IDENTIFICATION AND CHARACTERIZATION OF ESTROGEN RECEPTOR-
REGULATED GENE EXPRESSION PROGRAMS

BY

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DISSERTATION

Submitted in partial fulfillment of the requirements
for the degree of Doctor of Philosophy in Cell and Developmental Biology
in the Graduate College of the
University of Illinois at Urbana-Champaign, 2010

Urbana, Illinois

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ABSTRACT

The physiological effects of natural and synthetic estrogens are mediated by estrogen receptor alpha (ERα), and estrogen receptor beta (ERβ). Within the nucleus of target cells, ERα and ERβ serve as ligand-activated transcription factors to stimulate or repress the transcription of estrogen receptor regulated genes. ERα and ERβ may be co-expressed in estrogen-responsive cells, but may also be differentially expressed in a cell- and tissue-specific manner. In addition, within a given context these two receptors have different ligand binding and transcriptional activities. Taken together, these attributes underlie differences in target gene regulation, and overall, different physiological actions by ER subtypes. The work described here is an attempt to understand the roles of ERα and ERβ in target tissues (e.g. bone, breast, uterus) including the gene networks and cell signaling pathways under ER regulation. We have also characterized the regulation of one of the ER-regulated genes, *Carbonic Anhydrase XII*, and examined its regulation by ERα through use of a conserved distal enhancer.

The work described here reports the characterization of individual gene regulatory actions of ERα and ERβ. To investigate the individual actions of ERα or ERβ, we utilized Affymetrix oligonucleotide arrays to profile transcripts regulated by 17β-estradiol (E2) in U2OS-ERα and U2OS-ERβ cells. These cell lines were constructed by stable integration of ERα or ERβ into human osteoblast-like U2OS osteosarcoma cells and initially characterized for ER subtype expression, E2-binding, and cellular responses to E2, including proliferation, motility, and adhesion. Cells expressing apo-ERα or apo-ERβ did not show significant alteration in adhesion or proliferation after addition of E2,

however there was a significant stimulation of migration in E2-treated ERβ-expressing cells. U2OS-ERα, and U2OS-ERβ cells were treated with 10 nM E2 for 0, 4, 8, 24, and 48 hours and total RNA was collected and hybridized to Affymetryx U95Av2 GeneChips and subjected to a Confidence Score to determine E2-regulated RNAs. Of the *ca.* 100 stimulated or repressed genes identified, some were stimulated by E2 equally through ERα and ERβ, whereas others were selectively stimulated via ERα or ERβ. The E2-regulated genes showed three distinct temporal patterns of expression over the 48 hour time course studied. Among stimulated genes, ERα-containing cells exhibited a greater number of regulated transcripts, and overall magnitude of stimulation was increased as compared those regulated by ERβ. Of the functional categories of the E2-regulated genes, most numerous were those encoding cytokines and factors associated with immune response, signal transduction, and cell migration and cytoskeleton regulation, indicating that E2 can exert effects on multiple pathways in these osteoblast-like cell lines. Of note, E2 up-regulated several genes associated with cell motility selectively via ERβ, in keeping with the selective E2 enhancement of the motility of ERβ-containing cells. On genes regulated equally by E2 via ERα or ERβ, the phytoestrogen genistein preferentially stimulated gene expression via ERβ. These studies indicate both common as well as distinct target genes for these two ERs, and identify many novel genes not previously known to be under estrogen regulation.

We have examined the ER regulation of the *Carbonic Anhydrase XII (CA12)* gene, a gene identified as E2-regulated in the studies described above. We investigated the expression of *CA12* and its and regulation of by 17β-estradiol and selective estrogen receptor modulators in breast cancer cells, and characterize the ER usage of a distal

enhancer necessary for *CA12* gene regulation.   We find that *CA12* expression is highly correlated with ERα expression in human breast tumors. We demonstrate that E2 and SERMS increase *CA12* mRNA and protein in multiple breast cancer cell types expressing ERα, and that *CA12* regulation by estrogen is a primary transcriptional response mediated by ERα. By genome-wide chromatin immunoprecipitation (ChIP) and ChIP scanning of the *CA12* locus, we find E2-occupied ERα is recruited to a distal region 6.1 kb upstream of the CA12 transcription start site (TSS) *in vivo*. We find that E2 treatment results in recruitment of RNA polymerase II and steroid receptor coactivators SRC-2 and SRC-3 to the CA12 genomic locus and is correlated with increased histone H4 acetylation. Mutagenesis of an imperfect estrogen-responsive element within this -6.1kb distal enhancer region abolishes estrogen-dependent heterologous reporter activity. Chromosome conformation capture (3C) and chromatin immunoprecipitation assays demonstrate that this distal enhancer communicates with the transcriptional start site of the *CA12* gene via intra-chromosomal looping upon hormone treatment. This distal enhancer element is observed in the homologous mouse genomic sequence, and the expression of the mouse homolog, *Car12*, is rapidly and robustly stimulated by estradiol in the mouse uterus in vivo, suggesting that the ER regulation of *CA12* is mechanistically and evolutionarily conserved. Our findings highlight the crucial role of ER in regulation of the *CA12* gene, and provide insight into the transcriptional regulatory mechanism that accounts for the strong association of *CA12* and ER in human breast cancers. In addition, our findings imply that involvement of long distance enhancers in regulation of estrogen-responsive genes in breast cancer may be more frequent than previously appreciated.

For my family and my many generous teachers.

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION AND SIGNIFICANCE

Estrogen receptors as a window into a transcriptional basis for physiology

The proper coordination of cell- and tissue-specific responses throughout development and normal homeostasis of metazoans is achieved, in part, by the dynamic control of gene expression by hormones. This understanding stems directly from the pioneering studies of estrogen action by Jensen, Gorski, and others in the 1950's and 1960's showed that hormonal effects could be elicited through regulation of gene expression (1-3). In mammals, the effects of estrogenic steroid hormones are largely, if not exclusively, mediated by two members of the nuclear receptor superfamily, estrogen receptor alpha (ERα), and estrogen receptor beta (ERβ). By binding and activation of these receptors, estrogens are potent modulators of the development, homeostasis, and pathophysiology of many tissues such as the breast, male and female reproductive tracts, adipose, and skeletal, cardiovascular, immune, and central nervous systems (4-8).

Estrogen action and endocrine therapies for breast cancer

While the diverse actions of estrogens are of great interest, the pathophysiological role of estrogen receptors in breast cancer has been an area of intense interest. Breast cancer is the most common malignant diagnosis for women in the United States with approximately 190,000 diagnoses and 40,000 deaths estimated in 2009 (9). Unfortunately, the etiology of breast cancer is only partially understood, but the influence of estrogens on breast tumor growth has been known since the early 20th century.

Cumulative exposure of estrogens to the breast epithelium underlies the majority of known risk factors for breast cancer (10, 11). Estrogens stimulate proliferation of normal mammary cells, providing increased cell mass and opportunities for accrual of gross chromosomal aberrations and point mutations (12, 13). Once tumors are established, estrogens appear to stimulate breast cancer cell proliferation in most patients with primary breast tumors (11, 14). Preventive and adjuvant therapies targeting the actions of estrogens, either by lowering the available amount of endogenous estrogens or blocking estrogen receptor action, have contributed to the recent decline in breast cancer mortality (8, 15, 16). The decision to employ these therapies is clearly influenced by the relative presence or absence of ERα expression in breast tumor samples (5, 17, 18). ERα is expressed in approximately 70 percent of breast cancers, and unbiased clustering of tumor expression profiles reveals ERα expression status to be the foremost factor partitioning gene expression profiles (19-21). Patients with tumors expressing ERα generally have a more indolent disease and ERα-expressing tumors are more often well-differentiated and are associated with other favorable prognostic characteristics (18, 22). The expression of well-characterized ERα target genes such as *Progesterone Receptor*, *pS2/Trefoil Factor1*, and *Cathepsin D* have been used as markers of estrogen action *in vivo*, and thus surrogates for ERα expression, but their expression alone cannot fully predict ERα expression status or response to endocrine therapies. Regardless, the use of RNA expression profiling and next-generation sequencing of individual breast tumors continues to evolve, providing better classifications of tumor behavior, a greater number of genetic determinants and downstream effectors, thereby providing a more sophisticated understanding of treatment decisions (19, 21, 23-28).

Unlike ERα, the roles of the more recently discovered ERβ in breast cancer cells are still being uncovered, and ERβ appears to negatively modulate the actions of ERα. When co-expressed in the same cell, ERβ appears to negatively influence ERα binding in a target gene-specific manner, as well as affect global gene expression profiles and cancer cell proliferation. This is consistent with the relative decline of ERβ:ERα levels with disease progression, and is suggestive of an overall dampening effect of ERβ on ERα actions (29-35).

Mechanisms of Classical Estrogen Receptor Action in a Target Cell

ERα and ERβ are prototypic steroid hormone receptors, and like all nuclear receptor (NR) superfamily members, are intracellular ligand-regulated transcription factors which stimulate and repress expression of target genes by sensing classical hormones or other signaling mediators (36-38). ERα and ERβ share a modular domain structure with variable homologies of the respective domains between the ER subtypes. They share high homology between their DNA-binding domains which contain two zinc-finger motifs (DBD; 95% amino acid identity), and substantial homology between their ligand-binding domains (LBD; 56% amino acid identity) (39-43). The two receptor subtypes are expressed differentially in estrogen-target tissues and also differ in their abilities to bind certain ligands and activate specific responsive promoters or enhancers, suggesting that they may have widely-divergent biological actions *in vivo* (5, 41, 44).

In the cell, unliganded ERα largely localizes to the nucleus and is complexed with chaperone proteins (45-48). Upon binding of endogenous hormone (e.g. 17β-estradiol; E2), the ER undergoes conformational changes and exhibits protein surfaces allowing for

stable self-dimerization, as well as contact with specific regulatory regions of DNA termed estrogen-responsive elements (EREs) (39, 49). EREs were classically considered to be comprised of perfect or imperfect inverted palindromes of 5'-Pu-GGTCA-3' with three intervening nucleotides, however considerable diversity of ER binding elements have been described (50-53). In addition, activated ER may also directly bind other DNA-bound proteins which then bind or "tether" ER to chromatin and (53-57). Like other transcriptional regulators, the chromatin-bound ERα serves to recruit other transcription factors and RNA polymerase II to target genes in a response element- and signal-specific manner to regulate gene expression (38, 58, 59).

ERα may be able to directly bind members of the general transcription machinery, but principally regulates transcription through the dynamic recruitment of coregulator protein complexes which may (1) have histone acetyltransferase, methyltransferase, or deacetylase activites, (2) act as ATP-dependent nucleosome remodeling proteins, or (3) mediate contacts with other coregulators and/or the general transcription machinery (59-64). Ligand-bound ERα recruits these regulatory complexes by presentation of two activation domains, denoted as activation function 1 (AF1) and activation function 2 (AF2) located on the N- and C- terminal portions of the receptor, respectively. AF1 is constitutively active and may act independently or synergistically with AF2 when bound with agonist. Importantly, AF1 is modulated by phosphorylation via growth factor-mediated signaling cascades (65-67) and the relative strength of AF1 transactivation ability is dependent on both the context of coregulator proteins available to partner with and the promoter context of the target gene (68-70). AF2 activity, located in the ligand binding domain, is ligand dependent and profoundly influenced by the nature of ligand

bound. Estrogen-bound ER is able to present a binding surface for coactivators such as

the p160 family of proteins which have both acetyltransferase activity and serve as

scaffold proteins for recruitment of other transcription factors such as p300 or CBP,

DRIP/TRAP complexes, and ultimately the general transcription machinery and RNA

polymerase II. Pure anti-estrogenic ligands, often referred to as selective estrogen

receptor down-regulators (SERDS), such as the synthetic antiestrogen ICI 182,780 put

the receptor into a conformation which fail to recruit coactivators to gene sites and also

induce pronounced degradation of the receptor. Mixed agonist/antagonists like the

selective estrogen receptor modulators (SERMS) tamoxifen and raloxifene put the

receptor into a range of conformations which are akin to antiestrogen-bound ER, but

show a mixed ability to recruit coactivators and corepressors depending on the cell- and

promoter context (71-74). Thus, the estrogen receptor dynamically nucleates specific

transcriptional regulatory complexes and displays a complex pharmacology which is

influenced by the receptor itself (e.g. subtype, expression level, post-translational

modifications), the nature of the ligand (agonist, antagonist, SERM, SERD), coregulatory

effectors such as the type of DNA element bound, and coregulator proteins available for

binding (38, 68, 71, 74-77).

Estrogen Receptor Biology and Genomic Complexity

While the biology of the estrogen receptor displays a relatively complex

pharmacology involving the conformation, post-translational modification and location of

the receptor, ligand, allosteric DNA response elements, and cofactor recruitment

described above, this understanding is based on the investigation of relatively few

examples of known ERα-target genes studied *in vitro* and even fewer target genes studied *in vivo*. The wider adoption of technological advances such as microarrays and next-generation sequencing has allowed the examination of E2-responsive RNAs and ER binding sites on a genome-wide scale and has allowed a much greater understanding of estrogen receptor actions (53, 78-83).

mRNA expression profiling of breast cancer and human osteosarcoma cell lines expressing ERα, ERβ, or both subtypes has served to greatly expand our understanding of the 'transcriptome' under ER control (29, 78, 84-90). Work from the Katzenellenbogen group has demonstrated that in ERα-expressing MCF-7 cells, E2 is able to regulate as many as ~1500 transcripts (5% of expressed transcripts), and the majority of regulated transcripts are actually down-regulated. Consistent with E2-induced proliferation of MCF-7 cells, bioinformatic analysis of biological processes (e.g. Gene Ontology) regulated by specific E2-regulated transcripts showed clear up-regulation of pro-proliferation genes such as growth factors and cell cycle regulators, and down-regulation of pro-apoptotic and growth inhibitory factors (78). When considering this work with other research groups, a picture of E2-regulated gene expression emerges. In general, E2-stimulated genes appear to be regulated early, within 8 hours, while down-regulated genes appear to be regulated at later times. Approximately 20% of all regulated transcripts in MCF-7 cells are considered direct targets as shown by co-administration of the translational inhibitor cycloheximide (78, 86). Not surprisingly, there is divergence of E2-targets in ERα-expressing cell lines derived from different tissues or cell lineages (e.g MCF-7, T47-D, and U2OS-ERα), suggesting that expression programs underlie the divergent phenotypes (86, 90, 91). This also underscores the necessity of different

cofactors (e.g. FOXA1) present within a given cell which influence ER activities (80, 91-95). In addition, expression profiling has uncovered the necessity of some DNA bound transcription factors such as AP-1 for ER-mediated gene regulation (89, 96). ERα and ERβ appear to regulate a widely diverse set of genes (~70% divergent) when expressed in a given cell (29, 31, 84, 85, 87). Again this highlights the differential abilities of ERα and ERβ to recruit requisite cofactors and underscores their different biological effects in tissues. Expression studies have been extended to examine the activities of SERMs, which have both agonistic and antagonistic actions within a given cell. The mixed agonist/antagonist trans-hydroxytamoxifen acted as a partial or full agonist for approximately 50% of E2-stimulated genes and 75% of down-regulated genes (79, 97). Importantly, the identification of ER-regulated genes in these cell-based assays has been used to develop an outcome predictor able to define clinically relevant subgroups within clinically defined ER+ (21).

The genome-wide identification of ER binding sites has allowed a refinement of the classical ERE, as well as additional genomic sequences associated with, and perhaps necessary for, ER binding and activity (53, 80, 81, 89, 91, 94, 95). We now know that a majority of the ER binding sites in enhancers are a considerable distance (>5 kb) from known and putative ER-target genes (53, 80, 81, 98). The concept that distant transcription factor binding sites can be physically close to their regulated promoters in '*cis*' or '*trans*' is well-appreciated, but still poorly understood. In fact, the three-dimensional organization of genomes has been considered to be both functionally important for gene expression and relatively plastic in response to developmental or environmental cues (99-102). The use of so called chromosome conformation capture

("3C") and variants of this technique have suggested mechanisms of intra- and inter-chromosomal communication between these distal ER-binding enhancers and individual ER-regulated genes such as *pS2/Trefoil Factor1, GREB1, Progesterone Receptor*, and *Carbonic Anhdrase XII* (80, 103-106). Coupling ChIP and chromatin conformation techniques with next-generation sequencing has further advanced the study of enhancers and their usage on a genome-wide scale (98, 107-110). Specifically, the recent development of the chromatin interaction analysis by paired-end tag sequencing (ChIA-PET) has allowed for an unbiased, genome-wide method for detecting protein-based chromatin interactions *in vivo* (98). This technique has been used to examine both ERα binding sites with similar base-pair resolution as existing methods (e.g. ChIP-Seq), as well as interrogate the physical proximity of ER binding sites to one another and E2-regulated genes regardless of genomic distance or position within a chromosome. The work by Cheung and Ruan (98) found that activated ERα is recruited to multiple, often distal binding sites which physically communicate with each other in both simple duplex and complex looping of intervening chromatin. ERα-bound loops are associated with binding of additional factors (e.g. FOXA1) and RNAPII, and appear to be "anchored" at E2-target genes, with intervening non-target genes looped out. These interactions have been proposed to provide a physical partitioning of genes for differential regulation and act as centers to provide sufficient local concentrations of necessary factors for transcriptional regulation. It is easy to anticipate that the further advancement of methods such as ChIA-PET coupled with cell-based imaging will allow for continued appreciation of the ability of estrogen receptors to physically and functionally coordinate genomic actions.

References

1. **Jensen EV, Greene GL, Closs LE, DeSombre ER, Nadji M** 1982 Receptors reconsidered: a 20-year perspective. Recent Prog Horm Res 38:1-40
2. **Notides A, Gorski J** 1966 Estrogen-induced synthesis of a specific uterine protein. Proc Natl Acad Sci U S A 56:230-5
3. **O'Malley BW** 1995 Thirty years of steroid hormone action: personal recollections of an investigator. Steroids 60:490-8
4. **Couse JF, Korach KS** 1999 Estrogen receptor null mice: what have we learned and where will they lead us? Endocr Rev 20:358-417
5. **Gruber CJ, Tschugguel W, Schneeberger C, Huber JC** 2002 Production and actions of estrogens. N Engl J Med 346:340-52
6. **Nilsson S, Gustafsson JA** 2002 Estrogen receptor action. Crit Rev Eukaryot Gene Expr 12:237-57
7. **Nilsson M, Dahlman-Wright K, Gustafsson JA** 2004 Nuclear receptors in disease: the oestrogen receptors. Essays Biochem 40:157-67
8. **Deroo BJ, Korach KS** 2006 Estrogen receptors and human disease. J Clin Invest 116:561-70
9. **Jemal A, Siegel R, Ward E, Hao Y, Xu J, Thun MJ** 2009 Cancer statistics, 2009. CA Cancer J Clin 59:225-49
10. **Feigelson HS, Ross RK, Yu MC, Coetzee GA, Reichardt JK, Henderson BE** 1998 Sex steroid hormones and genetic susceptibility to breast and prostate cancer. Drug Metab Rev 30:421-34
11. **Henderson BE, Ross RK, Pike MC, Casagrande JT** 1982 Endogenous hormones as a major factor in human cancer. Cancer Res 42:3232-9
12. **Yue W, Santen RJ, Wang JP, Li Y, Verderame MF, Bocchinfuso WP, Korach KS, Devanesan P, Todorovic R, Rogan EG, Cavalieri EL** 2003 Genotoxic metabolites of estradiol in breast: potential mechanism of estradiol induced carcinogenesis. J Steroid Biochem Mol Biol 86:477-86
13. **Yue W, Wang JP, Li Y, Bocchinfuso WP, Korach KS, Devanesan PD, Rogan E, Cavalieri E, Santen RJ** 2005 Tamoxifen versus aromatase inhibitors for breast cancer prevention. Clin Cancer Res 11:925s-30s
14. **Henderson BE, Ross R, Bernstein L** 1988 Estrogens as a cause of human cancer: the Richard and Hinda Rosenthal Foundation award lecture. Cancer Res 48:246-53
15. **McKean-Cowdin R, Feigelson HS, Ross RK, Pike MC, Henderson BE** 2000 Declining cancer rates in the 1990s. J Clin Oncol 18:2258-68
16. **Johnston SR, Dowsett M** 2003 Aromatase inhibitors for breast cancer: lessons from the laboratory. Nat Rev Cancer 3:821-31
17. **Osborne CK** 1998 Tamoxifen in the treatment of breast cancer. N Engl J Med 339:1609-18
18. **Ali S, Coombes RC** 2002 Endocrine-responsive breast cancer and strategies for combating resistance. Nat Rev Cancer 2:101-12

19. **Gruvberger S, Ringner M, Chen Y, Panavally S, Saal LH, Borg A, Ferno M, Peterson C, Meltzer PS** 2001 Estrogen receptor status in breast cancer is associated with remarkably distinct gene expression patterns. Cancer Res 61:5979-84

20. **Liu ET, Sotiriou C** 2002 Defining the galaxy of gene expression in breast cancer. Breast Cancer Res 4:141-4

21. **Oh DS, Troester MA, Usary J, Hu Z, He X, Fan C, Wu J, Carey LA, Perou CM** 2006 Estrogen-regulated genes predict survival in hormone receptor-positive breast cancers. J Clin Oncol 24:1656-64

22. **Ali S, Coombes RC** 2000 Estrogen receptor alpha in human breast cancer: occurrence and significance. J Mammary Gland Biol Neoplasia 5:271-81

23. **Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D** 2000 Molecular portraits of human breast tumours. Nature 406:747-52

24. **Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lonning P, Borresen-Dale AL** 2001 Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 98:10869-74

25. **van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH** 2002 Gene expression profiling predicts clinical outcome of breast cancer. Nature 415:530-6

26. **Ramaswamy S, Perou CM** 2003 DNA microarrays in breast cancer: the promise of personalised medicine. Lancet 361:1576-7

27. **van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, Witteveen A, Glas A, Delahaye L, van der Velde T, Bartelink H, Rodenhuis S, Rutgers ET, Friend SH, Bernards R** 2002 A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 347:1999-2009

28. **Sotiriou C, Pusztai L** 2009 Gene-expression signatures in breast cancer. N Engl J Med 360:790-800

29. **Chang EC, Frasor J, Komm B, Katzenellenbogen BS** 2006 Impact of estrogen receptor beta on gene networks regulated by estrogen receptor alpha in breast cancer cells. Endocrinology 147:4831-42

30. **Frasor J, Chang EC, Komm B, Lin CY, Vega VB, Liu ET, Miller LD, Smeds J, Bergh J, Katzenellenbogen BS** 2006 Gene expression preferentially regulated by tamoxifen in breast cancer cells and correlations with clinical outcome. Cancer Res 66:7334-40

31. **Chang EC, Charn TH, Park SH, Helferich WG, Komm B, Katzenellenbogen JA, Katzenellenbogen BS** 2008 Estrogen Receptors alpha and beta as determinants of gene expression: influence of ligand, dose, and chromatin binding. Mol Endocrinol 22:1032-43

32. **Lin CY, Strom A, Li Kong S, Kietz S, Thomsen JS, Tee JB, Vega VB, Miller LD, Smeds J, Bergh J, Gustafsson JA, Liu ET** 2007 Inhibitory effects of estrogen receptor beta on specific hormone-responsive gene expression and association with disease outcome in primary breast cancer. Breast Cancer Res 9:R25

33. **Lindberg MK, Moverare S, Skrtic S, Gao H, Dahlman-Wright K, Gustafsson JA, Ohlsson C** 2003 Estrogen receptor (ER)-beta reduces ERalpha-regulated gene transcription, supporting a "ying yang" relationship between ERalpha and ERbeta in mice. Mol Endocrinol 17:203-8

34. **Strom A, Hartman J, Foster JS, Kietz S, Wimalasena J, Gustafsson JA** 2004 Estrogen receptor beta inhibits 17beta-estradiol-stimulated proliferation of the breast cancer cell line T47D. Proc Natl Acad Sci U S A 101:1566-71

35. **Hartman J, Strom A, Gustafsson JA** 2009 Estrogen receptor beta in breast cancer--diagnostic and therapeutic implications. Steroids 74:635-41

36. **Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, et al.** 1995 The nuclear receptor superfamily: the second decade. Cell 83:835-9

37. **Yamamoto KR** 1985 Steroid receptor regulated transcription of specific genes and gene networks. Annu Rev Genet 19:209-52

38. **Yamamoto KR, Darimont BD, Wagner RL, Iniguez-Lluhi JA** 1998 Building transcriptional regulatory complexes: signals and surfaces. Cold Spring Harb Symp Quant Biol 63:587-98

39. **Kumar V, Green S, Stack G, Berry M, Jin JR, Chambon P** 1987 Functional domains of the human estrogen receptor. Cell 51:941-51

40. **Couse JF, Lindzey J, Grandien K, Gustafsson JA, Korach KS** 1997 Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. Endocrinology 138:4613-21

41. **Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, Nilsson S, Gustafsson JA** 1997 Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. Endocrinology 138:863-70

42. **Enmark E, Gustafsson JA** 1999 Oestrogen receptors - an overview. J Intern Med 246:133-8

43. **Hall JM, Couse JF, Korach KS** 2001 The multifaceted mechanisms of estradiol and estrogen receptor signaling. J Biol Chem 276:36869-72

44. **Gruber DM, Sator MO, Huber JC** 1997 Hormone replacement therapy and breast cancer. Lancet 350:1628

45. **Gannon F, Gorski J** 1976 Nuclear binding of the estrogen receptor in whole uteri. Steroids 28:669-97

46. **Pratt WB, Toft DO** 1997 Steroid receptor interactions with heat shock protein and immunophilin chaperones. Endocr Rev 18:306-60

47. **Stenoien DL, Mancini MG, Patel K, Allegretto EA, Smith CL, Mancini MA** 2000 Subnuclear trafficking of estrogen receptor-alpha and steroid receptor coactivator-1. Mol Endocrinol 14:518-34

48. **Stenoien DL, Patel K, Mancini MG, Dutertre M, Smith CL, O'Malley BW, Mancini MA** 2001 FRAP reveals that mobility of oestrogen receptor-alpha is ligand- and proteasome-dependent. Nat Cell Biol 3:15-23

49. **Kumar V, Chambon P** 1988 The estrogen receptor binds tightly to its responsive element as a ligand-induced homodimer. Cell 55:145-56

50. **Klinge CM** 2001 Estrogen receptor interaction with estrogen response elements. Nucleic Acids Res 29:2905-19

51. **Gruber CJ, Gruber DM, Gruber IM, Wieser F, Huber JC** 2004 Anatomy of the estrogen response element. Trends Endocrinol Metab 15:73-8

52. **Vega VB, Lin CY, Lai KS, Kong SL, Xie M, Su X, Teh HF, Thomsen JS, Yeo AL, Sung WK, Bourque G, Liu ET** 2006 Multiplatform genome-wide identification and modeling of functional human estrogen receptor binding sites. Genome Biol 7:R82

53. **Lin CY, Vega VB, Thomsen JS, Zhang T, Kong SL, Xie M, Chiu KP, Lipovich L, Barnett DH, Stossi F, Yeo A, George J, Kuznetsov VA, Lee YK, Charn TH, Palanisamy N, Miller LD, Cheung E, Katzenellenbogen BS, Ruan Y, Bourque G, Wei CL, Liu ET** 2007 Whole-genome cartography of estrogen receptor alpha binding sites. PLoS Genet 3:e87

54. **Petz LN, Ziegler YS, Schultz JR, Kim H, Kemper JK, Nardulli AM** 2004 Differential regulation of the human progesterone receptor gene through an estrogen response element half site and Sp1 sites. J Steroid Biochem Mol Biol 88:113-22

55. **Petz LN, Ziegler YS, Schultz JR, Nardulli AM** 2004 Fos and Jun inhibit estrogen-induced transcription of the human progesterone receptor gene through an activator protein-1 site. Mol Endocrinol 18:521-32

56. **Harrington WR, Sheng S, Barnett DH, Petz LN, Katzenellenbogen JA, Katzenellenbogen BS** 2003 Activities of estrogen receptor alpha- and beta-selective ligands at diverse estrogen responsive gene sites mediating transactivation or transrepression. Mol Cell Endocrinol 206:13-22

57. **Cheung E, Acevedo ML, Cole PA, Kraus WL** 2005 Altered pharmacology and distinct coactivator usage for estrogen receptor-dependent transcription through activating protein-1. Proc Natl Acad Sci U S A 102:559-64

58. **Levine M, Tjian R** 2003 Transcription regulation and animal diversity. Nature 424:147-51

59. **Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M, Gannon F** 2003 Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. Cell 115:751-63

60. **McKenna NJ, Lanz RB, O'Malley BW** 1999 Nuclear receptor coregulators: cellular and molecular biology. Endocr Rev 20:321-44

61. **Stenoien DL, Nye AC, Mancini MG, Patel K, Dutertre M, O'Malley BW, Smith CL, Belmont AS, Mancini MA** 2001 Ligand-mediated assembly and real-time cellular dynamics of estrogen receptor alpha-coactivator complexes in living cells. Mol Cell Biol 21:4404-12

62. **McKenna NJ, O'Malley BW** 2002 Combinatorial control of gene expression by nuclear receptors and coregulators. Cell 108:465-74

63. **Rosenfeld MG, Lunyak VV, Glass CK** 2006 Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. Genes Dev 20:1405-28

64. **Green KA, Carroll JS** 2007 Oestrogen-receptor-mediated transcription and the influence of co-factors and chromatin state. Nat Rev Cancer 7:713-22

65. **Le Goff P, Montano MM, Schodin DJ, Katzenellenbogen BS** 1994 Phosphorylation of the human estrogen receptor. Identification of hormone-regulated sites and examination of their influence on transcriptional activity. J Biol Chem 269:4458-66

66. **Bunone G, Briand PA, Miksicek RJ, Picard D** 1996 Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. Embo J 15:2174-83

67. **Lannigan DA** 2003 Estrogen receptor phosphorylation. Steroids 68:1-9

68. **Tzukerman MT, Esty A, Santiso-Mere D, Danielian P, Parker MG, Stein RB, Pike JW, McDonnell DP** 1994 Human estrogen receptor transactivational capacity is determined by both cellular and promoter context and mediated by two functionally distinct intramolecular regions. Mol Endocrinol 8:21-30

69. **Webb P, Nguyen P, Shinsako J, Anderson C, Feng W, Nguyen MP, Chen D, Huang SM, Subramanian S, McKinerney E, Katzenellenbogen BS, Stallcup MR, Kushner PJ** 1998 Estrogen receptor activation function 1 works by binding p160 coactivator proteins. Mol Endocrinol 12:1605-18

70. **Webb P, Nguyen P, Valentine C, Lopez GN, Kwok GR, McInerney E, Katzenellenbogen BS, Enmark E, Gustafsson JA, Nilsson S, Kushner PJ** 1999 The estrogen receptor enhances AP-1 activity by two distinct mechanisms with different requirements for receptor transactivation functions. Mol Endocrinol 13:1672-85

71. **Katzenellenbogen BS, Montano MM, Ediger TR, Sun J, Ekena K, Lazennec G, Martini PG, McInerney EM, Delage-Mourroux R, Weis K, Katzenellenbogen JA** 2000 Estrogen receptors: selective ligands, partners, and distinctive pharmacology. Recent Prog Horm Res 55:163-93; discussion 194-5

72. **Shang Y, Brown M** 2002 Molecular determinants for the tissue specificity of SERMs. Science 295:2465-8

73. **Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M** 2000 Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell 103:843-52

74. **Katzenellenbogen BS, Katzenellenbogen JA** 2002 Biomedicine. Defining the "S" in SERMs. Science 295:2380-1

75. **Katzenellenbogen BS, Katzenellenbogen JA** 2000 Estrogen receptor transcription and transactivation: Estrogen receptor alpha and estrogen receptor beta: regulation by selective estrogen receptor modulators and importance in breast cancer. Breast Cancer Res 2:335-44

76. **Katzenellenbogen BS, Choi I, Delage-Mourroux R, Ediger TR, Martini PG, Montano M, Sun J, Weis K, Katzenellenbogen JA** 2000 Molecular mechanisms of estrogen action: selective ligands and receptor pharmacology. J Steroid Biochem Mol Biol 74:279-85

77. **Turgeon JL, McDonnell DP, Martin KA, Wise PM** 2004 Hormone therapy: physiological complexity belies therapeutic simplicity. Science 304:1269-73

78. **Frasor J, Danes JM, Komm B, Chang KC, Lyttle CR, Katzenellenbogen BS** 2003 Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology 144:4562-74

79. **Frasor J, Stossi F, Danes JM, Komm B, Lyttle CR, Katzenellenbogen BS** 2004 Selective estrogen receptor modulators: discrimination of agonistic versus antagonistic activities by gene expression profiling in breast cancer cells. Cancer Res 64:1522-33

80. **Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, Eeckhoute J, Shao W, Hestermann EV, Geistlinger TR, Fox EA, Silver PA, Brown M** 2005 Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. Cell 122:33-43

81. **Carroll JS, Meyer CA, Song J, Li W, Geistlinger TR, Eeckhoute J, Brodsky AS, Keeton EK, Fertuck KC, Hall GF, Wang Q, Bekiranov S, Sementchenko V, Fox EA, Silver PA, Gingeras TR, Liu XS, Brown M** 2006 Genome-wide analysis of estrogen receptor binding sites. Nat Genet 38:1289-97

82. **Welboren WJ, Sweep FC, Span PN, Stunnenberg HG** 2009 Genomic actions of estrogen receptor alpha: what are the targets and how are they regulated? Endocr Relat Cancer 16:1073-89

83. **Welboren WJ, van Driel MA, Janssen-Megens EM, van Heeringen SJ, Sweep FC, Span PN, Stunnenberg HG** 2009 ChIP-Seq of ERalpha and RNA polymerase II defines genes differentially responding to ligands. Embo J 28:1418-28

84. **Monroe DG, Getz BJ, Johnsen SA, Riggs BL, Khosla S, Spelsberg TC** 2003 Estrogen receptor isoform-specific regulation of endogenous gene expression in human osteoblastic cell lines expressing either ERalpha or ERbeta. J Cell Biochem 90:315-26

85. **Kian Tee M, Rogatsky I, Tzagarakis-Foster C, Cvoro A, An J, Christy RJ, Yamamoto KR, Leitman DC** 2004 Estradiol and selective estrogen receptor modulators differentially regulate target genes with estrogen receptors alpha and beta. Mol Biol Cell 15:1262-72

86. **Lin CY, Strom A, Vega VB, Kong SL, Yeo AL, Thomsen JS, Chan WC, Doray B, Bangarusamy DK, Ramasamy A, Vergara LA, Tang S, Chong A, Bajic VB, Miller LD, Gustafsson JA, Liu ET** 2004 Discovery of estrogen receptor alpha target genes and response elements in breast tumor cells. Genome Biol 5:R66

87. **Stossi F, Barnett DH, Frasor J, Komm B, Lyttle CR, Katzenellenbogen BS** 2004 Transcriptional profiling of estrogen-regulated gene expression via estrogen receptor (ER) alpha or ERbeta in human osteosarcoma cells: distinct and common target genes for these receptors. Endocrinology 145:3473-86

88. **Monroe DG, Secreto FJ, Subramaniam M, Getz BJ, Khosla S, Spelsberg TC** 2005 Estrogen receptor alpha and beta heterodimers exert unique effects on estrogen- and tamoxifen-dependent gene expression in human U2OS osteosarcoma cells. Mol Endocrinol 19:1555-68

89. **Kininis M, Chen BS, Diehl AG, Isaacs GD, Zhang T, Siepel AC, Clark AG, Kraus WL** 2007 Genomic analyses of transcription factor binding, histone acetylation, and gene expression reveal mechanistically distinct classes of estrogen-regulated promoters. Mol Cell Biol 27:5090-104

90. **Stender JD, Frasor J, Komm B, Chang KC, Kraus WL, Katzenellenbogen BS** 2007 Estrogen-regulated gene networks in human breast cancer cells: involvement of E2F1 in the regulation of cell proliferation. Mol Endocrinol 21:2112-23

91. **Krum SA, Miranda-Carboni GA, Lupien M, Eeckhoute J, Carroll JS, Brown M** 2008 Unique ERalpha cistromes control cell type-specific gene regulation. Mol Endocrinol 22:2393-406

92. **Eeckhoute J, Carroll JS, Geistlinger TR, Torres-Arzayus MI, Brown M** 2006 A cell-type-specific transcriptional network required for estrogen regulation of cyclin D1 and cell cycle progression in breast cancer. Genes Dev 20:2513-26

93. **Eeckhoute J, Keeton EK, Lupien M, Krum SA, Carroll JS, Brown M** 2007 Positive cross-regulatory loop ties GATA-3 to estrogen receptor alpha expression in breast cancer. Cancer Res 67:6477-83

94. **Lupien M, Eeckhoute J, Meyer CA, Wang Q, Zhang Y, Li W, Carroll JS, Liu XS, Brown M** 2008 FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription. Cell 132:958-70

95. **Lupien M, Eeckhoute J, Meyer CA, Krum SA, Rhodes DR, Liu XS, Brown M** 2009 Coactivator function defines the active estrogen receptor alpha cistrome. Mol Cell Biol 29:3413-23

96. **DeNardo DG, Kim HT, Hilsenbeck S, Cuba V, Tsimelzon A, Brown PH** 2005 Global gene expression analysis of estrogen receptor transcription factor cross talk in breast cancer: identification of estrogen-induced/activator protein-1-dependent genes. Mol Endocrinol 19:362-78

97. **Hodges LC, Cook JD, Lobenhofer EK, Li L, Bennett L, Bushel PR, Aldaz CM, Afshari CA, Walker CL** 2003 Tamoxifen functions as a molecular agonist inducing cell cycle-associated genes in breast cancer cells. Mol Cancer Res 1:300-11

98. **Fullwood MJ, Liu MH, Pan YF, Liu J, Xu H, Mohamed YB, Orlov YL, Velkov S, Ho A, Mei PH, Chew EG, Huang PY, Welboren WJ, Han Y, Ooi HS, Ariyaratne PN, Vega VB, Luo Y, Tan PY, Choy PY, Wansa KD, Zhao B, Lim KS, Leow SC, Yow JS, Joseph R, Li H, Desai KV, Thomsen JS, Lee YK, Karuturi RK, Herve T, Bourque G, Stunnenberg HG, Ruan X, Cacheux-Rataboul V, Sung WK, Liu ET, Wei CL, Cheung E, Ruan Y** 2009 An oestrogen-receptor-alpha-bound human chromatin interactome. Nature 462:58-64

99. **Kosak ST, Groudine M** 2004 Form follows function: The genomic organization of cellular differentiation. Genes Dev 18:1371-84

100. **Misteli T** 2007 Beyond the sequence: cellular organization of genome function. Cell 128:787-800

101. **Meaburn KJ, Misteli T** 2008 Locus-specific and activity-independent gene repositioning during early tumorigenesis. J Cell Biol 180:39-50

102. **Rajapakse I, Perlman MD, Scalzo D, Kooperberg C, Groudine M, Kosak ST** 2009 The emergence of lineage-specific chromosomal topologies from coordinate gene regulation. Proc Natl Acad Sci U S A 106:6679-84

103. **Deschenes J, Bourdeau V, White JH, Mader S** 2007 Regulation of GREB1 transcription by estrogen receptor alpha through a multipartite enhancer spread over 20 kb of upstream flanking sequences. J Biol Chem 282:17335-9

104. **Barnett DH, Sheng S, Charn TH, Waheed A, Sly WS, Lin CY, Liu ET, Katzenellenbogen BS** 2008 Estrogen receptor regulation of carbonic anhydrase XII through a distal enhancer in breast cancer. Cancer Res 68:3505-15

105. **Pan YF, Wansa KD, Liu MH, Zhao B, Hong SZ, Tan PY, Lim KS, Bourque G, Liu ET, Cheung E** 2008 Regulation of estrogen receptor-mediated long range transcription via evolutionarily conserved distal response elements. J Biol Chem 283:32977-88

106. **Boney-Montoya J, Ziegler YS, Curtis CD, Montoya JA, Nardulli AM** 2009 Long-Range Transcriptional Control of Progesterone Receptor Gene Expression. Mol Endocrinol

107. **Dostie J, Richmond TA, Arnaout RA, Selzer RR, Lee WL, Honan TA, Rubio ED, Krumm A, Lamb J, Nusbaum C, Green RD, Dekker J** 2006 Chromosome Conformation Capture Carbon Copy (5C): a massively parallel solution for mapping interactions between genomic elements. Genome Res 16:1299-309

108. **Lieberman-Aiden E, van Berkum NL, Williams L, Imakaev M, Ragoczy T, Telling A, Amit I, Lajoie BR, Sabo PJ, Dorschner MO, Sandstrom R, Bernstein B, Bender MA, Groudine M, Gnirke A, Stamatoyannopoulos J, Mirny LA, Lander ES, Dekker J** 2009 Comprehensive mapping of long-range interactions reveals folding principles of the human genome. Science 326:289-93

109. **Visel A, Rubin EM, Pennacchio LA** 2009 Genomic views of distant-acting enhancers. Nature 461:199-205

110. **Schoenfelder S, Sexton T, Chakalova L, Cope NF, Horton A, Andrews S, Kurukuti S, Mitchell JA, Umlauf D, Dimitrova DS, Eskiw CH, Luo Y, Wei CL, Ruan Y, Bieker JJ, Fraser P** Preferential associations between co-regulated genes reveal a transcriptional interactome in erythroid cells. Nat Genet 42:53-61

CHAPTER 2

TRANSCRIPTIONAL PROFILING OF ESTROGEN-REGULATED GENE EXPRESSION VIA ESTROGEN RECEPTOR ALPHA OR ESTROGEN RECEPTOR BETA IN HUMAN OSTEOSARCOMA CELLS: DISTINCT AND COMMON TARGET GENES FOR THESE RECEPTORS[1]

Abstract

Estrogens exert many important effects in bone, a tissue that contains both estrogen receptors alpha and beta (ERα and ERβ). To compare the actions of these receptors, we generated U2OS human osteosarcoma cells stably expressing ERα or ERβ, at levels comparable to those in osteoblasts, and we characterized their response to estradiol (E2) over time using Affymetrix GeneChip microarrays to determine the expression of approximately 12,000 genes, followed by quantitative PCR verification of the regulation of selected genes. Of the *ca.* 100 regulated genes we identified, some were stimulated by E2 equally through ERα and ERβ, whereas others were selectively stimulated via ERα or ERβ. The E2-regulated genes showed three distinct temporal patterns of expression over the 48 h time course studied. Of the functional categories of the E2-regulated genes, most numerous were those encoding cytokines and factors associated with immune response, signal transduction, and cell migration and cytoskeleton regulation, indicating that E2 can exert effects on multiple pathways in these osteoblast-like cell lines. Of note, E2 up-regulated several genes associated with cell motility selectively via ERβ, in keeping with the selective E2 enhancement of the

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motility of ERβ-containing cells. Of genes regulated equally by E2 via ERα or ERβ, the

phytoestrogen genistein preferentially stimulated gene expression via ERβ. These studies

indicate both common as well as distinct target genes for these two ERs, and identify

many novel genes not previously known to be under estrogen regulation.

Introduction

Estrogen is a pleiotropic hormone with multiple actions in reproductive tissues

(such as breast, uterus and ovary) and in many non-reproductive tissues including bone,

the central nervous system and the cardiovascular system. In the skeleton, estrogen

effects range from regulation of bone growth during puberty to bone remodeling in the

adult [reviewed in (1-4)]. The pivotal role of estrogens in the maintenance of bone tissue

has long been known from clinical studies where estradiol (E2) deficiency in

postmenopausal or ovariectomized women caused a rapid loss of trabecular bone;

moreover, in both men and women, estrogen deficiency is associated with an age-related

sustained bone loss that can lead to osteoporosis. Both situations can be reversed by

hormone replacement therapy (1-4).

Estrogen exerts its effects on bone and other target tissues by interacting with two

different members of the nuclear receptor superfamily of hormone-regulated transcription

factors, named estrogen receptor alpha (ERα) and beta (ERβ) (5-7). These two receptors

are encoded by different genes, on human chromosome 6 and 14, respectively. ERα and

ERβ have similar modular domain structures and very high amino acid identity in their

DNA-binding domains (97%), whereas they are more divergent in their N-terminal A/B

domains (only 18% amino acid identity) and in their ligand-binding domains (59% amino

acid identity). After the binding of hormone to these receptors, the hormone-receptor complexes bind to specific sequences on the DNA (EREs, Estrogen Response Elements) or interact with other transcription factors without direct ERα or ERβ binding to DNA (i.e., at AP-1, Sp1, and other sites) (8-13). In both cases, liganded-ERs recruit coregulator proteins and components of the transcriptional machinery to regulate the transcription of target genes (14-16). Recently, the importance of membrane-initiated signaling in the actions of estrogens has been highlighted by studies *in vitro* and *in vivo*, where membrane/cytoplasmic ERs as well as nuclear ERs seem to be involved in regulating E2 action and gene transcription in bone and other target cells (1, 17-19).

In bone, ERs are present in osteoblasts and chondrocytes, and at somewhat lower levels in osteoclasts, bone marrow stromal cells, osteocytes and bone cell precursors (20, 21). The levels of ERs in bone are generally about 10-fold lower than in reproductive tissues, such as uterus, and their levels can be affected by many parameters including cell differentiation state (22-24). Estrogen seems to act directly on osteoblasts and probably, as well, on osteoclasts and precursors of both cell types, and indirectly by regulating cytokine production in osteoblasts and bone marrow stromal cells, which in turn affects the actions and formation of osteoclasts (1, 25).

The aims of this study were to determine the effects of E2 on gene expression in a human osteoblastic cell line and, via transcriptional profiling, to better understand the comparative roles of ERα and ERβ in mediating the effects of this hormone. In particular, our interest was to elucidate whether the two receptors elicit the same and/or different transcriptional responses on a range of endogenous cellular genes. For this purpose we have generated U2OS human osteosarcoma cells stably expressing either

ERα or ERβ and examined several clonal cell lines containing similar, relatively low, physiological levels of functional receptor. Using Affymetrix GeneChip microarrays, which allow the examination of >12,000 genes, we observed that the hormone-regulated genes divided into three distinct temporal patterns and into several functional categories, indicating that E2 exerts effects on multiple regulatory pathways in these cells. In this study, we report the identification of genes that were commonly regulated by E2 through ERα and ERβ, as well as some that were preferentially or exclusively regulated by one or the other ER subtype, and many novel genes not previously known to be under regulation by this hormone. We discuss the relationship of some of these genes to the biological effects of E2 we observed in these cells.

Materials and Methods

Cell culture

U2OS human osteosarcoma cells were stably transfected with human ERα (encoding amino acids 1–595) or ERβ (encoding amino acids 1–530). Each ER cDNA (26) was subcloned into the pcDNA3.1+ expression vector (Invitrogen, Carlsbad, CA) which contains a neomycin resistance gene. Clones were selected with the antibiotic G418 (800 μg/ml) and 20 clones per ER subtype were screened for ER expression and for transactivation ability with an estrogen response element containing reporter gene (2ERE-tk-CAT). Four clones of each (denoted as ERα clones 1-4 and ERβ clones 1-4) were chosen for further characterization and for the gene expression studies. The U2OS-ER cells were routinely grown in Minimal Essential Medium with phenol red (Sigma Chemical Co., St. Louis, MO) supplemented with 15% fetal calf serum (Hyclone, Logan,

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UT), 100 units penicillin/ml, 100 µg streptomycin/ml and 400 µg G418/ml. Before use in experiments, cells were grown in Minimal Essential Medium without phenol red and supplemented with 5% charcoal-dextran treated fetal calf serum for at least 4 days before the start of E2 treatment. MCF-7 human breast cancer cells were grown as previously described (27).

Hormone binding and Western immunoblot assays

Whole cell extracts were prepared in cell lysis buffer (20 mM Tris pH 7.4, 0.5 M NaCl, 1 mM DTT, 10% glycerol, 50 µg/ml leupeptin, 50 µg/ml aprotinin, 2.5 µg/ml pepstatin A, and 0.2 mM phenylmethylsulfonyl fluoride) using freeze/thaw procedure. Total protein concentration was determined using the BCA kit (Pierce Biotechnology, Rockford, IL). Whole cell extracts were incubated in duplicate with a range of ^{3}H-E2 concentrations alone or with 100-fold excess unlabeled E2 for 1 h on ice. Hydroxylapatite (HAP) slurry was added and incubated for an additional 15 min on ice. The slurry was washed twice and its radioactivity then determined by scintillation counting. The amount of E2 binding in pmol was calculated per mg protein.

Western blots of whole cell extracts used the ERα specific antibody H222 and the ERβ specific antibody UCG40 (kindly provided by Geoffrey L. Greene, University of Chicago) and were done as previously described (28).

Hormone treatments, DNA microarrays, and analysis of microarray data

U2OS-ERα or ERβ containing cells were maintained in culture for 48 h prior to cell harvest and RNA collection. During this period, cells were treated with 10 nM E2 (Sigma Chemical Co., St. Louis, MO) for 4, 8, 24 or 48 h, and three separate samples were collected for each time point. Control cell samples were also treated with 0.1%

ethanol control vehicle for 48 h. In this way, cell density was similar in all samples, consistent with our observations (as discussed in Results), that E2 does not affect proliferation of these cells. Additional independent time course experiments were conducted to generate RNA samples for quantitative RT-PCR verification of gene regulation, as detailed in the next section below. Total RNA was prepared using Trizol Reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. RNA was further purified using RNeasy columns (Qiagen, Valencia, CA) and treatment with RNase free-DNase I (Qiagen, Valencia, CA).

Total RNA from each sample was used to generate cRNA, which was labeled with biotin as recommended by Affymetrix (Santa Clara, CA). Each cRNA was then hybridized on an Affymetrix human Hu-U95A GeneChip, which contains oligonucleotide probe sets representing approximately 12,500 human genes and ESTs. After washing, the chips were scanned and analyzed using MicroArray Suite 5.0 software (Affymetrix, Santa Clara, CA). Average intensities for each GeneChip were globally scaled to a target intensity of 150. Data were then analyzed using GeneSpring version 5.0.1 software (Silicon Genetics, San Carlos, CA). Data were first normalized on a per chip basis by dividing each measurement by the 50th percentile of all measurements on that chip, and then E2-treated samples were normalized to the mean of the vehicle treated control samples.

We applied a confidence score (CS) to evaluate which genes were estrogen-regulated (as described in (29); adapted from (30)). The CS is based on four parameters: fold change (FC), p-value (PV), percentage of present calls (PC) and expression level (EL), (CS=FC+PV+PC+EL). For each parameter, arbitrary scores were assigned. For

fold change, a score of 5 was assigned if the fold change was greater than 1.95, 2 if the fold change was between 1.5 and 1.95 and a penalty of –0.5 if it was under 1.5. For p value, a score of 3 was assigned if p-value was less than 0.05, 2 if was between 0.05 and 0.1, and a penalty of –0.5 if it was greater than 0.1. If present calls were assigned to more than 50% of the samples the score was 3, between 25-50% was 1, and if less than 25% a penalty of –0.5 was applied. For the expression level, a score of 3 was applied if it was greater than 30, a score of 1 if it was between 15 and 30 and a penalty of –0.5 if expression level was less than 15. The confidence scores ranged from –2 to 14, and genes with a CS value of 11 or higher were considered to be significantly E2-regulated genes. To determine ERα or ERβ preference in gene regulation, the average fold change for one of the receptors had to be 2.0 or greater, whereas fold change was less than 1.3-fold for the other receptor at all time points.

Estrogen-regulated genes were assigned to functional categories according to LocusLink, OMIM, PubMed (www.ncbi.nlm.nih.gov), GeneCards (bioinfo.weizmann.ac.il/cards) and GenMAPP databases. The entire microarray data set will be available through the Gene Expression Omnibus (GEO [http://www.ncbi.nlm.nih.gov/geo]).

Quantitative real-time PCR

Real-time PCR was carried out on *ca.* 25 genes to verify E2 regulation as assessed by microarray data analysis, and to evaluate mRNA levels of ERα or ERβ in U2OS stably transfected cells. Three independent time-course experiments using 3 separate cell samples each for ERα or ERβ containing cells either E2 or control vehicle treated at each time point, were conducted. The primers used are listed in Table 2.1. One μg of total

RNA from each sample was reverse transcribed in a total volume of 20 μl using 200 U

reverse transcriptase, 50 pmol random hexamers and 1 mM dNTPs (New England

Biolabs, Beverly, MA). The resulting cDNA was then diluted to a total volume of 100 μl.

Each real-time PCR reaction consisted of 5 μl of diluted RT product, 1x SYBR Green

PCR Master Mix (Applied Biosystems, Foster City, CA), and 50 nM of forward and

reverse primers. Reactions were carried out in an ABI Prism 7700 Sequence Detection

System (Applied Biosystems) for 40 cycles (95 °C for 15 sec, 60 °C for 1 min) following

an initial 10 min incubation at 95°C. The fold change in expression was calculated using

the ΔΔ Ct comparative threshold cycle method (31) with the ribosomal protein 36B4

mRNA as an internal control. As described in (31), gene expression is normalized to an

endogenous reference gene (36B4) and the fold change in gene expression is then

determined relative to the vehicle treated control.

Cell motility and cell adhesion assays

Cell motility assays used 48-well Boyden microchemotaxis chambers according to

the manufacturer's instructions (Neuroprobe, Cabin John, MD). Briefly, the cells were

grown in 5% CD-FCS MEM medium without phenol red for at least 4 days until ~70-

80% confluency. Cells were treated for either 24 h or 48 h with E2 or control (0.1%)

ethanol vehicle, harvested, and 50 μl of cell suspension (10^5 cells/50 μl serum-free MEM-

minus phenol red and containing 0.5% BSA) were placed in the open-bottom wells of the

upper compartment. Each pair of wells was separated by a poly-vinylpyrrolidone-free

polycarbonate porous membrane (8 μm pores) precoated with gelatin (0.2 mg/ml in PBS).

28 μl of the chemoattractant (MEM with 5% FBS, and MEM with no FBS as control)

were placed into the lower compartment wells of the chamber. The Boyden chambers

were then incubated for 5 h at 37°C. Cells were then fixed on the membrane with methanol, stained with Diff-Quick staining kit (Baxter Health Corp., McGraw Park, IL), mounted onto glass slides, and counted under a 100x microscope objective.

Cell adhesion assays were performed in 96-well plates coated for 2 h at 37°C with 20-40 μg/ml of different coating substrates (poly-L-lysine, BSA, collagen type I, fibronectin, or laminin). Cells were treated with 10 nM E2 for 24 or 48 h, and after blocking non-specific sites with 1% BSA for 2 h at 37°C, cells were harvested, resuspended in MEM minus phenol red containing 0.1% BSA at 10^5 cells/ml, and 100 μl of cell suspension was seeded per well. Cells were allowed to adhere for 30 min and then the medium was gently removed, the wells washed once with MEM minus phenol red and the cells then fixed with methanol. Cells were stained with 0.1% crystal violet for 30 min at room temperature and washed multiple times with double distilled water. Stain was then extracted from the cells with 5% TritonX-100 overnight at room temperature and absorbance monitored at 570 nm.

Results

Generation and characterization of U2OS cell lines stably expressing ERα or ERβ

U2OS human osteosarcoma cells, an ER-negative osteoblast-like cell line, were stably transfected with either ERα or ERβ using a pcDNA3.1+ plasmid containing a neomycin resistance gene as a vector for stable integration. Positive clones were selected with G418 (800 μg/ml), and after two rounds of selection, various clones were tested for the presence of ER mRNA by real time PCR and for ER protein by Western blot with ERα-specific or ERβ-specific antibodies (Fig. 2.1). The levels of ERα and ERβ mRNA

in U2OS-ER cell clones, as determined by real-time quantitative PCR using standard curves with known amounts of ERα and ERβ cDNA (Fig. 2.1A), were compared with the parental U2OS cells and with the ERα-containing human breast cancer cell line MCF-7. In the eight clones (ERα 1-4, ERβ 1-4) used for most of the studies reported in this paper, the number of copies of ERα and ERβ mRNA were similar: 8410±777 per 10 ng total RNA for ERα and 9112±1304 for ERβ. These ERα mRNA levels are *ca.* 20% of that measured in our MCF-7 breast cancer cells. In addition, as seen in Fig. 2.1A, no ERβ mRNA was detected in the ERα-expressing U2OS cells and likewise no ERα mRNA was present in the U2OS ERβ-expressing cells. The parental U2OS cells lacked detectable levels of either receptor mRNA.

Hormone binding (Scatchard) assays were performed on extracts from ERα clone 1 and ERβ clone 1 cells to ensure the presence of receptor able to bind hormone. These assays demonstrated similar levels of ^{3}H-E2 binding in these U2OS-ERα and U2OS-ERβ cells (~0.1 pmol/mg protein, Fig.2.1B). These levels are about 20% of that found in MCF-7 cells. Western blots (Fig. 2.1C) with H222 monoclonal antibody for ERα and UCG40 polyclonal antibody for ERβ showed ERα or ERβ of the correct size (*ca.* 65 and 56 kDa, respectively), and the presence of only ERβ in the ERβ clones (β clones 1-4 shown) and only ERα in the ERα clones (α clone 1 shown). Further characterization by estrogen responsive ERE-tk-CAT reporter assays demonstrated that U2OS-ERα and ERβ cells possessed E2-dependent transcriptionally functional receptors (data not shown). The level of ERα and ERβ in these clones is consistent with previous reports indicating the *ca.* 10-fold lower ER level in bone versus uterine tissue (23, 24).

We examined whether E2 affected the motility and adhesion properties of the

U2OS-ERα and ERβ-containing cells. Motility was assessed using a Boyden chamber

chemotactic assay and 5% FBS as a general chemotactic stimulus. With E2 treatment for

24 or 48 h, the ERβ-containing cells showed a 3-fold increase in motility; in contrast,

although the ERα and ERβ-containing cells showed similar motility, E2 elicited no

change in motility of the ERα-containing cells (Fig. 2.1D). We also evaluated whether

E2 treatment for 24 or 48 h affected the adhesive properties of these cells, and found that

it did not. An adhesion assay was employed that allows a comparison of the rapid

adhesion (in 30 min) of the cells to various substrates (poly-L-lysine, BSA, collagen type

I, fibronectin, or laminin). Adhesion to collagen type I is shown as an example (Fig.

2.1E), since it is a major component of bone matrix, and it is seen that E2 had no effect

on the adhesion of the cells. This was the case with all of the substrates tested, indicating

that this property is largely E2-independent.

Gene expression profiling and time courses of gene regulation using Affymetrix

GeneChip microarrays

To characterize changes in gene expression in response to E2 treatment, U2OS-

ERα (clone 1) and U2OS-ERβ (clone 1) cells were treated with 10 nM E2 for times from

0 to 48 h, and RNA was harvested, purified, and derivatized for gene profiling.

Affymetrix Hu-U95A GeneChips, which contain probes for approximately 12,000 human

genes, were used. As described in *Materials and Methods,* a confidence score (CS) was

applied to discriminate genes robustly and reproducibly regulated by E2. The CS takes

into account several parameters for each gene and weights the fold change increase or

decrease versus control, the reproducibility of the output (p-value), the expression level

related to the intensity of the signal on the chip and a fidelity score based on a decision matrix which compares hybridization of the perfect match probes versus the mismatch for each probe set.

We considered E2-regulated genes to be those with a confidence score equal to or greater than 11 (out of a maximum score of 14), and we included in our further studies only genes up- or down-regulated more than 2 fold. Using these criteria, we identified 105 genes that were E2-regulated through ERα and/or ERβ. This list of genes was subjected to gene tree cluster analysis using a standard correlation algorithm available in the GeneSpring software.

In the gene cluster analysis (Fig. 2.2), up-regulated genes are shown in red, down-regulated genes in blue and unchanging genes in yellow. We observed that many more genes were stimulated than inhibited (85 vs 20) in ERα- or ERβ-containing cells, and in further analyses, we focused on the up-regulated genes, since these were more numerous, were the most robustly regulated and gave good reproducibility of validation by real-time PCR.

When the E2-regulated genes were categorized according to their time patterns of expression, three major groupings were identified: early (stimulated only at 4-8 h), early and late (stimulated from 4 through 48 h) and late (stimulated only at 24-48 h) (Fig. 2.3). For ERα-containing cells, the early and late pattern was the most frequently represented (49% of E2-stimulated genes through ERα), followed by the late (46%) and early (5%) patterns. In U2OS-ERβ cells, the distribution was slightly different with 43%, 41% and 16%, respectively, in these three time patterns.

Of the 85 up-regulated genes, 52 were commonly regulated by ERα and ERβ (Fig. 2.4 top). These genes, their functions, and their time patterns of regulation are listed in Table 2.2. As shown in Fig. 2.4 top, 24 genes were stimulated selectively by ERα, whereas 9 genes were stimulated selectively by ERβ. Thus, the two receptors showed substantial overlap in regulated genes (61% of the genes), but both also have some different target genes. The estrogen-stimulated genes regulated selectively through one of the two ER subtypes are listed in Table 2.3, along with their temporal pattern of regulation and their function. Many new E2 target genes were also identified, as indicated in Tables 2.2 and 2.3.

Of note, our method of analysis demonstrated E2 regulation of some known E2-target genes (i.e. pS2/TFF1, PDZK1, GREB1, WISP2, keratin 19, angiotensinogen, NHERF/SLC9A3R2, BMP6, connexin 43/GJA1), suggesting the validity of our analyses. Further, regulation of several bone-related factors (BMP6, CD34, CD164, PTHLH) by E2 was observed (Tables 2.2 and 2.3).

Functional classification of E2-regulated genes

To classify E2 up-regulated genes into functional groups (Fig. 2.4) we chose eight categories: cytokines/immune response, signal transduction, cell motility/cytoskeleton regulation, growth factors/hormones, apoptosis/cell proliferation, housekeeping, nucleic acid processing, and other/unknown. We then assigned each gene to one major category representing its primary function (even though many genes encode proteins that have several physiological functions), based on mining of several databases (LocusLink, PubMed, GeneCards, OMIM, GenMapp).

Many of the genes commonly regulated through ERα and ERβ encode proteins associated with cytokine/immune response, signal transduction, and cell motility and cytoskeleton regulation (56% of all genes). Several ERα-selective genes were associated with nucleic acid processing. Very few genes regulated by the ERs were related to cell cycle, proliferation or apoptosis, supporting our observations (not shown) that E2 had no effect on the proliferation rate of these cells.

Hormone-regulated genes encoding cytokines and factors associated with immune response

Three members of the Natural Killer cell lectin-type receptors (NKG family), NKG2-C, -E and –F (KLRC2, KLRC3 and KLRC4) were robustly regulated by E2 through both receptors (Table 2.2). NKGs are activating receptors of natural killer cells that mediate HLA-E recognition (32-34). CD34 (common to both ERα and ERβ up-regulation, Table 2.2) and sialomucin/CD164 (ERα selective, Table 2.3) are cell surface antigens characteristic of human hematopoietic progenitors. CD164 is an adhesion receptor that inhibits proliferation of CD34+ hematopoietic progenitors (35, 36). Interleukin-8 (IL-8), a cytokine known for its angiogenic and chemotactic properties, is thought to be involved in bone cell function under interleukin-1β and TNFα control in bone marrow stromal cells and osteoblasts (37, 38). It was also regulated by E2 in both ERα and ERβ containing U2OS cells (Table 2.2).

Cyclooxygenase-2 (Cox-2), a key enzyme regulating the production of prostaglandins, and also regulating angiogenesis, tumor cell invasion and inflammatory responses (39), was markedly up-regulated by E2 via both ERα and ERβ (Table 2.2).

Cox-2 has been shown recently to be under estrogenic regulation mediated through the PI3K/Akt pathway (40).

Hormone regulated genes associated with cell motility and cytoskeletal function

This category is of particular interest because, using cell motility assays, we observed an increase in motility of U2OS-ERβ containing cells after E2 treatment (Fig. 2.1D). Among the ERβ selectively regulated genes, there are two candidates that might be involved in this response. The first is autotaxin (ENPP2), a known tumor autocrine motility factor that has been shown to stimulate the migration of melanoma cells via a G protein-coupled phosphoinositide 3-kinaseγ and Cdc42 and Rac1-dependent pathway. This regulation possibly involves Paxillin and FAK (focal adhesion kinase), two key players in cell adhesion and motility control (41, 42). Autotaxin has 5-nucleotide pyrophosphatase and phosphodiesterase activity, and it acts in the extracellular space as a lysophospholipase D with production of lysophosphatidic acid (LPA), which seems to be the tumor cell motility inducer (43). The experiments described in the section below demonstrate that autotaxin is very selectively regulated by ERβ. Another candidate associated with the motility increase by ERβ might be Rap1GEF (GFR), a guanine nucleotide exchange factor that constitutively activates Rap1 (44) and has been shown to be involved in cell motility by modulating integrin function (45, 46).

Quantitative real-time PCR validation and verification of gene expression regulation in multiple cell clones and using ER subtype-selective ligands

Table 2.1 shows the primers used, and Tables 2.2 and 2.3 show the *ca.* 25 genes that we verified for their E2 regulation by quantitative PCR (names in italics). It is of note that many of these are genes newly identified as being regulated by E2. In addition,

we verified their E2 regulation in several clones (4 each) of U2OS ERα and/or ERβ-containing cells (clones 1-4) and used ER subtype-selective ligands to confirm common regulation by both ERs or selective gene regulation by ERα or ERβ. The Tables indicate generally good correspondence in gene regulation between the microarray and the quantitative RT-PCR analyses using several ERα- or ERβ-containing cell clones. RT-PCR, however, is often more sensitive, with a greater dynamic range, such that fold changes found by RT-PCR were often greater than those obtained from microarray analysis.

Figure 5 shows real-time PCR validation of some genes that were commonly regulated by E2 through both ER subtypes. The time course of stimulation for four of these genes (GREB1, oligophrenin-1, interleukin-24 and carbonic anhydrase XII) is presented. All of these genes showed up-regulation by E2 by 4 h. There were robust, and very similar fold changes in mRNA for GREB1 mediated via ERα or ERβ over time (Fig. 2.5). Oligophrenin-1 also showed a pattern of mRNA change that was similar for ERα and ERβ in which a plateau in stimulation was achieved by 4 h for ERα and 8 h for ERβ. Interestingly, IL-24 was preferentially up-regulated by ERα, with ERβ being a much less effective mediating receptor. In contrast, the stimulation via ERβ was substantially greater for the carbonic anhydrase XII gene than was the stimulation via ERα. The ER mediation of all of these E2-induced changes is clearly suggested by the fact that the stimulation is fully reversed by an excess of the antiestrogen ICI182,780. In addition, and as expected, no stimulation was observed with this pure antiestrogen alone (Fig. 2.5).

Figure 2.6 shows the time course changes for two genes selectively regulated through ERβ, autotaxin and cystatin D. Their expression was highly stimulated via ERβ, with no significant change being elicited via ERα. The ICI antiestrogen reversal of the E2 stimulation of these genes supports the mediation by ER (Fig. 2.6).

To further examine the selectivity of gene regulation via ERα or ERβ, cells were treated with the ERα-selective ligand propylpyrazoletriol (PPT; (47-50)), the ERβ-selective ligand diarylpropionitrile (DPN; (51, 52)), or the soy phytoestrogen genistein which has a higher affinity for ERβ than ERα (53) (Fig. 2.7). The PDZK1 gene, which is equivalently stimulated by E2 via ERα or ERβ, showed stimulation by PPT in the ERα-containing cells and by DPN in the ERβ-containing cells, as expected. Interestingly, genistein, which has preferential binding affinity for ERβ (53), was considerably more effective in stimulating PDZK1 gene expression via ERβ (Fig. 2.7). At the cystatin D gene, E2 exclusively stimulated via ERβ, and the stimulation by genistein and DPN was only observed in the ERβ-containing cells, with no stimulation by PPT or genistein via ERα. These findings confirm the common regulation of PDZK1 through ERα and ERβ observed with E2, and the ERβ selectivity for E2 regulation of cystatin D expression.

Discussion

Estrogens exert profound effects on bone, a tissue that expresses ERα and ERβ. Yet the individual contributions of ERα and ERβ to gene regulation and physiological function in bone remain only partially understood. In this study, we have characterized a model system for examining the actions of the ER subtypes, ERα and ERβ, in a human

osteoblast-like cell type. These studies have enabled us to evaluate changes in the expression of *ca.* 12,000 genes by estradiol through ERα and/or ERβ. We have identified novel estrogen-regulated genes and genes regulated commonly as well as exclusively or preferentially through ERα or ERβ. Further, the identification of regulated genes allows some insight into the pathways and gene networks regulated by E2 through these two ERs.

Estrogen regulation of genes associated with cytokines, motility, cytoskeleton, and bone remodeling

Because estrogen plays important roles in bone remodeling and the control of osteoblast and osteoclast numbers (1), it is of note that E2 showed significant regulation of cytokines and of genes encoding proteins associated with regulation of the immune response. Our findings are consistent with the reports of estrogen regulation of genes encoding cytokines, growth factors and bone matrix proteins in wild type mouse trabecular bone and the importance of ERα and/or ERβ in the mediation of these effects, as evidenced by the use of ER knockout mice (54-56). We observed E2 up-regulation of genes encoding three members of the Natural Killer cell lectin-type receptors (NKG family, KLRC2, KLRC3 and KLRC4). These NKG family genes were all regulated by E2 at early as well as later times. Other factors associated with cytokine/immune function observed to be under estrogen regulation included CD34 antigen, defensin β1, IL13 receptor α2, interleukin 8, phospholipase A2, selenoprotein P1, and sialomucin/CD164. Many of these genes, as indicated in Tables 2.2 and 2.3, represent new E2-regulated genes not previously reported to be regulated by this hormone.

Another category of genes in which many members were regulated by E2, were those associated with regulation of cell motility and the cytoskeleton. We observed a three-fold increase in the motility of the ERβ-containing cells in response to E2 treatment. Hence, it is of note that two genes preferentially regulated by E2 via ERβ were autotaxin (ENPP2), a known tumor autocrine motility factor and phosphodiesterase, and Rap1 GEF, the guanine nucleotide exchange factor that constitutively activates Rap1 and is known to modulate integrin function (45, 46).

WISP-2, a connective tissue growth factor isolated from osteoblasts (57), was regulated by E2 via both receptor subtypes, and it might play a role in cytoskeletal regulation in these U2OS cells. Its stimulation by E2 has been reported in MCF-7 breast cancer cells (29, 58). In addition, oligophrenin-1, keratin 19, integrin α 6, cyritestin 1, connexin 43, and cadherin 19, all of which play roles in cell motility and the cytoskeleton, were for the most part not previously known to be regulated by estrogen in bone, although several are known to be under estrogen regulation in reproductive target cells (59, 60).

Bone morphogenetic protein 6 (BMP6), a potent osteogenic factor believed to play an important role in the bone-protective actions of estrogens, was observed to be up-regulated through both ERs, as was parathyroid hormone-like hormone (PTHLH/PTHrP), a homolog of PTH that functions as an autocrine growth inhibitor for osteoblast-like cells and as an anabolic agent in osteoporosis (61, 62). Neurotensin was another growth factor/hormone found to be up-regulated by E2 via ERα and ERβ. Cystatin D, an E2-responsive gene newly identified in these studies, was stimulated very preferentially via

ERβ. As this gene encodes a protein that is a secreted inhibitor of cysteine peptidases (cathepsins S and H), it might function in several roles in bone physiology.

ER regulation of other cellular functions: similarities and differences in estrogen target genes and temporal patterns of gene regulation in osteosarcoma and breast cancer cells

Estradiol up-regulated the expression of several genes encoding proteins associated with nucleic acid processing, such as the estrogen receptor coregulators RIP140 and TRAP240. However, in these U2OS cells, E2 regulated very few genes associated with apoptosis or cell proliferation, consistent with our findings that E2 had no effect on the proliferation rate of either the ERα or ERβ-containing U2OS cells (data not shown). In contrast, in a recently reported study in ER-positive human breast cancer cells, in which E2 stimulates cell proliferation, we observed that E2 stimulated the expression of positive proliferation regulators including multiple growth factors and genes involved in cell cycle progression, and E2 also down-regulated transcriptional repressors and anti-proliferative and pro-apoptotic genes (29). Hence, the categories of genes stimulated by E2 in the U2OS cells and in breast cancer cells are very different, and they reflect the quite different physiological effects of E2 on these target cells. Further, E2 down-regulated the expression of few genes in these ER-containing osteosarcoma cells, whereas E2 down-regulated the expression of more genes in ER-positive MCF-7 breast cancer cells than it up-regulated (29). Despite these marked differences in genes regulated in these two different types of estrogen target cells, some genes were stimulated by E2 in both U2OS and MCF-7 breast cancer cells. These included pS2, keratin 19, the sodium hydrogen exchanger regulatory factor (NHERF/EBP50), GREB1, PDZK1, RIP140, WISP2 and connexin 43, all previously shown to be under estrogen regulation,

as referenced in Tables 2.2 and 2.3. Some have important roles in cell cytoarchitecture and cytoskeletal regulation (e.g., NHERF/EBP50, connexin43), supporting the known effects of estrogen on the cytoskeleton of both mammary (63) and bone cells.

It is of interest that our time-course analysis in the U2OS cells (Fig. 2.3) revealed that genes regulated by E2 divided into 3 temporal patterns, denoted E, E+L,or L in Tables 2.2 and 2.3: those regulated at early times only (4 and/or 8 h), those regulated at both early and late times, and those showing increased expression only at late times (24 and/or 48h). In both MCF-7 and in ER-containing U2OS cells, approximately equal numbers of stimulated genes fell into the early and late, and late-only categories, whereas relatively few genes showed early-only regulation in the U2OS cells compared with the breast cancer cells where *ca.* one-third of the total stimulated genes showed the early-only expression pattern. We have used the protein synthesis inhibitor, cycloheximide, to begin to distinguish primary versus secondary effects of E2-ER complexes on regulation of some of the interesting genes we have identified in the U2OS cells. These studies reveal that some of the genes turned on early that show elevated expression over time and hence fall into the "early and late" category (such as GREB1, autotaxin, oligophrenin-1 and KLRC4) likely represent primary estrogen response genes since their increase in mRNA expression is not prevented by cycloheximide. In contrast, cycloheximide blocked the estrogenic stimulation of cystatin D and PDZK1, a late and an early and late gene, respectively, suggesting these are probably secondary response genes.

Novel estrogen-regulated genes and genes regulated selectively by ERα or ERβ

These studies have enabled us to compare the actions of ERα and ERβ. While the majority of E2-stimulated genes were regulated through both ER subtypes, we

identified a number of genes selectively regulated either by ERα or by ERβ, as well as

many novel estrogen-regulated genes (64). Most of the E2-regulated genes involved in

cytoskeleton regulation and motility, signal transduction, cytokine and immune response

and growth factors/hormones were commonly regulated through both receptors. These

included a marked up-regulation of cox-2, cyritestin 1, keratin 19, several interleukins

and integrin α6. The latter factor, integrin α6, forms laminin receptors when

heterodimerized with integrin β1 or β4 subunits, and it is one of the factors involved in

tumor invasion through basal membranes (65-67).

Important growth factors and hormones regulated by E2 through both receptors

included several that can act in either an autocrine or paracrine manner, including BMP6

and PTHLH, to effect the bone-protective actions of E2 (68). PTHLH, a homologue of

PTH (parathyroid hormone), acts as an anabolic agent in osteoporosis (62), and its

overproduction in breast and prostate cancers is associated with bone metastasis of these

tumors (69, 70). Hence, its up-regulation by E2 via either ERα or ERβ is of interest. Of

the genes we observed to be selectively regulated by one ER subtype, nearly three-fourths

were ERα-selective. These ERα-regulated genes included several associated with

nucleic acid processing, two zinc finger proteins, the coregulator TRAP240, and the

cytochrome P450 family member 2B6 (CYP2B6). Of the genes exclusively regulated by

ERβ were autotaxin and cystatin D, factors associated with motility/cytoskeleton and

cytokine/immune function.

A recent report of related studies in U2OS cells identified some similar and some

different gene targets for ERα and ERβ (71) as we observed in this report. There are

several important methodological differences between the two studies that could account

for some of the differences in findings. First, the levels of ERα and ERβ in the U2OS

cells used by Monroe et al (71) were very high, namely three times that of MCF-7 cells,

whereas we chose to use cells that contained ER at levels more comparable to those in

osteoblasts, about 20% that of MCF-7 cells. Also, the studies in ref. 71 examined E2

regulation only at 24 h, whereas we examined gene regulation over a time course of E2

treatment up to 48 h, so that we identified both early, early and late, and late-only

responding genes; hence, some different sets of genes would likely be identified in the

two studies. In addition, we used a confidence score that includes considerations of fold

change, p-values, expression level and present calls in defining estrogen-regulated genes,

whereas Monroe et al (71) used fold change only. Further, the studies reported in ref. 71

used an earlier version Affymetrix microarray chip that contains probes for about half of

the genes on the U95A array we used, and also uses fewer and different probe sets per

gene. Hence, it is not surprising that there are some differences in the observed pattern of

gene regulations between our study and that presented in ref. 71.

The differences in gene targets of ERα vs. ERβ, or of progesterone receptor-A vs.

progesterone receptor-B (72), may reflect differences in the activation functions of these

receptors, in particular their N-terminal activation regions, as well as the complexity of

gene regulation by hormone occupied-nuclear receptors. The latter involves a

combination of aspects (7, 16, 73): the diverse nature of target gene promoters (with

different response elements that may be simple or composite) where receptors may bind

directly to the DNA or act through tethering to other DNA-bound transcription factors;

the nature of the coregulators recruited to the hormone-receptor-gene promoter; and the

receptor protein conformations induced by different ligands in the context of associated coregulators and transcription factors.

These studies, which have allowed a comparison of the gene regulatory activities of ERα and ERβ, should assist in the characterization of genes and cellular signaling pathways regulated by E2 through ERα and/or ERβ. They highlight commonality but also significant differences in gene targets for these two ERs and have identified many novel genes not previously known to be under estrogen regulation. Several of these are likely to play significant roles in the bone maintenance and anti-osteoporosis effects of estrogens. It will be of interest in subsequent studies to use the preferentially regulated genes we have identified to explore the molecular basis for the ER subtype-selectivity in the regulation of these genes.

Abbreviations

The abbreviations used are: AP-1, activation protein-1; BMP6, bone morphogenetic protein 6; BSA, bovine serum albumin; CAT, chloramphenicol acetyltransferase; CS, confidence score; CYP2B6, cytochrome P450 family 2B6; DPN, diarylpropionitrile; E2, 17β-estradiol; ERE, estrogen response element; ERα, estrogen receptor alpha; ERβ, estrogen receptor beta; FAK, focal adhesion kinase; FBS, fetal bovine serum; GFR, guanine nucleotide exchange factor for Rap1; IL-24, interleukin 24; IL-8, interleukin 8; LPA, lysophosphatidic acid; NHERF, Na+/H+ exchanger regulatory factor; NKG, natural killer cell lectin-type receptors; PCR, polymerase chain reaction; PDZK1, PDZ domain containing 1; PPT, propylpyrazoletriol; PR, progesterone receptor; PTHLH, parathyroid hormone-like hormone, also known as PTHrP, parathyroid hormone-related peptide; RIP140, receptor interacting protein 140; TFF1, trefoil factor 1, also known as pS2; TNFα, tumor necrosis factor alpha; TRAP240, thyroid hormone receptor-associated protein, 240 kDa subunit; WISP2, WNT1 inducible signaling pathway protein 2.

Tables and Figures

Table 2.1. Primers used for real-time PCR

Gene	Abbrev.	Forward Primer	Reverse Primer
Acyl-CoA Oxidase 2	ACOX2	5'-TTACAGACCCTGACGCAATCC	5'-CCTTAGCAGCCTGGAGGTTGT
Adenosine Kinase	ADK	5'-GGGCTGCACCTTTCCTGAGAA	5'-AGCAATTAGGGCAGTGTCTGC
Angiotensinogen	AGT	5'-GCCCTTCACTGAGAGCGC	5'- TGAGACCCTCCACCTTGTCC
Autotaxin	ENPP2	5'-CTCCTTCATCCTGCCTCACC	5'-TCACGCACCCTAGCTGTGTG
Bone Morphogenetic Protein 6	BMP6	5'-CATCACGGCCACTAGCAATCT	5'-CATCCCTTGTCACCACGCT
Carbonic Anhydrase XII	CA12	5'-GCAGGTCCAGAAGTTCGATGA	5'-CAGACTCAGTCCTGCCGCA
Connexin 43	GJA1	5'-GCCAAAGACTGTGGGTCTCAA	5'-CCAGGAGGAGACATAGGCGAG
Cystatin D	CST5	5'-GACTTTGCCATCAGCGAGTACA	5'-TAGGCAGCCATCACCTGCA
Estrogen Receptor α	ERα/ESR1	5'-GAATCTGCCAAGGAGACTCGC	5'-ACTGGTTGGTGGCTGGACAC
Estrogen Receptor β	ERβ/ESR2	5'-TGTCTGCAGCGATTACGCA	5'-GCGCCGGTTTTTATCGATT
GREB1	GREB1	5'-CAGGCTTTTGCACCGAATCT	5'-CAAAGCGTGTCGTCTTCAGCT
Integrin Alpha 6	ITGA6	5'-AGATCCCGGCCTGTGATTAA	5'-CCCCACACGCTGTTTTCTG
Interleukin 24 / MDA-7	IL24	5'-ACTGGACACTTCACGCCCTT	5'-TCACTGGCGCTGCTTAAAGA
Interleukin 8	IL8	5'-CCAGGAGAAACCACCGGA	5'-GGAAGGCTGCCAAGAGAGC
Neurotensin	NTS	5'-CCAGCTGAGGAAACAGGAGAA	5'-TGAAAAGCCCTGCTGTGACA
NKG2F	KLRC4	5'-AGCACGTCATTGTGGCCATT	5'-GAAGCACAGGCCAGCAAAC
NOR1/CSMF/MINOR	NR4A3	5'-ACCCCGACTACACCAAGCTG	5'-TTGCTCGAGTAGCCCTCCAC
Oligophrenin 1	OPHN1	5'-GTGGGCTTCAAGTTTGTCAGG	5'-GTGCGGTACAACCCTTCTGTC
PDZK1	PDZK1	5'-CCACTTGACACCCCTCCAGAT	5'-TGTACTGTGGGCCCGTTCTT
Phospholipase A2, IVA	PLA2G4A	5'-TGGCCTTGGTGAGTGATTCAG	5'-TCAAGCCCAGCATGAAGTTG
Trefoil Factor 1 / pS2	TFF1	5'-ATACCATCGACGTCCCTCCA	5'-AAGCGTGTCTGAGGTGTCCG
RIP140	NRIP1	5'-AAGCGAAGCCCATTTGCA	5'-TTTGGGAGCTGGTAACTGCC
SKI-like	SKIL	5'-GGAAACTGGCAGTGCCTTTG	5'-CGGAGCATCAGGCTGAACATA
WISP2	WISP2	5'-GCACACCGAAGACCCACCT	5'-AGGTACATGGTGTCGGGCA
36B4	36B4	5'-GTGTTCGACAATGGCAGCAT	5'-GACACCCTCCAGGAAGCGA

Table 2.2. Genes up-regulated by estradiol through estrogen receptor alpha and estrogen receptor beta in U2OS cells. Genes are organized alphabetically. The Table lists genes determined to be E2-regulated based on a Confidence Score ≥11 and a fold change ≥2.0 from microarray analysis, as described in Materials and Methods, in ERα- or ERβ-containing cells with the other receptor giving gene stimulation ≥1.4 fold. Gene names in bold appear to be novel and not previously reported to be regulated by E2(†). Those in italics have been verified by real-time PCR and the real-time PCR values are shown as entries in parentheses under the microarray values. All values are mean ± SEM. Under Time Pattern, E, E+L, or L denote an early only, early and late, or late only time pattern of regulation by E2.

Name	Abbrev.	GenBank	Fold ERα (mean±SEM)	Fold ERβ (mean±SEM)	Time Pattern (ERα,ERβ)	Function	E2-Reg. Gene
Acyl-CoA Oxidase 2	ACOX2	X95190	6.72 ± 2.73 (3.61 ± 0.03)	2.00 ± 0.22 (2.32 ± 0.33)	E+L, L	housekeeping	†
Adenosine Kinase	ADK	U50196	2.75 ± 0.59 (2.47 ± 0.33)	1.68 ± 0.31 (2.07 ± 0.34)	E+L, L	signal transduction	†
Angiotensinogen	AGT	K02215	2.00 ± 0.49 (4.41 ± 0.32)	2.13 ± 0.19 (4.18 ± 0.50)	L, L	growth factors/ hormones	(74)
Baculoviral IAP-containing 3	BIRC3	U45878	2.47 ± 0.32	3.01 ± 1.17	L, E+L	apoptosis/ proliferation	†
Bone Morphogenetic Protein 6	BMP6	M60315	2.39 ± 0.41 (2.84 ± 0.15)	2.17 ± 0.27 (2.82 ± 0.09)	L, L	growth factors/ hormones	(75)
Cadherin 19, Type 2	CDH19	AF047826	6.49 ± 1.30	1.86 ± 0.48	E+L, E	cell motility/ cytoskeleton	†
Carbonic Anhydrase XII	CA12	AF037335	2.60 ± 0.24 (5.08 ± 1.04)	2.40 ± 0.04 (15.42 ± 4.22)	E+L, E+L	unknown/other	†
CD34 Antigen	CD34	S53911	1.94 ± 0.25	3.12 ± 1.05	L, E+L	cytokine/immune function	†
Clone IMAGE 446411		AJ011980	4.67 ± 1.34	6.98 ± 2.73	L, L	unknown/other	†
Coagulation Factor XIII, A1	F13A1	M14539	4.37 ± 1.08	5.54 ± 0.96	E+L, E+L	unknown/other	†
Connexin 43	GJA1	X52947	2.39 ± 0.30 (2.72 ± 0.13)	1.56 ± 0.06 (2.21 ± 0.36)	L, L	cell motility/ cytoskeleton	(60)
Cyclooxygenase-2	PTGS2	U04636	31.5 ± 1.88	6.64 ± 2.01	E+L, E+L	cytokine/immune function	(40)
Cyritestin 1	ADAM3A	X89656	4.73 ± 0.20	4.64 ± 2.33	E+L, E	cell motility/ cytoskeleton	†
Defensin Beta 1	DEFB1	AI309115	2.01 ± 0.21	1.54 ± 0.40	L, L	cytokine/immune function	†
Glycerol Kinase	GK	X78711	3.57 ± 1.28	4.72 ± 0.14	L, E+L	housekeeping	(40)

Table 2.2. (Cont.)

G-protein Coupled Receptor Kinase 5	GPRK5	L15388	2.13 ± 0.03	2.00 ± 0.26	E, E	signal transduction	†
GREB1 Protein	GREB1	AB011147	2.74 ± 0.27 (25 ± 3.65)	4.86 ± 1.88 (35.76 ± 4.22)	E+L, E+L	unknown/other	(76)
Hevin	SPARCL1	X86693	4.31 ± 2.37	3.22 ± 2.31	E+L, E+L	unknown/other	†
Homeobox HB9	HLXB9	U07664	2.23 ± 0.22	1.63 ± 0.27	E, E	nucleic acid processing	†
IL13 Receptor Alpha 2	IL13RA2	U70981	2.64 ± 0.39	1.80 ± 0.14	E+L, L	cytokine/immune function	†
Integrin Alpha 6	ITGA6	S66213	3.77 ± 0.89 (3.68 ± 0.36)	2.92 ± 0.12 (2.28 ± 0.43)	E+L, L	cell motility/ cytoskeleton	(77)
Interleukin 8	IL8	M28130	2.13 ± 0.21 (2.79 ± 0.52)	2.40 ± 0.02 (3.2 ± 0.40)	L, E	cytokine/immune function	(78)
Interleukin 24 / MDA-7	IL24	U16261	5.20 ± 1.25 (10.12 ± 1.56)	1.70 ± 0.34 (3.76 ± 0.92)	E+L, E	apoptosis/ proliferation	†
Keratin 19	KRT19	Y00503	8.99 ± 0.52	3.79 ± 1.32	E+L, L	cell motility/ cytoskeleton	(59)
Leucine-rich and Ig Domain 1	LRIG1	AL039458	2.50 ± 0.16	1.47 ± 0.10	E+L, E	signal transduction	†
Microseminoprotein Beta	MSMB	AA532495	14.0 ± 1.68	2.83 ± 0.74	E+L, L	growth factors/ hormones	(79)
Neurotensin	NTS	U91618	4.95 ± 1.44 (4.55 ± 0.15)	1.97 ± 0.97 (3.07 ± 0.08)	E+L, L	growth factors/ hormones	(80)
NHERF	SLC9A3R1	AF015926	1.97 ± 0.08	2.22 ± 0.10	L, E+L	signal transduction	(27)
NKG2C	KLRC2	AJ001684	8.13 ± 1.50	6.74 ± 1.08	E+L, E+L	cytokine/immune function	†
NKG2E	KLRC3	AJ001685	8.91 ± 0.54	7.14 ± 1.66	E+L, E+L	cytokine/immune function	†
NKG2F	KLRC4	AJ001683	2.94 ± 1.64 (9.75 ± 1.62)	2.67 ± 0.01 (14.42 ± 2.67)	E+L, E+L	cytokine/immune function	†
NOR1/CSMF/MINOR	NR4A3	X89894	1.38 ± 0.57 (2.74 ± 0.11)	2.00 ± 0.22 (4.17 ± 0.65)	L, E	nucleic acid processing	†
Oligophrenin 1	OPHN1	AJ001189	2.09 ± 0.37 (2.48 ± 0.44)	1.99 ± 0.23 (3.9 ± 1.03)	E+L, E+L	cell motility/ cytoskeleton	†
Parathyroid Hormone-like Hormone	PTHLH/ PTHrP	M24351	2.29 ± 0.01	2.80 ± 0.35	E+L, E+L	growth factors/ hormones	(81)
PDZK1 Protein	PDZK1	AF012281	2.33 ± 0.77 (7.96 ± 1.35)	5.76 ± 0.47 (8.23 ± 1.31)	E+L, E+L	signal transduction	(76)
Phosphodiesterase 4B	PDE4B	L20971	2.10 ± 0.05	1.40 ± 0.06	E, E+L	signal transduction	†
Phospholipase A2, IVA	PLA2G4A	M72393	2.91 ± 1.38 (2.04 ± 0.12)	2.79 ± 1.90 (4.31 ± 0.45)	E+L, L	cytokine/immune function	†
Protein Phosphatase 1, Subunit 12B	PPP1R12B	AB007972	2.97 ± 0.05	2.32 ± 0.11	E+L, E+L	signal transduction	(29)
pS2	TFF1	AA314825	3.38 ± 0.21 (20.35 ± 3.62)	3.17 ± 0.96 (8.27 ± 1.55)	L, L	cytokine/immune function	(82)
Putative Tumor Suppressor	FUS2	U73167	2.28 ± 0.15	2.17 ± 0.17	L, L	signal transduction	†
Ras Protein Specific GRF 1	RASGRF1	S62035	2.03 ± 0.04	1.94 ± 0.01	L, L	signal transduction	(29)
Ras-like Protein A	RALA	M29893	2.04 ± 0.06	1.45 ± 0.28	L, L	signal transduction	†
Ret/Ptc2 Fusion		HG4677-HT5102	2.27 ± 0.13	2.03 ± 0.37	L, L	unknown/other	(29)
Rho-related BTB Domain Containing 3	RHOBTB3	AB020685	2.06 ± 0.03	1.62 ± 0.36	E+L, L	signal transduction	†

Table 2.2. (Cont.)

RIP140	NRIP1	X84373	2.88 ± 0.12 (3.35 ± 0.45)	1.54 ± 0.10 (2.45 ± 0.18)	E+L, L	nucleic acid processing	(83)
Selenoprotein P1	SEPP1	Z11793	2.14 ± 0.17	2.18 ± 0.33	L, L	cytokine/immune function	†
SKI-like	SKIL	X15217	2.51 ± 0.22 (1.91 ± 0.20)	1.49 ± 0.51 (2.4 ± 0.31)	E+L, E	nucleic acid processing	†
Transcobalamin 1	TCN1	J05068	5.83 ± 0.59	11.6 ± 1.25	L, L	housekeeping	†
Transcortin	SERPINA6	J02943	6.24 ± 0.25	2.63 ± 0.97	E+L, L	growth factors/ hormones	(84)
Transducin-like, Enhancer of Split 3	TLE3	M99438	2.36 ± 0.29	2.28 ± 0.01	E, E	unknown/other	†
UDP-glycosyltransferase 2, B10	UGT2B10	X63359	6.80 ± 2.32	2.25 ± 0.77	E+L, E+L	housekeeping	†
WISP2	WISP2	AF100780	3.89 ± 0.25 (42.2 ± 7.59)	2.81 ± 0.26 (14.19 ± 1.38)	L, L	cell motility/ cytoskeleton	(58)

Table 2.3. Estrogen-stimulated genes regulated selectively through one estrogen receptor subtype. E2-up-regulated genes were classified as ER-subtype selective when the Confidence Score was ≥11 and the fold change as determined by microarray analysis was 2.0 or greater whereas fold change was less than 1.3 fold for the other receptor at all time points. Gene names in bold appear to be novel and not previously reported to be regulated by E2(†). Those in italics have been verified by real-time PCR and the real-time PCR values are shown as entries in parentheses under the microarray values. All values are mean ± SEM. Under Time Pattern, E, E+L, or L denote an early only, early and late, or late only time pattern of regulation by E2.

Name	Abbrev.	GenBank	Fold (mean±SEM)	Time Pattern	Function	E2-Reg. Gene
ERα-selective						
Cytochrome P450, Family 2 B6	CYP2B6	M29874	6.10 ± 0.15	E+L	housekeeping	†
Phosphodiesterase 3B	PDE3B	D50640	3.50 ± 0.83	E+L	signal transduction	†
RIESKE Iron-Sulfur Protein		M23316	3.20 ± 0.72	E+L	unknown/other	†
Chromosome 18, orf 1	C18ORF1	AF009426	2.65 ± 0.65	E+L	unknown/other	†
Sodium Channel, Non-voltage Gated 1, β	SCNN1B	X87159	2.57 ± 0.27	E+L	signal transduction	†
Sialomucin	CD164	D14043	2.50 ± 0.05	L	cytokine/immune function	†
Zinc Finger Protein 267	ZNF267	X78925	2.41 ± 0.17	L	nucleic acid processing	†
PCF11p Homolog	PCF11	AB020631	2.42 ± 0.27	L	nucleic acid processing	†
Zinc Finger Protein 148	ZNF148	L04282	2.39 ± 0.28	L	nucleic acid processing	†
TR-associated Protein, 240 kDa	TRAP240	AB011165	2.21 ± 0.23	L	nucleic acid processing	†
CD2-associated Protein	CD2AP	AL050105	2.17 ± 0.12	L	cell motility/ cytoskeleton	†
DKFZp586K1 Protein	DKFZp586K1	AL049382	2.16 ± 0.21	L	unknown/other	†
KIAA0626 Gene Product	KIAA0626	AB014526	2.16 ± 0.35	L	unknown/other	†
Bcl-2-associated Transcription Factor	BTF	D79986	2.13 ± 0.05	L	apoptosis/ proliferation	†
DKFZp434D193		U79263	2.13 ± 0.35	E+L	unknown/other	†
Hbc647 mRNA	hbc647	U68494	2.12 ± 0.06	L	unknown/other	†
PKC Binding Protein 1	PRKCBP1	U48251	2.12 ± 0.37	L	signal transduction	(85)
ATPase, Lysosomal, V1 Subunit D	ATP6V1D	AA877795	2.09 ± 0.13	L	unknown/other	†
KIAA0191 Protein	KIAA0191	D83776	2.09 ± 0.03	E+L	unknown/other	†

Table 2.3. (Cont.)

|						
Kinesin Family Member 5B	KIF5B	X65873	2.07 ± 0.58	L	cell motility/ cytoskeleton	†
PRP4 pre-mRNA Processing Factor 4B	PRPF4B	U48736	2.07 ± 0.01	E+L	nucleic acid processing	†
KIAA0372 Gene Product	KIAA0372	AB002370	2.01 ± 0.15	L	unknown/other	†
KIAA1033 Protein	KIAA1033	AB028956	2.01 ± 0.22	L	unknown/other	†
Clone wg82b12.x1		AI768188	2.00 ± 0.17	L	unknown/other	†
ERβ-selective						
Clone 23892		AF035317	7.24 ± 1.24	E+L	unknown/other	†
Autotaxin	ENPP2	L35594	5.73 ± 1.77 (8.98 ± 0.78)	E+L	cell motility/ cytoskeleton	†
Cystatin D	CST5	X70377	3.70 ± 1.27 (8.17 ± 1.02)	L	cytokine/immune function	†
HSU43279		U43279	3.53 ± 0.79	E+L	unknown/other	†
RA-inducible Endogenous Retroviral DNA		M64936	3.37 ± 0.59	E+L	unknown/other	†
Rap1 GEF	GFR	D87467	2.41 ± 0.17	E	signal transduction	†
Stromal Antigen 2	STAG2	Z75331	2.28 ± 0.51	E+L	unknown/other	†
Putative Mitochondrial Space Protein	PSORT	AF050198	2.2 ± 0.09	E	unknown/other	†
Hypothetical Protein	FLJ31564	W27600	2.2 ± 0.38	E+L	unknown/other	†
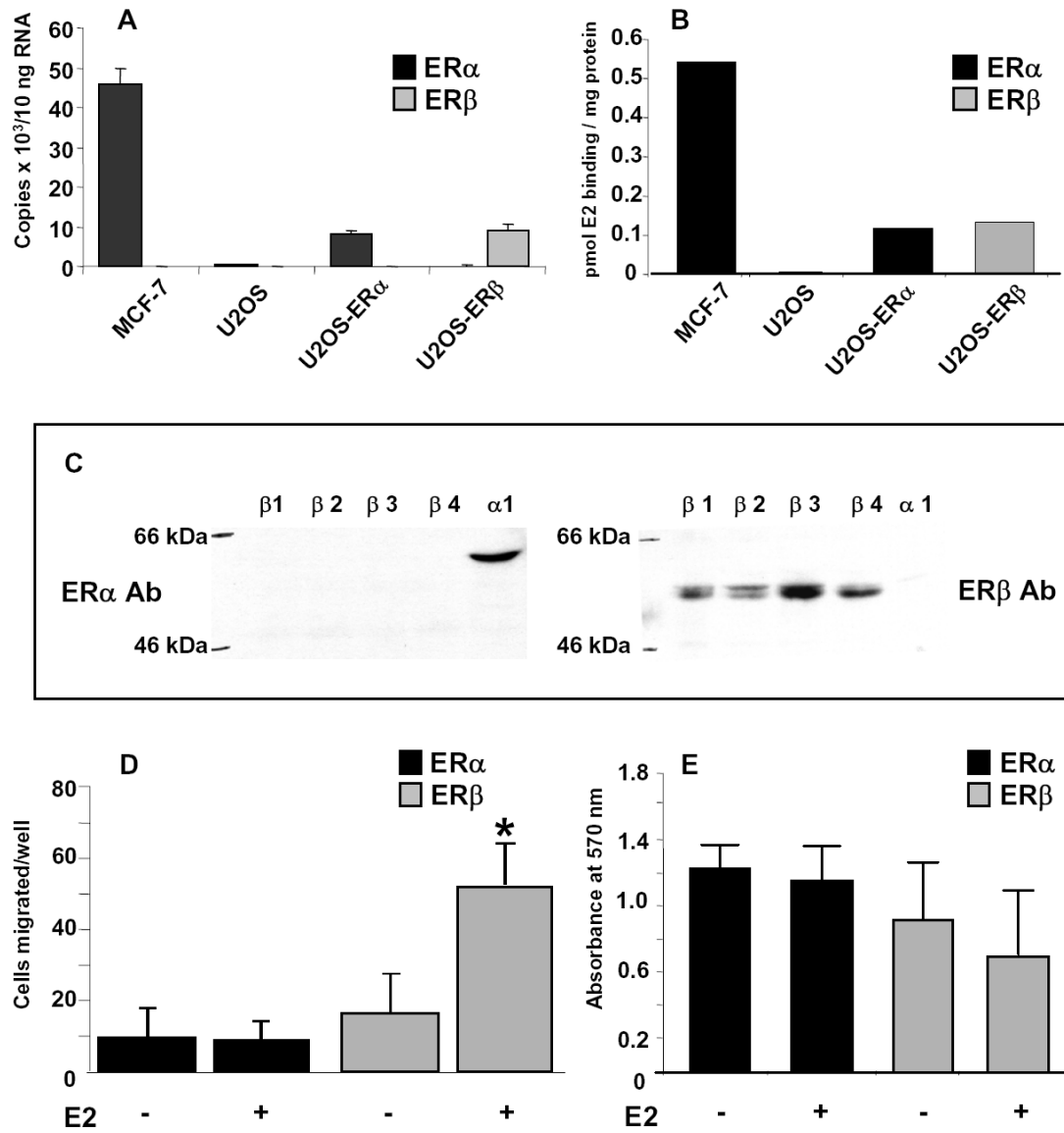
Figure 2.1. Characterization of U2OS human osteosarcoma cells stably expressing ERα or ERβ.

(**A**) Estrogen receptor mRNA copy number/10 ng total RNA as assessed by real-time

PCR in ERα clones 1-4 and in ERβ clones 1-4. (**B**) E2 binding sites/mg protein

evaluated by E2 binding assay in parental U2OS and U2OS-ER cell lines (ERα clone 1

Figure 2.1. (Cont.)

and ERβ clone 1) in comparison with the MCF-7 breast cancer cell line. The mean of

closely corresponding duplicate determinations is shown. **(C)** Western blot for ERα and

ERβ protein in different clones of stably transfected U2OS cells. **(D)** Motility (Boyden

chamber assay) of cells (ERα clone 1 and ERβ clone 1) was assayed with 5% FBS as a

general chemoattractive factor. E2 treatment was for 48 h. Similar findings were obtained

with 3 other ERα and ERβ clones. E) Adhesion assay was performed in 96-well plates

coated with various substrates. Shown is 30 min adhesion on collagen type I measured as

absorbance at 570 nm. E2 treatment was for 48 h. No effect of E2 on cell adhesion was

also seen in repeat experiments with 3 other ERα and ERβ clones. $*p<0.01$ for E2-treated

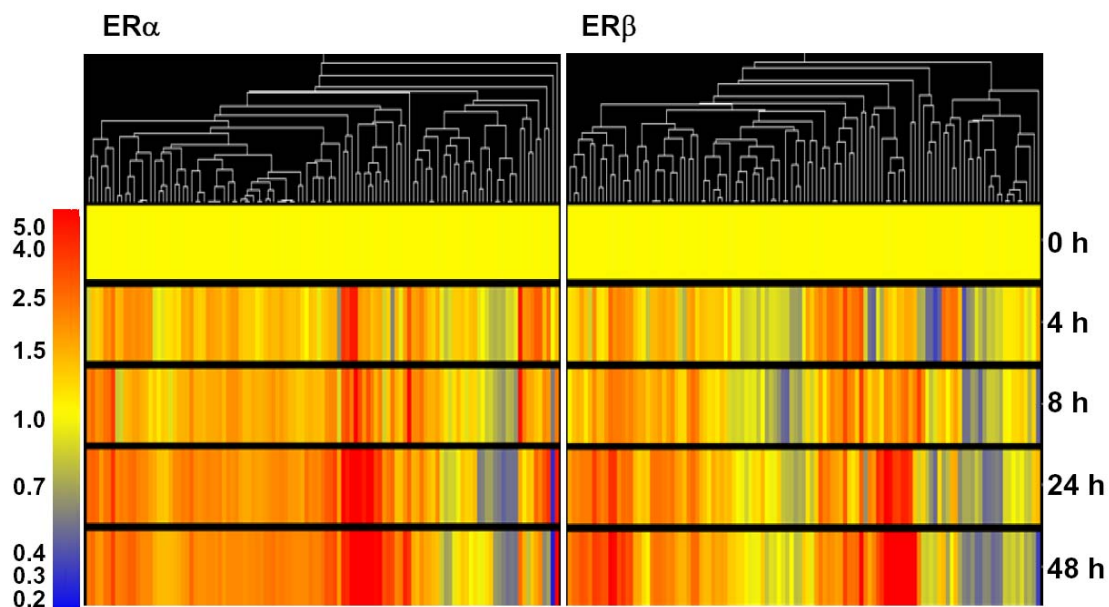cells vs. cells treated with control vehicle.

Figure 2.2. Cluster analysis of E2-regulated genes in U2OS-ERα or -ERβ containing cells.

After applying normalization and confidence score analyses, all the E2-regulated genes in U2OS-ER containing cells (ERα clone 1 and ERβ clone 1) were clustered using a standard correlation algorithm (GeneSpring software). Up-regulated genes are shown in red, down-regulated genes in blue and non-changing genes in yellow. The color scale corresponding to fold change in gene expression is shown at the left. The different time points of the E2 (10nM) time-course treatment are indicated at the right. The gene expression values shown are the average fold change of independent samples, each run on a separate microarray chip, for each time point.
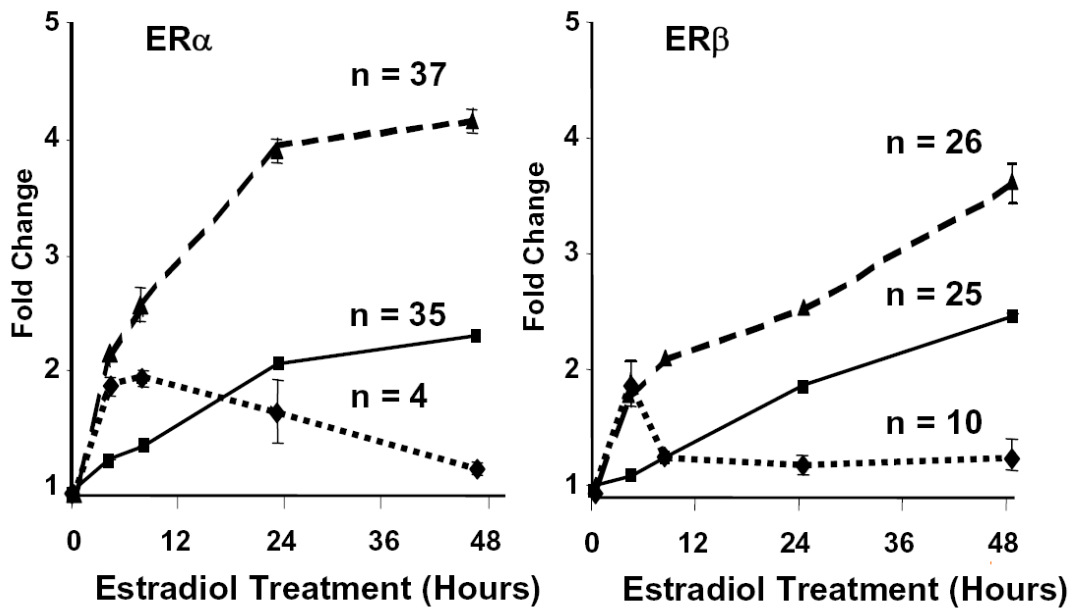
Figure 2.3. Time-course patterns of E2-stimulated genes in U2OS-ERα and U2OS-ERβ cells as identified from microarray analysis.

All E2 up-regulated genes were assigned to one of three categories. For "Early only" regulated genes (dotted broken line with diamonds), the fold change was 2-fold or greater at 4 and/or 8 h only. For "Early and Late" regulated genes (dashed line with triangles), the fold change was 2-fold or greater at 4 and/or 8 h and at 24 and/or 48 h. And for "Late only" regulated genes (solid line with squares), the fold change was 2-fold or greater at 24 and/or 48 h only. Once genes were assigned to one of the three time-course patterns, the mean fold-change ± SEM was calculated and plotted for all genes in that pattern. On some points, error bars are too small to be visible.
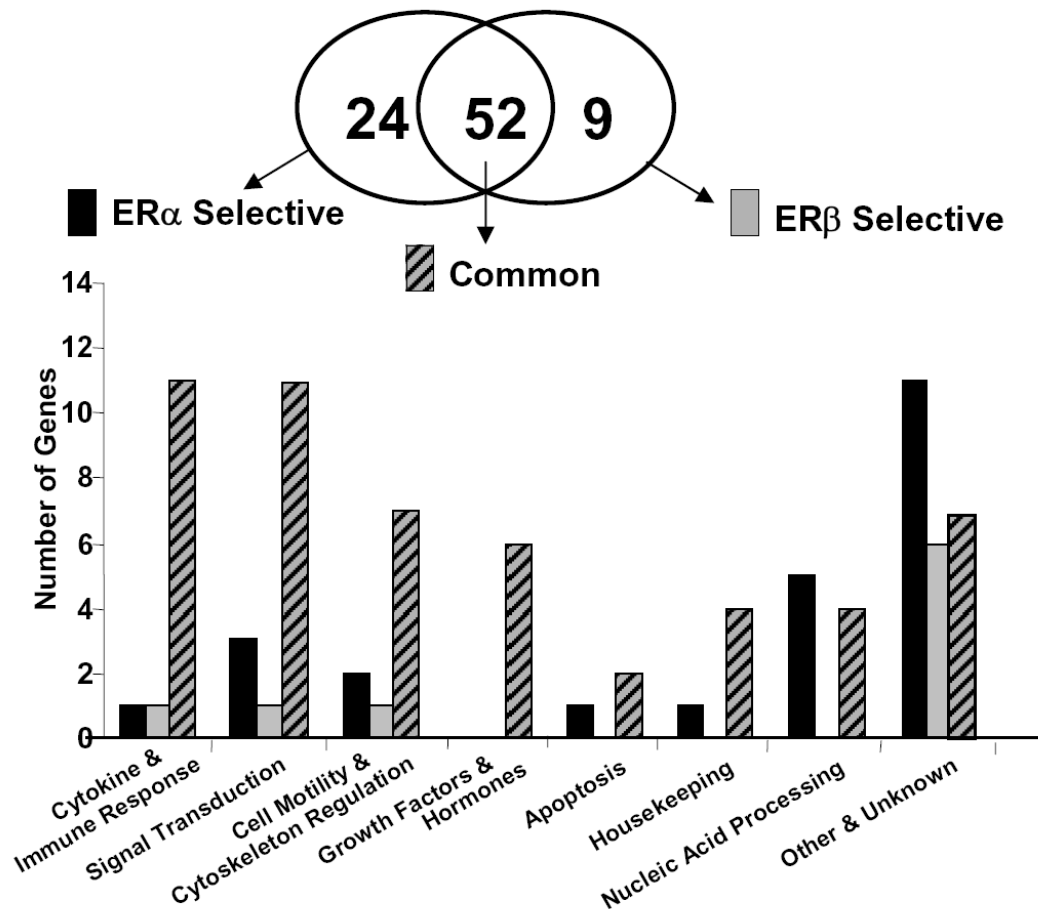
Figure 2.4. Functional classification of E2-stimulated genes.

E2 up-regulated genes were classified as ER-subtype selective when the fold change from

the microarray data for one receptor was 2.0-fold or greater whereas for the other

receptor it was less than 1.3-fold at all time points. All the genes identified were then

categorized in functional groups according to their main known function based on

LocusLink, OMIM, PubMed, GeneCards and GenMAPP databases.

Figure 2.5. Real-Time PCR validation of genes regulated commonly by both ERα and ERβ.

Estradiol (10nM) time-course and ICI 182,870 (1µM) treatments were performed in three independent experiments to confirm DNA microarray data and assess the estrogen receptor-dependent mechanism of the gene regulation through reversal by a 100-fold excess of the antiestrogen (ICI+E2). Values are mean fold change + SEM. *indicates $p < 0.05$ for gene stimulation by E2 vs. vehicle control.
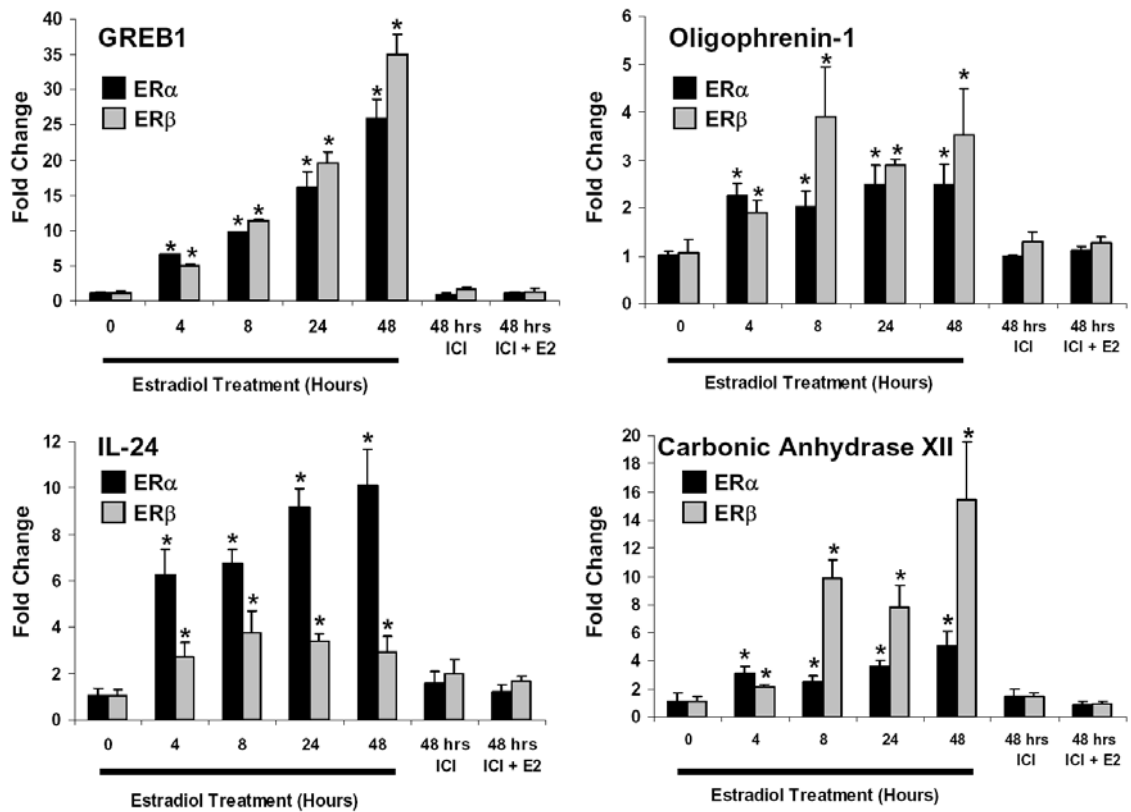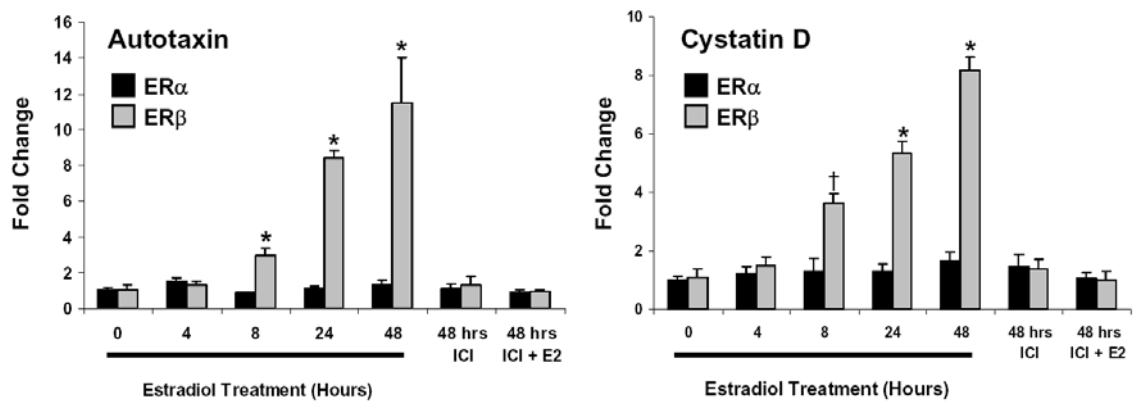
Figure 2.6. Real-Time PCR validation of genes regulated selectively through ERβ.
Estradiol (10nM) time-course and ICI 182,870 (1µM) treatments were performed in three

independent experiments to confirm DNA microarray data and assess the estrogen

receptor-dependent mechanism of the gene regulation through reversal by a 100-fold

excess of the antiestrogen (ICI+E2). Values are mean fold change + SEM. *indicates

$p<0.05$ and † indicates $p=0.06$ for gene stimulation in ERβ- vs. ERα-containing cells.
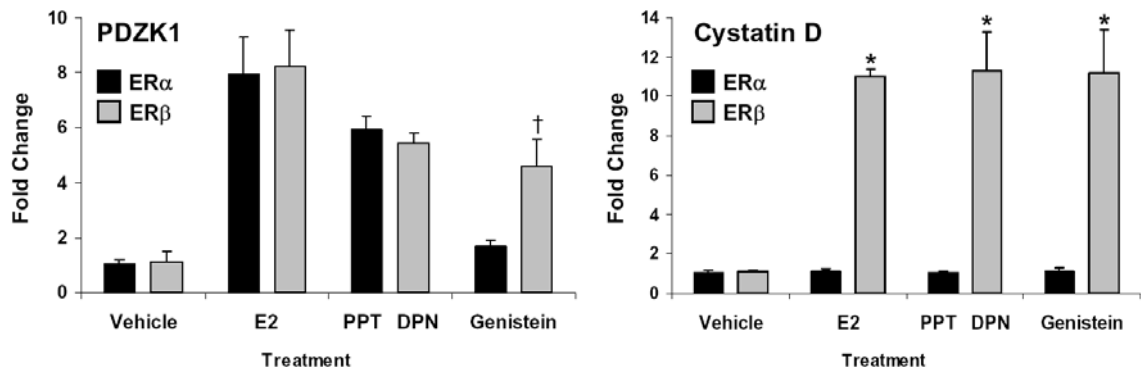
Figure 2.7. Real-Time PCR analysis of gene regulation by estradiol, the ERα-selective ligand PPT, the ERβ-selective ligand DPN, and Genistein.

Estradiol (10nM), PPT (100nM), DPN (100nM), and Genistein (1μM) treatments were performed for 48 h in three separate experiments to examine the ability of ER-subtype selective ligands to regulate gene expression in cells containing either ERα or ERβ. Values are mean fold change + SEM. *indicates p<0.05 and † indicates p=0.07 for gene stimulation in ERβ- vs. ERα-containing cells.

References

1.   **Manolagas SC, Kousteni S, Jilka RL** 2002 Sex steroids and bone. Recent Prog Horm Res 57:385-409
2.   **Manolagas SC** 2000 Birth and death of bone cells: basic regulatory mechanisms and implications for the pathogenesis and treatment of osteoporosis. Endocr Rev 21:115-137
3.   **Riggs BL, Khosla S, Melton LJ, 3rd** 2002 Sex steroids and the construction and conservation of the adult skeleton. Endocr Rev 23:279-302
4.   **Riggs BL, Khosla S, Melton LJ, 3rd** 1998 A unitary model for involutional osteoporosis: estrogen deficiency causes both type I and type II osteoporosis in postmenopausal women and contributes to bone loss in aging men. J Bone Miner Res 13:763-773
5.   **Katzenellenbogen BS, Montano MM, Ediger TR, Sun J, Ekena K, Lazennec G, Martini PGV, McInerney EM, Delage-Mourroux R, Weis K, Katzenellenbogen JA** 2000 Estrogen receptors: Selective ligands, partners, and distinctive pharmacology. Recent Prog. Horm. Res. 55:163-195
6.   **Pettersson K, Gustafsson JA** 2001 Role of estrogen receptor beta in estrogen action. Annu. Rev. Physiol. 63:165-192
7.   **McDonnell DP, Norris JD** 2002 Connections and regulation of the human estrogen receptor. Science 296:1642-1644
8.   **McDonnell DP** 1999 The molecular pharmacology of SERMs. Trends Endocrinol. Metab. 10:301-311
9.   **Klinge CM** 2001 Estrogen receptor interaction with estrogen response elements. Nucleic Acids Res 29:2905-2919
10.   **Kushner PJ, Agard DA, Greene GL, Scanlan TS, Shiau AK, Uht RM, Webb P** 2000 Estrogen receptor pathways to AP-1. J Steroid Biochem Mol Biol 74:311-317
11.   **Safe S** 2001 Transcriptional activation of genes by 17 beta-estradiol through estrogen receptor-Sp1 interactions. Vitam. Horm. 62:231-252
12.   **Saville B, Wormke M, Wang F, Nguyen T, Enmark E, Kuiper G, Gustafsson JA, Safe S** 2000 Ligand-, cell-, and estrogen receptor subtype (alpha/beta)-dependent activation at GC-rich (Sp1) promoter elements. J. Biol. Chem. 275:5379-5387
13.   **Paech K, Webb P, Kuiper GGJM, Nilsson S, Gustafsson J-A, Kushner PJ, Scanlan TS** 1997 Differential ligand activation of estrogen receptors ERα and ER at AP1 sites. Science 277:1508-1510
14.   **Hall JM, Couse JF, Korach KS** 2001 The multifaceted mechanisms of estradiol and estrogen receptor signaling. J. Biol. Chem. 276:36869-36872
15.   **Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M** 2000 Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell 103:843-852
16.   **McKenna NJ, O'Malley BW** 2002 Combinatorial control of gene expression by nuclear receptors and coregulators. Cell 108:465-474

17. **Kousteni S, Bellido T, Plotkin LI, O'Brien CA, Bodenner DL, Han L, Han K, DiGregorio GB, Katzenellenbogen JA, Katzenellenbogen BS, Roberson PK, Weinstein RS, Jilka RL, Manolagas SC** 2001 Nongenotropic, sex-nonspecific signaling through the estrogen or androgen receptors: dissociation from transcriptional activity. Cell 104:719-730

18. **Kousteni S, Han L, Chen JR, Almeida M, Plotkin LI, Bellido T, Manolagas SC** 2003 Kinase-mediated regulation of common transcription factors accounts for the bone-protective effects of sex steroids. J Clin Invest 111:1651-1664

19. **Kelly MJ, Levin ER** 2001 Rapid actions of plasma membrane estrogen receptors. Trends Endocrinol Metab 12:152-156

20. **Eriksen EF, Colvard DS, Berg NJ, Graham ML, Mann KG, Spelsberg TC, Riggs BL** 1988 Evidence of estrogen receptors in normal human osteoblast-like cells. Science 241:84-86

21. **Hoyland JA, Mee AP, Baird P, Braidman IP, Mawer EB, Freemont AJ** 1997 Demonstration of estrogen receptor mRNA in bone using in situ reverse-transcriptase polymerase chain reaction. Bone 20:87-92

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

23. **Bodine PV, Henderson RA, Green J, Aronow M, Owen T, Stein GS, Lian JB, Komm BS** 1998 Estrogen receptor-alpha is developmentally regulated during osteoblast differentiation and contributes to selective responsiveness of gene expression. Endocrinology 139:2048-2057

24. **Arts J, Kuiper GG, Janssen JM, Gustafsson JA, Lowik CW, Pols HA, van Leeuwen JP** 1997 Differential expression of estrogen receptors alpha and beta mRNA during differentiation of human osteoblast SV-HFO cells. Endocrinology 138:5067-5070

25. **Spelsberg TC, Subramaniam M, Riggs BL, Khosla S** 1999 The actions and interactions of sex steroids and growth factors/cytokines on the skeleton. Mol Endocrinol 13:819-828

26. **McInerney EM, Weis KE, Sun J, Mosselman S, Katzenellenbogen BS** 1998 Transcription activation by the human estrogen receptor subtype β (ERβ) studied with ERβ and ERα receptor chimeras. Endocrinology 139:4513-4522

27. **Ediger TR, Kraus WL, Weinman EJ, Katzenellenbogen BS** 1999 Estrogen receptor regulation of the Na+/H+ exchange regulatory factor. Endocrinology 140:2976-2982

28. **Lazennec G, Alcorn JL, Katzenellenbogen BS** 1999 Adenovirus-mediated delivery of a dominant negative estrogen receptor gene abrogates estrogen-stimulated gene expression and breast cancer cell proliferation. Mol. Endocrinol. 13:969-980

29. **Frasor J, Danes JM, Komm B, Chang K, Lyttle CR, Katzenellenbogen BS** 2003 Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology 144:4562-4574

30. **Jelinsky SA, Harris HA, Brown EL, Flanagan K, Zhang X, Tunkey C, Lai K, Lane MV, Simcoe DK, Evans MJ** 2003 Global transcription profiling of estrogen activity: estrogen receptor alpha regulates gene expression in the kidney. Endocrinology 144:701-710

31. **Livak KJ, Schmittgen TD** 2001 Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 25:402-408

32. **Braud VM, Allan DS, O'Callaghan CA, Soderstrom K, D'Andrea A, Ogg GS, Lazetic S, Young NT, Bell JI, Phillips JH, Lanier LL, McMichael AJ** 1998 HLA-E binds to natural killer cell receptors CD94/NKG2A, B and C. Nature 391:795-799

33. **Lazetic S, Chang C, Houchins JP, Lanier LL, Phillips JH** 1996 Human natural killer cell receptors involved in MHC class I recognition are disulfide-linked heterodimers of CD94 and NKG2 subunits. J Immunol 157:4741-4745

34. **Lanier LL, Corliss B, Wu J, Phillips JH** 1998 Association of DAP12 with activating CD94/NKG2C NK cell receptors. Immunity 8:693-701

35. **Zannettino AC, Buhring HJ, Niutta S, Watt SM, Benton MA, Simmons PJ** 1998 The sialomucin CD164 (MGC-24v) is an adhesive glycoprotein expressed by human hematopoietic progenitors and bone marrow stromal cells that serves as a potent negative regulator of hematopoiesis. Blood 92:2613-2628

36. **Watt SM, Chan JY** 2000 CD164--a novel sialomucin on CD34+ cells. Leuk Lymphoma 37:1-25

37. **Chaudhary LR, Avioli LV** 1994 Dexamethasone regulates IL-1 beta and TNF-alpha-induced interleukin-8 production in human bone marrow stromal and osteoblast-like cells. Calcif Tissue Int 55:16-20

38. **Akoum A, Lawson C, McColl S, Villeneuve M** 2001 Ectopic endometrial cells express high concentrations of interleukin (IL)-8 in vivo regardless of the menstrual cycle phase and respond to oestradiol by up-regulating IL-1-induced IL-8 expression in vitro. Mol Hum Reprod 7:859-866

39. **Nithipatikom K, Isbell MA, Lindholm PF, Kajdacsy-Balla A, Kaul S, Campell WB** 2002 Requirement of cyclooxygenase-2 expression and prostaglandins for human prostate cancer cell invasion. Clin Exp Metastasis 19:593-601

40. **Pedram A, Razandi M, Aitkenhead M, Hughes CC, Levin ER** 2002 Integration of the non-genomic and genomic actions of estrogen. Membrane-initiated signaling by steroid to transcription and cell biology. J Biol Chem 277:50768-50775

41. **Lee HY, Bae GU, Jung ID, Lee JS, Kim YK, Noh SH, Stracke ML, Park CG, Lee HW, Han JW** 2002 Autotaxin promotes motility via G protein-coupled phosphoinositide 3-kinase gamma in human melanoma cells. FEBS Lett 515:137-140

42. **Jung ID, Lee J, Yun SY, Park CG, Choi WS, Lee HW, Choi OH, Han JW, Lee HY** 2002 Cdc42 and Rac1 are necessary for autotaxin-induced tumor cell motility in A2058 melanoma cells. FEBS Lett 532:351-356

43. **Umezu-Goto M, Kishi Y, Taira A, Hama K, Dohmae N, Takio K, Yamori T, Mills GB, Inoue K, Aoki J, Arai H** 2002 Autotaxin has lysophospholipase D activity leading to tumor cell growth and motility by lysophosphatidic acid production. J Cell Biol 158:227-233

44. **de Rooij J, Rehmann H, van Triest M, Cool RH, Wittinghofer A, Bos JL** 2000 Mechanism of regulation of the Epac family of cAMP-dependent RapGEFs. J Biol Chem 275:20829-20836

45. **Reedquist KA, Ross E, Koop EA, Wolthuis RM, Zwartkruis FJ, van Kooyk Y, Salmon M, Buckley CD, Bos JL** 2000 The small GTPase, Rap1, mediates CD31-induced integrin adhesion. J Cell Biol 148:1151-1158

46. **Bos JL, De Bruyn K, Enserink J, Kuiperij B, Rangarajan S, Rehmann H, Riedl J, De Rooij J, Van Mansfeld F, Zwartkruis F** 2003 The role of Rap1 in integrin-mediated cell adhesion. Biochem Soc Trans 31:83-86

47. **Stauffer SR, Coletta CJ, Tedesco R, Nishiguchi G, Carlson K, Sun J, Katzenellenbogen BS, Katzenellenbogen JA** 2000 Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists. J. Med. Chem. 43:4934-4947

48. **Kraichely DM, Sun J, Katzenellenbogen JA, Katzenellenbogen BS** 2000 Conformational changes and coactivator recruitment by novel ligands for estrogen receptor-alpha and estrogen receptor-beta: correlations with biological character and distinct differences among SRC coactivator family members. Endocrinology 141:3534-3545

49. **Harrington WR, Sheng S, Barnett DH, Petz LN, Katzenellenbogen JA, Katzenellenbogen BS** 2003 Activities of estrogen receptor alpha- and beta-selective ligands at diverse estrogen responsive gene sites mediating transactivation or transrepression. Mol Cell Endocrinol 206:13-22

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

51. **Meyers MJ, Sun J, Carlson KE, Marriner GA, Katzenellenbogen BS, Katzenellenbogen JA** 2001 Estrogen receptor-beta potency-selective ligands: Structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. J. Med. Chem. 44:4230-4251

52. **Sun J, Baudry J, Katzenellenbogen JA, Katzenellenbogen BS** 2003 Molecular basis for the subtype discrimination of the estrogen receptor-beta-selective ligand, diarylpropionitrile. Mol Endocrinol 17:247-58

53. **Kuiper GG, Lemmen JG, Carlsson B, Corton JC, Safe SH, van der Saag PT, van der Burg B, Gustafsson JA** 1998 Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. Endocrinology 139:4252-4263

54. **Lindberg MK, Moverare S, Eriksson AL, Skrtic S, Gao H, Dahlman-Wright K, Gustafsson JA, Ohlsson C** 2002 Identification of estrogen-regulated genes of potential importance for the regulation of trabecular bone mineral density. J. Bone Miner. Res. 17:2183-2195

55. **Mueller SO, Korach KS** 2001 Estrogen receptors and endocrine diseases: lessons from estrogen receptor knockout mice. Curr Opin Pharmacol 1:613-619

56. **Windahl SH, Andersson G, Gustafsson JA** 2002 Elucidation of estrogen receptor function in bone with the use of mouse models. Trends Endocrinol. Metab. 13:195-200

57. **Kumar S, Hand AT, Connor JR, Dodds RA, Ryan PJ, Trill JJ, Fisher SM, Nuttall ME, Lipshutz DB, Zou C, Hwang SM, Votta BJ, James IE, Rieman DJ, Gowen M, Lee JC** 1999 Identification and cloning of a connective tissue growth factor-like cDNA from human osteoblasts encoding a novel regulator of osteoblast functions. J Biol Chem 274:17123-17131

58. **Inadera H, Dong HY, Matsushima K** 2002 WISP-2 is a secreted protein and can be a marker of estrogen exposure in MCF-7 cells. Biochem Biophys Res Commun 294:602-608

59. **Choi I, Gudas LJ, Katzenellenbogen BS** 2000 Regulation of keratin 19 gene expression by estrogen in human breast cancer cells and identification of the estrogen responsive gene region. Mol Cell Endocrinol 164:225-237

60. **Yu W, Dahl G, Werner R** 1994 The connexin43 gene is responsive to oestrogen. Proc Royal Soc Lond B Biol Sci 255:125-132

61. **Pasquini GM, Davey RA, Ho PW, Michelangeli VP, Grill V, Kaczmarczyk SJ, Zajac JD** 2002 Local secretion of parathyroid hormone-related protein by an osteoblastic osteosarcoma (UMR 106-01) cell line results in growth inhibition. Bone 31:598-605

62. **Horwitz MJ, Tedesco MB, Gundberg C, Garcia-Ocana A, Stewart AF** 2003 Short-term, high-dose parathyroid hormone-related protein as a skeletal anabolic agent for the treatment of postmenopausal osteoporosis. J Clin Endocrinol Metab 88:569-575

63. **Vic P, Vignon F, Derocq D, Rochefort H** 1982 Effect of estradiol on the ultrastructure of the MCF-7 human breast cancer cells in culture. Cancer Res. 42:667-673

64. **Stossi F, Barnett DH, Frasor J, Katzenellenbogen BS** Transcriptional profiling of estrogen-regulated gene expression in human osteosarcoma cells stably expressing estrogen receptor alpha or estrogen receptor beta. 85th Annual Meeting of The Endocrine Society, Philadelphia, PA, June 2003 (Abstract)

65. **Hynes RO, Marcantonio EE, Stepp MA, Urry LA, Yee GH** 1989 Integrin heterodimer and receptor complexity in avian and mammalian cells. J Cell Biol 109:409-420

66. **Isberg RR, Leong JM** 1990 Multiple beta 1 chain integrins are receptors for invasin, a protein that promotes bacterial penetration into mammalian cells. Cell 60:861-871

67. **Daemi N, Vallet T, Thomasset N, Jacquier MF, Zebda N, Dore JF, Sordat B, Remy L** 1995 Expression of the alpha 6, beta 1 and beta 4 integrin subunits, basement membrane organization and proteolytic capacities in low and high metastatic human colon carcinoma xenografts. Invasion Metastasis 15:103-115

68. **Hughes FJ, Collyer J, Stanfield M, Goodman SA** 1995 The effects of bone morphogenetic protein-2, -4, and -6 on differentiation of rat osteoblast cells in vitro. Endocrinology 136:2671-2677

69. **Shen X, Falzon M** 2003 PTH-related protein modulates PC-3 prostate cancer cell adhesion and integrin subunit profile. Mol Cell Endocrinol 199:165-177

70. **Hoey RP, Sanderson C, Iddon J, Brady G, Bundred NJ, Anderson NG** 2003 The parathyroid hormone-related protein receptor is expressed in breast cancer bone metastases and promotes autocrine proliferation in breast carcinoma cells. Br J Cancer 88:567-573

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

72. **Richer JK, Jacobsen BM, Manning NG, Abel MG, Wolf DM, Horwitz KB** 2002 Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. J Biol Chem 277:5209-5218

73. **Katzenellenbogen BS, Katzenellenbogen JA** 2002 Defining the "S" in SERMs. Science 295:2380-2381

74. **Zhao YY, Sun KL, Ashok K** 1998 Analysis of a cis-acting element involved in regulation by estrogen of human angiotensinogen gene expression. Biochemistry and Biophysics (Shanghai) 30:445-448

75. **Rickard DJ, Hofbauer LC, Bonde SK, Gori F, Spelsberg TC, Riggs BL** 1998 Bone morphogenetic protein-6 production in human osteoblastic cell lines. Selective regulation by estrogen. J Clin Invest 101:413-422

76. **Ghosh MG, Thompson DA, Weigel RJ** 2000 PDZK1 and GREB1 are estrogen-regulated genes expressed in hormone-responsive breast cancer. Cancer Res 60:6367-6375

77. **Sager R, Anisowicz A, Neveu M, Liang P, Sotiropoulou G** 1993 Identification by differential display of alpha 6 integrin as a candidate tumor suppressor gene. Faseb J 7:964-970

78. **Kanda N, Watanabe S** 2001 17beta-estradiol, progesterone, and dihydrotestosterone suppress the growth of human melanoma by inhibiting interleukin-8 production. J Invest Dermatol 117:274-283

79. **Nolet S, Mbikay M, Chretien M** 1991 Prostatic secretory protein PSP94: gene organization and promoter sequence in rhesus monkey and human. Biochim Biophys Acta 1089:247-249

80. **Watters JJ, Dorsa DM** 1998 Transcriptional effects of estrogen on neuronal neurotensin gene expression involve cAMP/protein kinase A-dependent signaling mechanisms. J Neurosci 18:6672-6680

81. **Thiede MA, Harm SC, Hasson DM, Gardner RM** 1991 In vivo regulation of parathyroid hormone-related peptide messenger ribonucleic acid in the rat uterus by 17 beta-estradiol. Endocrinology 128:2317-2323

82. **Brown AM, Jeltsch JM, Roberts M, Chambon P** 1984 Activation of pS2 gene transcription is a primary response to estrogen in the human breast cancer cell line MCF-7. Proc Natl Acad Sci U S A 81:6344-6348

83. **Thenot S, Charpin M, Bonnet S, Cavailles V** 1999 Estrogen receptor cofactors expression in breast and endometrial human cancer cells. Mol Cell Endocrinol 156:85-93

84. **Zajchowski DA, Kauser K, Zhu D, Webster L, Aberle S, White FA, 3rd, Liu HL, Humm R, MacRobbie J, Ponte P, Hegele-Hartung C, Knauthe R, Fritzemeier KH, Vergona R, Rubanyi GM** 2000 Identification of selective estrogen receptor modulators by their gene expression fingerprints. J Biol Chem 275:15885-15894

85. **Peddada SD, Lobenhofer EK, Li L, Afshari CA, Weinberg CR, Umbach DM** 2003 Gene selection and clustering for time-course and dose-response microarray experiments using order-restricted inference. Bioinformatics 19:834-841

CHAPTER 3

ESTROGEN RECEPTOR REGULATION OF CARBONIC ANHYDRASE XII

THROUGH A DISTAL ENHANCER IN BREAST CANCER[1]

Abstract

The expression of *carbonic anhydrase XII* (*CA12*), a gene that encodes a zinc-metalloenzyme responsible for acidification of the microenvironment of cancer cells, is highly correlated with estrogen receptor α (ERα) in human breast tumors. Here, we show that *CA12* is robustly regulated by estrogen via ERα in breast cancer cells, and that this regulation involves a distal estrogen-responsive enhancer region. Upon addition of estradiol, ERα binds directly to this distal enhancer *in vivo*, resulting in the recruitment of RNA polymerase II and steroid receptor coactivators SRC-2 and SRC-3, and changes in histone acetylation. Mutagenesis of an imperfect estrogen-responsive element within this enhancer region abolishes estrogen-dependent activity, and chromosome conformation capture (3C) and chromatin immunoprecipitation assays demonstrate that this distal enhancer communicates with the transcriptional start site of the *CA12* gene via intra-chromosomal looping upon hormone treatment. This distal enhancer element is observed in the homologous mouse genomic sequence, and the expression of the mouse homolog, *Car12*, is rapidly and robustly stimulated by estradiol in the mouse uterus in vivo, suggesting that the ER regulation of *CA12* is mechanistically and evolutionarily conserved. Our findings highlight the crucial role of ER in regulation of the *CA12* gene,

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and provide insight into the transcriptional regulatory mechanism that accounts for the strong association of *CA12* and ER in human breast cancers.

Introduction

Estrogen receptor alpha (ERα), a hormone-regulated transcription factor and member of the superfamily of nuclear receptors (1, 2), is expressed in approximately 70 percent of breast cancers (3). As the major regulator of the phenotypic properties of these breast cancers, ERα markedly influences the pattern of breast cancer gene expression and, perhaps more than any other protein, it defines the distinctly different gene signatures of ER-positive and ER-negative breast cancers (4-7). In our recent breast cancer gene expression profiling studies, we observed the *carbonic anhydrase XII* gene (*CA12*) to be robustly stimulated by estradiol (E2) in several ER-containing breast cancer cells (8, 9). Further, from our examination of transcriptional profiling data sets in ER-positive and ER-negative breast tumors, we found CA12 to show one of the most highly significant positive correlations with ER expression (10-12).

Carbonic anhydrase XII is a transmembrane, extracellular enzyme and member of the family of zinc-metalloenzymes that catalyze the reversible hydration of CO_2 to form bicarbonate ($H_2O + CO_2 \longleftrightarrow H^+ + HCO_3^-$), thereby regulating the microenvironment acidity and tumor malignant phenotype (13-16). CA12 was originally identified as a protein overexpressed in renal cancer cells (13), but is now known to be also overexpressed in some other cancers, including breast cancer (17, 18). While both CA12 and the closely related tumor-associated carbonic anhydrase IX (CA9) are thought to be regulated by hypoxia, only CA12, and not CA9, exhibits a strong positive correlation

with ER expression in breast tumors (4, 19) suggesting that CA12 might be under ER regulation. CA12 expression in breast tumors is associated with positive ERα status, lower grade disease, lower relapse rates, and better overall patient survival (20-22).

To understand the mechanistic basis underlying this strong association between ER positivity and CA12 expression, we have in the work reported here, explored the regulation of CA12 by the ER. Our results document that the CA12 gene is under primary transcriptional up-regulation by the estrogen-occupied ER and that this regulation in breast cancer cells is mediated by ER action through a distal enhancer that we newly characterize. Upon estrogen stimulation, this enhancer binds ERα through an imperfect estrogen response element and recruits p160 coactivators. Furthermore, by chromosomal looping this ER-dependent enhancer communicates with the promoter of the *CA12* gene, markedly enhancing transcription of the *CA12* gene. Our findings define a mechanistic basis for the robust coexpression of CA12 and ER in breast cancer.

Materials and Methods

Cell Culture and Experimental Treatments

MCF-7 cells were maintained in Minimal Essential Medium (MEM) (Sigma Chemical Co., St Louis, MO) supplemented with 5% calf serum (HyClone, Logan, UT), 100 μg/ml penicillin/streptomycin (Invitrogen, Carlsbad, CA), and 25 μg/ml gentamicin (Invitrogen). T47D cells were routinely maintained in MEM and antibiotics supplemented with 5% fetal bovine serum (Atlanta Biologicals, Atlanta, GA) and bovine insulin (6 ng/ml; Sigma, St. Louis, MO). All cells were grown in phenol red-free MEM

supplemented with 5% charcoal-dextran-treated serum for at least five days prior to use in experiments.

Animal Care and Treatments

Eight week old ovariectomized C57BL/6 mice were obtained from Harlan Co. (Indianapolis, IN) and housed under controlled conditions of light and temperature with free access to standard chow and water. All experiments were conducted in accordance with the principles and procedures of the NIH Guide for the Care and Use of Laboratory Animals and were approved by the University of Illinois Institutional Animal Care and Use Committee. At 16 days after ovariectomy, mice were injected sc with E2 (0.5 µg/animal) dissolved in DMSO then diluted 1:10 in corn oil or with control vehicle DMSO:corn oil alone. At 4 or 24 h after hormone or vehicle injection, uteri were removed, weighed after removal of associated fat, and snap-frozen in liquid nitrogen for RNA isolation.

RNA Isolation, Reverse Transcription, and Real-Time PCR

Cell and whole uterine total RNA was prepared using TRIzol reagent (Invitrogen) according to the manufacturer's recommendations. RNA samples were reverse transcribed in a total volume of 20 µl using 200 units of reverse transcriptase, 50 pmol random hexamer, and 1 mM deoxynucleotide triphosphates (New England Biolabs, Beverly, MA). The resulting cDNA was then diluted to a volume of 500 µl nuclease-free water. Real-time PCR was performed on an ABI Prism 7900HT instrument using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) according to the manufacturer's recommendations. Briefly, each PCR contained: 1x master mix, 4 µl of the diluted cDNA reaction, and 50 nM forward and reverse primers designed to yield 80-

to 125-bp amplicons. PCR was carried out through 40 cycles (95°C for 15 sec, 60°C for 1 min) following an initial 10 min incubation at 95°C. Relative expression levels were calculated as described previously, using acidic ribosomal protein 36B4 mRNA as an internal control (9). Real-time PCR of ChIP samples was performed in a similar manner, with appropriate primers.

Small Interfering RNA Studies

siRNA duplexes targeting ERα (forward, UCAUCGCAUUCCUUGCAAAdTdT; reverse, UUUGCAAGGAAUGCGAUGAdTdT, and control (GL3 luciferase, #D-001400-01) were obtained from Dharmacon (Lafayette, CO) and transfected into cells at a final concentration of 20 nM using DharmaFECT transfection reagent as per the manufacturer's recommendations at 72 h prior to ligand treatment.

Immunoblotting

Whole cell lysates of MCF-7 cells were prepared using 1x Cell Lysis Buffer (Cell Signaling Technology, Beverly, MA) in the presence of Complete Mini protease inhibitor cocktail (Roche Applied Science, Indianapolis, IN). Protein concentration of whole cell lysates was determined by BCA Protein Assay (Pierce, Rockford, IL). Proteins (20 µg) were boiled in 2x Laemlli buffer and separated by electrophoresis using 10% polyacrylamide gels containing sodium dodecyl sulfate (SDS-PAGE) at 150 volts for 50 min and were then transferred to a nitrocellulose membrane (Pall Corp., Pensacola, FL), using the wet transfer method, at 100 volts for 90 min. Membranes were blocked with 5% milk in TBS. Rabbit-anti-human CA12 primary antibody (13) was incubated with blocked membrane overnight at 4°C. The blot was then washed with TBS containing 0.1% Tween-20 prior to incubation with HRP-conjugated secondary antibody (Zymed

Antibodies, South San Francisco, CA). The blot was incubated with Super Signal West Femto ECL reagents (Pierce, Rockford, IL) and exposed to film in order to observe protein bands.

Genomic Cloning, Mutagenesis, and Luciferase Reporter Assays

The indicated genomic DNA associated with estrogen receptor binding and intervening regions was amplified by PCR from human genomic DNA (Roche Molecular Biochemicals, Indianapolis, IN) using specific primers and cloned into either pGL3-Promoter or pGL3-Basic luciferase vectors (Promega, Madison, WI) using the MluI and BglII sites. Site-directed mutagenesis was performed using QuikChange II kit (Stratagene, LaJolla, CA) as per manufacturer's directions. All constructs were sequenced to verify their correctness. Briefly, 1000 ng pGL3 reporter vector and 25 ng pRL-SV40 were co-transfected into MCF-7 cells in 24-well plates using Lipofectamine 2000 in OptiMEM as per manufacturer's instructions (Invitrogen, Carlsbad, CA). Cells were transfected for 6 h, washed, and treated with indicated ligands for 16 h prior to cell lysis in 1x passive lysis buffer (Promega, Madison, WI) and measurement of luciferase activity in MLX Microtiter Plate Luminometer (Dynex Technologies, Chantilly, VA).

Chromatin Immunoprecipitation (ChIP) Assays

Whole-genome ERα binding sites were mapped in MCF-7 cells treated with 10 nM E2 for 45 min using a chromatin immunopreciptation-Paired End diTag (ChIP-PET) cloning and sequencing strategy described previously (23), from which data was obtained on ERα binding sites near the *CA12* gene. Standard ChIP assays were performed essentially as previously described (24, 25) with a few noted modifications. Following the addition of ethanol vehicle or ligands for indicated times, MCF-7 cells were

crosslinked using 1% formaldehyde at 37°C for 10 min, washed twice with PBS, and

harvested in ice-cold PBS plus 1x protease inhibitor cocktail (Roche) and 10 mM DTT.

Cell pellets were first resuspended in nuclei isolation buffer (50 mM Tris-HCl, pH 8.0. 60

mM KCl, 0.5% NP40, protease inhibitor and 10 mM DTT), centrifuged at 1000 g for 3

min, and resuspended in lysis buffer (0.5% SDS, 10 mM EDTA, 0.5 mM EGTA, 50 mM

Tris-HCl, pH 8.0, protease inhibitor and 10 mM DTT). Nuclei were sonicated (Fisher

Scientific, Sonic Dismembrator Model 100) at 80% maximum power three times for 10

seconds and the sonicate was centrifuged at 14,000 g. The supernatant was 1:4 diluted by

dilution buffer (1% Triton X-100, 2mM EDTA, 150mM NaCl, 20 mM Tris-HCl, pH 8,

protease inhibitor and 10 mM DTT) and precleared with 15 µl preimmune IgG (Santa

Cruz Inc.), 2 µg salmon sperm DNA, 50 µl 25% protein A-agarose slurry (Santa Cruz

Inc.). Complexes were incubated at 4°C overnight with 2-5 µg antibody, then pulled

down at 4°C for 1 h with 60 µl of 25% protein A-agarose slurry and 2 µg salmon sperm

DNA. Antibodies used were: for ERα (HC-20, Santa Cruz Biotech), RNA Polymerase

II (N-20, Santa Cruz Biotech), SRC-2 (M-343, Santa Cruz Biotech), SRC-3 (H-270,

Santa Cruz Biotech) and acetylated histone H4 (07-329, Upstate Biotech). Precipitates

were sequentially washed with 1 ml washing buffer (0.1% SDS, 1% Triton X-100, 2 mM

EDTA, 20 mM Tris-HCl (pH 8.0), 150 mM NaCl), 1 ml washing buffer II (0.1% SDS,

1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl (pH 8.0), 500 mM NaCl), 1 ml

washing buffer III (0.25 mM LiCl, 1% NP40, 1% sodium deoxycholate, 1 mM EDTA, 10

mM Tris-HCl (pH 8.0)) and twice with 1 ml TE (1 mM EDTA, 10 mM Tris-HCl [pH

8.0]). Chromatin complexes were incubated at room temperature for 20 min with 100-300

µl elution buffer (1% SDS, 0.1 M NaHCO3). The crosslinking was reversed by

incubating at 65°C overnight with 200 mM NaCl and 200 mg/ml proteinase K (Invitrogen Corp.). DNA was purified with QIAquick columns (Qiagen) and amplified by real-time PCR.

Chromosome Conformation Capture (3C) Assays

MCF-7 cells were grown as per protocol for ChIP assay above and treated with indicated ligands for 45 min prior to fixation in 2% formaldehyde at 37°C for 10 min. The formaldehyde was quenched with addition of 0.125 M glycine and cells were lysed in lysis buffer (10 mM Tris-HCl pH 8.0, 10 mM NaCl, 0.2% NP-40, 1X Complete Protease Inhibitors (Roche) at 4°C for 90 min. Nuclei were resuspended in 1X New England Biolabs Buffer 2, 0.3 % SDS and incubated at 37°C for 60 min while rotating. Triton-X was added to a final concentration of 1.8 % to sequester the SDS and incubated at 37°C for 60 min while rotating. The chromatin was then digested overnight using MseI (New England Biolabs) or BtgI (New England Biolabs) at 37°C while rotating. SDS was added to a final volume of 1.6% and the samples were heated at 65°C for 20 min. Two µg aliquots of the chromatin samples were diluted in ligation buffer containing 1% Triton-X and incubated at 37°C for 1 h. The temperature was lowered to 16°C and T4 Ligase (New England Biolabs) was added and samples were incubated overnight. The ligated DNA was purified using phenol/chloroform extraction and analyzed using PCR amplification. Resulting PCR products were sequenced and mapped back to the USCS Genome Browser for verification.

Results

CA12 Regulation by Estrogen is a Primary Transcriptional Response Mediated by the Estrogen Receptor

In our prior transcriptional profiling microarray analyses of gene expression stimulation by E2 in ER-positive breast cancer and osteosarcoma cells (8, 9, 26), we observed a very marked up-regulation of *CA12* gene expression by E2. To investigate *CA12* regulation by estrogen in breast cancer in greater detail, and to elucidate the mechanism underlying this regulation, we first examined the time course of CA12 mRNA and protein increases in response to E2 and SERMs in ERα-positive MCF-7 and T47D breast cancer cells. *CA12* RNA was significantly stimulated after 2 h of E2 exposure and continued to rise to maximal stimulation levels by 4 and 8 h in MCF-7 and T47D cells, respectively, and remained greatly elevated throughout the 48 h of treatment in both cell lines (Fig. 3.1A). Increases in CA12 protein levels were detected as early as 2-4 h, and continued to rise throughout the time course of treatment (Fig. 3.1B), consistent with the early and sustained stimulation of *CA12* RNA by E2. The SERMs, trans-hydroxy-tamoxifen (Tam) and raloxifene (Ral), induced *CA12* RNA 3-4 fold, approximately 40% that obtained with E2 (Fig. 3.1C), and in like manner, Tam and Ral stimulated CA12 protein to approximately one-third that of E2 (Fig. 3.1D). The ER full antagonist and Selective Estrogen Receptor Down-regulator (SERD), fulvestrant (ICI 182,780), had no stimulatory effect on *CA12* RNA, and it was able to inhibit the E2-, Tam-, and Ral-mediated stimulation of *CA12* (Fig. 3.1C). Of the other steroid receptor ligands examined, only dihydrotestosterone was able to mildly stimulate *CA12*, possibly

through androgen receptor or because of its low affinity for ER (27), whereas the glucocorticoid receptor and progesterone receptor agonists, hydrocortisone and medroxyprogesterone acetate, respectively, did not regulate *CA12* expression (Fig. 3.1C).

We next examined the requirement of the estrogen receptor itself for E2-mediated stimulation of *CA12* mRNA. MCF-7 cells were pre-treated for 60 min with vehicle or a two log molar excess of fulvestrant prior to treatment with E2, and *CA12* mRNA, and protein levels were assessed after 2 or 8 h, respectively (Fig. 3.2A and 3.2B). Fulvestrant did not increase *CA12* mRNA or protein, but it was able to fully inhibit the E2-mediated up-regulation. We also examined the requirement of ER in *CA12* mRNA stimulation by siRNA-mediated depletion of ERα from MCF-7 cells. MCF-7 cells were transfected with siRNA demonstrated to deplete cells of >95% of ERα (data not shown). Loss of ER at 72 h post-transfection reduced the basal *CA12* mRNA level, likely due to possible ligand-independent ER transactivation activity, and abolished the E2- and SERM-mediated stimulation of *CA12* (Fig. 3.2C). These results indicate that ER is required for and SERM regulation of the *CA12* gene.

In addition to the ER itself, we also determined the requirement of on-going transcription and translation for E2 stimulation of *CA12*. MCF-7 cells were pre-treated for 60 min with the RNA polymerase inhibitor actinomycin D or the translational inhibitor cycloheximide for 4 h prior to treatment with E2 (Fig. 3.2A). The E2-mediated stimulation of *CA12* mRNA (or Tam- or Ral-mediated increase in *CA12* mRNA, not shown) was inhibited by actinomycin D, but not cycloheximide, suggesting that on-going transcription, but not synthesis of new protein factors, is necessary for *CA12* mRNA

stimulation by the ER. Taken together, these results suggest that *CA12* regulation by estrogen is a primary transcriptional response mediated by the ER.

Because of our observations of the requirement for ER for *CA12* regulation, we examined *CA12* expression in primary ER-positive breast tumors by analysis of several gene expression data sets from ER-positive breast tumors (Fig. 3.2D). These analyses reveal a very positive correlation of *CA12* expression with ERα expression in primary breast tumors, shown in the scatter plots in Fig. 3.2D. Our findings highlighting the crucial role of ER in *CA12* up-regulation may account for the robust coexpression of *CA12* and ERα observed in human breast cancers.

E2-Bound ER is Recruited to a Distal Region Upstream of the CA12 Transcription Start Site In Vivo

The ER primarily functions as a signal-activated transcriptional transactivator through direct binding to DNA response elements or other protein transcription factors (24, 28). To examine the role of ER in regulating *CA12* mRNA, we utilized a series of chromatin immunoprecipitation (ChIP) experiments to interrogate the recruitment and binding of ER to chromatin. Genome-wide chromatin immunoprecipitation paired-end ditag (ChIP-PET) experiments using an antibody against ERα to capture DNA loci bound by ER after 45 min of E2 exposure in MCF-7 cells showed a cluster of ER binding DNA fragments approximately 6 kilobases upstream from the transcriptional start site of the *CA12* gene (Fig. 3.3A and 3.3B).   Further examination of this ChIP-PET cluster of bound DNA fragments at ~ 6 kb revealed a cluster of five overlapping fragments, and two single upstream ChIP-PET DNA fragments considered to be experimental noise (Fig. 3.3B; (23)).

To validate and further examine the extent of ER binding within the *CA12* genomic region, we performed ChIP scanning for ERα in MCF-7 cells treated for 45 min with vehicle or 10 nM E2 and amplified recovered ChIP DNA fragments using 100 bp primer sets tiled approximately every 500 bp from -6.5 kb to the transcriptional start site of the *CA12* gene (Fig. 3.3C). Of note, within this region there are four predicted imperfect EREs and multiple response elements for factors to which ERα is known to tether (e.g. AP-1), suggesting multiple putative ER binding sites. ChIP scanning for ERα binding revealed robust E2-induced binding at the ~-6 kb binding region previously shown to bind ERα through the genome-wide ChIP-PET experiments, and low-level binding of ERα at the proximal promoter region, but near-background level binding at intervening positions (Fig. 3.3C). These ChIP assays further define the one robust ERα binding region at a distal region approximately 6 kb upstream from *CA12*.

E2- and SERM-Induced Transcription Factor Recruitment and Chromatin Modifications to CA12 Genomic Regions In Vivo

To better understand the regulation of the *CA12* gene, we further examined the recruitment of ER, coactivators, RNAPII, and permissive histone modifications at the enhancer, proximal promoter, and additional loci in MCF-7 cells treated with vehicle, 10 nM E2, or 100 nM trans-hydroxy-tamoxifen. ChIP experiments coupled with qRT-PCR showed specific and robust recruitment of both E2- and Tam-bound ERα at the enhancer region after 45 min, with minimal binding to a region upstream of the enhancer or to an intermediate position (denoted *Middle*) at approximately -4 kb (Fig. 3.4). The binding of ER to the enhancer region in E2- or Tam-treated cells remained elevated over vehicle at 4 and 24 h of exposure. At the proximal promoter (TSS), the largest subunit of RNA

polymerase II was bound to a certain degree in the absence of hormone, but increased seven-fold after 45 min of E2, consistent with a well-established role of E2-mediated formation of productive transcriptional complexes. Consistent with the lower potency of Tam in stimulation of *CA12* gene expression, Tam-treated cells showed less RNAPII recruitment at the TSS. RNAPII was not appreciably recruited to areas upstream of the TSS with either E2 or Tam treatments.

We also examined the recruitment of both of the coregulators SRC-2 and SRC-3 to the enhancer region in the presence of E2 (Fig. 3.4), and found a large change in recruitment of both at 45 min, and also at the 4 h and 24 h times monitored. In the presence of Tam, coactivator recruitment at the enhancer was comparable with that of vehicle, suggesting that the Tam-bound ER does not efficiently recruit p160 coregulators, as others have observed (29). To determine the degree of chromatin modifications consistent with gene activation, we also examined the level of histone H4 tail acetylation (AcH4), considered to be a general marker of acetylated, "relaxed" histones permissive of transcriptional regulation. We observed a marked increase of AcH4 at the enhancer region in both E2 and Tam-treated cells (E2 > Tam), which was greatest at 45 min and decreased at later times. This E2- and SERM-mediated increase in AcH4 was noted at the TSS and also, interestingly, at the middle and particularly at the upstream regions shown not to bind ER. These are supportive of findings by others showing a high level of histone lysine acetylation, consistent with chromatin remodeling, at the active enhancer and promoter of stimulated genes (30), as well as more broadly in nearby regions.

Estrogen Regulation of the Cloned CA12 Enhancer Is Mediated By An Imperfect Estrogen-Response Element

The *in vivo* recruitment of agonist-bound ERα and coactivator proteins, and histone modifications associated with transactivation, suggest that the identified region ~6 kb upstream of the TSS is an enhancer for the *CA12* gene. Sequence analysis of putative transcription factor binding sites revealed one imperfect ERE with a 1 bp mismatch at approximately -6047 (relative to TSS; Fig. 3.5A). To understand the cis elements involved in recruiting ERα and facilitating transactivation of *CA12*, we cloned the approximately 6.9 kb fragment of genomic DNA containing the putative upstream enhancer spanning a region approximating the captured DNA fragments binding ERα (ChIP-PET experiment, Fig. 3.3B) to just upstream of the *CA12* TSS (-6832 – +46, Chr15:61461083-61467960). In addition, we also cloned a truncated 1.8 kb fragment approximating the overlapping ChIP-PET cluster of ERα binding fragments (-6832 – -4999), and both full-length (-6832 – +46) and truncated (-6832 – -4999) genomic fragments were then subcloned into luciferase reporter vectors to assay putative estrogen responsiveness (Fig. 3.5A). After transfection into MCF-7 cells and exposure to 10 nM E2 for 16 h, the full-length reporter was stimulated approximately 8x over vehicle-treated transfectants or empty vector (Fig. 3.5B). In addition, the truncated reporter approximating the greatest overlap of ChIP-PET fragments (-6832 – -4999) was able to stimulate reporter activity upon treatment with E2 comparable to that of the full-length reporter (-6832 – +46; Fig. 3.5B), implying that the E2-responsive region is located in the far-upstream genomic region.

As noted above, the ER-binding region at ~ -6 kb has one imperfect ERE (Fig. 3.5A), which seemed a likely putative cis-regulatory element to direct ERα-dependent transactivation. To examine this, we mutated both the single consensus half-ERE (Mut. 1.0), or both the consensus half-ERE and imperfect arm of the ERE (Mut. 1.1). As seen in Fig. 3.5A and 3.5B, mutation of either one or both arms of the ERE abolished the ability of E2 to stimulate the -6832 – -4999 reporter, strongly suggesting that ER regulation of the cloned *CA12* enhancer is dependent on an imperfect (1 mismatch) ERE at -6047 relative to the TSS.

The ER-binding Distal Enhancer Communicates With the Transcriptional Start Site of the CA12 Gene via Intra-Chromosomal Looping Upon Estrogen Treatment In Vivo

The observed *in vivo* recruitment of E2-bound ER to a putative distal enhancer region, and the E2- and ERE-dependent activation of the cloned ER-binding enhancer in transiently-transfected MCF-7 cells together suggest that the E2-mediated stimulation of CA12 is via an ER-dependent upstream enhancer approximately 6 kb upstream from the *CA12* gene. To test the *in vivo* utilization of this enhancer, we employed chromosome conformation capture (3C) assays to examine the putative communication of the -6 kb enhancer and the *CA12* proximal promoter. Briefly, after 45 min of 10 nM E2 treatment, MCF-7 cells were fixed with formaldehyde and chromatin was isolated, digested with *Mse I*, and subjected to dilute intramolecular ligation before de-crosslinking, DNA isolation, and PCR amplification of DNA fragments of interest. Chromatin regions which are in close proximity with each other at the time of fixation were interrogated using PCR primers complementary to genomic DNA from the enhancer or proximal promoter regions. Specifically, primers were used in variable combinations to examine

the presence of enhancer DNA only, proximal promoter DNA only, or all possible DNA species only produced by intramolecular ligation of DNA derived from the respective enhancer and promoter regions after fixation and digestion (Fig. 3.6, top).

As shown in Figure 3.6A (middle), we observed a ligase- and E2-dependent 780 PCR product amplified using the "A" forward primer from the enhancer region and "F" reverse primer from the proximal promoter region of CA12 (Fig. 3.6A and 3.6B). Normally separated by approximately 6 kb, the DNA amplified in the resulting PCR fragment was purified, sequenced, and mapped via BLAT to the UCSC human genome browser, and resulting sequence analysis indicated the E2- and ligase-dependent product was derived from both *CA12* enhancer and promoter regions (Fig. 3.6A, bottom). The estrogen-enhanced communication of the distal enhancer and proximal promoter in the 3C assay strongly suggests that this ER-binding enhancer is functionally active *in vivo*. Taken together with the ChIP assays in which we did not observe appreciable *in vivo* recruitment of ERα or RNAPII in the intervening regions between the ER-binding enhancer and RNAPII-binding promoter, these 3C data suggest that the ER-binding enhancer transactivates the *CA12* gene via intrachromosomal looping.

Conservation of the CA12 Enhancer in Mammalian Genomes and of CA12 Regulation by Estrogen Receptor

Analysis of the upstream region of *CA12* by Vertebrate Multiz Alignment reveals a high degree of multi-species conservation within the newly-described, ERE-containing enhancer region (Fig. 3.6B, left). Within the enhancer region (Fig. 3.6B), there is only one bp mismatch between the human and mouse 15-mer ERE. To determine whether there is estrogen regulation of *Car12*, the mouse ortholog of the CA12 gene,

ovariectomized female mice were treated with E2 or vehicle, and uteri were collected at 4 or 24 h post-injection (Fig. 3.6B, right). At both time points examined, *Car12* gene expression was robustly stimulated by E2. These results provide strong evidence that the ER regulation of *CA12* is both mechanistically and evolutionarily conserved.

Discussion

Our studies reveal that the ER robustly up-regulates *CA12* gene expression in breast cancer cells and that this transcriptional regulation is mediated by a hormone-responsive enhancer located approximately 6.5 kb upstream of the start site of transcription of the *CA12* gene. This marked regulation of *CA12* by the ER may account for the strong coexpression of ER and *CA12* that is observed in breast tumors.

Dynamic signal-specific assembly of transcription factors at enhancers is an increasingly recognized aspect of biological control of genes essential for developmental and hormonal response programs. Recent studies have suggested that the majority of estrogen-responsive genes may be under the control of ER-binding sites at a considerable distance (>5 kb) from the target RNA-coding loci (23, 31, 32), but there has been only limited evidence that they function as genomic regulatory elements for these relatively distant hormone-regulated genes. Here we describe regulation of the *Carbonic Anhydrase XII* (*CA12*) gene by agonist bound ER through a long-range distal enhancer that we have characterized through in vivo ChIP-scanning across the *CA12* genomic region and ChIP-Paired End Ditag analysis, and by the ability of this element to strongly activate hormone dependent expression of a reporter. This enhancer contains an imperfect estrogen response element which we show to be essential for its ER regulation through

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mutagenesis and transfection studies. Chromosome conformation capture (3C) and ChIP assays demonstrate a physical interaction between this distal enhancer and the *CA12* promoter in breast cancer cells upon E2 treatment, indicating a direct role for the enhancer in *CA12* expression.

Associated with the recruitment of ligand-occupied ER to hormone-dependent enhancers is the recruitment of coregulators (2, 33, 34), some with histone acetyl transferase activity, resulting in distinct changes in histone acetylation status and chromatin conformational changes. In the case of CA12 gene regulation, we observed markedly increased recruitment to the enhancer of the p160 coregulators, SRC2 and SRC3 that have HAT activity. Of note, increased histone H4 acetylation status was observed not only at the enhancer and promoter regions, but also broadly throughout the upstream 5'-flanking region from the promoter to the enhancer and even at a more upstream region, suggesting that chromatin changes are effected over a broad region after receptor occupancy by ligand. As expected, RNA polymerase recruitment was only observed at the promoter.

Selective estrogen receptor modulators (SERMs) such as tamoxifen and raloxifene, shown to be effective in both the prevention and the treatment of breast cancer (35, 36) often have mixed agonist-antagonist activities on estrogen-regulated genes in breast cancer (26, 37, 38). Tamoxifen was a weak stimulator of CA12 RNA and protein expression compared to E2. In keeping with this, tamoxifen was less effective in increasing recruitment of ER to the enhancer, in recruiting RNA polymerase II to the CA12 promoter, and in augmenting the acetylation of histone H4. As observed previously for estrogen-regulated gene expression by SERMs (29), tamoxifen did not

recruit the SRC coregulators, suggesting that other coregulators are likely involved in eliciting the weak agonistic activity of tamoxifen on this gene.

Interestingly, the distal enhancer element displays synteny with the homologous mouse genomic sequence, and its robust stimulation by E2 in the mouse uterus highlights that ER regulation of *CA12* is mechanistically and evolutionarily conserved. Other approaches, including bioinformatic coupled with genome-wide nuclear receptor binding site analyses, have suggested the likely conservation of gene regulatory mechanisms at other estrogen responsive genes across mammalian species (23, 32).

Gene regulation by long distance enhancers has been observed recently for other nuclear receptors, such as the androgen receptor in its control of the prostate-specific antigen (PSA) gene (39). Also, recent reports have documented long distance enhancer regulation of GREB1 (gene regulated in breast cancer- 1), encoding a protein with an unknown function but suggested to contribute to the enhancement of proliferation of MCF-7 cells by E2 (40, 41). In the case of GREB1, its stimulation by E2 is mediated by the binding of ER to three consensus EREs spread over approximately 20 kb of upstream flanking sequences (41, 42).

CA12 is a membrane zinc metalloenzyme that is present in a variety of normal tissues but is overexpressed in some cancers (13, 20, 21, 43-47). In MCF-7 cells, we find that CA12 and CA9 are the only carbonic anhydrases that are expressed (DH Barnett and BS Katzenellenbogen, data not shown). CA12 and CA9 mRNA and protein levels are stimulated by hypoxia in a variety of cancer cell lines, and their expression is down-regulated by return to normoxia (13, 17, 47, 48). Of note, however, we find that only CA12, and not CA9, is regulated by estrogen, and likewise, only CA12, and not CA9,

exhibits a strong positive correlation with ER expression in breast tumors (4, 19). The activity of CA12 as a metalloenzyme, catalyzing the reversible hydration of carbon dioxide to form bicarbonate, is likely involved in modulating a variety of physiological processes including transport of carbon dioxide and other solutes, as well as acidification of microenvironments that can modulate the tumor malignant phenotype (16, 49, 50). That CA12 expression in breast tumors is associated with lower grade disease, positive ERα status, and lower relapse rates and better overall patient survival (20-22), suggests that the estrogen receptor regulation of CA12 expression may be an important parameter in this more optimal breast tumor phenotype.

Gene expression microarray profiling has documented that ERα is a master transcriptional regulator of the phenotype and behavior of approximately 70 percent of human breast cancers, and that the gene expression signatures in ERα-positive and ERα-negative breast tumors are profoundly different (5-7, 19). CA12 is one of the genes whose expression is most highly correlated with ERα in breast cancer. In fact, for comparison with the correlations shown in Fig. 3.2D for CA12 and ERα, we examined the association of ERα with progesterone receptor, a well-characterized ER target gene and useful clinical marker, in the same three studies of primary breast cancer gene expression. Interestingly, the correlation of progesterone receptor with ER in the studies by van de Vijer (0.296), Miller (0.287), and Wang (0.374) is considerably less than the correlation of CA12 with ER in the same data sets (Fig. 3.2D), highlighting the robust association of CA12 and ER expression. Our findings reveal a transcriptional regulatory mechanism that likely underlies this robust coexpression of CA12 and ERα in human breast cancers. In addition, our findings imply that involvement of long distance

enhancers in regulation of estrogen-responsive genes in breast cancer may be more frequent than previously appreciated.

Abbreviations:

E2, 17β-estradiol ; ERα, estrogen receptor alpha; Ful, ICI182,780, fulvestrant; SERM, selective estrogen receptor modulator; Ral, raloxifene; Tam, *trans*-hydroxytamoxifen.
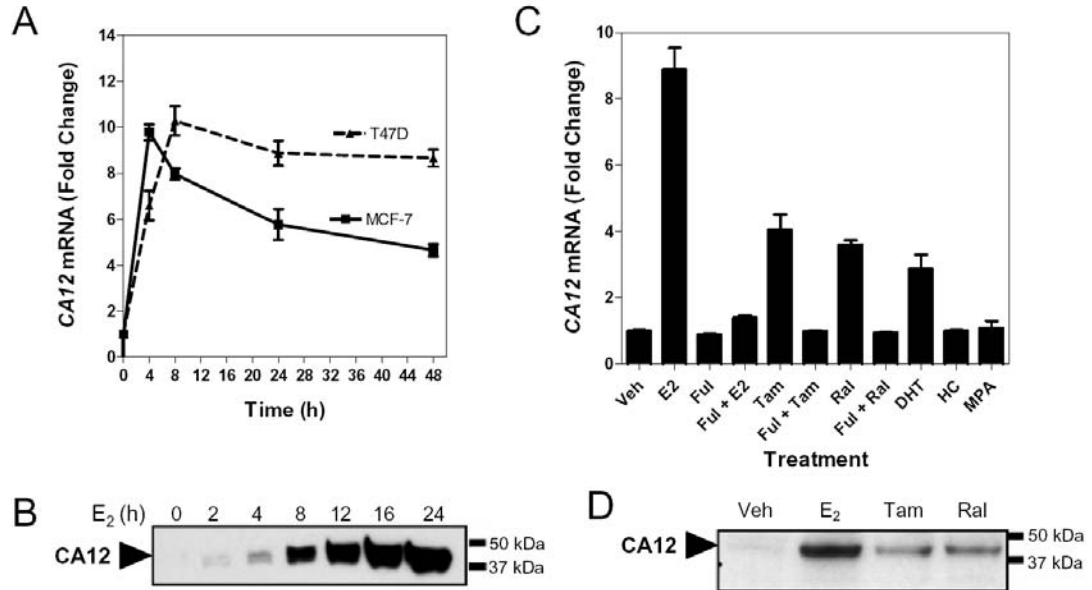
Figure 3.1. Various Estrogen Receptor Ligands Increase CA12 Levels in Breast Cancer Cells

(A) *CA12* mRNA is induced in a time-dependent manner by estradiol (E2) in ERα -expressing MCF-7 and T47D breast cancer cells. Cells were treated with 10nM E2 for 0-48 h. RNA was isolated, reverse transcribed, and cDNA measured by quantitative PCR (qPCR) using primers for *CA12* and internal control *36B4* mRNA. (B) CA12 protein levels are induced by E2 in a time dependent manner. MCF-7 cells were treated with 10 nM E2 for 0-24 h and total cellular lysates were used for CA12 immunoblotting. (C) *CA12* mRNA is induced by ER agonists. MCF-7 cells were treated for 8 h with vehicle (0.1% EtOH) or with intracellular receptor ligands E2 (10 nM), fulvestrant (ICI 182,780, Ful; 1 uM), Ful + E2, trans-hydroxytamoxifen (Tam; 100 nM), Ful + Tam, raloxifene

Figure 3.1. (Cont.)

(Ral; 100 nM), Ful + Ral, dihydrotestosterone (DHT; 10nM), hydrocortisone (HC, 10 nM), or medroxyprogesterone acetate (MPA; 10 nM). Cells were then harvested and qRT-PCR performed as above. **(D)** E2 and the SERMs induce CA12. MCF-7 cells were treated for 8 h with 10nM E2, 100 nM Tam, or 100 nM Ral and CA12 protein levels assessed by immunoblotting as above.
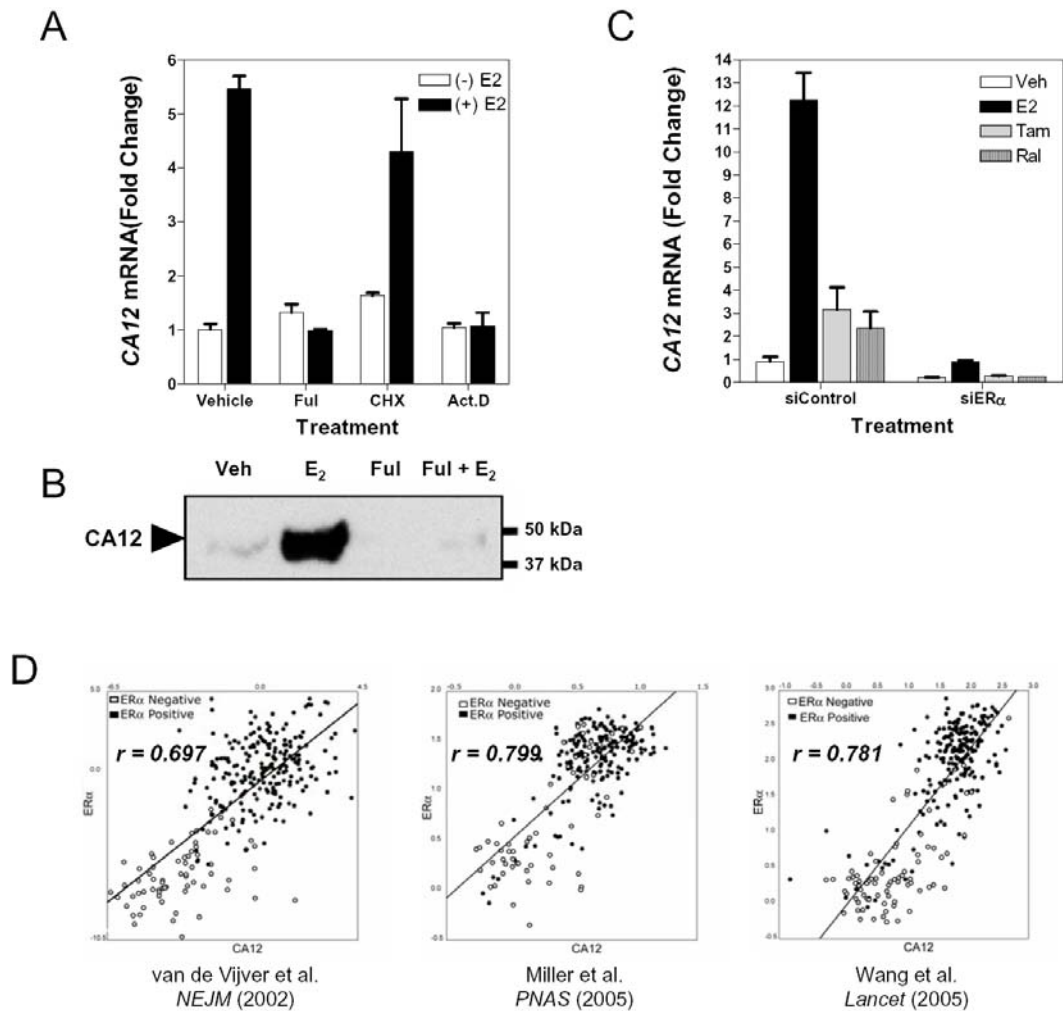
Figure 3.2. E2 Stimulation of *CA12* Gene Expression is Sensitive to Actinomycin D and Fulvestrant (ICI 182,780), but not Cycloheximide, and requires ERα. Strong Association of CA12 and ERα in breast tumor data sets.

(A) *CA12* mRNA induction by E2 is blocked by pre-treatment with the transcriptional inhibitor actinomycin D, or the pure ER antagonist fulvestrant, but not the translational inhibitor cycloheximide. MCF-7 cells were pre-treated for 60 min. with 0.1% DMSO,

Figure 3.2. (Cont.)

1 uM Ful, 10 ug/mL cycloheximide (CHX), or 5 uM Actinomycin D (Act.D) and then

0.1% EtOH or 10 nM E2 was added for 2 h. qRT-PCR for *CA12* mRNA was performed.

(B) ER antagonist Fulvestrant blocks E2 stimulation of CA12. MCF-7 cells were treated

for 8 h with 0.1% EtOH, 10 nM E2, 1 uM Ful, or both E2 and Ful prior to cell lysis and

immunoblotting for CA12. **(C)** *CA12* mRNA induction is ER-dependent. MCF-7 cells

were transfected with 5 nM siControl or 5 nM siRNA against ERα for 72 h. Cells were

then treated for 4 h with 0.1% EtOH, 10 nM E2, 100 nM Tam, or 100 nM Ral prior to

RNA isolation and qRT-PCR analysis. **(D)** Scatter plots and correlation between CA12

and ERα RNA expression in breast tumors from the indicated studies. The plots were

generated from the Oncomine Database. The *x* and *y* axes represent fold change in

expression for CA12 and ERα (ESR1), respectively.
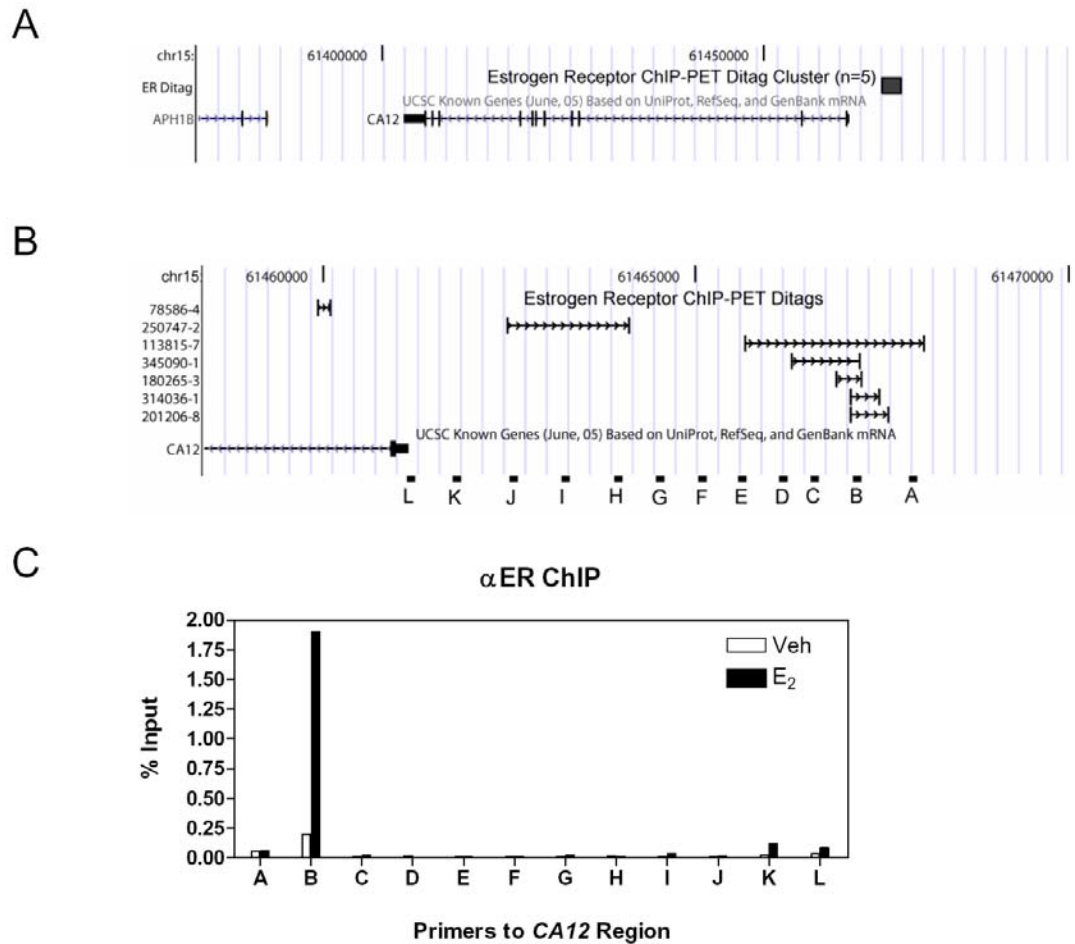
Figure 3.3. E2-Occupied ER is Recruited to a Distal Region 6.5 kb Upstream of the

CA12 **Transcription Start Site** *In Vivo*.

(**A**) Whole-genome ERα binding sites were mapped in MCF-7 cells treated with 10 nM

E2 for 45 min using ChIP-PET strategy (23) and mapped to the CA12 genomic region in

the UCSC Genome Browser (Hg17). A singular ChIP-PET cluster approximately 6 kb 5'

to *CA12* transcriptional start site (top) is shown, with a higher resolution map with

individual fragments indicated (bottom). (**B**) and (**C**) ChIP scanning of the *CA12*

Figure 3.3. (Cont.)

genomic region *in vivo* validates ChIP-PET identification of putative *CA12* enhancer.

(B) A schematic representation of Chromosome 15 and primer set locations (A-L)

immediately 5' to the *CA12* transcriptional start site. **(C)** E2-occupied ERα is recruited

to an upstream region ca. 6 kb 5' to the *CA12* gene. MCF-7 cells were treated for 45 min

with control 0.1% EtOH or 10 nM E2, subjected to ERα ChIP, and immunoprecipitated

DNA amplified using PCR primers as denoted in **(B)** and recovered DNA represented as

percent of input.
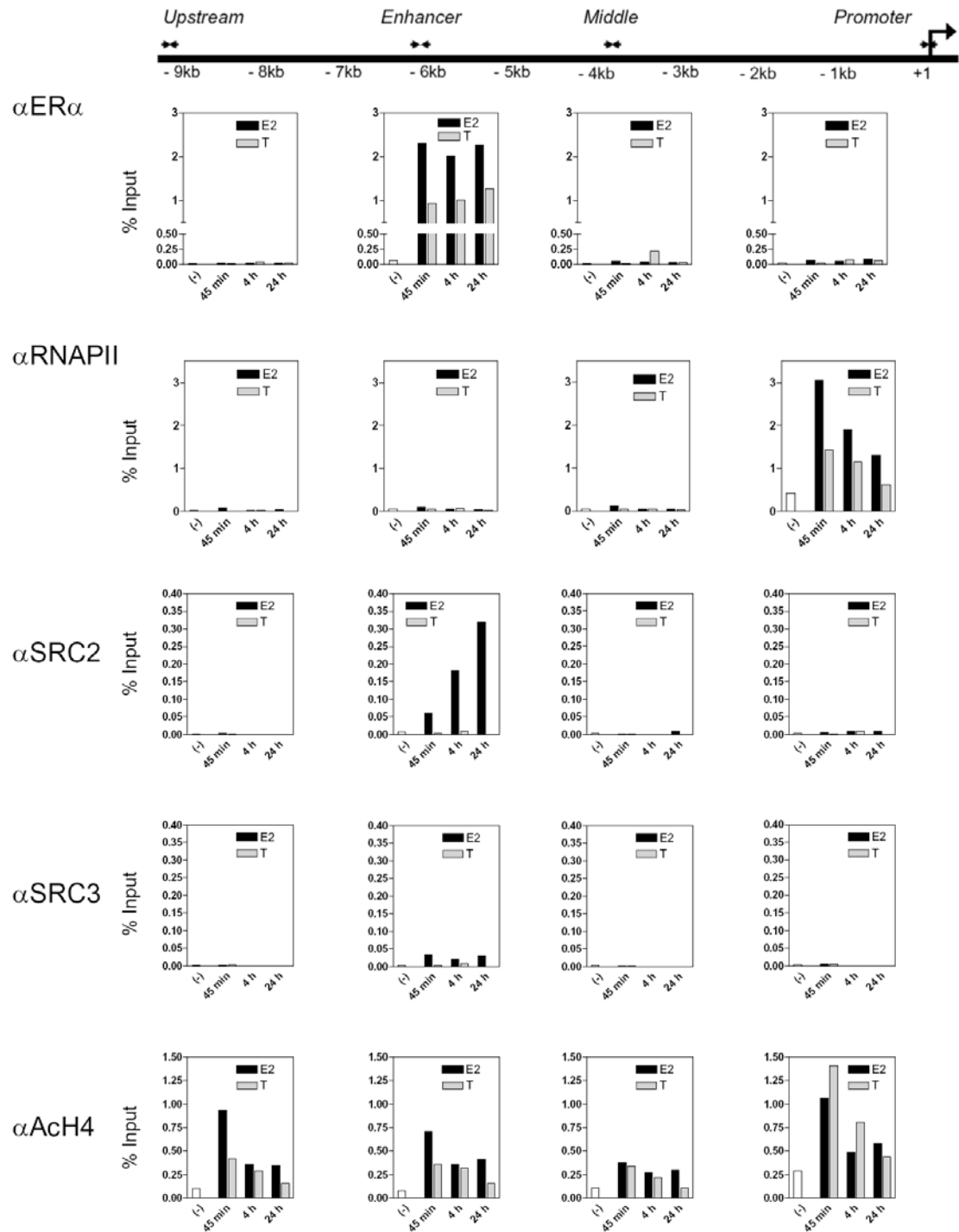
Figure 3.4.

Figure 3.4. (Cont.)

E2- and SERM-Induced Transcription Factor Recruitment and Chromatin

Modifications to the CA12 Genomic Region *In Vivo*

E2- and Tam-treated MCF-7 cells were examined for recruitment of ERα, RNA

Polymerase II, SRC-2, and SRC-3 binding and acetylated H4 modifications within the

CA12 genomic region using various primer sets at denoted positions (Top, schematic).

Immunoprecipitated DNA, expressed as % input, from experiments using specific

indicated antibodies (left) and amplified using primer sets as denoted above.
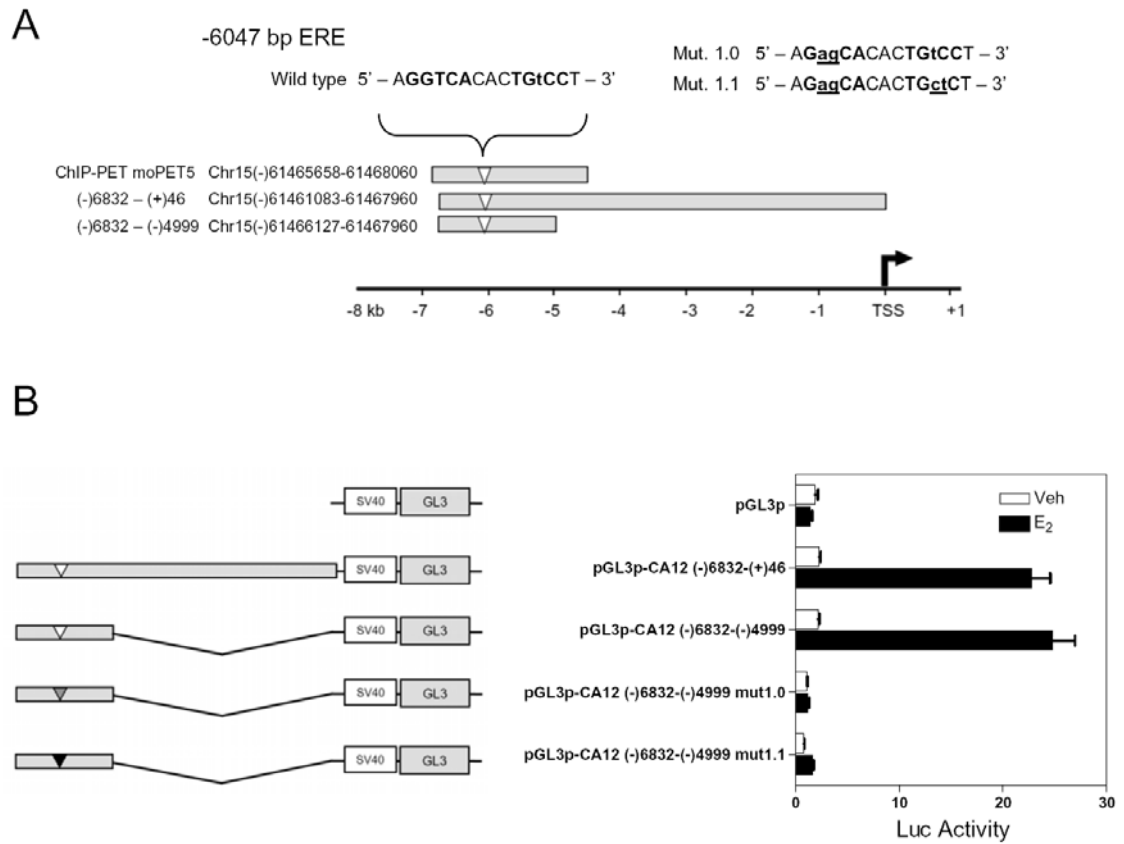
Figure 3.5. Estrogen Regulation of the Cloned *CA12* Enhancer Is Mediated By An Imperfect Estrogen-Response Element

 (A) A schematic representation indicates genomic regions identified as binding ERα by ChIP scanning and ChIP-PET analysis (ChIP-PET moPET5) and cloned genomic regions used for cis-element reporter assays. Indicated at the open triangle position is the wild type imperfect ERE with sequence shown above (above, left), as well as mutated sequences used (above, right; mutated nucleotides underlined and bases differing from wild type in lower case).

Figure 3.5. (Cont.)

(B) Cloned *CA12* enhancer reporter activity is dependent on an imperfect ERE.
Indicated full length, truncated, mutated, and empty reporter plasmids (left, 1 µg ea.)
were cotransfected with pRL-SV40 (25 ng) into MCF-7 cells in 24-well plates. At 24 h
after transfection, cells were treated for 16 h with 0.1% EtOH or 10 nM E2, lysed, and
assayed for Firefly and *Renilla* luciferase activities (right). Open triangle indicates wild
type imperfect ERE ca. 6 kb upstream of CA12 TSS, grey triangle indicates mutant 1.0,
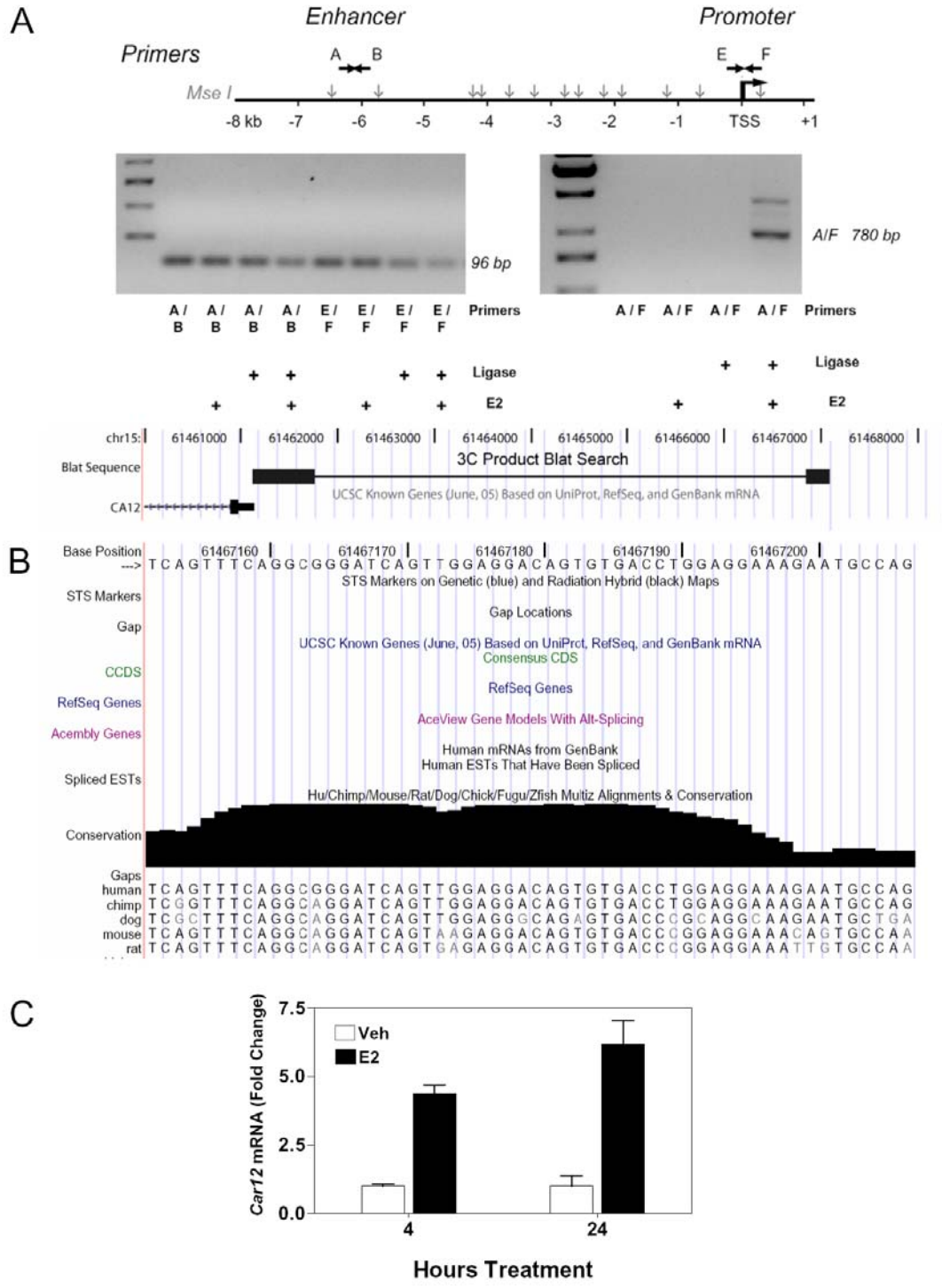and black triangle indicates mutant 1.1.

Figure 3.6.

Figure 3.6. (Cont.)

ER-binding Distal Enhancer Communicates With the Transcriptional Start Site of the *CA12* Gene via Intra-chromosomal Looping Upon Estrogen Treatment *In Vivo*, and Enhancer Shows Conservation Between Humans and Mice. **(A)** *Top*, Schematic of the *CA12* genomic region, *Mse I* restriction enzyme cut sites, and primer positions for chromosome conformation capture (3C). *Middle, CA12* enhancer and promoter show enhanced communication upon E2 treatment in MCF-7 cells *in vivo*. Cells were treated for 45 min with 0.1% EtOH or 10 nM E2, cross-linked, and subjected to 3C analysis using indicated primer pairs. *Bottom,* E2- and ligase-dependent 3C PCR product was purified, sequenced, and mapped to the human genome by UCSC BLAT indicating proximal and distal DNA communication. **(B)** Vertebrate Multiz Alignment shows conservation of the -6 kb CA12 ERE-containing enhancer region. **(C)** *Car12* mRNA is induced by E2 in the mouse uterus. Eight week-old female mice were ovariectomized and at 16 days post-ovariectomy, they were injected with vehicle or E2. Uteri were collected 4 or 24 h later and total RNA was isolated and subjected to qRT-PCR for *Car12* mRNA.

References

1. **Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, Blumberg B, Kastner P, Mark M, Chambon P, Evans RM** 1995 The nuclear receptor superfamily: the second decade. Cell 83:835-9

2. **Rosenfeld MG, Lunyak VV, Glass CK** 2006 Sensors and signals: a coactivator/corepressor/epigenetic code for integrating signal-dependent programs of transcriptional response. Genes Dev 20:1405-28

3. **Stierer M, Rosen H, Weber R, Hanak H, Spona J, Tuchler H** 1993 Immunohistochemical and biochemical measurement of estrogen and progesterone receptors in primary breast cancer. Correlation of histopathology and prognostic factors. Ann Surg 218:13-21

4. **Gruvberger S, Ringner M, Chen Y, Panavally S, Saal LH, Borg A, Ferno M, Peterson C, Meltzer PS** 2001 Estrogen receptor status in breast cancer is associated with remarkably distinct gene expression patterns. Cancer Res 61:5979-84

5. **Sorlie T, Perou CM, Tibshirani R, Aas T, Geisler S, Johnsen H, Hastie T, Eisen MB, van de Rijn M, Jeffrey SS, Thorsen T, Quist H, Matese JC, Brown PO, Botstein D, Eystein Lonning P, Borresen-Dale AL** 2001 Gene expression patterns of breast carcinomas distinguish tumor subclasses with clinical implications. Proc Natl Acad Sci U S A 98:10869-74

6. **Perou CM, Sorlie T, Eisen MB, van de Rijn M, Jeffrey SS, Rees CA, Pollack JR, Ross DT, Johnsen H, Akslen LA, Fluge O, Pergamenschikov A, Williams C, Zhu SX, Lonning PE, Borresen-Dale AL, Brown PO, Botstein D** 2000 Molecular portraits of human breast tumours. Nature 406:747-52

7. **Oh DS, Troester MA, Usary J, Hu Z, He X, Fan C, Wu J, Carey LA, Perou CM** 2006 Estrogen-regulated genes predict survival in hormone receptor-positive breast cancers. J Clin Oncol 24:1656-64

8. **Stossi F, Barnett DH, Frasor J, Komm B, Lyttle CR, Katzenellenbogen BS** 2004 Transcriptional profiling of estrogen-regulated gene expression via estrogen receptor (ER) alpha or ERbeta in human osteosarcoma cells: distinct and common target genes for these receptors. Endocrinology 145:3473-86

9. **Frasor J, Danes JM, Komm B, Chang KC, Lyttle CR, Katzenellenbogen BS** 2003 Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. Endocrinology 144:4562-74

10. **Wang Y, Klijn JG, Zhang Y, Sieuwerts AM, Look MP, Yang F, Talantov D, Timmermans M, Meijer-van Gelder ME, Yu J, Jatkoe T, Berns EM, Atkins D, Foekens JA** 2005 Gene-expression profiles to predict distant metastasis of lymph-node-negative primary breast cancer. Lancet 365:671-9

11. **van de Vijver MJ, He YD, van't Veer LJ, Dai H, Hart AA, Voskuil DW, Schreiber GJ, Peterse JL, Roberts C, Marton MJ, Parrish M, Atsma D, Witteveen A, Glas A, Delahaye L, van der Velde T, Bartelink H, Rodenhuis S, Rutgers ET, Friend SH, Bernards R** 2002 A gene-expression signature as a predictor of survival in breast cancer. N Engl J Med 347:1999-2009

12. **Miller LD, Smeds J, George J, Vega VB, Vergara L, Ploner A, Pawitan Y, Hall P, Klaar S, Liu ET, Bergh J** 2005 An expression signature for p53 status in human breast cancer predicts mutation status, transcriptional effects, and patient survival. Proc Natl Acad Sci U S A 102:13550-5

13. **Tureci O, Sahin U, Vollmar E, Siemer S, Gottert E, Seitz G, Parkkila AK, Shah GN, Grubb JH, Pfreundschuh M, Sly WS** 1998 Human carbonic anhydrase XII: cDNA cloning, expression, and chromosomal localization of a carbonic anhydrase gene that is overexpressed in some renal cell cancers. Proc Natl Acad Sci U S A 95:7608-13

14. **Ulmasov B, Waheed A, Shah GN, Grubb JH, Sly WS, Tu C, Silverman DN** 2000 Purification and kinetic analysis of recombinant CA XII, a membrane carbonic anhydrase overexpressed in certain cancers. Proc Natl Acad Sci U S A 97:14212-7

15. **Parkkila S, Rajaniemi H, Parkkila AK, Kivela J, Waheed A, Pastorekova S, Pastorek J, Sly WS** 2000 Carbonic anhydrase inhibitor suppresses invasion of renal cancer cells in vitro. Proc Natl Acad Sci U S A 97:2220-4

16. **Potter CP, Harris AL** 2003 Diagnostic, prognostic and therapeutic implications of carbonic anhydrases in cancer. Br J Cancer 89:2-7

17. **Ivanov S, Liao SY, Ivanova A, Danilkovitch-Miagkova A, Tarasova N, Weirich G, Merrill MJ, Proescholdt MA, Oldfield EH, Lee J, Zavada J, Waheed A, Sly W, Lerman MI, Stanbridge EJ** 2001 Expression of hypoxia-inducible cell-surface transmembrane carbonic anhydrases in human cancer. Am J Pathol 158:905-19

18. **Goonewardene TI, Sowter HM, Harris AL** 2002 Hypoxia-induced pathways in breast cancer. Microsc Res Tech 59:41-8

19. **van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH** 2002 Gene expression profiling predicts clinical outcome of breast cancer. Nature 415:530-6

20. **Wykoff CC, Beasley N, Watson PH, Campo L, Chia SK, English R, Pastorek J, Sly WS, Ratcliffe P, Harris AL** 2001 Expression of the hypoxia-inducible and tumor-associated carbonic anhydrases in ductal carcinoma in situ of the breast. Am J Pathol 158:1011-9

21. **Watson PH, Chia SK, Wykoff CC, Han C, Leek RD, Sly WS, Gatter KC, Ratcliffe P, Harris AL** 2003 Carbonic anhydrase XII is a marker of good prognosis in invasive breast carcinoma. Br J Cancer 88:1065-70

22. **Creighton CJ, Cordero KE, Larios JM, Miller RS, Johnson MD, Chinnaiyan AM, Lippman ME, Rae JM** 2006 Genes regulated by estrogen in breast tumor cells in vitro are similarly regulated in vivo in tumor xenografts and human breast tumors. Genome Biol 7:R28

23. **Lin CY, Vega VB, Thomsen JS, Zhang T, Kong SL, Xie M, Chiu KP, Lipovich L, Barnett DH, Stossi F, Yeo A, George J, Kuznetsov VA, Lee YK, Charn TH, Palanisamy N, Miller LD, Cheung E, Katzenellenbogen BS, Ruan Y, Bourque G, Wei CL, Liu ET** 2007 Whole-genome cartography of estrogen receptor alpha binding sites. PLoS Genet 3:e87

24. **Shang Y, Hu X, DiRenzo J, Lazar MA, Brown M** 2000 Cofactor dynamics and sufficiency in estrogen receptor-regulated transcription. Cell 103:843-52

25. **Metivier R, Penot G, Hubner MR, Reid G, Brand H, Kos M, Gannon F** 2003 Estrogen receptor-alpha directs ordered, cyclical, and combinatorial recruitment of cofactors on a natural target promoter. Cell 115:751-63

26. **Frasor J, Chang EC, Komm B, Lin CY, Vega VB, Liu ET, Miller LD, Smeds J, Bergh J, Katzenellenbogen BS** 2006 Gene expression preferentially regulated by tamoxifen in breast cancer cells and correlations with clinical outcome. Cancer Res 66:7334-40

27. **Schmidt WN, Katzenellenbogen BS** 1979 Androgen-uterine interactions: an assessment of androgen interaction with the testosterone- and estrogen-receptor systems and stimulation of uterine growth and progesterone-receptor synthesis. Mol Cell Endocrinol 15:91-108

28. **Green KA, Carroll JS** 2007 Oestrogen-receptor-mediated transcription and the influence of co-factors and chromatin state. Nat Rev Cancer 7:713-22

29. **Shang Y, Brown M** 2002 Molecular determinants for the tissue specificity of SERMs. Science 295:2465-8

30. **Palmer MB, Majumder P, Green MR, Wade PA, Boss JM** 2007 A 3' enhancer controls snail expression in melanoma cells. Cancer Res 67:6113-20

31. **Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, Eeckhoute J, Shao W, Hestermann EV, Geistlinger TR, Fox EA, Silver PA, Brown M** 2005 Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. Cell 122:33-43

32. **Carroll JS, Meyer CA, Song J, Li W, Geistlinger TR, Eeckhoute J, Brodsky AS, Keeton EK, Fertuck KC, Hall GF, Wang Q, Bekiranov S, Sementchenko V, Fox EA, Silver PA, Gingeras TR, Liu XS, Brown M** 2006 Genome-wide analysis of estrogen receptor binding sites. Nat Genet 38:1289-97

33. **McKenna NJ, O'Malley BW** 2002 Combinatorial control of gene expression by nuclear receptors and coregulators. Cell 108:465-74

34. **Lonard DM, O'Malley B W** 2007 Nuclear receptor coregulators: judges, juries, and executioners of cellular regulation. Mol Cell 27:691-700

35. **Fisher B, Costantino JP, Wickerham DL, Redmond CK, Kavanah M, Cronin WM, Vogel V, Robidoux A, Dimitrov N, Atkins J, Daly M, Wieand S, Tan-Chiu E, Ford L, Wolmark N** 1998 Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. J Natl Cancer Inst 90:1371-88

36. **Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, Atkins JN, Bevers TB, Fehrenbacher L, Pajon ER, Jr., Wade JL, 3rd, Robidoux A, Margolese RG, James J, Lippman SM, Runowicz CD, Ganz PA, Reis SE, McCaskill-Stevens W, Ford LG, Jordan VC, Wolmark N** 2006 Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. Jama 295:2727-41

37. **Hodges LC, Cook JD, Lobenhofer EK, Li L, Bennett L, Bushel PR, Aldaz CM, Afshari CA, Walker CL** 2003 Tamoxifen functions as a molecular agonist inducing cell cycle-associated genes in breast cancer cells. Mol Cancer Res 1:300-11

38. **Frasor J, Stossi F, Danes JM, Komm B, Lyttle CR, Katzenellenbogen BS** 2004 Selective estrogen receptor modulators: discrimination of agonistic versus antagonistic activities by gene expression profiling in breast cancer cells. Cancer Res 64:1522-33

39. **Wang Q, Carroll JS, Brown M** 2005 Spatial and temporal recruitment of androgen receptor and its coactivators involves chromosomal looping and polymerase tracking. Mol Cell 19:631-42

40. **Rae JM, Johnson MD, Scheys JO, Cordero KE, Larios JM, Lippman ME** 2005 GREB 1 is a critical regulator of hormone dependent breast cancer growth. Breast Cancer Res Treat 92:141-9

41. **Deschenes J, Bourdeau V, White JH, Mader S** 2007 Regulation of GREB1 transcription by estrogen receptor alpha through a multipartite enhancer spread over 20 kb of upstream flanking sequences. J Biol Chem 282:17335-9

42. **Sun J, Nawaz Z, Slingerland JM** 2007 Long-range activation of GREB1 by estrogen receptor via three distal consensus estrogen-responsive elements in breast cancer cells. Mol Endocrinol 21:2651-62

43. **Karhumaa P, Parkkila S, Tureci O, Waheed A, Grubb JH, Shah G, Parkkila A, Kaunisto K, Tapanainen J, Sly WS, Rajaniemi H** 2000 Identification of carbonic anhydrase XII as the membrane isozyme expressed in the normal human endometrial epithelium. Mol Hum Reprod 6:68-74

44. **Kivela A, Parkkila S, Saarnio J, Karttunen TJ, Kivela J, Parkkila AK, Waheed A, Sly WS, Grubb JH, Shah G, Tureci O, Rajaniemi H** 2000 Expression of a novel transmembrane carbonic anhydrase isozyme XII in normal human gut and colorectal tumors. Am J Pathol 156:577-84

45. **Kivela AJ, Parkkila S, Saarnio J, Karttunen TJ, Kivela J, Parkkila AK, Pastorekova S, Pastorek J, Waheed A, Sly WS, Rajaniemi H** 2000 Expression of transmembrane carbonic anhydrase isoenzymes IX and XII in normal human pancreas and pancreatic tumours. Histochem Cell Biol 114:197-204

46. **Parkkila S, Parkkila AK, Saarnio J, Kivela J, Karttunen TJ, Kaunisto K, Waheed A, Sly WS, Tureci O, Virtanen I, Rajaniemi H** 2000 Expression of the membrane-associated carbonic anhydrase isozyme XII in the human kidney and renal tumors. J Histochem Cytochem 48:1601-8

47. **Wykoff CC, Beasley NJ, Watson PH, Turner KJ, Pastorek J, Sibtain A, Wilson GD, Turley H, Talks KL, Maxwell PH, Pugh CW, Ratcliffe PJ, Harris AL** 2000 Hypoxia-inducible expression of tumor-associated carbonic anhydrases. Cancer Res 60:7075-83

48. **Ivanov SV, Kuzmin I, Wei MH, Pack S, Geil L, Johnson BE, Stanbridge EJ, Lerman MI** 1998 Down-regulation of transmembrane carbonic anhydrases in renal cell carcinoma cell lines by wild-type von Hippel-Lindau transgenes. Proc Natl Acad Sci U S A 95:12596-601

49.   **Sly WS, Hu PY** 1995 Human carbonic anhydrases and carbonic anhydrase deficiencies. Annu Rev Biochem 64:375-401
50.   **Breton S** 2001 The cellular physiology of carbonic anhydrases. Jop 2:159-64

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PUBLICATIONS AND PRESENTATIONS

ARTICLES

14.   Funk CC, **Barnett DH**, Bergamaschi A, Katzenellenbogen BS Estrogen Receptor Regulation of LRIG1 Represses ERBB2/ HER-2 Activities in Breast Cancer. <u>In Preparation.</u>

13.   ***Barnett DH**, *Lee C-H, Bergamaschi A, Katzenellenbogen BS Differential Ligand Regulation of Transcriptional Elongation of the *Immediate Early X-1 (IEX-1)* Gene Poised Promoter by the Estrogen Receptor. <u>In Preparation</u>. *** indicates co-authorship and equal contribution**.

12. Sheng S, **Barnett DH**, Katzenellenbogen BS (2008) Differential Estradiol and Selective Estrogen Receptor Modulator (SERM) Regulation of Keratin 13 Gene Expression and its Underlying Mechanism in Breast Cancer Cells. *Molecular and Cellular Endocrinology* 296, 1-9. PubMed PMID: 18951949

11. **Barnett DH**, Sheng S, Charn T-H, Waheed A, Sly WS, Lin C-Y, Liu ET, Katzenellenbogen BS (2008) Estrogen Receptor Regulation of the *Carbonic Anhydrase XII* Gene Through a Distal Enhancer in Breast Cancer. *Cancer Research* 68, 3505-15. PubMed PMID: 18451179.

10. Lin CY, Vega VB, Thomsen JS, Zhang T, Kong SL, Xie M, Chiu KP, Lipovich L, **Barnett DH**, Stossi F, Yeo A, George J, Kuznetsov VA, Lee YK, Charn TH, Palanisamy N, Miller LD, Cheung E, Katzenellenbogen BS, Ruan Y, Bourque G, Wei CL, Liu ET (2007) Whole-genome Cartography of Estrogen Receptor Alpha Binding Sites. *PLoS Genetics* 3(6):e87. PubMed PMID: 17542648

9. Lipinski RJ, Cook CH, **Barnett DH**, Gipp, JJ Peterson RE, Bushman W (2005) Sonic Hedgehog Signaling Regulates the Expression of Insulin-like Growth Factor Binding Protein-6 During Fetal Prostate Development. *Developmental Dynamics*, 233, 829-836. PubMed PMID: 15906375.

8. [*]Stossi F, [*]**Barnett DH**, Frasor J, Komm B, Lyttle R, Katzenellenbogen BS (2004) Transcriptional Profiling of Estrogen-Regulated Gene Expression via Estrogen Receptor (ER) Alpha or ER Beta in Human Osteosarcoma Cells: Distinct and Common Target Genes for These Receptors. *Endocrinology* 145, 3473-3486. PubMed PMID: 15033914.
[*] **indicates co-authorship and equal contribution**.

7. Harrington WR, Sheng S, **Barnett DH**, Petz L, Katzenellenbogen JA, Katzenellenbogen BS (2003) Activities of Estrogen Receptor Alpha- and Beta-Selective Ligands at Diverse Estrogen Responsive Gene Sites Through Which the Receptor Mediates Transactivation or Transrepression. *Molecular and Cellular Endocrinology* 206, 13-22. PubMed PMID: 12946570.

6. Perrin RJ, Payton JE, **Barnett DH**, Wraight CL, Woods WS, Ye L, George JM (2003) Epitope Mapping and Specificity of the Anti-alpha-Synuclein Monoclonal Antibody Syn-1 in Mouse Brain and Cultured Cell Lines. *Neuroscience Letters* 349, 133-135. PubMed PMID: 12943986.

5. Frasor J, **Barnett DH**, Danes JM, Hess R, Parlow AF, Katzenellenbogen BS (2003) Response-Specific and Ligand Dose-Dependent Modulation of Uterine Estrogen Receptor Alpha Activity by Estrogen Receptor Beta in the Uterus. *Endocrinology* 144, 3159-3166. PubMed PMID: 12810572.

4. **Barnett DH**, Huang HY, Wu XR, Laciak R, Shapiro E, Bushman W (2002) The Human Prostate Expresses Sonic Hedgehog During Fetal Development. *Journal of Urology* 168, 2206-2210. PubMed PMID: 12394760.

3. Lamm ML, Catbagan WS, Laciak RJ, **Barnett DH**, Hebner CM, Gaffield W, Walterhouse D, Iannaccone PM, Bushman W (2002) Sonic Hedgehog Activates Mesenchymal *Gli1* Expression During Prostate Ductal Bud Formation. *Developmental Biology* 249, 249-366. PubMed PMID: 12221011.

2. Lamm ML, Podlasek CA, **Barnett DH**, Lee J, Clemens JQ, Hebner CM, Bushman W (2000) The Mesenchymal Factor *Bone Morphogenetic Protein-4* Restricts Ductal Budding and Branching Morphogenesis in the Developing Prostate. *Developmental Biology* 232, 301-314. PubMed PMID: 11401393.

1. Podlasek, CA, **Barnett DH**, Clemens JQ, Bak PM, Bushman W (1999) Expression of *Sonic Hedgehog* in the Urogenital Sinus Epithelium is Required for Prostate Development. *Developmental Biology* 209, 28-39. PubMed PMID: 10208740.

MEETING ABSTRACTS and PRESENTATIONS

29. Stender JD. Funk CC, Charn TH, **Barnett DH**, Stossi F, Katzenellenbogen BS (2008). PITX1, an Estrogen Regulated Transcription Factor, Coordinates Gene-Specific Regulation by the Estrogen Receptor in Breast Cancer Cells. 90th Annual Meeting of The Endocrine Society, San Francisco, CA USA, 2008. Proceedings.

28. Katzenellenbogen BS, **Barnett DH,** Chang EC, Kim K, Madak-Erdogan Z, Park SH, Stender JD, Stossi F. (2008). Integration of Nuclear and Extranuclear Signaling in Global Gene Regulation by Estrogen Receptors. Keystone Symposium on Nuclear Receptors: Steroid Sisters, Whistler, Canada, March 2008.

27. **Barnett DH**, Sheng S, Charn TH, Lin CY, Liu ET, Katzenellenbogen BS (2007) Estrogen Receptor Use of a Distal Enhancer for Gene Regulation: Carbonic Anhydrase XII in Breast Cancer. 89th Annual Meeting of The Endocrine Society, Toronto, Canada, June 2– 5, 2007. Proceedings.

26. Katzenellenbogen BS, **Barnett DH,** Chang EC, Madak-Erdogan Z, Park SH, Stender JD, Stossi F, Katzenellenbogen JA (2007). Integration of nuclear and extranuclear signaling by estrogen receptors, EMBO Conference on Nuclear Receptors in Health and Disease, Italy, May 2007

25. Katzenellenbogen BS, Chang, EC, Stossi F., Park S-E, Mussi P, **Barnett DH**, Madak-Erdogan Z, Morrow C, Hess R, Katzenellenbogen JA (2006).   Novel modulators of estrogen receptor function. Keystone Symposium, Nuclear Receptors: Steroid Sisters, Banff, Alberta, Canada, March 2006.

24. Kim DW, **Barnett DH**, Gunther JR, Katzenellenbogen BS, Katzenellenbogen JA (2005) Inhibitors of Carbonic Anhydrase XII Suitable For F-18 Radiolabeling. 16th International Symposium on Radiopharmaceutical Chemistry, Iowa City, IA, June 24-28, 2005. Proceedings.

23.   **Barnett DH**, Frasor J, Stossi F, Sengupta S, Harrington WR, Katzenellenbogen BS (2004) Carbonic Anhydrase XII: An Estrogen-Regulated Gene in Diverse Targets of Estrogen Action. 86th Annual Meeting of The Endocrine Society, New Orleans, LA, USA, June 16 – 19, 2004. Proceedings.

22.   Frasor J, **Barnett DH**, Danes JM, Katzenellenbogen BS (2004) The selective estrogen receptor modulators (SERMs) tamoxifen and raloxifene have differential effects on gene regulation in breast cancer cells. 86th Annual Meeting of The Endocrine Society, New Orleans, LA, USA, June 16 – 19, 2004. Proceedings.

21.   Stossi F[*], **Barnett DH**[*], Frasor J, Katzenellenbogen BS (2004) Transcriptional Profiling of Estrogen-Regulated Gene Expression Via Estrogen Receptor Alpha or Estrogen Receptor Beta in Human Osteosarcoma Cells: Distinct and Common Target Genes for These Receptors. Keystone Symposium - Nuclear Receptors: Steroid Sisters, Keystone, CO, USA, February 28 – March 4, 2004. Proceedings.
      [*] indicates co-authorship and equal contribution.

20. Katzenellenbogen BS, Frasor J, Park S-E, Stossi F, **Barnett DH**, Stender J, Harrinton WR, Chang EC, and Sheng S, (2004) Estrogen Receptors: Structure-Activity Relationships and Regulation of Gene Expression. Keystone Symposium - Nuclear Receptors: Steroid Sisters, Keystone, CO, USA, February 28 – March 4, 2004. Proceedings.

19. Harrington WR, Sheng S, **Barnett DH**, Katzenellenbogen JA, Katzenellenbogen BS (2003) Activities of Estrogen Receptor Alpha and Beta Subtype-Selective Ligands at Diverse Estrogen Receptive Gene Sites Mediating Transactivation or Transrepression. Jensen Symposium: Nuclear Receptors and Endocrine Disruptors, Cincinnati, OH, USA, December 5 – 7, 2003. Proceedings.

18. Katzenellenbogen BS, Frasor J, Rai D, Stossi F, Harrington W, Park S, Rajendran R, **Barnett DH**, Chang E, Sheng S. Estrogen actions: 2003 perspective of estrogen receptor mechanisms and cellular regulation. Abstract, Society for the Study of Reproduction Thirty-Sixth Annual Meeting, Cincinnati, OH, July 2003.

17. Stossi F, **Barnett DH**, Frasor J, Katzenellenbogen BS (2003) Transcriptional Profiling of Estrogen-Regulated Gene Expression in Human Osteosarcoma Cells Stably Expressing Estrogen Receptor Alpha or Estrogen Receptor Beta. 85th Annual Meeting of The Endocrine Society, Philadelphia, PA, USA, June 19-22, 2003. Proceedings , p. 332.

16. Harrington WR, Sheng S, **Barnett DH**, Katzenellenbogen JA, Katzenellenbogen BS (2002) Activities of Estrogen Receptor Alpha and Beta Subtype-Selective Ligands at a Range of Estrogen Receptive Gene Sites. Keystone Symposium: Nuclear Receptor Superfamily, Snowbird, UT, USA, April 13 – 19, 2002. Proceedings.

15. Lamm ML, Catbagan WS, Laciak RJ, **Barnett** DH, Hebner CM, Gaffield W, Walterhouse D, Iannaccone P, Bushman W: Sonic Hedgehog Activates Mesenchymal *Gli1* Expression During Prostate Ductal Bud Formation. Society for Developmental Biology 61st Annual Meeting, Madison, WI, July 21-25, 2002. Proceedings.

14. Lamm ML, **Barnett DH**, Catbagan W, Laciak R, Bushman W (2001) Hedgehog Signaling in Fetal Prostate Development. The Gordon Research Conference on Developmental Biology, Procter Academy, Andover, NH, June 24-29, 2001. Proceedings.

13. Lamm ML, Fan L, **Barnett DH**, Catbagan WS, Laciak RJ, Hebner CM, Bushman W (2001) Sonic Hedgehog-Gli Signaling is Recapitulated in the LNCaP Xenograft Tumor. 96th Annual Meeting of the American Urological Association, Anaheim, CA, USA, June 2-7, 2001. *Journal of Urology* 165 (5 SUPPL.). 2001.

12. **Barnett DH** and W Bushman (2001) Hedgehog-Interacting Protein in Prostate Development and Models of Prostate Cancer. 1st Annual NIEHS GLI Program Project Retreat, Children's Memorial Institute for Education and Research/Northwestern University Medical School, Evanston, IL March 20, 2001. Proceedings

11. Lamm ML, Podlasek CA, **Barnett DH**, Lee J, Clemens JQ, Hebner CM, Bushman W (2000) The Mesenchymal Factor Bone Morphogenetic Protein-4 Restricts Branching Morphogenesis in the Developing Prostate. Fall Symposium 2000 Meeting of the Society for Basic Urological Research, Ft. Myers, FL, USA, November 9-12, 2000. Proceedings.

10. **Barnett DH**, Lamm ML, Lee J, Bushman W (2000) The *Veratrum* Alkaloid Cyclopamine Arrests Prostate Development by Disrupting the Sonic Hedgehog-Patched-GLI Signaling Pathway. 95th Annual Meeting of the American Urological Association, Atlanta, GA, USA, April 29-May 4, 2000. *Journal of Urology* 163 (4 SUPPL.). 1999. 34.

9. **Barnett DH**, Lamm ML, Lee J, Bushman W (2000) Arrest of Hedgehog-Dependent Prostate Development by Cyclopamine. 11th Annual Scientific Poster Session, Robert H. Lurie Comprehensive Cancer Center of Northwestern University, Chicago, IL USA, June 11, 2000. Proceedings.

8. **Barnett DH**, Lamm ML, Lee J, Bushman W (1999) Cyclopamine Arrests Prostate Development by Inhibiting Sonic Hedgehog Signaling. 20th Annual Minisymposium on Reproductive Biology, Evanston, IL, October 18, 1999. Proceedings.

7. Lamm ML, Podlasek CA, Clemens JQ, Lee J, **Barnett DH**, Bushman W (1999) *Bone Morphogenetic Protein-4* is a Negative Regulator of Prostate Growth. 94th Annual Meeting of the American Urological Association, Dallas, TX, USA, May 1-6, 1999. *Journal of Urology* 161 (4 SUPPL.). 1999. 125.

6. Podlasek CA, **Barnett DH**, Clemens JQ, Bushman W (1997) Prostate Development is Induced by *Sonic Hedgehog* in the Urogenital Sinus Epithelium. 92nd Annual Meeting of the American Urological Association, New Orleans, LA, USA, April 12-17, 1997. *Journal of Urology* 157. (4 SUPPL.). 1997. 189.

5. Podlasek CA, **Barnett DH**, Clemens JQ, Bushman W (1997) Prostate Development is Induced by *Sonic Hedgehog*. Rachford Lectures on Transcriptional Control of Embryogenesis, Cincinnati, OH, 1997. Proceedings.

4. Podlasek CA, **Barnett DH**, Clemens JQ, Bushman W (1997) Prostate Development is Induced by *Sonic Hedgehog* in the Urogenital Sinus Epithelium. Gordon Conference in Developmental Biology, Andover, NH, 1997. Proceedings.

3. **Barnett D**, Olsen B, Nixon K, Bertrand-Garcia R (1995) Genetic analysis of two zebra stripe chloroplast mutants in maize. Annual Meeting of the Botanical Society of America and the American Institute of Biological Sciences, San Diego, California, USA, August 6-10, *1995 American Journal of Botany* 82 (6 Supplement). 70.

2. **Barnett D**, Olsen B, Nixon K, Bertrand-Garcia R (1995) Analysis of chloroplasts in two zebra stripe mutants. 20th Annual West Coast Undergraduate Research Conference, Proceedings. 4.

1. **Barnett D**, Olsen B, Nixon K, Bertrand-Garcia R (1995) Analysis of chloroplasts in two zebra stripe mutants. 37th Annual Maize Genetics Conference, Proceedings. 71.

AWARDS and HONORS

Named to "Teachers Ranked as Excellent" for University of Illinois College of Medicine (2008)
Carle Foundation / University of Illinois at Urbana-Champaign Thomas A. Buetow Memorial Grant (2007)
The Endocrine Society Fellows and Students Workshop Award (2007)
NIH Reproductive Biology Pre-Doctoral Training Fellowship (T32HD07028, 2004-2006)
The Endocrine Society Travel Grant Award for Exceptional Research (2004)
University of Illinois at Urbana-Champaign Graduate College Travel Grant (2004)
NIH Reproductive Biology Training Grant Travel Award (2003)
NIH Toxicology Pre-Doctoral Training Fellowship (T32ES07326, 2002-2004)
Best Poster Award, 92nd Annual Meeting of the American Urological Association (1997)
Colorado College Representative to West Coast Undergraduate Research Conference in Biological Sciences (1995)
Howard Hughes Medical Institute Undergraduate Research Fellowship (Summer and Fall 1994)
The Colorado College Dean's List (1992-1993, 1994-1995)

PROFESSIONAL and COMMUNITY SERVICE

Member, Psychiatry Clerkship Curriculum Review Committee   2009
        University of Illinois College of Medicine, Urbana-Champaign
Member, Medicine and Society Clerkship Curriculum Review Committee   2009
        University of Illinois College of Medicine, Urbana-Champaign
Chairperson, American Cancer Society Man-to-Man Prostate Cancer Support Group   2008 – Present
Institutional Representative, American Physician Scientists Association   2007 – 2009
Student Member, HeRMES Student-Operated Free Clinic, Champaign-Urbana, IL   2007 – Present
Chair (2005) and Vice-Chair (2006 – Present), Physicians for Social Responsibility   2005 – Present
        University of Illinois College of Medicine, Urbana-Champaign

Oral Session Chairperson, 9th Biennial Allerton Reproductive Biology Conference 2005
Trainee Liaison, NIH Reproductive Biology Training Program 2004 – 2006
    University of Illinois College of Medicine, Urbana-Champaign
Chairperson, Medical Scholars Program Advisory Committee 2001 – 2003
    University of Illinois College of Medicine, Urbana-Champaign

TEACHING EXPERIENCE

Medical Pharmacology, University of Illinois College of Medicine at Urbana-Champaign   Fall 2008, 2009
Medical Histology, University of Illinois College of Medicine at Urbana-Champaign   Fall, Spring 2008
Medical Neuroscience, University of Illinois College of Medicine at Urbana-Champaign   Spring 2008

PROFESSIONAL SOCIETIES

American Physician Scientists Association 2006
The American Society for Cell Biology, Student Member 2005
The Endocrine Society, Student Associate 2003
American Association for the Advancement of Science, Student Member 2002
Physicians for Social Responsibility 2001