activation function-2 (AF-2) in the C-terminal E and F domains share 59% and 18% aa homology, respectively (1,2). AF-2 interacts with corepressors and coactivators in a ligand-dependent manner. The high degree of homology in the LBD allows ER $\alpha$  and ER $\beta$  to form heterodimers which can bind to estrogen response elements (EREs) (3). Once activated by E<sub>2</sub> or an estrogen-like compound, ERs form homodimers



**Figure 1:** Illustration of the domains and percent homology between human ER $\alpha$  and ER $\beta$ . The percent aa homology between the A-F domains is indicated in the ER $\beta$  diagram. The aa position of the end of each domain is also indicated (2,4).

of ER $\alpha$ /ER $\alpha$  or ER $\beta$ /ER $\beta$  and heterodimers of ER $\alpha$ /ER $\beta$  and bind with high affinity to EREs in the promoters of target genes. In addition to EREs, ER $\alpha$  and ER $\beta$  can interact directly with other DNA-bound transcription factors, *e.g.* Sp1 or AP1 sites and up-regulate transcription through a tethering mechanism (5,6).

ER $\alpha$  and ER $\beta$  are not distributed uniformly in the tissues of the body. While some tissues express exclusively ER $\alpha$  or ER $\beta$ , other tissues express both ER subtypes (1). In the rat, ER $\alpha$  was detected by RT-PCR in ovary, uterus, testis, prostate, epididymis, bladder, pituitary, liver, kidney, adrenal, and heart (7). ER $\beta$  was found in rat ovary, uterus, testis, prostate, epididymis, bladder, pituitary, lung, several regions of the brain, and in the spinal cord (7). ER $\alpha$  and ER $\beta$  are expressed in both normal human breast and in breast cancers (8-11). In fact, ratios of the ER subtype have been shown to be altered during carcinogenesis. Expression of both ER $\alpha$  and ER $\beta$  in breast cancer indicates a poor prognosis (12). It has been suggested that over-expression of ER $\beta$  when occupied by an antiestrogen, activates the AP-1 transcription factor at AP-1 response element sites leading to gene expression that promotes cell proliferation (12). On the other hand, Jan-Ake Gustafsson has proposed that ER $\alpha$  has a proliferative role in the cell, while ER $\beta$  may have an anti-proliferative role (13). Recently it has been recognized that ER $\beta$  is the major subtype present in the normal mammary gland and in benign breast cancer (14). Hence, loss of ER $\beta$  may cause breast cancer cell proliferation (15). ER $\beta$  has at least 6 isoforms due to 2 different transcription start sites and alternative splicing (16). The role of these splice variants is of great interest in breast carcinogenesis because these variants can form heterodimers with ER $\alpha$ . Sequestering of

ER $\alpha$  by ER $\beta$  isoforms, may lead to over-expression of growth factor receptors in the cell, allowing the normally non-proliferative cells to proliferate (17). ER $\beta$ cx is an example of a splice variant of ER $\beta$  capable of sequestering ER $\alpha$  (18). hER $\beta$ 1 (530 aa long form) is identical to ER $\beta$ cx in exons 1-7. However, exon 8 is completely different containing 26 unique aa residues in place of the 61 aa residues that normally encode part of helix 11 and helix 12. This mutation abolishes the AF-2 domain and amino acids responsible for ligand binding. While ER $\beta$ cx does not bind to consensus EREs, it does preferentially bind to ER $\alpha$  rather than ER $\beta$ , resulting in a dominant negative effect on ER $\alpha$  gene transactivation (18).

### **Breast Cancer, Lung Cancer, and Estrogen**

Breast cancer is the most frequently diagnosed cancer in women in the western world (19). Of the total cancer burden worldwide, breast cancer comprises 1/5 of the total (20). This figure includes a 33% increase in breast cancer incidence in the 1990s, which comprised a total of 1,050,346 new cases in 2000 (20). The progression of genetic events that results in formation of a breast tumor are still not clear. However, it well established that exposure to estrogens over a lifetime places women at a higher risk for developing breast cancer than men (21-24). Estrogens are hormonal regulators of cell growth and differentiation. They also regulate the physiological functions of many peripheral tissues in the male and female reproductive system. Tissues regulated by estrogens include ovaries, vagina, mammary gland, and uterus in females and testis, prostate, and epididymis in males (25). The primary circulating form of estrogen in premenopausal women, *i.e.*, 17 $\beta$ -Estradiol (E<sub>2</sub>) is needed for the growth and development

of the mammary gland and it has been implicated in the promotion of breast cancer (26). In fact, many human breast cancers are hormone-dependent in early stages and later progress to a hormone independent stage if not treated. In these early stages, tumors are responsive to antiestrogens, some of which are also referred to as Selective Estrogen Receptor Modulators (SERMs) because they have mixed estrogen agonist/antagonist activity, such as tamoxifen (TAM). ER $\alpha$  is over-expressed in breast tumors and is used as a prognostic indicator of TAM therapy (27). About two-thirds of patients with ER $\alpha$ -positive tumors will react favorably to treatment with TAM, as indicated by enhanced patient survival (17).

Results from case-control studies, which examine the relationship between diseases and risk factors after disease occurrence, indicated that women may be more susceptible to the carcinogenic potential of smoking on the lungs (28). However, cohort studies, which identify a patient group that is tracked over time for future development of disease after exposure to a causative agent, have resulted in conflicting data (28). Despite these contradictory results, much like breast cancer, estrogens are considered a risk factor for lung cancer in females (29). Overall, the risk of lung cancer in females has been suggested to be 2-fold higher in comparison to male smokers (30). Tumor xenografts and lung cancer cells in culture have been shown to proliferate in response to E<sub>2</sub> (29,31). Additionally, lung epithelial cells and lung cancer cells express both ER $\alpha$  and ER $\beta$  (reviewed in (32)). In fact, treatment of transgenic mice expressing an ERE regulated luciferase gene with E<sub>2</sub> resulted in a ~15 fold increase in relative luciferase activity in the lung (33).



Lung carcinomas are divided into small cell lung carcinoma (SCLC) and non-small cell lung carcinoma (NSCLC) with NSCLC comprising the majority of lung cancers including adenocarcinomas, squamous cell carcinomas, large cell carcinomas, and adeno-squamous cell carcinomas. Female smokers have a 2.5-fold increased risk of developing lung adenocarcinoma compared to male smokers (34). In fact, adenocarcinomas comprise approximately three-fourths of primary lung tumors in females and only one third in males (35-37).

Although the presence of both ER subtypes has been detected in lung cells, ER $\alpha$  has been determined not to play a significant role in the progression lung cancers (38,39). However, ER $\beta$  is predominantly expressed in normal lung and lung tumors and thought to mediate the actions of E<sub>2</sub> (7,29). Studies in ER $\beta$  knockout mice ( $\beta$ ERKO) support a role for ER $\beta$  in the normal lung.  $\beta$ ERKO mice display abnormal alveolar development (40). In lung tumors, ER $\beta$  has been shown to be an essential contributor to the development of NSCLC (38,39). Use of the classical ER antagonist ICI 182,780 on ER $\beta$  expressing lung tumors reduced the proliferation of NSCLC and this effect is proposed to be mediated through ER $\beta$  (38). Recently, the analysis of ER $\alpha$  and ER $\beta$  expression and activity in human lung adenocarcinoma cell lines and normal lung fibroblasts revealed that E<sub>2</sub> stimulated proliferation in lung cancer cells derived only from female patients (32). Lung adenocarcinoma cell lines derived from females were inhibited by 4-hydroxytamoxifen (4-OHT) and ICI 182,780 treatment, in agreement with previous studies. In contrast, lung adenocarcinoma cell lines derived from male patients, although expressing equal amounts of ER $\alpha$  and ER $\beta$  as those from females, were unresponsive to the effects of E<sub>2</sub>, 4-OHT and ICI 182,780 (32).

In general, the mechanisms by which estrogens may promote both breast and lung tumors are not yet fully understood. However, the presence of ER in both tumor types and the strong association of a proliferative response to E<sub>2</sub> makes breast and lung cancers potential targets of therapies designed to prevent the tumorigenic effects of estrogens.

### **ERE and Coactivator Interactions with ER**

The effects of estrogens in the cell depends on the genes regulated by ER; this in turn depends on the affinity of ER interaction with the target gene promoter (41,42). The nucleotide sequence of the ERE impacts ER $\alpha$  and ER $\beta$  conformation which leads to a change in the coactivator binding pocket affecting the recruitment of coactivators to the ER-ERE (43). Deviation from the perfect consensus ERE correlates with decreasing affinity of ER-ERE binding (42).

Coactivators interact with DNA bound liganded-ER leading to recruitment of chromatin remodeling complexes, “loosening” of nucleosomal structure, recruitment of general transcription factors, and increased transcription of target genes (44). The SRC/p160 family of coactivators, which interacts directly with the ER, is composed of three members: SRC-1, SRC-2/TIF-2/GRIP1, and SRC-3/ACTR/AIB1 (44). These SRC/p160 family members contain several conserved domains including a central domain with three LXXLL motifs. The LXXLL motif forms an amphipathic  $\alpha$ -helix that interacts with the LBD and the N-terminus of ligand-bound ER (45). Additionally, the C-termini of SRC-1 and SRC-3 contain histone acetyltransferase (HAT) activity (46,47). The HAT activity in the C-terminus results in acetylation of lysine groups on the histone/nucleosomal complex leading to an opening of the chromatin. p160 coactivators

also interact with the AF-1 domain of ER $\alpha$  (48). Once bound to the ER-ERE complex, the SRC/p160 coactivators can recruit subsequent proteins involved in opening of the chromatin through two separate transcription activation domains, AD-1 and AD-2 (49). AD-1 mediates the recruitment of CBP/p300 coactivators and acetyltransferases while the AD-2 domain recruits Coactivator-associated Arginine Methyltransferase (CARM1) (49) and Protein Arginine Methyltransferase 1 (PRMT1), secondary protein-modifying enzymes (50). Overall, this recruitment leads to opening of the chromatin by remodeling complexes, such as the SWI/SNF complex and upregulation of transcription (51).

### **Selective Estrogen Receptor Modulators**

SERMs act as mixed estrogen agonist/antagonists when bound to the LBD of ERs. They mediate their effects on ER function through direct binding to ER in the LBD (52). The differential effects in SERM activity, *i.e.*, agonist in some tissues such as bone and uterus and antagonist in breast are attributed to the recruitment of corepressors instead of coactivators to the ERs and tissue specific expression of coregulators which bind to the SERM-ER complex to mediate downstream effects (52). Examples of SERMs include 4-OHT, the active metabolite of tamoxifen (53) and raloxifene (RAL). 4-OHT is an antagonist in breast and an agonist in uterine tissue (54). Thus, while efficacious in treating ER $\alpha$  positive breast cancer, 4-OHT increases the risk of endometrial cancer (54). The differential effect of 4-OHT in endometrial tissue has been attributed to a high level of SRC-1 expression in the endometrium allowing the 4-OHT-ER complex to recruit SRC-1 and coactivate genes whereas tissues with low SRC-1 result in corepressor recruitment to the 4-OHT-ER complex and no transcription (55).

RAL differs from 4-OHT in that it can act as an estrogen agonist in bone and liver, but it functions as an antagonist in uterus and breast (56). Ideally, a perfect SERM could not only provide a way to target ER positive breast tumors, but would serve as an alternative to hormone replacement therapy (HRT) that might protect women against postmenopausal breast or endometrial cancer (57). To add a layer of complexity to the ER-SERM story, ER $\alpha$  and ER $\beta$  both have unique biological responses to estrogens (including phytoestrogens and endocrine disruptors) and SERMs (2). It has been suggested that the asymmetrical ratios of ER heterodimers and homodimers expressed in tissues containing both subtypes may be an indicator of a subtle and yet undescribed way to regulate cell-type-specific and tissue-type-specific response to estrogens and SERMs (58).

In recent years, Diesel Exhaust Particles (DEP) have received attention for their potential role as endocrine disrupting agents. DEP are mostly fine particles ranging in size from 0.2-0.5  $\mu\text{m}$  and are composed of a variety of organic compounds and heavy metals (59). Both antiestrogenic and estrogenic effects of DEPs have been shown in MCF-7 cells (60,61). Antiandrogenic effects have also been reported in a prostate cancer cell line, PC3/AR (62). The effects of DEPs are thought to be mediated in part by polycyclic aromatic hydrocarbons (PAHs) which are a major component of DEPs and are agonist for the AhR receptor (59,60,62). It is known that AhR agonists can direct both antiestrogenic (63,64) and also antiandrogenic effects (60,62) through signaling between ER and AhR

## **Mitochondrial Function and Maintenance**

Eukaryotic mitochondria contain several metabolic pathways that are required for homeostasis and energy production. These include ATP synthesis via oxidative phosphorylation, heme biosynthesis,  $\beta$ -oxidation, metabolism of certain amino acids, cholesterol synthesis, and formation and exportation of iron-sulfur clusters (65).

Mitochondria also mediate apoptosis by integrating cell death signals (65).

Mitochondrial DNA (mtDNA) is a 16.5 kb circular genome encoding 13 mRNAs, 2 rRNAs, and 22 tRNAs (65,66). The mRNAs encode protein subunits of respiratory complex I (7 subunits), III (1 subunit), IV (3 subunits), and V (2 subunits) which are required for proper function of each complex (66). The remaining subunits of the respiratory complex as well as other proteins involved in mtDNA metabolism (~100 proteins) and mitochondrial function are nuclear encoded (67,68). Once translated in the cytoplasm, these proteins are targeted to the mitochondria through a signal sequence and imported into the mitochondria via translocases (69).

Mitochondrial transcription is initiated at two promoters ( $P_L$  and  $P_H$ ) located in the D-loop regulatory region (65). Transcription is initiated at these sites through the binding of mitochondrial RNA (mtRNA) polymerase and the mitochondrial transcription factors mitochondrial transcription factor A (Tfam) and mitochondrial transcription factor B1 (TFB1) and mitochondrial transcription factor B2 (TFB2), hereafter referred to as TFBs (65,70). Both Tfam and TFBs are nuclear-encoded mitochondrial proteins whose transcription is regulated by the transcription factor nuclear respiratory factor 1 (NRF-1) (67).

## **Estrogen Activity in Mitochondria**

Several studies have revealed that estrogens may exert direct or indirect effects on mitochondrial function in a variety of tissues with particular attention to their neuroprotective effects (71,72). It is known that E<sub>2</sub> can protect against ATP depletion within the cell, reactive oxygen species (ROS) generation caused by exposure to 3-nitropropionic acid, and mitochondrial membrane potential decline (73). Recently, ERβ was localized to the mitochondria in several cell types of the brain including primary cerebral cortical and hippocampal neurons, primary cardiomyocytes, and murine hippocampal cells (74). The mitochondrial localization was independent of the differentiation state of the cells. The localization of ERβ was demonstrated by a variety of complementary techniques including immunocytochemistry, immunoblotting, and mass spectrometry (74). The mitochondrial localization of ERα and ERβ has also been detected in MCF-7 human breast cancer cells where treatment with E<sub>2</sub> enhanced ER mitochondrial localization in a concentration- and time-dependent manner (75). An increase in transcript levels of the mtDNA-encoded genes cytochrome *c* oxidase subunits I and II was also detected with E<sub>2</sub> treatment. This increase was inhibited by the ER specific antagonist ICI 182,780 demonstrating that these observations were ER dependent (75).

Mitochondrial proteins are targeted to the mitochondria through mitochondrial targeting protein sequences (mTPS), and they are post-translationally imported into the mitochondria. To determine if ERα and ERβ contain mitochondrial targeting sequences, Chen *et al.* analyzed the primary aa sequence of human ERα and human ERβ using the TargetP program (75). The authors reported that aa sequence 220–270 of ERβ contains a

number of positively charged regions characteristic of mTPS (score of 0.73 out of 1.0). The secondary structure program "Predator" indicated that the aa sequences 221–230 and 251–260 have a tendency to form  $\alpha$ -helical structures characteristic of mTPS. An internal sequence (aa 231–280) of human ER $\alpha$  also contained a concentration of positively charged residues consistent with a mitochondrial targeting protein sequence (mTPS). However, the TargetP program predicted that this region had lower probability to be an mTPS, *i.e.*, score of 0.45 (75). The presence of a targeting sequence was suggested to explain how a normally nuclear protein, *i.e.*, ER, could also be located in mitochondria (75).

A second paper by Chen *et al.* further examined the localization of ER to mitochondria by determining that both nuclear and mitochondrial proteins extracted from MCF-7 cells bound to several different mitochondrial EREs (mtEREs) detected in the D-loop regulatory region and to selected nuclear EREs (76). Competition binding experiments demonstrated that the binding was sequence-specific and enhanced by E<sub>2</sub> in a time- and concentration-dependent manner. Through antibody binding in supershift assays, the authors concluded that ER $\beta$  was the predominant protein binding to mtEREs in MCF-7 cell extracts (76). However, this recent body of literature regarding mitochondrial-localized ER has generated controversy. One group reported that ER $\beta$  was not detectable in mouse liver mitochondria as analyzed by MALDI-TOF mass spectrometry (77). On the other hand, the mitochondrial localization of ER may be cell-specific because ER $\beta$  was recently identified in human heart mitochondrial protein extracts by MALDI-TOF (78).

## **Lessons from Knockout and Ovariectomized Mice**

The role of estrogens in mitochondrial function is well-established from studies with male ER $\alpha$  knockout mice (ERKO) and ovariectomized (Ovx) rodents. In a model of cardiac ischemia-reperfusion, the mitochondria from male ERKO mice contained noticeable ultrastructural damage, and a reduced capacity to metabolize 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), indicating a decrease in respiratory chain function (79). These mice demonstrated fragmented, swollen mitochondria with a loss of matrix space, granular amorphous bodies, and ruptured cristae (79). In a similar examination of mitochondrial function in an ischemia-reperfusion model, Ovx or E<sub>2</sub>-supplemented Ovx rats were compared to sham rats. The Ovx animals showed myocardial mitochondrial damage and a decrease in respiratory chain function (80). The ability of the respiratory chain to metabolize MTT was restored in Ovx rats fed a diet rich in phytoestrogens and was blocked by the classical ER antagonist ICI 182,780 (81). Likewise, mitochondrial structure was preserved in Ovx animals on the phytoestrogen diet and cotreatment with ICI 182,780 resulted in a loss of mitochondrial ultrastructural integrity (81). Inclusive, these studies suggest an important role for ER in maintaining mitochondrial structure and function in the ischemia-reperfusion model.

Two studies examined the role of ER $\beta$  in the cardiac tissues of knockout mice.  $\beta$ ERKO mice develop hypertension beginning at approximately six months of age (82). These mice also demonstrate an increase in mortality and a rise in markers of heart failure. Additionally, ER $\beta$  deletion leads to impairment of Ca<sup>2+</sup> channels in chronic heart failure after a heart attack (83). Vascular smooth muscle cells and blood vessels from



$\beta$ ERKO mice have also demonstrated functional abnormalities such as abnormal vascular contraction (84). Unfortunately, the ultrastructure, function, and E<sub>2</sub> responsiveness of cardiac mitochondria was not examined in either of these studies.

The cytochrome P450 enzyme aromatase produces E<sub>2</sub>. A targeted disruption of exon 9 in this gene in Aromatase knockout mice (ArKO), leads to a deficiency in E<sub>2</sub> production (85). ArKO have been shown to have a lower diastolic blood pressure (BP) and higher heart rate (HR) than wild type control mice. The lower BP was proposed to be the result of lower vascular tone (85). In cultures of primary vascular smooth muscle cells from ArKO mice, apoptosis stimulated by TNF- $\alpha$  was increased and this upregulation was corrected by exogenous E<sub>2</sub> exposure (86). To date, only one study has looked at the ultrastructure of the mitochondria in ArKO mice, and respiratory chain function has not been examined in these mice. Mitochondria from the cardiac interstitial cells of ArKO mice have been reported to be smaller in size compared to wild type animals and contain less organized tubular structure and less defined cristae. The deficient mitochondrial features observed were reversed by supplementation with E<sub>2</sub> (87).

### **Nuclear Respiratory Factor-1 and Mitochondrial Regulation**

Nuclear Respiratory Factor-1 (NRF-1) was discovered in 1989 through examination of the cytochrome *c* promoter, which contained several transcription factor binding sites including a recognition site for NRF-1 (88). This 68 kDa protein contains a C-terminal transcriptional activation domain made up of clusters of hydrophobic aa such as glutamine (89,90). NRF-1 homodimerizes and binds to palindromic NRF-1 sites in the promoters of nuclear-encoded mitochondrial genes (91). NRF-1 can be phosphorylated

on serine residues in its N-terminal domain leading to an increase in DNA-binding and enhanced trans-activation function (92). NRF-1 target genes have been predominantly identified by characterization of NRF-1 binding sites in the promoter regions of these genes. The major group of proteins first identified and mediated by NRF-1 includes the subunits of the five respiratory chain complexes. Additionally a number of other genes involved in the maintenance and function of the mitochondrion have been recognized including assembly factors for the respiratory apparatus, parts of the mtDNA transcription and replication machinery, mitochondrial and cytosolic enzymes of heme biosynthesis, and components of mitochondrial protein import (reviewed in (93)). Due to its ability to regulate the transcription of the nuclear-encoded mitochondrial transcription factors Tfam, TFB1, and TFB2, NRF-1 has been suggested to play a key role in integrating nucleo-mitochondrial interactions (67).

Several studies have shown a correlation between an increase in NRF-1 and an increase in mitochondrial biogenesis. For example, skeletal muscle has been shown to raise both NRF-1 and PGC-1 $\alpha$  levels in response to exercise training (94). In agreement with this study, corresponding results were obtained in cultured myocytes subjected to calcium treatment which simulates exercise-induced mitochondrial biogenesis (95). HeLa cells depleted of mitochondria display an increase in NRF-1 and Tfam which is thought to be a compensatory response (96). Another study examining the regulation of Tfam in connection to a decrease in mtDNA found that methylation of the NRF-1 binding site in the Tfam promoter significantly decreased activity of the promoter construct in transient transfection assays (97).

Perhaps the strongest evidence for a definitive link between NRF-1 and mitochondrial function and maintenance has come from the NRF-1 knockout mouse. A targeted disruption of the NRF-1 gene resulted in lethality between embryonic days 3.5 and 6.5 in the homozygous null embryos (98). The null embryos displayed a reduced amount of mtDNA and a loss of mitochondrial membrane potential. The decrease in mtDNA was proposed to occur between fertilization and the blastocyst stage since heterozygous mothers exhibited normal levels of mtDNA (98). Thus, the decrease in mtDNA may result from a loss of NRF-1-dependent mtDNA maintenance (98). A similar pattern of embryonic lethality is seen in Tfam knockout mice between embryonic day 8.8 to 10.5 (99). This indicates that the earlier lethality observed in the NRF-1 knockout mice may be the result of a combination of loss of mtDNA maintenance and other NRF-1 dependent pathways (99).

### **NRF-1 and Breast Cancer**

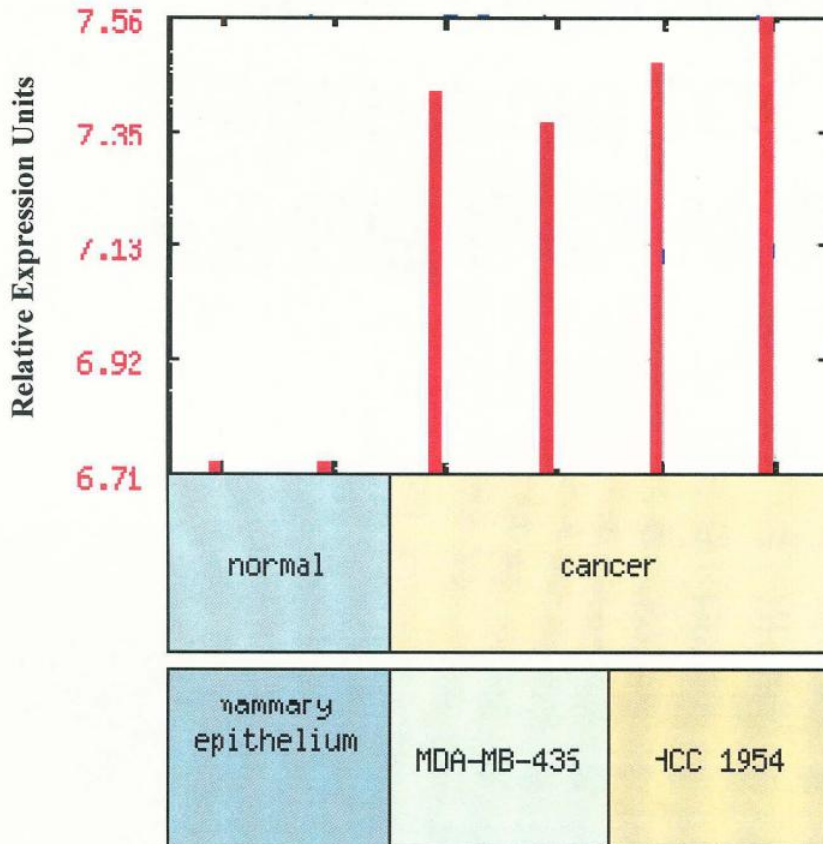
To date, there are no reports examining a role for NRF-1 in breast cancer. However, many scientists in the field of breast cancer research have performed microarray analysis in both breast cancer cell lines and breast cancer tissues. A closer examination of the data from these studies yielded some very interesting information. In order to mine data from previously published microarray data sets, two search methods were utilized. I used Oncomine (<http://www.oncomine.org/main/index.jsp>) to examine NRF-1 expression from microarray data sets in this database. I also searched available Affymetrix Geodata sets available on NCBI (<http://www.ncbi.nlm.nih.gov>). The first microarray data set examined involved a comparison of normal mammary epithelium

compared to two breast cancer cell lines, MDA-MB-436 (ER $\alpha$  and ER $\beta$  negative (100)) and HCC 1954 (ER $\alpha$  positive (101)). In this study, the two cancer cells lines clearly expressed more NRF-1 compared to the normal mammary epithelium (Figure 2).

A second microarray study examined the gene expression profiles of ER positive breast cancer cell lines treated with E<sub>2</sub> for 24 h (102). The cell lines examined were MCF-7, T47-D, and BT-474 and the data was studied through NCBI Geo DataSets. In this microarray study, it is suggestive that NRF-1 expression was slightly stimulated by E<sub>2</sub> in the MCF-7 and T47-D but not the BT-474 cell lines (Figure 3). Since all three cell lines express ER $\alpha$  and ER $\beta$ , the reason for a lack of responsiveness in the BT-474 cells is unknown.

Moving to an *in vivo* model of breast cancer, mammary tumorigenesis was studied in an MMTV-neu mouse model by microarray profiling (103). In this set of experiments, gene expression profiles were examined in preneoplastic glands and tumors of MMTV-neu mice (103). An increase in NRF-1 expression is seen in the preneoplastic tissue compared to normal tissue and in the tumor compared to both the normal and preneoplastic tissue (Figure 4) (103). This increase in NRF-1 expression corresponding to an increase in tumor progression suggests that upregulation of mitochondrial function/activity via NRF-1 may play a role in tumor progression.

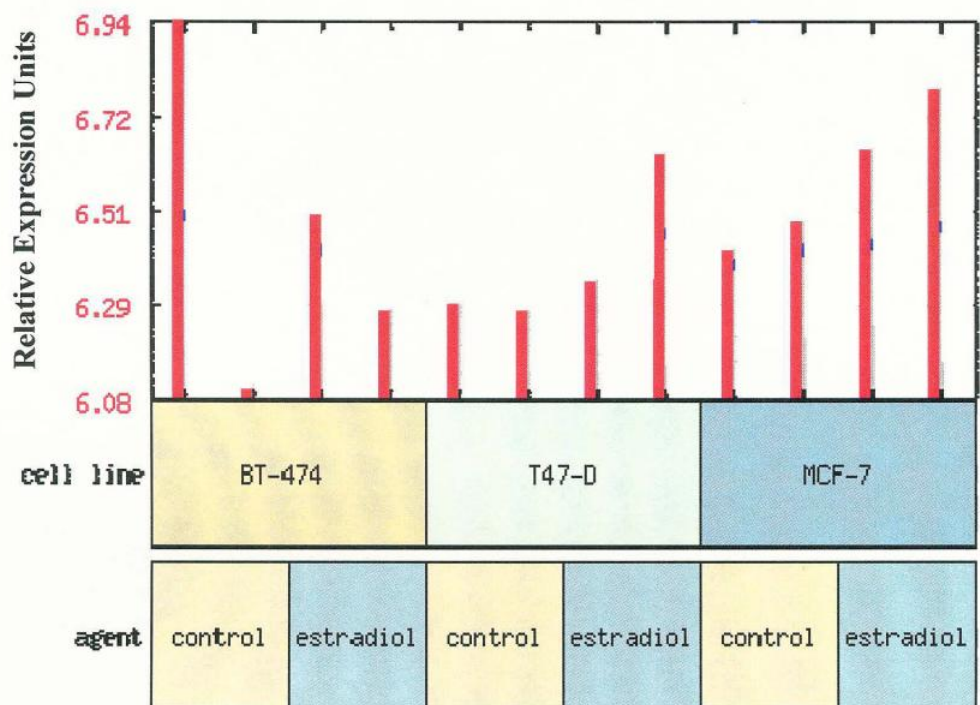
Expression patterns in human breast tumors reflect the trend demonstrated in breast cancer cell lines and the MMTV-neu mouse model, *i.e.*, NRF-1 expression is increased in higher tumor grades. In the first of two studies that I obtained by searching in the Oncomine database, gene expression profiles were examined in primary breast



**Figure 2: NRF-1 expression is increased in breast cancer cell lines compared to normal mammary epithelium.** Affymetrix microarray analysis was used to evaluate gene expression levels in normal mammary epithelium compared to two breast cancer cell lines. The Red bars correspond with the increase in gene expression in a sample.

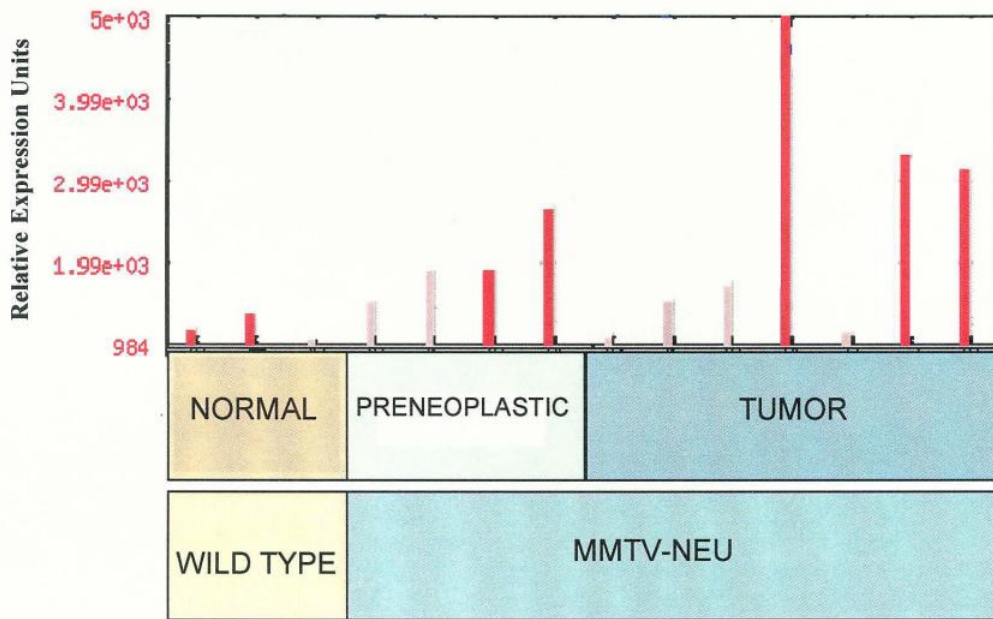
This data set is available at:

[http://www.ncbi.nlm.nih.gov/projects/geo/gds/profileGraph.cgi?&dataset=ABoksz&dataset=UU3134&labels=21246p1m5p1p1p1&gmax=7.560000&gmin=6.710000&title=GD S820++204652\\_s\\_at%20/%20nuclear%20respiratory%20factor%201](http://www.ncbi.nlm.nih.gov/projects/geo/gds/profileGraph.cgi?&dataset=ABoksz&dataset=UU3134&labels=21246p1m5p1p1p1&gmax=7.560000&gmin=6.710000&title=GD S820++204652_s_at%20/%20nuclear%20respiratory%20factor%201)



**Figure 3: NRF-1 expression is increased in response to E<sub>2</sub> in some ER positive breast cancer cell lines.** Cells were treated with E<sub>2</sub> for 24 h and then examined by Affymetrix Microarray. The data is presented in relative expression units. The Red bars correspond with the increase in gene expression in a sample. This data set is available at:

[http://www.ncbi.nlm.nih.gov/projects/geo/gds/profileGraph.cgi?&dataset=zA3OPOTcY2dn&dataset=4UZWXWX1XZ\\_2&labels=80914p1p1p1p1p1p1p1p1p1p1&gmax=6.936474&gmin=6.079675&title=GDS1549+204652\\_s\\_at%20nuclear%20respiratory%20factor%201](http://www.ncbi.nlm.nih.gov/projects/geo/gds/profileGraph.cgi?&dataset=zA3OPOTcY2dn&dataset=4UZWXWX1XZ_2&labels=80914p1p1p1p1p1p1p1p1p1p1&gmax=6.936474&gmin=6.079675&title=GDS1549+204652_s_at%20nuclear%20respiratory%20factor%201)



**Figure 4: NRF-1 is increased in preneoplastic mammary tissue and mammary tumors in a MMTV-neu mouse model.** Microarray analysis was performed to examine gene expression profiles in preneoplastic glands and tumors of MMTV-neu mice (103). The expression of NRF-1 was extracted from the Affymetrix data sets available on NCBI. The Red bars correspond with the increase in gene expression in a sample. Pink bars represent expression levels that may have stray cross-hybridization and are therefore considered questionable. This data set is available at:

[http://www.ncbi.nlm.nih.gov/geo/gds/gds\\_browser.cgi?gds=1222](http://www.ncbi.nlm.nih.gov/geo/gds/gds_browser.cgi?gds=1222)

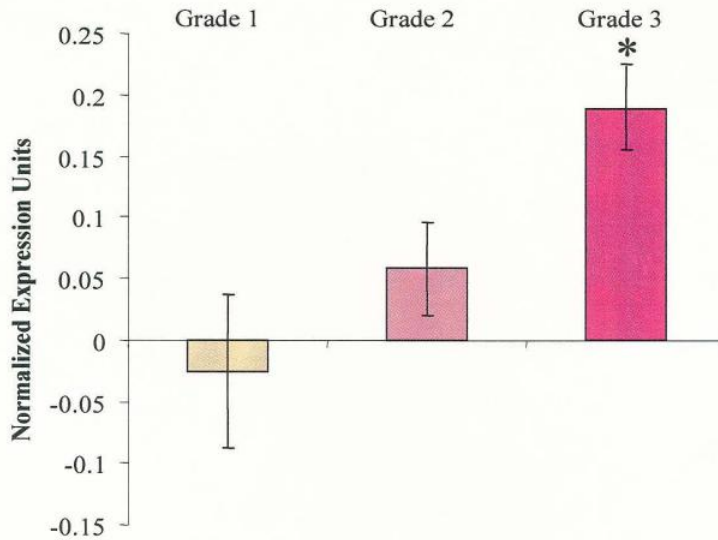
tumors from 117 patients using microarrays (104). For microarray profiling, the tumors were divided into groups based on tumor grade. The NRF-1 expression data displays a positive association with increasing tumor grade with statistical significance between Grade 1 and Grade 3 (Figure 5A). The goal of a second large microarray study was to identify genes that would predict distant metastasis of lymph-node-negative primary breast cancer using 286 lymph-node-negative human breast tumor samples (105). I analyzed NRF-1 mRNA expression from these microarray data. My analysis showed that NRF-1 expression was significantly higher in ER $\alpha$ -positive compared to ER $\alpha$ -negative tumors (Figure 5B).

### **E<sub>2</sub>-Induced Changes in Mitochondria in Normal Tissues**

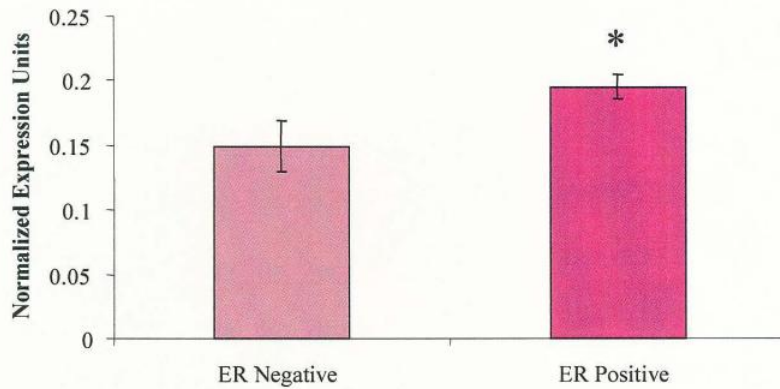
Estrogens protective effects in the brain have been proposed to be mediated mainly through the mitochondria (106). In neuronal cells there is evidence that estrogens may promote cell viability by increasing the efficiency of respiration and maintaining ATP levels (107). Estrogens have been shown to increase Complex IV activity in the rat hippocampus (108), a pituitary cell line, GH<sub>4</sub>C<sub>1</sub> (109), whole brain mitochondria (106), and primary hippocampal neuron cultures (106). An increase in Complex IV activity was also accompanied by an increase in cytochrome *c* oxidase subunit IV (COIV) protein levels (106). Subsequent studies revealed the regulation of mitochondrial transcripts such as cytochrome *c* oxidase subunit I (COI), cytochrome *c* oxidase subunit II (COII), cytochrome *c* oxidase subunit III (COIII), NADH dehydrogenase subunit 1 (NDI), and the six ATPase subunits by E<sub>2</sub> (108-112). More recently, the protein expression NRF-1 was shown to be increased in the cerebral blood vessels of Ovx rats after E<sub>2</sub> treatment for



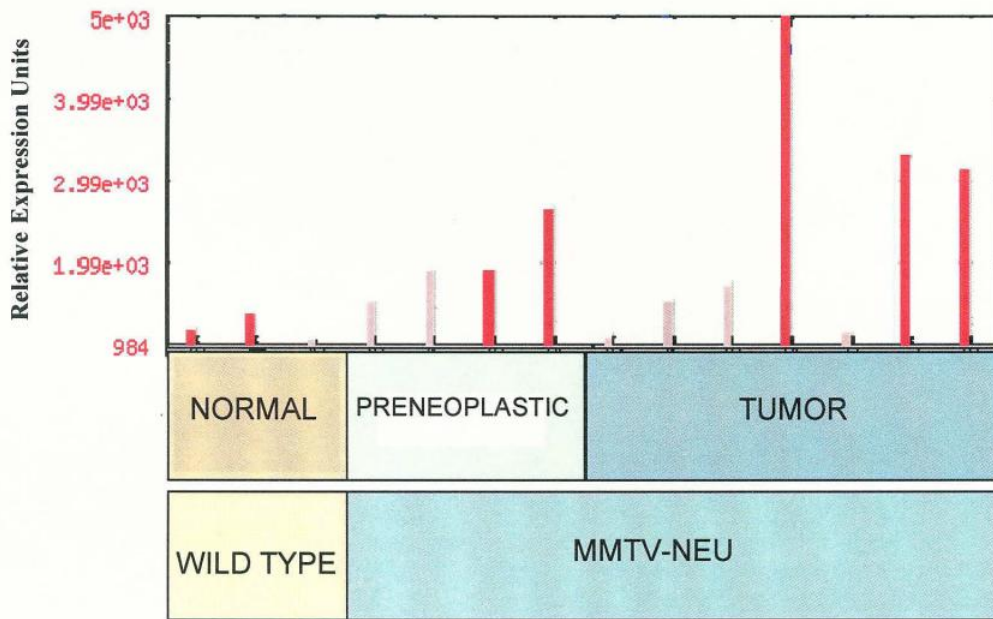
A.



B.



**Figure 5: NRF-1 mRNA expression is increased in higher tumor grades and in ER positive human breast tumors.** (A) Gene expression profiles were examined in primary breast tumors from 117 patients using microarray (104). NRF-1 mRNA expression was analyzed from the microarray data and displays a trend of increased NRF-1 mRNA with increasing tumor grade.  $p < 0.003$  between Grade 1 and Grade 3. (B) Gene-expression profiling was completed in 286 lymph-node-negative tumor samples to predict distant metastasis of lymph-node-negative primary breast cancer (113). NRF-1 mRNA expression was analyzed from the microarray data and displays an increase in ER positive tumors.  $p < 0.037$



**Figure 4: NRF-1 is increased in preneoplastic mammary tissue and mammary tumors in a MMTV-neu mouse model.** Microarray analysis was performed to examine gene expression profiles in preneoplastic glands and tumors of MMTV-neu mice (103). The expression of NRF-1 was extracted from the Affymetrix data sets available on NCBI. The Red bars correspond with the increase in gene expression in a sample. Pink bars represent expression levels that may have stray cross-hybridization and are therefore considered questionable. This data set is available at:

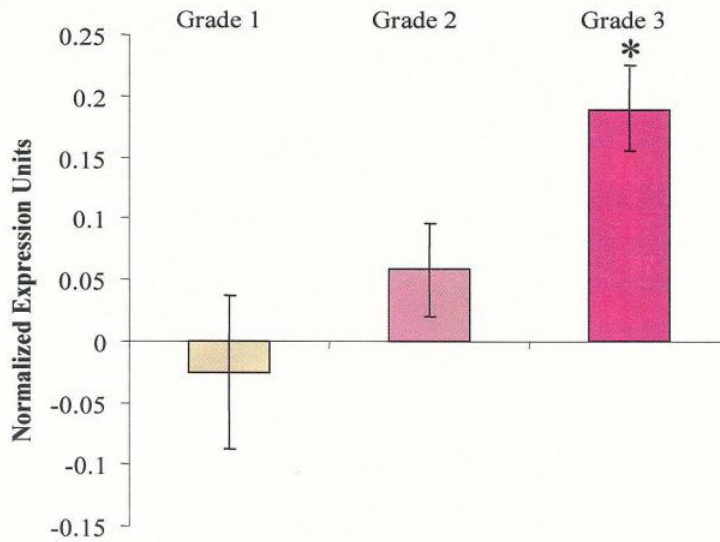
[http://www.ncbi.nlm.nih.gov/geo/gds/gds\\_browser.cgi?gds=1222](http://www.ncbi.nlm.nih.gov/geo/gds/gds_browser.cgi?gds=1222)

tumors from 117 patients using microarrays (104). For microarray profiling, the tumors were divided into groups based on tumor grade. The NRF-1 expression data displays a positive association with increasing tumor grade with statistical significance between Grade 1 and Grade 3 (Figure 5A). The goal of a second large microarray study was to identify genes that would predict distant metastasis of lymph-node-negative primary breast cancer using 286 lymph-node-negative human breast tumor samples (105). I analyzed NRF-1 mRNA expression from these microarray data. My analysis showed that NRF-1 expression was significantly higher in ER $\alpha$ -positive compared to ER $\alpha$ -negative tumors (Figure 5B).

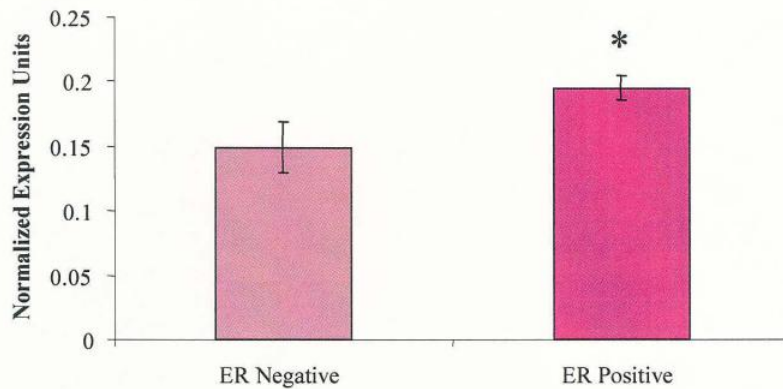
### **E<sub>2</sub>-Induced Changes in Mitochondria in Normal Tissues**

Estrogens protective effects in the brain have been proposed to be mediated mainly through the mitochondria (106). In neuronal cells there is evidence that estrogens may promote cell viability by increasing the efficiency of respiration and maintaining ATP levels (107). Estrogens have been shown to increase Complex IV activity in the rat hippocampus (108), a pituitary cell line, GH<sub>4</sub>C<sub>1</sub> (109), whole brain mitochondria (106), and primary hippocampal neuron cultures (106). An increase in Complex IV activity was also accompanied by an increase in cytochrome *c* oxidase subunit IV (COIV) protein levels (106). Subsequent studies revealed the regulation of mitochondrial transcripts such as cytochrome *c* oxidase subunit I (COI), cytochrome *c* oxidase subunit II (COII), cytochrome *c* oxidase subunit III (COIII), NADH dehydrogenase subunit 1 (NDI), and the six ATPase subunits by E<sub>2</sub> (108-112). More recently, the protein expression NRF-1 was shown to be increased in the cerebral blood vessels of Ovx rats after E<sub>2</sub> treatment for

A.



B.



**Figure 5: NRF-1 mRNA expression is increased in higher tumor grades and in ER positive human breast tumors.** (A) Gene expression profiles were examined in primary breast tumors from 117 patients using microarray (104). NRF-1 mRNA expression was analyzed from the microarray data and displays a trend of increased NRF-1 mRNA with increasing tumor grade.  $p < 0.003$  between Grade 1 and Grade 3. (B) Gene-expression profiling was completed in 286 lymph-node-negative tumor samples to predict distant metastasis of lymph-node-negative primary breast cancer (113). NRF-1 mRNA expression was analyzed from the microarray data and displays an increase in ER positive tumors.  $p < 0.037$

2 weeks (114). These blood vessels also displayed increased expression of COI and COIV, an increase in Complex IV activity, and an ICI 182,780 inhibited increase in Complex IV activity (114). In agreement with this information, previous studies reported that the respiratory function of mitochondria from whole brain was increased by treatment with E<sub>2</sub> *in vivo* as measured by oxygen consumption (106). However, the mechanism of these effects, including the E<sub>2</sub>-induced increase in Complex IV activity and protein, has not been described.

When NRF-1 expression was examined in normal tissues, breast tissue had a higher expression of NRF-1 compared to the other tissues examined with the exception of lung (115). NRF-1 expression was shown to be responsive to E<sub>2</sub> treatment in normal uterus in microarray experiments. Using an Affymetrix Geo DataSet from Moggs *et al*, NRF-1 expression was demonstrated in response to a single injection of 400 µg/kg E<sub>2</sub> in immature 19 to 20 day old Alpk:APfCD-1 mice. Alpk:APfCD-1 mice are immunodeficient due to a lack of alpha protein kinase 1. Microarray analysis was performed to analyze gene expression profiles at varying time points (116). NRF-1 mRNA expression is increased in E<sub>2</sub> treated samples as soon as 2 h after exposure to E<sub>2</sub>. This response tapers off by 24 h (Figure 8).

Electron microscopy has been used to examine the structure and number of mitochondria as previously discussed in the case of the ERKO rats. Changes in mitochondrial structure in response to E<sub>2</sub> have been reported in MCF-7 cells, mammary gland, and uterine smooth muscle. MCF-7 cells were examined by transmission electron microscopy for their response to E<sub>2</sub> (10 nM) over time (117). It was reported that when MCF-7 cells were cultured in 10% Fetal Calf Serum (FCS) containing media, the cells

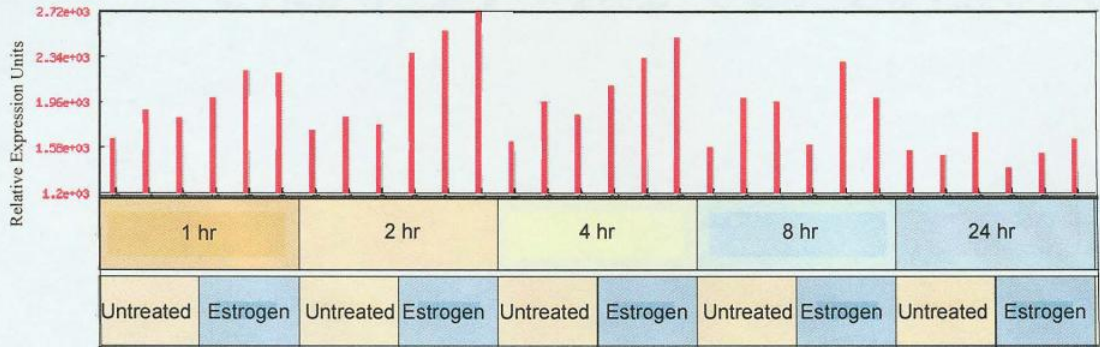
had normal round mitochondria that were large and clear. However, after both 5 and 15 days of culture in media containing 10% dextran coated charcoal (DCC) treated FCS (DCC-FCS), the cells appeared smaller with small dark mitochondria. After 4 days in E<sub>2</sub> containing DCC-FCS, the mitochondria were again large and clear with thin and numerous cristae. A similar effect was seen in the ER positive T47D breast cancer cells, but not the ER null BT20 breast cancer cells (117). The results of these experiments support a role for ER in the maintenance of mitochondrial structure. These results also agree with the observations recorded in Ovx mice, in which, a lack of estrogen lead to a loss of matrix area and reduced mitochondrial respiratory chain function (MRC) (80,114).

A study examining the effects of E<sub>2</sub> and progesterone in Ovx mice used electron microscopy to examine the structure of cells in the parenchyma and blood vessels of the mammary gland (118). While examining the epithelial cells of the mammary ducts and buds, it was observed that the Ovx and progesterone treated mice displayed a small number of mitochondria. However, in E<sub>2</sub> and E<sub>2</sub>+Progesterone treated Ovx mice, there were numerous mitochondria (118). This data suggests a role for E<sub>2</sub> in increasing the number of mitochondria in the cell.

The fine structure of the uterine smooth muscle was examined in Ovx rats treated with a single injection of E<sub>2</sub> and sacrificed at 6, 12, 24, 48, 72, and 96 h. No change in the mitochondria was noted at 6 and 12 h. However, an increase in the number of mitochondria was seen between 24 and 48 h followed by an increase in mitochondrial size at 72 and 96 h (119,120). As with the previously discussed study in mammary



**Figure 6: NRF-1 expression in normal tissue.** Gene expression patterns were examined in a number of normal tissues using microarrays.



**Figure 7: NRF-1 expression in uterine tissue in response to E<sub>2</sub>.** Gene expression patterns were examined in response to a single injection of 400 µg/kg E<sub>2</sub> in immature 19 to 20 day old Alpk:APfCD-1 mice. Microarray analysis was performed to examine gene expression profiles at varying time points (116). The Red bars correspond with an increase in gene expression in a sample. This data set is available at:

[http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=geo&cmd=search&term=GDS1058\[A  
CCN\]](http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=geo&cmd=search&term=GDS1058[A<br/>CCN])



gland, the results of the examination of the uterine smooth muscle suggest a role for E<sub>2</sub> in increasing mitochondrial number.

Inclusively, from the aforementioned studies it is clear that E<sub>2</sub> has an impact on the number and morphology of mitochondria in several cells types. However, the mechanisms guiding this change have not yet been determined.

### **Goals of the Project**

The overall goal of this project was to test the hypothesis that the previously reported E<sub>2</sub>-induced increases in mitochondrial activity and mitochondrial subunit mRNA expression are a result of an increase in the transcription of NRF-1 mediated directly by ER in response to E<sub>2</sub>. In addition, I hypothesized that this upregulation of NRF-1 by ER is mediated directly through an ERE in the NRF-1 promoter. My experimental design included cloning of the promoter region of NRF-1 into a luciferase reporter vector and testing the responsiveness of regions with and without the ERE and a mutated ERE to examine the contribution of the ERE to NRF-1 induced expression.

Since SERMs can have differential effects based on the coactivator expression patterns in each cell type and gene context, *e.g.*, the ERE sequence and adjacent transcription factor binding sites, the expression of NRF-1 was examined in response to the SERMs 4-OHT and RAL. Additionally, since NRF-1 is known to be important to cardiovascular function, Human Umbilical Vein Endothelial Cells (HUVEC) were treated with E<sub>2</sub>, 4-OHT, and RAL and their response examined. Diesel Exhaust Particle Extracts (DEPE), known antagonists of genomic estrogen responses in MCF-7 cells were used in conjunction with E<sub>2</sub>, 4-OHT, and RAL to determine their impact on NRF-1 expression

due to the known risk increase in cardiovascular disease in people living in high traffic areas (121). Lastly, to further extend these studies, a known phytoestrogen, resveratrol (122,123), was tested for its ability to induce NRF-1 and protect against DEPE induced antagonism of the NRF-1 gene. Resveratrol has been previously reported to have cardioprotective effects (124,125). Here I tested the hypothesis that resveratrol would protect human umbilical vein endothelial cells against DEPE- induced damage.

Inclusively, these studies have provided more information on how NRF-1 is regulated by E<sub>2</sub>, SERMs, an environmental pollutant, and a phytoestrogen.

## CHAPTER II

### REGULATION OF NUCLEAR RESPIRATORY FACTOR-1 EXPRESSION BY 17 $\beta$ -ESTRADIOL

#### INTRODUCTION

Experimental evidence demonstrates that liver and brain mitochondria of normal, but not Ovx, female rats generate less ROS and have higher respiratory potential resulting from decreased oxidative damage (126). The decrease in ROS and higher respiratory potential may help explain the observed increased longevity of females in most mammalian species (127). Although the sex differences in mitochondrial function are likely mediated by estrogens, the mechanism(s) underlying these effects remain ill-defined. Therefore, a goal in the present study was to elucidate one of the pathways that may contribute to the observed estrogen-regulated increase in mitochondrial function.

In addition to the already discussed genomic effects of ER that occur within 3-6 h after estrogen administration, ER has rapid, membrane-initiated, non-genomic effects that take place within seconds-minutes of estrogen treatment (reviewed in (128,129)). In order to study the mechanism of ER induced changes in NRF-1 expression, the contribution of non-genomic ER must be considered. Non-genomic effects of ER have been best characterized in endothelial cells in which E<sub>2</sub> rapidly inhibits calcium influx (130), increases intracellular Ca<sup>++</sup> (131) and cAMP (132), and stimulates NO release resulting in vasodilation (133), and thus accounting, in part for the observed

cardioprotective effects of estrogens. ER is known to mediate non-genomic effects through the Mitogen Activated Protein Kinase (MAP Kinase) pathway in addition to genomic effects in MCF-7 cells (134,135). The membrane-associated ERs that mediate these non-genomic effects are transcribed from the same messages used to generate nuclear ER $\alpha$  and ER $\beta$ , and are inhibited by the ER antagonist ICI 182,780 (136). Membrane-associated ER conveys these rapid intracellular effects by activating G-coupled proteins, adenylate cyclase production, and inositol phosphate production (136). By activating networks including the MAPK and phosphoinositide-3 (PI3) Kinase pathways, non-genomic ER rapidly induces a number of changes within the cell including changes in gene expression through phosphorylation of transcription factors and coregulators (129,137).

As previously discussed, NRF-1 is upregulated in cerebral blood vessels of Ovx rats chronically treated with E<sub>2</sub>, suggesting that estrogen may regulate NRF-1 transcription (114). Coordination of the expression of the mitochondrial and nuclear-encoded genes required for mitochondrial biogenesis and function has been proposed to be mediated by the ability of hormone receptors present within the mitochondria to induce expression of genes encoded in the mtDNA (138). However, this hypothesis has been debated in the literature. Here, I tested the hypothesis that the E<sub>2</sub>-induced effects such as upregulation of mitochondrial activity and an increase in mitochondrial subunit mRNA expression previously demonstrated are a result of the upregulation of NRF-1 by ER in response to E<sub>2</sub>.

## MATERIALS AND METHODS

### **Cell Culture and Treatment Description**

MCF-7 breast adenocarcinoma, H1793 lung adenocarcinoma, and MCF-10A non-tumorigenic epithelial cell were purchased from the American Type Culture Collection (ATCC). According to the ATCC, MCF-7 cells were derived from the pleural effusion of a 69 year old female with breast cancer. MCF-7 cells express both ER $\alpha$  and ER $\beta$  in equivalent amounts as our lab has previously reported (32). MCF-7 cells were maintained in Iscove's Modified Eagle's Medium (IMEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. For all treatments, MCF-7 cells were placed in phenol red free IMEM supplemented with 10% charcoal stripped (CCS)-FBS for 72-96 h prior to treatment.

NCI-H1793 cells were derived from the non-small cell lung adenocarcinoma of a 52 year old non-smoking female, according to the ATCC. These cells express predominantly ER $\beta$  with approximately 60% less ER $\alpha$  than ER $\beta$  (32). H1793 cells were maintained in media containing 50% Dulbecco's Modified Eagle's Medium (DMEM) and 50% FK-12 medium supplemented with 0.005 mg/ml insulin, 0.01 mg/ml transferrin, 30 nM sodium selenite, 10 nM hydrocortisone, 10 nM 17 $\beta$ -estradiol, 10 mM HEPES, 2 mM L-glutamine, 5% FBS, and 1% penicillin/streptomycin. Prior to treatment the cells were placed in DMEM:FK-12 media supplemented with 30 nM sodium selenite, 10 mM HEPES, 2 mM L-glutamine, 5% CCS-FBS, and 1% penicillin/streptomycin for 48 hours.

MCF-10A cells are a "normal" breast non-tumorigenic epithelial cell line that was originally derived from a subcutaneous mastectomy and spontaneously immortalized (139). They express low levels of ER $\beta$  and no ER $\alpha$  (139). MCF-10A cells were

maintained in media consisting of 50% DMEM and 50% FK-12 medium supplemented with 5% horse serum, 20 ng of EGF per ml, 10 µg of insulin per ml, and 0.5 µg of hydrocortisone per ml of media. For all treatments, MCF-10A cells were placed in phenol red free IMEM supplemented with 10% charcoal stripped (CCS)-FBS for 48 h prior to treatment.

### **RNA Isolation, RT-PCR and Quantitative Real Time RT-PCR (QRT-PCR)**

RNA was extracted using Trizol (Invitrogen) according to the manufacturer's protocol. RNA concentration was determined, and 2 µg of RNA was used to generate cDNA. RNA was subjected to a DNase I digestion at 37°C for the PCR analysis using the COI and NDI mitochondrial primers. The High Capacity cDNA archive kit (PE Applied Biosystems) was used to reverse transcribe total RNA using random hexamers. Taqman primers and probes for NRF-1, Tfam, and the control gene, 18S rRNA, were purchased as Assays-on-Demand™ Gene Expression Products (PE Applied Biosystems). Measurement of COI, NDI, and 18S using SYBR Green (PE Applied Biosystems) was performed using previously published primer sequences for COI and NDI listed in Table 1 (140). The expression of each target gene was determined in triplicate and normalized using 18S. QRT-PCR for NRF-1 and 18S Assays-on-Demand was performed in the ABI PRISM 7900 SDS 2.1 (PE Applied Biosystems) using relative quantification with standard thermal cycler conditions. QRT-PCR for COI, NDI, and 18S was also performed in the ABI PRISM 7900 SDS 2.1 using absolute quantification. Analysis and fold differences were determined using the comparative CT method. Fold change was

calculated from the  $\Delta\Delta C_T$  values with the formula  $2^{-\Delta\Delta C_T}$  and data are presented as relative to expression in EtOH-treated samples unless otherwise stated.

### **Transient Transfection of MCF-10A Cells**

MCF-10A cells were grown to 60% confluency in 6-well plates. Prior to transfection, MCF-10A cells were placed in IMEM with 10% CCS-FBS. The cells were transfected with 10 ng of pCMV-ER $\alpha$  (provided by B.S. Katzenellenbogen) using FuGene 6 at a 3:1 FuGene 6 to DNA ratio. Twenty-four h post-transfection, the cells were treated for 4 h with E<sub>2</sub> and the mRNA harvested as described in the previous section.

### **Protein Isolation and Western Blot Analysis**

Whole cell extracts (WCE) were collected in 300  $\mu$ L modified Radioimmunoprecipitation (RIPA) buffer 50 mM Tris-HCl, pH 7.4; 1% Nonidet P-40; 0.25% Na-deoxycholate; 150 mM NaCl; 1 mM EDTA; 1 mM phenylmethylsulfonyl fluoride (PMSF); aprotinin, leupeptin and pepstatin, each at 1  $\mu$ g/ml; 1 mM Na<sub>3</sub>VO<sub>4</sub>; 1 mM NaF. To obtain membrane preparations, the cells were collected in TSE Buffer (25 mM Tris, 250 mM Sucrose, 1mM EDTA, and 1 X protease inhibitors (Roche). Cells were lysed, subjected to centrifugation at 600 rpm for 10 min, and the supernatant collected and subject to centrifugation for 20 min at 100,000 rpm. The pellet was resuspended in TSE Buffer. Protein concentrations were determined using the

<b>Primer Name</b>	<b>Primer Sequence</b>
COI Forward	5'- TACGTTGTAGCCCACTTCCACT -3'
COI Reverse	5'- GGATAGGCCGAGAAAGTGTTGT -3'
NDI Forward	5'- ACACTAGCAGAGACCAACCGAA -3'
NDI Reverse	5'- GGGAGAGTGCATATGTTGT -3'

**Table 1: SYBR Green Primer Sequences for QRT-PCR.** All primers were used at a concentration of 5  $\mu$ M with standard cycling conditions.



resuspended in TSE Buffer. Protein concentrations were determined using the Bio-Rad DC Protein Assay.

Thirty micrograms of protein were separated on 8% or 15% SDS-PAGE gel using a BioRad casting and electrophoresis system. The protein was transferred onto a PVDF membrane (BioRad) at 100 volts for 1 h for NRF-1 detection and 0.5 h for COI and cytochrome *c* oxidase subunit IV (COIV) detection. The blots were blocked in 5% dry milk in TBS-Tween (20 mM Tris, 137 mM NaCl, and 0.1% Tween) and probed with either the polyclonal NRF-1 antibody (Rockland Scientific), monoclonal COI antibody (MitoScience), or monoclonal COIV antibody (MitoScience). The NRF-1 blots were stripped by incubation in Stripping Buffer (100 mM  $\beta$ -mercaptoethanol, 2% sodium dodecyl sulfate, 62.5 mM Tris-HCl, pH 6.7) at 50<sup>0</sup>C for 30 minutes with intermittent agitation. The membranes were then washed in TBS-Tween and re-blocked in 5% milk prior to incubation with  $\alpha$ -tubulin (Neomarkers) as a loading control. Protein Disulfide Isomerase (PDI) (Sigma) was used as a loading control for COI and COIV. A secondary horse radish peroxidase (HRP) conjugated anti-rabbit or anti-mouse antibody (Pierce) was used followed by treatment with PicoWest Chemiluminescent Reagent (Pierce). Kodak Film (Eastman Kodak) was used for image capture followed by development in a Kodak X-OMAT Film Processor.

Un-Scan-It (Silk Scientific) was used to quantitate the integrated optical densities (IOD) for each band. The IOD for NRF-1 was divided by the concordant  $\alpha$ -tubulin or PDI in the same blot. The EtOH value was set to 1 in all hormone treatment experiments.

## **Mitochondrial Biogenesis**

Two methods were used to examine mitochondrial biogenesis, semi-quantitative analysis of mtDNA copy number and oxygen (O<sub>2</sub>) consumption. For semi-quantitative PCR, MCF-7 cells were plated at 50% confluency in a 12-well plate. The cells were placed in phenol free IMEM containing 10% CCS-FBS for 72 h followed by treatment with 10 nM E<sub>2</sub> for 24, 48, and 72 h. The cells were harvested using the Wizard Genomic DNA Isolation Kit (Promega). 1 ng of genomic DNA was subject to PCR for a 250 bp segment of mtDNA and a 150 bp segment of 18S for 27 cycles and the product was resolved on a 2% agarose gel. Semi-quantitative PCR analysis was performed using GoTaq Polymerase from Promega. Each reaction included 1X Green GoTaq Flexi Buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 1 μM mtDNA forward primer, 1 μM mtDNA reverse primer, 1 μM 18S forward primer, 1 μM 18S reverse primer, 1.25 units GoTaq, and nuclease free water to a total volume of 20 uL. The PCR primers, designed using Primer3, are shown in Table 2. Gel images were scanned and Un-Scan-It was used to quantitate the IOD for each band. The IOD for mtDNA was divided by the concordant 18S in each lane. The EtOH value was set to 1 in all hormone treatment experiments.

O<sub>2</sub> consumption was measured using a Strathkelvin Instruments Electrode. For these experiments, MCF-7 cells were propagated as described previously in 150 mm cultured dishes. Upon reaching 50% confluency, the cells were placed in phenol red-free IMEM with 10% CCS-FBS for 48 h. Subsequently, a final concentration of 10 nM E<sub>2</sub> was added to the cells for 2, 4, or 6 d. The media was replaced every 48 h with fresh IMEM containing 10% CCS-FBS and EtOH or 10 nM E<sub>2</sub> where appropriate. Prior to the O<sub>2</sub> consumption measurements, cells were removed from the plates and cell number was

Primer Name	Primer Sequence
mtDNA Forward	5'-TACCCATCATAATCGGAGGC-3'
mtDNA Reverse	5'-TGAAATTGATGGCCCCTAAG-3'
18S Forward	5'-GTAACCCGTTGAACCCCAT-3'
18S Reverse	5'-CCATCCAATCGGTAGTAGCG-3'

**Table 2: PCR Primers Used for Mitochondrial Biogenesis Experiments**

ascertained using trypan blue exclusion to identify viable cells. O<sub>2</sub> consumption rates were measured using 5 x 10<sup>6</sup> cells suspended in a total volume of 0.5 ml of phenol red-free IMEM medium containing 10% CCS-FBS. Readings were collected for 10 min or until all O<sub>2</sub> had been consumed. Rates of O<sub>2</sub> consumption are calculated using a software package: 782 System ver. 3.0 (Strathkelvin Instruments).

### **siRNA Knockdown of ER $\alpha$ , ER $\beta$ , and NRF-1**

siRNA knockdown of ER $\alpha$  and ER $\beta$  was performed in MCF-7 cells using siRNA duplexes obtained from either New England Biolabs (ER $\alpha$ ) or Millipore (ER $\beta$ ). Both siRNAs were transfected using TransPass<sup>TM</sup> R1 (New England Biolabs). MCF-7 cells were cultured in 12-well plates until 50% confluent. 6  $\mu$ l of TransPass<sup>TM</sup> R1 was added to 100  $\mu$ l of OPTI-MEM. A final concentration of 25 nM of ER $\alpha$  and 100 nM of ER $\beta$  was added to the diluted transfection reagent and incubated for 20 min. 500  $\mu$ l of IMEM containing 10% CCS-FBS was added to the transfection mixture. The MCF-7 cells were then washed twice with IMEM containing 10% CCS-FBS and the transfection mixture was added to the cells. The cells were incubated with the transfection mixture for 24 h, and then the media was replaced with IMEM containing 10% CCS-FBS. The cells were incubated another 48 h, and they were treated with 10 nM E<sub>2</sub> or EtOH and RNA or protein harvested.

MCF-7 cells were cultured in 6-well plates in order to generate RNA, WCE, and genomic DNA examining the effect of siRNA knockdown of NRF-1. Upon reaching 60% confluency, the growth media was replaced with IMEM containing 10% CCS-FBS for the transfection. 8  $\mu$ l of siRNA targeting NRF-1 (Santa Cruz) or Control siRNA

(Santa Cruz) was diluted in 100  $\mu$ L of OPTI-MEM. In a separate tube, 8  $\mu$ l of siRNA Transfection Reagent (Santa Cruz) was diluted in 100  $\mu$ L of OPTI-MEM. The siRNA and Transfection Reagent Solutions were combined and incubated at room temperature for 45 min. For each transfection, 0.8 ml of IMEM containing 10% CCS-FBS was added to the transfection mixture. The media was removed from the cells and the transfection complex added. After 24 h, the media was replaced with fresh IMEM containing 10% CCS-FBS and incubated another 24 h. A total of 48 h post-transfection, the cells were treated with 10 nM E<sub>2</sub> for 48 h.

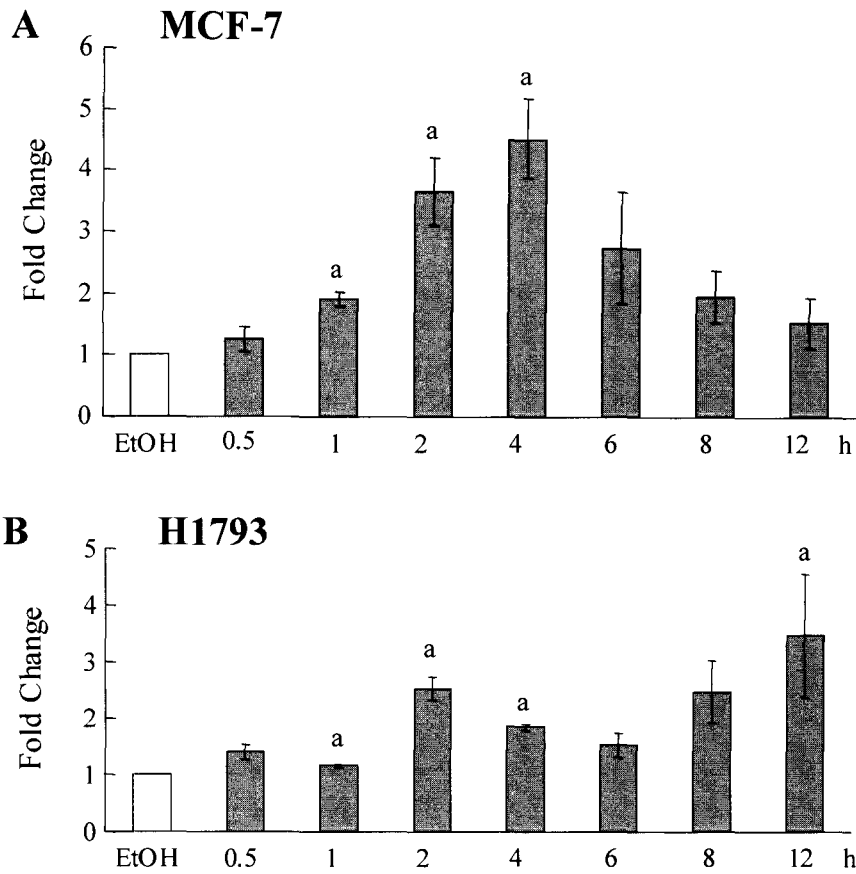
### **Statistics**

Statistical analyses were performed using GraphPad Prism (San Diego, CA.). Two-tailed Student's *t* tests were used for the pair-wise comparison of experimental groups. Statistical significance was defined at  $\geq 95\%$  confidence interval (95% CI) or  $P \leq 0.05$ . Bar graphs represent the mean  $\pm$  standard error of the mean (SEM) for the number of independent experiments indicated in each figure legend.

## RESULTS

### **E<sub>2</sub>-Induced NRF-1 Transcription is Mediated by Genomic ER**

NRF-1 protein expression was reported to be increased in the cerebral blood vessels of Ovx rats after two weeks of E<sub>2</sub> treatment (114). However, this study did not examine whether this E<sub>2</sub> effect was a primary estrogen response, *i.e.*, mediated directly by E<sub>2</sub> at the transcription level, or a result of a long term alteration in gene expression patterns, *i.e.*, a secondary or tertiary response, due to E<sub>2</sub> exposure. To determine if NRF-1 is a direct E<sub>2</sub> target gene, estrogen-responsive H1793 human lung adenocarcinoma (32) and MCF-7 human breast adenocarcinoma (141) cells were treated with E<sub>2</sub> and NRF-1 expression measured by QRT-PCR. NRF-1 mRNA expression was increased by E<sub>2</sub> treatment in both cell lines with a more robust induction in MCF-7 compared to H1793 cells. MCF-7 cells displayed a significant increase in NRF-1 mRNA at 1 h after E<sub>2</sub> treatment, and this response peaked at 5-fold induction at 4 h (Figure 8A). H1793 cells also displayed a significant increase in NRF-1 mRNA at 1 h after E<sub>2</sub> treatment and this response peaked at 2.5-fold at 2 h (Figure 8B). H1793 cells showed an apparent second phase of induction at 12 h (Figure 8B).



**Figure 8: E<sub>2</sub> treatment induces an increase in NRF-1 mRNA expression in MCF-7 and H1793 cells.** (A) MCF-7 and (B) H1793 cells were treated with EtOH or 10 nM E<sub>2</sub> for the times indicated. The total mRNA was isolated and QRT-PCR was performed as described in Material and Methods. Values are the average of 3-6 separate experiments  $\pm$  SEM. a=  $P < 0.05$  compared to EtOH.

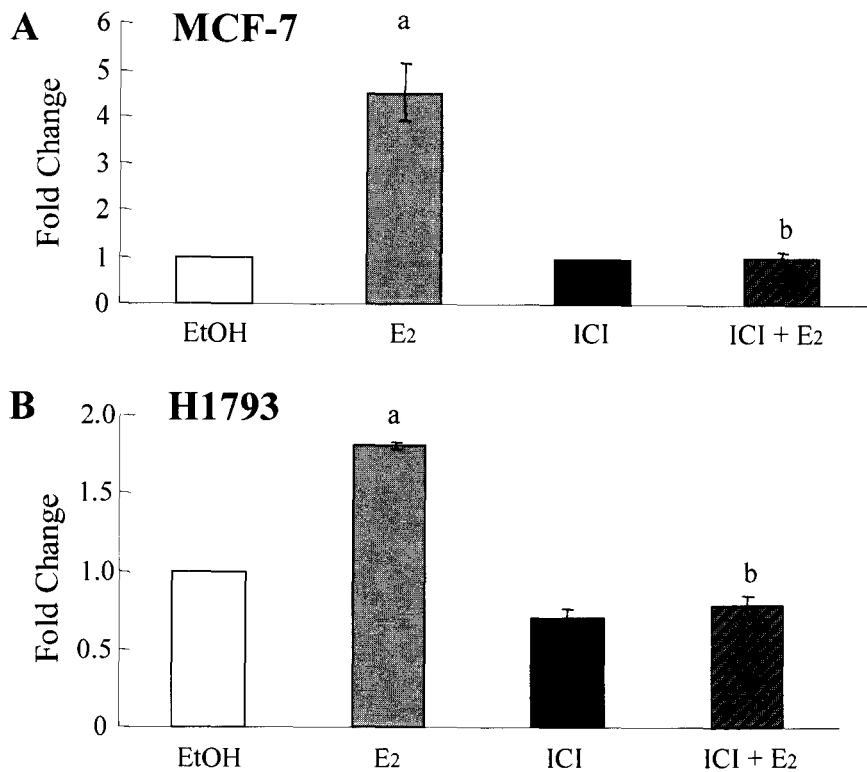
ICI 182,780 is a well-established antagonist of genomic ER that decreases nuclear ER $\alpha$  concentration (142), prevents coactivator recruitment (143), inhibits dimerization of the receptor (144), and enhances ER $\alpha$ , but not ER $\beta$ , proteosomal degradation (143). To determine if the E<sub>2</sub>-induced increase in NRF-1 is directly mediated by ER, MCF-7 and H1793 cells were pre-treated for 6 h with 100 nM ICI 182,780 prior to treatment with 10 nM E<sub>2</sub>. This pre-incubation time was selected because previous studies had demonstrated a ~80% decrease in ER $\alpha$  protein levels as early as 4 h post-treatment with ICI 182,780 (143). ICI 182,780 blocked the E<sub>2</sub>-induced increase in NRF-1 mRNA, indicating that ER mediated this response (Figure 9A and B).

In addition to treatment with ICI-182,780, MCF-7 cells were treated with a Proteolysis Targeting Chimeric Molecule (Protac) known as E<sub>2</sub>-Penta (Figure 10A) (145). In general, Protacs are hybrid compounds constructed of an E3 ubiquitin ligase conjugated to a targeting molecule (146). In this case, E<sub>2</sub> has been conjugated to an E3 ubiquitin ligase. Binding of the E<sub>2</sub>-Penta Compound (EPC) to ER results in targeting of ER to the Proteasome. Treatment with the EPC inhibited the E<sub>2</sub>-induced increase in MCF-7 cells further indicating that ER mediates this response (Figure 10B).

### **NRF-1 is a Primary Estrogen-Responsive Gene Mediated by Genomic ER**

The transcriptional inhibitor Actinomycin D (Act D) and the protein synthesis inhibitor cycloheximide (CHX) were used to determine if the E<sub>2</sub>-ER mediated increase in NRF-1 was a direct effect of ER at the genomic level or required synthesis of a secondary estrogen-responsive protein. Notably, Act D, but not CHX, inhibited the E<sub>2</sub>-induced





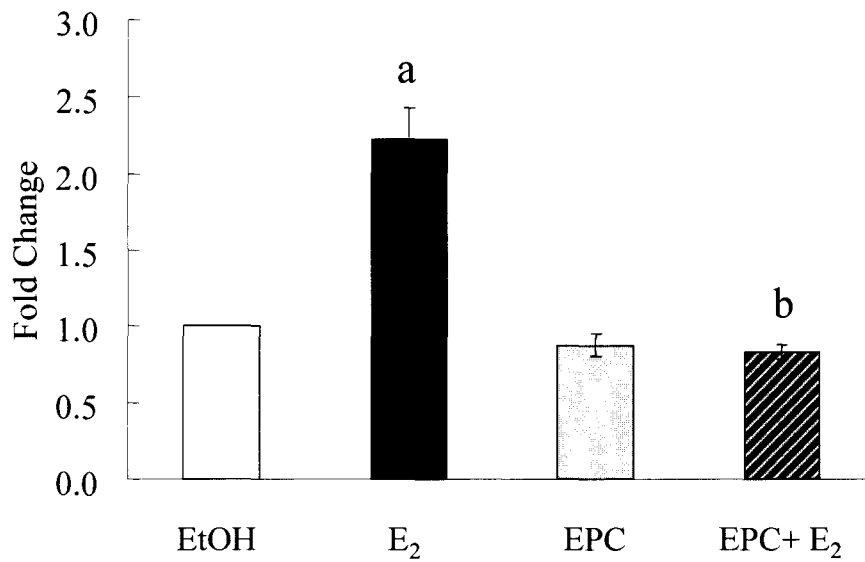
**Figure 9: ICI 182,780 blocks the E<sub>2</sub>-induced increase in NRF-1 mRNA in MCF-7**

**and H1793 cells.** (A) MCF-7 and (B) H1793 cells were treated with 100 nM ICI

182,780 or EtOH for 6 h prior to treatment with 10 nM E<sub>2</sub> for 4 h. The total mRNA was isolated and QRT-PCR was performed as described in Material and Methods. Values are the average of 3-6 separate experiments ± SEM.

a =  $P < 0.05$  compared to EtOH;

b =  $P < 0.05$  compared to E<sub>2</sub>



**Figure 10: EPC blocks the E<sub>2</sub>-induced increase in NRF-1 mRNA in MCF-7 cells.**

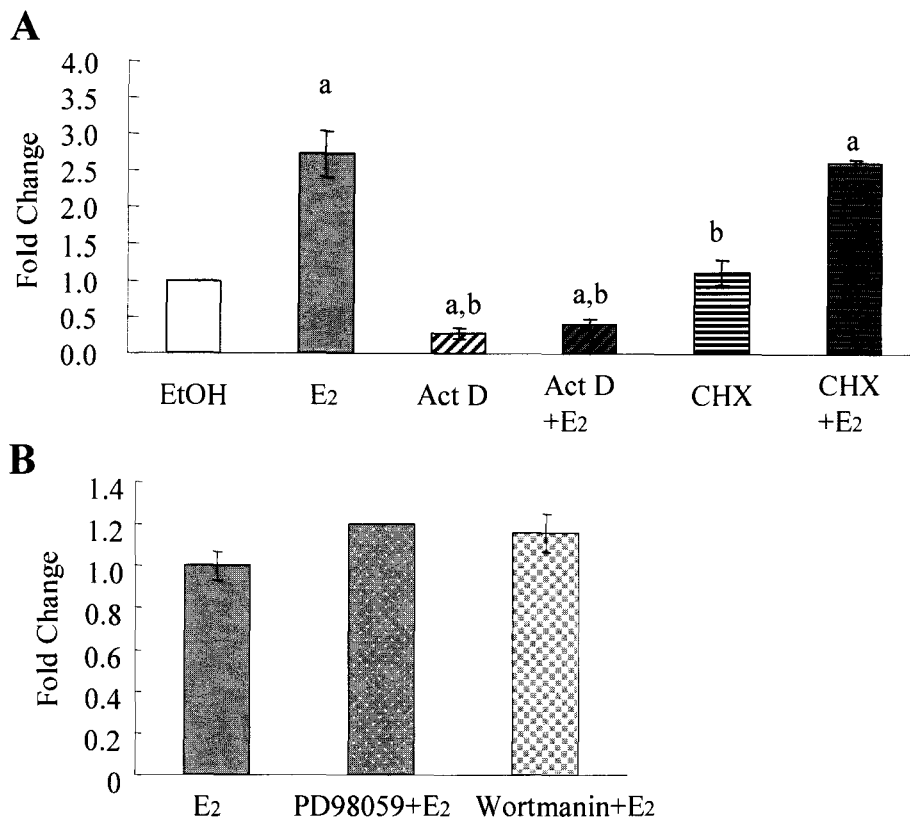
MCF-7 cells were pre-treated with 25  $\mu$ M EPC for 48 h followed by the addition of either EtOH or 10 nM E<sub>2</sub> for 4 h. The total mRNA was isolated and QRT-PCR was performed as described in Material and Methods. Values are the average of 3-4 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH; b =  $P < 0.05$  compared to E<sub>2</sub>

increase in NRF-1 mRNA (Figure 11A), indicating that the *de novo* expression of an E<sub>2</sub>-induced protein was not required for increased NRF-1 transcription. I concluded that NRF-1 is a primary E<sub>2</sub>-responsive gene.

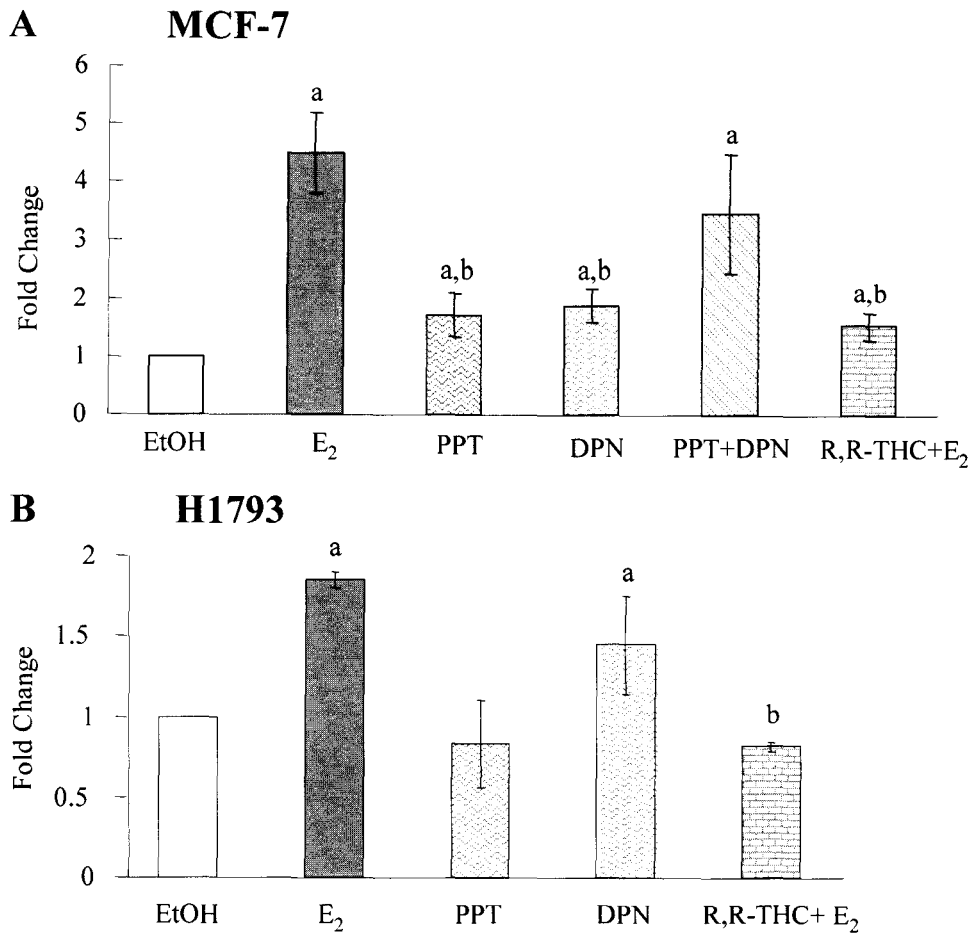
To determine if the E<sub>2</sub>-induced increase in NRF-1 mRNA is mediated by non-genomic ER activity, MCF-7 cells were pretreated for 1 h with the PI3 Kinase and MAP kinase kinase (MEK) inhibitors Wortmannin and PD98059, respectively. Neither inhibitor altered the E<sub>2</sub>-induced increase in NRF-1 mRNA (Figure 11B). This indicates that the E<sub>2</sub>-induced increase in NRF-1 is mediated by genomic actions of ER and not its nongenomic functions through the PI3K/Akt and MAPK pathways.

### **Both ER $\alpha$ and ER $\beta$ Increase NRF-1 Transcription**

Since both ER $\alpha$  and ER $\beta$  proteins are expressed in MCF-7 (32,147) and H1793 cells (32), the observed ER-dependent upregulation of NRF-1 by E<sub>2</sub> could be mediated by both or either ER subtype. To test the ER subtype specificity of the E<sub>2</sub>-induced increase in NRF-1 in MCF-7 and H1793 cells, cells were treated with ER $\alpha$  and ER $\beta$  selective agonists, propyl pyrazole triol (PPT) (148) and diarylpropionitrile (DPN) (149), respectively. PPT or DPN alone yielded ~ 50% of the E<sub>2</sub> response in MCF-7 (Figure 12A). The increase in NRF-1 mRNA induced by a combination of PPT and DPN was not statistically different from the E<sub>2</sub>-stimulated increase in NRF-1 transcription perhaps due to the large error bars. Together, these data indicate that both subtypes of ER contribute equally to NRF-1 activation. Treatment with an ER $\beta$  selective antagonist, the R,R-enantiomer of tetrahydrochrysenes (R,R-THC) (150), in combination with E<sub>2</sub> in



**Figure 11: E<sub>2</sub>-induced NRF-1 transcription is mediated by genomic ER.** (A) MCF-7 cells were pretreated with 10  $\mu$ g/ml Act D or CHX for 1 h followed by 4 hs of 10 nM E<sub>2</sub>. (B) MCF-7 cells were pre-treated with 50  $\mu$ M PD98059 or 50 nM Wortmannin for 1 h prior to incubation with 10 nM E<sub>2</sub> for 4 h. The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH; b =  $P < 0.05$  compared to E<sub>2</sub>

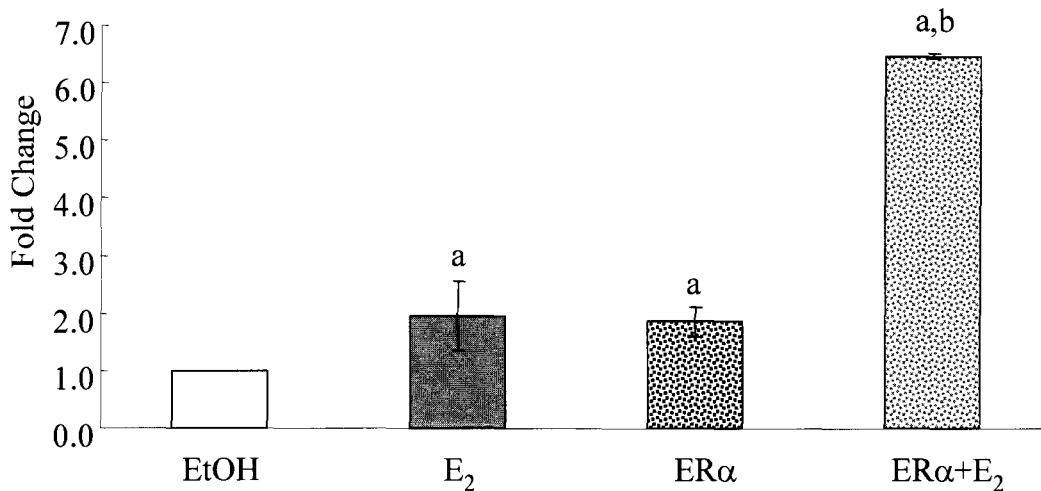


**Figure 12: Both ER $\alpha$  and ER $\beta$  upregulate NRF-1 gene transcription.** (A) MCF-7 or (B) H1793 cells were treated with EtOH, 10 nM E<sub>2</sub>, 10 nM DPN, 10 nM PPT, 10 nM R,R-THC, or both PPT and DPN for 4 h. The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH; b =  $P < 0.05$  compared to E<sub>2</sub>

MCF-7 cells resulted in a ~50% repression of the E<sub>2</sub>-induced increase in NRF-1 mRNA (Figure 12A). Our lab previously reported that the MCF-7 cells we use express equivalent amounts of ER $\alpha$  and ER $\beta$  (32). The PPT, DPN, and R,R-THC data support an equal contribution for each ER subtype in the E<sub>2</sub>-regulation of NRF-1 in MCF-7 cells. In contrast, PPT had no significant effect on NRF-1 expression in H1793 cells. Importantly, DPN induced an increase in NRF-1 mRNA in H1793, and co-treatment of H1793 with E<sub>2</sub> and R,R-THC inhibited NRF-1 expression (Figure 12B). These data indicate that ER $\beta$  mediates E<sub>2</sub>-stimulated NRF-1 expression in H1793 and are in agreement with the observation that ER $\beta$  protein expression is more than twice that of ER $\alpha$  in H1793 cells (32).

MCF-10A cells are a non-tumorigenic mammary epithelial cell line that express ER $\beta$  but not ER $\alpha$  (139). To further explore the ER subtype contribution to NRF-1 activation, MCF-10A cells were treated with E<sub>2</sub> in the presence or absence of transiently transfected ER $\alpha$  (Figure 13). Treatment with E<sub>2</sub> resulted in a small but significant increase in NRF-1 mRNA expression. Transfection with ER $\alpha$  alone resulted in an increase in basal NRF-1 mRNA expression equal to the increase stimulated by E<sub>2</sub> treatment in the untransfected cells. This could be the result of residual hormones in the media activating the large amount of ER $\alpha$  now present in the cell. When ER $\alpha$  transfected MCF-10A cells were treated with E<sub>2</sub>, a 6-fold induction in NRF-1 mRNA was observed. These data indicate that both ER $\alpha$  and ER $\beta$  contribute to the E<sub>2</sub>-induction of NRF-1 mRNA expression in MCF-10A cells.

From the MCF-7, H1793, and MCF-10A experiments I concluded that the induction of NRF-1 in response to E<sub>2</sub> does not appear to be ER subtype specific.



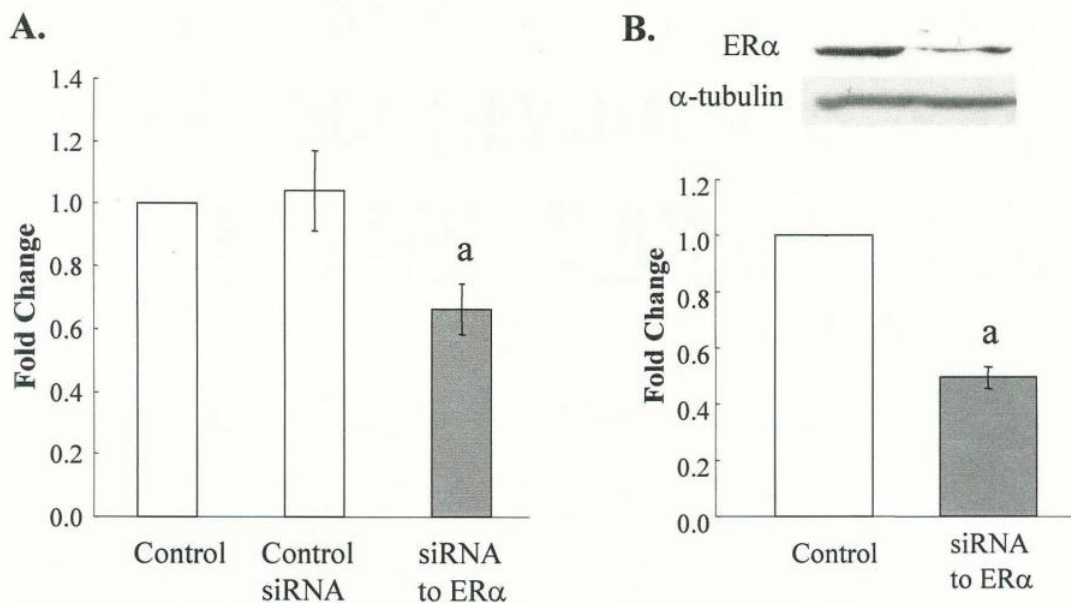
**Figure 13: Both ERα and ERβ upregulate NRF-1 gene transcription in MCF-10A**

**cells.** MCF-10A or MCF-10A cells transiently transfected with ERα were treated with EtOH or 10 nM E<sub>2</sub> for 4 h. The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3 separate experiments ± SEM. a =  $P < 0.05$  compared to EtOH; b =  $P < 0.05$  compared to E<sub>2</sub> alone or ERα alone

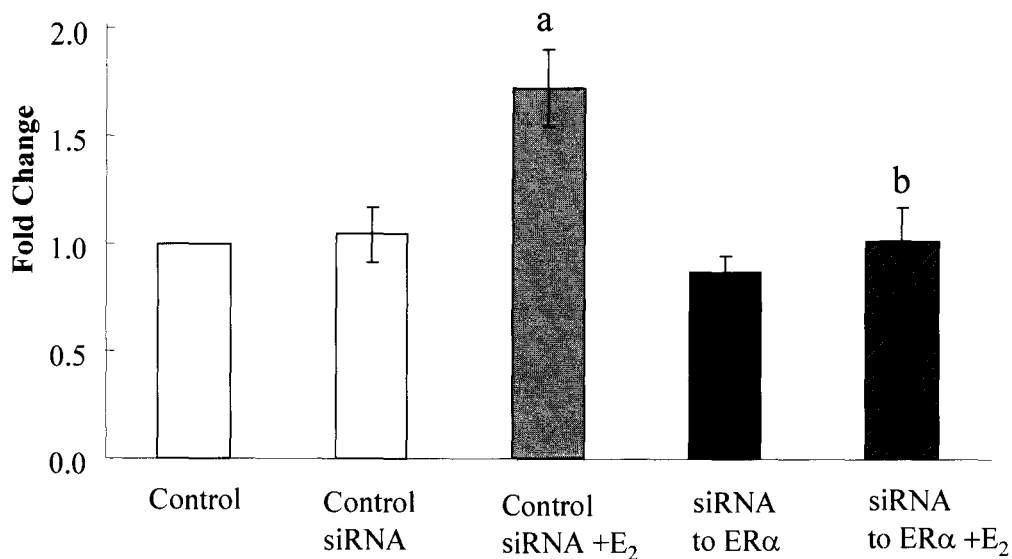
### **siRNA Knockdown of ER $\alpha$ and ER $\beta$ Inhibits the E<sub>2</sub>-induced Increase in NRF-1**

MCF-7 cells were transfected with siRNA targeting ER $\alpha$  or ER $\beta$  in order to further examine the subtype contribution to the E<sub>2</sub>-induced increase in NRF-1. Transfection of MCF-7 cells with siRNA targeting ER $\alpha$  lead to approximately a 50% decrease in both ER $\alpha$  mRNA and protein production (Figure 14). Subsequently, the ER $\alpha$ -siRNA transfected cells were treated for 4 h with E<sub>2</sub> and NRF-1 mRNA levels examined. In the presence of the siRNA targeting ER $\alpha$ , no increase in NRF-1 mRNA was seen with E<sub>2</sub> treatment (Figure 15). In a similar manner, MCF-7 cells were transfected with siRNA targeting ER $\beta$ . ER $\beta$  was decreased by 30% at the mRNA levels (Figure 16). Knockdown of ER $\beta$  resulted in a loss of the E<sub>2</sub>-mediated increase in NRF-1 mRNA (Figure 17). The observed significant decrease in NRF-1 in response to knockdown of both ER $\alpha$  and ER $\beta$  suggests that heterodimers of ER $\alpha$ /ER $\beta$  may be predominantly mediating the activation of this promoter.

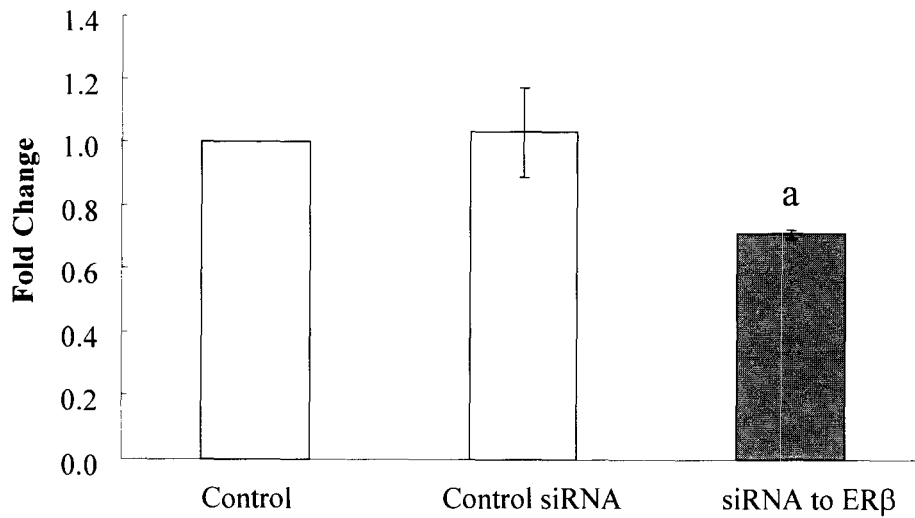




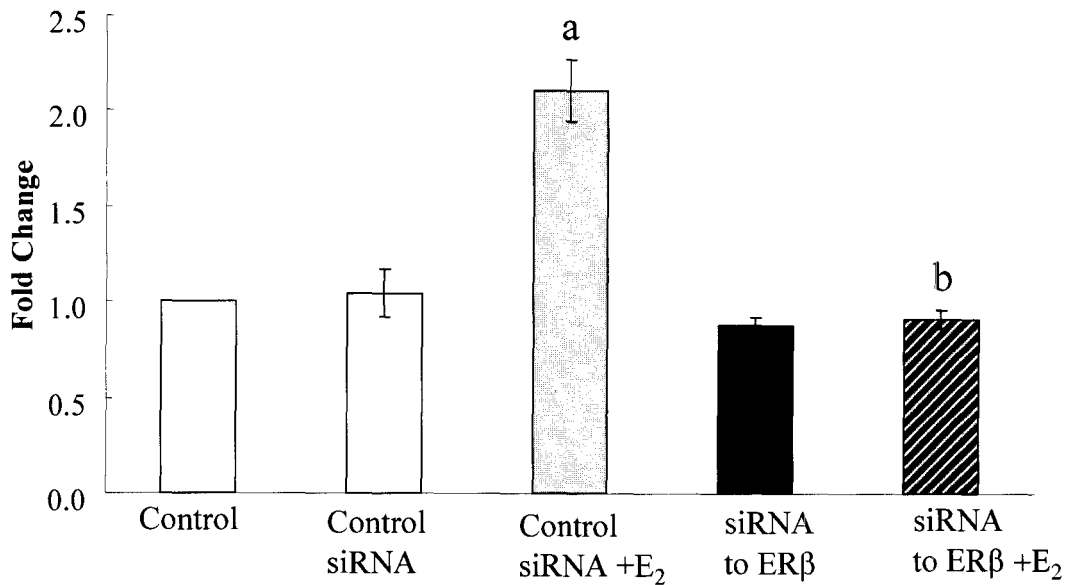
**Figure 14: siRNA knockdown of ER $\alpha$ .** (A) MCF-7 cells were not transfected or transfected with Control siRNA or siRNA targeting ER $\alpha$ . The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3-6 separate experiments  $\pm$  SEM. (B) A representative western blot of MCF-7 cells transfected with Control siRNA or siRNA targeting ER $\alpha$  is shown above a summary graph of the data normalized to  $\alpha$ -tubulin in the same blot. Values are the average of 3 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to Control



**Figure 15: siRNA knockdown of ER $\alpha$  inhibits the E<sub>2</sub>-induced increase in NRF-1 mRNA.** MCF-7 cells were not transfected or transfected with Control siRNA (treated with EtOH or E<sub>2</sub>) or siRNA targeting ER $\alpha$  (treated with or without E<sub>2</sub>). The cells were treated for 4 h and QRT-PCR was performed for NRF-1. The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3-6 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to Control siRNA; b =  $P < 0.05$  compared to E<sub>2</sub>



**Figure 16: siRNA knockdown of ERβ.** (A) MCF-7 cells were not transfected or transfected with Control siRNA or siRNA targeting ERβ. The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3-6 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to Control



**Figure 17: siRNA knockdown of ERβ inhibits the E<sub>2</sub>-induced increase in NRF-1 mRNA.** MCF-7 cells were not transfected or transfected with Control siRNA (treated with EtOH or E<sub>2</sub>) or siRNA targeting ERα (treated with or without E<sub>2</sub>). The cells were treated for 4 h and QRT-PCR was performed for NRF-1. The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3-6 separate experiments ± SEM. a =  $P < 0.05$  compared to Control siRNA; b =  $P < 0.05$  compared to E<sub>2</sub>

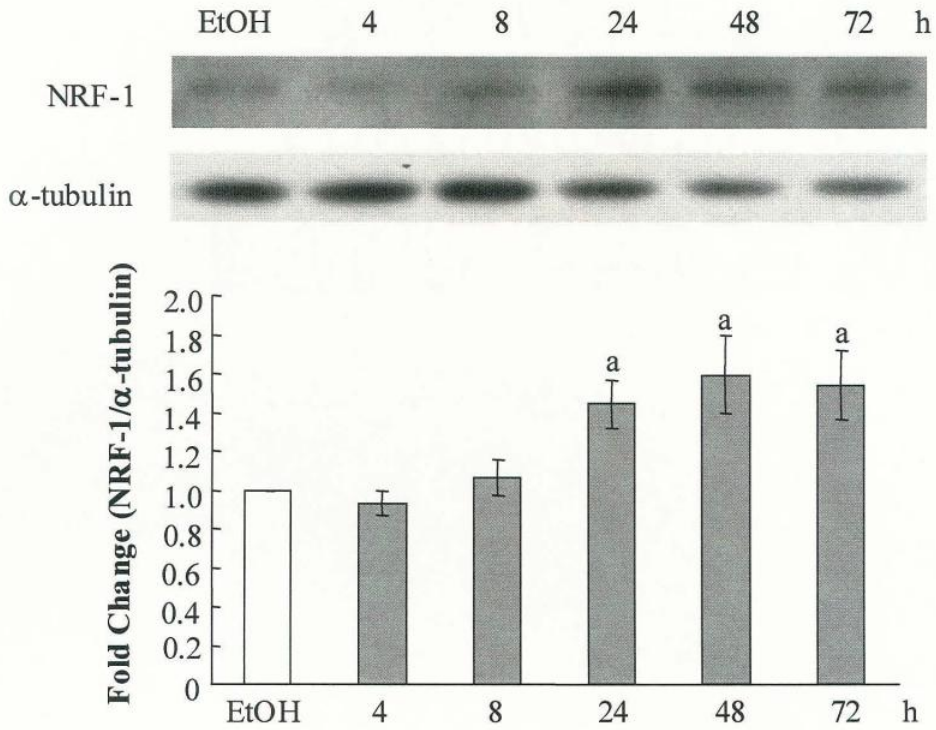
### **NRF-1 Protein Expression is Increased by E<sub>2</sub>**

Western blot analysis of NRF-1 protein expression after E<sub>2</sub> treatment revealed a time-dependent increase in NRF-1 in MCF-7 cells which was significant above control at 24 h of E<sub>2</sub> treatment (Figure 18). These results agree with the increase seen in NRF-1 mRNA expression with E<sub>2</sub> treatment but are delayed in time, as seen for other E<sub>2</sub>-target genes (151).

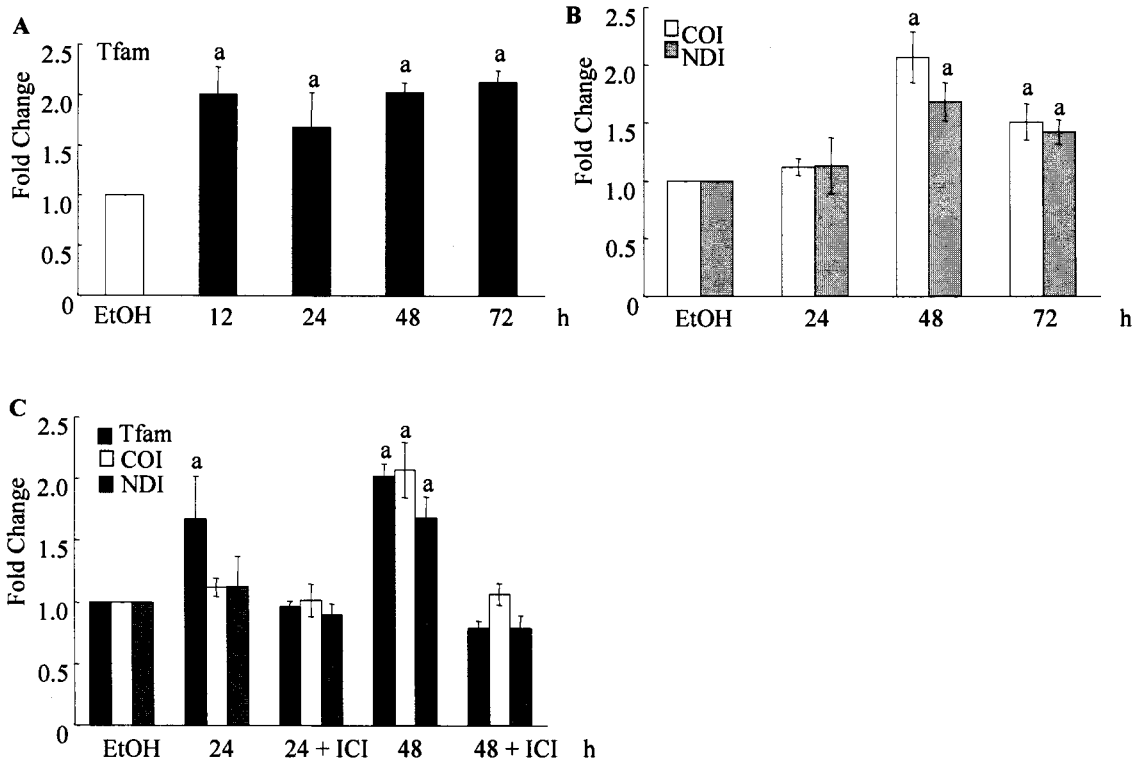
### **The Expression of Tfam, COI, and NDI mRNAs are Increased by E<sub>2</sub>**

The expression of Tfam was analyzed by QRT-PCR to determine if the E<sub>2</sub>-induced increase in NRF-1 resulted in a downstream upregulation in the transcription of a NRF-1 target gene, nuclear-encoded *TFAM*. Subsequently, QRT-PCR was used to examine an induction in the expression of two mitochondrial-encoded mRNAs, COI and NDI, which are targets of Tfam. Tfam mRNA was increased 12-72 h after E<sub>2</sub> treatment (Figure 19A) and ICI 182,780 inhibited this response (Figure 19C). The expression of mtDNA-encoded COI and NDI mRNAs was stimulated 48 to 72 h after E<sub>2</sub> treatment (Figure 19B), and this increase was similarly inhibited by ICI 182,780 (Figure 19C). Together these data demonstrate that the E<sub>2</sub>-induced increase in NRF-1 results in a downstream increase in its target gene *TFAM* and mtDNA-encoded Tfam target genes *MTCOI* and *MTNDI* in an ER-dependent manner in MCF-7 cells.

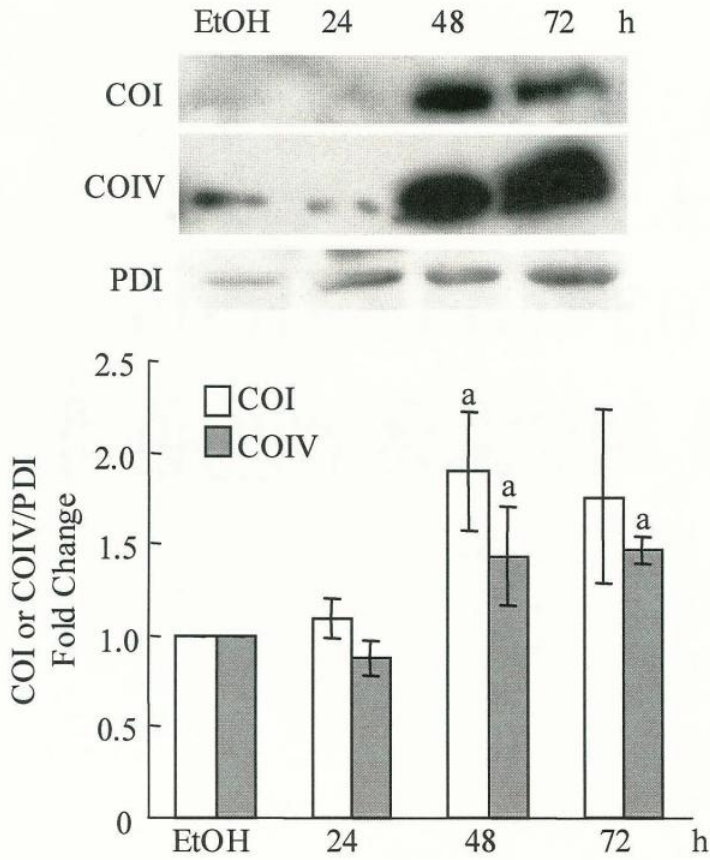
To further examine the downstream effects of E<sub>2</sub>-induced NRF-1 upregulation, the expression of COI (mtDNA encoded) and COIV (nuclear encoded) proteins was evaluated (Figure 20A). These proteins are both subunits of Complex IV in the electron



**Figure 18: NRF-1 protein levels are increased by E<sub>2</sub> treatment.** A representative western blot is shown for NRF-1 expression in 10 nM E<sub>2</sub>-treated MCF-7 cells above a summary graph for NRF-1 normalized to  $\alpha$ -tubulin from the same blot. Values are the average of 3 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH.



**Figure 19: Secondary gene expression is increased in  $E_2$ -treated MCF-7 cells.** (A) MCF-7 cells were treated with EtOH or 10 nM  $E_2$  for the indicated times and QRT-PCR was performed for Tfam (A, black bars) or COI and NDI (B, white bars and C, grey bars). Where indicated, cells were pretreated with ICI 182,780 for 6 h (C). The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3-6 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH.



**Figure 20: Protein expression of secondary genes is increased in E<sub>2</sub>-treated MCF-7 cells.** A representative western blot of COI and COIV mRNA expression in 10 nM E<sub>2</sub>-treated MCF-7 cells is shown above a summary graph of the data normalized to PDI in the same blots. Values are the average of 3 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH.



transport chain. COI is transcriptionally regulated by Tfam and TFBS while COIV is a target of NRF-1 (67). E<sub>2</sub> induced a significant increase in both proteins after 48 and 72 h.

### **Mitochondrial Biogenesis is Increased by E<sub>2</sub>**

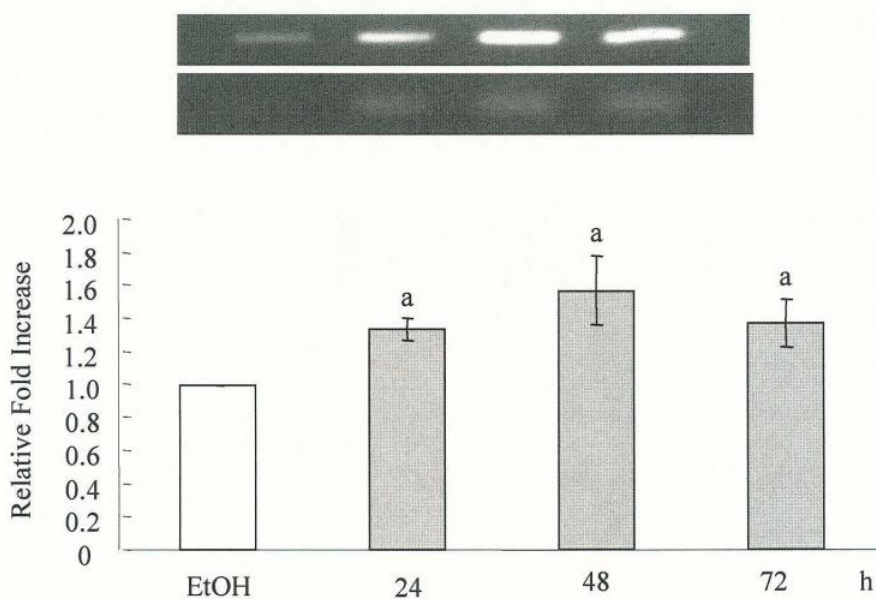
To determine if the previously described E<sub>2</sub>-induced increase in mRNA and protein levels in NRF-1 and its downstream targets lead to a subsequent induction in mitochondrial biogenesis, I measured two endpoints of mitochondrial biogenesis: mtDNA copy number and O<sub>2</sub> consumption. mtDNA copy number was assayed by semi-quantitative PCR using primers that recognized a 250 bp region of the mtDNA normalized to primers for the *18S* gene. E<sub>2</sub> significantly increased mtDNA copy number after 24, 48, and 72 h (Figure 21). Because Tfam is essential for mtDNA replication (99), these data are consistent with the increase in E<sub>2</sub>-induced Tfam mRNA beginning at 12 h.

O<sub>2</sub> consumption was used as a measure of MRC function in E<sub>2</sub>-treated MCF-7 cells. First, in preliminary experiments, I examined how serum starvation (IMEM with 10% CCS-FBS for 4 d) followed by treatment with or without 0.1% EtOH affected O<sub>2</sub> consumption. These experiments were completed to determine if EtOH or extended starvation had an effect on O<sub>2</sub> consumption in the MCF-7 cells. This concentration of EtOH was chosen because it is the highest amount of total EtOH added to cell culture media as vehicle for the E<sub>2</sub> treatment. I wanted to ensure that the EtOH itself was not affecting O<sub>2</sub> consumption. Additionally, I compared the rate of O<sub>2</sub> consumption in MCF-7 cells in CCS-FBS-containing media to those for MCF-7 cells grown normal growth media consisting of IMEM supplemented with 10% FBS. It was previously reported that treatment with serum devoid of steroids induced a shrinkage in mitochondria size and a

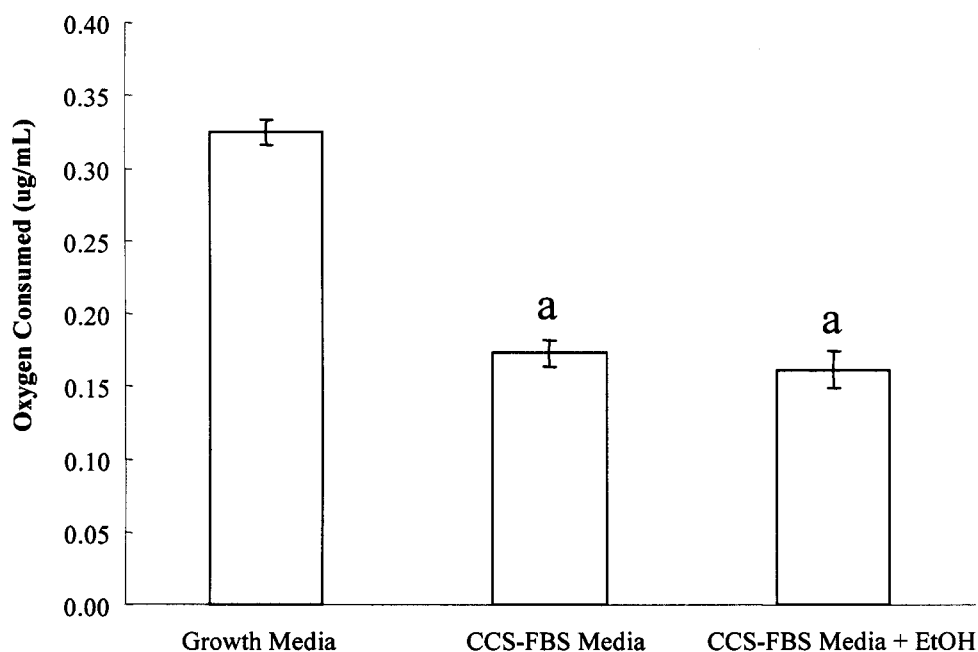
loss of well defined cristae, implying a decrease in mitochondrial activity, that was resolved by 4 d of culture in 10% FCS-containing media (117). Therefore, it is possible that placing the cells in CCS-FBS-containing media alone may affect their ability to consume O<sub>2</sub>.

EtOH added to 10% CCS-FBS IMEM did not affect the rate of O<sub>2</sub> consumption when compared to 10% CCS-FBS IMEM alone (Figure 22). However, growth in 10% CCS-FBS supplemented media did result in a decrease in O<sub>2</sub> consumption when compared to the Growth Media. Clearly, the absence of hormones in the CCS-FBS media lead to a decrease in mitochondrial O<sub>2</sub> consumption. This is consistent with previous reports indicating a lack of hormones in culture media or endogenous circulating E<sub>2</sub> leads to impaired MRC activity (80,114,117).

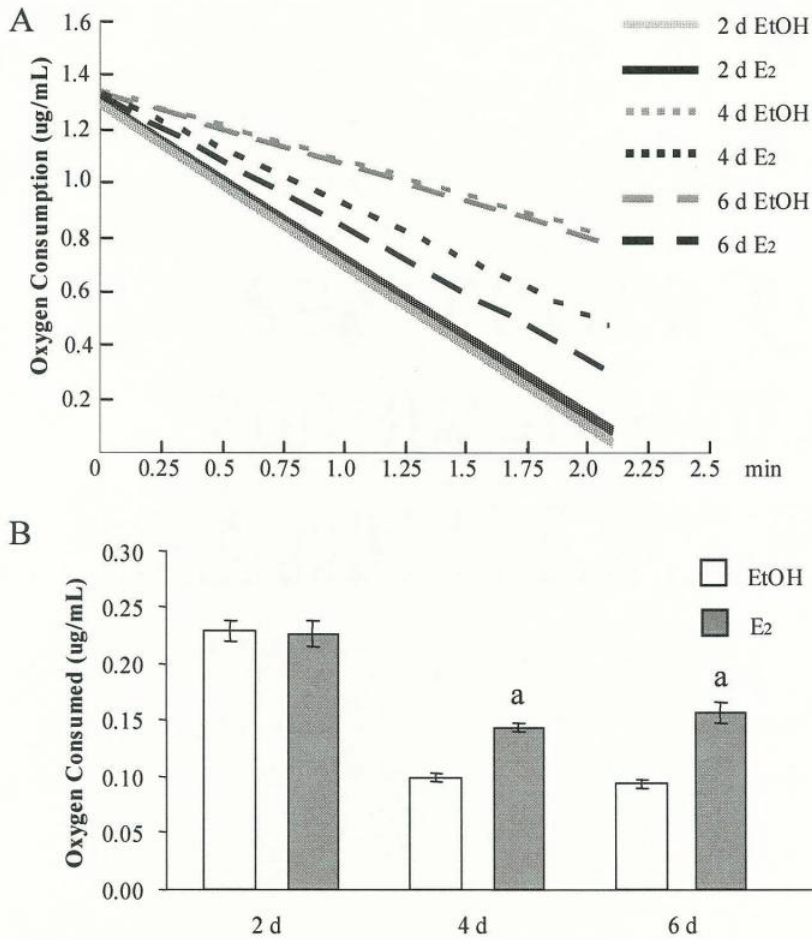
Subsequently, cells were treated with E<sub>2</sub> for 2, 4, or 6 days and O<sub>2</sub> consumption measured. A significant increase in O<sub>2</sub> consumption was seen after 4 and 6 d of E<sub>2</sub> treatment (Figure 23). This is consistent with the upregulation of COI and COIV proteins beginning at 48 h which, in concert with increased expression of MRC proteins resulting from NRF-1, Tfam, and TFB activity would lead to an increase in oxygen consumption in the cell. These data also agree with previous reports detailing a loss of respiratory chain function in the absence of E<sub>2</sub> (80,114,117).



**Figure 21: E<sub>2</sub> increases mtDNA copy number.** (A) MCF-7 cells were treated with EtOH or 10 nM E<sub>2</sub> for the indicated times. A summary graph of 4 experiments evaluating mtDNA normalized to *18S* expression by semi-quantitative PCR is shown along with a representative agarose gel. a =  $P < 0.05$  compared to EtOH



**Figure 22: EtOH does not affect O<sub>2</sub> consumption rates.** O<sub>2</sub> consumption was measured in MCF-7 cells grown in either 10% FBS containing IMEM (Growth Media), IMEM supplemented with 10% CCS-FBS (CCS-FBS Media), or IMEM containing 10% CCS-FBS and 0.1% EtOH for 4 d. The average rate of O<sub>2</sub> consumption from 3-4 separate experiments is shown  $\pm$  SEM in  $\mu\text{g}/\text{mL}$ . a =  $P < 0.05$  compared to Growth Media.



**Figure 23: E<sub>2</sub> increases O<sub>2</sub> consumption in MCF-7 cells.** (A) O<sub>2</sub> consumption was measured in MCF-7 cells treated with EtOH or 10 nM E<sub>2</sub> for the indicated number of days and representative slopes are shown. (B) The average rate of O<sub>2</sub> consumption from 4-6 separate experiments is shown ± SEM in ug/mL. a = *P* < 0.05 compared to EtOH for the same time point

### **siRNA Knockdown of NRF-1 in MCF-7 Cells**

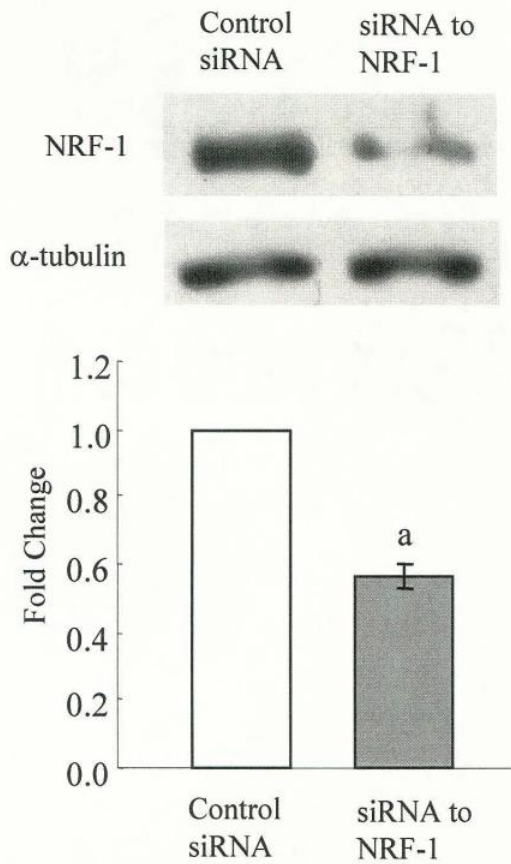
siRNA knockdown of NRF-1 followed by E<sub>2</sub> treatment was utilized in order to ensure that the E<sub>2</sub>-ER mediated positive effects on mitochondrial activity and gene expression were indeed controlled by NRF-1. MCF-7 cells were first transfected with siRNA targeting NRF-1 and WCE extracts were prepared and analyzed by Western Blot for NRF-1 protein expression. NRF-1 protein expression was decreased by 50% compared to control siRNA (Figure 24).

QRT-PCR was performed to examine secondary gene expression in parallel to experiments done in Figure 15. MCF-7 cells were transfected with Control siRNA or siRNA targeting NRF-1 and the expression of Tfam, COI, and NDI was examined in response to E<sub>2</sub>. Previously, all three genes were significantly up-regulated by E<sub>2</sub> treatment after 48 h. However, siRNA knockdown of NRF-1 followed by treatment with E<sub>2</sub> resulted in no increase in secondary gene expression. In these cells, Tfam, COI, and NDI were all significantly decreased in comparison to the nontransfected MCF-7 cell samples. Only, Tfam and NDI were significantly decreased compared to the Control siRNA (Figure 25). In addition to a lack of increased secondary gene mRNA, protein expression of two secondary genes was not increased by E<sub>2</sub> in the NRF-1 knockdown MCF-7 cells. Previously, a significant increase in COI and COIV was observed after 48 h of E<sub>2</sub> treatment in MCF-7 cells (Figure 16). However, MCF-7 cells transfected with siRNA to NRF-1 followed by E<sub>2</sub> treatment did not respond with a rise in protein expression (Figure 26).

Finally, in order to demonstrate that the ER mediated positive effects are directed through NRF-1, mtDNA copy number was measured in MCF-7 cells transfected with

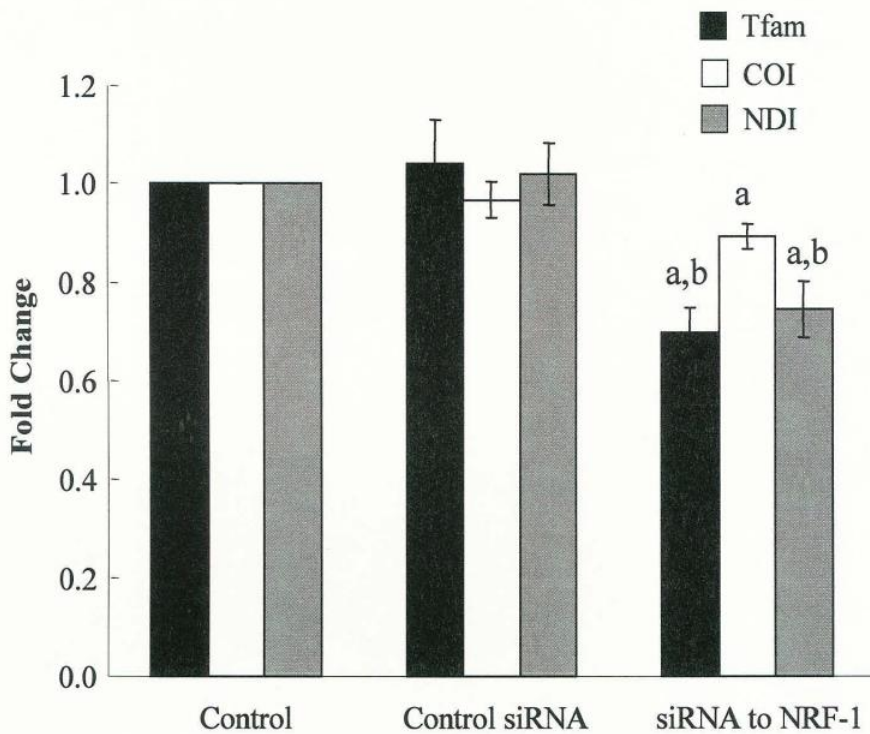
siRNA targeting NRF-1. Previously, mtDNA was observed to be increased by E<sub>2</sub>-treatment of MCF-7 cells at 48 h. It was assumed that the increase in mtDNA was due to an NRF-1 induced increase in Tfam and other mitochondrial transcription factors. To test this hypothesis, MCF-7 cells were transfected with siRNA targeting NRF-1 and an increase in mtDNA was measured at 48h of E<sub>2</sub>-treatment. siRNA knockdown of NRF-1 inhibited the increase in mtDNA observed in prior experiments (Figure 27).

Overall, the results from the NRF-1 knockdown experiments indicate that the positive effects on mitochondrial gene expression through ER are being mediated by the ER controlled increase in NRF-1 expression.

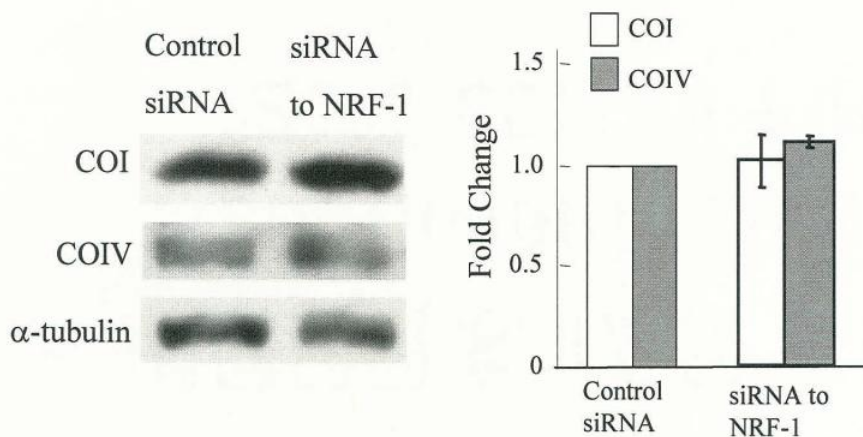


**Figure 24: Transfection with siRNA targeting NRF-1 decreases NRF-1 protein expression.** A representative western blot of MCF-7 cells transfected with Control siRNA or siRNA targeting NRF-1 is shown above a summary graph of the data normalized to  $\alpha$ -tubulin in the same blots. Values are the average of 3 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to Control siRNA

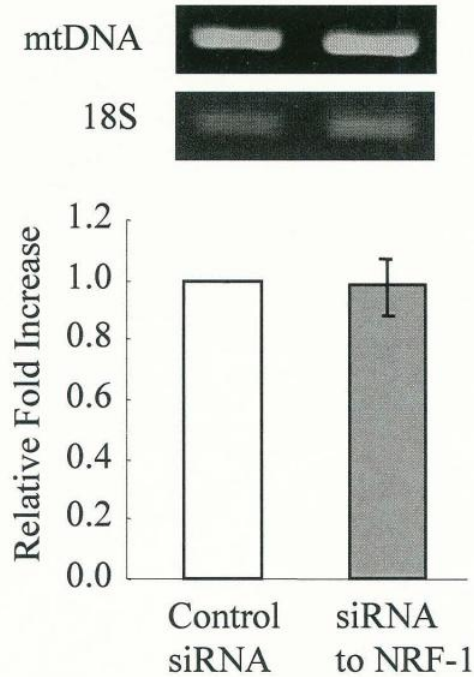




**Figure 25: Secondary gene expression is not increased in E<sub>2</sub>-treated MCF-7 cells transfected with siRNA targeting NRF-1.** MCF-7 cells were not transfected or transfected with Control siRNA (treated with EtOH) or siRNA targeting NRF-1 (treated with E<sub>2</sub>). The cells were treated for 48 h and QRT-PCR was performed for Tfam, COI, and NDI. The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3-6 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to Control; b =  $P < 0.05$  compared to Control siRNA



**Figure 26: Protein expression of secondary genes is not increased in E<sub>2</sub>-treated MCF-7 cells transfected with siRNA targeting NRF-1.** MCF-7 cells were transfected with Control siRNA (treated with EtOH) or siRNA targeting NRF-1 (treated with E<sub>2</sub>). WCE were made from 48 hr treated samples as described in Materials and Methods. A representative western blot of COI and COIV protein expression is shown next to a summary graph of the data normalized to  $\alpha$ -tubulin in the same blot. Values are the average of 3 separate experiments  $\pm$  SEM.



**Figure 27: The E<sub>2</sub>-induced increase in mtDNA copy number is inhibited in MCF-7 cells transfected with siRNA targeting NRF-1.** MCF-7 cells were transfected with Control siRNA (treated with EtOH) or siRNA targeting NRF-1 (treated with E<sub>2</sub>). Genomic DNA was made from 48 hr treated samples as described in Materials and Methods. A summary graph of 3 experiments evaluating mtDNA normalized to 18S expression by semi-quantitative PCR is shown along with a representative agarose gel.

## DISCUSSION

While it is clear that estrogens have protective effects on mitochondrial function in multiple cell types, it is unclear how these effects are mediated. Several theories have been postulated regarding the action of ER on mitochondrial structure and function. The results of my research demonstrate that E<sub>2</sub> directly activates a genomic mechanism of ER action that impacts mitochondrial function. E<sub>2</sub> increases transcription of NRF-1 which is an established integrator of nuclear and mitochondrial interactions (67). The increase in NRF-1 mRNA and protein in response to E<sub>2</sub> agrees with the report that NRF-1 protein was increased in the cerebral blood vessels of E<sub>2</sub>-treated Ovx rats (114). These results may explain previously observed increases in mitochondrial activity in response to E<sub>2</sub> (106,108,109,114), but do not rule out a role for ER action within the mitochondria as postulated by other investigators (110,152). A role for nongenomic E<sub>2</sub> action in mediating NRF-1 transcription is unlikely because inhibitors of the two predominant nongenomic signaling pathways in MCF-7 cells (153,154), MAP Kinase and PI3 Kinase, had no effect on E<sub>2</sub>-induced NRF-1 expression. Likewise, E<sub>2</sub> increased the transcription of genes regulated by NRF-1: *TFAM*, and subsequently, in a time-delayed manner, mtDNA-encoded Tfam-regulated COI and NDI mRNA. These data are in agreement with a report demonstrating a 2-fold increase in COI mRNA with E<sub>2</sub> treatment in MCF-7 cells (75); however, there was a time difference noted between that study and my results. I detected an increase in COI at 48 h whereas the previous report (75) indicated this increase at 12 and 24 h after E<sub>2</sub>-treatment of MCF-7 cells. The use of a 10-fold higher E<sub>2</sub> concentration (100 nM, non-physiologic) in the previous study may account for the

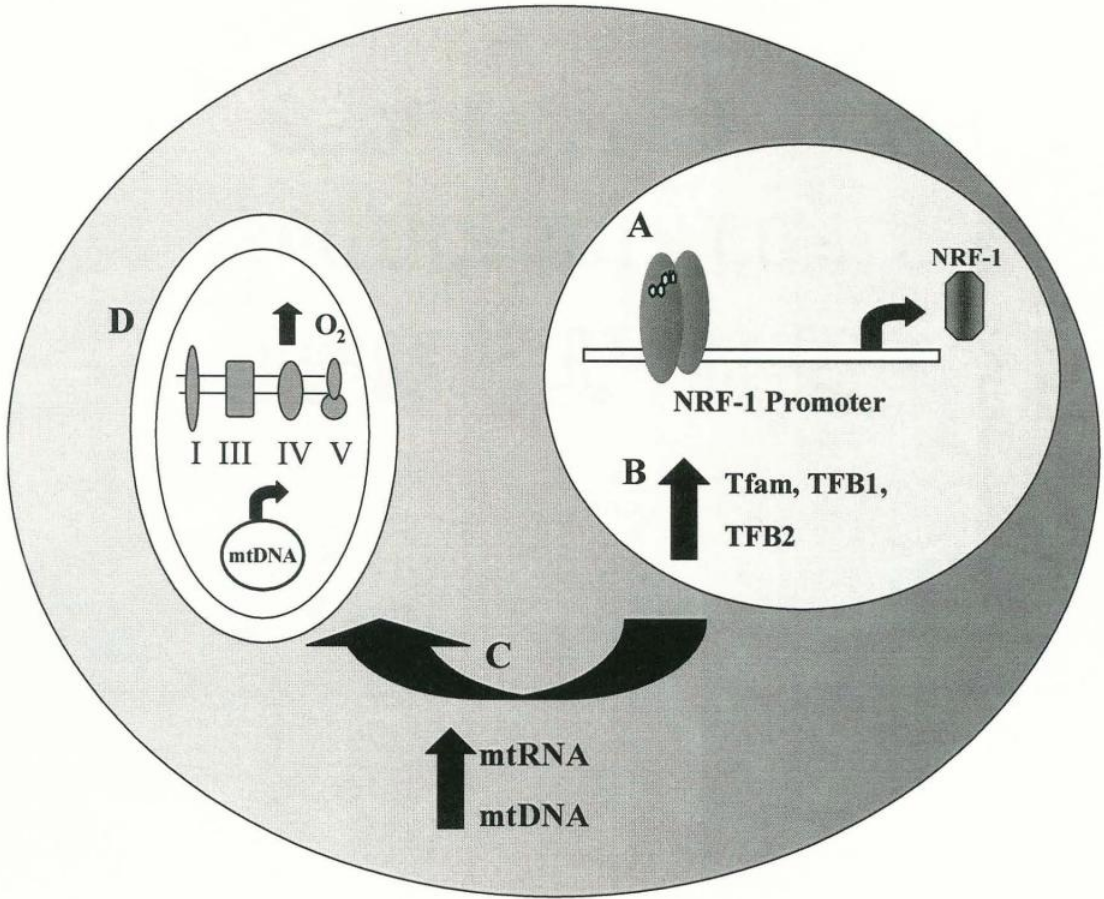
difference in induction times.

Consequent to the increase in NRF-1 and in secondary gene expression, including increased COI and COIV proteins, an increase in mtDNA and O<sub>2</sub> consumption was observed. The increase in mtDNA in concert with COI and NDI suggests a pathway by which E<sub>2</sub>-induced NRF-1 and a subsequent increased Tfam activates both transcription and mtDNA replication as detailed in Figure 28. In parallel, E<sub>2</sub> increased O<sub>2</sub> consumption which is an index of MRC activity. Whether this increase is due to increased MRC activity or an increase in mitochondrial biogenesis leading to more mitochondria per cell remains to be determined.

It is plausible that the secondary gene increases and increases in mitochondrial biogenesis are controlled by a yet undetermined protein and not NRF-1. siRNA studies targeting NRF-1 were undertaken in order to further solidify the relationship between an E<sub>2</sub>-induced increase in NRF-1 by ER leading to the positive effects on secondary mitochondrial gene expression and increases in mtDNA. These studies confirm that NRF-1 is essential for mediating the downstream effects of E<sub>2</sub>-ER (Figures 25-27). An increase in secondary gene expression, secondary gene protein production, and mtDNA was absent when NRF-1 was removed from the system despite the presence of E<sub>2</sub>.

In summary, my studies identified NRF-1 as a primary estrogen target gene. The E<sub>2</sub>-stimulated increase in NRF-1 was followed in a time-dependent manner by increases in NRF-1 regulated Tfam mRNA and then, in turn, by Tfam-regulated COI and ND1 mRNA expression. In concert with these changes in mRNA and protein expression, O<sub>2</sub> consumption and mtDNA copy number were increased by E<sub>2</sub>, indicating an increase in

mitochondrial biogenesis. Together these results suggest an important role for E<sub>2</sub> in upregulating mitochondrial activity in estrogen-responsive cells.



**Figure 28: A model of the regulation of NRF-1 by  $E_2$  and the subsequent downstream effects of this stimulation.** (A)  $E_2$ -occupied ER binds to the ERE in the NRF-1 promoter leading to an increase in NRF-1 promoter activity. (B) NRF-1 subsequently increases the transcription of its nuclear-encoded target genes including Tfam and TFBS. (C) Tfam and TFBS are imported into the mitochondria where Tfam increases mtDNA replication and both Tfam and TFBS increase mtDNA transcription. (D) Resulting increases in the levels of MRC proteins leads to an increase in  $O_2$  consumption through Complex IV.

## CHAPTER III

### ANALYSIS OF THE NUCLEAR RESPIRATORY FACTOR-1 PROMOTER REGION AND CONTRIBUTION OF THE PUTATIVE ESTROGEN RESPONSE ELEMENT

#### INTRODUCTION

Expression of NRF-1 has been shown to be increased by a variety of environmental stimuli and hormones. NRF-1 mRNA was induced by electrical stimulation in cardiomyocytes (155), exogenous addition of a creatine analogue (156), activation of AMP kinase (156), and stimulated following bouts of exercise in skeletal muscle (94). Thyroid hormone ( $T_3$ ) increases NRF-1 gene expression through a hormone responsive element in the NRF-1 promoter (157,158). While glucocorticoids and estrogens have both been suggested to play a critical role in mitochondrial upregulation and mitochondrial gene expression (76,159), no one has evaluated if these hormones directly regulate NRF-1 transcription. Interestingly, a recent study revealed that NRF-1 protein expression was increased in cerebral blood vessels of Ovx rats chronically treated with  $E_2$  compared to Ovx control, suggesting that estrogen may regulate NRF-1 transcription (114).

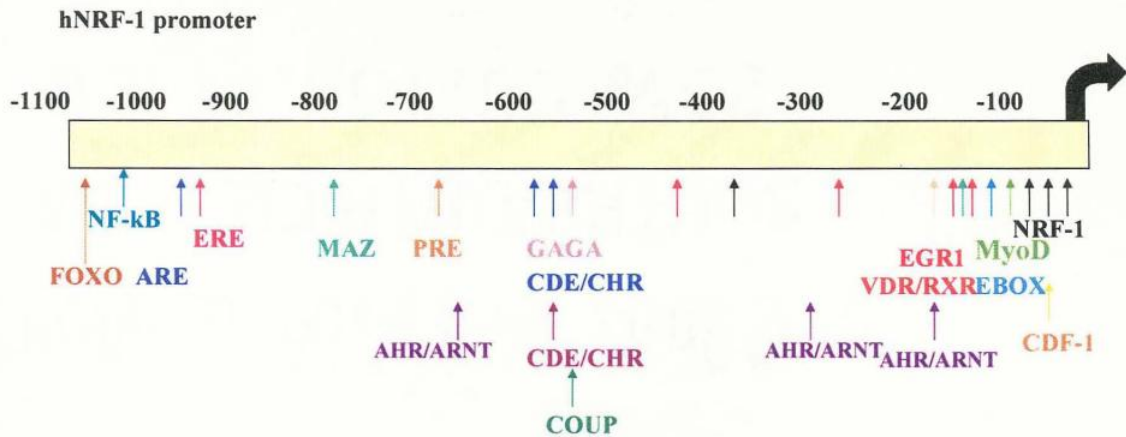
The Gene2Promoter program (160) was used to analyze the NRF-1 gene. Gene2Promoter scans the starting region of a target gene for promoter motifs and predicts the region most likely to contain the promoter in that gene. Gene2Promoter predicted the NRF-1 promoter to be within the -600 bp region of the NRF-1 start site. Using



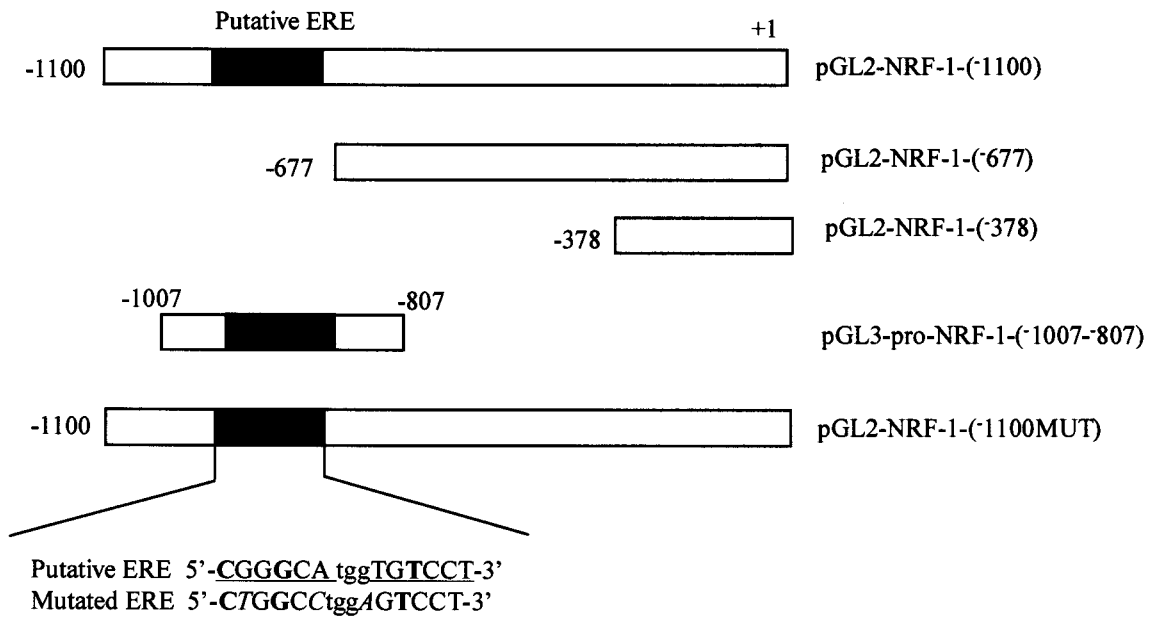
MatInspector (161), an extended -5000 bp region 5' of the NRF-1 start site was examined for putative transcription factor binding sites. However, only one ERE was detected at -963 to -944 of the NRF-1 transcriptional start site. A brief diagram detailing the -1100 bp region of the NRF-1 promoter is shown in Figure 29 listing some of the many putative transcription factor binding sites predicted by MatInspector. For the purposes of this Aim, I decided to expand my cloning efforts beyond the predicted -600 bp promoter region out to the putative NRF-1 ERE at -963 to -944 bp. It was previously reported that a -1000 bp region of the hNRF-1 gene contained the promoter based on transient transfections in COS, HeLa, and L6 Cells (162). In order to determine the contribution and function of the putative ERE, several approaches were taken. First, the -1100 bp region was cloned and inserted into a pGL2-basic-luciferase vector. Subsequently deletions were generated to examine smaller fragments of the promoter that did not contain the ERE. Next, the ERE was eliminated from the -1100 pGL2-construct using site directed mutagenesis, and finally, the 200 bp region surrounding the putative ERE was cloned into a pGL3-promoter-luciferase vector. By cloning the 200 bp surrounding the ERE, I was able to examine the activity of the ERE separated from the rest of the promoter. In Figure 30, the pGL2-basic-luciferase and pGL3-promoter-luciferase constructs tested in transient transfection assays are diagramed.

The putative NRF-1 ERE (5'-CGGGCA**tg**TGTCCT-3') differs from the palindromic consensus ERE (5'-AGGTCAgagTGACCT-3) by 2 bp changes in the 5' half-site and one bp change in the 3' half-site indicated in bold. These changes, while reducing the affinity of ER for the ERE, do not eliminate the site. In fact, all three of the changes in the ERE site in the NRF-1 promoter occur at nucleotide positions previously

identified as lower in importance to the binding of ER $\alpha$  (163). An adaptation of the original description detailing the importance of each position of the ERE is shown in Figure 31 along with the putative NRF-1 ERE and the proposed bp changes that were used to eliminate the ERE site in site-direct mutagenesis experiments (42,163).



**Figure 29: 5' promoter region of the hNRF-1 gene.** The -1100- +1 region of the hNRF-1 promoter was analyzed using MatInspector to identify transcription factor binding sites as indicated. Some of the less common sites shown include CDF-1 = Cell cycle-dependent element; CDE/CHR =Cell cycle gene homology region (CDE/CHR tandem elements regulate cell cycle dependent repression); EGR1 = early growth response 1; MyoD: Myogenic regulatory factor MyoD (myf3)  
EBox:Member of b-zip family, induced by ER damage/stress, binds to the ERSE in association with NF-Y; TCF/LEF-1: involved in the Wnt signal transduction pathway.



**Figure 30: Promoter deletion and ERE mutant constructs of the hNRF-1 promoter.**

The diagram shows the structures of the pGL2-basic- and pGL3-promoter- luciferase expression vectors containing various lengths of 5'-flanking regions of the human NRF-1 gene promoter and a construct in which the ERE was mutated. The ERE is underlined in the wt putative NRF-1 ERE. The nucleotides in bold are different from the consensus ERE and the nucleotides in italics indicate mutations compared to the wt NRF-1 ERE in the mutated pGL2-NRF-1-(-1100MUT) construct.

**Consensus ERE (5'-3')**

**A** **G****G****T** **C****A** T G G **T****G****A****C****C****T**

**Putative NRF-1 ERE**

**C** **G****G****G** **C****A** T G G **T****G****T****C****C****T**

**Mutated NRF-1 ERE**

**C** **T** **G****G** **C****C** T G G **A** **G** **T****C****C****T**

**Figure 31: Comparison of the nucleotide sequence of consensus ERE with the putative NRF-1 ERE and mutated NRF-1 ERE.** The height of the nucleotides is relative to importance for ER $\alpha$  binding. The color scheme and model are based on motif screening of ERE binding sites in Chromosomes 21 and 22 by Carroll *et al* (163). The importance of each bp has been review in detail by Klinge (42). Nucleotides in black are different from the consensus ERE. The nucleotides changed in the synthetic mutated NRF-1 ERE are circled.

## MATERIALS AND METHODS

### **Cloning of the NRF-1 Promoter**

Primers were designed to the -1100 bp region of the NRF-1 promoter using Primer3 and are listed in Table 3. The -1100 bp fragment of hNRF-1 (NM\_005011) was cloned from human genomic DNA (Roche) into the pCR-Blunt II-Topo vector (Invitrogen). After sequencing, restriction digestion (*XhoI/SacI*) was used to subclone the -1100 promoter fragment into the pGL2-basic-luciferase vector (Promega). Primers were then designed to clone two smaller fragments of the NRF-1 promoter at approximately 300 bp intervals. The primers designed resulted in a -677 bp fragment and a -378 fragment (see Table 3). Primers were also designed to clone the 200 bp region surrounding the putative NRF-1 ERE. The -677, -378, and 200 bp fragments were inserted into the pCR-Blunt II-Topo vector, sequenced, and subsequently digested (*XhoI/SacI*), and placed into either the pGL2-basic-luciferase plasmid or the pGL3-promoter-luciferase plasmid. The plasmids are named after their corresponding fragments as noted in Figure 30.

The pGL2-NRF-1(-1100) mutant ERE (pGL2-NRF-1(-1100MUT)) was generated using the QuickChange Mutagenesis Kit (Stratagene) according to the manufacturer's protocol using the primers listed in Table 3. The primers introduced 2 bp changes into the 5'-half site and 1 bp change in the 3'-half site abolishing the palindromic ERE as described in Figure 31. Presence of the correct mutation was confirmed by sequencing.

<b>Primer Name</b>	<b>Primer Sequence</b>
NRF-1-1100 Forward	5'-GAGTCTCCTGACGACACTAA-3'
NRF-1 -1100 Reverse	5'-GAAGTTCTACTCAGAGCGGC-3'
NRF-1 -677 Forward	5'- TATCTGCACAGCACGAGAC-3'
NRF-1 -677 Reverse	5'- GAAGTTCTACTCAGAGCGGC-3'
NRF-1 -378 Forward	5'-CGCCGCTTCTCCGGGGCGTC-3'
NRF-1 -378 Reverse	5'- GAAGTTCTACTCAGAGCGGC-3'
NRF-1 -1007- -807 Forward	5'-GAGTCTCCTGACGACACTAA-3'
NRF-1-1007- -807 Reverse	5'-TAACGGTGGAGACACACGAG-3'
Mutant ERE Forward	5'-CCCCTCTGCCTGGCCTGGAGTCCTGTAAT-3'
Mutant ERE Reverse	5'-GCCTCCCAAAGTGCTGGTATTACAGGA-3'

**Table 3: Primer Sequences for Cloning and Mutagenesis of the NRF-1 Promoter Constructs**

## Dual Luciferase Assays in MCF-7 and HEK-293 Cells with NRF-1 Promoter

### Constructs

MCF-7 cells were plated in 24-well plates at a density of  $1 \times 10^5$  cells/well in growth media consisting of IMEM with 10% FBS. HEK-293 cells were plated in 24-well plates at a density of  $1 \times 10^5$  cells/well in growth media consisting of Alpha MEM (Invitrogen) with 10% FBS. After 24 h in growth media, the cells were placed in 10% CCS-FBS supplemented media and transfected. The MCF-7 cells were transfected with pGL2-NRF-1(-1100), pGL2-NRF-1(-1100MUT), pGL2-NRF-1(-677), pGL2-NRF-1(-378), pGL3-pro-NRF-1(-1007-807), pGL3-pro-luciferase, or pGL2-basic-luciferase parental vector using FuGene 6 at a 3:1 FuGene 6 to DNA ratio. Each well received 250 ng of the luciferase reporter plasmid and 5 ng of a *Renilla* luciferase internal control reporter (pRL-tk) from Promega. Since HEK-293 cells are ER null, transient transfections in HEK-293 cells proceeded as described for the MCF-7 cells, however either 10 ng of pCMV-ER $\alpha$  (provided by B.S. Katzenellenbogen) or pcDNA3.1-ER $\beta$  (provided by Jan-Ake Gustafsson) were also cotransfected with the reporter and *Renilla* vector. Twenty-four hours after transfection, triplicate wells were treated with EtOH (vehicle control), 10 nM E<sub>2</sub>, 100 nM ICI 182,780, or a combination of ICI 182,780 and E<sub>2</sub>. The cells were harvested 30 h post-treatment using Promega's Passive Lysis buffer. Luciferase and *Renilla* luciferase activities were determined using Promega's Dual Luciferase assay in a Plate Chameleon Luminometer (BioScan) (32). Firefly luciferase was normalized by *Renilla* luciferase to correct for transfection efficiency. Fold induction was determined by dividing the averaged normalized values from each



treatment by the EtOH value for each transfection condition within that experiment. Values were averaged from multiple experiments as indicated in the Figure legends.

### **Chromatin Immunoprecipitation Assays**

Chromatin Immunoprecipitation (ChIP) Assays followed methods previously described by Metiever *et al.* (164) and detailed in a flow chart in Figure 32. For the ChIP assays, MCF-7 cells were grown to 50% confluency in 150 mm plates and placed in 10% CCS-FBS supplemented IMEM media for 72 h prior to treatment. Approximately  $4 \times 10^6$  cells per immunoprecipitation (IP) were treated with 2.5  $\mu\text{M}$   $\alpha$ -amanitin for 2 h. The medium was then removed and replaced with media containing 10 nM  $\text{E}_2$  or EtOH for 60 min. Chromatin-protein complexes were crosslinked by incubation with 1.5% formaldehyde for 5 min at 37°C, and the cells were collected after 2 washings with PBS in Collection Buffer (100 nM Tris [pH 9.4], 100 nM Dithiothreitol (DTT), and 1 X Protease Inhibitors (Roche)). The samples were then incubated on ice for 15 min and then at 30°C for 15 min. The cells were disrupted sequentially by vortexing and 5 min of centrifugation at  $3000 \times g$  at 4°C with 5 ml Buffer A (10 mM EDTA, 0.5 mM EGTA, 10 mM HEPES [pH 6.5] and 0.25% Triton X-100) and then 5 ml Buffer B (1 mM EDTA, 0.5 mM EGTA, 10 mM HEPES [pH 6.5] and 200 mM NaCl). The cells were sonicated 15 times for 10 s at a setting of 1 in a (Sonifier Cell Disruptor, Branson, Danbury, CT) in 200  $\mu\text{l}$  lysis buffer [10 mM EDTA, 50 mM Tris-HCl [pH 8.0], 1% SDS]. 20  $\mu\text{l}$  of extract was removed and a final concentration of 200 mM NaCl was added to the sample which was then incubated overnight at 65°C to facilitate decrosslinking of the protein-DNA complexes. 5  $\mu\text{l}$  was run on a 0.8% agarose gel to ensure that the sonication resulted in

fragments in the range of 2-0.5 kb. The remainder of the sonicated sample was diluted 2.5-fold in IP buffer (2 mM EDTA, 100 mM NaCl, 20 mM Tris-HCl [pH 8.1], and 0.5% Triton X-100). The samples were precleared by a 2 h incubation with 30  $\mu$ L Protein A/G beads containing salmon sperm DNA (Upstate). The samples were then incubated with either anti-ER $\alpha$  (HC-20) antibody, anti-ER $\beta$  antibody (H-150), or normal rabbit IgG (all from Santa Cruz) overnight. Antibody-protein-DNA complexes were collected by a 2 h incubation with 50  $\mu$ L Protein A/G beads. The beads were washed by incubation with agitation for 10 min per wash with the following buffers: Low Salt Buffer (2 mM EDTA, 20 mM Tris-HCl [pH 8.0], 0.1% SDS, 1% Triton X-100, 150 mM NaCl), High Salt Buffer (2 mM EDTA, 20 mM Tris-HCl [pH 8.0], 1% Triton X-100, 500 mM NaCl), LiCl Buffer (1 mM EDTA, 10 mM Tris-HCl [pH 8.0], 1% NP-40, 1% Deoxycholate, 0.25 M LiCl), and TE (10 mM Tris, pH 8.0, 1 mM EDTA). Immune complexes were eluted by incubation with Elution Buffer (1% SDS with 0.1 M NaHCO<sub>3</sub>) for 30 min with vortexing every 5 min. A final concentration of 200 mM NaCl was added to the eluted fraction and the samples were incubated overnight at 65°C. The DNA was purified using the Qiagen PCR Clean-Up Kit (Qiagen), and probed for target sequences. Each PCR reaction included 1X Green GoTaq Flexi Buffer, 1.5 mM MgCl<sub>2</sub>, 0.2 mM of each dNTP, 1  $\mu$ M reverse primer, 1  $\mu$ M forward primer, 1.25 units GoTaq, 3-7  $\mu$ L eluted DNA, and nuclease free water to a total volume of 20  $\mu$ L. Primers for the putative NRF-1 ERE probed the -761 to -1032 bp region (see Table 4). As a positive control, primers flanking the established ERE in the human pS2 (Trefoil Factor 1; *TFF1*) gene promoter were used (164). As a negative control, primers were designed to a distal region (-3060 to -3208) of

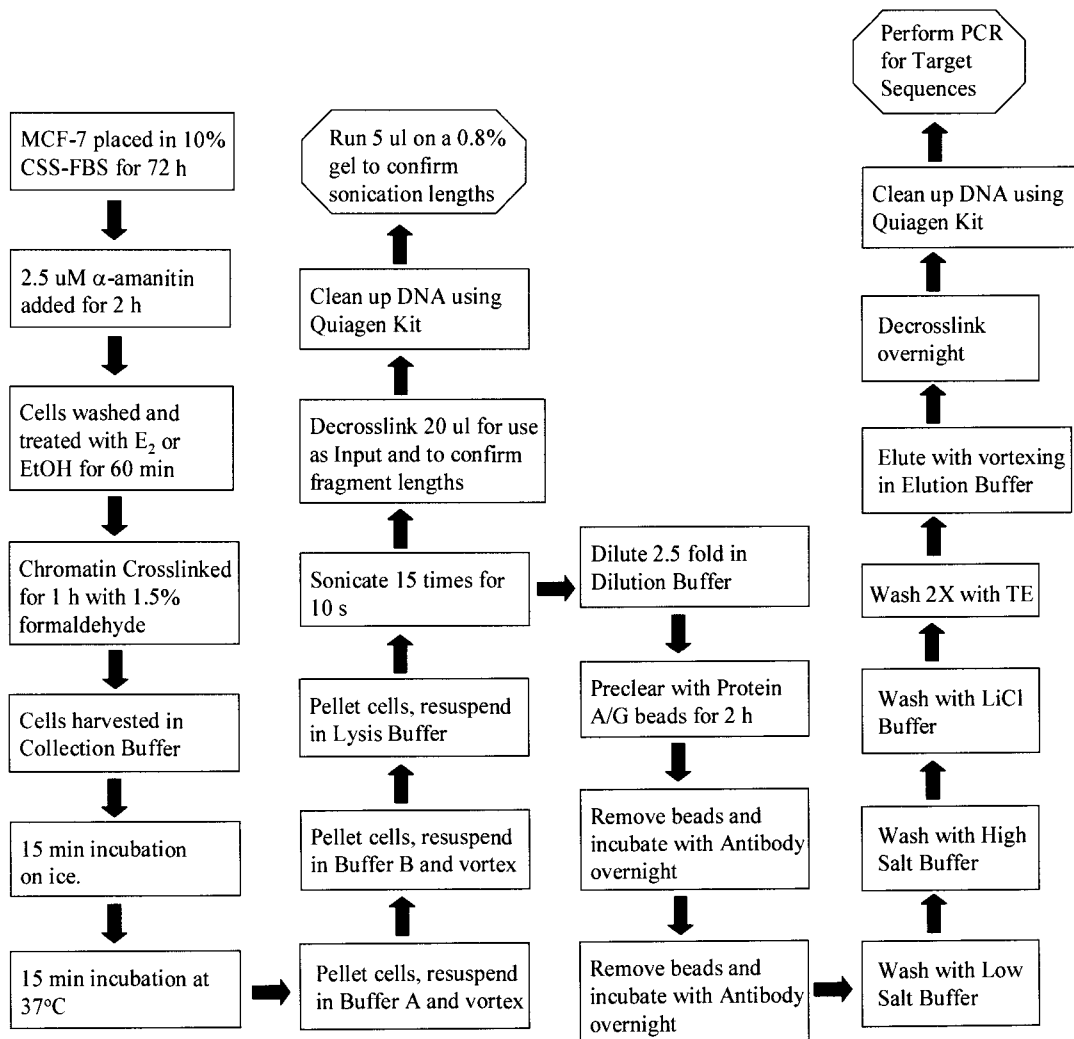
the NRF-1 promoter (see Table 4). PCR products were resolved on 2% agarose gels and stained with ethidium bromide.

### **ER $\alpha$ and ER $\beta$ Proteins**

Recombinant human ER $\alpha$  (165) and recombinant human ER $\beta$ 1 (166) were expressed by baculovirus infection of Sf21 cells and were used for electrophoretic mobility shift assay (EMSA). The concentration of ER $\alpha$  and ER $\beta$  was determined by specific [<sup>3</sup>H]E<sub>2</sub> binding in a hydroxyapatite assay (167) and was 150 fmol/ $\mu$ g dimer concentration for ER $\alpha$  and 95 fmol/ $\mu$ g dimer concentration for ER $\beta$ 1.

### **EMSA and ER-NRF-1-ERE K<sub>i</sub> Determination**

The DNA oligonucleotide sequences used in EMSA were as follows: putative NRF-1-ERE from the human NRF-1 promoter 5'-GGAAGCCGGGCATGGTIGTCCTGT-3' a consensus ERE, *i.e.*, EREc38 (5'-AGGTCACAGTGACCT-3') (168). EMSA was performed as previously described (58). In brief, oligomers were fill-in labeled with [<sup>32</sup>P] $\alpha$ -dTTP (NEN) using the Klenow fragment of *E. coli* Pol. I. as previously described (169). Baculovirus-expressed ER $\alpha$  and ER $\beta$  (41) were preincubated with E<sub>2</sub> for 1h at 4<sup>0</sup> C prior to addition of the [<sup>32</sup>P]EREc38. All reactions used a fixed concentration of ER $\alpha$  (1.2 nM) or ER $\beta$  (1.5 nM) and included 60 mM KCl, 0.75 mg/ $\mu$ l purified BSA (New England Biolabs); 25  $\mu$ g/ml poly(dI-dC) (Sigma); and 15% (v/v) glycerol in a final volume of 20  $\mu$ l. The ER-[<sup>32</sup>P]ERE-ER reaction was incubated for 30 min at room temperature prior to separation on 4% non-denaturing polyacrylamide gels (169). Dried EMSA gels were analyzed using a Packard Instruments Instant Imager and associated



**Figure 32: Flow Chart Describing the ChIP Method.** ChIP experiments proceeded as detailed above and in Materials and Methods.

<b><u>Primer Name</u></b>	<b><u>Primer Sequence</u></b>
NRF-1 ChIP Forward	5'- GGTCCCAGGACTCAAACAA-3'
NRF-1 ChIP Reverse	5'- CAGGTGCCTGAGAAGTAGGG-3'
pS2 ChIP Forward	5'- GGCCATCTCTCACTATGAATCACTTCTGC-3'
pS2 ChIP Reverse	5'-GGCAGGCTCTGTTTGCTTAAAGAGCG-3'
NRF-1 Distal ChIP Forward	5'-ACCCTTGTGGAAACAGCATC-3'
NRF-1 Distal ChIP Reverse	5'-AAACGGACTGGGCTTACCTT-3'

**Table 4: Primers Used for ChIP Experiments**

software, Packard Imager for Windows v2.04 (16).

To measure  $K_i$  in competition assays, reactions included 1.2 nM ER $\alpha$  or 1.5 nM ER $\beta$ , 1.1 nM [ $^{32}$ P]EREc38 (5'-CCAGGTCAGAGTGACCTGAGCTAAAATAACACATT-3') (168), 100 nM E $_2$ , and 0.5 nM – 20,000 nM unlabeled NRF-1-ERE (169). The concentrations of free and ER-bound [ $^{32}$ P]EREc38 were fitted to the one-site binding model (determination coefficient  $R^2 > 0.93$  and  $0.98$  for ER $\alpha$  and ER $\beta$  with no ligand,  $0.97$  and  $0.94$  with E $_2$ ). The value of the IC $_{50}$  was determined by equation of the Pseudo-Hill plot:  $\log \%/(100-\%) = \text{nlog}\{[I] + \text{nlogIC}_{50}$ , where  $\%$ = percent competition of specific binding, I=competitor (Figure 32B, left panel). The  $K_i$  was calculated from the IC $_{50}$ , using the equation of Cheng and Prusoff (170):  $K_i = \text{IC}_{50}/(1 + [\text{radioligand}]/K_d)$ .

### **Statistics**

All statistical analyses were performed as described in Chapter II.

## RESULTS

### **Luciferase Reporter Assays with E<sub>2</sub> and ICI 182,780**

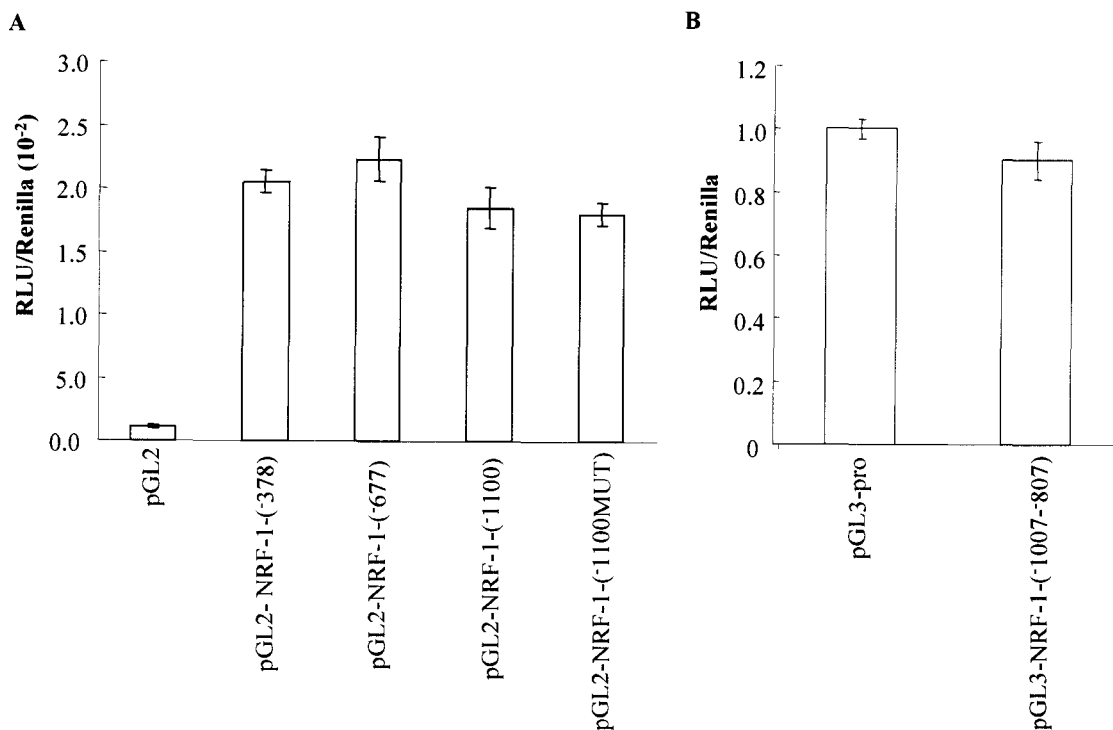
MCF-7 cells were first transfected with the NRF-1 promoter constructs and parental vectors and the basal activity examined (Figure 33). The pGL2-NRF-1-(<sup>-378</sup>), pGL2-NRF-1-(<sup>-677</sup>), pGL2-NRF-1-(<sup>-1100</sup>), and pGL2-NRF-1-(<sup>-1100MUT</sup>) all displayed a ~20-fold increase in activity compared to the parental vector, pGL2-basic-luciferase which contains no promoter elements controlling the luciferase gene (Figure 33A). This increase in activity confirms the presence of a promoter region within all of the constructs. The pGL3-pro-luciferase parental vector and the pGL3-NRF-1-(<sup>-1007-<sup>-</sup>807</sup>) displayed equivalent activity (Figure 33B). This activity level is much higher when compared to the activity levels seen in the pGL2 constructs due to the presence of a strong SV40 promoter in the pGL3-pro-luciferase vector. This vector is designed to test putative enhancer sequences placed in front of the SV40 driven luciferase gene.

The effects of E<sub>2</sub> on promoter function were first assayed by transient transfection in MCF-7 cells (Figure 34). E<sub>2</sub> stimulated an increase in luciferase activity from the pGL2-NRF-1-(<sup>-1100</sup>) construct which contains the putative ERE but not the pGL2-NRF-1-(<sup>-677</sup>) or (<sup>-378</sup>) constructs lacking the ERE. Treatment with ICI 182,780 had no effect on luciferase activity from the full length promoter construct (Figure 34); however, cotreatment of cells with E<sub>2</sub> and ICI 182,780 ablated the E<sub>2</sub>-induced increase in luciferase activity. Transfection of the pGL3-pro-NRF-1-(<sup>-1007-<sup>-</sup>807</sup>) construct yielded an increase in luciferase activity in the presence of E<sub>2</sub> that was blocked by cotreatment with ICI 187,780 (Figure 35). This response was smaller than that seen in the full length promoter

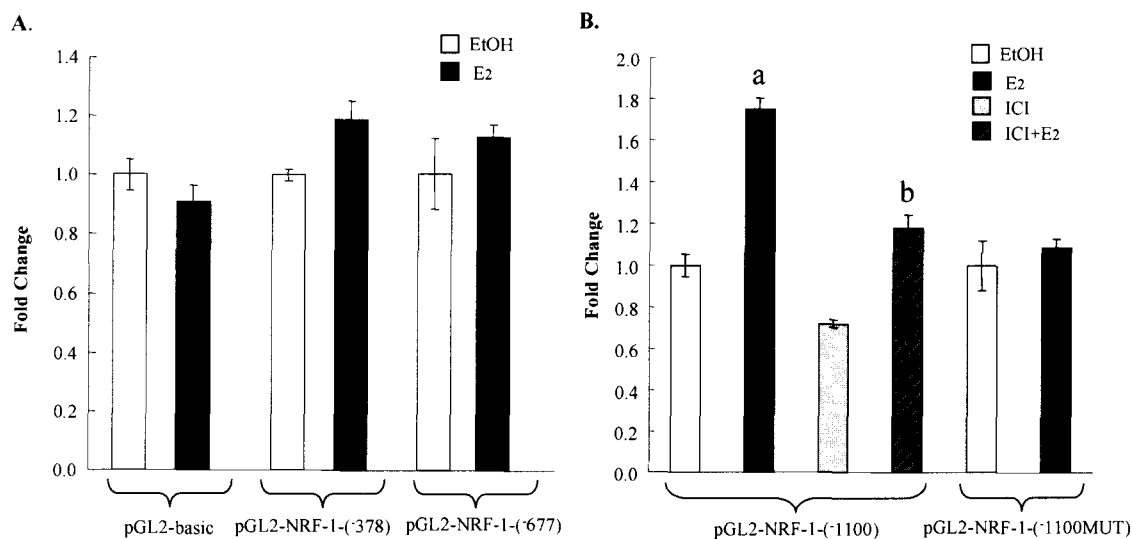
construct. These data indicate that the region from -1007 to -807 containing the putative ERE is estrogen-responsive.

Mutagenesis was performed to eliminate the putative ERE from the pGL2-NRF-1(-1100) construct to further evaluate the role of the putative ERE in the estrogen response. As demonstrated in Figure 34B, when nucleotides within the core ERE were mutated within the pGL2-NRF-1(-1100) construct, E<sub>2</sub> did not stimulate luciferase activation. These results indicate that the E<sub>2</sub>-induced reporter activity is mediated by ER interaction with the NRF-1 promoter region containing the putative ERE in transient transfection assays.

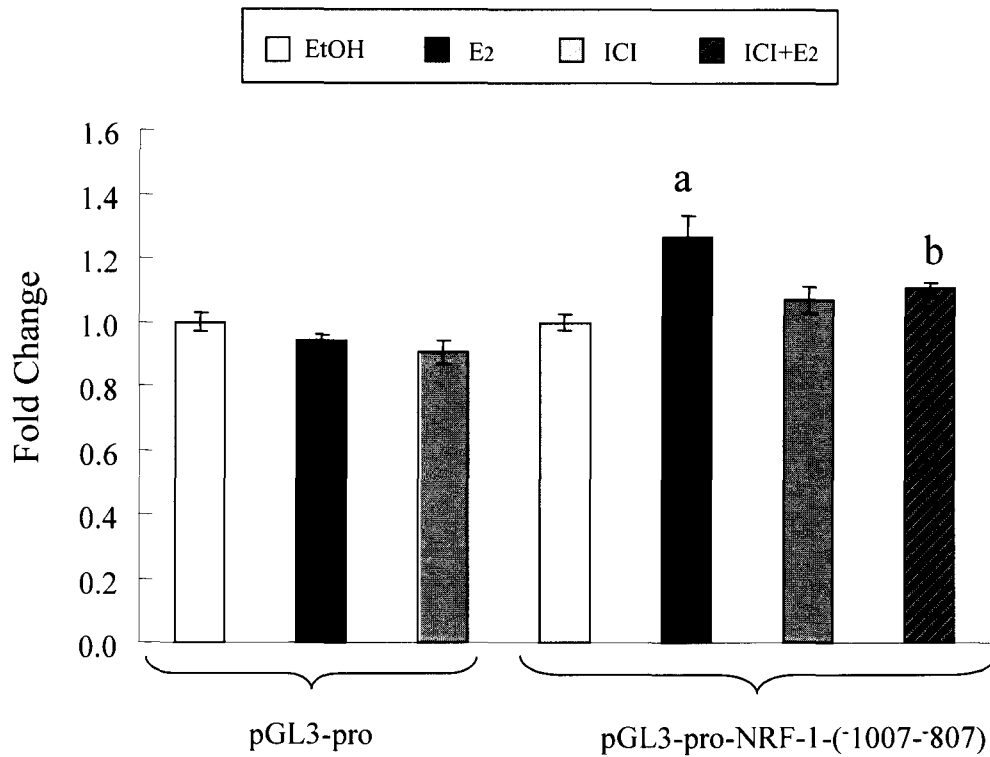




**Figure 33: Activity of the NRF-1 promoter constructs in MCF-7 cells.** (A) MCF-7 cells were transfected with the indicated parental or pGL2-NRF-1 promoter constructs and luciferase and *Renilla* values measured as described in Materials and Methods. (B) MCF-7 cells were transfected with the indicated pGL2-basic-luciferase or pGL3-pro-luciferase parental vector or with the pGL3-NRF-1 (-1007 - 807) construct. The cells were harvested 48 h post-transfection. WCE were prepared and dual luciferase assays were performed as described in Materials and Methods. Values are relative luciferase units (RLU)/*Renilla*. Values are the mean  $\pm$  SEM of 3.



**Figure 34: Effect of E<sub>2</sub> on luciferase activity from NRF-1 promoter constructs in transiently transfected MCF-7 cells.** MCF-7 cells were transfected with the indicated pGL2-NRF-1 promoter constructs and *Renilla* luciferase. Twenty-four h post-transfection the cells were treated with EtOH, 10 nM E<sub>2</sub>, 100 nM ICI 182,780, or 100 nM ICI 182,780 plus 10 nM E<sub>2</sub> for 30 h as described in Materials and Methods. 30 h after treatment, WCE were prepared and dual luciferase assays were performed as described in Materials and Methods. Values are luciferase/*Renilla* luciferase activity normalized to the EtOH control for each construct. Values are the mean ± SEM of 4. a =  $P < 0.05$  compared to EtOH; b =  $P < 0.05$  compared to E<sub>2</sub>

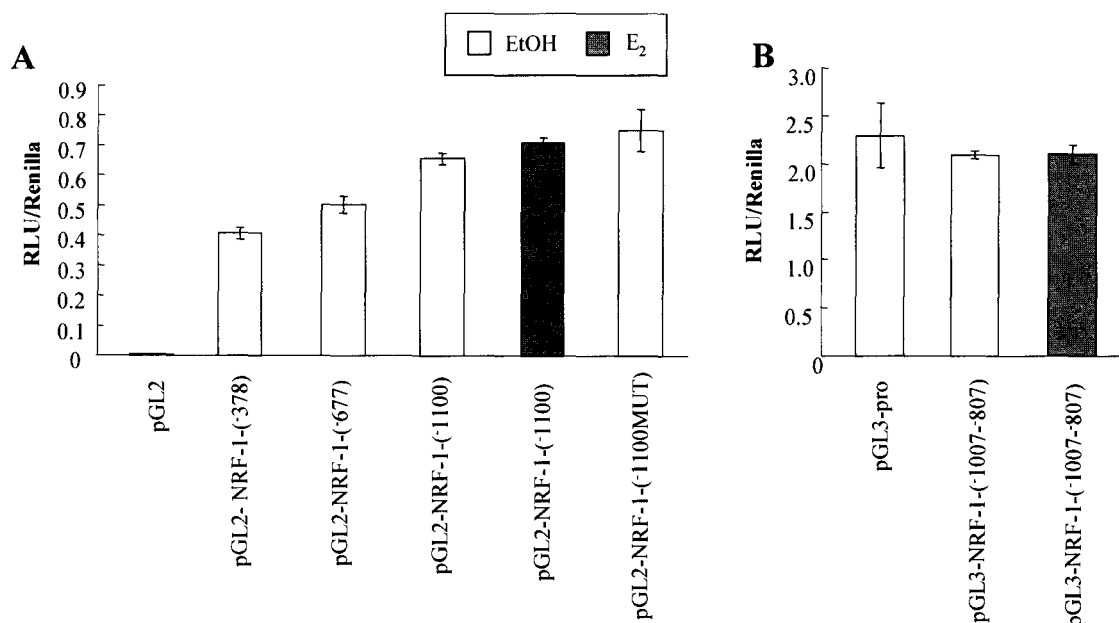


**Figure 35: Effect of E<sub>2</sub> on luciferase activity from pGL3-pro-NRF-1-(1007-807) in transiently transfected MCF-7 cells.** MCF-7 cells were transfected with pGL3-pro-luciferase or pGL3-pro-NRF-1-(1007-807) and *Renilla* luciferase. 24 h post-transfection, the cells were treated with EtOH, 10 nM E<sub>2</sub>, 100 nM ICI 182,780, or 100 nM ICI 182,780 plus 10 nM E<sub>2</sub> for 30 h. WCE were prepared and dual luciferase assays were performed as described in Materials and Methods. Values are luciferase/*Renilla* luciferase activity normalized to the EtOH control for each construct. Values are the mean ± SEM of 3 experiments. a =  $P < 0.05$  compared to EtOH; b =  $P < 0.05$  compared to E<sub>2</sub>

Transient transfections with the pGL2-basic-luciferase constructs were subsequently repeated in HEK-293 ER-null cells with either ER $\alpha$  or ER $\beta$  cotransfected as described in Materials and Methods. These transfections allow the contribution of each ER subtype to the luciferase induction to be further examined. Prior to cotransfection with ER $\alpha$  or ER $\beta$ , the basal activity of the NRF-1 promoter constructs were examined in HEK-293 cells (Figure 36A). As seen in the MCF-7 control experiment (Figure 33A), the pGL2-NRF-1 constructs demonstrate an increase in basal luciferase activity compared to the parental vector (pGL2-basic-luciferase) indicating the presence of a promoter within the constructs. In contrast to the MCF-7 results, in which equal activity was seen between the pGL2-NRF-1-( $\sim$ 378), pGL2-NRF-1-( $\sim$ 677), pGL2-NRF-1-( $\sim$ 1100), and pGL2-NRF-1-( $\sim$ 1100MUT) plasmids, the HEK-293 cells demonstrate an increasing amount of activity with increasing length of the promoter. These differences are likely a cell-specific difference in the expression of transcription factors and coregulators (171-173). When pGL2-NRF-1-( $\sim$ 1100) transfected HEK-293 cells were treated with E<sub>2</sub> in the absence of transfected ER, no increase in relative luciferase activity was seen (Figure 36A). In parallel with the MCF-7 control transfections, pGL3-pro-luciferase and pGL3-pro-NRF-1-( $\sim$ 1007- $\sim$ 807) were transfected into HEK-293 cells in the absence of ER (Figure 36B). Both plasmids had equivalent basal activity in HEK-293 cells, and treatment of pGL3-pro-NRF-1-( $\sim$ 1007- $\sim$ 807) with E<sub>2</sub> did not result in an increase in luciferase activity.

In the presence of cotransfected ER $\alpha$ , E<sub>2</sub> stimulated an increase in luciferase activity from the pGL2-NRF-1-( $\sim$ 1100) plasmid construct which contains the putative ERE but not the pGL2-NRF-1-( $\sim$ 677), ( $\sim$ 378), or ( $\sim$ 1100MUT) constructs lacking the

ERE (Figure 37A and B). Cotreatment of cells with E<sub>2</sub> and ICI 182,780 ablated the E<sub>2</sub>-induced increase in luciferase activity. This agrees with data from the MCF-7 transfections. However, E<sub>2</sub> did not stimulate a significant increase in any of the constructs in the presence of ERβ (Figure 37C and D). When pGL3-pro-NRF-1-(1007-807) was transfected in the presence of ERα or ERβ, a significant increase in luciferase activity was seen in the presence of E<sub>2</sub> (Figure 38A and B). The increase seen in the ERα transfected cells was greater than the increase observed in ERβ cotransfected HEK-293 cells, 1.8- and 1.3-fold, respectively. The increase in E<sub>2</sub> was ablated by cotreatment with ICI 182,780, further indicating the ER specificity of the induction (Figure 38A and B).



**Figure 36: Basal activity of the NRF-1 promoter constructs in HEK-293 cells. (A)**

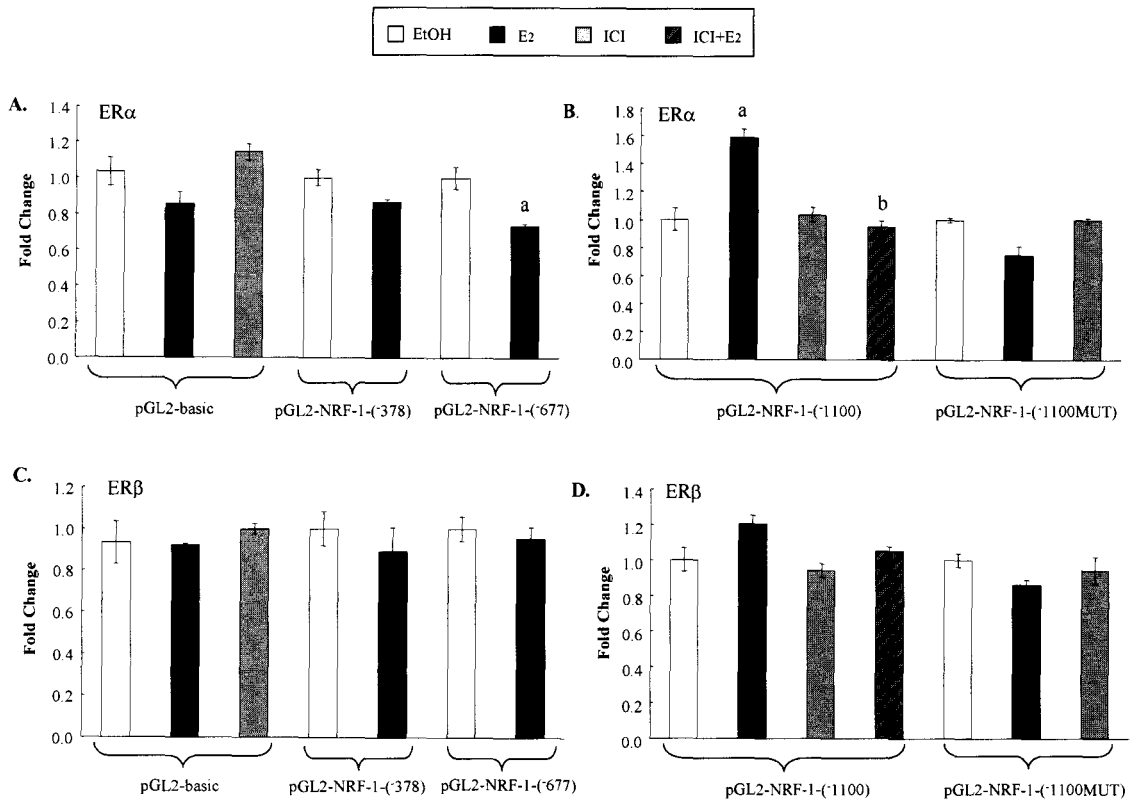
HEK-293 cells were transfected with the indicated parental or pGL2-NRF-1 promoter constructs and *Renilla* luciferase and treated with EtOH or 10 nM E<sub>2</sub> 24 h post-transfection.

(B) HEK-293 cells were transfected with the indicated parental vector or pGL3-NRF-1 construct and treated with EtOH or 10 nM E<sub>2</sub> 24 h post-transfection. WCE

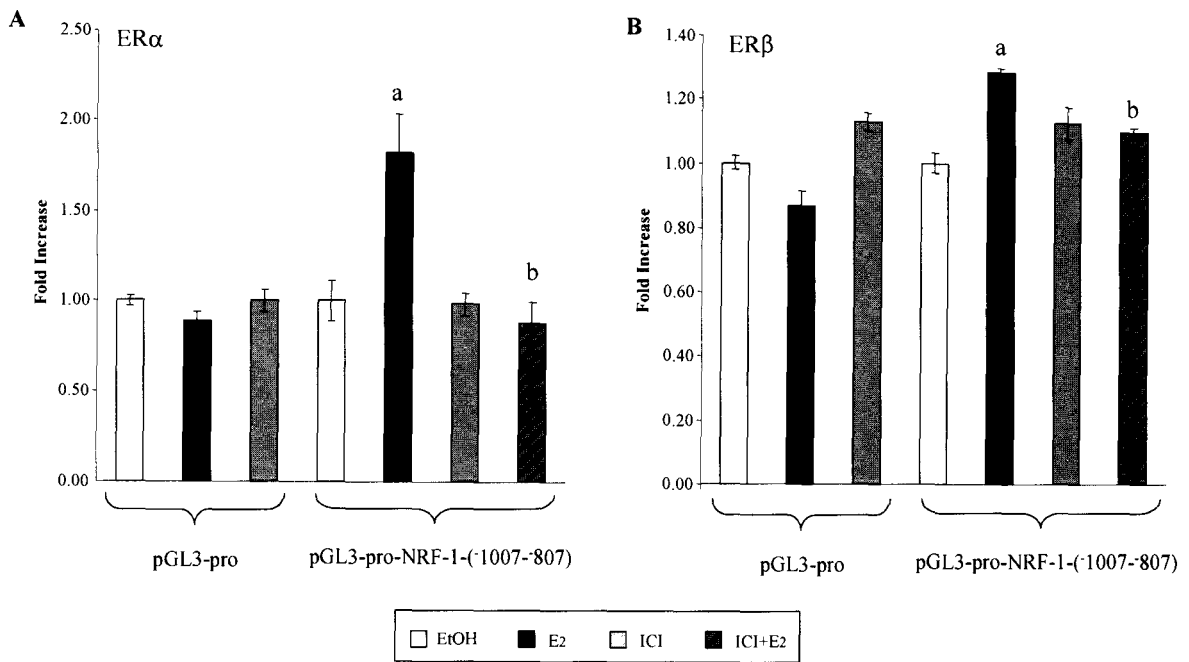
were prepared 30 h after treatment and dual luciferase assays were performed as

described in Materials and Methods. Values are relative luciferase units (RLU)/*Renilla*.

Values are the mean  $\pm$  SEM of 3 experiments.



**Figure 37: Effect of E<sub>2</sub> on luciferase activity from NRF-1 promoter constructs in transiently transfected HEK-293 cells.** (A) and (B) HEK-293 cells were transfected with the indicated pGL2-NRF-1 promoter constructs, ER $\alpha$ , *Renilla* luciferase, and treated with EtOH, 10 nM E<sub>2</sub>, 100 nM ICI 182,780, or 100 nM ICI 182,780 plus 10 nM E<sub>2</sub> for 24 h as described in Materials and Methods. (C) and (D) HEK-293 cells were transfected and treated as listed for (A) and (B) with the exception that ER $\beta$  was transfected in place of ER $\alpha$ . WCE were prepared 30 h after treatment and dual luciferase assays were performed as described in Materials and Methods. Values are luciferase/*Renilla* luciferase activity normalized to the EtOH control for each construct. Values are the mean  $\pm$  SEM of 4. a =  $P < 0.05$  compared to EtOH; b =  $P < 0.05$  compared to E<sub>2</sub>



**Figure 38: Effect of E<sub>2</sub> on luciferase activity from pGL3-pro-NRF-1-(1007-807) in transient transfections in HEK-293 cells.** (A) HEK-293 cells were transfected with ER $\alpha$ , *Renilla* luciferase, and either pGL3-pro-luciferase or pGL3-pro-NRF-1-(1007-807). The cells were treated with EtOH, 10 nM E<sub>2</sub>, 100 nM ICI 182,780, or 100 nM ICI 182,780 plus 10 nM E<sub>2</sub> 24 h post-transfection. (B) HEK-239 cells were transfected and treated as listed for (A) with the exception that ER $\beta$  was transfected in place of ER $\alpha$ . WCE were prepared 30 h after treatment and dual luciferase assays were performed as described in Materials and Methods. Values are luciferase/*Renilla* luciferase activity normalized to the EtOH control for each construct. Values are the mean  $\pm$  SEM of 3 experiments. a =  $P < 0.05$  compared to EtOH; b =  $P < 0.05$  compared to E<sub>2</sub>

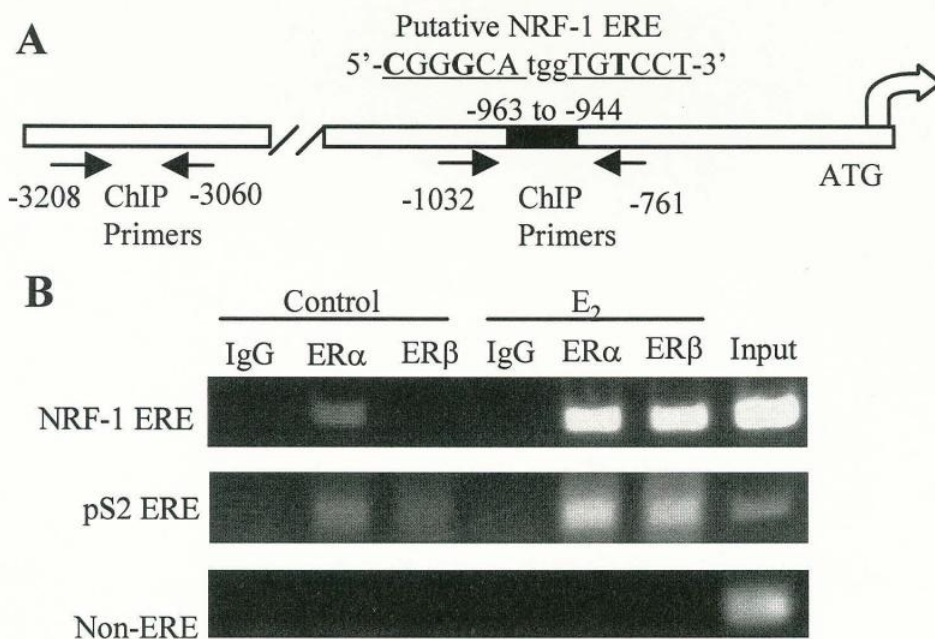


### **ER Binds to the Putative NRF-1 ERE *in vivo* and *in vitro***

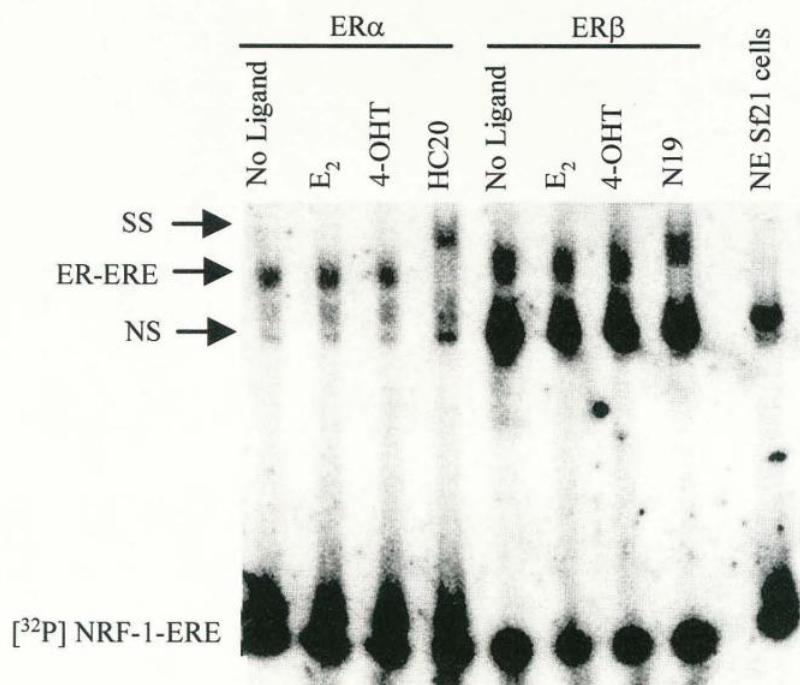
ChIP assays were used to evaluate whether ER $\alpha$  and ER $\beta$  bind directly to the putative ERE (-944 to -963) in the NRF-1 promoter. The location of the NRF-1 ChIP primers is shown in Figure 39A. pS2 is an E<sub>2</sub>-responsive gene that contains a well characterized ERE which has been shown to recruit both ER $\alpha$  and ER $\beta$  in the presence of E<sub>2</sub> in MCF-7 cells (174), hence serving as a positive control. A distal region of the NRF-1 promoter (-3060 to -3208) was used as a negative control. I observed that both ER $\alpha$  and ER $\beta$  are present on the putative NRF-1 and pS2 EREs in MCF-7 cells after E<sub>2</sub> treatment (Figure 39B). No binding of ER $\alpha$  or ER $\beta$  was indicated in the negative control in the presence or absence of E<sub>2</sub>.

EMSA performed by Dr. Margarita M. Ivanova indicated that ER $\alpha$  and ER $\beta$  bind directly to the putative ERE in the NRF-1 promoter without ligand and in the presence of E<sub>2</sub> and 4-OHT (Figure 40).

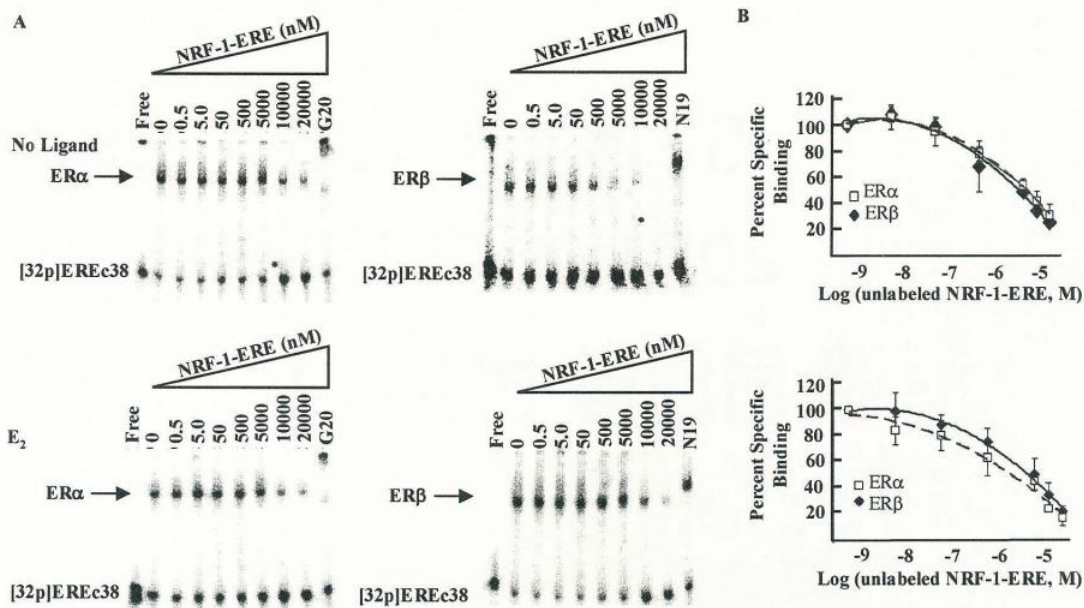
Deviation from the perfect consensus ERE correlates with decreasing affinity of ER-ERE binding (42). To determine the  $K_i$  of the ER-NRF-1-ERE interaction, competition EMSAs were performed (Figure 41). As shown, the NRF-1-ERE had ~1,000-fold lower binding affinity for ER than EREc38. The binding affinity was similar for unliganded ER $\alpha$  and ER $\beta$  with a  $K_i = 668 \pm 76.2$  nM and  $699 \pm 71$  nM, respectively and  $1056 \pm 98$  nM and  $895 \pm 51$  nM for E<sub>2</sub>-ER $\alpha$  and for E<sub>2</sub>-ER $\beta$ , respectively.



**Figure 39: ER $\alpha$  and ER $\beta$  bind to the putative NRF-1 ERE *in vivo*.** (A) A diagram of the NRF-1 promoter and putative ERE (-963 to -944) with the location of the ChIP primers shown. ERE half-sites are underlined and letters in bold indicate deviations from the consensus ERE. (B) MCF-7 cells were treated with EtOH or E<sub>2</sub> for 1 h and ChIP was performed as described in Materials and Methods.



**Figure 40: ER $\alpha$  and ER $\beta$  bind to the putative NRF-1 ERE *in vitro*.** Baculovirus-expressed ER $\alpha$  and ER $\beta$  were incubated with [<sup>32</sup>P]-labeled NRF-1 ERE in the presence of E<sub>2</sub>, 4-OHT, or no ligand, as indicated. EMSA was performed as described in Materials and Methods. NS = non-specific binding; SS = supershift with the indicated ER antibodies.



**Figure 41: ER $\alpha$  and ER $\beta$  bind the NRF-1 ERE with reduced affinity in comparison to a palindromic ERE.** (A) Competitive EMSAs were performed using unliganded (top) or E<sub>2</sub>-liganded ER $\alpha$  (left) and ER $\beta$  (right) incubated with 1.1 nM [<sup>32</sup>P]EREc38 plus increasing concentrations of nonlabeled NRF-1-ERE as indicated and described in Materials and Methods. ER $\alpha$  and ER $\beta$  antibodies G20 and N19, respectively, were included in the indicated binding reactions to demonstrate the specificity of the bound complex (SS). (B) Competitive binding curves are shown for ER $\alpha$  (open boxes) and ER $\beta$  (filled diamonds), and the IC<sub>50</sub> was calculated as described in Materials and Methods.

## DISCUSSION

Results of previous experiments (Chapter I, Figure 12A) suggested that both subtypes of ER could contribute to the E<sub>2</sub>-stimulated increase in NRF-1 mRNA transcription. Transient transfections in MCF-7 cells revealed that endogenous ER could induce activity in constructs containing the NRF-1 ERE. Using HEK-293 cells, the contribution of ER $\alpha$  and ER $\beta$  were examined. Cotransfection with ER $\alpha$  and the full length pGL2-NRF-1-(-1100) promoter construct in the presence of E<sub>2</sub> resulted in a significant increase in luciferase activity; however, this effect was not seen when ER $\beta$  was cotransfected with pGL2-NRF-1-(-1100). In a similar manner, cotransfection of ER $\alpha$  or ER $\beta$  with pGL3-pro-NRF-1-(-1007-7807) resulted in a significant increase in luciferase activity. This increase was greater in HEK-293 cells cotransfected with ER $\alpha$  than ER $\beta$ : 1.8- and 1.3-fold, respectively. The differences in fold induction are likely due to the different transcriptional activities of the two ER subtypes. ER $\alpha$  is known to be a more potent inducer of reporter activity while ER $\beta$  is a weaker inducer of reporter activity (58). Overall, the results of the transient transfection assays utilizing promoter deletion analysis and site-directed mutagenesis revealed that the ERE in the promoter of the NRF-1 gene is capable of mediating the E<sub>2</sub>-induced increase in luciferase activity observed in the full length pGL2-NRF-1-(-1100) construct.

In order to examine the direct interaction of ER with the NRF-1 ERE, both *in vivo* and *in vitro* methods were used. EMSA experiments revealed direct binding of ER $\alpha$  and ER $\beta$  to the NRF-1 ERE *in vitro*. However, the affinity of this interaction measured in EMSA experiments was low. Despite the low affinity of ER $\alpha$  and ER $\beta$  binding to the

NRF-1 ERE, *in vivo* ChIP assays revealed that the putative NRF-1 ERE is occupied by both ER $\alpha$  and ER $\beta$  in response to E<sub>2</sub>, similar to the pS2 promoter, an established ER target gene (164). This suggests that genomic ER $\alpha$  and ER $\beta$  bind to the NRF-1 ERE in response to E<sub>2</sub> in MCF-7 cells *in vivo*.

Inclusively, the experiments presented in this chapter demonstrate that I have identified an ERE in the promoter of the NRF-1 gene that binds ER $\alpha$  and ER $\beta$  *in vivo* and with low affinity *in vitro*. This adds NRF-1 to the list of genes regulated by non-palindromic, low affinity ER-ERE binding.

## CHAPTER IV

### REGULATION OF NUCLEAR RESPIRATORY FACTOR-1 IN MCF-7 AND H1793 CELLS BY 4-HYDROXYTAMOXIFEN AND RALOXIFENE

#### INTRODUCTION

As previously discussed, SERMs have the ability to act as agonists in a cell-type and gene-type specific manner. Many researchers have evaluated the gene induction patterns in cells and tissues in response to E<sub>2</sub>; however, relatively little experimental work has been done in to identify genes uniquely up-regulated by TAM and RAL, two of the mostly commonly used clinical SERMs. One study has been published that specifically looked at unique gene regulation by ER subtypes in combination with TAM and RAL. Studies in U2OS osteosarcoma cells stably expressing either ER $\alpha$  or ER $\beta$  demonstrated that very few of the genes identified as upregulated by ER $\alpha$  or ER $\beta$  individually were regulated by both ER $\alpha$  and ER $\beta$  (27% of the total ER $\alpha$  and ER $\beta$  upregulated genes) in TAM- and RAL- treated cells (175). This suggests that each subtype of ER regulates a unique set of genes in response to SERMs (175). In agreement with the previous set of data, an additional study examining the effect of co-expression of both ER subtypes in U2OS cells showed that TAM regulated 27% of the total amount of upregulated genes observed exclusively through the presence of both subtypes (176). This suggests a

unique gene expression pattern regulated by heterodimers of ER $\alpha$ /ER $\beta$  compared to either individual subtype (176). The possibility that heterodimers could uniquely regulate a subset of genes can complicate the interpretation of existing data.

MCF-7 cells were classically considered to be ER $\alpha$  positive and ER $\beta$  negative. Recently, this was found not to be true of all MCF-7 cells (147). In our own lab, MCF-7 cells express equivalent amounts of ER $\alpha$  and ER $\beta$  protein (32). This means that conclusions drawn from microarray studies in different labs that did not comment on the receptor status of their MCF-7 cell line may result in variation in the data. This may be speculative, but it demonstrates the difficulty in not only looking at the sparse amount of existing data on TAM and RAL gene regulation but in designing studies to more clearly examine these interactions.

In the following set of experiments, I examined the effect of 4-OHT and RAL on the expression of NRF-1. Originally, I used 4-OHT treatment of MCF-7 cells as a negative control, expecting no effect on basal NRF-1 transcription and that 4-OHT would inhibit E<sub>2</sub>-induced NRF-1 expression, i.e., have antagonist activity similar to ICI 182,780. However, 4-OHT did not act in the expected manner. Thus, the following set of experiments was undertaken to determine the mechanism by which 4-OHT induced NRF-1 transcription. In addition to 4-OHT, a second clinically used SERM, RAL was also used since it has lower agonist activity than 4-OHT (52).



## MATERIALS AND METHODS

### **RNA Isolation, RT-PCR and QRT-PCR**

All RNA Isolations, RT-PCR, and QRT-PCR were performed as described in Chapter II.

### **Transient Transfection and Dual Luciferase Assays in MCF-7 and HEK-293 Cells with NRF-1 Promoter Constructs**

All transient transfections and luciferase assays were performed as described in Chapter III.

### **Chromatin Immunoprecipitation Assay**

All ChIP assays were performed as described in Chapter III

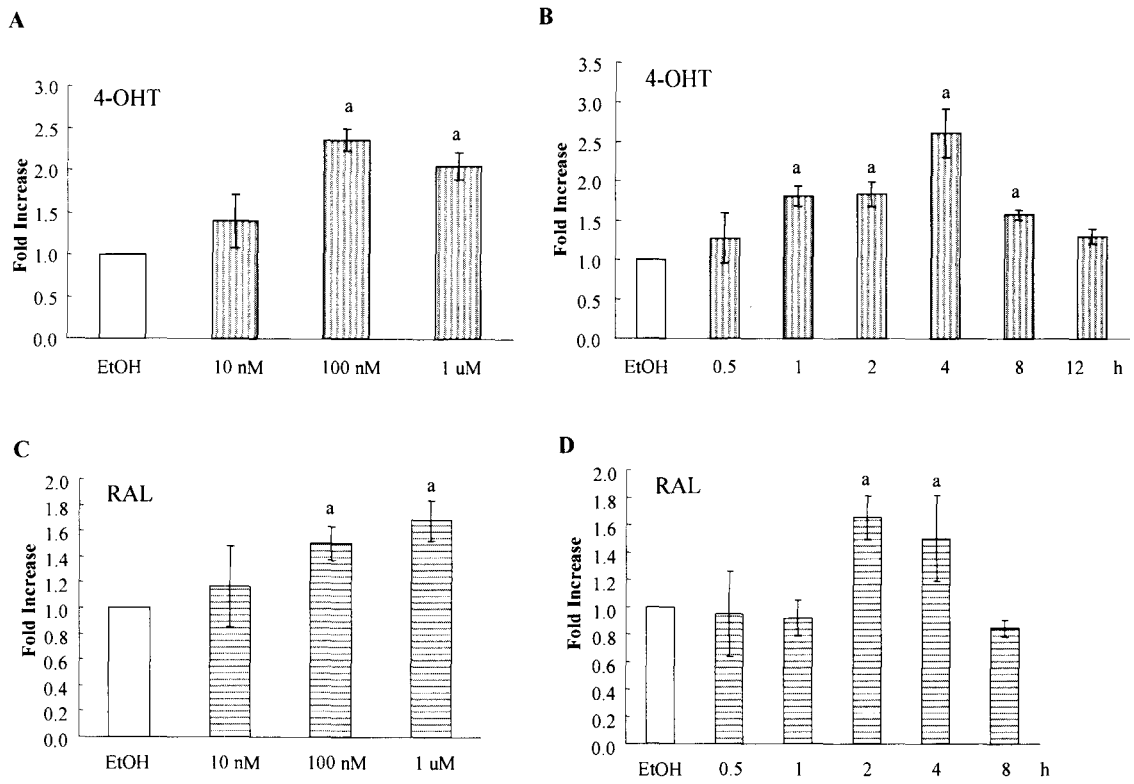
### **Statistics**

All statistical analyses were performed as described in Chapter II.

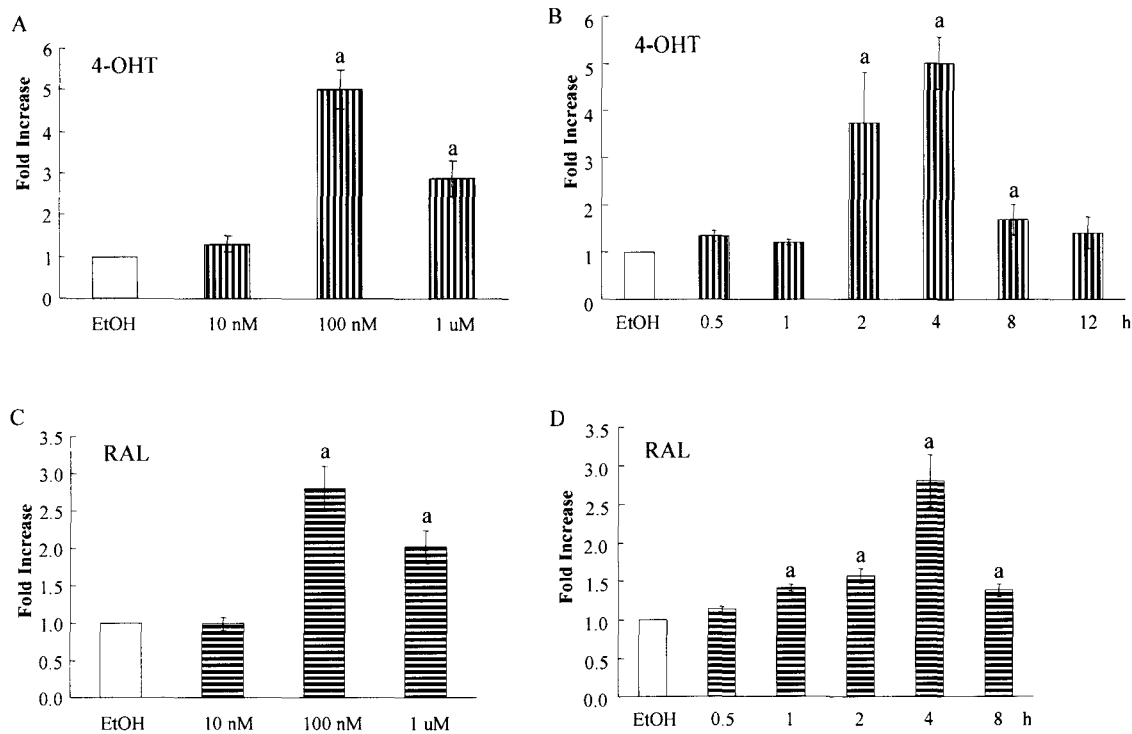
## RESULTS

### **4-OHT and RAL Increase NRF-1 mRNA in a Concentration- and Time-Dependent Manner in MCF-7 and H1793 Cells**

Both MCF-7 and H1793 cells were treated with increasing concentrations, *i.e.*, 10 nM-1  $\mu$ M, of either 4-OHT or RAL. Both 4-OHT and RAL increased NRF-1 mRNA in a concentration-dependent manner in MCF-7 cells (Figure 42A and C). A significant increase was seen beginning at 100 nM for both SERMs in MCF-7 cells. H1793 cells also demonstrated an increase in NRF-1 mRNA in a concentration-dependent manner with maximum induction occurring at 100 nM 4-OHT and RAL (Figure 43A and C). A time course using the 100 nM concentration was then performed for both 4-OHT and RAL in MCF-7 and H1793 cells. In MCF-7 cells, 4-OHT displayed a time-dependent increase in NRF-1 mRNA beginning at 1 h, peaking at 4 h, and ending by the 12 h time point (Figure 42B). H1793 cells displayed a time-dependent 4-OHT-mediated rise in NRF-1 mRNA starting at 2 h, culminating at 4 h, and ending by the 12 h time point (Figure 43B). In MCF-7 cells, RAL did not increase NRF-1 mRNA levels until 2 h post-treatment and the induction ended by the 8 h time point (Figure 42D). H1793 cells displayed an earlier RAL-stimulated increase in NRF-1 mRNA beginning at 1h, peaking at 4 h, and ending by the 8 h time point (Figure 43D).



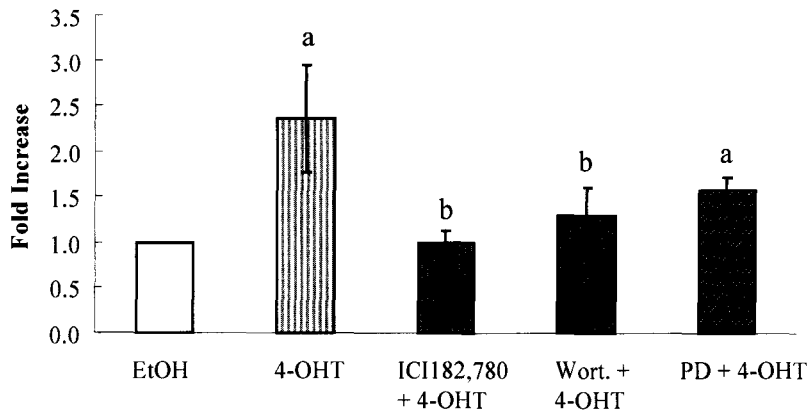
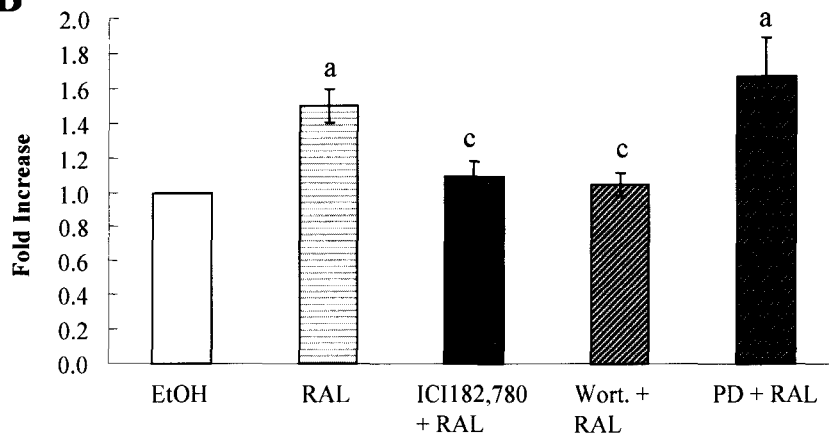
**Figure 42: 4-OHT and RAL increase NRF-1 mRNA in a concentration- and time - dependent manner in MCF-7 cells.** MCF-7 cells were treated with increasing amounts of 4-OHT (A) or RAL (C). MCF-7 cells were treated for the indicated times with 100 nM 4-OHT (B) or 100 nM RAL (D). The total mRNA was isolated and QRT-PCR was performed as described in Material and Methods. Values are the average of 3-6 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH



**Figure 43: 4-OHT and RAL increase NRF-1 mRNA in a concentration- and time-dependent manner in H1793 cells.** H1793 cells were treated with increasing amounts of 4-OHT (A) or RAL(C). H1793 cells were treated for the indicated times with 100 nM 4-OHT (B) or 100 nM RAL (D). The total mRNA was isolated and QRT-PCR was performed as described in Material and Methods. Values are the average of 3-6 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH

Both 4-OHT and RAL have been shown to act through both genomic and nongenomic mechanisms (177-180). MCF-7 cells were treated with 4-OHT or RAL plus ICI 182,780 to first determine if the observed 4-OHT- and RAL- induced increases in NRF-1 mRNA were mediated by ER. Pretreatment with ICI 182,780 followed by treatment with ICI 182,780 plus 4-OHT or RAL decreased NRF-1 transcription compared to either 4-OHT or RAL alone (Figure 44A and B). This indicates that ER is involved in mediating the observed stimulation in NRF-1 mRNA. To determine if the 4-OHT- and RAL- induced increase in NRF-1 mRNA are mediated by nongenomic ER activity, MCF-7 cells were pretreated for 1 h with the PI3 Kinase and MAP Kinase inhibitors Wortmannin and PD98059, respectively. Treatment with PD98059 did not significantly decrease the NRF-1 induction compared to 4-OHT or RAL alone indicating that the MAP Kinase pathway is not involved in mediating the NRF-1 induction by these SERMs (Figure 44A and B). However, treatment with Wortmannin inhibited both the 4-OHT- and RAL- induced increase in NRF-1 (Figure 44A and B). This data indicates involvement of the PI3 Kinase pathway in induction of NRF-1 transcription in response to either 4-OHT or RAL in MCF-7 cells.

As show in Figure 43, NRF-1 mRNA increased in response to 4-OHT and RAL in H1793 cells as it did in MCF-7 cells (Figure 42). The effect of using Wortmannin and PD98059 in concert with 4-OHT and RAL was also tested in H1793 cells. In parallel to the results in MCF-7 cells, the 4-OHT induction of NRF-1 mRNA is inhibited by both ICI 182,780 and Wortmannin (Figure 45A). This indicates a role for nongenomic signally through the PI3 Kinase pathway. In contrast, cotreatment with ICI 182,780 or

**A****B****Figure 44: 4-OHT- and RAL- induced NRF-1 transcription is mediated by****nongenomic ER in MCF-7 cells.** (A) MCF-7 cells were pre-treated with 50  $\mu$ M

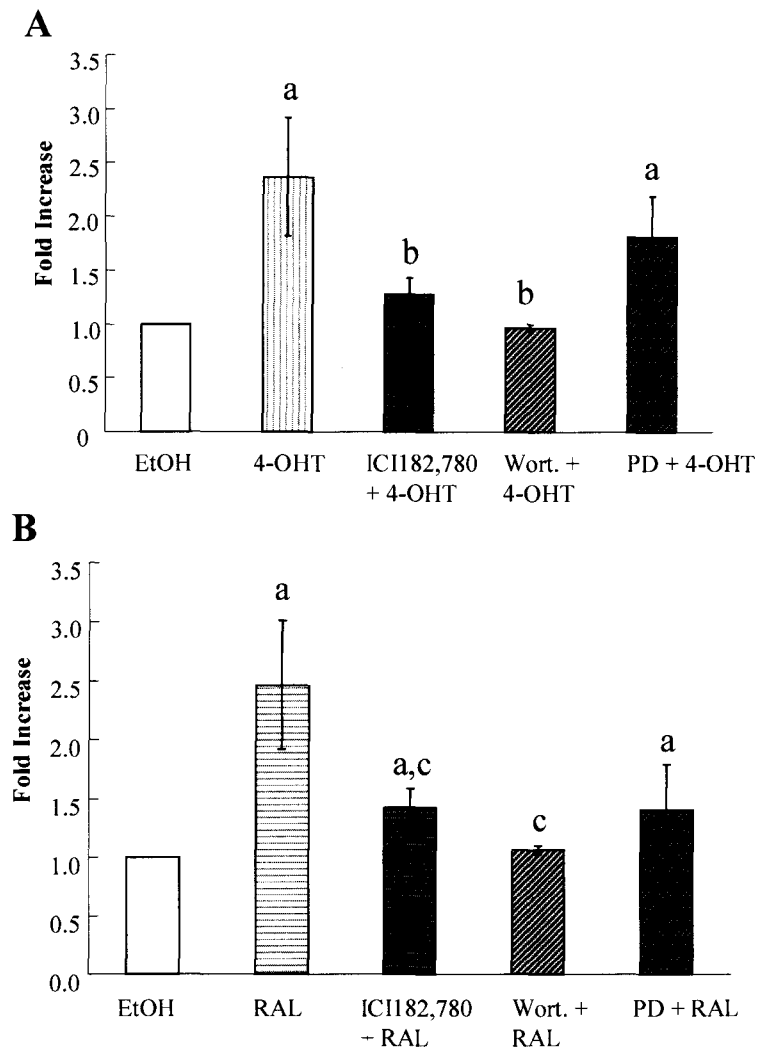
PD98059 (PD) or 50 nM Wortmannin (Wort) for 1 h prior to incubation with 100 nM 4-

OHT for 4 h. (B) MCF-7 cells were pre-treated with PD or Wort for 1 h prior to

incubation with 100 nM RAL for 4 h. The total mRNA was isolated following treatment

and QRT-PCR was performed as described in Material and Methods. Values are the

average of 3 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH; b =  $P < 0.05$ compared to 4-OHT; c =  $P < 0.05$  compared to RAL



**Figure 45: 4-OHT- and RAL- induced NRF-1 transcription is mediated by**

**nongenomic ER action in H1793 cells.** (A) H1793 cells were pre-treated with 50  $\mu$ M PD98059 (PD) or 50 nM Wortmannin (Wort) for 1 h prior to incubation with 100 nM 4-OHT for 4 h. (B) H1793 cells were pre-treated with PD or Wort for 1 h prior to incubation with 100 nM RAL for 4 h. The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH; b =  $P < 0.05$  compared to 4-OHT; c =  $P < 0.05$  compared to RAL

PD98059 with RAL only partially blocked the RAL induced increase in NRF-1 mRNA levels (Figure 45B). Wortmannin inhibited the RAL stimulated induction of NRF-1 (Figure 45B).

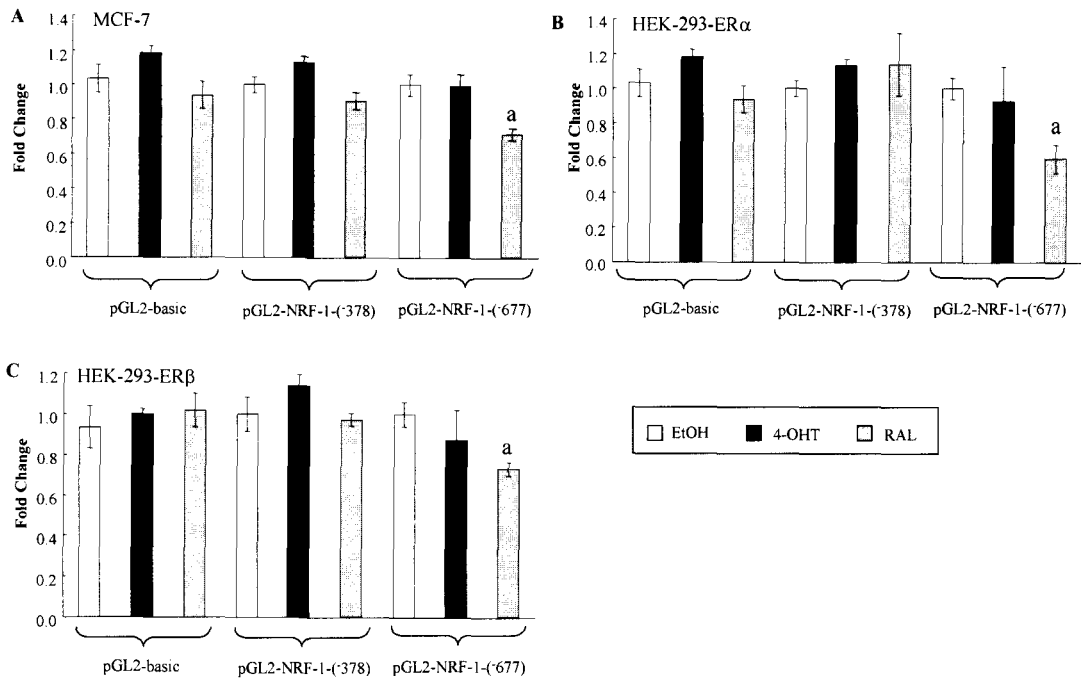
These data indicate a role for nongenomic signaling through the PI3 Kinase pathway in mediating the 4-OHT and RAL induction of NRF-1 in MCF-7 cells. In H1793 cells, 4-OHT parallels the results in MCF-7. In these cells 4-OHT is signaling through the PI3 Kinase pathway. However, RAL appears to be acting through the PI3 Kinase and MAP Kinase nongenomic pathways in H1793 cells.

### **Dual Luciferase Assays in MCF-7 and HEK-293 Cells with NRF-1 Promoter**

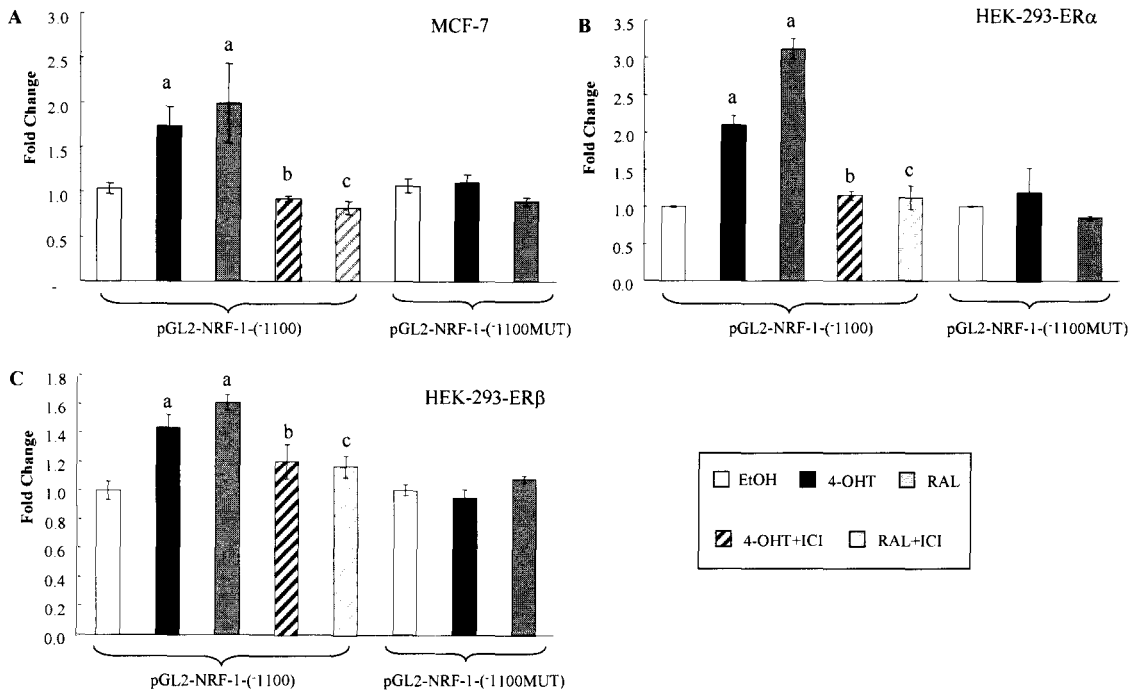
#### **Constructs in the Presence of SERMs**

To further examine the agonist activity of 4-OHT and RAL on the NRF-1 promoter, MCF-7 cells were transfected with each of the NRF-1 promoter constructs and treated with 4-OHT or RAL (Figure 46A and 47A). The NRF-1 promoter constructs were also transfected into HEK-293 cells cotransfected with either ER $\alpha$  (Figure 46B and 47B) or ER $\beta$  (Figure 46C and 47C). In the MCF-7, HEK-293-ER $\alpha$ , and HEK-ER $\beta$  transfections, 4-OHT and RAL did not have an effect on the reporter activity of the non-ERE containing reporters, pGL2-basic, pGL2-NRF-1(-378), pGL2-NRF-1(-677), or pGL2-NRF-1(-1100MUT) (Figure 46 and 47). The pGL2-NRF-1(-1100) and pGL3-NRF-1(-1007-807) constructs containing the ERE sequence showed increased luciferase activity in response to 4-OHT and RAL in MCF-7, HEK-293-ER $\alpha$  and HEK-293-ER $\beta$  transfections that was inhibited by ICI 182,780 (Figures 47 and 48). Notably, this





**Figure 46: Effect of SERMs on luciferase activity from non-ERE containing NRF-1 promoter constructs.** MCF-7 (A), HEK-293-ER $\alpha$  (B), and HEK-293-ER $\beta$  (C) cells were transfected with the indicated pGL2-NRF-1 promoter constructs and *Renilla* luciferase. Twenty-four h later, the cells were treated with EtOH, 100 nM 4-OHT, or 100 nM RAL. WCE were prepared 30 h after treatment and dual luciferase assays were performed as described in Materials and Methods. Values are luciferase/*Renilla* luciferase activity normalized to the ETOH control for each construct. Values are the mean  $\pm$  SEM of 4. a =  $P < 0.05$  compared to EtOH



**Figure 47: Effect of SERMs on luciferase activity from ERE-containing NRF-1**

**promoter constructs.** MCF-7 (A), HEK-293-ER $\alpha$  (B), and HEK-239-ER $\beta$  (C) cells

were transfected with the indicated pGL2-NRF-1 promoter constructs. Twenty-four h

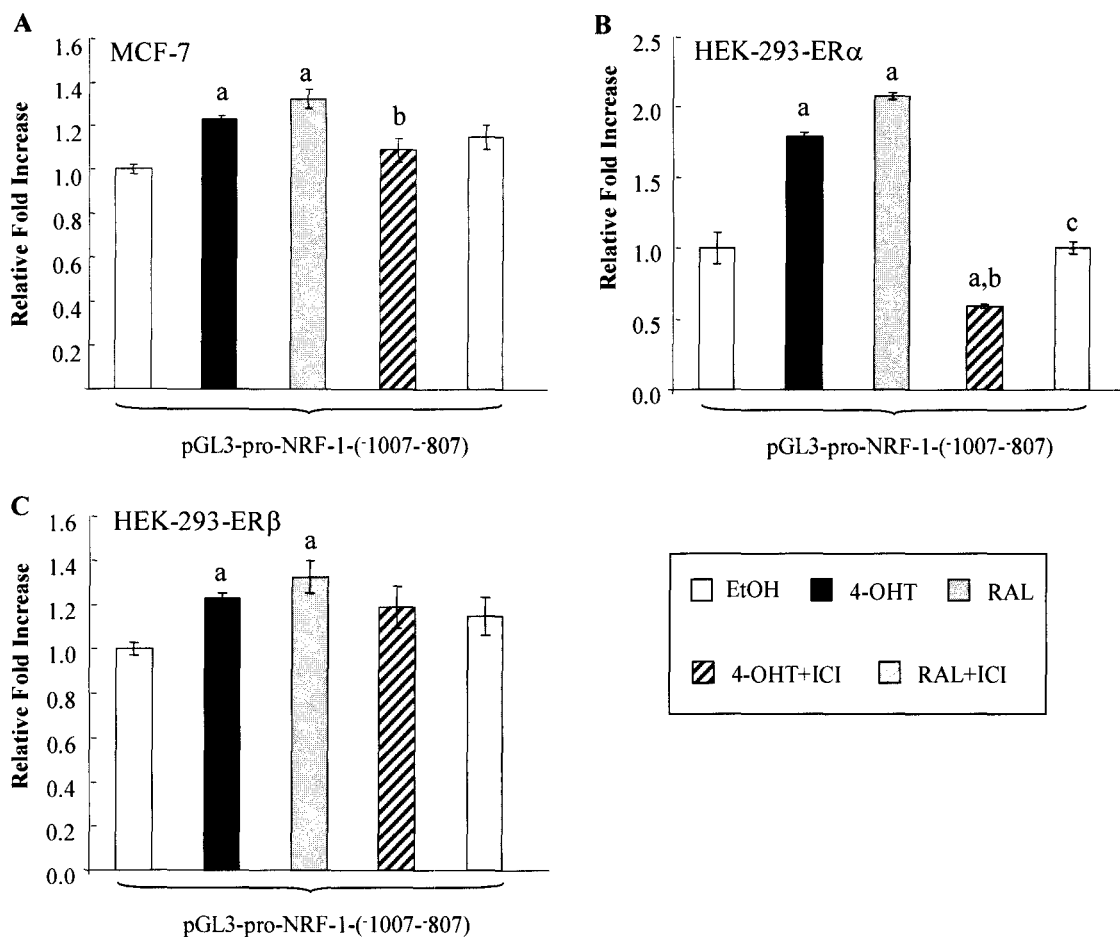
later, the cells were treated with EtOH, 100 nM 4-OHT, or 100 nM RAL. WCE were

prepared 30 h after treatment and dual luciferase assays were performed as described in

Materials and Methods. Values are luciferase/*Renilla* luciferase activity normalized to

the ETOH control for each construct. Values are the mean  $\pm$  SEM of 4. a =  $P < 0.05$

compared to EtOH; b =  $P < 0.05$  compared to 4-OHT; c =  $P < 0.05$  compared to RAL

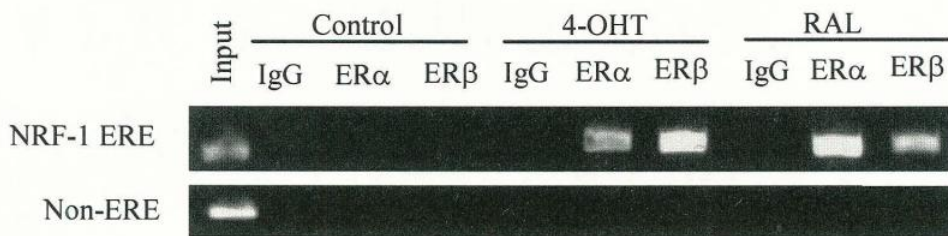


**Figure 48: Effect of SERMs on luciferase activity from the pGL3-pro-NRF-1-(1007-807) construct.** MCF-7 (A), HEK-293-ER $\alpha$  (B), and HEK-293-ER $\beta$  (C) cells were transfected with pGL3-pro-NRF-1-(1007-807). Twenty-four h later, the cells were treated with EtOH, 100 nM 4-OHT, 100 nM RAL, ICI 182,780+4-OHT, or ICI 182,780+RAL. WCE were prepared 30 h after treatment and dual luciferase assays were performed as described in Materials and Methods. Values are luciferase/*Renilla* luciferase activity normalized to the EtOH control for each construct. Values are the mean  $\pm$  SEM of 3 experiments. a =  $P < 0.05$  compared to EtOH; b =  $P < 0.05$  compared to 4-OHT; c =  $P < 0.05$  compared to RAL

effect was not detected in the pGL-NRF-1-(1100MUT) construct, indicating that the ERE is mediating the 4-OHT- and RAL- inducible response (Figure 47).

### **ChIP Assays Examining the ER Occupancy of the NRF-1 Promoter in the Presence of SERMs**

Previously, ChIP assays revealed that both ER $\alpha$  and ER $\beta$  occupied the NRF-1 promoter region containing the ERE *in vivo* in the presence of E<sub>2</sub> (Figure 49). Thus far, the mRNA and transfection data have suggested a mixed genomic/nongenomic mode of 4-OHT and RAL- induced ER action on the NRF-1 promoter. ChIP assays were performed to look at the *in vivo* binding of ER $\alpha$  and ER $\beta$  in the presence of 4-OHT and RAL. ChIP indicated an increase in the NRF-1 promoter occupancy by both ER $\alpha$  and ER $\beta$  after MCF-7 cell treatment with either 4-OHT or RAL (Figure 49).



**Figure 49: ER $\alpha$  and ER $\beta$  bind to the putative NRF-1 ERE *in vivo* in the presence of 4-OHT and RAL.** MCF-7 cells were treated with EtOH, 100 nM 4-OHT, or 100 nM RAL for 1 h and ChIP was performed as described in Materials and Methods.

## DISCUSSION

The goal of the experiments described in this chapter was to examine the effects of 4-OHT and RAL on the expression of NRF-1 mRNA and the effects of these ligands on the NRF-1 luciferase reporter plasmids. The observed increases in NRF-1 mRNA in MCF-7 cells are lower than those observed by E<sub>2</sub> treatment. As previously seen, E<sub>2</sub> treatment yielded a ~4-fold increase in NRF-1 (Figure 8) while 4-OHT results in a ~2.5-fold increase and RAL only stimulated a ~1.7 fold increase (Figures 42 and 43 ). While 4-OHT and RAL may increase mRNA expression, they act as weaker inducers of NRF-1 transcription in MCF-7 cells in comparison to E<sub>2</sub>. This effect is not true in H1793 cells. H1793 cells displayed a ~2.0-fold increase in NRF-1 mRNA in response to E<sub>2</sub> whereas both 4-OHT and RAL induced a ~2.5 fold increase in NRF-1 mRNA. In a cell type specific manner, 4-OHT and RAL are increasing NRF-1 mRNA more in H1793 cells and acting equally efficient as inducers.

Both genomic and nongenomic mechanisms of 4-OHT and RAL action through ER have been documented (177,181). The observed mixed effect of these two modes of activation was not expected. The time of NRF-1 mRNA increase, 1 h for 4-OHT and 2 h for RAL in MCF-7 cells, suggests a genomic mechanism as does the promoter occupancy of the NRF-1 ERE by ER $\alpha$  and ER $\beta$  in ChIP experiments. Additionally, data from the transient transfection experiments suggests a role for genomic ER binding to the NRF-1 ERE. Nongenomic effects would be expected in a more rapid time frame of sec to min.

In contrast to the effects of E<sub>2</sub>, the Wortmannin treatment results clearly point to a contribution of the PI3 Kinase pathway to the 4-OHT and RAL induced increase in NRF-1 mRNA in MCF-7 cells. Wortmannin treatment of H1793 cells in conjunction with 4-OHT indicated a contribution of the PI3 Kinase pathway to the 4-OHT stimulated increase in NRF-1 mRNA, similar to results achieved in MCF-7 cells. However, RAL appears to be acting through genomic and both the PI3 Kinase and MAP Kinase nongenomic pathways in H1793 cells.

The differences seen in activation of the NRF-1 gene in response to different ER ligands may be due to several cell-type specific differences such as coregulator expression (171-173) or AF-1 and AF-2 contributions (182-186). For example, the agonist properties of 4-OHT in endometrial tissue are mediated by high levels of SRC-1 expression allowing the formation of a 4-OHT-ER-SRC-1 complexes on the promoters of targets genes (55). In other tissues, such as breast, with lower SRC-1 activity the 4-OHT-ER complex recruits corepressors (55). To date, there is no experimental data in the literature comparing coregulator expression patterns in MCF-7 and H1793 cells. In addition to the potential for unique coregulator expression patterns to influence gene activation, coregulator recruitment is affected by the conformation of the receptor induced by both the ligand and the ERE to which the E<sub>2</sub>-ER complex is bound (166).

The contributions of AF-1 and/or AF-2 on the transcriptional control vary in a cell- and promoter- type manner (182-186). For example, ER $\alpha$  in MCF-7 cells are believed to have signaling mediated more through AF-1 than AF-2 (184,186), while HEK-203 cells signal through AF-1 and AF-2 equally (187). Using different promoter constructs, 4-OHT was also demonstrated to act through both AF-1 and AF-2 on some

promoters whereas on other promoters, the AF-1 and AF-2 function independently. AF-2 activity was not required in all of the promoters tested (183). In addition to cell- and promoter- type dependence of AF-1 and AF-2 activity, the contribution of AF-1 and AF-2 toward ER $\alpha$  activity has also been demonstrated to be dependent on the cell differentiation stage (186). AF-1 is the predominant AF mediating ER transcriptional activity in differentiated cell lines. However, more de-differentiated cells mediate signaling through AF-2. For example, AF-2 is functional in cells that have undergone the epithelial-mesenchymal transition, but not AF-1 (186).

Overall, I conclude that both 4-OHT and RAL are acting as ER agonists on the NRF-1 promoter in both MCF-7 and H1793 cells. The agonist effect is mediated by both genomic and nongenomic mechanisms. However, the exact contribution of coregulator differences as well as AF-1 vs. AF-2 signaling in these cell lines on the NRF-1 induction of mRNA has yet to be determined.



## CHAPTER V

### REGULATION OF NUCLEAR RESPIRATORY FACTOR-1 EXPRESSION BY TAMOXIFEN AND RALOXIFENE IN HUMAN UMBILICAL VEIN ENDOTHELIAL CELLS

#### INTRODUCTION

The cardiovascular system is an important target of estrogen action, much like the reproductive tissues, brain, liver and bone, through both genomic and nongenomic pathways (reviewed in (188)). Expression of both ER $\alpha$  and ER $\beta$  have been demonstrated using RT-PCR in the coronary arteries of both male and female cynomolgus monkeys (189). ER $\alpha$  has been demonstrated in human coronary arteries (190) and ER $\beta$  has been observed in primary human cardiomyocytes (74). Studies have also linked a decrease in mitochondrial respiratory chain activity and a loss of mitochondrial ultrastructure to a lack of ER in ERKO and  $\beta$ ERKO mice (79,81).

The proposed protective effects of estrogens in the cardiovascular system have been challenged by results from the Women's Health Initiative/ Heart and Estrogen/Progestin Replacement Study (WHI and HERS). These studies showed no significant improvement of CHD in postmenopausal women receiving hormone replacement therapy (HRT), which included a mix of conjugated equine estrogens (CEE) and medoxyprogesterone acetate (MPA) (191). Instead, HRT increased the incidence of CHD (192) and both HRT and ERT increased venous thromboembolism (191,193). In postmenopausal women with pre-existing CHD given ERT, the cardioprotective benefits

were tempered by the potential increase in breast cancer events due to estrogen exposure (194,195). In contrast to the results from the WHI trial, animal models of stroke and vascular function have shown an E<sub>2</sub>-protective effect (196). One of the criticisms of the WHI/HERS studies is that the participants were, in general, many years post-menopausal at the initiation of HRT treatment (196,197). Studies addressing the time lapse between the onset of menopause and initiation of HRT treatment in primates revealed that estrogen treatment started immediately following ovariectomy reduced atherosclerotic plaque formation in primates fed a moderately atherogenic diet (198). However, if administration of ERT was postponed for two years after ovariectomy (the equivalent of ~six years in humans), there was no reduction in coronary lesions (198). An additional critique of the HRT trial has been the use of oral CEE since CEEs contain a minimum of 10 estrogens in the form of sulfate esters and several equine estrogens not secreted by human ovaries (199). In contrast, human ovaries secrete E<sub>2</sub> and estrone (E<sub>1</sub>) (199). Since each estrogen has a unique activity profile for ER $\alpha$  and ER $\beta$ , CEE may not produce the same effects as the naturally occurring estrogens (196). More studies taking into account the time between the onset of menopause and the beginning of HRT and the form and route of administration of HRT/ERT are needed (200). Lastly, the statistical evaluations in the initial studies have been criticized and re-analysis by one scientist did not support an increase risk of cardiovascular disease, stroke or venous thromboembolism in patients receiving HRT in the WHI trial (197). In conclusion, despite much research, the mechanisms through which E<sub>2</sub> conveys its protective effect in the vasculature has yet to be fully elucidated.

SERMs, *i.e.*, 4-OHT and RAL, have been suggested to have cardioprotective effects without the negative side effects of HRT (137,201,202). With regard to cardiovascular disease, TAM has been shown to lower serum lipids and it may protect against CHD (52). However the Study of Tamoxifen and Raloxifene (STAR) trial found no cardioprotective benefit with 4-OHT or RAL use (203). In contrast, RAL was shown to increase proliferation in a cellular model of venous tissue, human umbilical vein endothelial cells (HUVEC) (204). Additionally, clinical trials using RAL have demonstrated a reduction in plasma LDL levels and fibrinogen levels (205). However, in agreement with the STAR trial, the Raloxifene Use for the Heart (RUTH) clinical trial revealed that RAL did not significantly affect the risk of CHD, but increased the risk of venous thromboembolism and fatal stroke in postmenopausal women (206). However, the same critiques that were suggested for the WHI HRT study can also be applied to the RUTH study since the average age of the participants was 68 years with 39% being over 70 and the average time postmenopause was 19 years (207). Ideally, SERMs could provide an alternative to HRT/ERT that would be beneficial in preventing CHD and stroke (57).

Recent studies in rats have shown that E<sub>2</sub> decreased ROS production and increased mitochondrial energy production in intact cerebral blood vessels *in vitro* and *in vivo* (114). Importantly, these studies identified an increase in NRF-1 protein in the cerebral blood vessels examined (93). As discussed in Chapter II, my research identified NRF-1 as a primary estrogen responsive gene in MCF-7 breast adenocarcinoma and H1793 lung adenocarcinoma cells. The mechanism of NRF-1 activation in response to E<sub>2</sub> was genomic, *i.e.*, the result of an ER-mediated increased gene transcription, and

resulted in transcription of secondary NRF-1 target genes and an increase in mitochondrial activity. The ability of NRF-1 to increase mitochondrial activity is important in tissues such as the heart which consume large amounts of ATP. Therefore, I wanted to examine the ability of E<sub>2</sub>, 4-OHT, and RAL to potentially change NRF-1 expression in HUVEC.

As discussed in Chapter I, DEP have received attention for their potential role as endocrine disrupting agents in recent years. Diesel Exhaust Particulate Extracts (DEPE) have displayed both antiestrogenic and antiandrogenic properties in cell culture models (60-62). It was previously reported that the speed, stopping and starting patterns, and the load that a vehicle is carrying can affect the composition of the DEPE generated (208). Therefore, it was important to evaluate the antiestrogenic effects of DEPE generated under different engine loads and speeds at which a diesel truck was run. In the following studies, DEPE were collected from a Japanese diesel truck run on a chassis dynamometer at either varying speeds: 20, 50, or 80 km/h (referred to as S20, S50, and S80) or at a constant speed with varying capacity loads 0, 50, or 75% (referred to as L0, L50, and L75) by Dr. Ryoichi Kizu and colleagues in Japan (60,208). The six DEPE samples were extracted from the filter on which they were collected (60,208) and sent to Dr. Klinge's lab.

In addition to testing the effects of an environmental pollutant, I wanted to examine the effects of a phytoestrogen previously studied in our lab (123) on expression of NRF-1 in HUVEC. Resveratrol is a phytoestrogen found in the skin of grapes that has been shown to bind and activate ER (122,209). In addition to binding to ER and activating transcription in a genomic manner, resveratrol has also been shown to activate

the MAP Kinase pathway (59-61). Recently, long term (15 wk) treatment of male mice with 200 or 400 mg/kg resveratrol was demonstrated to significantly increase their aerobic capacity, O<sub>2</sub> consumption (210). Interestingly, resveratrol increased mitochondrial number and size and increased the expression of genes mediating oxidative phosphorylation and mitochondrial biogenesis including NRF-1 in the gastrocnemius muscle (210). Studies using mouse embryonic fibroblasts (MEFs) from Sirtuin 1 knockout mice (Sirt<sup>-/-</sup> MEFs) showed that the increase in mitochondrial activity was largely explained by a resveratrol-mediated decrease in PGC-1 $\alpha$  acetylation which increased the activity of this coregulator (210). PGC-1 $\alpha$  is a known coactivator for NRF-1. An increase in both NRF-1 and PGC-1 $\alpha$  activity would further promote the transcription of NRF-1 target genes (67). In general, previous studies have suggested a chemopreventative and cardioprotective role for resveratrol (124,125,211). If DEPE can oppose the positive increase in NRF-1 induced by E<sub>2</sub>, it would be relevant to examine if resveratrol could protect against these negative effects.

In the following set of experiments, I have shown that E<sub>2</sub> and SERMs increase NRF-1 transcription which was followed by a subsequent increase in mitochondrial Complex IV activity in HUVEC. DEPE inhibited NRF-1 expression and prevented an increase in NRF-1 stimulated by E<sub>2</sub>, 4-OHT, and RAL. Resveratrol increased NRF-1 mRNA expression through an apparent genomic ER mechanism and conveyed a small amount of protection against the decrease in basal NRF-1 mRNA induced by some DEPE.

## MATERIALS AND METHODS

### **Cell Culture and Treatments**

HUVEC were obtained from Cambrex Bioscience and maintained in Endothelial Cell Basal Medium-2 (EBM-2) supplemented with hydrocortisone, human fibroblast growth factor, vascular epidermal growth factor, insulin growth factor-1, ascorbic acid, human epidermal growth factor, Gentamicin sulfate, amphotercin-B, heparin, and 2% FBS provided in a supplemental kit with the media from Clonetics (hereafter referred to as EGM-2 media). Prior to treatment, HUVEC were placed in EBM-2 media containing 2% CCS-FBS. HUVEC used in these experiments were between passage number 4 and 8.

At the time this work was completed, the particulate composition of the DEPE was known for L0, L50, and L75. The distribution of PAHs is given in Table 5.

### **RNA Isolation, RT-PCR and Quantitative Real Time RT-PCR (QRT-PCR)**

RNA isolation and subsequent processing to cDNA and analysis by QRT-PCR was performed as described in Chapter II.

### **Bromodeoxyuridine (BrdU) Proliferation Enzyme Linked Immunosorbant**

#### **Assay (ELISA)**

The Bromodeoxyuridine (BrdU) Cell Proliferation Enzyme Linked Immunosorbant Assay (ELISA) Kit (Roche) is a colorimetric immunoassay for the quantification of cell proliferation. It is based upon the incorporation of a pyrimidine

analogue, BrdU, in place of thymidine into DNA during DNA synthesis. The presence of BrdU is detected through binding of an anti-BrdU-POD Fab-fragment which subsequently reacts with a substrate generating a detectable colorimetric change.

For the BrdU assay, HUVEC were seeded at a density of 6000 cells/ well in a 96-well plate. The cells were treated for 48 h with the indicated treatments. To each well, 10 µl of BrdU labeling solution was added, and the cells were incubated for an additional 2 h allowing time for the incorporation of BrdU into the cellular DNA. The labeling solution was removed and the cells were incubated in 200 µl/well FixDent Solution provided with the kit. The cells were incubated at room temperature for 30 min. The fixing solution was removed and 100 µl/well of anti-BrdU-POD solution was added for 90 min. The cells were rinsed three times with Washing Solution followed by addition of 100µl/well Substrate Solution and incubated at room temperature for 10-20 min. Absorbance was measured at 370 nm. Each treatment was performed in quadruplicate and the values were averaged. All values were compared with those in the wells treated with vehicle (EtOH) control, which was set to 1.0.

#### **Complex IV Activity Assay**

The MitoProfile Microplate Assay Kit for Human Complex IV Activity was purchased from MitoSciences. It was used to determine the activity of Complex IV from cell or tissue extracts. In this protocol, Complex IV was immunocaptured and its activity determined colorimetrically via the oxidation of reduced cytochrome *c*. This is measured as a decrease in absorbance at 550 nm.

HUVEC were seeded at 60% confluency in 6-well plates. The cells were grown in EBM-2 media supplemented with 2% CCS-FBS for 24 h. The cells were treated in EBM-2 media with 2% CCS-FBS for 6 d. The treatment was reapplied every 2 d. The cells were harvested in 100  $\mu$ l of Solution 1 with detergent (included in the kit). Protein concentrations were determined using the Bio-Rad DC Protein Assay, and 20  $\mu$ g of protein/well was diluted in 200  $\mu$ l Solution 2. The diluted protein was incubated in a 96-well plated containing bound antibody for Complex IV. After a 3 h incubation, the wells were washed and 200  $\mu$ l of Assay Solution added. Absorbance was measured every 5 min for 75 min. The slope of the reaction leveled off after 75 min. A linear range between 20 min and 60 min was examined for all treatments. The rate of Complex IV activity was determined by calculating the slope of the line between these two points resulting in OD/min change. One experiment was completed and assayed in quadruplicate. The EtOH from this experiment was set to 1.



Concentration (pmole/mg DEPE))			
Compound	L0	L50	L75
pyrene	409 ± 108	806 ± 254	740 ± 195
benz[ <i>a</i> ]anthracene	77.3 ± 38.7	111 ± 82.5	147 ± 63.3
chrysene	112 ± 58.2	118 ± 90.6	286 ± 140
benzo[ <i>b</i> ]fluoranthene	95.7 ± 13.4	68.6 ± 18.2	152 ± 60.9
benzo[ <i>k</i> ]fluoranthene	11.6 ± 3.70	2.70 ± 1.00	17.5 ± 7.00
benzo[ <i>a</i> ]pyrene	19.4 ± 2.20	1.90 ± 0.70	4.30 ± 0.30
benzo[ <i>b</i> ]chrysene	0.70 ± 0.20	0.10 ± 0.10	N.D.*
perylene	7.00 ± 0.40	1.40 ± 0.50	1.20 ± 0.30
dibenz[ <i>a,h</i> ]anthracene	10.0 ± 4.90	6.60 ± 2.20	6.10 ± 6.50
benzo[ <i>ghi</i> ]perylene	13.6 ± 2.00	1.40 ± 2.40	5.20 ± 5.80
indeno[1,2,3- <i>cd</i> ]pyrene	4.00 ± 1.70	N.D.*	N.D.*
dibenzo[ <i>a,e</i> ]pyrene	0.30 ± 0.30	0.10 ± 0.30	0.10 ± 0.30
coronene	0.50 ± 0.40	N.D.*	N.D.*
-----			
Sum (13 PAHs)	761 ± 217	1118 ± 383	1359 ± 447
-----			
*Not detected, mean ± SD (three separate extract samples).			

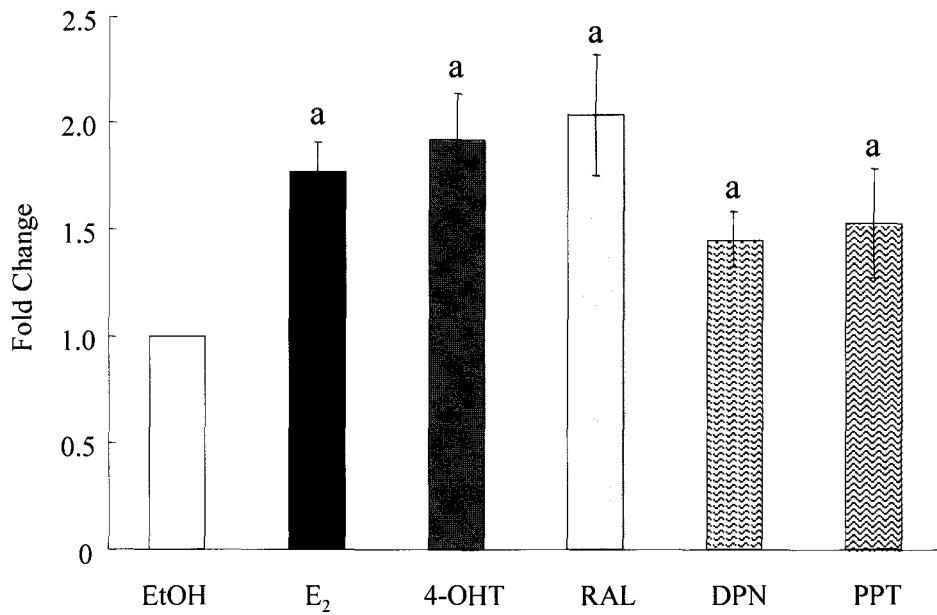
**Table 5: Concentrations of PAHs Having Four or More Rings in the DEPE Samples**

## RESULTS

### **E<sub>2</sub>, 4-OHT and RAL Increase NRF-1 Transcription in HUVEC**

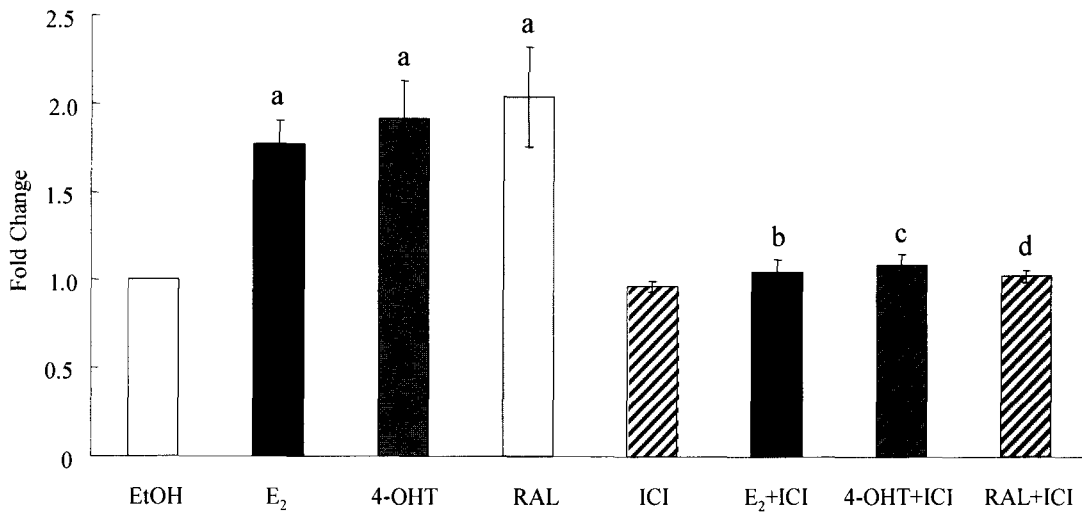
As reported in Chapter II, I established that E<sub>2</sub> stimulated NRF-1 gene expression through genomic ER action. In addition to E<sub>2</sub>, 4-OHT and RAL stimulate the expression of NRF-1 in HUVEC (Figure 50). HUVEC express both ER $\alpha$  and ER $\beta$  (212). To determine if the increase in NRF-1 transcription in response to E<sub>2</sub>, 4-OHT, and RAL was mediated by both ER subtypes or by only one, cells were treated with either PPT, selective for ER $\alpha$  (148), or DPN, a selective agonist for ER $\beta$  (149). Both PPT and DPN stimulated the transcription of NRF-1 in HUVEC (Figure 50), indicating that ER $\alpha$  and ER $\beta$  transcriptionally activate NRF-1. Interestingly, the increase in NRF-1 mRNA was not significantly different in the DPN and PPT treated HUVEC when compared to the E<sub>2</sub> treated cells. This reveals that activation of either subtype can result in complete induction of NRF-1 RNA in comparison to E<sub>2</sub> treated cells.

To further evaluate if ER is mediating the E<sub>2</sub>-, 4-OHT-, and RAL- induced increase in NRF-1 message levels, HUVEC were pretreated with ICI 182,780 for 6 h prior to addition of E<sub>2</sub>, 4-OHT, and RAL. This pre-incubation time was selected because previous studies had demonstrated a ~80% decrease in ER $\alpha$  protein levels as early as 4 h post-treatment with ICI 182,780 (143). ICI 182,780 inhibited the E<sub>2</sub>-, 4-OHT-, and RAL- induced increases in NRF-1 mRNA (Figure 51) indicating that ER mediates the induction response previously observed.



**Figure 50: E<sub>2</sub>, 4-OHT, and RAL activate NRF-1 mRNA expression in HUVEC.**

HUVEC were treated with EtOH, 10 nM E<sub>2</sub>, 100 nM 4-OHT, 100 nM RAL, 10 nM DPN, or 10 nM PPT for 4 h. The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH



**Figure 51: ICI 182,780 blocks the E<sub>2</sub>, 4-OHT, and RAL stimulated NRF-1 mRNA induction in HUVEC** HUVEC were treated with 100 nM ICI 182,780 (ICI) for 6 h prior to treatment with 10 nM E<sub>2</sub>, 100 nM 4-OHT and 100 nM RAL for 4 h, as indicated. The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3-6 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH; b =  $P < 0.05$  compared to E<sub>2</sub>; c =  $P < 0.05$  compared to 4-OHT; d =  $P < 0.05$  compared to 4-OHT

Signaling pathways including the MAP Kinase and PI3 Kinase pathways have been shown to be activated by nongenomic E<sub>2</sub> activity in HUVEC (213,214). To determine if E<sub>2</sub>, 4-OHT, and RAL are acting through these nongenomic pathways, HUVEC were pre-treated with the MAP Kinase inhibitor PD98059 or the PI3 Kinase inhibitor Wortmannin, and then treated with E<sub>2</sub>, 4-OHT, and RAL. Neither PD98059 nor Wortmannin inhibited the E<sub>2</sub>-, 4-OHT- or RAL- induced increase in NRF-1 mRNA (Figure 50). From these results, I conclude that 4-OHT and RAL are acting through classical genomic ER to mediate an increase in NRF-1 mRNA, results that parallel the effect of E<sub>2</sub> in these cells.

### **Secondary Gene Expression is Increased in Treated HUVEC**

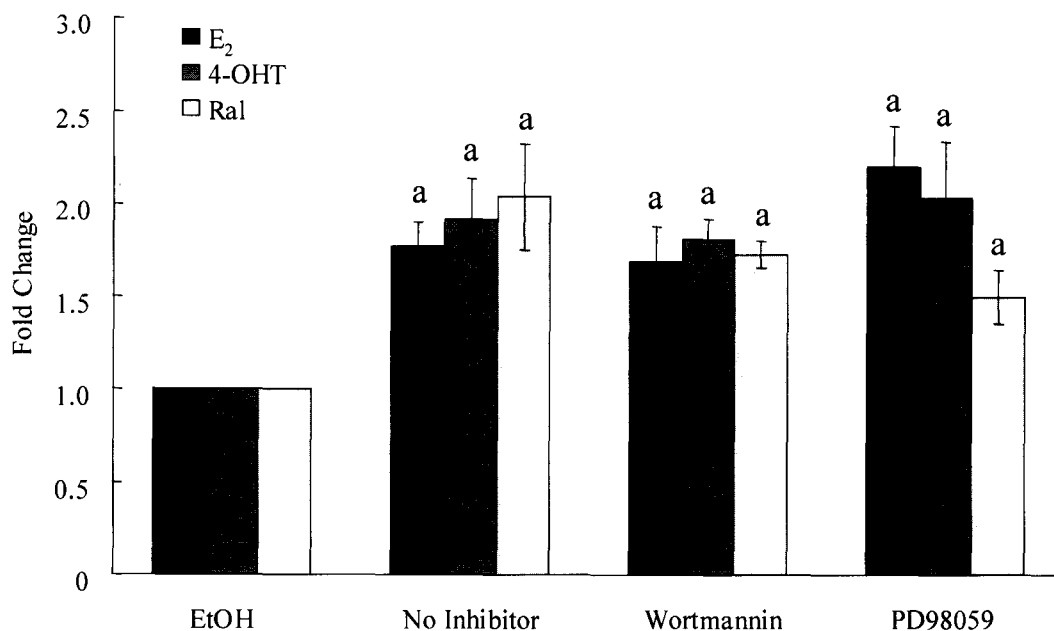
QRT-PCR was performed to look at the effect of 48 h of treatment with E<sub>2</sub>, 4-OHT, or RAL in the presence or absence of ICI 182,780 on expression of Tfam in HUVEC. This time point was chosen because Tfam, as well as its target genes COI and NDI, were all significantly increased in an E<sub>2</sub> dependent manner in MCF-7 cells after 48 h of treatment (Chapter II, Figure 19). As previously discussed, Tfam is a downstream target of NRF-1 which is involved in transcription of the mtDNA. E<sub>2</sub>, 4-OHT, and RAL all stimulated an increase in Tfam mRNA (Figure 53). The observed increase is inhibited by pretreatment for 6 h with ICI 182,780 followed by cotreatment with ICI 182,780 in the E<sub>2</sub> and RAL samples. However, treatment with both 4-OHT and ICI 182,780 resulted in a decrease that was not significant compared to the control, but was also not significant when compared to the 4-OHT treatment alone.

In parallel to experiments performed in MCF-7 cells in Chapter II, QRT-PCR was used to examine the mRNA expression of two Tfam target genes, COI and NDI, which are encoded in the mtDNA. COI and NDI mRNA were both induced by E<sub>2</sub>, 4-OHT, and RAL (Figure 53). Treatment with ICI 182,780 in the presence of E<sub>2</sub>, 4-OHT, and RAL was able to significantly decrease expression when compared to treatment in the absence of ICI 182,780. These results indicate that the observed increase in NRF-1 in the presence of all three ligands leads to a downstream effect on NRF-1 and Tfam target genes.

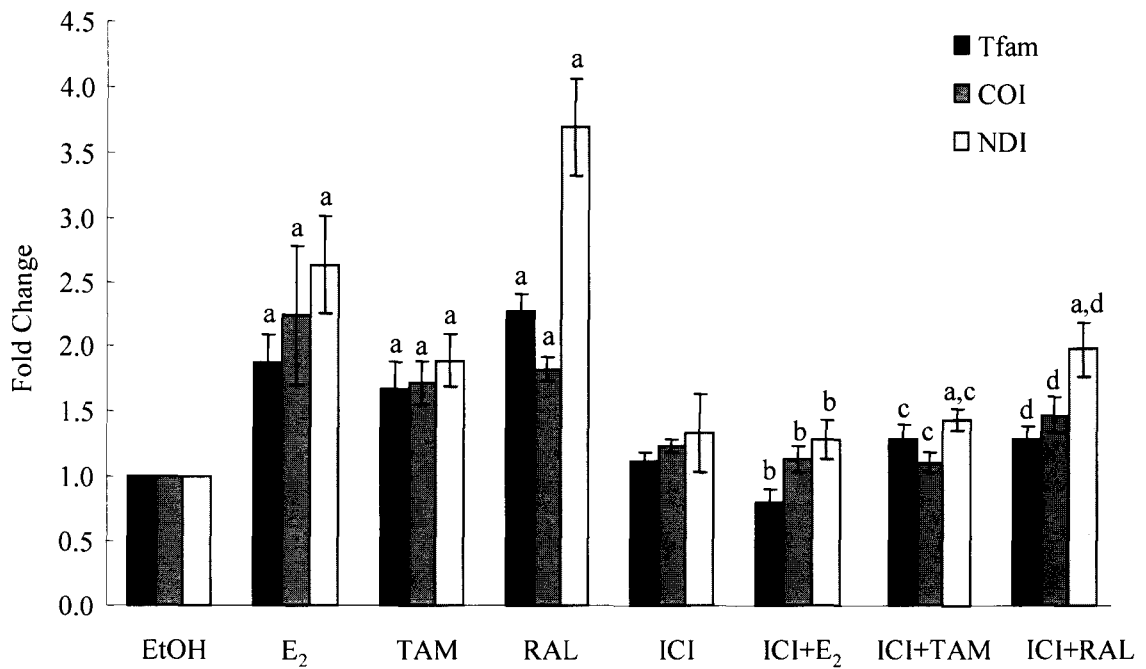
### **Complex IV Activity in Treated HUVEC**

In the mitochondrial respiratory chain, Complex IV serves as the terminal electron acceptor, consuming O<sub>2</sub> and generating H<sub>2</sub>O. To determine if the observed increase in NRF-1 mRNA lead to a subsequent increase in mitochondrial activity, Complex IV activity was determined using an EIA that colorimetrically measures the oxidation of reduced cytochrome *c*. E<sub>2</sub>, 4-OHT and RAL increased Complex IV activity and the increase in Complex IV activity caused by E<sub>2</sub> was reduced by cotreatment with ICI 182,780 (Figure 54).

These data agree with the O<sub>2</sub> consumption data (Chapter II, Figure 23). The results suggest that E<sub>2</sub>-treatment leads to an increase in NRF-1 gene expression, and also stimulates a downstream increase in MRC function. Additionally, 4-OHT and RAL are also observed to induce an increase in Complex IV activity. This suggests that 4-OHT and RAL are acting in a similar manner to E<sub>2</sub>, not only in their capacity to induce gene transcription, but also to stimulate an increase in MRC activity.

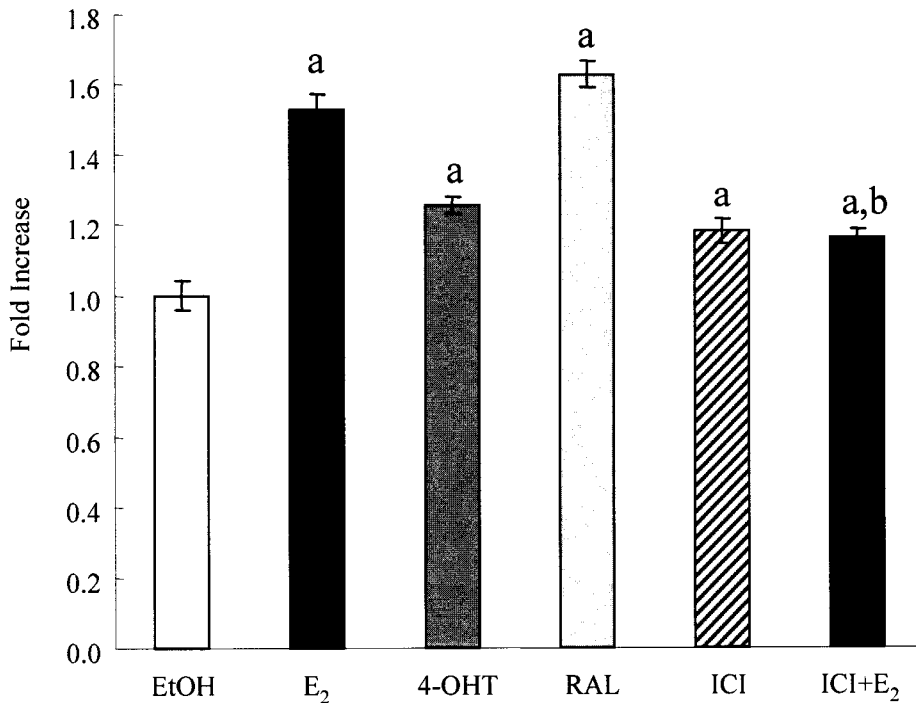


**Figure 52: Genomic ER is responsible for the increase in NRF-1 mRNA induced by E<sub>2</sub>, 4-OHT, and RAL.** HUVEC were pre-treated with 50 nm Wortmannin or 50 μm PD98059 for 1 h followed by 4 h treatment with 10 nm E<sub>2</sub>, 100 nM 4-OHT, or 100 nM RAL in the presence of Wortmannin or PD98059. The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3 separate experiments ± SEM. a = *P* < 0.05 compared to EtOH



**Figure 53: Secondary gene mRNA expression is increased in response to treatment with E<sub>2</sub>, 4-OHT, and RAL.** HUVEC were treated with 10 nM E<sub>2</sub>, 100 nM 4-OHT, or 100 nM RAL. Cells were also pretreated for 6 h with ICI 182,780 followed by addition of 10 nM E<sub>2</sub>, 100 nM 4-OHT, or 100 nM RAL for 48 h. The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH; b =  $P < 0.05$  compared to E<sub>2</sub>; c =  $P < 0.05$  compared to TAM; d =  $P < 0.05$  compared to RAL





**Figure 54: Effect of E<sub>2</sub>, 4-OHT, and RAL on Complex IV activity in HUVEC.**

HUVEC were treated with 10 nM E<sub>2</sub>, 100 nM 4-OHT, 100 nM RAL, 100 nM ICI 182,780 or 10 nM E<sub>2</sub> and 100 nM ICI 182,780 for 6 d. Post-treatment, Complex IV activity was measured as described in Materials and Methods. The EtOH control was set to 1. The EtOH value was originally  $1.13 \times 10^{-4}$  OD/min. Values are the Average of one experiment assayed in quadruplicate  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH; b =  $P < 0.05$  compared to E<sub>2</sub>

### **DEPE Inhibit NRF-1 mRNA Expression**

DEPE were reported to act as antagonists of E<sub>2</sub> at the transcriptional level (60). They are also prevalent in industrialized cities and thought to pose a significant health risk (59,60). A recent epidemiological study suggested that living in a high traffic area may be an important risk factor for CHD (121). Since NRF-1 is important to cardiovascular health through maintenance of mitochondrial function (79-81,93), I wanted to determine the effect of DEPE on the transcription of NRF-1. HUVEC were treated with the highest concentration (10 mg/mL) of each DEPE and NRF-1 mRNA expression was quantified by real time PCR. L50, L75, and S80 significantly inhibited basal NRF-1 expression by 25-40% (Figure 55).

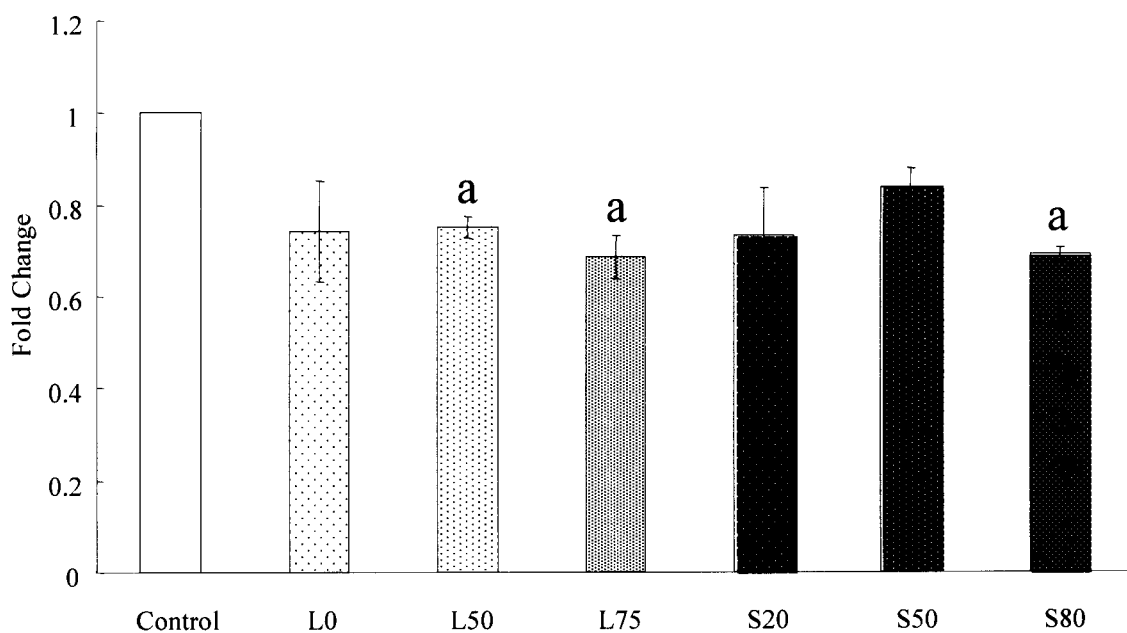
A concentration-response titration for L50, L75, S50, and S80 was performed in conjunction with a single concentration of E<sub>2</sub> (10 nM) to determine the optimal inhibitory concentration of these DEPE. At only the maximum concentration tested (10 mg/ml), L50 alone significantly inhibited basal NRF-1 mRNA expression. In contrast, L75 inhibited basal levels of NRF-1 transcription at all three concentrations tested (Figure 56). In conjunction with E<sub>2</sub>, both L50 and L75 inhibited the E<sub>2</sub>-induced increase in NRF-1 at all three concentrations. However, the combination of L50 plus E<sub>2</sub> still resulted in an increase in NRF-1 mRNA compared to the effect of the L50 treatment alone, whereas L75 inhibited NRF-1 expression to levels equivalent to that seen with L75 alone, i.e., significantly below basal (per the a and b designations) (Figure 56). S50 significantly blocked basal expression of NRF-1 at only the middle concentration tested (1mg/ml), and S80 was able to suppress basal NRF-1 mRNA transcription at only the 10 mg/ml concentration (Figure 57). However, S50 exhibited a concentration-dependent inhibition

of the E<sub>2</sub> induced stimulation of NRF-1 at the 1 mg/ml and 10 mg/ml concentrations (Figure 57). All concentrations of S80 inhibited the E<sub>2</sub>-induced increase in NRF-1 mRNA (Figure 57).

Next, I determined if the DEPE exhibiting the concentration that exhibited the greatest inhibition on NRF-1 transcription, L75 and S80, had an impact on the observed E<sub>2</sub>, 4-OHT-, and RAL- induced increase in NRF-1 mRNA expression in HUVEC. L75 and S80 inhibited the E<sub>2</sub>, 4-OHT-, and RAL- mediated increase in NRF-1 mRNA (Figure 58). I conclude that, in regards to the NRF-1 mRNA expression, L75 and S80 appear to act as antagonist on the E<sub>2</sub>, 4-OHT-, and RAL- induced stimulation of NRF-1.

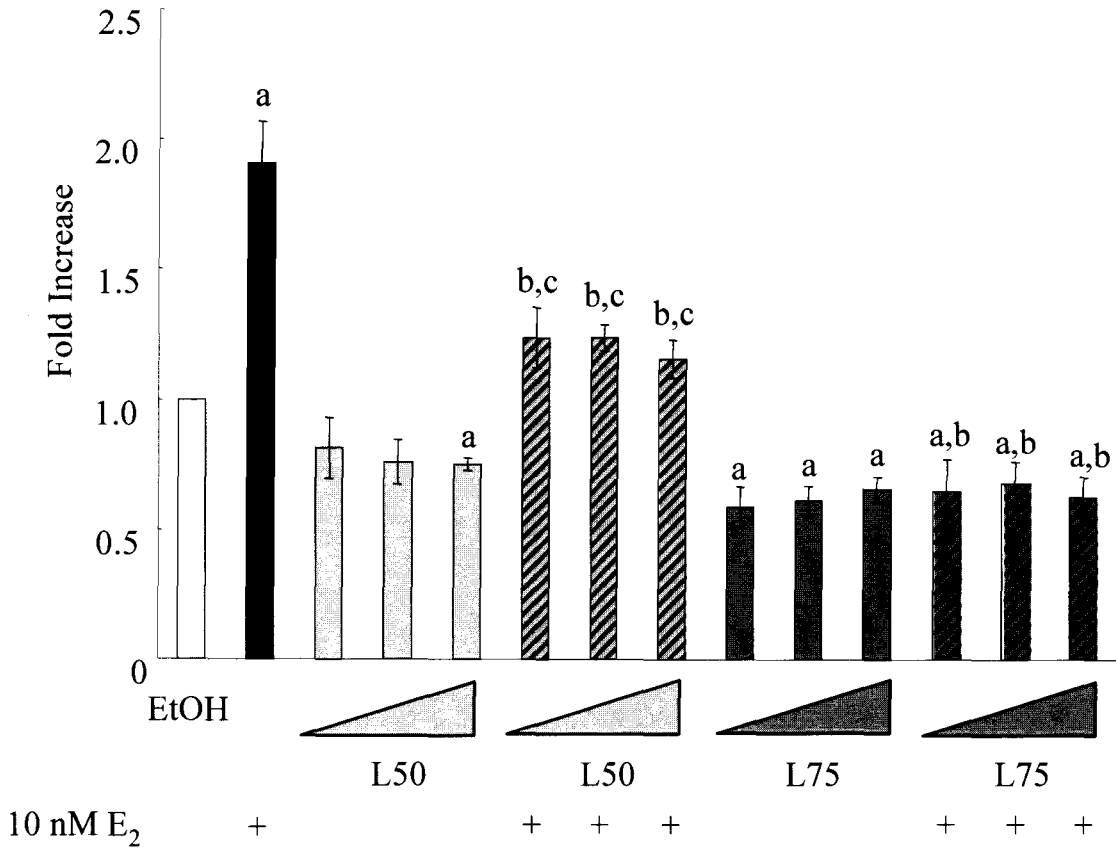
#### **DEPE Do Not Stimulate Cell Proliferation in HUVEC as Assessed in BrdU Assays**

BrdU assays were conducted to determine if DEPE were stimulating cell proliferation. E<sub>2</sub> treatment of HUVEC displayed an increase in DNA proliferation (Figure 59). However, 4-OHT, RAL, L50, L75, S50, and S80 treatment did not result in any change in cell proliferation. This indicated that the changes in NRF-1 levels do not correspond with an effect on cell proliferation at a 48 h time point.



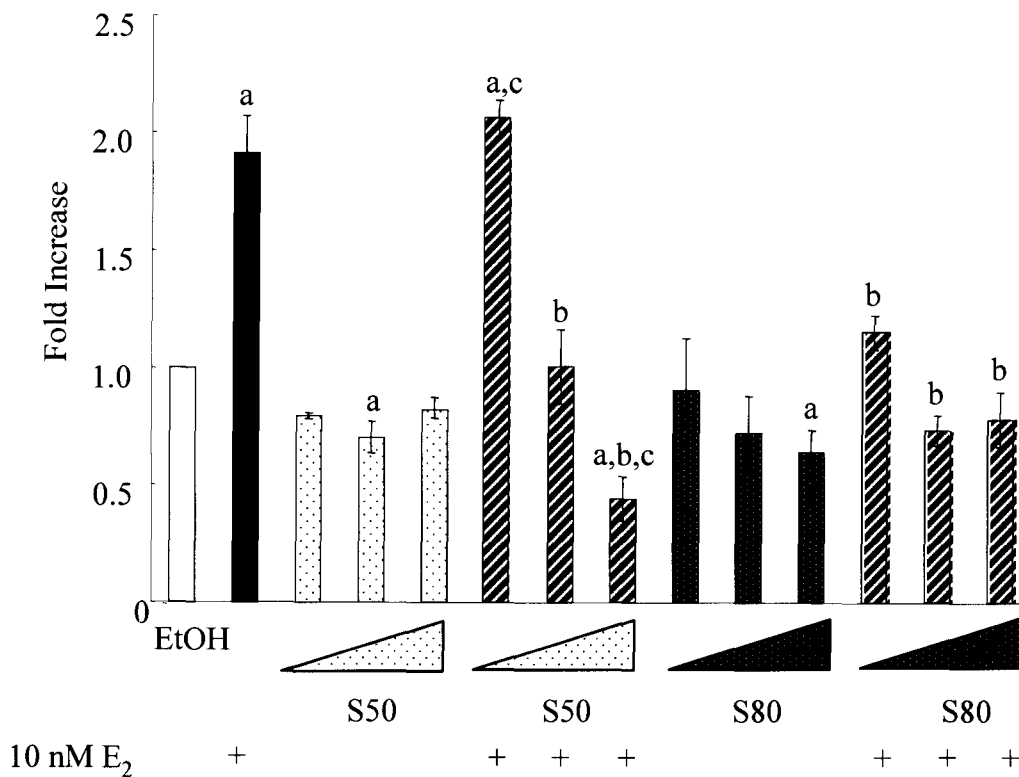
**Figure 55: L50, L75, and S80 inhibit basal transcription of NRF-1 in HUVEC.**

HUVEC were treated with 10 mg/mL of each DEPE indicated for 4 h. The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH. b =  $P < 0.05$  compared to  $E_2$  alone or  $ER\alpha$  alone

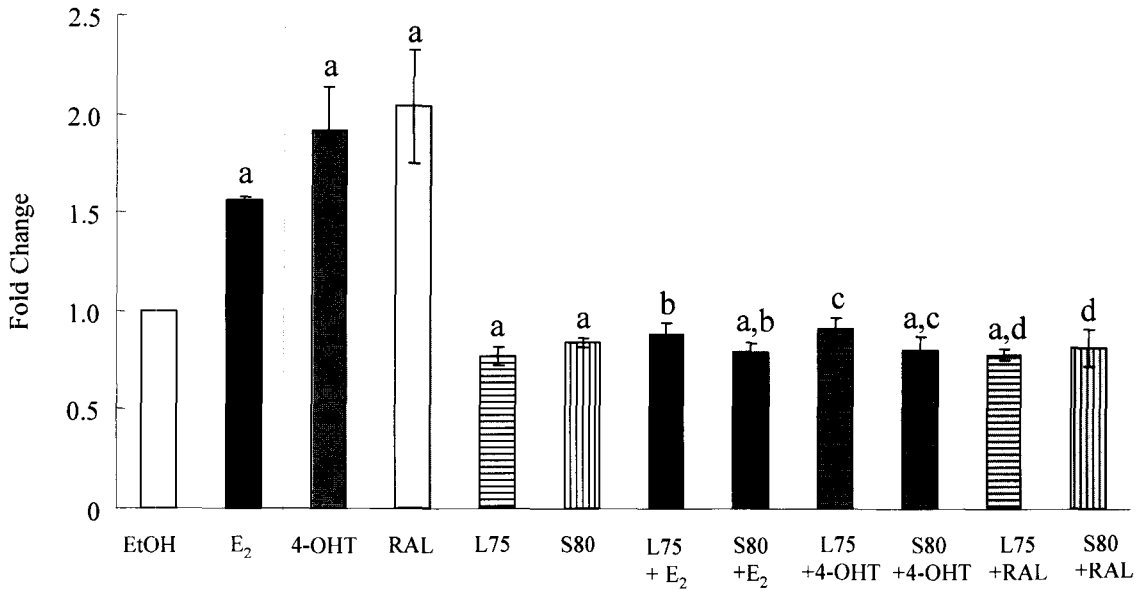


**Figure 56: L50 and L75 inhibit the E<sub>2</sub>-induced increase in NRF-1 at all**

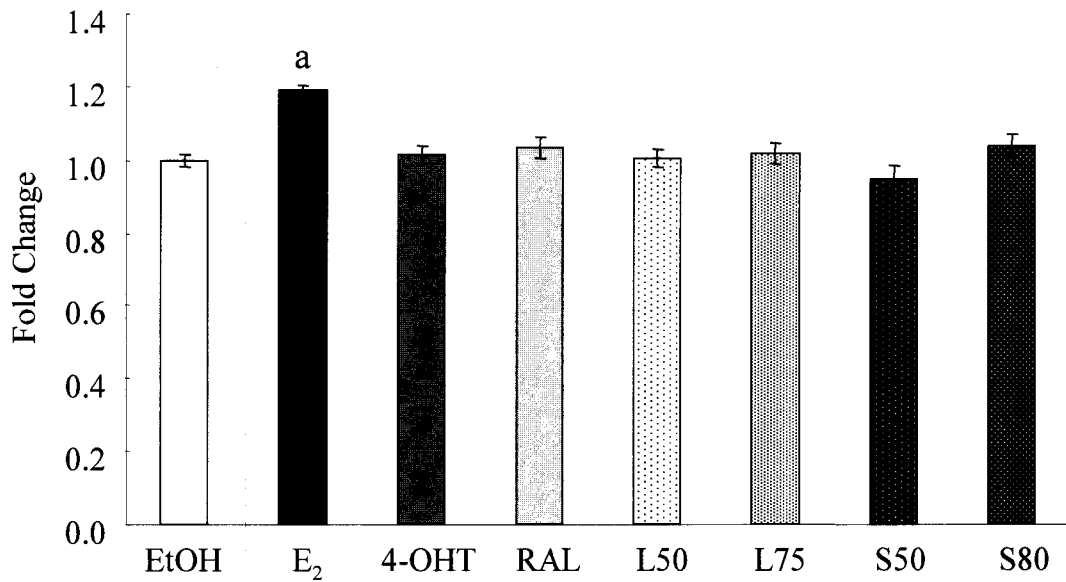
**concentrations tested in HUVEC.** HUVEC were treated with 0.1, 1.0, or 10 mg/mL of L50 or L75 in the presence or absence of 10 nM E<sub>2</sub> for 4 h. The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH; b =  $P < 0.05$  compared to E<sub>2</sub>; c =  $P < 0.05$  compared to the corresponding concentration without the addition of E<sub>2</sub>



**Figure 57: S80 inhibits the E<sub>2</sub>-induced increase in NRF-1 at all concentrations tested in HUVEC.** HUVEC were treated with 0.1, 1.0, or 10 mg/mL of S50 or S80 in the presence or absence of 10 nM E<sub>2</sub> for 4 h. The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH. b =  $P < 0.05$  compared to E<sub>2</sub>; c =  $P < 0.05$  compared to the corresponding concentration without the addition of E<sub>2</sub>



**Figure 58: L75 and S80 inhibit the E<sub>2</sub>-, 4-OHT-, and RAL-induced increase in NRF-1 mRNA in HUVEC.** HUVEC were treated with 10 nM E<sub>2</sub>, 100 nM 4-OHT, and 100 nM RAL alone or in combination with 10 mg/mL of L75 or S80 in the indicated combinations for 4 h. The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH; b =  $P < 0.05$  compared to E<sub>2</sub> alone; c =  $P < 0.05$  compared to 4-OHT alone; d =  $P < 0.05$  compared to RAL alone



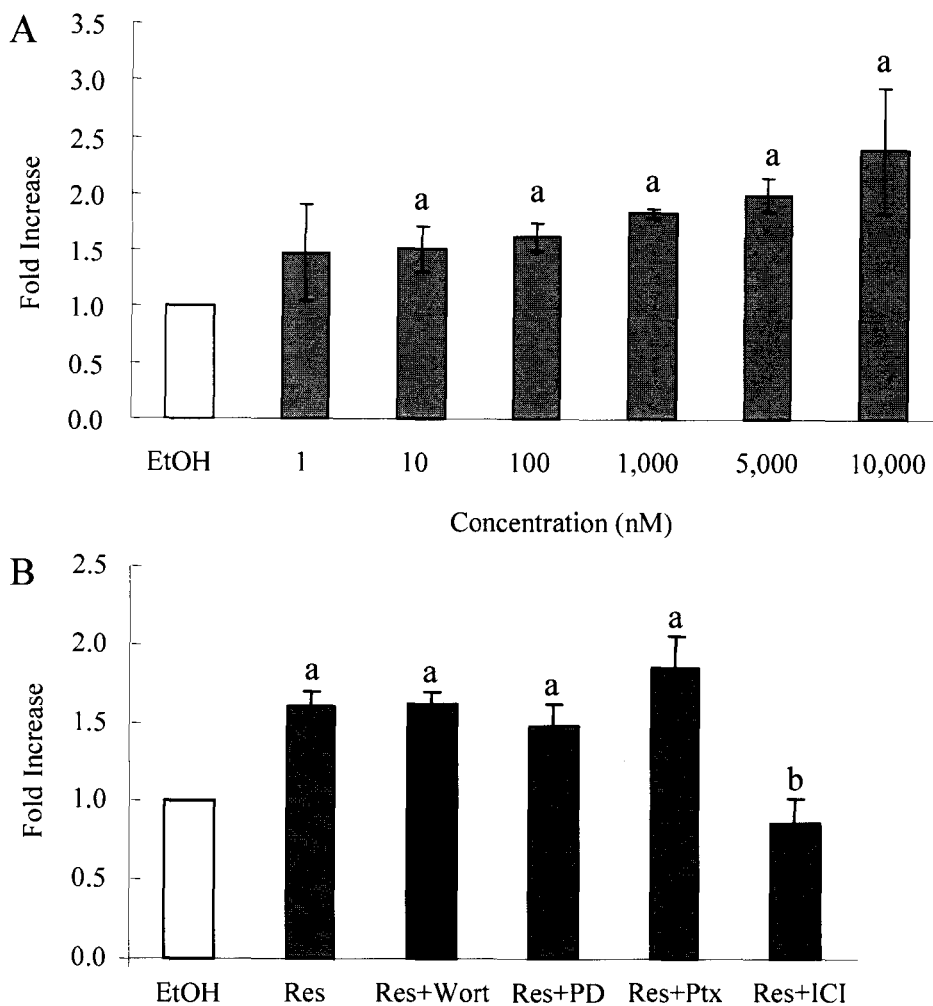
**Figure 59: DEPE do not induce cell death in HUVEC.** HUVEC were treated with EtOH, 10 nM E<sub>2</sub>, 100 nM 4-OHT, 100 nM RAL, 10 mg/ml L50, 10 mg/ml L75, 10 mg/ml S50, or 10 mg/ml S80 for 48 h prior to performing the BrdU assay. Cells were assayed to look for a proliferative response as described in Materials and Methods. Values are the average of 3 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH



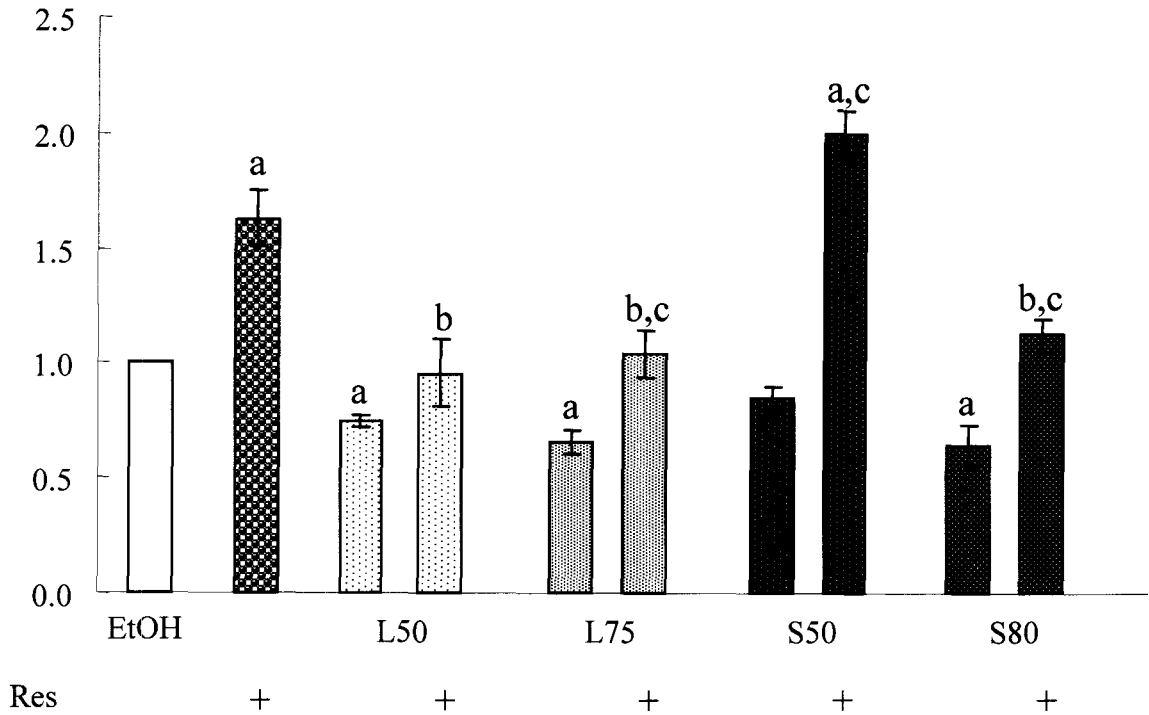
## **Resveratrol Induces an Increase in NRF-1 Through Activation of Genomic ER**

Resveratrol, a phytoestrogen found in grape skins (209), has been shown to bind to and activate ER with low affinity (122) and have cardioprotective capabilities (124,125). Resveratrol increased NRF-1 mRNA expression in a concentration-dependent manner (Figure 60A). To determine if nongenomic ER signaling or genomic ER activation was responsible for mediating this response, Wortmannin, PD98059, Pertussis Toxin, and ICI 182,780 were used in combination with resveratrol to treat HUVEC. Only ICI 182,780 inhibited the resveratrol-induced increase in NRF-1 mRNA, indicating that genomic ER is responsible for the increase in NRF-1 (Figure 60B).

To determine if resveratrol could convey protection against the observed decrease in NRF-1 transcription by DEPE, HUVEC were pretreated with 100 nM resveratrol for 24 h prior to the addition of 10 mg/ml of 4 different DEPE for 4 h. As anticipated from data shown in Figures 55 and 56, L50, L75, and S80 all inhibited basal expression of NRF-1 mRNA (Figure 61). The resveratrol pre-treatment was able to prevent the reduction in basal level expression of NRF-1 by L50, L75, and S80. In the case of L75 and S80, resveratrol pre-treatment lead to an increase in NRF-1 when compared to the respective DEPE treatment alone. However, the expression of NRF-1 seen with the combination of resveratrol and L50, L75, and S80 was significantly lower compared to the sample treated with resveratrol alone. S50 treatment did not inhibit basal NRF-1 transcription and did not block the induction of NRF-1 mRNA by resveratrol (Figure 61).



**Figure 60: Resveratrol increases NRF-1 mRNA expression in a concentration-dependent manner through genomic ER activity in HUVEC.** (A) HUVEC were treated with increasing concentrations of resveratrol (Res). (B) HUVEC were pretreated with 100 nM ICI 182,780 for 6 h or 50 nM Wort or 50  $\mu$ M PD or 50 nM Pertussis Toxin (Ptx) for 1 hr followed by the addition of 10 nM resveratrol for 4 h. The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH; b =  $P < 0.05$  compared to Res alone



**Figure 61: Resveratrol prevents basal suppression of NRF-1 transcription by L75 and S80 in HUVEC.** HUVEC were incubated with 100 nM Res for 24 h prior to the addition of 10mg/ml of the indicated DEPE for 4 h. The total mRNA was isolated following treatment and QRT-PCR was performed as described in Material and Methods. Values are the average of 3 separate experiments  $\pm$  SEM. a =  $P < 0.05$  compared to EtOH; b =  $P < 0.05$  compared to Res alone; c =  $P < 0.05$  compared to treatment with the respective DEPE alone

## DISCUSSION

Estrogens and SERMs have been proposed to have cardioprotective effects; however the mechanisms by which these effects are conveyed remain to be elucidated. I propose that one of these protective mechanisms may be an increase in NRF-1 leading to a subsequent increase in mitochondrial number and activity. Here I report that E<sub>2</sub> and the SERMs 4-OHT and RAL increased NRF-1 gene transcription in HUVEC. Additionally, I observed that DEPE inhibited basal NRF-1 expression and DEPE also inhibited the ability of E<sub>2</sub>, 4-OHT, and RAL to induce NRF-1 expression. The phytoestrogen resveratrol can act in a protective manner to prevent some of the decreases seen using the DEPE extracts.

Although nongenomic signaling by E<sub>2</sub> and SERMS in HUVEC is well established (214), my studies showed that E<sub>2</sub> and SERMs increase NRF-1 gene transcription independent of the MAP Kinase and PI3 Kinase pathways. Instead, this series of experiments revealed that NRF-1 induction was stimulated through genomic ER in response to both E<sub>2</sub> and the SERMs 4-OHT and RAL. This contrasts the results from MCF-7 and H1793 cells in Chapter IV. While the E<sub>2</sub> mediated increase in NRF-1 mRNA was activated through genomic ER in all three cell lines, only HUVEC demonstrated a genomic mode of NRF-1 mRNA induction through 4-OHT and RAL. Previous data (Figures 44 and 45) indicated involvement of the PI3 Kinase pathway in induction of NRF-1 transcription in response to either 4-OHT or RAL in MCF-7 cells. In MCF-7 cells, the data suggested a role for genomic ER signaling and nongenomic signaling in 4-OHT and RAL treated samples. Both nongenomic and genomic signaling was also

observed in 4-OHT and RAL treated H1793 cells. It is not known why genomic signaling appears to be the predominant mode of NRF-1 mRNA activation in HUVEC. However, this observation does indicate the complexity in signaling pathways between diverse cell types in regards to NRF-1 activation. The observation that 4-OHT and RAL can act as inducers of a protein important for mitochondrial maintenance, and therefore important in tissues such as the heart that require large amounts of energy, is unique. While the series of molecular events that lead to cardiovascular disease and dysfunction are complex and multifaceted, a down- or up- regulation in an important integrator of nucleo-mitochondrial interactions such as NRF-1 is notable. The upregulation of NRF-1 by E<sub>2</sub>, 4-OHT, and RAL could be one mechanism by which these compounds convey their cardioprotective effects, although the extent of these effects is still controversial.

It was interesting to note that the HUVEC increased transcription of NRF-1 mRNA in response to both PPT and DPN in an equivalent manner (Figure 49). In experiments done in MCF-7 cells, the activation of NRF-1 correlated with the expression levels of either subtype (Figure 12). HUVEC express both ER $\alpha$  and ER $\beta$  (215). Both PPT and DPN induced an increase in NRF-1 mRNA that was not statistically different from the E<sub>2</sub>-stimulated response. This indicates that either subtype can fully induce NRF-1 mRNA expression in this cell line. The importance of ER $\alpha$  vs. ER $\beta$  in the vasculature has been debated in the literature. Studies in ERKO and Ovx rodents have suggested an important role for ER $\alpha$  in maintaining mitochondrial structure and function in an ischemic reperfusion model (79). It has also been reported that  $\beta$ ERKO mice show a decrease in vascular function compared to wild type animals implying a role for ER $\beta$  in the vasculature (84). In a recent report examining a model of trauma-hemorrhage, PPT

and DPN were used to demonstrate that the protective effects  $E_2$  were mediated through  $ER\beta$  in the cardiovascular (216).

DEPE are known to have both antiandrogenic and antiestrogenic effects in cell culture models (60-62). It is also well known that high levels of particulate matter (PM) in air pollution are associated with an increased risk in cardiovascular disease. Acute exposure to air pollution has been reported to increase cardiac disease mortality (217), and inhalation of high urban levels of concentrated ambient fine PM and ozone for 2 h caused conduit arterial vasoconstriction in healthy adults (218). However, the mechanisms mediating the effects of airborne PM pollutants are complex. Results from the present study allow me to propose that one component of the observed increase in cardiovascular disease mediated by PM may be a decrease in NRF-1 expression and a loss of the beneficial effects of  $E_2$  when these particles are present. The subtle decrease in mitochondrial activity that could result from this scenario may contribute to the observed increase in cardiovascular risk in urban areas (59,60).

The resveratrol data presented in this Chapter suggests that resveratrol mediates a genomic activation of ER in HUVEC leading to an increase in NRF-1 mRNA. This contrasts recent data from mouse studies suggesting a role for resveratrol in increasing mitochondrial biogenesis and activity by upregulating PGC-1 $\alpha$  through SIRT1 (210). SIRT1 is a protein deacetylase known to be activated by resveratrol (210,219). In recent studies, resveratrol-induced SIRT1 activity was shown to activate PGC-1 $\alpha$  by deacetylation without change of PGC-1 $\alpha$  expression (210,219) leading to an increase in genes related to mitochondrial biogenesis and oxidative phosphorylation, including NRF-1 and the NRF-1 target gene Tfam (210). An increase in liver mitochondrial number was

also observed in male mice fed high doses (400 mg/kg/day) of resveratrol for 15 wks.

(219). In microarray data, mice feed a diet high in resveratrol displayed double the NRF-1 mRNA levels compared to the mice feed a diet free of resveratrol (210). These microarrays were performed using muscle tissue. It is interesting to note that the authors did not detect and increase in mitochondrial biogenesis stimulated by resveratrol in the heart under the conditions tested (210). As previously mentioned, the mode of gene activation is likely not only to be multifaceted but differ in between tissue types. Therefore, while the aforementioned studies provide important insight into the action of resveratrol in mitochondrial function in these mice, I do not think they dismiss a potential role for an ER mediated induction of NRF-1 in other cell types, *i.e.*, HUVEC.

The results presented in this Chapter have demonstrated a role for genomic ER in regulating NRF-1 expression in response to E<sub>2</sub>, 4-OHT, RAL, and resveratrol in HUVEC. I have also demonstrated a negative impact of DEPE on NRF-1 expression and a loss of the beneficial effects of E<sub>2</sub>, 4-OHT, and RAL in the presence of the DEPE. Resveratrol was able to protect against the decrease in basal level NRF-1 expression resulting from DEPE treatment. Together these results lay the foundation for future experiments further examining the impact of ER-induced NRF-1 expression on cardiovascular function.

## CHAPTER VI

### CONCLUDING DISCUSSION

There is a clear effect of estrogens on the function and maintenance of mitochondria as demonstrated by numerous studies (79,80,87,106,108-112,114,117-120). How this beneficial effect is mediated is not well defined and may be multifaceted. For example, E<sub>2</sub> can act directly as an antioxidant (220,221) and in this capacity does not require ER. However, a role for ER in mediating many of the positive effects of E<sub>2</sub>, such as an increase in Complex IV activity and increases in mitochondrial O<sub>2</sub> consumption and number has also been discussed at length in the literature (79,81-83,110,114). Conversely, the mechanism by which ER is mediating these beneficial effects has not been well defined. In this body of work, I have attempted to elucidate one of the potential mechanism by which ER can induce the positive increases in mitochondrial function and maintenance previously described (79,81-83,110,114). I have examined the ability of E<sub>2</sub> and other ER ligands to increase the expression of an important integrator of nucleo-mitochondrial interactions, NRF-1, that serves to coordinate a global increase in mitochondrial function.

On a physiological level, one might question the relevance of a small E<sub>2</sub>-induced increase in the expression of NRF-1 and mitochondrial activity in general. Indeed, why would it be evolutionarily beneficial for NRF-1 expression to be enhanced by E<sub>2</sub>? There



are, of course, several lines of thought on this matter. During the course of a lifetime, women are exposed to fluctuating levels of  $E_2$  (22). On a cyclical basis,  $E_2$  is secreted by the ovary during the proliferative phase of the menstrual cycle leading to an increase in cell proliferation in the stroma and endometrial glands (222,223). The increase in  $E_2$  production lasts 10-14 d and is accompanied by a subsequent rise in progesterone levels after ovulation (222,223). During pregnancy there is an increase in circulating  $E_2$  resulting in a variety of phenotypic changes, *i.e.*, increase in breast ductal tissue in preparation for lactation and an increase in uterine volume. Both increases require an increase in available energy; therefore, an increase in mitochondrial number and activity would be beneficial to support these physiological changes. In addition to the changes in breast and uterus during pregnancy, there is also a greater demand on the cardiovascular system due to an increase in blood volume (reviewed in (224,225)). Again, it would be advantageous to upregulate mitochondria in the cardiovascular system in response to  $E_2$  in order to help fill the increased demand in energy. In line with this reasoning, a mitochondrial disorder normally asymptomatic during regular activity resulted in a high rate of preeclampsia in a family carrying the mitochondrial disorder (226). This report suggested that several characteristic features of preeclampsia, including vasoconstriction and disturbed ion transport may, in fact, result from previously undiagnosed mitochondrial dysfunction (226). While, NRF-1 was shown to be increased in the cerebral blood vessels of Ovx rats chronically treated with  $E_2$  (114). However, no one has examined how  $E_2$  affects the expression of NRF-1 in human cardiovascular samples.

Ischemia-reperfusion and trauma-hemorrhage models have demonstrated the importance of both  $E_2$  and ER in protecting against tissue damage (79-81,216). While

mouse knockout studies demonstrated that ER( $\alpha$  or  $\beta$ - or both) can mediate these protective effects (79-81,216), exactly how ER was able to convey these effects was not identified. Several theories have emerged in the literature. Some researchers have proposed that the protective effects of E<sub>2</sub> are mediated by ER localized to the mitochondria (74,76,138,152), and they propose that E<sub>2</sub> acts directly on mtEREs to increase the expression of mitochondrial-encoded genes to mediate this effect (76,138). However, this theory seems unlikely since upregulating the expression of only the few subunits encoded by the mitochondrial genome would not result in functioning complexes. Only one nuclear-encoded mitochondrial gene, cytochrome *c* oxidase subunit VII (COVII) is known to have a functional ERE in the promoter (227). It has been suggested that more nuclear-encoded mitochondrial genes could contain EREs (110), although this has not been demonstrated to date. Even if the existence of a few more mitochondrial genes containing ERE were found, it would certainly not account for a global upregulation of mitochondrial function. Of course, ER regulates gene transcription via tethering mechanisms involving other transcription factors such as AP-1 and Sp1 bound to their response elements and responsive elements in addition to EREs may be involved in regulation of gene transcription involved in OXPHOS. On the other hand, others have proposed that mitochondrial-localized ER conveys its mitochondrial protective effects by increasing the activity of manganese superoxide dismutase (MnSOD) in the mitochondria and protecting against an increase in ROS (228). However, this mechanism would not explain previously demonstrated increases in mitochondrial cristae area, Complex IV activity and protein levels, and O<sub>2</sub> consumption. In this Dissertation, I have presented scientific evidence supporting an alternative

hypothesis for the previously observed beneficial effects of E<sub>2</sub> on mitochondrial function. I propose that ER regulation of NRF-1 could explain the increases in mitochondrial function and maintenance of mitochondrial ultrastructure previously observed in response to E<sub>2</sub>. While my research does not rule out a role for mitochondrial-localized ER, it does present a new theory of ERs mechanism of mitochondrial protection.

Interestingly, although I did not originally anticipate that 4-OHT and RAL would be positive regulators of NRF-1 via ER activation because these SERMs have ER antagonist activity in MCF-7 and H1793 cells, here I report the novel observation that 4-OHT and RAL increase NRF-1 transcription. The activation of NRF-1 in response to E<sub>2</sub>, 4-OHT, and RAL in three different cell types further emphasizes the complexity of predicting gene responses and applying models of activation across cell and tissue types. Both SERMs have been proposed to have cardioprotective effects (52,57,137,201,202), although the extent of these effects is currently unclear (204,207,221). However, the ability of 4-OHT and RAL to upregulate NRF-1 mRNA levels, secondary gene expression, and Complex IV activity in HUVEC suggests a potential to increase mitochondrial activity leading to a possible cardioprotective effect. In line with this hypothesis, the ability of DEPE to antagonize the stimulatory effects of E<sub>2</sub>, 4-OHT and RAL on NRF-1 expression suggests a mechanism by which diesel PM pollution may increase the risk for cardiovascular disease. Inclusive, the effects of 4-OHT, RAL, and DEPE on NRF-1 expression provide an interesting area of study into the contribution of these compounds to cardiovascular disease. More importantly, it would be interesting to know in future experiments if the ER agonist effects of 4-OHT and RAL or the antiestrogenic activity of DEPE will be seen in other tissues. While there is little data on

the neuroprotective role of SERMs, there is evidence in the literature to suggest that they may mediate some beneficial effects (reviewed in (229)).

In addition to the observations made in the DEPE experiments, it is important to note a potential cardioprotective role for resveratrol in protecting against the DEPE reduction in NRF-1 basal levels. While resveratrol is regarded as a cardioprotective agent (124,125,211), the mechanisms by which these effects are conveyed are not fully understood. The nongenomic effects of resveratrol in cardioprotection have been examined in some detail previously (215,230). However, in this Dissertation, I have suggested a new mechanism through which resveratrol may convey cardioprotection by stimulating genomic transcription of NRF-1. Future studies of the downstream effects of this stimulation, as well as the coordinated effort of nongenomic and genomic mechanisms, will be needed to completely understand the cardioprotective effects of resveratrol.

Understanding the mechanisms involved in mitochondrial dysfunction is important in a variety of pathological conditions such as, heart disease, neurodegenerative disorders, and cancer. It has been suggested that administration of E<sub>2</sub> or a SERM may provide beneficial effects in these various conditions (110,137,188,196,201,202,229). However, until the mechanisms regulating the effects of E<sub>2</sub> and their mediation by ER are determined, developing treatment regimens using E<sub>2</sub> or SERMs will be difficult to approach. The work contained within this Dissertation is unique because it addresses a potentially important pathway for ER mediation of the advantageous E<sub>2</sub> effect seen on mitochondrial function in multiple cell types.

## REFERENCES

1. Kuiper, G. G., Shughrue, P. J., Merchenthaler, I., and Gustafsson, J. A. (1998) *Front. Neuroendocrinol.* **19**, 253-286
2. Kuiper, G. G., Enmark, E., Peltö-Huikko, M., Nilsson, S., and Gustafsson, J.-A. (1996) *Proc. Natl. Acad. Sci. USA* **93**, 5925-5930
3. Cowley, S. M., Hoare, S., Mosselman, S., and Parker, M. G. (1997) *J. Biol. Chem.* **272**, 19858-19862
4. Katzenellenbogen, B. S., Montano, M. M., Ekena, K., Herman, M. E., and McInerney, E. M. (1997) *Breast Cancer Res. Treat.* **44**, 23-38
5. Sun, G., Porter, W., and Safe, S. (1998) *Mol. Endocrinol.* **12**, 882-890
6. Paech, K., Webb, P., Kuiper, G. G., Nilsson, S., Gustafsson, J., Kushner, P. J., and Scanlan, T. S. (1997) *Science* **277**, 1508-1510
7. Kuiper, G. G., Carlsson, B., Grandien, J., Enmark, E., Haggblad, J., Nilsson, S., and Gustafsson, J.-A. (1997) *Endocrinology* **138**, 863-870
8. Dotzlaw, H., Leygue, E., Watson, P. H., and Murphy, L. C. (1997) *J. Clin. Endocrinol. Metab.* **82**, 2371-2374
9. Leygue, E., Snell, L., Dotzlaw, H., Hole, K., Hiller-Hitchcock, T., Roughley, P. J., Watson, P. H., and Murphy, L. C. (1998) *Cancer Res* **58**(7), 1348-1352
10. Dotzlaw, H., Leygue, E., Watson, P. H., and Murphy, L. C. (1999) *Cancer Res.* **59**, 529-532

11. Leygue, E., Dotzlaw, H., Watson, P. H., and Murphy, L. C. (1999) *Cancer Res* **59**, 1175-1179.
12. Speirs, V., Parkes, A. T., Kerin, M. J., D.S., W., Carleton, P. J., Fox, J. N., and Atkin, S. L. (1999) *Cancer Res.* **59**, 525-528
13. Weihua, Z., Saji, S., Makinen, S., Cheng, G., Jensen, E. V., Warner, M., and Gustafsson, J. A. (2000) *Proc Natl Acad Sci U S A* **97**, 5936-5941
14. Saji, S., Jensen, E. V., Nilsson, S., Rylander, T., Warner, M., and Gustafsson, J. A. (2000) *Proc Natl Acad Sci U S A* **97**, 337-342
15. Roger, P., Sahla, M. E., Makela, S., Gustafsson, J. A., Baldet, P., and Rochefort, H. (2001) *Cancer Res* **61**, 2537-2541.
16. Ramsey, T. L., Risinger, K. E., Jernigan, S. C., Mattingly, K. A., and Klinge, C. M. (2004) *Endocrinology* **145**(1), 149-160.
17. Gustafsson, J., and Warner, M. (2000) *J Steroid Biochem Mol Biol* **74**, 245-248.
18. Ogawa, S., Inoue, S., Watanabe, T., Orimo, A., Hosoi, T., Ouchi, Y., and Muramatsu, M. (1998) *Nucl. Acids Res.* **26**, 3505-3512
19. Pecorelli, S., Favalli, G., Zigliani, L., and Odicino, F. (2003) *Int J Gynaecol Obstet* **82**(3), 369-379.
20. Boyle, P. (1997) *Lancet* **349 Suppl 2**, SII23-26.
21. Fishman, J., Osborne, M. P., and Telang, N. T. (1995) *Ann N Y Acad Sci.* **768**, 91-100
22. Hankinson, S. E., Willett, W. C., Manson, J. E., Colditz, G. A., Hunter, D. J., Spiegelman, D., Barbieri, R. L., and Speizer, F. E. (1998) *J Natl Cancer Inst* **90**(17), 1292-1299

23. Russo, J., and Russo, I. H. (1999) *Oncol Res* **11**(4), 169-178
24. Henderson, B. E., and Feigelson, H. S. (2000) *Carcinogenesis* **21**(3), 427-433
25. Couse, J. F., and Korach, K. S. (1999) *Endocr Rev* **20**, 358-417
26. Liao, D. Z., Pantazis, C. G., Hou, X., and Li, S. A. (1998) *Carcinogenesis* **19**(12), 2173-2180
27. Encarnacion, C. A., Ciocca, D. R., McGuire, W. L., Clark, G. M., Fuqua, S. A., and Osborne, C. K. (1993) *Breast Cancer Res Treat* **26**(3), 237-246.
28. Patel, J. D., Bach, P. B., and Kris, M. G. (2004) *Jama* **291**(14), 1763-1768
29. Stabile, L. P., Davis, A. L., Gubish, C. T., Hopkins, T. M., Luketich, J. D., Christie, N., Finkelstein, S., and Siegfried, J. M. (2002) *Cancer Res* **62**(7), 2141-2150.
30. Shields, P. G. (2000) *Curr Oncol Rep* **2**(3), 257-262.
31. Stabile, L. P., Lyker, J. S., Gubish, C. T., Zhang, W., Grandis, J. R., and Siegfried, J. M. (2005) *Cancer Res* **65**(4), 1459-1470
32. Dougherty, S. M., Mazhawidza, W., Bohn, A. R., Robinson, K. A., Mattingly, K. A., Blankenship, K. A., Huff, M. O., McGregor, W. G., and Klinge, C. M. (2006) *Endocr Relat Cancer* **13**(1), 113-134
33. Ciana, P., Di Luccio, G., Belcredito, S., Pollio, G., Vegeto, E., Tatangelo, L., Tiveron, C., and Maggi, A. (2001) *Mol Endocrinol* **15**(7), 1104-1113.
34. Siegfried, J. M. (2001) *Lancet Oncol.* **2**(8), 506-513
35. Sekine, I., Nagai, K., Tsugane, S., Yokose, T., Kodama, T., Nishiwaki, Y., Suzuki, K., and Kuriyama, T. (1999) *Jpn J Cancer Res* **90**(2), 129-135

36. Finkelstein, D. M., Ettinger, D. S., and Ruckdeschel, J. C. (1986) *J Clin Oncol* **4**(5), 702-709
37. Albain, K. S., Crowley, J. J., LeBlanc, M., and Livingston, R. B. (1991) *J Clin Oncol* **9**(9), 1618-1626
38. Omoto, Y., Kobayashi, Y., Nishida, K., Tsuchiya, E., Eguchi, H., Nakagawa, K., Ishikawa, Y., Yamori, T., Iwase, H., Fujii, Y., Warner, M., Gustafsson, J. A., and Hayashi, S. I. (2001) *Biochem Biophys Res Commun* **285**(2), 340-347.
39. Mollerup, S., Jorgensen, K., Berge, G., and Haugen, A. (2002) *Lung Cancer* **37**(2), 153-159
40. Patrone, C., Cassel, T. N., Pettersson, K., Piao, Y. S., Cheng, G., Ciana, P., Maggi, A., Warner, M., Gustafsson, J. A., and Nord, M. (2003) *Mol Cell Biol* **23**(23), 8542-8552
41. Kulakosky, P. C., Jernigan, S. C., McCarty, M. A., and Klinge, C. M. (2002) *J. Mol. Endocrinol.* **29**, 137-152
42. Klinge, C. M. (2001) *Nucleic Acids Res* **29**(14), 2905-2919.
43. Hall, J. M., McDonnell, D. P., and Korach, K. S. (2002) *Mol Endocrinol* **16**(3), 469-486.
44. McKenna, N. J., and O'Malley, B. W. (2002) *Cell* **108**(4), 465-474.
45. Shiau, A. K., Barstad, D., Loria, P. M., Cheng, L., Kushner, P. J., Agard, D. A., and Greene, G. L. (1998) *Cell* **95**(7), 927-937.
46. Spencer, T. E., Jenster, G., Burcin, M. M., Allis, C. D., Zhou, J., Mizzen, C. A., McKenna, N. J., Onate, S. A., Tsai, S., Tsai, M. J., and O'Malley, B. W. (1997) *Nature* **389**, 194-198



47. Chen, H., Lin, R. J., Schiltz, L., Chakravarti, D., Nash, A., Nagy, L., Privalsky, M. L., Nakatani, Y., and Evans, R. M. (1997) *Cell* **90**, 569-580
48. Klinge, C. M. (2000) *Steroids* **65**, 227-251
49. Chen, D., Ma, H., Hong, H., Koh, S. S., Huang, S. M., Schurter, B. T., Aswad, D. W., and Stallcup, M. R. (1999) *Science* (5423), 2174-2177
50. Koh, S. S., Chen, D., Lee, Y. H., and Stallcup, M. R. (2001) *J Biol Chem* **276**, 1089-1098
51. Aalfs, J. D., and Kingston, R. E. (2000) *Trends Biochem Sci* **25**(11), 548-555.
52. MacGregor, J. I., and Jordan, V. C. (1998) *Pharmacol Rev* **50**, 151-196
53. Katzenellenbogen, B. S., Norman, M. J., Eckert, R. L., Peltz, S. W., and Mange, W. F. (1984) *Cancer Res.* **44**, 112-119
54. Fisher, B., Costantino, J. P., Redmond, C. K., Fisher, E. R., Wickerham, D. L., and Cronin, W. M. (1994) *J Natl Cancer Inst* **86**, 527-537
55. Jepsen, K., Hermanson, O., Onami, T. M., Gleiberman, A. S., Lunyak, V., McEvelly, R. J., Kurokawa, R., Kumar, V., Liu, F., Seto, E., Hedrick, S. M., Mandel, G., Glass, C. K., Rose, D. W., and Rosenfeld, M. G. (2000) *Cell* **102**(6), 753-763
56. Bryant, H. U., and Dere, W. H. (1998) *Proc Soc Exp Biol Med* **217**(1), 45-52.
57. Gustafsson, J. A. (1998) *Curr Opin Chem Biol* **2**(4), 508-511.
58. Tyulmenkov, V. T., Jernigan, S. C., and Klinge, C. M. (2000) *Mol. Cell. Endocrinol* **165**, 151-161
59. WHO. (1996) *Environmental Health Criteria* **171**, Geneva, Switzerland

60. Okamura K, K. R., Toriba A, Klinge CM, Hayakawa K. (2002) *Polycyclic Aromatic Compounds* **22**, 747-759
61. Meek, M. D. (1998) *Environ Res* **79**(2), 114-121.
62. Kizu, R., Okamura, K., Toriba, A., Mizokami, A., Burnstein, K. L., Klinge, C. M., and Hayakawa, K. (2003) *Toxicol Sci.* **76**(2), 299-309
63. Klinge, C. M., Kaur, K., and Swanson, H. I. (2000) *Arch. Biochem. Biophys.* **373**, 163-174
64. Safe, S. (2001) *Toxicol Lett.* **120**(1-3), 1-7
65. Garesse, R., and Vallejo, C. G. (2001) *Gene* **263**(1-2), 1-16
66. Fernandez-Silva, P., Enriquez, J. A., and Montoya, J. (2003) *Exp Physiol* **88**(1), 41-56
67. Scarpulla, R. C. (2002) *Biochim Biophys Acta* **1576**(1-2), 1-14
68. Attardi, G., and Schatz, G. (1988) *Annu Rev Cell Biol* **4**, 289-333
69. Herrmann, J. M., and Neupert, W. (2000) *Biochim Biophys Acta* **1459**(2-3), 331-338
70. Fisher, R. P., Lisowsky, T., Parisi, M. A., and Clayton, D. A. (1992) *J Biol Chem* **267**(5), 3358-3367
71. McEwen, B. S. (2001) *J Appl Physiol* **91**(6), 2785-2801
72. Wise, P. M. (2002) *Trends Endocrinol Metab* **13**(6), 229-230
73. Wang, J., Green, P. S., and Simpkins, J. W. (2001) *J Neurochem* **77**(3), 804-811
74. Yang, S. H., Liu, R., Perez, E. J., Wen, Y., Stevens, S. M., Jr., Valencia, T., Brun-Zinkernagel, A. M., Prokai, L., Will, Y., Dykens, J., Koulen, P., Simpkins, J. W.,

- Chen, J. Q., Delannoy, M., Cooke, C., and Yager, J. D. (2004) *Proc Natl Acad Sci U S A* **101**(12), 4130-4135
75. Chen, J. Q., Delannoy, M., Cooke, C., and Yager, J. D. (2004) *Am J Physiol Endocrinol Metab* **286**(6), E1011-1022
76. Chen, J. Q., Eshete, M., Alworth, W. L., and Yager, J. D. (2004) *J Cell Biochem* **93**(2), 358-373
77. Schwend, T., and Gustafsson, J. A. (2006) *Biochem Biophys Res Commun* **343**(3), 707-711
78. Yang, S. H., Prokai, L., and Simpkins, J. W. (2006) *Biochem Biophys Res Commun.* **345**(3), 917-918
79. Zhai, P., Eurell, T. E., Cooke, P. S., Lubahn, D. B., and Gross, D. R. (2000) *Am J Physiol Heart Circ Physiol* **278**(5), H1640-1647.
80. Zhai, P., Eurell, T. E., Cotthaus, R., Jeffery, E. H., Bahr, J. M., and Gross, D. R. (2000) *Am J Physiol Heart Circ Physiol.* **279**(6), H2766-2775
81. Zhai, P., Eurell, T. E., Cotthaus, R. P., Jeffery, E. H., Bahr, J. M., and Gross, D. R. (2001) *Am J Physiol Heart Circ Physiol.* **281**(3), H1223-1232
82. Zhu, Y., Bian, Z., Lu, P., Karas, R. H., Bao, L., Cox, D., Hodgins, J., Shaul, P. W., Thoren, P., Smithies, O., Gustafsson, J. A., and Mendelsohn, M. E. (2002) *Science* **295**(5554), 505-508.
83. Pelzer, T., Loza, P. A., Hu, K., Bayer, B., Dienesch, C., Calvillo, L., Couse, J. F., Korach, K. S., Neyses, L., and Ertl, G. (2005) *Circulation.* **111**(12), 1492-1498

84. Zhu, Y., Bian, Z., Lu, P., Karas, R. H., Bao, L., Cox, D., Hodgins, J., Shaul, P. W., Thoren, P., Smithies, O., Gustafsson, J. A., and Mendelsohn, M. E. (2002) *Science* **295**(5554), 505-508
85. Head, G. A., Obeyesekere, V. R., Jones, M. E., Simpson, E. R., and Krozowski, Z. S. (2004) *Endocrinology*. **145**(9), 4286-4291
86. Ling, S., Dai, A., Dilley, R. J., Jones, M., Simpson, E., Komesaroff, P. A., and Sudhir, K. (2004) *Circulation*. **109**(4), 537-543
87. Toda, K., Takeda, K., Okada, T., Akira, S., Saibara, T., Kaname, T., Yamamura, K., Onishi, S., and Shizuta, Y. (2001) *J Endocrinol* **170**(1), 99-111
88. Evans, M. J., and Scarpulla, R. C. (1989) *J Biol Chem* **264**(24), 14361-14368
89. Chau, C. M., Evans, M. J., and Scarpulla, R. C. (1992) *J Biol Chem* **267**(10), 6999-7006
90. Gugneja, S., Virbasius, C. M., and Scarpulla, R. C. (1996) *Mol Cell Biol* **16**(10), 5708-5716
91. Virbasius, C. A., Virbasius, J. V., and Scarpulla, R. C. (1993) *Genes Dev* **7**(12A), 2431-2445
92. Gugneja, S., and Scarpulla, R. C. (1997) *J Biol Chem* **272**(30), 18732-18739
93. Kelly, D. P., and Scarpulla, R. C. (2004) *Genes Dev* **18**(4), 357-368
94. Murakami, T., Shimomura, Y., Yoshimura, A., Sokabe, M., and Fujitsuka, N. (1998) *Biochim Biophys Acta* **1381**(1), 113-122
95. Ojuka, E. O., Jones, T. E., Han, D. H., Chen, M., and Holloszy, J. O. (2003) *Faseb J* **17**(6), 675-681

96. Miranda, S., Foncea, R., Guerrero, J., and Leighton, F. (1999) *Biochem Biophys Res Commun* **258**(1), 44-49
97. Choi, Y. S., Kim, S., Kyu Lee, H., Lee, K. U., and Pak, Y. K. (2004) *Biochem Biophys Res Commun*. **314**(1), 118-122
98. Huo, L., and Scarpulla, R. C. (2001) *Mol Cell Biol* **21**(2), 644-654
99. Larsson, N. G., Wang, J., Wilhelmsson, H., Oldfors, A., Rustin, P., Lewandoski, M., Barsh, G. S., and Clayton, D. A. (1998) *Nat Genet*. **18**(3), 231-236
100. Zhao, C., Lam, E. W., Sunter, A., Enmark, E., De Bella, M. T., Coombes, R. C., Gustafsson, J. A., and Dahlman-Wright, K. (2003) *Oncogene*. **22**(48), 7600-7606
101. Mecham, B. H., Klus, G. T., Strovel, J., Augustus, M., Byrne, D., Bozso, P., Wetmore, D. Z., Mariani, T. J., Kohane, I. S., and Szallasi, Z. (2004) *Nucleic Acids Res*. **32**(9), e74
102. Rae, J. M., Johnson, M. D., Scheys, J. O., Cordero, K. E., Larios, J. M., and Lippman, M. E. (2005) *Breast Cancer Res Treat*. **92**(2), 141-149
103. Landis, M. D., Seachrist, D. D., Montanez-Wiscovich, M. E., Danielpour, D., and Keri, R. A. (2005) *Oncogene*. **24**(33), 5173-5190
104. van 't Veer, L. J., Dai, H., van de Vijver, M. J., He, Y. D., Hart, A. A., Mao, M., Peterse, H. L., van der Kooy, K., Marton, M. J., Witteveen, A. T., Schreiber, G. J., Kerkhoven, R. M., Roberts, C., Linsley, P. S., Bernards, R., and Friend, S. H. (2002) *Nature*. **415**(6871), 530-536
105. Wang, Y., Klijn, J. G., Zhang, Y., Sieuwerts, A. M., Look, M. P., Yang, F., Talantov, D., Timmermans, M., Meijer-van Gelder, M. E., Yu, J., Jatkoe, T., Berns, E. M., Atkins, D., and Foekens, J. A. (2005) *Lancet*. **365**(9460), 671-679

106. Nilsen, J., and Brinton, R. D. (2004) *Curr Drug Targets CNS Neurol Disord* **3**(4), 297-313
107. Simpkins, J. W., Wang, J., Wang, X., Perez, E., Prokai, L., and Dykens, J. A. (2005) *Curr Drug Targets CNS Neurol Disord* **4**(1), 69-83
108. Bettini, E., Pollio, G., Santagati, S., and Maggi, A. (1992) *Neuroendocrinology* **56**(4), 502-508
109. Van Itallie, C. M., and Dannies, P. S. (1988) *Mol Endocrinol.* **2**(4), 332-337
110. Chen, J. Q., Yager, J. D., and Russo, J. (2005) *Biochim Biophys Acta* **14**, 14
111. Law, S. W., Apostolakis, E. M., Samora, P. J., O'Malley, B. W., and Clark, J. H. (1994) *J Steroid Biochem Mol Biol.* **51**(3-4), 131-136
112. Chen, J., Gokhale, M., Li, Y., Trush, M. A., and Yager, J. D. (1998) *Carcinogenesis.* **19**(12), 2187-2193
113. Wang, K., Gan, L., Jeffery, E., Gayle, M., Gown, A. M., Skelly, M., Nelson, P. S., Ng, W. V., Schummer, M., Hood, L., and Mulligan, J. (1999) *Gene* **229**(1-2), 101-108.
114. Stirone, C., Duckles, S. P., Krause, D. N., and Procaccio, V. (2005) *Mol Pharmacol* **7**, 7
115. Ramaswamy, S., Tamayo, P., Rifkin, R., Mukherjee, S., Yeang, C. H., Angelo, M., Ladd, C., Reich, M., Latulippe, E., Mesirov, J. P., Poggio, T., Gerald, W., Loda, M., Lander, E. S., and Golub, T. R. (2001) *Proc Natl Acad Sci U S A.* **98**(26), 15149-15154

116. Moggs, J. G., Tinwell, H., Spurway, T., Chang, H. S., Pate, I., Lim, F. L., Moore, D. J., Soames, A., Stuckey, R., Currie, R., Zhu, T., Kimber, I., Ashby, J., and Orphanides, G. (2004) *Environ Health Perspect.* **112**(16), 1589-1606
117. Vic, P., Vignon, F., Derocq, D., and Rochefort, H. (1982) *Cancer Res* **42**(2), 667-673
118. Matsumoto, M., Nishinakagawa, H., Kurohmaru, M., and Hayashi, Y. (1995) *J Vet Med Sci* **57**(1), 39-44
119. Bo, W. J., Odor, D. L., and Rothrock, M. (1968) *Am J Anat.* **123**(2), 369-384
120. Bo, W. J., Odor, D. L., and Rothrock, M. L. (1969) *Anat Rec.* **163**(1), 121-131
121. Hoffmann, B., Moebus, S., Stang, A., Beck, E. M., Dragano, N., Mohlenkamp, S., Schmermund, A., Memmesheimer, M., Mann, K., Erbel, R., and Jockel, K. H. (2006) *Eur Heart J.* **27**(22), 2696-2702
122. Gehm, B. D., McAndrews, J. M., Chien, P. Y., and Jameson, J. L. (1997) *Proc. Natl. Acad. Sci. U. S. A.* **94**, 14138-14143
123. Bowers, J. L., Tyulmenkov, V. V., Jernigan, S. C., and Klinge, C. M. (2000) *Endocrinology* **141**, 3657-3667
124. Bagchi, D., Das, D. K., Tosaki, A., Bagchi, M., and Kothari, S. C. (2001) *Drugs Exp Clin Res* **27**(5-6), 233-248.
125. German, J. B., and Walzem, R. L. (2000) *Annu Rev Nutr* **20**, 561-593.
126. Borrás, C., Sastre, J., Garcia-Sala, D., Lloret, A., Pallardo, F. V., and Vina, J. (2003) *Free Radic Biol Med* **34**(5), 546-552
127. Vina, J., Borrás, C., Gambini, J., Sastre, J., and Pallardo, F. V. (2005) *Sci Aging Knowledge Environ* **2005**(23), pe17

128. Nilsson, S., and Gustafsson, J. A. (2002) *Crit Rev Eukaryot Gene Expr* **12**(4), 237-257
129. Kelly, M. J., and Levin, E. R. (2001) *Trends Endocrinol Metab* **12**(4), 152-156
130. Mueck, A. O., Seeger, H., and Lippert, T. H. (1996) *Int. J. Clin. Pharmacol. Ther.* **34**(10), 424-426
131. Goetz, R. M., Thatte, H. S., Prabhakar, P., Cho, M. R., Michel, T., and Golan, D. E. (1999) *Proc Natl Acad Sci U S A* **96**(6), 2788-2793
132. Farhat, M. Y., Lavigne, M. C., and Ramwell, P. W. (1996) *FASEB J.* **10**(5), 615-624
133. Kauser, K., and Rubanyi, G. M. (1997) *J. Vasc. Res.* **34**, 229-236
134. Improta-Brears, T., Whorton, A. R., Codazzi, F., York, J. D., Meyer, T., and McDonnell, D. P. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 4686-4691
135. Chen, C. C., Lee, W. R., and Safe, S. (2004) *J Cell Biochem.* **93**(5), 1063-1074
136. Razandi, M., Pedram, A., Greene, G. L., and Levin, E. R. (1999) *Mol. Endocrinol.* **13**, 307-319
137. Bian, Z., Nilsson, S., and Gustafsson, J. A. (2001) *Trends Cardiovasc Med* **11**(5), 196-202
138. Chen, J. Q., and Yager, J. D. (2004) *Ann N Y Acad Sci* **1028**, 258-272
139. Soule, H. D., Maloney, T. M., Wolman, S. R., Peterson, W. D., Jr., Brenz, R., McGrath, C. M., Russo, J., Pauley, R. J., Jones, R. F., and Brooks, S. C. (1990) *Cancer Res.* **50**(18), 6075-6086



140. Piechota, J., Tomecki, R., Gewartowski, K., Szczesny, R., Dmochowska, A., Kudla, M., Dybczynska, L., Stepien, P. P., and Bartnik, E. (2006) *Acta Biochim Pol.* **53**(1), 157-168
141. Riggs, K. A., Wickramasinghe, N. S., Cochrum, R. K., Watts, M. B., and Klinge, C. M. (2006) *Cancer Res* **66**(20), 10188-10198
142. Dauvois, S., White, R., and Parker, M. G. (1993) *J Cell Sci* **106 ( Pt 4)**, 1377-1388
143. Wijayaratne, A. L., and McDonnell, D. P. (2001) *J. Biol. Chem.* **276**(38), 35684-35692
144. Fawell, S. E., White, R., Hoare, S., Sydenham, M., Page, M., and Parker, M. G. (1990) *Proc Natl Acad Sci U S A* **87**(17), 6883-6887.
145. Zhang, D., Baek, S. H., Ho, A., and Kim, K. (2004) *Bioorg Med Chem Lett.* **14**(3), 645-648
146. Sakamoto, K. M. (2005) *Methods Enzymol.* **399**, 833-847
147. Fuqua, S. A., Schiff, R., Parra, I., Friedrichs, W. E., Su, J. L., McKee, D. D., Slentz-Kesler, K., Moore, L. B., Willson, T. M., and Moore, J. T. (1999) *Cancer Res* **59**, 5425-5428
148. Stauffer, S. R., Coletta, C. J., Tedesco, R., Nishiguchi, G., Carlson, K., Sun, J., Katzenellenbogen, B. S., and Katzenellenbogen, J. A. (2000) *J Med Chem.* **43**(26), 4934-4947
149. Meyers, M. J., Sun, J., Carlson, K. E., Marriner, G. A., Katzenellenbogen, B. S., and Katzenellenbogen, J. A. (2001) *J Med Chem* **44**(24), 4230-4251.

150. Sun, J., Meyers, M. J., Fink, B. E., Rajendran, R., Katzenellenbogen, J. A., and Katzenellenbogen, B. S. (1999) *Endocrinology* **140**, 800-804
151. Silberstein, G. B., Van Horn, K., Hrabeta-Robinson, E., and Compton, J. (2006) *J Endocrinol.* **190**(2), 225-239
152. Chen, J. Q., Delannoy, M., Cooke, C., and Yager, J. D. (2004) *Am J Physiol Endocrinol Metab.* **286**(6), 1011-1022
153. Lim, K. T., Cosgrave, N., Hill, A. D., and Young, L. S. (2006) *Breast Cancer Res.* **8**(3), R33
154. Li, X., Zhang, S., and Safe, S. (2006) *J Steroid Biochem Mol Biol.* **98**(2-3), 122-132
155. Xia, Y., Buja, L. M., Scarpulla, R. C., and McMillin, J. B. (1997) *Proc Natl Acad Sci U S A* **94**(21), 11399-11404
156. Bergeron, R., Ren, J. M., Cadman, K. S., Moore, I. K., Perret, P., Pypaert, M., Young, L. H., Semenkovich, C. F., and Shulman, G. I. (2001) *Am J Physiol Endocrinol Metab* **281**(6), E1340-1346
157. Weitzel, J. M., Radtke, C., and Seitz, H. J. (2001) *Nucleic Acids Res* **29**(24), 5148-5155
158. Rodriguez-Pena, A., Escriva, H., Handler, A. C., and Vallejo, C. G. (2002) *FEBS Lett* **514**(2-3), 309-314
159. Demonacos, C. V., Karayanni, N., Hatzoglou, E., Tsiriyiotis, C., Spandidos, D. A., and Sekeris, C. E. (1996) *Steroids* **61**(4), 226-232
160. Scherf, M., Klingenhoff, A., and Werner, T. (2000) *J Mol Biol* **297**(3), 599-606

161. Quandt, K., Frech, K., Karas, H., Wingender, E., and Werner, T. (1995) *Nucleic Acids Res* **23**(23), 4878-4884
162. Gopalakrishnan, L., and Scarpulla, R. C. (1995) *J Biol Chem.* **270**(30), 18019-18025
163. Carroll, J. S., Liu, X. S., Brodsky, A. S., Li, W., Meyer, C. A., Szary, A. J., Eeckhoute, J., Shao, W., Hestermann, E. V., Geistlinger, T. R., Fox, E. A., Silver, P. A., and Brown, M. (2005) *Cell* **122**(1), 33-43
164. Metivier, R., Penot, G., Hubner, M. R., Reid, G., Brand, H., Kos, M., and Gannon, F. (2003) *Cell* **115**(6), 751-763
165. Klinge, C. M., Studinski-Jones, A. L., Kulakosky, P. C., Bambara, R. A., and Hilf, R. (1998) *Mol. Cell. Endocrinol.* **143**, 79-90
166. Klinge, C. M., Jernigan, S. C., Mattingly, K. A., Risinger, K. E., and Zhang, J. (2004) *J Mol Endocrinol* **33**(2), 387-410
167. Pavlik, E. J., and Coulson, P. B. (1976) *J. Steroid Biochem.* **7**, 357-368
168. Klinge, C. M., Peale, F. V., Jr., Hilf, R., Bambara, R. A., and Zain, S. (1992) *Cancer Res.* **52**, 1073-1081
169. Tyulmenkov, V. V., and Klinge, C. M. (2001) *Mol Cell Endocrinol* **182**(1), 109-119.
170. Cheng, Y., and Prusoff, W. H. (1973) *Biochem Pharmacol* **22**, 3099-3108
171. Mahajan, M. A., and Samuels, H. H. (2005) *Endocr Rev.* **26**(4), 583-597
172. Tremblay, G. B., and Giguere, V. (2002) *Crit Rev Eukaryot Gene Expr.* **12**(1), 1-22

173. Katzenellenbogen, B. S., and Katzenellenbogen, J. A. (2000) *Breast Cancer Res.* **2**(5), 335-344
174. Berry, M., Nunez, A. M., and Chambon, P. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 1218-1222
175. Kian Tee, M., Rogatsky, I., Tzagarakis-Foster, C., Cvorovic, A., An, J., Christy, R. J., Yamamoto, K. R., and Leitman, D. C. (2004) *Mol Biol Cell* **15**(3), 1262-1272
176. Monroe, D. G., Secreto, F. J., Subramaniam, M., Getz, B. J., Khosla, S., and Spelsberg, T. C. (2005) *Mol Endocrinol* **19**(6), 1555-1568
177. Yada-Hashimoto, N., Nishio, Y., Ohmichi, M., Hayakawa, J., Mabuchi, S., Hisamoto, K., Nakatsuji, Y., Sasaki, H., Seino-Noda, H., Sakata, M., Tasaka, K., and Murata, Y. (2006) *Menopause*. **13**(6), 935-941
178. Mori-Abe, A., Tsutsumi, S., Takahashi, K., Toya, M., Yoshida, M., Du, B., Kawagoe, J., Nakahara, K., Takahashi, T., Ohmichi, M., and Kurachi, H. (2003) *J Endocrinol*. **178**(3), 417-426
179. Visram, H., and Greer, P. A. (2006) *Cancer Biol Ther*. **5**(12), 1677-1682
180. Gururaj, A. E., Rayala, S. K., Vadlamudi, R. K., and Kumar, R. (2006) *Clin Cancer Res*. **12**(3 Pt 2), 1001s-1007s
181. Acconcia, F., Barnes, C. J., and Kumar, R. (2006) *Endocrinology*. **147**(3), 1203-1212
182. Berry, M., Metzger, D., and Chambon, P. (1990) *EMBO J*. **9**(9), 2811-2818
183. Tzukerman, M. T., Esty, A., Santiso-Mere, D., Danielian, P., Parker, M., Stein, R., B., Pike, J. W., and McDonnell, D. P. (1994) *Mol. Endocrinol*. **8**, 21-30

184. Flouriot, G., Brand, H., Denger, S., Metivier, R., Kos, M., Reid, G., Sonntag-Buck, V., and Gannon, F. (2000) *Embo J* **19**(17), 4688-4700
185. Metivier, R., Petit, F. G., Valotaire, Y., and Pakdel, F. (2000) *Mol Endocrinol* **14**, 1849-1871
186. Merot, Y., Metivier, R., Penot, G., Manu, D., Saligaut, C., Gannon, F., Pakdel, F., Kah, O., and Flouriot, G. (2004) *J Biol Chem.* **279**(25), 26184-26191
187. Moverare, S., Dahllund, J., Andersson, N., Islander, U., Carlsten, H., Gustafsson, J. A., Nilsson, S., and Ohlsson, C. (2003) *Mol Pharmacol* **64**(6), 1428-1433
188. Chambliss, K. L., Yuhanna, I. S., Anderson, R. G., Mendelsohn, M. E., and Shaul, P. W. (2002) *Mol Endocrinol* **16**(5), 938-946.
189. Register, T. C., and Adams, M. R. (1998) *J Steroid Biochem Mol Biol* **64**, 187-191
190. Kim-Schulze, S., McGowan, K. A., Hubchak, S. C., Cid, M. C., Martin, M. B., Kleinman, H. K., Greene, G. L., and Schnaper, H. W. (1996) *Circulation* **94**(6), 1402-1407
191. Hulley, S., Furberg, C., Barrett-Connor, E., Cauley, J., Grady, D., Haskell, W., Knopp, R., Lowery, M., Satterfield, S., Schrott, H., Vittinghoff, E., and Hunninghake, D. (2002) *Jama* **288**(1), 58-66.
192. Fletcher, S. W., and Colditz, G. A. (2002) *Jama* **288**(3), 366-368.
193. Nelson, H. D., Humphrey, L. L., Nygren, P., Teutsch, S. M., and Allan, J. D. (2002) *Jama* **288**(7), 872-881.
194. Moerman, C. J., Van Hout, B. A., Bonneux, L., and Witteman, J. C. (2000) *J Intern Med* **248**(2), 143-150

195. Reis, S. E., Holubkov, R., Young, J. B., White, B. G., Cohn, J. N., and Feldman, A. M. (2000) *J Am Coll Cardiol* **36**(2), 529-533
196. Turgeon, J. L., McDonnell, D. P., Martin, K. A., and Wise, P. M. (2004) *Science* **304**(5675), 1269-1273
197. Clark, J. H. (2006) *Nucl Recept Signal.* **4**, e023
198. Wagner, J. D. (2000) *J Reprod Med* **45**, 245-258
199. Bhavnani, B. R. (2003) *J Steroid Biochem Mol Biol.* **85**(2-5), 473-482
200. Creasman, W. T., Hoel, D., and Disaia, P. J. (2003) *Am J Obstet Gynecol.* **189**(3), 621-626
201. Gol, M., Baris, N., Guneri, S., and Posaci, C. (2006) *Am J Obstet Gynecol.* **194**(5), 1249-1254
202. Vogelvang, T. E., van der Mooren, M. J., Mijatovic, V., and Kenemans, P. (2006) *Drugs.* **66**(2), 191-221
203. Vogel, V. G., Costantino, J. P., Wickerham, D. L., Cronin, W. M., Cecchini, R. S., Atkins, J. N., Bevers, T. B., Fehrenbacher, L., Pajon, E. R., Jr., Wade, J. L., 3rd, Robidoux, A., Margolese, R. G., James, J., Lippman, S. M., Runowicz, C. D., Ganz, P. A., Reis, S. E., McCaskill-Stevens, W., Ford, L. G., Jordan, V. C., and Wolmark, N. (2006) *Jama.* **295**(23), 2727-2741
204. Doshida, M., Ohmichi, M., Tsutsumi, S., Kawagoe, J., Takahashi, T., Du, B., Mori-Abe, A., Ohta, T., Saitoh-Sekiguchi, M., Takahashi, K., and Kurachi, H. (2006) *J Biol Chem.* **281**(34), 24270-24278

205. de Valk-de Roo, G. W., Stehouwer, C. D., Meijer, P., Mijatovic, V., Kluft, C., Kenemans, P., Cohen, F., Watts, S., and Netelenbos, C. (1999) *Arterioscler Thromb Vasc Biol* **19**(12), 2993-3000.
206. Barrett-Connor, E., Mosca, L., Collins, P., Geiger, M. J., Grady, D., Kornitzer, M., McNabb, M. A., and Wenger, N. K. (2006) *N Engl J Med*. **355**(2), 125-137
207. Wenger, N. K., Barrett-Connor, E., Collins, P., Grady, D., Kornitzer, M., Mosca, L., Sashegyi, A., Baygani, S. K., Anderson, P. W., and Moscarelli, E. (2002) *Am J Cardiol*. **90**(11), 1204-1210
208. Okamura, K., Kizu, R., Toriba, A., Murahashi, T., Mizokami, A., Burnstein, K. L., Klinge, C. M., and Hayakawa, K. (2004) *Toxicology*. **195**(2-3), 243-254
209. Celotti, E., Ferrarini, R., Zironi, R., and Conte, L. S. (1996) *J. Chromatogr.* **730**, 47-52
210. Lagouge, M., Argmann, C., Gerhart-Hines, Z., Meziane, H., Lerin, C., Daussin, F., Messadeq, N., Milne, J., Lambert, P., Elliott, P., Geny, B., Laakso, M., Puigserver, P., and Auwerx, J. (2006) *Cell*. **127**(6), 1109-1122
211. Bradamante, S., Barenghi, L., and Villa, A. (2004) *Cardiovasc Drug Rev*. **22**(3), 169-188
212. Cartharius, K., Frech, K., Grote, K., Klocke, B., Haltmeier, M., Klingenhoff, A., Frisch, M., Bayerlein, M., and Werner, T. (2005) *Bioinformatics*. **21**(13)
213. Montiel, M., de la Blanca, E. P., and Jimenez, E. (2006) *Cell Physiol Biochem*. **18**(1-3), 123-134
214. Sumanasekera, W. K., Zhao, L., Ivanova, M., Morgan, D. D., Noisin, E. L., Keynton, R. S., and Klinge, C. M. (2006) *Cell Tissue Res*. **324**(2), 243-253

215. Klinge, C. M., Blankenship, K. A., Risinger, K. E., Bhatnagar, S., Noisin, E. L., Sumanasekera, W. K., Zhao, L., Brey, D. M., and Keynton, R. S. (2005) *J Biol Chem* **280**(9), 7460-7468
216. Hsieh, Y. C., Yu, H. P., Suzuki, T., Choudhry, M. A., Schwacha, M. G., Bland, K. I., and Chaudry, I. H. (2006) *J Mol Cell Cardiol* **41**(3), 511-521
217. Pope, C. A., 3rd, Burnett, R. T., Thurston, G. D., Thun, M. J., Calle, E. E., Krewski, D., and Godleski, J. J. (2004) *Circulation* **109**(1), 71-77
218. Brook, R. D., Brook, J. R., Urch, B., Vincent, R., Rajagopalan, S., and Silverman, F. (2002) *Circulation* **105**(13), 1534-1536
219. Baur, J. A., Pearson, K. J., Price, N. L., Jamieson, H. A., Lerin, C., Kalra, A., Prabhu, V. V., Allard, J. S., Lopez-Lluch, G., Lewis, K., Pistell, P. J., Poosala, S., Becker, K. G., Boss, O., Gwinn, D., Wang, M., Ramaswamy, S., Fishbein, K. W., Spencer, R. G., Lakatta, E. G., Le Couteur, D., Shaw, R. J., Navas, P., Puigserver, P., Ingram, D. K., de Cabo, R., and Sinclair, D. A. (2006) *Nature* **444**(7117), 337-342
220. Schwenke, D. C. (1998) *Semin Reprod Endocrinol* **16**(4), 281-308.
221. Moosmann, B., and Behl, C. (1999) *Proc Natl Acad Sci U S A* **96**, 8867-8872
222. Gilardi, K. V., Shideler, S. E., Valverde, C. R., Roberts, J. A., and Lasley, B. L. (1997) *Biol Reprod* **57**(2), 335-340
223. Slayden, O. D., and Brenner, R. M. (2004) *Arch Histol Cytol.* **67**(5), 393-409
224. van Oppen, A. C., Stigter, R. H., and Bruinse, H. W. (1996) *Obstet Gynecol.* **87**(2), 310-318



225. Del Bene, R., Barletta, G., Mello, G., Lazzeri, C., Mecacci, F., Parretti, E., Martini, E., Vecchiarino, S., Franchi, F., and La Villa, G. (2001) *Bjog*. **108**(4), 344-352
226. Torbergsen, T., Oian, P., Mathiesen, E., and Borud, O. (1989) *Acta Obstet Gynecol Scand*. **68**(2), 145-148
227. Watanabe, T., Inoue, S., Hiroi, H., Orimo, A., Kawashima, H., and Muramatsu, M. (1998) *Mol. Cell. Biol*. **18**, 442-449
228. Pedram, A., Razandi, M., Wallace, D. C., and Levin, E. R. (2006) *Mol Biol Cell*. **17**(5), 2125-2137
229. Dhandapani, K. M., and Brann, D. W. (2002) *Biol Reprod*. **67**(5), 1379-1385
230. Das, S., Fraga, C. G., and Das, D. K. (2006) *Free Radic Res*. **40**(10), 1066-1075

## CURRICULUM VITAE

**KATHLEEN A. MATTINGLY**

**117 North Clifton Avenue**

**Louisville, KY 40206**

**Ktravi01@aol.com**

**(502) 893-6575**

---

### **I. Education**

2000 B. A. Biology, Minor in Chemistry; Bellarmine University, Louisville, KY

2004 M. S. Biochemistry and Molecular Biology; University of Louisville,  
Louisville, KY

M. S. Title: "Maximizing Expression of Flag-tagged Estrogen  
Receptor Beta and Development of a Dual  
Expression Baculoviral System for the Expression of  
Estrogen Receptor Alpha and Beta"

2007 Ph. D. Biochemistry and Molecular Biology; University of Louisville,  
Louisville, KY.

Ph. D. Title: "Regulation of Nuclear Respiratory Factor-1  
Expression by 17 $\beta$ -Estradiol: A New Mechanism for  
Coordinating Mitochondrial Gene Expression"

### **II. Honors and Awards**

2000 Bellarmine University Department of Natural Science Award for  
Excellence

2000 Graduated Magna Cum Laude from Bellarmine University

2005 Recipient of a Women in the Endocrine Society (WE) Travel  
Award to attend the 2005 Endocrine Society meeting in San Diego

### III. Research Experience

- 2001 (spring) Research Assistant, William Dean, Ph.D. (Advisor), Department of Biochemistry and Molecular Biology, Louisville, KY.
- 2001 (summer) Research Assistant, Mark Brennen, Ph.D. (Advisor), Department of Biochemistry and Molecular Biology, Louisville, KY.
- 2001 (summer) Research Assistant, Thomas Geoghegan, Ph.D. (Advisor), Department of Biochemistry and Molecular Biology, Louisville, KY.
- 2004 Was selected to attend the Edward A. Smuckler Memorial Workshop on the Pathobiology of Cancer in Snowmass, Colorado sponsored by the American Association for Cancer Research
- 2001-2007 Graduate Student, Carolyn M. Klinge, Ph.D. (Mentor), Department of Biochemistry and Molecular Biology, Louisville, KY.

### IV. Funding Sources

American Heart Association Ohio Valley Affiliate Pre-doctoral fellowship #315087B to Kathleen A. Mattingly "Estrogen receptor heterodimers" 7/1/03-6/30/05

### V. Teaching Experience

- 2001-2002 Teaching Assistant, Graduate Biochemistry 656 Robert Gray (Course Director), Department of Biochemistry and Molecular Biology; University of Louisville, Louisville, KY.
- 2003-2006 Tutor, Medical Biochemistry, Course for Freshman medical students. University of Louisville, Louisville, KY.

### VI. Students Mentored

- Summer 2002 Mellani Lefta, Sophomore, Eastern Kentucky University, "Characterization of Flag-tagged recombinant human estrogen receptor beta (ER $\beta$ )"
- Summer 2003 Candice Brown, Junior, University of Louisville, "Coactivator

Interaction with Estrogen Receptor  $\beta$  in Electromobility Shift Assays"

- Fall 2004 Smita Ranjan, Graduate Student, "Characterization of HA-tagged *versus* non-tagged estrogen receptor alpha"
- Fall 2004 Lauren Thomas, High School Science Fair Project; "Isolating Flag-Tagged Proteins Using Affinity Chromatography"
- Fall 2004 Erin Gibson, 6<sup>th</sup> Grade Science Fair Project; "Bacterial Growth in Our Home"
- Spring 2005 Matt Hernberger, Graduate Rotation Student, "Analyzing Baculovirus Infection Rates in Sf21 Cells"
- Fall 2005 Erin Gibson, 7<sup>th</sup> Grade Science Fair Project; "Antibiotic Resistance in Our Home"
- Summer 2006 Andrew Barnes, Senior Student, University of Louisville, "Utilizing PCR to Analyze Mitochondrial DNA Copy Number in 17 $\beta$ -Estradiol Treated Cells"
- Summer 2006 Katherine B. Visanescu, First Year Medical Student, University of Louisville, School of Medicine, "Regulation of Nuclear Respiratory Factor-1 (NRF-1) Expression by 17 $\beta$ -Estradiol and Diesel Exhaust Particulate Extracts (DEPE)"

## VII. Peer-reviewed Publications

1. Ramsey, T.L., Jernigan, S.C., Risinger, K.E., Mattingly, K.A., and Klinge, C.M. Estrogen receptor beta isoforms exhibit differences in ligand-activated transcriptional activity in an estrogen response element sequence-dependent manner. *Endocrinology* 145: 149-160, 2004.
2. Klinge, C.M., Jernigan, S.C., Mattingly, K.A., Risinger, K.E., and Zhang, J. Allosteric regulation of the functional interaction of estrogen receptors alpha and beta with coactivators, corepressors, and co-integrators by estrogen response element sequence. *J. Mol. Endocrinol.* 33: 387-410, 2004.
3. Dougherty, S.M., Mazhawidza, W., Bohn, A.S., Robinson, K.A., Mattingly, K.A., Blankenship, K.A., Huff, M.O., McGregor, W.G., and Klinge, C.M. Gender difference in the activity but not expression of estrogen receptors alpha and beta in human lung adenocarcinoma cells. *Endocrine-Rel. Cancer* 13: 113-134, 2006.

4. Ivanova, M. M., Mattingly, K. A., Klinge, C. M. Estrogen receptor beta yield from baculovirus lytic infection is higher than from stably transformed Sf21 cells. *Appl Microbiol Biotechnol* 21: in press, 2007.

## **IX. Abstracts and Presentations**

1. Mattingly, K.A. and Klinge, C.M. Optimizing production of full-length, recombinant human estrogen receptor beta (ER $\beta$ ) in Sf21 insect cells using a baculovirus expression system. (Abstract GR39) Research!Louisville, 2002.

2. Mattingly, K.A., Risinger, K.E., and Klinge, C.M. Estrogen receptor interaction with coregulators is influenced by estrogen response element sequence. (Abstract 22) Great Lakes Nuclear Receptor Conference held at Medical College of Ohio in Toledo, OH. November 15, 2003.

3. Mattingly, K.A. and Klinge, C.M. Maximizing expression of Flag-tagged ERbeta and development of a dual expression baculoviral system for ERalpha/ERbeta. Poster Abstract 13 at the Mid West Molecular Endocrinology meeting, University of Indiana, Indianapolis, IN. May 20-21, 2004.

4. Mattingly, K.A., Jernigan, S.C., Risinger, K.E., and Klinge, C.M. Estrogen receptor interaction with coregulators is influenced by estrogen response element sequence (Abstract 43) Third Annual Retreat of the James Graham Brown Cancer Center, University of Louisville School of Medicine, September 23, 2004.

5. Mattingly, K.A., Risinger, K.E., and Klinge, C.M. Estrogen Receptor Interaction with Coregulators is Influenced by Estrogen Response Element Sequence. Research!Louisville, 2004.

6. Mattingly, K.A., Risinger, K.E., and Klinge, C.M. Estrogen receptor interaction with coregulators is influenced by estrogen response element sequence. IMD3 Symposium, University of Louisville, March, 2005.

7. Mattingly, K.A., Risinger, K.E., and Klinge, C.M. Estrogen receptor interaction with coregulators is influenced by estrogen response element sequence Annual Endocrine Society Meeting, San Diego, CA, June, 2005.

8. Mattingly, K.A., Ivanova, M.M., and Klinge, C.M. Regulation of Nuclear Respiratory Factor 1 by 17 $\beta$ -Estradiol. Research!Louisville, 2006.

9. Mattingly, K.A., Ivanova, M.M., and Klinge, C.M. Regulation of Nuclear Respiratory Factor 1 by 17 $\beta$  -Estradiol. Fifth Annual Retreat of the James Graham Brown Cancer Center, University of Louisville School of Medicine.

10. Mattingly, K.A., Ivanova, M.M., and Klinge, C.M. Regulation of Nuclear Respiratory Factor 1 by 17 $\beta$  -Estradiol. IMD3 Symposium, University of Louisville, March, 2007.