The Molecular Pharmacology of Endogenous and Therapeutic Estrogen Receptor Modulators in the Breast and Skeleton

by

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Department of Pharmacology and Cancer Biology
Duke University

Date:_______________________

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Donald McDonnell, Supervisor

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Mark Dewhirst

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Tso-Pang Yao

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Pharmacology and Cancer Biology in the Graduate School of Duke University

2009
ABSTRACT

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Abstract

Estrogens and the estrogen receptor (ER) have been implicated in the etiology of breast cancer and osteoporosis. However, the mechanisms by which this receptor-ligand complex manifest their regulatory activities in these processes is not completely understood. The development and subsequent definition of the molecular mechanism of action of selective ER modulators (SERMs), compounds with differential relative agonist/antagonist activity, has uncovered an unanticipated complexity in this signaling pathway. Furthermore, these analyses indicate that it is likely that in addition to the classical steroidal estrogens, which exhibit agonist properties, endogenous compounds exist that interact with ER and function as physiological SERMs. Recently, 27-hydroxycholesterol (27HC) was identified as an endogenous ER ligand with tissue-specific estrogenic/anti-estrogenic activities. Indeed, we determined that 27HC exhibited the three basic properties of a SERM: 1) it bound competitively with estradiol (E2) to both genetic subtypes of ER, ERα and ERβ; 2) it induced a unique conformation of ER that is likely related to its biological activity; and 3) it displayed tissue-specific ER modulatory activity in the cardiovascular system, breast, and bone. In particular, we undertook a series of in vivo studies to show that a pathological elevation of 27HC was associated with decreased bone quantity, an effect that was partially rescued by E2 supplementation. The ability of 27HC to decrease bone density in the absence of endogenous estrogens suggests that the circulating level of 27HC may be of critical importance in determining osteoporosis risk in post-menopausal women. Interestingly, cholesterol-lowering statins have been shown to improve bone density; thus, given the
stoichiometric relationship between circulating cholesterol and 27HC, our data provide a possible explanation for the observed bone sparing actions of this class of drugs.

In general, it is considered that SERM activity can be explained by the ability to induce differential alterations in ER structure and the impact that this has on the recruitment of functionally distinct cofactors. The results of our studies reveal a much more complex picture and suggest that some SERM pharmacology can be ascribed to actions in pathways that do not include ER. Specifically, we have determined that the SERM 4-hydroxy-tamoxifen (4OHT) can bind to and activate the aryl hydrocarbon receptor (AHR). Given that AHR controls the expression of E2-metabolizing enzymes, our finding that 4OHT regulates AHR in the context of breast cancer could have important pharmacological and pathological implications. Interestingly, our preliminary *in vitro* data indicate that the ability of 4OHT to inhibit osteoclast (OC) differentiation, and thus aid in preserving bone density in post-menopausal women, is primarily dependent on expression of AHR, not ER. Conversely, the inhibitory activity of raloxifene (RAL), another SERM, on OC differentiation was absolutely dependent on ER. Thus, the activity of 4OHT in bone is likely to be a composite response requiring its actions on both ER and AHR.

Many new aspects of the estrogen and ER signaling pathways have been uncovered as we learn more about ligands that modulate ER by altering its conformation and thus its ability to engage in protein-protein interactions. Collectively, our findings demonstrate that the intersection between cholesterol metabolism, ER signaling, and the AHR pathway will have important consequences in regulating cellular function, and may be involved in the development or progression of multiple disease states.
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<th>Definition</th>
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<tr>
<td>22HC</td>
<td>22-hydroxycholesterol</td>
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<tr>
<td>27HC</td>
<td>27-hydroxycholesterol</td>
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<tr>
<td>3MC</td>
<td>3-Methylcholanthrene</td>
</tr>
<tr>
<td>4OHT</td>
<td>4-hydroxytamoxifen</td>
</tr>
<tr>
<td>ActD</td>
<td>Actinomycin D</td>
</tr>
<tr>
<td>AEIP</td>
<td>Anti-estrogen interacting peptide</td>
</tr>
<tr>
<td>AF (AF-1, AF-2)</td>
<td>Activation function</td>
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<tr>
<td>AHR</td>
<td>Aryl hydrocarbon receptor</td>
</tr>
<tr>
<td>AHRR</td>
<td>Aryl hydrocarbon receptor repressor</td>
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<td>AI</td>
<td>Aromatase inhibitor</td>
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<tr>
<td>AIB1</td>
<td>Amplified in breast cancer 1</td>
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<td>AIP</td>
<td>Aryl hydrocarbon receptor interacting protein</td>
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<td>ANF</td>
<td>α-naphthoflavone</td>
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<td>ANOVA</td>
<td>One way analysis of variance</td>
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<td>AP-1</td>
<td>Activator protein 1</td>
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<td>AR</td>
<td>Androgen receptor</td>
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<tr>
<td>ARNT</td>
<td>Aryl hydrocarbon receptor nuclear translocator</td>
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<tr>
<td>ASC2</td>
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<td>ATF4</td>
<td>Activating transcription factor 4</td>
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<td>bHLH</td>
<td>Basic helix-loop-helix</td>
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<td>BMD</td>
<td>Bone mineral density</td>
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<td>BNF</td>
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<tr>
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<td>-------------</td>
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<tr>
<td>BrdU</td>
<td>Bromodeoxyuridine</td>
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<td>CBP/p300</td>
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<td>CNOT1</td>
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<td>COMT</td>
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<td>E2F1</td>
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<tr>
<td>E3</td>
<td>Estriol</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme linked immunosorbent assay</td>
</tr>
<tr>
<td>EMSA</td>
<td>Electrophoretic mobility shift assay</td>
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<td>v-erb-a erythroblastic leukemia viral oncogene homolog 4</td>
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<tr>
<td>ERE</td>
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<tr>
<td>ERK</td>
<td>Extracellular signal-regulated kinase</td>
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<td>ERRγ</td>
<td>Estrogen related receptor γ</td>
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<td>ET/HT</td>
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<td>Hepatocyte nuclear factor</td>
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<td>laminin alpha 3 subunit</td>
</tr>
<tr>
<td>LBD</td>
<td>Ligand binding domain</td>
</tr>
<tr>
<td>LDL/HDL</td>
<td>Low density lipoprotein / high-density lipoprotein</td>
</tr>
<tr>
<td>LRRTM4</td>
<td>leucine rich repeat transmembrane neuronal 4</td>
</tr>
<tr>
<td>Luc</td>
<td>Luciferase</td>
</tr>
<tr>
<td>LXR</td>
<td>Liver X receptor</td>
</tr>
<tr>
<td>M-CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>M2H</td>
<td>Mammalian 2-hybrid</td>
</tr>
<tr>
<td>MAPK</td>
<td>Mitogen activated protein kinase</td>
</tr>
<tr>
<td>MCP-1</td>
<td>Monocyte chemotactic protein 1</td>
</tr>
<tr>
<td>MMTV</td>
<td>Mouse mammary tumor virus</td>
</tr>
<tr>
<td>MNC</td>
<td>Multi-nucleated cell</td>
</tr>
<tr>
<td>MNF</td>
<td>3-methoxy-4-nitroflavone</td>
</tr>
<tr>
<td>mRNA</td>
<td>Messenger ribonucleic acid</td>
</tr>
<tr>
<td>NCoR</td>
<td>nuclear receptor corepressor</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>NFκB</td>
<td>Nuclear Factor κ B</td>
</tr>
<tr>
<td>NO</td>
<td>Nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>Nitric oxide synthase</td>
</tr>
<tr>
<td>NQO1</td>
<td>Quinone reductase</td>
</tr>
<tr>
<td>NR</td>
<td>Nuclear receptor</td>
</tr>
<tr>
<td>OB</td>
<td>Osteoblast</td>
</tr>
<tr>
<td>OC</td>
<td>Osteoclast</td>
</tr>
<tr>
<td>OMM / IMM</td>
<td>Outer / inner mitochondrial membrane</td>
</tr>
<tr>
<td>OPG</td>
<td>Osteoprotegrin</td>
</tr>
<tr>
<td>Osx</td>
<td>Osterix</td>
</tr>
<tr>
<td>OVX</td>
<td>Ovariectomy, ovariectomized</td>
</tr>
<tr>
<td>OVX E</td>
<td>Ovariectomy plus estradiol treated</td>
</tr>
<tr>
<td>OVX P</td>
<td>Ovariectomy plus placebo treated</td>
</tr>
<tr>
<td>PANK3</td>
<td>Pantothenic acid kinase 3</td>
</tr>
<tr>
<td>PAS</td>
<td>Per-Arnt-Sim (period circadian protein / aryl hydrocarbon receptor nuclear translocator / single-minded protein)</td>
</tr>
<tr>
<td>PB</td>
<td>Probasin</td>
</tr>
<tr>
<td>PBX</td>
<td>Pre-B-cell leukemia homeobox</td>
</tr>
<tr>
<td>PBXIP1/HPIP</td>
<td>pre-B-cell leukemia homeobox interacting protein 1</td>
</tr>
<tr>
<td>PCR</td>
<td>Polymerase chain reaction</td>
</tr>
<tr>
<td>PI3K</td>
<td>Phosphotidylinositol (PI) 3-kinase</td>
</tr>
<tr>
<td>PKC</td>
<td>Protein kinase C</td>
</tr>
<tr>
<td>PLD</td>
<td>Phospholipase D</td>
</tr>
<tr>
<td>Term</td>
<td>Description</td>
</tr>
<tr>
<td>------</td>
<td>-------------</td>
</tr>
<tr>
<td>Pol II</td>
<td>RNA polymerase II</td>
</tr>
<tr>
<td>PPAR</td>
<td>Peroxisome proliferator-activated receptor</td>
</tr>
<tr>
<td>PR</td>
<td>Progesterone receptor</td>
</tr>
<tr>
<td>PREX2</td>
<td>PI-3,4,5-trisphosphate-dependent Rac exchange factor 2</td>
</tr>
<tr>
<td>PRMT1</td>
<td>Protein arginine methyltransferase 1</td>
</tr>
<tr>
<td>pS2</td>
<td>Trefoil factor 1</td>
</tr>
<tr>
<td>PTOV1</td>
<td>prostate tumor overexpressed 1</td>
</tr>
<tr>
<td>PV</td>
<td>Prunella vulgaris</td>
</tr>
<tr>
<td>PXR</td>
<td>Pregnane X receptor</td>
</tr>
<tr>
<td>qRT-PCR</td>
<td>Quantitative real time polymerase chain reaction</td>
</tr>
<tr>
<td>RAI14</td>
<td>Retinoic acid induced 14</td>
</tr>
<tr>
<td>RAL</td>
<td>Raloxifene</td>
</tr>
<tr>
<td>RANK(L)</td>
<td>Receptor Activator of Nuclear Factor κ B (Ligand)</td>
</tr>
<tr>
<td>RANTES</td>
<td>Regulated upon activation normal T-cell expressed and secreted</td>
</tr>
<tr>
<td>RAW</td>
<td>RAW264.7 murine monocyte cell line</td>
</tr>
<tr>
<td>RXR</td>
<td>Retinoid X receptor</td>
</tr>
<tr>
<td>SARM</td>
<td>Selective androgen receptor modulator</td>
</tr>
<tr>
<td>SDF-1</td>
<td>Stromal derived factor 1</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulfate polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>SERD</td>
<td>Selective estrogen receptor down-regulator</td>
</tr>
<tr>
<td>SERM</td>
<td>Selective estrogen receptor modulator</td>
</tr>
<tr>
<td>SF-1</td>
<td>Steroidogenic factor 1</td>
</tr>
<tr>
<td>SH3BP2</td>
<td>SH3-domain binding protein 2</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
</tr>
<tr>
<td>siRNA</td>
<td>Small interfering ribonucleic acid</td>
</tr>
<tr>
<td>SLC7A1</td>
<td>High affinity cationic amino acid transporter 1</td>
</tr>
<tr>
<td>SMAD3</td>
<td>Mothers against decapentaplegic homolog 3</td>
</tr>
<tr>
<td>SMRT</td>
<td>silencing mediator of retinoid and thyroid hormone receptors</td>
</tr>
<tr>
<td>SNK</td>
<td>Student Newman Keuls</td>
</tr>
<tr>
<td>SOCS2</td>
<td>Suppressor of cytokine signaling 2</td>
</tr>
<tr>
<td>Sp1</td>
<td>Specificity protein 1</td>
</tr>
<tr>
<td>SREBP</td>
<td>Sterol regulatory element binding protein</td>
</tr>
<tr>
<td>STAR</td>
<td>Steroidogenic acute regulatory protein</td>
</tr>
<tr>
<td>T4</td>
<td>thyroxine</td>
</tr>
<tr>
<td>TAM</td>
<td>Tamoxifen</td>
</tr>
<tr>
<td>TCDD/dioxin</td>
<td>2,3,7,8-tetrachlorodibenzo-p-dioxin</td>
</tr>
<tr>
<td>TGFβ (2)</td>
<td>Transforming growth factor β (2)</td>
</tr>
<tr>
<td>TMC3</td>
<td>transmembrane channel-like 3</td>
</tr>
<tr>
<td>TNFα</td>
<td>Tumor necrosis factor α</td>
</tr>
<tr>
<td>TRAcP</td>
<td>Tartrate-resistant acid phosphatase</td>
</tr>
<tr>
<td>TRAP220</td>
<td>thyroid hormone receptor-associated protein 220</td>
</tr>
<tr>
<td>UGT</td>
<td>Uridine diphosphate glycosyltransferase</td>
</tr>
<tr>
<td>WISP2</td>
<td>WNT1 inducible signaling pathway protein 2</td>
</tr>
<tr>
<td>XRE/DRE/AHRE</td>
<td>Xenobiotic/dioxin/aryl hydrocarbon receptor response element</td>
</tr>
<tr>
<td>β-gal</td>
<td>β-Galactosidase</td>
</tr>
<tr>
<td>µCT</td>
<td>Micro computed tomography</td>
</tr>
</tbody>
</table>
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Thank you all for making these past 5 years a time for discovering who I am and what I want to accomplish in life, and for letting me foster my curiosity in science and life.
1 Background

1.1 Thesis Research

The objectives of my studies were to gain a better understanding of estrogen receptor (ER) pharmacology, particularly in breast cancer and in the bone. We aspired to understand new mechanisms by which ER controls cellular behavior through ligand-specific process modulation and intersection with other signaling pathways.

1.2 Estrogen Signaling

1.2.1 Estrogens

![Diagram of estrogen structures]

Figure 1.1: The structures of the three estrogens

The three estrogens, estrone (E1), estradiol (E2), and estriol (E3), are the most abundant and well-studied endogenous agonists for ER (Figure 1.1). The biosynthesis of estrogen in the ovary is initiated in the early follicular stage of the reproductive cycle in response to follicle stimulating hormone (FSH), a pituitary hormone whose receptor is expressed in ovarian granulosa cells. Upon receipt of the signal, cholesterol is mobilized from the outer mitochondrial membrane (OMM) to the inner mitochondrial membrane (IMM) by steroid acute regulatory protein (STAR). At the IMM, cholesterol is converted to pregnenolone by the action of the cholesterol side chain cleavage enzyme cytochrome
P450 (CYP) 11A1 (CYP11A1). From there, pregnenolone is metabolized by numerous CYPs, hydroxysteroid dehydrogenases (HSDs), and hydroxysteroid reductases to produce estrogens, as well as other steroid hormones (Figure 1.2).

![Steroidogenesis Diagram](image)

**Figure 1.2: Steroidogenesis**

A diagram of the reactions in the steroidogenic pathway that contribute to the synthesis of endogenous estrogens, highlighting the enzymes that catalyze these reactions.

Estrogens can be metabolized through several mechanisms, including oxidation by CYPs, sulfation by sulfotransferases, glucuronidation by uridine diphosphateglucuronosyltransferases (UGT), and O-methylation by catechol O-methyltransferases (COMT) (Figure 1.3) (1). The primary site of E2 metabolism is the liver, where CYP1A2, CYP3A4, and CYP1B1 mediate 2- and 4-hydroxylation of E2. Outside the liver (in the
breast, uterus, placenta, brain, and pituitary) CYP1A1, and to some extent CYP3A4, converts E2 to the 2-hydroxylated form. COMT rapidly methylates 2-hydroxy-E2 to avoid free radical formation and is thus considered to be anti-tumorigenic. In the breast and uterus, CYP1B1 actively produces high levels of 4-hydroxy-E2. Unfortunately, 4-hydroxy-E2 is rapidly converted between semiquinone and quinone forms, which generates free radicals that cause DNA damage, and thus CYP1B1 metabolism of E2 is considered genotoxic. There is evidence to suggest that E2 can control the expression of its metabolic enzymes, although the data is not clear (1). Estrogens function through ER to control particular biological processes, as discussed below.

Figure 1.3: Estradiol Metabolism

The creation and metabolism of E2 is catalyzed by many members of the CYP family. E2 metabolites fall into two main categories, those that are genotoxic and those that may inhibit tumorigenesis. Reprinted with permission from (1).
1.2.2 The Estrogen Receptor

ER is a member of the nuclear receptor (NR) super-family of ligand-activated transcription factors. There are two genetic subtypes of ER, ERα and ERβ, that exhibit overlapping but distinct activities and expression patterns (2-5). They share the same modular structure: an N-terminal A/B domain, the C domain containing the zinc-finger DNA binding domain (DBD), a hinge region (D domain), the E domain which houses the ligand binding domain (LBD), and the C-terminal F domain (Figure 1.4). Given their high degree of homology in the LBD and DBD, it is not surprising that ERα and ERβ regulate mostly identical DNA response elements and bind many of the same endogenous and exogenous ligands (4, 6). Tissue distribution patterns and knockout mouse models of ER indicate that ERα and ERβ are not redundant, and instead suggest unique tissue-specific functions for each receptor subtype (2, 7). ERα is more ubiquitously expressed throughout the body, whereas the pattern of ERβ is more restricted (2). Together, ERα and ERβ control many important biological processes, including ovulation, mammary gland development, proper bone mineral density (BMD), cardiovascular function, aspects of mating behavior, and the immune system (7).
Schematic illustration of ER modular structure, highlighting the amino acid similarity between ERα and ERβ.

Residing in either the cytoplasm or the nucleus, signaling through ER is initiated by ligand binding or by ligand-independent processes such as phosphorylation. The transcriptional activity of ER is mediated by the activation functions (AFs), of which there are two in ERα (AF-1 and AF-2) but only one in ERβ (AF-2). AF-2 is located within the LBD and is primarily responsible for ligand-dependent activation of transcription, whereas AF-1 is thought to transduce ligand-independent transcriptional activation. When both are present, as in ERα, AF-1 and AF-2 can function synergistically or independently, depending on the cell and promoter context, thus adding a layer of complexity (8). The distinct biologies of ERα versus ERβ may stem from the divergent A/B and F domains, particularly since no obvious AF-1 domain is found in ERβ.

To activate transcription, the general transcriptional machinery must be recruited to DNA. Although it was once considered that ER directly interacted with the transcription machinery, it is now apparent that the protein-protein interaction surfaces on ER recruit coregulatory proteins, such as coactivators and corepressors, that bridge this interaction. Coregulatory proteins interact primarily with ER at the AF-2 domain, where
a hydrophobic pocket is formed predominantly by helix 12 (H12). This pocket binds the hydrophobic sequence LxxLL found in coactivators. Coactivators can promote transcription through several mechanisms: the acetylation of histones by steroid receptor coactivators (SRC) -1, -2, and -3, thyroid hormone receptor activating protein of 220kDa (TRAP220), and CREB-binding protein (CBP/p300); the methylation of histones by protein arginine methyltransferase 1 (PRMT1); RNA processing by RNA helicases such as p68; and the coupling of ERα to degradation machinery through the ubiquitin ligases E6-associated protein and ribosome production factor 1. Corepressors, the other class of coregulatory proteins, contain a similar domain termed a CoRNR box motif that binds to the same hydrophobic pocket in ER (9). In general, corepressor proteins either recruit or exhibit intrinsic histone deacetylase activity, thereby actively repressing transcription.

After binding to ligand, ER undergoes a conformational change that is critical in dictating the downstream biological response. Previously, it was thought that there were only two conformations that ER could adopt, an active and an inactive one. However, the discovery that different ligands confer unique responses made this model unlikely. Instead, it appears that each ligand induces a unique conformational change that allows for differential presentation of protein-protein interaction surfaces that are used to couple ER to other signaling pathways, therefore determining the cellular response to a particular ligand.

After binding to estrogen and undergoing a conformational change, ER dimerizes and binds to DNA within the regulatory regions of target genes. Direct binding of ER to DNA occurs at estrogen response elements (EREs), whereas ER can also interact
indirectly with DNA through binding to Fos and Jun at activator protein 1 (AP-1) elements \((10)\) or to specificity protein 1 (Sp1) family members at GC-rich DNA regions \((11)\). Although the ERE directs binding of ER to DNA, proximal DNA sequences influence the affinity of ER binding by impacting ER conformation in an undetermined way, as evidenced by distinct patterns of transcriptional activity at particular ERE-containing DNA elements \((12)\).

After initiating transcription, ER\(\alpha\) undergoes ligand-mediated degradation through the 26S proteosome. Although controversial, this degradation event has been linked to efficient transcriptional activity. The activity of particular coactivators, such as AIB1 (amplified in breast cancer 1), has been shown to be necessary for ER\(\alpha\) degradation under certain contexts \((13)\), therefore bringing forth the hypothesis that DNA-bound ER\(\alpha\) recruits the proteins that target it for destruction. The complete identity of these proteins, and whether they interact with ER\(\alpha\) in the same way as coactivators, has yet to be fully elucidated. Further, ER\(\beta\) does not undergo ligand-dependent degradation, despite efficient transcription, suggesting that perhaps another surface outside the coactivator binding groove may be responsible for recruitment of the degradation machinery.

Besides its well-studied role in modulating transcription of target genes, ER may also regulate other biological processes in both the nuclear and cytoplasmic compartments through both ligand-dependent and –independent mechanisms. These include association with the phosphotidylinositol-3-kinase (PI3K), insulin-like growth factor 1 (IGF-1), and extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathways \((14)\). Through interaction with these pathways, ER
activity can be regulated by direct phosphorylation or by phosphorylation of coactivator and corepressor proteins. The exact role of these phosphorylation events, and other potential post-translational modifications, on the signaling competency of ERα and its associated coregulatory proteins remains to be fully elucidated.

1.3 Breast Cancer

1.3.1 General information

Breast cancer is a malignancy of the epithelial cells comprising the mammary gland. It is the most common cancer in women, with approximately one in four being affected over a lifetime. In 2008, it was estimated that among women there would be 182,460 new cases of breast cancer and 40,480 estimated deaths from this disease (15). Mortality rates have been steadily decreasing in all age groups, likely due to increased early detection, better therapeutics, and successful prevention strategies. Many risk factors have been identified for breast cancer, including lifetime exposure to endogenous hormones, obesity, increased age, family history, and alcohol consumption. The primary treatments for breast cancer are surgery, radiation therapy, and systemic chemical therapy, which includes chemotherapeutics and endocrine/hormone therapy.

1.3.2 The role of estrogen and ER in breast cancer

Estrogens and ER have been implicated in many cancers, but none so clearly as breast cancer. In 1896 it was first suggested that ovariectomy (OVX) induced regression of metastatic breast cancer (16), and by 1900 it became clear that this surgery had a one-third response rate in metastatic breast cancer (17). The discovery of estrogen in ovarian follicular fluid set the stage for the development of compounds that either mimic or inhibit classic estrogenic responses (18).
In mammary epithelial cells, E2 signaling through ERα promotes cellular proliferation, which when unchecked can lead to pathologic disease. Even after menopause, when systemic E2 production is largely decreased, adipose tissues continue to make E2 and many breast tumors acquire the ability to locally produce E2 through expression of the aromatase enzyme (19, 20). In addition to promoting cell proliferation via alterations in gene expression, E2/ERα engages the ERK/MAPK and PI3K signaling pathways as complementary ways to increase cell proliferation and survival. As discussed earlier, some metabolites of E2 are DNA damaging agents that may increase DNA mutagenesis, leading to increased probability of cell transformation (20, 21).

Given the implication of E2/ERα signaling in the etiology of breast cancer, pharmaceutical efforts have uncovered many natural and synthetic modulators of ERα activity and E2 synthesis with a wide range of activities. The Selective ER Modulators (SERMs) are characterized by three important qualities: they bind competitively to ER, they induce a unique conformational change in ER that facilitates differential cofactor interactions, and they exhibit promoter- and tissue-specific activity. Tamoxifen (TAM) and raloxifene (RAL) are SERMs that have clinical utility in breast cancer, and the tissue-specific activity profiles of these compounds as compared to E2 are shown in Table 1.1. In the context of the breast, both TAM and RAL are considered ER antagonists based on their ability to block coactivator recruitment to ER and thus transcriptional activation, yet they have different biological consequences as assessed by their individual abilities to decrease the risk for different types of breast cancer. Whereas TAM decreases the risk for both invasive and noninvasive breast cancer, RAL only decreases the risk for invasive breast cancer, suggesting perhaps that differences exist in their mechanism of
action in the breast. A distinction between TAM and RAL is highlighted in the uterus, wherein TAM is an ER agonist and thereby increases the risk for endometrial/uterine cancer and endometriosis, but RAL is an ER antagonist.

**Table 1.1: The relative agonist activities of SERMs**

<table>
<thead>
<tr>
<th></th>
<th>Breast</th>
<th>Uterus</th>
<th>Bone</th>
<th>Cardiovascular System</th>
<th>Brain / Vasomotor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estradiol</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
<td>++</td>
</tr>
<tr>
<td>Tamoxifen</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Raloxifene</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Basedoxifene</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>-</td>
</tr>
</tbody>
</table>

Another class of ER ligands that has been developed clinically is the selective ER down-regulators (SERDs), which includes faslodex/ICI 182,780 (ICI) and GW5638. SERDs, similar to SERMs, bind competitively to ER, but have the additional activity of inducing rapid re-compartmentalization and degradation of ERα protein. This activity contributes to the efficacy of SERDs such as ICI as a second-line therapy for patients who have failed at least one endocrine therapy.

Besides inhibiting ER signaling at the level of ligand binding, another way to impact ER signaling is to reduce the concentration of its agonist, E2. As mentioned above, E2 synthesis from testosterone requires the aromatase enzyme, which also catalyzes the conversion of androstenedione to E1. Thus, aromatase inhibitors (AIs), such as anastrozole, exemestane, and letrozole, have been developed (22). However, only 40 – 50 % of patients respond to AIs, suggesting the presence of *de novo* or acquired resistance mechanisms of circumventing this particular block in E2 synthesis.
One hypothesis is that breast tissue expresses high levels of steroid-sulfatase expression, which converts $E_1$-$3$-sulfate into the un-sulfaconjugated form, whereby subsequent action of $17\beta$-HSD creates $E_2$ (23). Other rationales maintain that perhaps not all ER-positive breast cancers rely on $E_2$ and may instead rely on other endogenous estrogenic compounds (24), or that distinct molecules and signaling pathways, such as the epidermal growth factor receptor (EGFR)/MAPK pathway (25), compensate in the face of an AI and continue to induce cell proliferation and survival.

The success of anti-estrogen therapies in the treatment of breast cancer highlights the importance of $E_2$ and $ER\alpha$ in the development and progression of this disease, but both de novo and acquired resistance suggest that there is still much to learn. A more thorough understanding of the signaling pathways and how they interact and intersect will allow for the development of superior targeted therapies as well as better biomarkers that predict risks and likelihood of response to a given therapeutic.

1.4 *Aryl Hydrocarbon Receptor Signaling*

1.4.1 AHR / ARNT structure and signaling

![Figure 1.5: A schematic of the modular structure of AHR](image)

Figure 1.5: A schematic of the modular structure of AHR
The aryl hydrocarbon receptor (AHR) is a member of the basic helix-loop-helix (bHLH) Per-Arnt-Sim (PAS) family of transcription factors (Figure 1.5). In the apo state, AHR is in a cytoplasmic complex with heat shock protein 90 (Hsp90), p23, and AHR interacting protein (AIP) (Figure 1.6) (26). Hsp90 is critical for proper function of AHR as it facilitates the formation of a high affinity ligand binding conformation while it sequesters AHR in the cytoplasm (26). When a ligand binds to AHR, p23 and AIP are shed, and upon translocation to the nucleus, AHR exchanges Hsp90 for its heterodimerization partner, AHR nuclear translocator (ARNT) (27, 28). It is controversial as to whether ARNT solely resides in the nucleus and thus only interacts with AHR once it has been imported, or whether it binds to AHR in the cytoplasm and enhances nuclear translocation. The AHR/ARNT complex then binds to DNA at xenobiotic/dioxin response elements (XREs/DREs, also referred to as AHREs) in the regulatory regions of target genes, such as CYP1A1 and CYP1B1, UGT1A1 and UGT1A6 (29, 30), quinone reductase (NQO1) (31), and AHR repressor (AHRR) (32, 33).
Ligand (black triangle) binding induces nuclear localization, exchange of p23, AIP, and Hsp90 for ARNT, DNA binding, and modulation of target gene expression. Adapted with permission from (26).
In addition, AHR binds to the RelA subunit of NFκB, enabling formation of a transcription-competent complex on NFκB elements within regulatory regions of NFκB target genes such as c-MYC (34). This is one proposed mechanism by which AHR promotes cell proliferation and may be involved in AHR-driven tumorigenesis. However, this interaction represses AHR/ARNT activities by sequestration of AHR. Other transcriptional complexes, such as those involving AP-1 and Sp1 family members, have also been demonstrated, but the transcriptional outcomes are not clear and may differ in a cell- and promoter-specific manner (35). Outside the nucleus, activated AHR also binds to and impacts the activity of proteins within the growth factor and cytokine signaling pathways, including c-SRC, EGFR, and retinoblastoma protein (35).

Expression of AHR is tightly regulated in a cell- and tissue-specific manner, and varies widely during development and aging. In the liver, where xenobiotic detoxification is critical, high expression of AHR is maintained by hepatic nuclear factors (HNFs) (36). The immune system, which must also respond to and deal with exogenous molecules and substances, expresses high levels of AHR that are controlled by cytokine signaling. Hormonal disruption is known to impact AHR expression and activity, suggesting that the identified potential binding sites for NRs within the AHR promoter region may indeed be functional (36).

One of the most well-studied functions of AHR pertains to its role in mediating the effects of the environmental contaminant 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD or dioxin), and many hypotheses as to the normal physiological roles of AHR signaling come from delineation of the toxic side-effects associated with TCDD exposure. However, the availability of the Ahr−/− mouse has allowed investigators another avenue
for exploration of the physiological roles of AHR. The first identified abnormalities in the Ahr−/− mice were in the liver and immune system, but since then it has become clear that AHR expression in endothelial and hematopoietic cells is critically important for proper vasculature development (37). For our interests, the Ahr−/− are viable and fertile, but the females exhibit profound defects in reproductive system development. AHR is expressed in oocytes and follicular granulosa cells, where its expression is controlled by both FSH and E2 (38, 39). The Ahr−/− female mice exhibit reduced fertility, possibly because of low ovarian E2 levels stemming from the loss of coordinate regulation of aromatase expression by AHR and SF-1 (steroidogenic factor 1) (39). These female mice also have impaired and incomplete mammary gland development, either from a direct effect of AHR signaling in this tissue or secondary to the disruption of E2 production by the ovaries (40, 41). Either way, this leads to decreased lactation and therefore reduced pup survival.

1.4.2 AHR ligands

There are two main classes of AHR ligands, those that are synthetic and those that occur naturally. Synthetic AHR ligands have been studied most extensively and include planar hydrophobic halogenated aromatic hydrocarbons and polynuclear aromatic hydrocarbons. Further examination of AHR has allowed for the identification of a structurally diverse set of compounds that bind this receptor, albeit with varying affinity and degrees of agonist and antagonist behavior (Figure 1.7A). An interesting characteristic that has been uncovered from these studies is that many AHR ligands induce AHR-dependent expression of the enzymes for which they are metabolic substrates.
Figure 1.7: Ligands for AHR

(A) Classic exogenous AHR ligands. (B) Potential endogenous ligands for AHR. Reprinted, with permission, from the Annual Review of Pharmacology and Toxicology, Volume 43 © 2003 by Annual Reviews www.annualreviews.org (42).

Much evidence points to the existence of an endogenous ligand for AHR, including the demonstration of nuclear AHR staining in the absence of exogenous compound treatment and of cellular pathway disruptions when AHR expression has been experimentally reduced. Potential endogenous ligands comprise indoles, tetrapyroles, and amino acid metabolites (Figure 1.7B). However, the in vivo regulation of AHR by these ligands has yet to be conclusively established. Further, the role of
ligand-independent regulation of AHR by post-translational modifications such as phosphorylation has yet to be fully elucidated.

1.4.3 Cross-talk between AHR and ER

There is accumulating evidence for many points of cross-talk between the AHR and ER signaling pathways. In most instances, this cross-talk is antagonistic to ER signaling, and therefore ligand-activated AHR is often considered to be anti-estrogenic and perhaps anti-tumorigenic in E2-mediated pathologies. At the transcriptional level, ligand-activated AHR interferes with ERα binding to Sp1 family members (43). On gene targets such as BRCA1, the transcriptional effect depends on the liganded status of both AHR and ER, and thus AHR/ER interactions can either increase or inhibit BRCA1 expression (44). As part of a cullin 4B ubiquitin ligase complex, ligand-bound AHR recruits ER to the proteosome, where both receptors are subsequently degraded (45). Alternatively, ARNT, the requisite dimerization partner of AHR, interacts with ER in a ligand-dependent manner to coactivate transcription of ER target genes, rendering it unavailable to bind AHR and thus potentially inhibiting AHR responses (46). There is also evidence that AHR ligands, such as 3-methylcholanthrene (3MC) (47), 3,3′4,4′5-pentachlorobiphenyl (47), the trimeric form of indole-3-carbinol (48), and 6-methyl-1,3,8-trichlorodibenzoofuran (49) bind to and modulate ER function. Whether ER ligands conversely regulate AHR has yet to be demonstrated.

1.5 Bone Remodeling

1.5.1 Bone formation and growth

Continuous bone remodeling, comprising a balance of bone formation and resorption, is critical for proper maintenance of bone strength and regulation of calcium
flux. Mature osteoclasts (OCs), the bone resorbing cells, initiate the remodeling process, which occurs through mineral and organic bone matrix degradation. To begin, the OC attaches to the bone matrix to form a sealing zone that outlines the ruffled membrane border where resorption will occur. Once in place, acid and acidic collagenolytic enzymes are released, mineral and matrix degradation occurs, and the resorption products are transported by transcytosis into the circulation. Then the OC detaches and either migrates to another resorption site or undergoes apoptosis. Subsequently, osteoblasts (OBs), the bone forming cells, begin the process of laying down new bone by synthesizing collagenous organic matrix, composed primarily of type I collagen, which must be mineralized to form new bone. After this process, OBs that are buried within the matrix become osteocytes, some of which communicate with other cells within the bone through gap junctions and others form the blood-bone barrier that regulates mineral ion flux.

Bone development begins early in fetal life, and by the eighth week the skeletal pattern is formed in humans. A prevailing model is that most bone is formed by cartilage replacement in a process termed endochondral ossification. This process starts during the end of the first trimester, when blood vessels infiltrate the cartilage, allowing entry of OBs. These OBs form a collar of compact bone, from which a primary ossification center is formed. As the cartilage disintegrates, OBs infiltrate to lay down spongy bone. The bone marrow is housed within this spongy bone, which eventually becomes surrounded by cortical bone. After birth, secondary ossification centers form at the epiphyses (or ends of the bones), where a layer of cartilage is retained between the epiphyses and diaphyses as the growth plate. Linear bone growth occurs from this
growth plate until sex hormones, such as E2 and testosterone, cause ossification of this region. From thence, bones only grow in diameter and thickness (appositional growth) in response to mechanical stress. Just as growth plate closure is controlled by E2 and testosterone, these hormones also control aspects of bone remodeling that make their age-related decline detrimental to bone integrity, often leading to osteoporosis, as discussed in further detail below.

1.5.2 Osteoblasts and osteoclasts

Bone formation is the profession of OBs, derived from the multipotent mesenchymal cell lineage, from which adipocytes, chondrocytes, and myoblasts also develop (Figure 1.8) (50, 51). OBs play many important roles, including laying down osteoid, which gets mineralized into bone at sites of resorption, and controlling the maturation of bone resorbing OCs. Formation of an OB from a mesenchymal progenitor absolutely requires the transcription factors Runx2 and Osterix (Osx). Whereas Runx2 is also required for complete chondrocyte differentiation, Osx is specific to OBs. Other signaling pathways, such as those initiated by transforming growth factor β (TGFβ) and bone morphogenic protein, impinge on Runx2 expression and transcriptional activity in a bidirectional manner, depending on the initiating cytokine (52). Another important transcription factor in OB differentiation and function is ATF4 (activating transcription factor 4), which is required for OB import of the amino acids used in type I collagen synthesis (53) and for the expression of receptor activator of nuclear factor κB (NFκB) (RANK) ligand (RANKL), a protein expressed on pre-OBs that promotes differentiation and activation of OCs (54).
Mesenchymal progenitor cells differentiate into OBs, chondrocytes, myoblasts, and adipocytes under the control of multiple transcription factors. OCs are multi-nucleated tartrate-resistant acid phosphatase (TRAcP)-positive cells of the monocyte lineage that mediate bone resorption. Monocytes differentiate into OCs in response to RANKL secreted by pre-OBs in a monocyte-colony stimulating factor (M-CSF) permissive environment (Figure 1.9). RANKL binds RANK, a surface receptor expressed on OCs, thereby initiating a signaling pathway that along with many other cytokines promotes OC differentiation, activation, and survival. Once OC activation is no longer needed, OBs produce another physiological regulator of OC differentiation, osteoprotegrin (OPG), which acts as a decoy receptor for RANKL and leads to OC apoptosis. As with OBs, a multi-faceted transcriptional program controls OC differentiation (55).

**Figure 1.8: Mesenchymal cell differentiation**

Mesenchymal progenitor cells differentiate into OBs, chondrocytes, myoblasts, and adipocytes under the control of multiple transcription factors.
OC differentiation is controlled by signals from many cell types. Cytokines produced by T cells lead to expression of RANKL and M-CSF, which induce OC differentiation from precursor cells of the monocyte lineage. Reprinted with permission from (56).

### 1.5.3 A specific role for estrogen signaling in the bone

The menopausal loss of estrogen is associated with elevated bone turnover stemming from an increased number and activity of mature OCs, leading to an increased risk for osteoporosis (57-59). There are multiple inputs of E2 in the regulation of bone homeostasis, including at the level of T cells, OBs, and OCs, each of which is thought to express ER and respond to E2 stimulation (Figure 1.10). Much research has focused on cytokine regulation by E2, specifically determining that E2 down-regulates interleukin-6 (IL-6), tumor necrosis factor α (TNFα), and macrophage colony-stimulating factor (M-
CSF) (60-62). Together, these cytokines increase the number of pre-OCs present in the bone marrow (59, 63); therefore, down-regulation of expression of these cytokines by E2 is one mechanism by which E2 attenuates bone resorption.

![Diagram](image)

**Figure 1.10: E2 influences many aspects of bone remodeling**

Unfortunately, ERα and ERβ knockout mouse models have not been useful in understanding the action of ER in the context of the bone due to compensation by androgen signaling (64-66). Nevertheless, a recent mouse model in which ERα was selectively ablated in mature OCs was created, and these female mice showed high bone turnover and trabecular bone loss that was associated with increased OC numbers,
indicating that ERα signaling in OCs is of primary importance \((67)\). Further, it was determined that E2/ERα upregulates Fas ligand (FasL) in OCs, leading to OC apoptosis. A contrasting hypothesis exists, still implicating FasL, but instead positing that E2/ERα increases FasL in OBs, and then FasL acts in a paracrine manner to induce apoptosis of pre-OCs (see Figure 1.10) \((68)\).

Using in vitro models and recapitulation of the menopausal state in in vivo models, ways in which E2 may impinge on OC and OB differentiation, function, and survival have been hypothesized. To influence OC differentiation and function, E2 regulates OPG secretion by OBs \((69)\) and RANK expression by OCs \((70)\). Additionally, E2 negatively regulates the production of the pro-OC cytokine TNFα by bone marrow CD4+ T cells \((71)\), and positively regulates TGFβ expression, both of which contribute to inhibiting bone resorption \((59)\). Overall, E2 decreases OC differentiation and survival, likely through many complementary mechanisms \((72)\). Studies provide conflicting data as to the role of E2 in OBs; whereas some show that E2 directly increases the proliferation of OB precursors and/or mature OBs \((73-75)\), others demonstrate that E2 is anti-apoptotic in OBs \((76)\). Further, it is unclear whether E2 modulates the differentiation of mesenchymal progenitor cells into OBs \((74, 77)\). The general consensus in the field is that the primary effect of E2 is modulation of bone resorption by OCs rather than a direct effect on bone formation by OBs.

Although a clearly demonstrated set of actions of E2, ERα, and ERβ has not yet been put forward, the accumulating evidence suggests that these signaling molecules play a critical role in bone development and homeostasis through key interactions at multiple points, including the OBs, OCs, and T cells.
1.6 Cholesterol and 27-Hydroxycholesterol

Cholesterol is an integral part of the cell membrane as well as the precursor to steroid hormones and bile acids. Evidence is accumulating that cholesterol is also a key signaling molecule in and of itself. Cholesterol comes from two sources, diet and \textit{de novo} synthesis, each of which accounts for about half of the total amount in the body. Under master control of the transcription factor sterol regulated element binding protein (SREBP), three primary mechanisms regulate the cellular content of cholesterol: HMG-CoA reductase activity, acyl-CoA:cholesterol acetyltransferase activity, and the ratio of low- to high-density lipoprotein (LDL/HDL), which mediate uptake and reverse transport of cholesterol, respectively. When an imbalance occurs, cholesterol can be converted to bile acids in the liver and intestines, then excreted. However, the production of bile acids alone cannot compensate for extreme over-consumption of dietary cholesterol, thus leading to a hypercholesterolemic state.

Important intermediates in the synthesis of bile acids from cholesterol are the oxysterols, or hydroxylated metabolites of cholesterol. \(7\alpha\)-hydroxycholesterol is the primary oxysterol produced in the liver in the process of bile acid synthesis, but in the periphery an alternative pathway has been assigned critical importance in the elimination of cellular cholesterol. This alternative pathway begins with hydroxylation of cholesterol into 27-hydroxycholesterol (27HC) by the mitochondrial enzyme CYP27A1 (Figure 1.11) (78). 27HC can be further hydroxylated into 3\(\beta\)-hydroxy-5-cholestenoic acid (cholestenoic acid), which is secreted more efficiently as it is more polar (79). Both 27HC and cholestenoic acid can undergo \(7\alpha\)-hydroxylation by CYP7B1 in the endoplasmic reticulum (see Figure 1.11) (80); the products of these reactions can exit the
cell. In circulation, most oxysterols are esterified by lecithin cholesterol acyltransferase into 3β-acyl esters or diesters (81) and are transported by albumin and HDL to the liver, where they are eventually converted to bile acids (79, 82). CYP27A1 is widely expressed throughout the body, although most highly in macrophages (82). The importance of this particular enzyme, and thus 27HC, in regulating cholesterol homeostasis is highlighted by the fact that patients with mutations in CYP27A1 accumulate cholesterol in peripheral macrophages (83). However, the role of 27HC in regulating cholesterol synthesis is controversial (84, 85).

![Cholesterol metabolism](image.png)

**Figure 1.11: Cholesterol metabolism**

Cholesterol is extensively metabolized by many CYP enzymes, with individual reactions shown with numbers next to the enzyme that catalyzes that particular reaction. Reprinted, with permission, from the Annual Review of Biochemistry, Volume 72 © 2003 by Annual Reviews www.annualreviews.org (86).
The levels of 27HC do not change with diurnal or menstrual cycles, suggesting that circadian rhythm, estrogens, and progestins do not influence flux through this pathway of cholesterol elimination (87), despite demonstration that the CYP27A1 gene is transcriptionally regulated by estrogens and androgens (88). On the other hand, certain factors such as glucocorticoids, growth hormone, IGF-1, thyroxine (T4), and the NRs PXR (pregnane X receptor), PPAR (peroxisome proliferator-activated receptor), RXR (retinoid X receptor), and HNF4 have all been demonstrated to influence CYP27A1 at the level of mRNA expression or protein stability and may impact cholesterol metabolism through this pathway (89-92). Interestingly, there is a significant difference in circulating levels of 27HC among the genders, with males having more 27HC than females irrespective of serum cholesterol (approximately 450 nM versus 345 nM respectively), suggesting hormonal control (87). The concentration of 27HC is strongly positively correlated with serum cholesterol (93), but interestingly there is significant variability in the increased production of 27HC in response to a high fat/high cholesterol diet (94, 95).

1.7 27HC is an endogenous SERM

In 2007, the Mangelsdorf group published the first evidence that 27HC impacts ER signaling (95). This endogenous oxysterol competitively binds to ERα and ERβ with an affinity in the low micromolar range, similar to the circulating concentration. Given that CYP27A1 is highly expressed in macrophages and 27HC levels are robustly increased in macrophage-laden atherosclerotic plaques (81), 27HC is in a unique position to impact ER signaling in the cardiovascular system. E2/ER is important for nitric oxide (NO) synthesis in vascular endothelium and smooth muscle by way of up-regulating the
expression of endothelial and inducible NO synthases (eNOS and iNOS) \((96, 97)\). Generation of NO in the cardiovascular system is disease-preventative, leading to relaxation of smooth muscle, wound healing, and reduced thrombosis \((98)\). The importance of NO is confirmed by data linking cardiovascular pathologies with reduced NO synthesis \((98)\). *In vivo* studies demonstrated that elevation of 27HC attenuated these E2/ER-mediated responses in the cardiovascular system, specifically by reducing the induction of eNOS and iNOS and inhibiting the reendothelialization of artificially damaged blood vessels \((95)\). The effects of 27HC on eNOS and iNOS expression was dependent on ER\(\alpha\) and ER\(\beta\) as shown by genetic ablation of each receptor, but was not dependent on the presence of liver X receptor (LXR), the classic oxysterol receptor. The sum of the activities of 27HC suggest that this endogenous ligand can influence both the transcriptional and non-transcriptional ER signaling pathways.

These findings have important implications in the context of estrogen/hormone replacement therapy (ET/HT) for the prevention of cardiovascular disease. E2 is thought to protect against the development of new atherosclerotic lesions, but not against existing disease progression. Lesions are often present at the time of menopause, even if at the subclinical level \((99)\). As 27HC levels increase in atherosclerotic lesions as they pathologically progress, this creates a microenvironment in which 27HC could compete with E2, attenuating the cardio-protective effect of E2. Further studies are necessary to determine if 27HC impinges on ER signaling in other tissue systems as well.
2 27-hydroxycholesterol is an endogenous SERM

2.1 Introduction

2.1.1 Estrogen Receptor Signaling in the Breast

Estrogens function as mitogens in most ER-positive breast cancers. ER is expressed as two genetic isoforms, ERα and ERβ, which exhibit distinct but overlapping expression patterns and functions but share a common mechanism of action (3-5). Upon ligand binding, ER undergoes a conformational change that results in dimerization, DNA binding, recruitment of transcriptional coregulators, and modulation of target gene expression. In the context where both ERα and ERβ are expressed, ERβ functions to dampen the transcriptional activity of ERα (100). Therefore, the biological response of cells and tissues to ER modulation is a composite of the resulting activities of ERα and ERβ.

2.1.2 The pharmaceutical development of SERMs

The implication of estrogens and ER in breast cancer has led to the pharmaceutical development of SERMs and anti-estrogens. SERMs exhibit tissue- and promoter-specific agonist and antagonist behavior. The unique and compound-specific conformational changes in ER induced by SERM binding allows different protein-protein interaction surfaces to be exposed, leading to differential recruitment of coregulatory proteins and thus diverse biological outcomes. For example, the SERM TAM is an ER antagonist in the breast, but it functions as an agonist in the bone, cardiovascular system, and uterus. It is therefore useful in the treatment of breast cancer and osteoporosis, but can only be used under limited circumstances in pre-menopausal women (101).
There has always been a question in the field as to whether SERMs mimic an aspect of ER signaling that already exists endogenously, or if they are merely a product of pharmacological development. The identification of the oxysterol 27-hydroxycholesterol (27HC) as an endogenous molecule with SERM-like activity provides the first proof that the SERM concept is an extension of a natural process.

### 2.1.3 27-hydroxycholesterol, a primary oxysterol

Oxysterols are hydroxylated metabolites of cholesterol. Some oxysterols have been previously described as ligands for members of the NR family, most notably for LXR (102). These cholesterol derivatives are produced by many cell types throughout the body, both inside and outside of the liver. The acidic bile acid synthesis pathway is the predominant pathway outside the liver, and this pathway is initiated by CYP27A1 (103). This enzyme converts cholesterol to 27HC, the principal endogenous oxysterol. Cholesterol and 27HC competitively bind the active site of CYP27A1, so when cholesterol levels are limiting, 27HC remains in the active site and is further hydroxylated to cholestenoic acid.

### 2.2 Results

#### 2.2.1 27HC regulates ER transcriptional activity

It has been previously shown that 27HC is an ER ligand that inhibits both the genomic and nongenomic actions of E2 in the cardiovascular system (95). In this study, we focused on defining the molecular mechanisms underlying the distinct pharmacological actions of 27HC in a variety of cellular models of estrogen action. Our first objective was to evaluate the ability of 27HC to regulate the transcriptional activity of both ERα and ERβ. To measure transcriptional activation, we used a classic ERE fused to the luciferase (luc) coding region in an ER-negative mammalian cell line. In this
experiment, HeLa cells were transiently transfected with ERα or ERβ and an ERE-luciferase (3XERE-TATA-Luc) reporter. 27HC induced transactivation of both ERα and ERβ in a dose-dependent manner (Figure 2.1). Notably, the maximal activity of ERα and ERβ in the presence of 27HC did not reach that obtained after treatment with E2. The dose-response curve was right-shifted, indicative of the differing affinities for ERα (27HC Ki=1.32 µM, E2 Kd~0.2 nM) and for ERβ (27HC Ki=0.42 µM, Kd E2~0.5nM ) (4, 95, 104). Similar results were obtained in two additional ER-negative cell lines (CV-1 and HepG2, data not shown).

![Image](image.png)

**Figure 2.1: Induction of ER transcriptional activity by 27HC**

HeLa cells were transfected overnight with 3XERE-TATA-Luc and either an ERα or ERβ expression plasmid. Cells were treated overnight with increasing concentrations of E2 or 27HC, or co-treated with 0.5 nM E2 + increasing concentrations of 27HC. Adding 1 or 10 µM 27HC significantly reduces the transcriptional activity of ER in the presence of 0.5 nM E2 compared to 0.5 nM E2 alone (p<0.05). Mean±SEM from a representative experiment is shown. Adapted in part with permission from (24) Copyright 2008, The Endocrine Society.

The concentration range in which 27HC activated ER is physiologically relevant given that the circulating concentration of 27HC ranges from 0.15 – 0.73 µM in a healthy individual, and can reach a local concentration in the millimolar range within atherosclerotic plaques in an individual with severe cardiovascular disease (81, 95). In addition, 27HC levels in the mouse aorta were found to be 0.25 – 0.6 µM in the absence
of disease, and notably over 30% of this 27HC was unesterified and thus available to impact signaling pathways directly (95). Interestingly, the $K_M$ of 27HC for its catabolic enzyme, CYP7B1, is 24 $\mu$M, which is significantly higher than that required to saturate ER$\alpha$ or ER$\beta$ (95).

Given that 27HC can effectively compete with E2 for binding to ER$\alpha$ and ER$\beta$ at physiological concentrations in an \textit{in vitro} binding assay, we next evaluated its ability to antagonize transcriptional activation by E2. At a relevant concentration of E2 (0.5 nM), increasing concentrations of 27HC reduced the stimulation of transcriptional activity by E2 (Figure 2.1). These data were the first indication that 27HC may in fact be a classic partial agonist or a SERM, with both agonist and antagonist activities against ER$\alpha$ and ER$\beta$.

\textbf{2.2.2 27HC elicits a unique conformational change in ER}

The transcription assays indicated that 27HC functions as a partial agonist, eliciting a response similar to E2 albeit with lower efficacy. This prompted us to determine whether or not there were mechanistic differences between 27HC and E2 that may help to define its pharmacological identity. ER ligands elicit specific conformational changes within the receptor that dictate their biological response. These ligand-induced conformational changes can be identified and tracked using peptide conformational probes that bind to differentially exposed protein-protein interaction surfaces (105). Using peptides recognizing either the coactivator or corepressor binding surfaces on the receptor, it is possible to determine the likelihood that a given ligand will function as an agonist or an antagonist. We previously identified peptides that bind specifically to ER$\alpha$ and/or ER$\beta$ in the presence of agonist (E2), SERM (4-hydroxy-TAM, 4OHT), or antagonist (ICI) (106-108). Therefore, we used these peptides to see whether the
conformations of ERα and ERβ induced by 27HC resembled in any way those induced by other ER ligands. To do this, we analyzed by mammalian 2-hybrid (M2H) assay the interaction of ERα or ERβ with this set of peptides in the presence of vehicle, 27HC, E2, 4OHT, or ICI. In this assay, ERα and ERβ were fused to the VP16 transactivation domain, and the peptides were linked to the yeast Gal4 DNA-binding domain (Gal4DBD). Interaction between the receptor and peptides was assessed by measuring the transcriptional readout of a 5XGal4Luc3 luciferase reporter (Figure 2.2). Previous data demonstrated that the αII peptide binds to ERα when a ligand is occupying the LBD (108). For both ERα and ERβ, prior analysis revealed that the D30 peptide binds only pure agonist-activated receptor, bT1 binds only in the presence of 4OHT, and bI2 only binds in the presence of ICI (106, 107). As shown in Figure 2.2, 27HC recruited αII to ERα, suggesting that it occupies the LBD as expected from the competition assays. Further, 27HC also led to recruitment of D30 to ERα and ERβ, indicating that it induced an agonist-like receptor conformation. Neither 27HC-bound ERα nor ERβ adopted a conformation conducive to binding bT1 or bI2, confirming our expectations that the conformational change induced by 27HC is similar to E2 and distinct from 4OHT or ICI.
HepG2 cells were transfected overnight with VP16, VP16-ERα or -ERβ, a 5XGal4Luc3, the β-gal control, and the following peptides: pM (vector control), αII, D30, bT1, or bI2. Cells were treated overnight with vehicle, 1 µM 27HC, 1 nM E2, 100 nM 4OHT, or 100 nM ICI. No significant interaction occurred between VP16 and the peptides or pM and VP16-ERα or -ERβ. Mean±SEM from a representative experiment is shown. Adapted in part with permission from (24) Copyright 2008, The Endocrine Society.

As this was a limited set of conformation peptide probes, we next performed combinatorial peptide phage display to identify peptide probes that could be used to further evaluate the impact of 27HC on ER structure and how the structure differs from E2-activated receptor. For this study, we screened a peptide library containing the LxxLL motif found in NR coactivators (109). Since 27HC did not exhibit full agonist activity, we also screened a peptide library containing the CoRNR box motif characteristic of corepressor proteins (9, 110). Using a modified M13 phage display screen (106), we isolated peptides from both libraries that interacted with 27HC-bound ERα or ERβ and classified these interacting peptides based on the ligands that elicited an interaction between the peptide and the receptor. The binding profile of representative peptides is shown in Figure 2.3, and a list of peptide sequences is in Table 2.1.

Figure 2.2: 27HC induces a unique active conformation of ER

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Figure 2.3: Peptide profiling highlights the unique conformation of ER in the presence of 27HC

HepG2 cells were transfected overnight with VP16, VP16-ERα or -ERβ, a 5XGal4Luc3, the β-gal control, and the indicated peptides. Cells were treated overnight with vehicle, 10 µM 27HC, 100 nM E2, or 100 nM 4OHT. No significant interaction occurred between VP16 and the peptides or pM and VP16-ERα or -ERβ. Mean±SEM from a representative experiment is shown. Adapted in part with permission from (24) Copyright 2008, The Endocrine Society.
Table 2.1: Select peptide sequences

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<th>Peptide</th>
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<tr>
<td>CDD.64</td>
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<tr>
<td>CDD.30</td>
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<td>CDD.1</td>
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<td>CDD.91</td>
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</tr>
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</table>

The majority of the peptides recognized the conformation of ER bound by either 27HC or E2, with varying degrees of selectivity for each ligand. For ERα, some peptides interacted equally well with the receptor in the presence of 27HC or E2 (CDD.64). Importantly, we identified peptides that preferred binding to 27HC-bound ERα versus E2-bound receptor (CDD.11 and CDD.30), and were intrigued by the finding that this group consisted of peptides containing either an LxxLL or a CoRNR box motif. Interestingly, we also found peptides containing a CoRNR box motif that interacted preferentially with 27HC- and 4OHT-bound ERα but not E2-bound ERα (CDD.29). It is known that 4OHT-bound ERα recruits corepressor proteins, this being a key element of its ability to manifest antagonist activity.

In the case of ERβ, similar classes of peptides were identified. For example, there were peptides that bound with similar affinity to 27HC-ERβ and E2-ERβ (CDD.11, CDD.1) and peptides that preferred either the 27HC- or E2-bound conformation.
Interestingly, we identified a class of peptides unique to ERβ, which consisted of peptides that preferred the 27HC-bound ERβ conformation and that interacted better with ERβ in the absence of ligand than in the presence of E2 (CDD.22, CDD.6). This suggested that there are surfaces on ERβ that are occluded in the presence of E2, but not in the presence of 27HC; however, the biological significance of this remains to be determined.

Lastly, we identified proteins that bound with selectivity for ERα versus ERβ (CDD.29, CDD.16, and CDD.21). This was not surprising, as the receptors are not identical, are known to exhibit different biological activities in response to the same ligands, and do show some degree of ligand specificity. From these conformational studies using peptide probes, we concluded that binding of 27HC to ERα and ERβ leads to a unique conformational change in each receptor that may allow for the recruitment of both coactivator and corepressor proteins, and this may explain the partial agonist/antagonist activity of this oxysterol.

Peptides have been previously identified that bind to ERβ and inhibit its transcriptional activation, presumably by blocking recruitment of coactivators or transcriptional machinery (111). Given that we were able to identify peptides that had receptor and/or ligand selectivity, we wanted to see if these peptides could be used to inhibit the actions of E2 versus 27HC on ERα or ERβ. We chose to move forward with peptide CDD.30, as it had the best selectivity for 27HC-bound ERα and ERβ over E2-bound receptor (see Figure 2.4). Interestingly, addition of the peptide had little effect on the transcriptional activity of ERα, but it significantly inhibited the activity of ERβ. Although CDD.30 did slightly inhibit the action of E2-ERβ, it completely inhibited the transcriptional activity of ERβ in the presence of 27HC. This would suggest that in the
presence of both E2 and 27HC, CDD.30 would selectively inhibit responses initiated by 27HC through ERβ, although this has yet to be experimentally confirmed.

![Graph showing ERα and ERβ transcriptional activity](image)

**Figure 2.4: CDD.30 inhibits ERβ transcriptional activity**

HeLa cells were transfected with expression plasmids for ERα or ERβ, the CDD.30 peptide, and the 3XERE-TATA-luc luciferase reporter. Cells were treated overnight with vehicle or increasing concentrations of E2 or 27HC. Mean±SEM from a representative experiment is shown.

### 2.2.3 27HC-bound ER recruits coactivator peptides

In the presence of E2, the conformational change in ERα or ERβ allows for the recruitment of peptides containing the NR interaction motifs found within coactivators such as steroid receptor coactivator 1 (SRC1), activating signal integrator-2 (ASC2), AIB1, or glucocorticoid receptor (GR) interacting protein 1 (GRIP1) (106). We therefore investigated whether the conformational change induced by 27HC also allowed for the recruitment of these coactivator peptides. In cells transfected with VP16-ERα or -ERβ, coactivator peptides fused to Gal4DBD, and a 5XGal4Luc3 luciferase reporter, treatment with 27HC or E2, but not 4OHT, led to recruitment of SRC1-NR, ASC2-NR, AIB1-NR, and GRIP1-NR to ERα and ERβ (Figure 2.5). These data provide additional support for the hypothesis that 27HC is indeed an ER agonist capable of inducing an active AF-2 conformation and is likely to be a physiologically relevant estrogen in certain contexts.
Figure 2.5: 27HC-bound ER recruits coactivator peptides

HepG2 cells were transfected overnight with VP16, VP16-ERα or -ERβ, a 5XGal4Luc3, the β-gal control, and the indicated coactivator peptides. Cells were treated overnight with vehicle, 1 μM 27HC, 1 nM E2, or 100 nM 4OHT. No significant interaction occurred between VP16 and the peptides or pM and VP16-ERα or -ERβ. Mean±SEM from a representative experiment is shown. Adapted in part with permission from (24) Copyright 2008, The Endocrine Society.

2.2.4 27HC treatment increases ERα occupancy at the pS2 promoter

Although we have shown that 27HC, like E2, regulates ERα and ERβ transcriptional activity, we sought to determine whether activation by 27HC allows for receptor recruitment to DNA elements analogous to that seen with E2. From this point forward, we focused on ERα, as in vitro models of ERβ are limited due to a common loss of expression upon introduction of cells into culture conditions. Therefore, we performed chromatin immunoprecipitation (ChIP) in the ERα-positive MCF7 breast cancer cell line to analyze ERα recruitment to the well-characterized pS2 (trefoil factor 1) promoter region. Treatment for 45 minutes with E2 or 27HC led to a significant recruitment of ERα to the ERE-containing region of the pS2 promoter and not to a distal non-estrogen-responsive region as a negative control (Figure 2.6, data not shown).
Recruitment of ERα to the pS2 promoter region was analyzed in MCF7 cells treated with vehicle (Veh), 100 nM E2, or 10 µM 27HC for 45 minutes. Cells were harvested after cross-linking and subjected to immunoprecipitation with either rabbit IgG control (IgG) or ERα antibody (ERα). After reversal of the cross-linking, DNA was isolated and subjected to qRT-PCR analysis. Data is the Mean±SEM from triplicate amplification reactions from a representative experiment. There was significant recruitment of ERα to the ERE-containing region of the pS2 promoter in the presence of E2 (p<0.0001, t-test) and 27HC (p<0.05, t-test) when compared to vehicle. Adapted in part with permission from (24) Copyright 2008, The Endocrine Society.

2.2.5 27HC regulates endogenous ERα-target gene expression in MCF7 and T47D breast cancer cells

Given that 27HC displayed agonist activity in the above assays and allowed for the recruitment of ERα to DNA response elements, we sought to determine whether 27HC acts as an agonist in ERα-positive breast cancer cell lines. Using MCF7 cells, we analyzed by qRT-PCR (quantitative real time polymerase chain reaction) the ability of 27HC to regulate ERα target gene expression. Increasing concentrations of 27HC led to target gene regulation similar to treatment with increasing concentrations of E2, albeit with lower efficacy in some cases, which was a reflection of its partial agonist activity (Figure 2.7). Significant regulation of target genes occurred at physiologically relevant concentrations (0.5 – 1 µM).
Figure 2.7: 27HC has agonist activity on ERα target genes in MCF7 breast cancer cells

MCF7 cells were treated with vehicle or increasing concentrations of E2 or 27HC for either 8 hours (SDF-1, PR, pS2, and E2F1) or 24 hours (WISP2 and ERBB4). Cells were harvested and cDNA prepared from isolated RNA was subjected to qRT-PCR analysis. Data are normalized to 36B4 and expressed as mean fold change over vehicle±SEM. Adapted in part with permission from (24) Copyright 2008, The Endocrine Society.

ERα regulates target gene expression in a direct manner through interaction with an ERE, and also by indirect mechanisms involving interactions with Fos and Jun at AP-1 elements and with Sp1 family members at GC-rich motifs (112, 113). Given that the SERM 4OHT is able to activate transcription of ERα target genes under control of an AP1 element but not those under control of an ERE (114), we asked whether 27HC exhibited any selectivity with respect to the expression of genes using these two models of ER-mediated transcriptional regulation. Interestingly, we found that 27HC regulated target gene expression at 1) classical EREs such as in pS2 (trefoil factor 1) and WISP2 (WNT1-inducible signaling pathway protein 2), 2) AP1 elements such as in ERBB4 (v-erb-a erythroblastic leukemia viral oncogene homolog 4) and PR (progesterone receptor), and
3) Sp1 elements such as those in PR and E2F1 (E2F transcription factor 1) (Figure 2.7). Therefore, with respect to this activity of ERα, 27HC most closely resembled E2.

To ascertain that the effects of 27HC on ERα target gene expression were not limited to MCF7 cells, we analyzed gene expression in another ERα-positive breast cancer cell line, T47D. Similar transcriptional responses were observed upon treatment with either E2 or 27HC in these cells (Figure 2.8).

**Figure 2.8: 27HC has agonist activity in T47D breast cancer cells**

T47D cells were treated with vehicle, 1 nM E2, 1 µM 27HC, 100 nM 4OHT, or 100 nM ICI for either 8 hours (SDF-1, WISP2, RET, and ERBB4) or 24 hours (pS2). Cells were harvested and cDNA prepared from isolated RNA was subjected to qRT-PCR analysis. Data are normalized to 36B4 and expressed as mean fold change over vehicle±SEM. Adapted in part with permission from (24) Copyright 2008, The Endocrine Society.

In addition to gene expression, we analyzed the expression of PR at the protein level, this being a robust surrogate marker of ERα activation in MCF7 cells. As expected,
there was an increase in PR expression beginning at 4 hours upon treatment with E2 (Figure 2.8). A similar pattern was observed following treatment with 27HC, albeit with a slight temporal delay. 4OHT did not induce PR expression.

![Figure 2.9: 27HC increases the protein expression of PR](image)

MCF7 cells were treated for the indicated amount of time with vehicle (V), 1 nM E2, 10 μM 27HC, or 100 nM 4OHT. Whole cell extract was resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for PR or cytokeratin 18 (CYT18) as a loading control. Adapted in part with permission from (24) Copyright 2008, The Endocrine Society.

Although the most well characterized ERα transcriptional targets are up-regulated upon treatment with E2, many genes important for growth, differentiation, and signaling are down-regulated by E2, including ERBB4, IL1-R1 (IL1 receptor 1), SMAD3 (mothers against decapentaplegic homolog 3), and Id2 (inhibitor of differentiation 2) (115). Importantly, these E2 down-regulated genes were similarly down-regulated by 27HC (Figure 2.7 and 2.10), although the kinetics were not identical to those observed in E2-treated cells. It appeared that 27HC may more robustly regulate ERα-down-regulated genes than up-regulated ones, which was not surprising given that the 27HC-ERα complex recruited peptides containing the CoRNR box motif found in corepressors more efficiently than E2-ERα.
Figure 2.10: 27HC down-regulates target genes similarly to E2

MCF7 cells were treated with vehicle, 1 nM E2, or 1 µM 27HC for 1, 8, or 24 hours. Cells were harvested and cDNA prepared from isolated RNA was subjected to qRT-PCR analysis. Data are normalized to 36B4 and expressed as mean fold change over vehicle±SEM.

To confirm that the effects on gene expression observed after treatment with 27HC were in fact mediated through ERα, we used the ERα pure antagonist ICI. In both MCF7 and T47D cells co-treated with ICI and either 27HC or E2, gene regulation was abrogated (Figure 2.11 and data not shown). Cumulatively, these data confirm that 27HC is working through ERα in these breast cancer cell lines and that pharmacologically, it closely resembles E2.
Figure 2.11: Activation of ERα by 27HC is suppressed by ICI

MCF7 cells were treated with vehicle, 1 nM E2, or 1 µM 27HC for 8 or 24 hours in the absence or presence of 100 nM ICI. Cells were harvested and cDNA prepared from isolated RNA was subjected to qRT-PCR analysis. Data are normalized to 36B4 and expressed as mean fold change over vehicle±SEM. Adapted in part with permission from (24) Copyright 2008, The Endocrine Society.

2.2.6 27HC induces ERα protein degradation

An important hallmark of ERα agonists is their ability to induce receptor turnover. Treatment with E2 leads to ERα degradation, an event that is intimately linked to transcriptional activity (116-118). Therefore, we evaluated the ability of 27HC to regulate ERα turnover. In MCF7 cells, treatment with 27HC led to a decrease in ERα protein over 24 hours (Figure 2.12A). The percent ERα protein remaining is depicted in Figure 2.12B, where it is evident that 27HC mimicked E2, and was mechanistically distinct from 4OHT. 27HC did not induce degradation of ERα as robustly as E2, most likely reflecting the partial agonist activity of 27HC, and perhaps explaining the weaker induction of transcription.
The transcriptional coactivator AIB1 has been shown to be required for E2-mediated, but not ICI-mediated, ERα degradation in MCF7 cells (13). Therefore, we investigated the importance of AIB1 in 27HC-induced degradation of ERα. Using small interfering RNA (siRNA), we knocked down AIB1 expression and analyzed ERα protein levels after 8 hours of ligand treatment (Figure 2.12C). Identical to E2, AIB1 was required for 27HC-mediated degradation of ERα in MCF7 cells, suggesting that the mechanisms of E2- and 27HC-induced ERα turnover were likely very similar.

![Figure 2.12: Degradation of 27HC-bound ERα requires AIB1](image)

(A) MCF7 cells were treated for 1, 4, 8, or 24 hours with vehicle (V), 1 nM E2, 10 µM 27HC, or 100 nM 4OHT. Whole cell extract was resolved by SDS-PAGE, transferred to nitrocellulose, and blotted for ERα or CYT18 as a loading control. (B) Quantification of the data in A using ImageJ software. (C) MCF7 cells were transiently transfected with siRNA to AIB1 (siAIB1) or control (siControl) for 48 hours. Cells were then treated for 8 hours with vehicle (V), 1 nM E2 (E), 10 µM 27HC (H), 100 nM 4OHT (T), or 100 nM ICI (I). Whole cell extract was resolved by SDS-PAGE, transferred to nitrocellulose, and blotted for AIB1, ERα, or CYT18 as a loading control. Adapted in part with permission from (24) Copyright 2008, The Endocrine Society.

### 2.2.7 MCF7 cells proliferate in response to 27HC

In breast cancer cells, E2 is a mitogen that induces expression of CYCLIN D1, increases entrance into the S-phase of the cell cycle, and subsequently leads to cell
proliferation (119). Using gene expression analysis, we showed that, similar to E2, treatment of MCF7 cells with 27HC led to a robust induction of CYCLIN D1 expression (Figure 2.13A). Not surprisingly then, similar to E2, 27HC increased the number of cells cycling through S-phase as analyzed by BrdU (bromodeoxyuridine) incorporation (Figure 2.13B). Because 27HC acted as an ERα partial agonist, we hypothesized that the same behavior would be observed in the BrdU labeling assay. As shown in Figure 2.13B, co-treatment of cells with 27HC and E2 led to a dose-dependent decrease in BrdU incorporation to a level that represented the maximal activity of 27HC alone. Finally, over 6 days of treatment, 27HC and E2 increased total cell number in a dose-dependent manner compared to vehicle (Figure 2.13C). Furthermore, increasing concentrations of 27HC suppressed E2-mediated proliferation, an activity reflecting its partial agonist activity. We therefore concluded that the partial agonist activity of 27HC observed at the level of transcriptional activation and gene expression was also manifest at the level of cell proliferation.
Figure 2.13: 27HC increases proliferation of ERα-positive breast cancer cells

(A) *CYCLIN D1* expression was determined in MCF7 cells treated for 24 hours with vehicle or increasing concentrations of 27HC or E2. Cells were harvested and cDNA prepared from isolated RNA was subjected to qRT-PCR analysis. Data are normalized to 36B4 and expressed as mean fold change over vehicle±SEM. (B) MCF7 cells were treated for 24 hours with vehicle (Veh) or increasing concentrations of 27HC or E2 or a combination of both as indicated. Cells were harvested and assayed for BrdU incorporation per manufacturer’s protocol. Data are the mean±SEM from a representative triplicate experiment. (C) MCF7 cells were treated for 6 days with vehicle or increasing concentrations of 27HC or E2 or a combination of both as indicated. Cells were then harvested and cell number was quantitated. Data are the mean±SEM from one representative triplicate experiment. Adapted in part with permission from (24) Copyright 2008, The Endocrine Society.

2.3 Discussion

In this study we show that 27HC is an endogenous SERM that displays significant partial agonist activity in a variety of cellular models of ER action. As expected for a SERM, the relative estrogenic activity of 27HC varied when assessed by different endpoints. Interestingly, as compared to its antagonist activity described in the cardiovascular system, the pharmacological activity of 27HC most closely resembled E2
in cellular models of breast cancer. Both ligands activated the transcriptional activity of ERα and ERβ in exogenous reporter assays, and both activated ERα when assessed by qRT-PCR gene expression analysis of endogenous target genes. Additionally, both 27HC and E2 induced recruitment of ERα to DNA response elements and triggered ligand-dependent receptor degradation, an event that was dependent on AIB1 in MCF7 cells. Of specific importance was the observation that 27HC, like E2, induced the proliferation of ERα-positive breast cancer cells \textit{in vitro}. In all these instances, however, 27HC did not exhibit full agonist activity but rather showed classic partial agonist behavior. This was readily apparent in both the transcription and the proliferation assays, where 27HC antagonized the E2-induced activation of ERα and ERβ. The fact that 27HC had similar, yet distinct, behavior from E2 was highlighted by the apparent differences in the structures of ERα and ERβ in the presence of E2 versus 27HC. To explore these ligand-induced conformational changes, we used both previously identified and newly discovered peptide probes. Specifically, we were able to show in peptide-binding experiments that 27HC-activated ERα and ERβ adopted structures that share features in common with the E2 and 4OHT-bound conformations. Thus we believe that our findings support the idea that 27HC is similar in function to, yet distinct from, E2 in its ability to regulate ER activity in a variety of validated models of estrogen action, and that its overall activity is consistent with it being classified as a SERM. We plan to investigate whether oxysterols other than 27HC have similar actions in terms of ER regulation. Further, by searching for proteins that share similarity to our identified peptides, we discovered many potential novel ER-interacting proteins whose function in modulating ER signaling have yet to be uncovered (see Chapter 9).
We were intrigued by the apparent tissue-specific action of 27HC, given our finding that 27HC had agonist activity in the breast and the previous observation that it manifests antagonistic activity in the cardiovascular system. Such tissue-specific agonist and antagonist actions are classically thought to arise from differential cofactor recruitment. Therefore, it will be interesting to compare the cofactor preferences of 27HC-bound ER in the breast versus the vasculature. The fact that we were able to identify peptides that interacted in a highly specific manner with ERα or ERβ in the presence of 27HC suggests that there are distinct protein-protein interaction surfaces presented on the receptors in the presence of this ligand. Defining how this influences the differential recruitment of cofactors is an area of investigation that is currently underway.

2.3.1 27HC as an endogenous ER ligand

In the past, our laboratory and others have generated ER-indicator mice wherein ERE-β-galactosidase or ERE-luciferase were introduced transgenically (120-122). In these mice, it was reported that there was a significant level of E2-independent activation of ER-reporter activity that was blocked by treatment with ICI (120, 121). This basal activity is currently attributed to the ligand-independent activity of ER. However, it is interesting to speculate that the presence of 27HC, or a similar oxysterol, may be responsible for this basal ER-reporter activity, a hypothesis that could be tested by administration of specific CYP27A1 inhibitors (123) or by crossing the Cyp27a1−/− mouse with these ER-indicator mice and examining whether the basal activity is eliminated. These studies are currently planned in our laboratory.
2.3.2 Biological significance of 27HC action in breast cancer

In the breast, and specifically in breast cancer, E2 is a mitogen through its actions on ERα (124-126). We demonstrated that 27HC induces proliferation of ERα-positive breast cancer cells and that this proliferation correlated with increased CYCLIN D1 expression and an increase in cells in the S-phase of the cell cycle. This finding has important implications with respect to the treatment of ERα-positive breast cancer, particularly those treated with AIs. Although these therapies are extremely successful, resistance by an as yet unknown means is a significant clinical issue (127). Given that many breast cancer cells and tumors with acquired resistance in vitro and in vivo continue to rely on ERα (128), we suggest that the ability of 27HC to regulate proliferation may be a contributing factor. In this way, 27HC may act as an alternate estrogenic ligand that manifests its agonist behavior in a low-estrogen environment.

We examined microarray data from human breast tumor samples and found a trend between CYP7B1 expression and disease-free survival. Among those patients with ERα-positive breast tumors, increased expression of CYP7B1 was associated with an increase in disease-free survival. No such correlation was observed in patients with ERα-negative breast tumors. CYP7B1 metabolizes 27HC, therefore increased expression of this enzyme would facilitate the conversion of 27HC into downstream products and thereby lower the local concentration of 27HC. These findings support our hypothesis that 27HC plays an important role in the development and/or progression of ERα-positive breast cancer. It would be anticipated that ERα-positive breast tumors with lower CYP7B1 expression, and therefore higher 27HC levels, would have a growth advantage over those with lower expression, especially in situations of low estrogen, such as post-menopausal or anti-estrogen-treated women.
Interestingly, a survey of gene expression analyses in breast tumors revealed a link between \textit{CYP7B1} expression and ER-positivity itself. It was found that ER-positive tumors had decreased expression of \textit{CYP7B1} compared to ER-negative tumors (129). This may reflect a desire for tumor cells that rely on ER signaling for growth to increase local concentrations of 27HC, and one way to do this may be to decrease expression of its catabolic enzyme, \textit{CYP7B1}. However, whether this is a causal or correlative relationship remains to be determined.

Of all the cell types in the body, macrophages have the highest capacity to convert cholesterol to 27HC. This is interesting in light of the observation that an increased number of tumor-infiltrating macrophages is an independent risk factor for reduced survival among breast cancer patients (130). Many hypotheses exist as to how tumor-infiltrating macrophages are co-opted to enter the tumor microenvironment and how they increase tumorigenic behavior (131-133). However, a potential explanation stemming from our findings is that an increased macrophage presence in breast tumors elevates the local 27HC concentration and subsequently the activation of ER. Although estrogens have an established role in modulating the inflammatory response through repression of cytokine production by macrophages (134), their primary contribution in the etiology of breast cancer is considered to be their ability to function as mitogens. Thus, we hypothesize that the primary action of 27HC in the context of breast cancer is to induce cell proliferation through activation of ER, and that tumor-infiltrating macrophages may be an important source of 27HC in this environment.

There is an increased risk for breast cancer in obese individuals that has been linked to increased aromatase activity and resultant estrogen production in adipose tissue (135-138). In a healthy individual, 27HC levels decline alongside estrogen levels.
during menopause (139). However, circulating levels of 27HC positively correlate with cholesterol levels, and obesity often coincides with hypercholesterolemia (87). Thus, the increased level of 27HC in an obese post-menopausal woman may also contribute to the development of breast cancer through the potential mitogenic actions of 27HC in the breast.
3 27-hydroxycholesterol negatively impacts the skeleton

3.1 Introduction

This chapter outlines our findings with respect to 27HC signaling in the bone, and represents data gained from a collaboration between myself and Dr. Erik Nelson, a post-doctoral fellow in the McDonnell laboratory.

3.1.1 Estrogen receptor signaling in the bone

The menopausal loss of estrogen is associated with increased bone turnover, leading to an increased risk for osteoporosis (57). This increased bone turnover stems primarily from an increased number and activity of mature OCs, or bone-resorptive cells (58, 59). The signaling components that create the menopausal pro-resorptive environment have yet to be completely elucidated, and there are multiple mechanisms by which estrogens and ER may influence these pathways (Figure 3.1). Both OCs and OBs, the bone forming cells, express ER and are responsive to E2. Much research has focused the effect of cytokine regulation by E2 on bone parameters, and has specifically determined that E2 down-regulates IL-6, TNFα, and macrophage colony-stimulating factor (M-CSF) (60-62). Together, these cytokines increase the number of pre-OCs present in the bone marrow (59, 63), therefore down-regulation of the expression of these cytokines by E2 is one mechanism to attenuate bone resorption.
For complete OC differentiation, RANKL production by OBs is required. When OBs and OCs come in physical proximity, RANKL binds its receptor RANK, which is expressed on the OC surface, and initiates a signaling pathway leading to OC differentiation, activation, and survival. OB secretion of OPG, a physiological inhibitor of RANKL/RANK signaling, is augmented by E2 (69). E2 also increases RANK expression by OCs (70), but does not directly regulate RANKL expression. E2 may negatively regulate the production of the pro-OC cytokine TNFα by bone marrow T cells, as evidenced by the increase in TNFα-producing T cells after OVX (71). Additionally, E2 positively regulates TGFβ expression, and together this and the potential decrease in TNFα production contribute to the ability of E2 to inhibit bone resorption (59). Overall,
E2 decreases OC differentiation and survival, likely through many complementary mechanisms (72).

Studies provide conflicting data as to the role of E2 in OBs. Whereas some show that E2 directly increases the proliferation of OB precursors and/or mature OBs (73-75), others consider E2 to be anti-apoptotic and affect OB numbers in that way (140). Further, it is unclear whether E2 modulates the differentiation of mesenchymal progenitor cells into OBs or adipocytes (74, 77). The general consensus in the field is that the primary effect of E2 is modulation of bone resorption by OCs rather than a direct effect on bone formation by OBs.

### 3.1.2 Evidence that cholesterol impacts bone remodeling

27HC levels are positively correlated with those of cholesterol, thus studies on the biological consequences of changes in cholesterol levels may give insight into potential biological actions of 27HC. Links between cholesterol levels and BMD have been established, but there is conflicting data as to the association between lipid parameters such as HDL and LDL, total cholesterol, or body mass index, and whole body BMD, lumbar spine BMD, or markers of bone turnover. Whereas some studies showed a negative correlation between total serum cholesterol and BMD or bone turnover (141-145), others showed no such correlation (146, 147). To further support the potential role of 27HC and cholesterol in bone, low BMD was reportedly associated with increased risk for cardiovascular events (148, 149).

There is also much clinical data on statin treatment and BMD, although again the results are conflicting, perhaps due to inclusion criteria or different control parameters (body mass index, HDL/LDL ratio, ET/HT users). Studies of atorvastatin showed that, at a dose that lowered cholesterol levels, there was no change in BMD or indices of bone
metabolism \((150)\). Nevertheless, a recent review suggests that despite conflicting data, there is strong evidence that statin therapy has a positive effect on bone \((151)\).

Polymorphisms in genes crucial for proper statin response (leptin receptor) or regulation of bone remodeling (TNF\(\alpha\)) correlate with changes in BMD after 12 months of treatment with atorvastatin, suggesting that the efficacy of statins may be related to both their ability to lower cholesterol, and thus 27HC, and their ability (directly or indirectly) to down-regulate TNF\(\alpha\) production. Patients carrying the wild-type (Lys/Lys at position 656) allele of the leptin receptor have significantly higher BMD after treatment than those with the Lys/Asn polymorphism, although there was no difference in baseline BMD \((152)\). Similarly, only patients with the G/G genotype of TNF\(\alpha\) exhibited a significant increase in lumbar spine BMD after atorvastatin treatment \((153)\).

In combination therapy trials, atorvastatin showed additive effects with the bisphosphonate risedronate in terms of increasing lumbar spine BMD after 6 months of treatment, when compared to risedronate alone \((154)\). Simvastatin treatment for 2 months was also associated with decreased markers of bone turnover \((155)\), however longer studies (1 - 2 years) were inconsistent as to whether these markers ultimately lead to an increase in BMD \((156, 157)\).

Given the positive correlation between cholesterol and 27HC levels, we were interested in defining whether a portion of the activity attributed to cholesterol in the bone was due to changes in 27HC levels. 27HC is a SERM with partial agonist activity in cellular models of breast cancer, thus it is possible that 27HC impacts ER signaling in T cells, OBs, or OCs, thereby modifying bone turnover and ultimately influencing bone quantity and quality.
3.2 Results

3.2.1 Elevated 27HC does not alter T cell populations in adult female mice

Bone loss secondary to loss of endogenous estrogens is associated with an increase in the activation, differentiation, and proliferation of CD4+ T cells, suggesting that estrogens may inhibit T cell production, proliferation, and/or function (158). Given that 27HC is an endogenous SERM, we hypothesized that it might also impact the CD4+ T cell population. To ascertain this, we examined T cell populations in the Cyp7b1−/− mouse, which lacks expression of the primary catabolic enzyme for 27HC and therefore does not efficiently metabolize 27HC, leading to a 4–5-fold elevation in the circulating level of this oxysterol (95). T cell numbers in the spleen were lower in adult female Cyp7b1−/− mice compared to wild-type mice, but there was no difference in the bone marrow nor was there a change in the number of TNFα-producing T cells (Figure 3.2A, data not shown). Interestingly, when primary splenic, but not bone marrow-derived, CD4+ T cells were stimulated overnight, those from the Cyp7b1−/− mice responded more robustly (data not shown), implying that 27HC may alter the expression of proteins that regulate cytokine responsiveness in a manner that was maintained ex vivo. Further studies are warranted to identify those molecular changes that manifest increased T cell sensitivity to stimulation and to determine if there are alterations in production of cytokines other than TNFα.
Figure 3.2: 27HC may affect T cell populations, but not OC differentiation

(A) Whole spleen and flushed bone marrow were stained for CD4 and subjected to FACS to determine the percent CD4+ T cells in the total cellular population of the indicated organ. Data is the mean±SEM for n=3 adult nulliparous female mice. Cyp7b1/- mice had fewer CD4+ cells in the spleen than Cyp7b1+/+ controls (p<0.05, t-test). (B, C) RAW cells were treated with M-CSF, RANKL, and the indicated ligand for 8 days, after which the cells were assessed for differentiation into OC by TRAcP activity (left panels) or percent TRAcP+ MNCs (right panels). (B) 27HC treatment does not alter OC differentiation, whereas E2 treatment (100 nM for activity, 10 and 100 nM for cell count) significantly inhibits this process (*p<0.05, compared to vehicle) (ANOVA with SNK). (C) 27HC treatment does not block the ability of E2 to inhibit OC differentiation (**p<0.01, ***p<0.001, compared to vehicle, ANOVA with SNK). Data (n=4-6) is the mean±SEM from a representative experiment.
3.2.2 27HC has no measurable effect on *in vitro* osteoclast differentiation

Another important aspect of ER signaling in the bone is its ability to modulate OC differentiation, and thus limit the production of these bone resorbing cells. Stimulated with RANKL and M-CSF, RAW264.7 (RAW) murine monocytes differentiate into mature OCs, or multi-nucleated cells (MNCs) that are TRAcP+. Differentiation can be quantitated either by microscopy (TRAcP+ MNCs) or by assessing TRAcP activity. As expected, E2 dose-dependently decreased the differentiation of RAW cells into OCs (Figure 3.2B). However, treatment with 27HC did not influence RAW OC differentiation (Figure 3.2B), nor did it impact the ability of E2 to inhibit this differentiation process (Figure 3.2C). Therefore, 27HC does not impinge on ER signaling during OC differentiation *in vitro*. However, it may affect homing of OC precursors to the bone microenvironment, or modulate survival of precursors or mature OCs; these aspects of OC signaling will be tested in future studies.

3.2.3 27HC decreases the proliferation but increases the activity of primary osteoblast precursors

Although conflicting literature exists as to the effects of E2 on OB proliferation, we assessed the impact of ER signaling on these bone forming cells *in vitro*. Pre-OBs isolated from the calvaria of newborn mice were cultured in the presence of E2 for 10 days, during which time they were assessed for total cell number. We showed that E2 modestly increased the proliferation of these primary pre-OBs compared to control-treated cells (Figure 3.3A). Treatment with 27HC caused a dose-dependent decrease in proliferation, suggestive of ER antagonist behavior. Yet, the inhibitory effect of 27HC was not reversed by co-treatment with E2 (Figure 3.3B). These data suggest that 27HC and E2 act through different mechanisms to influence proliferation of OB precursors,
and that the pathway initiated by 27HC is irreversible or otherwise not affected by E2-mediated signaling.

**Figure 3.3: 27HC inhibits proliferation of OB precursors**

Primary pre-OBs were isolated from neonatal Cyp7b1+/+ mice. (A, B) Pre-OBs were assayed for proliferation over 10 days in culture with (A) vehicle or 10 nM E2, or (B) vehicle, 100 nM or 1 µM 27HC, or a combination of 10 nM E2 and 1 µM 27HC. E2 treatment significantly enhances proliferation at days 4 and 6 (**p<0.01, **p<0.001, compared to vehicle, ANOVA with SNK). 27HC treatment at 1 µM significantly inhibits proliferation at all time points, and co-treatment with E2 does not alter this response (compared to vehicle, ANOVA with SNK). Data is from a representative experiment performed in triplicate (mean±SEM). (C) Pre-OBs were treated for 36 hours with vehicle, 10 nM E2, or 1 µM 27HC in the presence or absence of 1 µM ICI. E2 and 27HC both increase alkaline phosphatase activity, and this increase in completely blocked by ICI (**p<0.01, **p<0.001 compared to vehicle, ANOVA with SNK). Data is from a representative experiment with n=6 (mean±SEM).

On the other hand, treatment with 27HC modestly increased primary pre-OB activity, as assessed by alkaline phosphatase activity, and this increase was completely blocked by co-treatment with the pure ER antagonist ICI (Figure 3.3C). Caution is necessary in interpreting these results, as alkaline phosphatase activity is a common marker of estrogen response in certain cell types, such as those from the endometrium.
Therefore, it is unclear whether alkaline phosphatase activity in this context measures OB activity or estrogen responsiveness. It would be prudent to examine whether 27HC alters the ability of OBs to deposit bone as a true measure of activity. Either way, 27HC functionally regulated signaling pathways in OBs, likely both through ER and independent of ER. It will be interesting to see whether 27HC impacts OB differentiation from mesenchymal progenitors, given its seemingly opposing effects on OB proliferation and activity.

**3.2.4 ER-dependent and -independent gene regulation by 27HC in primary osteoblast precursors**

We were intrigued by the finding that E2 and 27HC do not act in concert to regulate proliferation of primary pre-OBs, therefore we wanted to uncover molecular differences in the response to each ligand, particularly by analyzing specific gene expression. Receptors for TNFα are expressed on both OBs and OCs, where TNFα influences OB survival and promotes OC differentiation (159). The mRNA expression of TNFα was up-regulated by 27HC in a manner that was completely inhibited by co-treatment with E2 (Figure 3.4), suggesting perhaps coordinate regulation of the expression of this pro-OC factor, or conceivably convergent pathways initiated by 27HC and E2.
Figure 3.4: 27HC modulates gene expression in primary pre-OBs

Primary pre-OBs isolated from neonatal Cyp7b1+/− mice were treated for 24 hours with vehicle or 1 µM 27HC in the presence or absence of 100 nM E2. cDNA prepared from isolated RNA was subjected to qRT-PCR analysis of target gene expression. Data is normalized to cyclophilin and presented as fold induction over vehicle treated cells (mean±SEM). *p<0.05, **p<0.01, ***p<0.0001 compared to vehicle, ^p<0.01 compared to 1 µM 27HC (ANOVA with SNK). A representative experiment performed with n=4 is shown.

SDF-1 (stromal-derived factor 1), also known as chemokine (C-X-C motif) Ligand 12 (CXCL12), is regulated by both 27HC and E2 in cellular models of breast cancer (24) and, as the ligand for chemokine (C-X-C motif) receptor 4 (CXCR4), is important for homing of OC precursor cells to the bone microenvironment. To complicate matters, SDF-1 expression by OBs can be elevated by TNFα, which contributes to the creation of the chemotactic gradient of high SDF-1 in the bone microenvironment and comparably lower SDF-1 in circulation (160). We found that both 27HC and E2 regulate SDF-1 in primary pre-OBs (Figure 3.4). Specifically, 27HC and E2 increased SDF-1 expression, but in fitting with its ER partial agonist activity, co-treatment of 27HC and E2 led to SDF-1 expression only to the maximal level induced by 27HC alone.
Significantly, the 27HC-mediated increase in expression of the pro-inflammatory cytokine IL-7 was not affected by co-treatment with E2 (Figure 3.4), either implicating the SERM activity of 27HC in differential gene expression or implying that 27HC regulated this cytokine independent of ER. IL-7 is produced primarily by pre-OBs in the bone, where it acts both to inhibit OB differentiation and to augment T cell production of M-CSF and RANKL, thereby indirectly increasing OC differentiation and activity (161). Interestingly, E2 treatment blocks the induction of IL-7 in OVX mice, suggesting that E2 can regulate the production of this cytokine in vivo (162), but this may occur in a cell type other than the OB or may require a complex multi-cellular pathway to elicit this biological effect. A similar pattern of regulation was observed with RANKL expression in OB precursors in that it was increased by 27HC in a manner not affected by E2 treatment (Figure 3.4). Again, regulation of RANKL may be an example of tissue- and promoter-specific activities of ER ligands, or 27HC may regulate a unique, ER-independent signaling pathway. We saw no significant regulation of osteocalcin or OPG by either 27HC or E2 in primary pre-OBs.

To gain a better understanding of the similarities and differences in regulation of TNFα and SDF-1 by 27HC and E2, we used chemical and siRNA methods to interrogate the involvement of ER. When primary OB precursors were co-treated with 27HC and the pure ER antagonist ICI, up-regulation of SDF-1 was blocked, but there was no effect on the up-regulation of TNFα (Figure 3.5A), implying that 27HC regulates SDF-1 directly through ER and that modulation of TNFα expression is through a unique pathway that has yet to be identified. The inability of ICI to block 27HC-mediated expression of TNFα does not support the hypothesis that the ability of E2 to inhibit this activity is due to direct competition at the level of ERα, and instead points to separate convergent
pathways initiated by each ligand that are not influenced by ICI. Studies using siRNA to decrease ERα expression in primary pre-OBs supported this preliminary conclusion, specifically that ERα may be required for regulation of SDF-1 by 27HC, but of TNFα expression (Figure 3.5B). The mechanism by which 27HC modulates gene expression independent of ER remains to be determined, and is an active area of research within our laboratory.

Figure 3.5: ERα is required for 27HC-mediated induction of SDF-1

Primary pre-OBs isolated from neonatal Cyp7b1+/+ mice were used for gene expression analysis. (A) Cells were treated for 24 hours with vehicle or 1 μM 27HC in the presence or absence of 1 μM ICI. *p<0.05, **p<0.01 compared to vehicle (ANOVA with SNK). (B) Cells were transfected with siRNA to ERα (siERα) or mock control for 2 days, then treated for 24 hours with vehicle, 10 nM E2, or 1 μM 27HC. *p<0.05, **p<0.01, ***p<0.001 compared to mock-transfected vehicle-treated cells (ANOVA with SNK). cDNA prepared from isolated RNA was subjected to qRT-PCR analysis of target gene expression. Data (n=4) is normalized to cyclophilin and presented as fold induction over vehicle treated cells (mean±SEM).
3.2.5 Female Cyp7b1⁻/⁻ mice have decreased trabecular bone density

Given the effects of 27HC on proliferation, activity, and gene expression in pre-OBs, and the involvement of cholesterol and ER signaling in bone development and homeostasis, we were interested in examining the effect of altering 27HC levels on bone properties in vivo. To accomplish this, we utilized two genetic mouse models, the Cyp27a1⁻/⁻ mouse that cannot produce 27HC and the Cyp7b1⁻/⁻ mouse that does not efficiently metabolize 27HC and thus has elevated circulating levels of this oxysterol (95, 163). In adult intact female mice, we found a significant decrease in lumbar spine BMD in Cyp7b1⁻/⁻ mice compared to age-matched wild-type (Cyp7b1⁺/+ ) controls (Figure 3.6A). Conversely, there were no measurable differences in BMD between adult female Cyp27a1⁻/⁻ and wild-type (Cyp27a1⁺/+ ) mice (Figure 3.6B).

![Figure 3.6](image-url)

**Figure 3.6: Pathological elevation of 27HC decreases trabecular bone density**

The lumbar spine and femur from female mice at 10 weeks of age were harvested and subjected to BMD analysis by DEXA. Data (n=9-10) is the mean±SEM. (A) The BMD in the lumbar spine was significantly lower in the Cyp7b1⁻/⁻ mice compared to wild-type (ANOVA with SNK, p<0.001). (B) There were no significant differences in bone density between the Cyp27a1⁺/+ and Cyp27a1⁻/⁻ mice.
More detailed examination by µCT (micro-Computed Tomography) imaging of the distal femur from the Cyp7b1−/− mice revealed a 30% decrease in bone volume/total volume fraction (BV/TV, %), concurrent with a 17% increase in trabecular separation, as compared to wild-type controls (Figure 3.7A). The alterations in these parameters likely stem from the observed decrease in both trabecular number and thickness (Figure 3.7A). The loss in trabecular number was qualitatively examined using histological hematoxylin and eosin (H&E) staining of the proximal tibia (Figure 3.7B). Hence, the Cyp7b1−/− mice, which have pathologically elevated 27HC, have decreased trabecular bone quantity, and therefore may be more prone to fracture.

Interestingly, despite no measurable differences in BMD in the absence of 27HC at early adulthood, there were significant changes in the trabecular micro-architecture in female Cyp27a1−/− mice (Figure 3.8). The increased trabecular number is in direct contrast to data from the Cyp7b1−/− mice, providing strong support for a role for 27HC in controlling this biological parameter. On the other hand, female Cyp27a1−/− mice exhibited a decrease in trabecular thickness similar to Cyp7b1−/− mice. An increased trabecular number coincident with decreased thickness could imply increased trabecular formation stemming from increased OB proliferation, suggesting that this parameter may be responsive to 27HC both in vitro and in vivo.
Figure 3.7: Trabecular architecture is altered in the Cyp7b1\textsuperscript{-/-} mice

(A) Trabecular morphometry was assessed by \(\mu\)CT on the femur. Data (n=9-10) is the mean±SEM. *** \(p<0.001\), ** \(p<0.01\), * \(p<0.05\) by t-test. (B) H&E staining of the proximal tibia from representative Cyp7b1\textsuperscript{+/-} and Cyp7b1\textsuperscript{-/-} sham-operated female mice.
Trabecular morphometry was assessed by µCT on the femur. Data (n=7-8) is the mean±SEM. ** p<0.01 by t-test.

### 3.2.6 Loss of endogenous estrogen exacerbates the bone loss observed in female Cyp7b1⁻/⁻ mice

Our previous study defined 27HC as an ER partial agonist in the context of cellular models of breast cancer (24), therefore we were interested if this activity would also be evident in the bone. In this tissue, a partial agonist manifests antagonistic activity in the presence of normal estrogen levels, and agonist activity under estrogen-depleted conditions. As an example, the SERM TAM decreases BMD in pre-menopausal women, but partially blocks menopausal loss of BMD (164). Therefore, we hypothesized that 27HC would be bone-sparing in OVX mice, a model of the menopausal state in which the majority of endogenous E2 production is lost. To confirm the involvement of E2 and ER in changes brought on by OVX, we supplemented OVX mice with daily injections of either placebo or 10 µg/kg E2. Uterine weight measurements confirmed that the endogenous production of E2 was largely lost in OVX mice as evidenced by the
decrease in uterine weight, and further that supplementation of OVX mice with E2 reversed this effect (Figure 3.9). Interestingly, treatment of OVX Cyp7b1⁻/⁻ mice with E2 did not completely block uterine weight loss, suggesting that 27HC may act as a SERM in the uterus to functionally modulate ER signaling. More studies are necessary to delineate a potential role for 27HC in the uterus, and any potential impact this role would have on fertility. A previous study suggested a fertility defect in female Cyp7b1⁻/⁻ mice (165), but we see no evidence of this in our colony; this may be explained by differences in genetic background of the Cyp7b1⁻/⁻ mice in our colony (C57BL/6J-129SvEv) versus those reported in the literature to have a defect (C57BL/6J). As expected, mice that underwent OVX gained more body weight over the 28 day study period, most significantly in the wild-type OVX placebo group (Figure 3.9), but there were no discernable differences between the wild-type and Cyp7b1⁻/⁻ mice in terms of weight gain.
Figure 3.9: OVX decreases uterine weight and increases body weight

Uterine wet weight and weight gain (over the 28 day study) were assessed at the conclusion of the study. Mice underwent sham or OVX surgery at Day 1 (6 weeks old), then the OVX mice were assigned to either placebo (OVX P) or E2 (OVX E) treatment. Data (n=4-10) is the mean±SEM. *** p<0.001, * p<0.05 compared to the Cyp7b1+/+ sham group, ^^^ p<0.001 compared to the Cyp7b1-/- sham group (ANOVA with SNK).

After OVX, Cyp7b1-/-, but not wild-type, mice lost a significant amount of BMD in cortical (mid-femur) bone, but this loss was inhibited by treatment with E2 (Figure 3.10A). These data suggest that elevated 27HC increased the sensitivity of the cortical bone micro-environment to E2-deprivation, which has critical implications for the management of osteoporosis risk in post-menopausal women with high cholesterol, remembering that levels of 27HC are positively correlated with those of cholesterol. On the other hand, wild-type mice underwent OVX-induced bone loss in the lumbar spine region that was inhibited by treatment with E2. However, E2 treatment did not recover the low baseline lumbar spine BMD in the Cyp7b1-/- mice back to wild-type levels. The lack of further loss of BMD in the lumbar spine of the Cyp7b1-/- mice implies that 27HC
may be a partial agonist in this tissue, or that a critically low bone mass had already been reached or that the elevated 27HC completely saturated ER and therefore did not allow any effects of E2/ER signaling to be manifest. To support these hypotheses, E2 supplementation of intact female Cyp7b1−/− mice increased BMD in both the lumbar spine and cortical bone, suggesting that direct competition between E2 and 27HC for activation of ER could be occurring (Figure 3.10B).

**Figure 3.10: The mid-femur is sensitive to alterations in E2 and 27HC levels**

The lumbar spine and femur were harvested and subjected to BMD analysis by DEXA. (A) Mice were assigned to sham surgery (Sham), or OVX with placebo (OVX P) or E2 (OVX E) treatment for 28 days. Data (n=9-10) is the mean±SEM. *** p<0.001, ** p<0.01 compared to the Cyp7b1+/+ sham group, ^^^ p<0.001, ^^ p<0.01 compared to the Cyp7b1−/− sham group (ANOVA with SNK). (B) Cyp7b1−/− mice were treated with placebo or E2 for 28 days. Data (n=4-5) is the mean±SEM. *** p<0.001, * p<0.05 compared to the placebo group (ANOVA with SNK).
The fact that the Cyp7b1−/− mice exhibited loss of cortical BMD upon OVX makes it unlikely that 27HC is a partial ER agonist under this circumstance, although this is complicated by the lack of further BMD decrease in the lumbar spine. 27HC may influence many signaling pathways, thus making it difficult to thoroughly ascertain its effects on ER signaling in the bone. However, it is clear that 27HC does not mimic the action of SERMs with partial agonist activity in the bone, such as TAM and RAL, and thus should be considered an ER antagonist in this tissue.

3.2.7 ER-dependent and ER-independent aspects of bone architecture

Analysis of bone architecture by µCT allows for a determination of parameters that impact overall bone quality. OVX led to a decrease in bone volume/total volume fraction in both wild-type and Cyp7b1−/− mice, concurrent with a loss of trabecular number that was not significantly inhibited by treatment with E2 (Figure 3.11). Trabecular thickness was increased back to base-line by E2 treatment in OVX wild-type mice. Intriguingly, although trabecular thickness was not significantly altered by OVX in Cyp7b1−/− mice, treatment with E2 increased thickness up to wild-type levels. All together, the changes in these parameters correlated with an increase in trabecular separation after OVX in both wild-type and Cyp7b1−/− mice, which was not appreciably altered by E2 treatment. These data suggest that an ER-independent process (or processes) influences trabecular number in OVX Cyp7b1−/− female mice, and perhaps by a similar ER-independent mechanism 27HC impacts trabecular formation or destruction. On the other hand, E2/ER increased trabecular thickness, a parameter that 27HC antagonized in the presence of normal, but not supra-physiological, levels of E2. This could implicate direct competition for ER binding, although it remains equally possible that the two ligands regulate parallel pathways that both influence trabecular thickness.
Figure 3.11: Aspects of trabecular and cortical bone are modulated by both 27HC and E2

Trabecular morphometry was assessed by μCT on the femur. Data (n=9-10) is the mean±SEM. Mice were assigned to sham surgery (Sham), or OVX with placebo (OVX P) or E2 (OVX E) treatment for 28 days. ***p<0.001, **p<0.01, *p<0.05 compared to the Cyp7b1+/+ sham group, ^^^p<0.001, ^p<0.05 compared to the Cyp7b1-/− sham group (ANOVA with SNK).

To support the classification of ER-dependent and –independent activities of 27HC in the context of the bone, we evaluated by μCT the effect of supra-physiological E2 levels on bone parameters in intact female Cyp7b1-/− mice. Treatment of these mice with E2 increased uterine weight, indicating that circulating E2 levels were effectively increased (Figure 3.9). Importantly, E2 treatment of intact Cyp7b1-/− mice increased trabecular thickness and bone volume/total volume fraction, which are known or suspected ER-dependent processes (Figure 3.12A). Conversely, trabecular number, and thereby trabecular separation, were not significantly impacted by treatment.
Interestingly, cortical thickness increased in E2-treated *Cyp7b1<sup>−/−</sup>* mice, stemming from decreased endosteal circumference. This change mimics what was observed in a patient with a loss-of-function mutation in ER (166), suggesting that 27HC directly affects the ability of ER to maintain normal cortical thickness.

![Bone Volume / Total Volume](image1)

![Cortical Thickness](image2)

![Trabecular Thickness](image3)

![Trabecular Number](image4)

![Trabecular Separation](image5)

**Figure 3.12: E2 increases trabecular and cortical thickness in the face of high 27HC**

Trabecular morphometry was assessed by µCT on the femur. Mice were treated with placebo or E2 for 28 days. Data (n=4-5) is presented as the mean±SEM. ***p<0.001, **p<0.01 compared to the placebo group (ANOVA with SNK).

### 3.2.8 Elevated osteoclastic bone resorption and reduced bone formation underlie the low BMD in *Cyp7b1<sup>−/−</sup>* mice

The actions of OBs and OCs coordinately regulate homeostatic bone formation and resorption. The osteoporotic phenotype of the female *Cyp7b1<sup>−/−</sup>* mice is indicative of a disruption in this homeostasis, either resulting from decreased bone formation or increased bone resorption, or some combination thereof. As a marker of bone formation, we analyzed serum osteocalcin levels and found no difference between wild-type and *Cyp7b1<sup>−/−</sup>* mice (Figure 3.13A). After OVX, *Cyp7b1<sup>−/−</sup>* mice, but not wild-type
controls, tended to have lower osteocalcin levels, and this reached significance after E2 treatment. Since there was no change in osteocalcin in OVX wild-type mice treated with or without E2, we do not think that this parameter is controlled by E2/ER under normal conditions. Instead, our data suggest an E2- or ER-independent effect of 27HC on osteocalcin, which is somehow manifest most clearly in OVX mice. These in vivo data are consistent with our findings in primary OB cultures, specifically that 27HC decreased OB proliferation in a way not reversible by E2, and perhaps it is through this action that 27HC impinges on bone formation. Further studies are needed to determine if the effects of 27HC on bone formation are mediated by OBs and whether this leads to impaired bone mineralization or response to bone injury.

**Figure 3.13:** Markers of bone formation and resorption correlate with the overall bone phenotype

(A) Serum was collected and analyzed for osteocalcin by ELISA. (B) Urine was collected and analyzed for DPD crosslinks. For both, *p<0.05 compared to the sham-operated Cyp7b1+/+ group (ANOVA with SNK).

Deoxypyridinoline (DPD) crosslinks are excreted unmetabolized in urine during the process of bone resorption. At baseline, the Cyp7b1−/− mice have increased urine DPD compared to wild-type mice, and although this was not further increased by OVX, DPD levels were modestly decreased in these mice after treatment with E2, suggestive of an ER-dependent effect of 27HC on bone resorption (Figure 3.13B). The data from wild-
type mice support this conclusion, as E2 treatment completely inhibited the OVX-induced increase in bone resorption. Our in vitro data did not offer a mechanistic understanding of these physiological data, as differentiation of RAW cells into OCs was not altered by the presence of 27HC. It is possible, however, that 27HC influences OC activity, OC numbers in the bone, OC apoptosis, other factors that impinge on bone resorption, or other cell types that secrete signaling molecules that impact some aspect of OC biology. Gene expression analysis of the calvaria of the wild-type and Cyp7b1−/− mice subjected to OVX and treatment with E2 was uninformative (Supplemental Figure 3.14).

3.3 Discussion

3.3.1 27HC is a potential endogenous ER antagonist in the bone

Numerous links have already been established between cholesterol levels and bone quantity, however a mechanism of action has yet to be clearly elucidated. Our data presented here suggest that the creation of 27HC from cholesterol is an important link between this metabolic precursor and bone integrity. Female mice with a pathologic elevation of 27HC due to disruption of the catabolic enzyme Cyp7b1 have decreased BMD in the lumbar spine accompanied by significant defects in trabecular bone. Further, when these Cyp7b1−/− mice undergo OVX, they experience loss of cortical bone, as assessed in the mid-femur region. This implies that 27HC, while a partial agonist in the breast, is an ER antagonist in the bone as it is in the cardiovascular system, and thereby continues to be characterized as an endogenous SERM.

As with all genetic models, assigning a function to a particular signaling molecular must be done with caution. Although 27HC is over-produced in the Cyp7b1−/− mice, other changes are also evident, such as increased levels of 25-hydroxycholesterol and changes in bile acid metabolism in the liver which could impact steroidogenesis.
However, the cholesterol and E2 levels in these mice are not significantly different from those in wild-type mice (95). To pinpoint 27HC as the mediator of the bone phenotype in the Cyp7b1<sup>−/−</sup> mice, it would be prudent to treat wild-type mice with exogenous 27HC and ascertain whether the same phenotype occurs once circulating levels have been appropriately increased; this study is currently underway in our laboratory.

However, our study of the Cyp27a1<sup>−/−</sup> mice, which completely lack 27HC, provides further indirect evidence supporting a role for 27HC in the bone. These female mice, at adulthood, did not have significantly different BMD than wild-type mice, but did exhibit changes in trabecular bone architecture. The increased trabecular number could indicate a developmental impact of 27HC, an impact on normal age-related trabecular loss, or as evidenced by our in vitro data, an effect on OB proliferation; whether these depend on signaling through ER remains to be determined. On the contrary, both the absence and over-production of 27HC decreased trabecular thickness, a factor known to be under ER control, suggesting perhaps a critical balance of ER activity is necessary to maintain proper trabecular thickness.

### 3.3.2 ER-dependent and -independent actions of 27HC in the bone

Probing the biological effects of 27HC in primary T cells, primary OB precursors, and cultured OC precursors in vitro uncovered potential ER-dependent and ER-independent activities as assessed by gene regulation and differentiation. Even though ER is known to attenuate T cell differentiation and proliferation, we found only a minor effect of increasing 27HC concentration on T cell numbers in the spleen, and no effect in the bone marrow. Further, there was no measurable effect of 27HC itself on OC differentiation, nor did it influence the ability of E2 to inhibit this process. Lastly, in terms of cell-specific regulation, we found that 27HC regulated gene expression,
functional activity, and cell proliferation in primary pre-OBs. Given the in vivo data, it would not be surprising if 27HC did attenuate E2 signaling in OCs, or even modulated aspects of OC function other than differentiation, but it definitely appears that 27HC affects OB number and function both in vitro and in vivo. The effects of 27HC on OCs and OBs may also comprise both direct and indirect actions.

There were distinct factors apparently controlled in an ER-dependent and -independent manner within the bone, as assessed by the effect of exogenous E2 supplementation of Cyp7b1−/− mice. Trabecular thickness, BMD, and bone volume/total volume fraction all returned close to normal in the Cyp7b1−/− mice when treated with E2, but there was no significant change in trabecular number or trabecular separation. Interestingly, trabecular number is decreased by OVX, but this cannot be completely due to loss of endogenous E2 since E2 supplementation does not inhibit this decrease. It has been suggested that follicle-stimulating hormone (FSH), whose release from the pituitary is increased by OVX, or inhibins A or B directly lead to changes in bone volume (167). Further, FSH has been shown to regulate cytokine production by immune cells, and could thus complicate the ability to define the role of ER and E2 in OVX-induced bone loss. It is therefore prudent to remember that OVX does not solely remove endogenous E2; there are additional accompanying biological alterations that must be considered. Additionally, there are reported effects of E2 on trabecular bone in the ERα/ERβ double knockout mouse, suggesting perhaps that E2 signals in an ER-independent manner under certain circumstances (168). Another hypothesis is that different compartments in the bone utilize ERα versus ERβ and classical (ERE) versus non-classical (AP-1/Fos/Jun, NFκB, Sp1) signaling pathways to differing extents (66, 168), and that these pathways may be differentially regulated by 27HC.
3.3.3 A role for 27HC in bone development

The differentiation of mesenchymal progenitor cells into adipocytes, myoblasts, or OBs is under delicate control. During the aging process, changes in the balance between formation of adipocytes versus OBs in the bone microenvironment have been hypothesized to contribute to age-related bone loss, which coincides with an increase in bone adiposity (169). Oxysterols, such as 22-hydroxycholesterol (22HC), are thought to be osteo-inductive through modulation of hedgehog, Wnt, and PI3K signaling in progenitor cells (170). Given the structural similarity between 22HC and 27HC, it is not unreasonable to hypothesize that 27HC may also affect the differentiation of mesenchymal progenitor cells into OBs. Whether this control of differentiation is most important during development or post-natal homeostatic control of bone density remains to be seen. Analysis of trabecular and cortical bone by μCT imaging of young (10 day old) wild-type, Cyp7b1−/−, and Cyp27a1−/− mice will be necessary to characterize a potential role for 27HC signaling in early bone development. Interestingly, the role for ER in early bone development has not been as thoroughly characterized as its role in pubertal bone growth, growth plate closure, and menopausal bone loss. However, the data available suggest that ER is expressed in neonatal bone within OCs, OBs, and chondrocytes within the growth plate (171), and that an alteration in prenatal exposure to estrogens permanently impacts bone development and homeostasis (172).

3.3.4 Potential 27HC-mediated regulation of LXR in osteoblasts

Recent data suggest that both isoforms of LXR (LXRα and LXRβ) play an important role in homeostatic bone maintenance, although the data is limited (173). LXRα and LXRβ were found to be expressed in OBs and OCs, although LXRβ was the predominant subtype. mRNA expression of these receptors does not change during OB
or OC differentiation, nor does the absence of one subtype influence the expression level of the other subtype (173). Interestingly, differences in OC regulation were found between trabecular bone, where LXRβ was critical, and cortical bone, where LXRα had a more significant effect. Besides increasing OC survival and/or differentiation, LXRβ may negatively modulate markers of OB activity. On the other hand, LXRα mainly influenced OC activity, as evidenced by the increase to total BMD in Lxra−/− female mice (173).

The cognate ligand for LXR is 22HC, an oxysterol that is structurally related to 27HC. Although an initial study suggested that 27HC does not significantly regulate LXR (95), studies by our group and others indicate that 27HC increases the transcriptional activity of LXRβ and thus may be a second endogenous ligand for these receptors (Supplemental Figure 3.15) (174, 175). Therefore, it is possible that the ER-independent activities of 27HC in the context of the bone can be attributed to regulation of LXR, and in particular LXRβ. Further studies are necessary to ascertain the contribution of signaling through ER versus LXRβ in mediating the biological effects of 27HC.

3.3.5 Conclusion

Given the current increase in obese individuals (approximately 34% of Americans), and the fact that many (up to 75%) of those people have high cholesterol, it is increasingly important to understand the many ways that cholesterol impacts normal physiology. The direct correlation between cholesterol and 27HC levels suggests that a significant portion of the population is also experiencing a potentially pathologic elevation in 27HC. Further, obesity and cholesterol increase with age, and thus inversely correlate with estrogen levels. Our data suggest that there is high potential for 27HC to impact estrogen/ER signaling in the bone, and that removal of endogenous estrogens
exacerbates the effects of 27HC. A complete mechanistic understanding of the ways that 27HC alters signaling pathways through ER, LXR, and potentially other proteins as well, will allow a better diagnostic of the risk for osteoporosis in obese/hypercholesterolemic individuals, and will aid in appropriate selection or development of therapeutics.
3.4 Supplemental Data

![Gene expression analysis graphs]

Figure 3.14: Gene expression analysis

The calvaria from each mouse was harvested, flash frozen in liquid N₂, and stored at -80°C. RNA was isolated using Trizol extraction followed by DNase treatment. cDNA prepared from isolated RNA was subjected to qRT-PCR analysis for analysis of target gene expression. Data are presented as the normalized expression relative to cyclophilin and shown as the mean±SEM. *p<0.05 compared to Cyp7b1+/+ sham-operated mice, ^p<0.05 compared to Cyp7b1−/− sham-operated mice (ANOVA with SNK).
Figure 3.15: LXRα is activated by 27HC

Hela cells were transfected overnight with an LXRα expression plasmid, the xDR4-luc reporter, and the CMV-βgalactosidase (βgal) transfection control. Cells were treated overnight with vehicle or increasing concentrations of the LXRα agonist T0901317, 22(R)HC, or 27HC, then harvested and assayed for luciferase and β-gal activity. Vehicle treatment was not different from treatment with 10 pM 22(R)HC. Data is the mean±SEM for a representative triplicate experiment.
4 Anti-estrogen mediated ER degradation

4.1 Introduction

It has been well established that estrogens are a major contributor to the growth and metastasis of ER-positive breast cancer. These mitogens mediate their action through binding to one of two genetic isoforms of ER, ERα or ERβ (3, 5). Binding of E2 to ER induces an activating conformational change that leads to receptor dimerization, DNA binding, recruitment of transcriptional cofactors, and expression of target genes. Coactivator recruitment, which is necessary for transcriptional activity, is dependent on the E2-induced conformational change in ERα that results in the presentation of unique protein-protein interaction surfaces.

The clinical benefit of modulating ER signaling has been clearly established, and thus many diverse classes of ER modulators have been developed. SERMs, such as TAM, competitively bind to ER, alter the interaction of coactivators with the receptor, and in some cases recruit corepressor proteins that actively repress ER target gene expression, leading to context-specific agonist and antagonist activity. SERDs, such as Fulvestrant (ICI), also bind competitively to ER and disrupt coactivator-receptor interactions. However, they have an additional function in that they induce rapid degradation of ER protein. This specific activity is likely to contribute to their efficacy in patients who have already failed one endocrine therapy or in patients with metastatic breast cancer (176-181). Unfortunately, there exist clinical limitations to therapy with ICI stemming from poor bioavailability and pharmacokinetics/pharmacodynamics (176, 179-182). Thus, there is a need to develop better SERDs and to attain a more complete understanding of the regulation of ER protein levels in breast cancer cells.
4.1.1 Degradation differs between E2- and ICI-bound ERα

Interestingly, the ligand-induced conformations and pathways of degradation differ between ERα and ERβ, despite a high degree of structural similarity in their LBDs. The specific conformational changes induced by E2 lead only to degradation of ERα, not ERβ, through the ubiquitin-proteosome 26S pathway (116, 117). Further, ICI stabilizes ERβ (183), in stark contrast to the rapid degradation it induces of ERα. Together, this indicates that ERα and ERβ have subtle structural differences that dictate distinct protein-protein interactions and ultimately receptor degradation, and these differences can be exploited to further our understanding of ligand-mediated receptor turnover.

Degradation of ERα, but not ERβ, is essential for efficient E2-mediated transcriptional activity, and the induction of transcriptional activity is necessary for efficient E2-mediated degradation of ERα (118). Given the link between E2-mediated transcription and degradation, it was not surprising to find that an important ERα coactivator, AIB1, was involved in the E2-induced degradation of ERα in MCF7 cells (13). However, AIB1 is not required for ERα degradation by ICI (13), suggesting that the pathways triggered by E2 and ICI that lead to receptor degradation are not identical. Furthermore, it is known that E2 and ICI cause distinct ubiquitination patterns of ERα, perhaps due to the recruitment of different E3 ligase complexes to the ligand-bound receptor (118). A general characteristic that is associated with degradation is the exposure of hydrophobic patches on a protein surface (184, 185). Binding of E2 or ICI to ERα causes increased exposure of such hydrophobic patches on the receptor surface, but ICI increases the surface hydrophobicity of ERα significantly more than E2 (185). This indicates that although E2 and ICI treatments cause conformational changes in ERα that
increase its hydrophobicity and lead to receptor ubiquitination and degradation through the 26S proteosome pathway, the mechanisms targeting ERα for degradation are by inference distinct.

Indeed, recent data highlight the differences in ERα following binding of E2 or various SERDs (186). Not only does each SERD induce a unique conformation of ERα, they facilitate differential protein-protein interactions that lead to alternate trafficking of the receptor (186). Specifically, a peptide termed AEIP (anti-estrogen interacting peptide) interacts uniquely with ICI-bound ERα and can block the degradation of this ligand-receptor complex, suggesting that an endogenous protein with a motif similar to that of AEIP is required for efficient ICI-mediated degradation of ERα (186). This interaction motif is not required for E2- or other SERD-mediated degradation of ERα, again highlighting the unique actions of each ligand-ER complex.

4.1.2 The role of Helix 12 in ER degradation

Like all NRs, the LBD of ER consists of twelve alpha helices that function together to bind ligand and cofactors, and thus regulate ER transcriptional activity. Studies have demonstrated that the critical ligand-specific conformational change in ERα associated with receptor degradation is the change in position of H12 within the LBD (185, 187, 188). Due to its conformational flexibility, H12 plays a primary role in cofactor protein recruitment, which dictates ERα transcriptional activity. Deletion mutants that eliminate H12 or point mutations within this helix (D538A, E542A, D545A) block coactivator recruitment and stabilize ERα, leading to impaired E2-induced ERα transcriptional activity (116). Furthermore, 4OHT stabilizes ERα through a positional change in H12 within the LBD, a part of its efficacy in blocking ERα transcriptional activity. In contrast, ICI binding to ERβ abolishes all interactions between H12 and the
LBD and physically prevents H12 from associating with the coactivator binding site (188). A similar displacement of H12 by ICI may occur as well on ERα and be important for the rapid degradation of the receptor. This movement of H12 could expose the hydrophobic residues within this helix and account for the increase in surface hydrophobicity. The ligand-specific effects on H12 and the subsequent changes in ERα stability suggest that this helix may recruit the protein(s) necessary for ERα degradation. These conformational changes and their effects on ERα activity may explain the pharmacological differences between estrogens, SERDs, and SERMs.

Although both agonist- and antagonist-mediated ERα degradation involve the 26S proteosome pathway, it is clear that the mechanisms are distinct (13). Nevertheless, data suggest that both E2 and ICI depend on H12 and receptor degradation for their respective activities. ERα relies on the conformational changes induced by E2 binding for the recruitment of cofactors and degradation machinery, both of which are critical for and dependent upon efficient E2-mediated transcriptional activity. In contrast, ICI can inhibit E2-mediated transcriptional activation in breast cancer by inducing rapid degradation of ERα, among other mechanisms. Therefore, it is important to understand the links between ERα conformation, degradation, and transcriptional activity to aid in the development of better pharmaceuticals to block the mitogenic actions of E2-ERα in breast cancer.

4.2 Results

4.2.1 The importance of the LBD in mediating ER degradation

The differences between ERα and ERβ in terms of ligand-mediated receptor degradation can be utilized to gain a better understanding of the residues and protein-protein interaction surfaces on ERα that are required for ICI-induced degradation.
Chimeras of ERα and ERβ were used previously to define domains of both receptors that are involved in transcriptional activation (100), so we started with a similar approach to determine if the regions necessary for ERα degradation are sufficient to induce ligand-mediated degradation of ERβ.

First we confirmed that ERα and ERβ behaved appropriately in our system for studying receptor degradation. MCF7 cells endogenously express ERα, but not ERβ, so we transiently expressed ERβ with a v5 tag since there are not reliable antibodies available for the detection of ERβ. Treatment with E2 or ICI led to ERα degradation as expected, whereas ERβ remained stabilized in the presence of either ligand (Figure 4.1).

![Figure 4.1: The influence of ligand on ER protein stability](image)

MCF7 cells were transfected with pcDNA3.1v5-ERβ and pEGFP (green fluorescent protein) for 24 hours prior to treatment with vehicle, 100 nM E2, or 100 nM ICI for 4 hours. Whole cell extract was resolved by SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblotting for endogenous ERα (CYT18 as a loading control) or transfected v5-ERβ (GFP as a transfection control).

Given that the LBD has been implicated in ligand-mediated degradation of ERα, we wanted to begin with this region in making chimeras of ERα and ERβ. Therefore, we replaced the LBD of ERβ with that of ERα (Figure 4.2A). To start, we examined the transcriptional activity of ERα, ERβ, and ERβ-αLBD on a 3XERE-TATA-luc reporter. The three receptors were comparable in terms of activating transcription in the presence of E2, and none were significantly activated nor repressed by 4OHT (Figure 4.2B). It is known that ICI behaves like an inverse agonist on ERα by bringing the basal
transcriptional activity of this receptor below that in the presence of vehicle. This was not the case for ICI-bound ERβ, as ICI merely blocked transcriptional activation without any active repression below the basal level (Figure 4.2B). Interestingly, introduction of the LBD of ERα into ERβ transferred the ability of ICI to behave as an inverse agonist, and treatment of ERβ-αLBD with ICI resulted in transcriptional activity below basal (Figure 4.2B). This change in transcriptional activity correlated with the ability of ICI to induce degradation of ERβ-αLBD protein levels (Figure 4.2C). Although not as robust, E2 may also be able to induce degradation of ERβ-αLBD, however this result needs to be verified.

**Figure 4.2: Introduction of the LBD of ERα into ERβ allows for ICI-mediated degradation**

(A) The LBD of ERβ was replaced with that of ERα to create ERβ-αLBD. (B) Hela cells were transfected overnight with pcDNA3.1nv5-ERα, -ERβ, or -ERβ-αLBD along with 3XERE-TATA-luc and the CMV-βgal transfection control. After 24 hours, cells were treated for 24 hours with vehicle, 100 nM E2, 100 nM 4OHT, or 100 nM ICI and then harvested and assayed for luciferase and β-gal activity. The right panel is the same data magnified to show the effects of ICI. Data is the mean±SEM from a representative experiment. (C) MCF7 cells were transfected with pcDNA3.1nv5-ERβ or -ERβ-αLBD and pEGFP for 24 hours, then treated for 4 hours with vehicle, 100 nM E2, or 100 nM
ICI. Whole cell extract was resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for the V5 tag or GFP as a transfection control.

### 4.2.2 Specific residues in H12 are involved in ER degradation

Given the importance of H12 in transcriptional activation and turnover of ERα, we first examined the specific amino acid sequences of ERα and ERβ within the H12 region to identify any differences. A sequence alignment revealed two residues that differ between the two receptors in this region: Leucine (L) at position 536 of ERα versus Valine (V) at position 487 of ERβ, and aspartic acid (D) at position 545 of ERα versus asparagine (N) at position 496 of ERβ (Figure 4.3A). Therefore, we used a site-directed mutagenesis strategy to modify the residues in ERβ to match those present in ERα. Since the most dramatic difference in terms of amino acid identity was between D545 of ERα and N496 of ERβ, we focused our studies on that mutant.

We first evaluated ERβ-N496D and ERβ-V487L in terms of transcriptional activity, and were surprised to see that both mutants showed active repression in the presence of ICI, indicating that these two residues may play a role in the ability of ICI to actively repress the transcriptional activity of ERα (Figure 4.3B).

We next determined the ligand-bound stability of ERβ-N496D and compared it to wild-type ERβ. After treatment with E2 for 4 hours, neither ERβ nor ERβ-N496D were degraded (Figure 4.3C). Interestingly, after 4 hours of ICI treatment, ERβ-N496D underwent significant ligand-induced degradation (Figure 4.3C). Together, these data indicate that D545 of ERα is important in mediating ICI-induced receptor degradation, and that transfer of this residue into ERβ was sufficient to lead to receptor degradation after treatment with ICI, but not E2.
Figure 4.3: Mutation of N496 in ERβ allows for ICI-mediated degradation

(A) Sequence alignment of H12 and surrounding region of ERα and ERβ. (B) Hela cells were transfected with pcDNA3.1nv5-ERα, -ERβ, or -ERβ-N496D, or ERβ-V487L along with 3XERE-TATA-luc and the CMV-βgal transfection control. After 24 hours, cells were treated for 24 hours with vehicle, 100 nM E2, or 100 nM ICI and then harvested and assayed for luciferase and β-gal activity. (C) MCF7 cells were transfected with pcDNA3.1nv5-ERβ, or -ERβ-N496D and pEGFP for 24 hours, then treated for 4 hours with vehicle, 100 nM E2, or 100 nM ICI. Whole cell extract was resolved by SDS-PAGE, transferred to nitrocellulose, and immunoblotted for the V5 tag or GFP as a transfection control.

4.3 Discussion

Degradation of ERα is dependent on receptor conformation and is critical to E2-mediated transcriptional activity. Therefore, the conformational changes induced by different ligands and the corresponding differences in receptor degradation are pharmacologically important. Crystal structures of ligand-bound ERα and ERβ indicate that H12 is a highly mobile helix that adopts distinct conformations in response to ligand binding. These positions significantly alter the overall surface hydrophobicity and affect the availability of the coactivator/corepressor interaction domains. Protein-protein interactions at or near H12 are important for ligand-mediated degradation, as evidenced by the identification of a unique interaction motif on ERα that is responsible for ICI-induced degradation (186).
Using chimeras of ERα and ERβ, we showed that the LBD of ERα is sufficient for mediating E2- and ICI-mediated receptor degradation. Introduction of the LBD of ERα into ERβ allowed for E2- and ICI-induced degradation, which suggests that the differences between ERα and ERβ within the LBD account for their differences in degradation, but does not exclude the possibility that regions outside the LBD are also involved in this process.

Data suggest that H12 is the specific region within the LBD that dictates ligand-bound ER stability. Upon ligand binding, H12 moves to form the coactivator binding groove that allows for important protein-protein interactions that lead to biological responses. A crystal structure of ERβ bound to ICI suggests that H12 is highly mobile (188), but it is not certain that the same can be said for the structure of ICI-ERα. Within the region surrounding and containing H12, there are two residues that differ between ERα and ERβ, so we made mutations in ERβ to replace those residues with the corresponding residue from ERα. With these ERβ mutants, we found that both L536 and D545 of ERα are important in dictating the inverse activity of ICI in terms of transcription. Further, we found that D545 is sufficient for ICI-mediated degradation. When the corresponding residue on ERβ is mutated to aspartic acid, this mutant ERβ undergoes rapid ICI-mediated degradation. It remains to be seen if residue L536 of ERα is also important for this activity. Additionally, it is necessary to see if introduction of the particular residues of ERβ into ERα inhibits ICI-mediated degradation and its inverse agonist activity on transcription.

The goal of making these chimeras was to gain a better understanding of the structural requirements for anti-estrogen-mediated ER degradation. Once the protein-protein interaction surfaces that are required have been mapped, we can use phage
display technology to identify proteins that bind to those particular surfaces. Further, perhaps the surfaces/residues on ER required for degradation differ among the SERDs, and by identifying the interacting proteins we can further classify the SERDs, and potentially be able to link a particular protein-protein interaction with a biological outcome.
5 Biological regulation of the aryl hydrocarbon receptor by 4OHT in models of breast cancer and bone remodeling

5.1 Introduction

Breast cancer is the most common cancer diagnosed in women, leading to an estimated 40,000 deaths in 2007 in the U.S. alone (15). Among the most well-studied contributors to the growth and progression of breast cancer is the endogenous hormone E2 and its cognate receptor, ER. A member of the NR superfamily of ligand-inducible transcription factors, ER elicits biological changes in response to E2 by altering the transcriptome and interacting with rapid intracellular signaling pathways. There are two genetic forms of ER, alpha and beta, that exhibit overlapping but distinct expression patterns and biological functions (3-5). However, it is clear that ERα and ERβ impact each other's activity, with ERβ generally considered to repress the actions of ERα when the two receptors are co-expressed (100).

Drug discovery efforts have focused on the development of agents that inhibit the actions of ERα as treatment for ER-positive breast cancer and other estrogen-associated pathologies. The development of SERMs has led to a better understanding of the numerous actions of ERα, both within and outside of the reproductive tract. The SERMs, such as TAM and RAL, are an interesting class of molecules in that they can function as tissue-specific agonists and antagonists. For example, in relation to ER activity, TAM is an antagonist in the breast but an agonist in the bone and the uterus, whereas RAL is an antagonist in both the breast and the uterus but maintains agonist activity in the bone (101, 189). Both ligands have efficacy as breast cancer chemotherapeutics, but whereas TAM decreases the risk of both invasive and
noninvasive breast cancer, RAL only decreases the risk for invasive breast cancer (189). A prevailing hypothesis is that the tissue-specific activity attributed to these compounds is due to differential cofactor recruitment by the SERM-ER complex, which is dependent on the distinct ligand-induced conformation of ER that allows for the presentation of unique protein-protein interaction surfaces. The biological activity of SERMs then hinges on the relative and absolute expression level of both coactivator and corepressor proteins in different cell types (190).

However, in the past twenty years data has accumulated suggesting that anti-estrogens (SERMs) have off-target effects that contribute to their observed biological activity. Additionally, differences in ligand-induced ER structure and cofactor interaction have not been able to fully account for the different activities of SERMs. There are two potential types of off-target effects, one referring to the ability of the ligand to allow ER to interact in an ectopic manner with cofactor proteins that do not interact with E2-bound ER (ER-dependent actions), and the other referring to the ability of the ligand to interact with another protein entirely (ER-independent actions). An example of an ER-dependent off-target effect comes from studies with TAM, in which it was shown that the corepressor proteins NR corepressor (NCoR) and silencing mediator of retinoid and thyroid hormone receptors (SMRT) interact with TAM-bound ER in MCF-7 cells, resulting in the repression of target gene expression (191). Conversely, ER-independent actions of TAM and its metabolite 4OHT include reversible and irreversible inhibition of protein kinase C (PKC) (192), interaction with calmodulin (193) and regulation of phospholipase D (PLD) activity (194). Perturbation of this signaling pathway by increasing intracellular calcium and modulating the activity of PKC, calmodulin, and PLD may contribute to induction of apoptosis by TAM and 4OHT in an
ER-independent manner. Lastly, TAM and 4OHT bind to and inhibit the transcriptional activity of estrogen-related receptor gamma (ERRγ) (195), suggesting that SERMs have the potential to modulate other NRs or other ligand-binding proteins besides ER.

Recent gene expression studies have highlighted the differences among SERMs, distinguishing these compounds from E2 and from one another (196-198). From the observations of ER-independent actions of anti-estrogens, it followed that they may elicit unique gene transcription programs. Microarray data highlights that SERMs can regulate the expression of a subset of genes that are not responsive to estrogens, therefore making it likely that these actions are in part mediated by a factor other than ER (196-198). In such a study of unique genes regulated by SERMs, known targets of AHR were identified, including CYP1A1 and 1B1, TNFα, NQO1, and suppressor of cytokine signaling 2 (SOCS2) (199-202). This suggested that SERMs could potentially modulate the AHR signaling axis either directly or indirectly.

One of the most well-studied functions of AHR, a bHLH-PAS family transcription factor, pertains to its role in mediating the effects of the environmental contaminant TCDD, but the normal physiological functions of AHR are slowly being revealed. Once bound by agonist, AHR displaces an inhibitory Hsp90 complex, enters the nucleus, and binds to its required partner, ARNT. There, the AHR/ARNT complex controls transcription of target genes through direct DNA binding to AHREs or indirectly through interaction with Sp1 (203). Activated AHR also interacts with growth factor and cytokine signaling pathways, including c-SRC (204), EGFR (205), and the RelB subunit of NFκB (206). Relevant to this study, it has been demonstrated that AHR interacts directly with ER, and that ligand-activated AHR interferes with ER binding to common transcription factors such as Sp1 (43, 207). In general, agonist-activated AHR is thought
to inhibit ER activity, in some cases to induce degradation of ER at the protein level, and thus elicit an anti-estrogenic effect. Alternatively, ARNT interacts with ER in a ligand-dependent manner to coactivate transcription of ER target genes, rendering it unavailable to bind AHR and thus potentially inhibiting AHR responses (208).

Given the observation that several SERMs activate AhR target genes, we set out to investigate the potential role of AHR in mediating aspects of TAM pharmacology, with a specific focus on 4OHT, in both the breast and the bone. Understanding the unintended actions of SERMs, both favorable and unfavorable, is important for elucidating the exact mechanisms of action of each ligand. With this information, better pharmaceuticals can be developed that retain beneficial ER-dependent and ER-independent properties. Additionally, given that TAM has been extensively used clinically, an understanding of how it impacts AHR signaling may give us insight into the physiological roles of AHR.

5.2 Results

5.2.1 4OHT regulation of CYP1A1 expression is independent of ER

We began our studies in the MCF7 cell line, a well-characterized model of estrogen-dependent breast cancer. CYP1A1 was found to be robustly up-regulated by the SERMs TAM and RAL by microarray analysis (Table 5.1), and since the CYP1A1 promoter contains multiple consensus AHREs, we analyzed its expression and used this as a proxy for AHR activation. In our studies we used the classic AHR agonist β-naphthoflavone (BNF) as a control.
Since 4OHT is a well-characterized ligand for ER, we were interested in determining the role of ER in 4OHT-mediated regulation of AHR target genes. ER and AHR are known to physically interact, therefore it was possible that 4OHT-bound ER modulated the expression of this class of genes via an indirect mechanism. To test this, we used siRNA to knock-down expression of ERα in MCF7 cells and then examined the induction of CYP1A1 following 4OHT treatment. We were unable to detect ERβ in MCF7 cells, and thus assume ERα to be the only ER subtype present. Using two different siRNA constructs targeting ERα (siERα A and siERα C), ERα expression was reduced by greater than 90%. 4OHT treatment led to a significant increase in both CYP1A1 and CYP1B1 expression in control cells as well as in cells transfected with either siERα A or siERα C (Figure 5.1). Induction of the ER target gene PR by E2 was completely blocked by siERα A and siERαC, but not control siRNA (Figure 5.1). Interestingly, CYP1B1 has been reported to be partly regulated by an ERE in addition to AHREs (209), however our data suggest that AHR is more crucial in transducing the effects of 4OHT on this promoter in this particular context.

Table 5.1: Microarray studies identify AHR target genes as regulated by SERMs

<table>
<thead>
<tr>
<th>SERM</th>
<th>AHR Target Gene</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>RAL, 4OHT</td>
<td>CYP1A1</td>
<td>Frasor 2004</td>
</tr>
<tr>
<td>RAL, 4OHT</td>
<td>CYP1B1</td>
<td>Glidewell-Kenney 2005, Frasor 2004</td>
</tr>
<tr>
<td>TAM</td>
<td>TNFα</td>
<td>Glidewell-Kenney 2005</td>
</tr>
<tr>
<td>4OHT</td>
<td>NQO1</td>
<td>Montano MM 1998</td>
</tr>
<tr>
<td>TAM</td>
<td>SOCS2</td>
<td>Itoh 2005</td>
</tr>
</tbody>
</table>
ERα-positive MCF7 cells were transfected with either of two unique siRNA duplexes to ERα (siERα A and siERα C) or siRNA control (Mock). After 48 hours, the cells were treated for 4 hours with either vehicle, 100 nM 4OHT, or 1 nM E2. Cells were harvested and cDNA prepared from isolated RNA was subjected to qRT-PCR analysis. Data are normalized to 36B4 and expressed as mean fold change over vehicle ± SEM.

To further confirm that 4OHT does not require ER to activate AHR, we examined the ability of 4OHT to induce CYP1A1 and CYP1B1 expression in two ER-negative breast cancer cell lines, the MDA-MB-231 and SKBR3 cells. 4OHT treatment resulted in a significant increase in CYP1A1 and CYP1B1 expression in both cell lines (Figure 5.2A and B). These data suggest that 4OHT-mediated regulation of AHR target genes does not require ER expression, and thus regulation of AHR is an ER-independent off-target effect of 4OHT.
Figure 5.2: 4OHT increases expression of AHR target genes in ER-negative breast cancer cells

Activation of AHR was examined in the ERα-negative breast cancer cell lines (A) MDA-MB-231 treated for 4 hours with vehicle, 10 nM BNF, or 100 nM 4OHT or (B) SKBR3 treated for 4 hours with vehicle, 100 nM BNF, or 100 nM 4OHT. Cells were harvested and cDNA prepared from isolated RNA was subjected to qRT-PCR analysis. Data are normalized to 36B4 and expressed as mean fold change over vehicle±SEM.

4OHT is commonly used at a dose of 100 nM – 1 µM in studies of ER action, so we began our studies within this concentration range. However, concentrations of 3 – 200 nM 4OHT are achieved in circulation following treatment with the parent drug TAM (210, 211), so we analyzed AHR activity at doses that were expected to be clinically important. The data shown in Figure 5.3 demonstrate that at concentrations of 4OHT as low as 10 nM, there was significant induction of CYP1A1. The maximal activity elicited by 4OHT did not reach that of BNF, most likely due to differing affinities for AHR, but the time course of activation was similar (Figure 5.3). The activation of AHR leads to transient up-regulation of CYP1A1, perhaps explaining why these gene targets were not
noticed in studies with treatment times of 24 – 48 hours. We decided to use 100 nM – 1 
µM 4OHT, pharmacologically relevant doses, in the subsequent studies.

Figure 5.3: 4OHT increases CYP1A1 expression in a dose-dependent manner

MCF7 cells were treated for (left panel) 4 hours with vehicle or increasing concentrations 
of 4OHT or (right panel) with vehicle, 10 nM BNF, or 100 nM 4OHT for 1, 4, 8, or 24 
hours. Cells were harvested and cDNA prepared from isolated RNA was subjected to 
qRT-PCR analysis. Data are normalized to 36B4 and expressed as mean fold change 
over vehicle±SEM.

5.2.2 4OHT regulates AHR transcriptional activity and protein stability

Although increased expression of CYP1A1 is a well-characterized marker of AHR 
activation, it was important for us to show that AHR was required for 4OHT-induced up-
regulation of the chosen AHR target genes. Targeting siRNA to AHR (siAHR) blocked 
the induction of CYP1A1 and CYP1B1 by both 4OHT and BNF in ER-negative SKBR3 
cells (Figure 5.4) and ERα-positive MCF7 cells (Figure 5.5).
Figure 5.4: AHR is required for 4OHT-induced expression of AHR target genes in ER-negative breast cancer cells

SKBR3 cells were transfected with siRNA to AHR (siAHR) or siRNA control (Mock). After 48 hours, the cells were treated for 4 hours with either vehicle, 1 µM 4OHT, or 100 nM BNF. cDNA from RNA extracted from treated cells was analyzed by RT-qPCR, normalized to 36B4 expression, and presented as mean fold change over vehicle±SEM.
Figure 5.5: AHR is required for 4OHT-mediated induction of AHR target genes in ER-positive breast cancer cells

MCF7 cells were transfected with siRNA to AHR (siAHR) or siRNA control (Mock). After 48 hours, the cells were treated for 4 hours with either vehicle, 100 nM 4OHT, or 100 nM BNF. cDNA from RNA extracted from treated cells was analyzed by RT-qPCR, normalized to 36B4 expression, and presented as mean fold change over vehicle±SEM.

To assess whether 4OHT functions as a classical AHR agonist, we also examined the ability of 4OHT to modulate the expression of another AHR target gene, AHRR. AHRR plays an important role within a negative feedback loop that regulates AHR activity. It is not clear exactly how AHRR negatively regulates AHR activity, but the ability to repress signaling is known to require the N terminus of AHRR, which contains both the putative DNA binding and ARNT heterodimerization domains (212). In Figure 5.4, we clearly show that AHRR was also regulated by 4OHT in an AHR-dependent and ER-independent manner. Although not an exhaustive gene expression analysis study, our data strongly suggest that 4OHT can recapitulate many of the activities of classic AHR agonists in terms of target gene regulation.
In a process linked to DNA binding and transcriptional activation, AHR undergoes ubiquitin-dependent degradation through the 26S proteosome pathway in the presence of agonists such as BNF and TCDD to modulate the activity of the AHR pathway. Therefore we examined whether or not 4OHT induced AHR protein degradation. In ER-negative breast cancer cells, treatment with BNF or 4OHT for 6 hours led to a significant decrease in AHR protein expression (Figure 5.6). Thus, 4OHT modulated both AHR activation and degradation.

**Figure 5.6: Agonist-induced degradation of AHR is accomplished by 4OHT**

SKBR3 cells were treated for 6 hours with vehicle, 100 nM BNF, or 1 µM 4OHT. 60 µg whole-cell extract was resolved by SDS-PAGE, transferred to nitrocellulose, and subjected to immunoblotting for AHR or GAPDH (glyceraldehyde 3-phosphate dehydrogenase) as a loading control. A representative blot is shown.

### 5.2.3 The actions of 4OHT can be inhibited by AHR antagonists

We next sought to determine if AHR target gene regulation by 4OHT was inhibited by AHR antagonists. When treated in combination with the partial agonist ANF (α-naphthoflavone) or the pure antagonist MNF (3-methoxy-4-nitroflavone), 4OHT and BNF were unable to elicit maximal expression of CYP1A1 (Figure 5.7). Further, increasing concentrations of MNF competed in a dose-dependent manner with 4OHT to inhibit AHR (data not shown), suggesting that 4OHT may be a direct modulator of AHR.
CYP1A1 mRNA expression was examined in SKBR3 cells after treatment for 4 hours with vehicle, 1 µM 4OHT, or 10 nM BNF in the absence or presence of either 1 µM ANF or 1 µM MNF. cDNA from RNA extracted from treated cells was analyzed by RT-qPCR, normalized to 36B4 expression, and presented as mean fold change over vehicle±SEM.

5.2.4 4OHT directly increases AHR transcriptional potential

The question remained whether 4OHT-mediated up-regulation of CYP1A1 mRNA expression was a direct effect on the transcriptional activity of AHR. To ascertain this, we first made use of the transcriptional inhibitor actinomycin D (ActD). The induction of CYP1A1 in SKBR3 cells was blocked by co-treatment with ActD, proving that 4OHT-mediated up-regulation of CYP1A1 required active transcription and thereby suggested a direct effect of 4OHT on AHR transcriptional activity (Figure 5.8A).
Figure 5.8: 4OHT increases the transcriptional activity of AHR

(A) SKBR3 cells were pre-treated for 1 hour with either vehicle or 100 ng/ml ActD followed by treatment for 4 hours with vehicle, 100 nM BNF, or 1 µM 4OHT. cDNA from RNA extracted from treated cells was analyzed by RT-qPCR, normalized to 36B4 expression, and presented as mean fold change over vehicle±SEM. (B) SKBR3 cells were transfected overnight with pAHRE-TK-luc3 and then treated overnight with either vehicle, 100 nM 4OHT, or 100 nM BNF. After treatment, cells were harvested and assayed for luciferase activity. Data is the mean±SEM for a representative experiment performed in triplicate. The responses to 4OHT and BNF were significantly different from vehicle (p<0.05 by t test).

To definitively demonstrate whether 4OHT activated transcription through an AHRE, we utilized a reporter construct containing a consensus AHRE fused to luciferase, and transfected this into ER-negative breast cancer cells. We found that 4OHT and BNF increased AHR transcriptional activity as assessed by increased luciferase expression (Figure 5.8B). From this, we concluded that 4OHT increases expression of AHR target genes by increasing AHR transcriptional activity directly.

The most direct way a ligand affects target gene expression is by modulating the recruitment of the transcription factor to DNA elements. Using ChIP in SKBR3 cells, we found that treatment with 4OHT for 75 minutes increased AHR binding to the CYP1A1 promoter region, and that this increase in binding was concomitant with an increase in RNA polymerase II recruitment and acetylated histone H4 (Figure 5.9A and 5.9B). This effect was recapitulated in ERα-positive MCF7 cells (Figure 5.9C). Therefore, it appeared that 4OHT directly influenced the transcriptional activity of AHR in an ER-
independent manner by increasing occupancy of AHR at DNA response elements of target genes.

Figure 5.9: 4OHT increases the occupancy of AHR at the CYP1A1 promoter

(A) A schematic of the CYP1A1 promoter region, where 1 indicates the AHRE and C indicates the distal control region. SKBR3 (B) or MCF7 (C) cells were treated for 75 minutes with vehicle, 100 nM 4OHT, or 100 nM BNF. Cells were harvested after cross-linking and subjected to immunoprecipitation with either control IgG or antibodies to AHR, RNA polymerase II (Pol II), or acetylated histone H4 (Acetyl H4). After reversal of the cross-link, DNA was isolated and subjected to qRT-PCR analysis. There was no significant recruitment of AHR to a distal region of the CYP1A1 promoter.

5.2.5 4OHT directly binds to AHR

While competitive binding assays are used extensively for studying NRs, they are quite difficult for studies on AHR, and therefore electrophoretic mobility shift assays (EMSAs) have been used as a surrogate to examine ligand binding to AHR. An EMSA
assesses the essential hallmarks of AHR activation, which are dimerization with ARNT and heterodimeric binding to DNA. To accomplish this, we prepared recombinant human AHR and ARNT protein separately in vitro, then combined the proteins in the presence of vehicle, BNF, or 4OHT. The protein-ligand mixture was incubated with $^{32}$P-labeled oligo containing a single copy of a consensus AHRE and then resolved on a non-denaturing acrylamide gel. Both BNF and 4OHT increased binding of the AHR-ARNT complex to DNA as evidenced by retarded mobility of the radiolabeled probe (Figure 5.10). We confirmed that neither AHR nor ARNT bound to DNA alone under any treatment condition (data not shown). Despite leading to similar recruitment of AHR to the CYP1A1 promoter, 4OHT did not induce as robust binding of AHR/ARNT to the isolated AHRE as BNF, perhaps due to unique conformations of the liganded AHR/ARNT complex. It is well known that the ligand-bound structure of an NR can influence its ability to bind to response elements based on the surrounding DNA sequences, thus a similar phenomenon is plausible with AHR. It may also be that the 4OHT-bound AHR/ARNT complex is less stable on DNA, and therefore gives a lower signal by EMSA, although we did not observe the characteristic smear that appears when a complex falls apart during the electrophoresis procedure. The fact that 4OHT increased the DNA binding ability of the AHR/ARNT complex in a defined system makes it likely that 4OHT directly binds to AHR and influences its biological activity in a direct manner. As there is precedent for ligands binding to AHR outside the ligand-binding pocket, it will be necessary to use more detailed ligand binding studies to determine if 4OHT binds within the ligand-binding pocket or to a second site on the receptor (42, 214, 215). However, biophysical measurements, like microcalorimetry, are difficult to perform due to the instability of purified AHR.
AHR/ARNT binding to DNA was examined by EMSA. AHR and ARNT were translated \textit{in vitro} from pCDNA3.1nv5-AHR and pCDNA3.1nv5-ARNT. Proteins were allowed to heterodimerize in the presence of ligand prior to addition of radiolabeled oligo. Protein/DNA complexes resolved on a 5% non-denaturing acrylamide gel were dried and subjected to autoradiography.

\textbf{5.2.6 Many SERMs modulate AHR activity}

4OHT is not the only metabolite of TAM that is found in the serum or mammary tissue of breast cancer patients treated with TAM. Given the higher affinity of 4OHT than TAM for ER, it has generally been considered that 4OHT is the biologically active form of the pro-drug TAM (216). However, the circulating levels of both TAM and 4OHT are high enough to activate ER, so it is unclear what portion of the \textit{in vivo} biological activity is attributable to TAM versus 4OHT. Further, other metabolites such as N-desmethyl-TAM and the secondary metabolite endoxifen have been identified and partially characterized (217-219). Thus, we examined whether the parent compound TAM or the secondary metabolite endoxifen possessed the ability to regulate AHR.
signaling. In SKBR3 cells, we showed that 4OHT was the most active component over the specified dose range, although both TAM and endoxifen significantly induced CYP1A1 (Figure 5.11A). Interestingly, given the results of this dose curve analysis, both TAM and 4OHT would be predicted to activate AHR at the concentrations at which the drugs circulate in patients. This was not the case with endoxifen, which only exhibited AHR modulatory activity at exceedingly high concentrations relative to what has been measured in patients. Thus, both TAM and 4OHT exhibited a significant ER-independent ability to modulate the AHR signaling axis that we feel is clinically important.
Figure 5.11: Many SERMs possess the capability to regulate the transcriptional activity of AHR

(A) SKBR3 cells were treated for 4 hours with vehicle or increasing concentrations of TAM, 4OHT, or endoxifen (Endo). (B) MCF7 cells were treated for 4 hours with the vehicle or 100 nM ligand, unless otherwise indicated. For A and B, cDNA from RNA extracted from treated cells was analyzed by qRT-PCR, normalized to 36B4 expression, and presented as mean fold change over vehicle±SEM.
These findings prompted us to perform a preliminary examination of multiple NR modulators to see if the ability to regulate AHR was shared among this group of compounds. Interestingly, many other ER ligands including RAL, ICI, DPN (diarylpropionitrile), and RU58668 possessed the ability to regulate AHR in breast cancer cells, as assessed by up-regulation of CYP1A1 (Figure 5.11B). However, none of the ligands with an estradiol steroidal core were able to activate AHR, i.e. 17α- and 17β-ethinyl-E2 did not induce CYP1A1 expression (Figure 11B and data not shown). The ability of DPN to robustly activate AHR is intriguing given its widespread use as an ERβ-selective ligand (220-223). Our studies make it plausible that activities attributed to ERβ may in fact either be mediated by AHR, or may represent examples of cross-talk between the ERβ and AHR signaling pathways in systems such as the brain, prostate, and immune system. Further studies are thus warranted to determine the extent to which the biological activities of these ligands are mediated through AHR.

5.2.7 4OHT blocks osteoclast differentiation through AHR

TAM and RAL preserve BMD in post-menopausal women, and this is thought to be through tissue-specific agonist activity on ER (224). Estrogens and 4OHT inhibit OC differentiation, both directly and indirectly, as one part of the mechanism by which they preserve bone density (70, 225). AHR also plays a role in both OB and OC biology, but the physiological outcome of AHR modulation is not clear. AHR agonists have been shown to decrease OC differentiation and activity (226). Additionally, studies suggest that AHR is expressed and active in OBs, and further that the AHR agonist 3MC inhibits both OB proliferation and calcification of bones in vitro and in vivo (227, 228). We therefore wanted to test the possibility that a portion of the positive effects of 4OHT in the bone are through AHR. To accomplish this, we used an OC differentiation assay in
which RAW cells were induced to differentiate with M-CSF and RANKL. During this process, cells were first treated with vehicle or increasing concentrations of E2, BNF, RAL, 4OHT, or ICI and evaluated for OC differentiation. This confirmed that E2, 4OHT, and RAL suppress OC differentiation, and revealed that BNF also suppresses OC differentiation to a comparable degree as the ER ligands (Figure 5.12). Neither vehicle (ethanol or DMSO) nor ICI had a significant effect on OC differentiation.

![Figure 5.12: ER and AHR ligands inhibit OC differentiation](image)

Differentiating RAW cells were treated with vehicle (EtOH or DMSO) or increasing concentrations of E2, BNF, 4OHT, RAL, or ICI. Total TRAcP-positive MNCs were counted after 8 days. Data is the percent suppression compared to vehicle-treated cells from a representative experiment.

Next, we co-treated the cells during the differentiation process with E2, BNF, RAL, or 4OHT in the presence of either an ER antagonist (ICI) or an AHR antagonist (ANF). Although ICI and RAL robustly induced CYP1A1 expression in breast cancer cells, neither ligand significantly activated AHR in OCs, another example of context-selective differences between 4OHT/TAM and RAL (Figure 5.13).
**Figure 5.13: SERMs exhibit tissue-specific regulation of AHR**

*CYP1A1* expression was analyzed after treatment of differentiated RAW cells for 6 hours with vehicle or increasing concentrations of E2, BNF, 4OHT, RAL, or ICI. cDNA from RNA extracted from treated cells was analyzed by RT-qPCR. Data is the mean±SEM for a representative experiment performed in triplicate.

Surprisingly, we found that the ability of 4OHT to suppress OC differentiation was significantly inhibited by co-treatment with ANF (Figure 5.14A), indicating that 4OHT may require AHR for maximal activity in inhibiting OC differentiation, and may not solely work through ER. In contrast, the ability of RAL to inhibit OC differentiation required only ER expression and did not depend on AHR functionality. These data were independently verified (Figure 5.14B). These data indicate that 4OHT and RAL may differ in their preferential signaling through ER versus AHR in different tissue contexts, thereby extending the SERM context beyond ER to AHR. Further *in vitro* and *in vivo* studies are necessary to determine the extent of AHR involvement in mediating the biological effects of 4OHT, and perhaps other NR modulators as well.
Differentiating RAW cells were treated with vehicle, 100 nM E2, 100 nM BNF, 100 nM 4OHT, or 100 nM RAL in the presence of either 1 µM ICI or 1 µM ANF. Total TRAcP-positive multinucleated cells were counted after 8 days. Data is presented as the percent suppression compared to vehicle-treated cells from two representative experiments.

5.3 Discussion

TAM has been used clinically for more than 30 years to treat breast cancer, and for more than 10 years to reduce the risk of breast cancer in women at high risk of developing this disease. The activities of TAM, 4OHT, and endoxifen in breast cancer have primarily been attributed to their ability to inhibit ER signaling. Although clearly
an ER antagonist in the breast, the ability of TAM to exhibit context-selective agonist properties enables it to function as an anti-resorptive agent in the bone. The mechanisms underlying the increased risk for endometriosis and endometrial cancer, blood clots, and stroke are not well understood. Thus, it is not entirely clear how TAM, RAL, and other SERMs exhibit tissue-specific activities through a single signaling pathway. Differential cofactor expression and tissue-selective kinase signaling pathways could certainly play a role in this activity; however, it is also quite likely that there exist mechanisms of action of SERMs that are unrelated to their ability to bind ER. We identified one such ER-independent mechanism of action of TAM and its metabolites.

We show here that 4-OHT regulates AHR in an ER-independent manner in cellular models of breast cancer. In these models, 4-OHT induced many AHR target genes, including CYP1A1, CYP1B1, and AHRR in an AHR-dependent and ER-independent manner. 4-OHT directly affected the transcriptional activity of AHR, as shown by sensitivity to the transcriptional inhibitor actinomycin D. Further, 4-OHT increased AHR binding to the CYP1A1 promoter region in a manner analogous to the known AHR agonist BNF. Importantly, we found that 4-OHT bound directly to AHR as assessed by EMSA, which recapitulates ligand binding, dimerization with ARNT, and heterodimeric binding to an AHRE.

Not all SERMs share a common mechanism of action in the bone, although they all lead to preservation of BMD. We were therefore curious whether the ability of 4-OHT to regulate AHR was involved in its actions in the skeleton. As 4-OHT and RAL inhibit osteoclastogenesis, we examined the requirement of ER versus AHR in mediating the effects of each of these ligands, using E2 and BNF as controls for ER and AHR, respectively. Using a chemical approach, we discovered that a significant portion of the
ability of 4OHT to block osteoclastogenesis required AHR, whereas RAL depended entirely on ER expression for its function in this tissue context. The extent to which other SERMs with bone protective activity act through AHR remains to be determined, but this presents a potential explanation for differing mechanisms of action among SERMs in the bone.

5.3.1 4OHT regulation of AHR highlights a previously unappreciated activity of 4OHT at the intersection of the AHR and ER signaling pathways

It is known that the AHR and ER pathways engage in cross-talk through direct interaction, and that they also impact the other's activity by competing for transcriptional regulators such as Sp1. Further, studies show that agonist-activated AHR induces proteosome-dependent degradation of ER protein (229). Together, this suggests that AHR activation may be anti-estrogenic and could thus have anti-tumorigenic activity in the breast. However, the exact role of AHR in the breast is not clear. AHR is required for the proper development of the mammary gland and its differentiation during pregnancy, thereby positing AHR as an important regulator of both cell proliferation and differentiation (40). Further, a microarray analysis comparing gene expression in normal breast to breast carcinoma showed that CYP1A1 is down-regulated in the cancerous state, suggesting that AHR activity is decreased during tumorigenesis (data not shown). Despite cross-talk and independent roles in mammary gland physiology, an intriguing hypothesis emerges related to the ability of AHR activation to directly lead to E2 metabolism, and thus control the ER signaling pathway in this way.

E2 metabolism is controlled in a large part by CYP enzymes [for minireview, see (1)]. In particular, actions initiated by CYP1A1 and CYP1B1 lead to the production of hydroxy-, methoxy-, and quinone- derivatives of E2. CYP1A1 converts E2 to 2-hydroxy-
E2, which is transformed by COMT to form 2-methoxy-E2, an anti-tumorigenic metabolite (230). On the other hand, 4-hydroxylation of E2 by CYP1B1 leads to E2-3,4-quinone, which is associated with DNA adduct formation that can result in DNA damage and genotoxic stress and thereby potentially promote or accelerate tumorigenesis (231). Although shown in rodent models, the importance of adduct formation in humans remains controversial. AHR up-regulation of CYP1A1 versus CYP1B1 expression varies in a cell-specific manner, therefore influencing the pathway by which E2 is metabolized in a particular cell (232). The implication that 4OHT may directly affect the local metabolism of E2 through activation of AHR offers another mechanism by which 4OHT is effective in the treatment of breast cancer. On the other hand, the possibility that 4OHT increases the metabolism of E2 to the pro-tumorigenic E2-3,4-quinone through AHR offers a mechanism by which resistance to 4OHT could arise. Here, the 4OHT- and AHR-dependent increase in DNA damaging agents would increase genomic instability, leading to accumulation of mutations within the tumor that allow growth signaling to occur in the face of ER inhibition.

In spite of success in many in vitro and in vivo models of breast cancer, many SERMs and anti-estrogens have shown disappointing activity in the clinic. For example, fulvestrant (ICI), a high affinity antagonist with no agonist activity on ER in both in vitro and in vivo models of breast cancer, is not superior to TAM as a first line of therapy in breast cancer (180). It does have limited efficacy against TAM-resistant tumors (180), stressing that the mechanisms of action of the two compounds differ, but a lack of a robust response in patients perhaps points to potential pharmacokinetic hindrances. For instance, ICI can be inactivated by the UGT family of glucuronidation enzymes. The most highly expressed UGT family member in the breast is UGT1A4, which is capable of
producing significant amounts of fulvestrant-glucuronide (233). Interestingly, UGT1A4 is regulated largely by AHR through AHREs located within its promoter (234). Recent studies have shown that TAM, 4OHT, and endoxifen can also be glucuronidated as a pathway of metabolism and excretion (235). It is therefore possible that by activating AHR, SERMs and anti-estrogens are concomitantly inducing their own metabolism and excretion, thereby reducing their efficacy. The ability of each therapeutic to induce AHR, and thus up-regulate metabolic enzymes, could contribute to disappointing in vivo efficacy that is not well modeled in rodents with vastly different metabolic systems and AHR signaling pathways (236, 237). Thus, the tissue-specific expression levels of AHR and UGT family members could greatly influence the efficacy of anti-hormonal therapy in inhibiting breast cancer growth or progression.

5.3.2 Potential impacts on other estrogen-responsive tissues

4OHT does not activate AHR solely in breast cancer and OC cells; it also activated AHR in cell lines from tissues such as the liver, endometrium, and bone (data not shown). It is interesting to speculate on the role(s) 4OHT-activated AHR may play in the bone and the endometrium, two tissues where AHR plays important but unspecified roles. Although many SERMs display ER agonist activity in the bone, leading to preservation of BMD under estrogen-deprived conditions, differences have been suggested for the mechanisms of action for these therapeutics. It is possible that some differences in SERM activity in the bone microenvironment stem from the ability to modulate the AHR signaling pathway. Despite potentially conflicting data, it is obvious that AHR plays a role in bone development and homeostasis, and thus it is crucial to uncover what portion of the actions of 4OHT, and perhaps other SERMs, in bone are
through AHR, and to determine whether this ER-independent effect is therapeutically desirable.

Pre-menopausal women are given TAM with great caution due to the increased risk of developing endometrial cancer or uterine sarcoma. In the Breast Cancer Prevention Trial, premenopausal women taking TAM had an increased risk for leiomyomas, endometriosis, and ovarian cysts (238). TAM is an ER agonist in the endometrium and causes increased steroidogenesis in the ovary, thereby increasing local E2 production and leading to abnormal proliferation (239, 240). Similar to the bone, the precise role of AHR in the endometrium is unclear. In a mouse model of endometriosis, short term treatment of mice with the AHR agonist dioxin did not increase the growth of the endometriotic lesions, but instead inhibited the proliferative actions of E2 (241). On the other hand, mice exposed to dioxin at different developmental stages showed reduced expression of PR and transforming growth factor β2 (TGFβ2), both of which mimic what is seen in women with endometriosis (242). While still controversial, some epidemiological studies show an association between increased risk of endometriosis and heavier body burden of dioxin and dioxin-like compounds (243, 244). Lastly, we recently published a study showing that the Chinese herb *prunella vulgaris* (PV) inhibited the growth of endometriotic implants in mice, potentially through activation of AHR (245). While clearly AHR is expressed in the endometrium and is ligand-responsive, the role AHR plays in endometriosis is not yet fully understood. Since TAM therapy is associated with increased risk for endometriosis, studies are warranted to define the role of AHR in mediating the actions of TAM and 4OHT in this disease.

The availability of an *Ahr*−/− mouse has made possible studies designed to further understand the role of AHR in development, homeostatic processes, and disease
progression. Although viable, these mice exhibit fertility and mammary gland development defects (40, 246), poor development of the immune system and the liver (247), and various pathologies of the skin, heart, uterus, and lung (248). A great potential exists for cross talk between AHR and ER in both normal biology and in disease states, and studies can thus be undertaken with genetic mouse models to assign biological responses of 4OHT, and perhaps other SERMs, to actions through ER versus AHR.
6 Prunella Vulgaris, an AHR modulator in the endometrium

6.1 Introduction

This chapter represents a collaborative study between our laboratory and that of Dr. Bruce Lessey, with figures 1 – 3 contributed by the Lessey laboratory and figures 4 – 6 representing our work.

6.1.1 Endometriosis

Endometriosis is an inflammatory disease that affects 5 - 10 % of women within the reproductive years and is generally characterized by the presence of endometrial glands and stromal tissue outside the uterine cavity (249). Endometriosis gives rise pelvic pain and infertility, and it is believed to be an estrogen-dependent disease (249). Common treatments for endometriosis include gonadotropin-releasing hormone analogues, oral contraceptives, or progestins; however, each of these treatments comes with a host of side-effects associated with altering the estrogen signaling axis.

Endometriotic tissue is distinguished from normal endometrium by altered expression of ER and PR, elevated steroidogenesis, and increased production of pro-inflammatory cytokines (250-252). It is the increase in local de novo production of E2, and a coordinate decrease in its conversion to the less active form, E1, that underlie the progression of this disease, although these are not thought to be contributing factors in the initial development of endometriosis (253). E2 synthesis in the endometrium is partly controlled by SF-1, an NR that is absent in normal endometrium but is significantly over-expressed in endometriotic lesions due to decreases in promoter methylation (254). Increased expression of cyclooxygenase-2 results in increased prostaglandin E2 production, which activates SF-1 to control many steps within the E2
biosynthesis pathway, from cholesterol transport to the mitochondria to expression of the aromatase enzyme (255).

The inflammatory response observed in endometriosis is characterized by infiltration of macrophages, granulocytes, and natural killer cells. A milieu of IL-1β, IL-6, IL-8, TNF, monocyte chemotactic protein 1 (MCP-1), and regulated upon activation normal T cell expressed and secreted (RANTES) attract immune cells and promote the adhesion and survival of endometriotic fragments to extra-uterine surfaces (256). These cells and secreted cytokines and chemokines are also important contributors to angiogenesis.

6.1.2 Estrogen receptor action in the endometrium

Estrogens are essential for reproductive function in the mammary gland, the ovaries, and the uterus. However, in some cases the actions of unopposed estrogen can lead to breast cancer, endometriosis, or endometrial cancer (250, 257, 258). In the normal endometrium, E2 and progesterone coordinately regulate the ovulatory cycle. Progesterone is a differentiation signal for the epithelial and stromal cells, and controls the production of E2 through indirect regulation of 17βHSD. In contrast, in endometriotic tissue, PR expression decreases and progesterone resistance develops, thereby removing an important control on E2 production and ER signaling.

The development of SERMs and the use of ET/HT has highlighted both the positive and negative aspects of activation of ER, such as preservation of BMD or worsening of uterine fibroids. SERMs are characterized by their unique activity profile in terms of agonist/antagonist activity on ER. For example, TAM is an antagonist in the breast, but an agonist in the bone and the uterus. RAL, on the other hand, mimics TAM in the breast and bone, but is instead an antagonist in the uterus.
6.1.3 Aryl hydrocarbon receptor action in the endometrium

Although studies have yet to provide clear evidence that AHR activation is either positive or negative in terms of the development and progression of endometriosis, it seems apparent that AHR plays an important role in both the epithelial and stromal cells of the endometrium, as revealed by studies of the classic AHR ligand TCDD. Short term treatment of mice with TCDD did not increase the growth of endometriotic lesions, but instead inhibited the proliferative actions of E2 (241). On the other hand, exposing mice to TCDD at various developmental stages led to decreased PR and TGFβ2 expression, both of which are observed in women with endometriosis (242). PR-mediated expression of TGFβ2 down-regulates expression of matrix metalloproteinases (MMPs) and mediates the anti-inflammatory response needed to maintain pregnancy. Thus the loss of this signaling axis leads to increased MMP expression, local tissue invasion, and a profound inflammatory response. Many cytokines, such as TNF, IL-6, and IL-1β are elevated upon treatment with TCDD, leading to an active inflammatory response (242). Exposure to TCDD during pregnancy is associated with spontaneous abortion, probably due in part to the pro-inflammatory environment (259). TCDD, presumably through AHR, also increases angiogenesis at sites of endometriotic tissue implantation (260). Lastly, although controversial, some epidemiological studies show an association between elevated risk for endometriosis and exposure to TCDD and TCDD-like compounds (242-244, 261).

Another aspect of AHR involvement in the endometrium pertains to its ability to regulate E2 production and metabolism. AHR can transcriptionally regulate expression of the aromatase enzyme, and thereby control the local production of E2 in the endometrium (262). In addition, two primary AHR target genes, CYP1A1 and CYP1B1,
directly metabolize E2. The balance of these two actions within the endometrium will be crucial in defining whether AHR contributes to or alleviates E2 overproduction in this tissue. Further studies are thus warranted to ascertain the positive and/or negative roles of AHR in the endometrium.

6.1.4 Alternative medicines for reproductive diseases

Many alternative medicines have been identified that modulate ER, including primrose oil, black cohosh, and soy protein (263-265). However, many of these alternative medicines have not been evaluated in clinical trials, nor ascribed a specific mechanism of action. Given that some alternative medicines have been used for many years, they often have well-defined biological functions. Elucidating the mechanism of action will shed light on pathways important for various afflictions, and aid in the development of novel combination treatments for disease.

The botanical herb *Prunella vulgaris* (PV) has long been used as a remedy for multiple conditions due to its anti-oxidant and anti-microbial functions (266-270). In a large screen of Chinese herbs, PV was found to have anti-estrogenic activity as well (245). Based on this property, and its previous use to treat dysmenorrhea, we sought to describe its mechanism of action in the context of the endometrium. As many herbs, supplements, and environmental toxins also modulate the activity of the AHR, we also sought to determine whether PV regulates AHR. Since AHR and ER can physically interact, in many cases leading to inhibition of ER signaling, the potential for intersection of PV, AHR, and ER in the endometrium is great, and thus warranted our in vitro and in vivo studies presented below.
6.2 Results

6.2.1 The anti-estrogenic activity of PV

To evaluate the anti-estrogenic actions of PV, we used the ECC-1 cell line, a model of human endometriotic tissue that responds to estrogenic stimuli by inducing alkaline phosphatase activity. For our purposes, we used diethylstilbestrol (DES) instead of E2, since it does not activate AHR, leads to similar responses as E2, and is not metabolized by cells in vitro or in vivo. PV was the only herb tested that significantly reduced alkaline phosphatase activity, and even approached the activity of the pure ER antagonist ICI (Figure 6.1.A). PV significantly decreased cell proliferation, but importantly did not affect cell viability (Figure 6.1.B and 6.1.C). Surprisingly, ICI did not completely block DES-induced endometrial cell proliferation, suggesting either that DES has an ER-independent activity, or that saturating concentrations of ICI were not achieved.

Another marker of estrogenic action in the endometrium is the up-regulation of the connective tissue growth factor (CCN) family member Cyr61 (cystein-rich angiogenic inducer 61). Treatment of ECC-1 cells with DES increased Cyr61 expression, and this was blocked by co-treatment with PV or ICI as a control (Figure 6.2).
Figure 6.1: The anti-estrogenic activity of PV in ECC-1 cells

(A) PV and ICI inhibit alkaline phosphatase activity induced by DES. (B) Proliferation of ECC-1 cells treated with DES, DES+PV, or DES+ICI. (C) Cell viability comparing increasing concentrations of PV over 4 days of treatment. PV only significantly affected cell viability at the highest dose tested (500 µg/ml). *p<0.01, ** and ***p<0.001. Reprinted with permission from (245).

Figure 6.2: Cyr61 expression is inhibited by PV

Protein expression of CYR61 was determined by immunoblot analysis after treatment with DES in the presence or absence of ICI or PV. β-actin (ACTB) is included as a loading control. Reprinted with permission from (245).
6.2.2 PV decreases the growth of endometriotic implants

The RAG-2γ(c) knockout mouse is a validated model of endometriosis (271). In this model, human endometriotic implants placed subcutaneously grow under the influence of E2 pellets. We examined the effect on implant growth in mice treated for one month with placebo, E2, E2 plus PV, or E2 plus progesterone. Co-treatment with either progesterone or PV reduced the weight of the implants compared to E2 alone (Figure 6.3). Interestingly, the uterine wet weight did not differ between treatment groups. Importantly, 2 weeks of treatment with PV did not affect the reproductive potential of the mice, in terms of ability of get pregnant after an overnight mating with a male or the number of pups per litter.

![Figure 6.3: PV decreases the growth of endometriotic implants](image)

The wet weight of the xenografted implants of endometrium tissue was determined after mice were treated for one month with E2, E2+PV, or E2+progesterone (P). PV was delivered in the form of tea. *p<0.05, **p<0.01. Reprinted with permission from (245).

6.2.3 PV activates AHR signaling in the endometrium

There exist many mechanisms by which PV could be inhibiting the effects of E2 in the endometrium, including direct inhibition of ER or indirect inhibition of ER signaling through activation of AHR. To test the second possibility, we investigated the
ability of PV to modulate the expression of the AHR target genes CYP1A1, CYP1B1, and AHRR in ECC-1 cells. As seen in Figure 6.4, PV increased expression of these AHR target genes in a dose-dependent manner. Further, the actions of PV on AHR were inhibited by the pure AHR antagonist MNF (Figure 6.5A). The ability of PV to modulate expression of AHR target genes was dependent on the expression of AHR itself (Figure 6.5B). Lastly, using an isolated AHR response element, we were able to show that PV mimicked the actions of BNF by increasing AHR transcriptional activity (Figure 6.6).

**Figure 6.4: PV increases the expression of AHR target genes**

ECC-1 cells were treated for 4 hours with 100 nM BNF or increasing concentrations of PV (0.5 – 50 µg/ml). Cells were harvested and cDNA prepared from isolated RNA was subjected to qRT-PCR analysis. Data are normalized to 36B4 and expressed as mean fold change over vehicle±SEM. Adapted with permission from (245).
Figure 6.5: AHR is required for PV-mediated induction of AHR target genes

(A) ECC-1 cells were co-treated with 100 nM BNF, 1 µM MNF, or PV and increasing concentrations of MNF. (B) ECC-1 cells were transfected with either mock siRNA or siRNA targeting AHR (siAHR), then treated with 100 nM BNF or 50 µg/ml PV. Cells were harvested and cDNA prepared from isolated RNA was subjected to qRT-PCR analysis. Data are normalized to 36B4 and expressed as mean fold change over vehicle±SEM. Adapted with permission from (245).
Figure 6.6: AHR antagonist inhibits the PV-mediated increase in AHR transcriptional activity

ECC-1 cells were transfected with the AHRE-TK-luc3 reporter construct, then treated overnight with 1 µM BNF or increasing concentrations of PV. Cells were harvested and luciferase activity was determined and normalized to β-gal control. Data are the mean±SEM from two independent triplicate experiments. Adapted with permission from (245).

6.3 Discussion

In the area of hormonal disturbances, herbal medicines have become more commonly used, especially in the wake of the publishing of the Women’s Health Initiative studies. As there are many side effects of herbal remedies, and often serious negative interactions with traditional medicines (272), there is much concern about the use of these alternative remedies without prior consultation with a physician. However, given proper studies, alternative medicine can offer a complement to traditional Western medicine, particularly in the area of reproductive medicine.

One such herbal remedy is PV, a Chinese herb with strong anti-estrogenic activity in models of endometriosis. We discovered that this herb, while not affecting cell viability, inhibited cell proliferation and blocked ER activity. By placing endometriotic implants into RAG-2γ(c) knockout mice, we showed that oral consumption of PV significantly reduced the growth of the xenograft. Analysis of the extracted implants
suggested that ER actions in this tissue were inhibited in the PV-treated mice. Importantly, treatment with PV did not affect the reproductive ability of the mice, suggesting that PV may be a viable treatment option for endometriosis in pre-menopausal women.

Many herbs, compounds found in common food, and environmental toxins activate AHR. These include curcumin, red clover, flavinoids, resveratrol, and dioxin (273-277). Therefore, it was important to determine if PV could affect AHR activity, particularly since AHR is thought to play a role in the endometrium. Our studies showed that PV activated AHR as assessed by increased expression of AHR target genes in an *in vitro* model of endometriosis in a manner that required AHR expression and could be inhibited by AHR antagonists. This suggests that the biological effects of PV may be in part mediated by modulation of AHR signaling in the endometrium.

Cross-talk between the ER and AHR signaling pathways has already been described, adding another layer of complexity to the possible mechanisms of action of PV. It may be that the anti-estrogenic effects are indirect by way of AHR modulation. AHR induces ubiquitin-mediated degradation of ER protein (45), forms a complex with ARNT and ER at ER target gene promoters, and influences the binding of E2 to ER (278). On the other hand, AHR and ER can interact at ARHEs, thereby modulating expression of AHR target genes as well (278). These many intersecting layers of co-regulation of the ER and AHR signaling pathways complicate the elucidation of the mechanism of action of PV, but the biological endpoint of inhibiting endometriotic growth is clear. Further *in vivo* studies are necessary to understand the biological implications of the activation of AHR and inhibition of ER signaling by PV.
7 Conclusions and Future Implications

7.1 27HC is an endogenous SERM

The identification of 27HC as an endogenous SERM answers a prevailing question in the field, namely whether or not the context-selective pharmacological manipulation of ER signaling mimicked a naturally occurring activity. 27HC fits all the criteria for being a bona fide SERM: it competitively binds to ERα and ERβ, elicits a unique conformational change in both receptors, and acts in a context-specific manner to modulate the ER signaling pathway. Specifically, 27HC acts an antagonist in the cardiovascular system to attenuate the protective effects of E2 and ER, an agonist in cellular models of breast cancer where it mimics the actions of E2 on ERα, and an antagonist in the bone to decrease bone quantity. The role of 27HC in modulating the actions of ERα and ERβ in other target tissues, such as the lung, brain, and endometrium, remains to be determined. Further, the contribution of signaling by 27HC through ERα versus ERβ has yet to be comprehended.

7.1.1 Biological significance of 27HC action in breast cancer

Although 27HC was found to be a partial agonist in cellular models of breast cancer, studies are necessary to confirm this activity in vivo. 27HC may be an important contributor to breast cancer by manifesting agonist activity in a low E2 environment, such as in post-menopausal women and in particular those with high cholesterol. On the other hand, 27HC may antagonize E2 signaling in pre-menopausal women by virtue of its partial agonist activity, and thus influence breast cancer development or progression in this population as well. This finding has implications in the context of resistance to endocrine therapeutics. Despite initial success, resistance to both anti-estrogens and AIs
does occur, and often these recurrent breast cancers continue to express ER, suggesting continued dependence on estrogen signaling (279). The ability of 27HC to increase breast cancer cell proliferation makes plausible the hypothesis that 27HC acts as an alternative estrogen whose levels would not be affected by an AI. It is generally considered that the elevated total production of estrogen by breast stromal adipocytes explains the increased risk of breast cancer in obese/hypercholesterolemic patients. However, 27HC levels are likely increased in these individuals, and this molecule has ER partial agonist activity, suggesting an alternate explanation for the link between obesity/hypercholesterolemia and increased breast cancer risk.

The high production of 27HC by macrophages may place 27HC as a primary growth stimulus in the etiology of cancers with high numbers of infiltrating macrophages, such as breast and lung cancer. In both diseases, infiltrating macrophages are associated with reduced patient survival (130, 131). These macrophages produce cytokines that de-repress genes inhibited by ER and increase expression of genes involved in matrix degradation and motility (131, 132). However, an unexplored question is whether these tumor-infiltrating macrophages provide a local estrogenic source by production of 27HC in the tumor microenvironment. The in vivo implications of this hypothesis, and the overall role of 27HC in breast cancer development and/or progression, are currently being pursued in the laboratory using genetic and pharmacological manipulation of circulating 27HC levels and of macrophage production of 27HC.

### 7.1.2 The role of 27HC in regulating bone resorption and formation

The skeleton is under exquisite control by estrogen signaling, both during development and throughout adulthood. Estrogen helps maintain the proper balance
between bone formation and bone resorption, as evidenced by the increase in bone turnover after menopause that elevates a woman’s risk for osteoporosis. Resident bone marrow macrophages likely produce high levels of 27HC, and may provide a local source of this SERM in the bone environment. Here, we found that elevated 27HC negatively impacted bone quantity in vivo, through potential effects on both OBs and OCs. Further, 27HC actively antagonized ER signaling as evidenced by the ability of E2 treatment to block some, but not all, of the deleterious effects of elevated 27HC on bone. These data could alter how we assess and manage osteoporosis risk, and may promote increased monitoring of cholesterol, and perhaps of 27HC as well. Further, the widespread use of cholesterol-lowering statins may have a profound impact on the bone, particularly in post-menopausal women, through their ability to coincidentally lower 27HC levels. To better link cholesterol with the effects of 27HC in the bone, studies are underway to treat mice with elevated 27HC with statins, and after confirming a decrease in circulating 27HC level, to analyze changes in bone density. Further, we are interested in directly implicating 27HC in the Cyp7b1−/− phenotype by treating wild-type mice with exogenous 27HC or feeding them a high cholesterol diet to elevate the circulating level of this oxysterol and then analyzing bone quantity. In another avenue, our preliminary studies suggest that 27HC may play a role in bone development by impacting the growth plate, therefore we are analyzing bone development prior to and during puberty in mice with varying levels of 27HC. If this hypothesis holds, it would have critical implications for risk assessment and treatment of children with high cholesterol, as this situation could lead to profound changes in bone development.
7.2 The biological regulation of AHR by SERMs

7.2.1 4OHT regulates AHR in breast cancer

TAM has been used clinically for more than 30 years to treat breast cancer, and for more than 10 years to reduce the risk of breast cancer in women at high risk of developing this disease. The activities of TAM, and its metabolites 4OHT and endoxifen, in breast cancer have primarily been attributed to their ability to inhibit ER signaling. Although clearly an ER antagonist in the breast, TAM has agonist activity in other ER target tissues and as such preserves BMD while increasing the risk for endometriosis and endometrial cancer, blood clots, and stroke. However, it is not entirely clear how TAM, RAL, and other SERMs exhibit tissue-specific activities through a single signaling pathway. Differential cofactor expression and tissue-selective kinase signaling pathways could certainly play a role in these phenotypes, however it is also quite likely that there exist mechanisms of action of SERMs that are unrelated to their ability to bind ER. Many studies have proposed such ER-independent actions of particular SERMs, including functions in regulating kinase signaling pathways and calcium signaling. Further, there is evidence for cross-reactivity of many SERMs with other NRs, such as 4OHT-mediated antagonism of ERRγ. Through many avenues, we were intrigued by potential cross-talk between ER, SERMs, and AHR signaling, including direct protein-protein interactions between ER and AHR, regulation of ER by direct binding of AHR ligands, and microarray data suggesting an overlap in genes regulated by the SERM 4OHT and the classic AHR agonist TCDD.

Using breast cancer cells as a model, we found that there is indeed another layer of cross-talk between the ER and AHR signaling pathways mediated by SERMs, and potentially other anti-estrogens as well. In this model, 4OHT directly binds to and
regulates AHR, increasing its transcriptional activity and thereby increasing the expression of AHR target genes such as *CYP1A1* and *CYP1B1*. Coincidentally, these enzymes are key players in E2 metabolism, leading one to question whether one aspect of the efficacy of 4OHT in the treatment of breast cancer stems from an ability to increase metabolism of E2. However, these enzymes can also produce pro-tumorigenic genotoxic metabolites of E2, which through increasing DNA mutagenesis may lead to cancer progression while on treatment. The impact of estrogen metabolism on the efficacy of TAM therapy can be assessed by correlating treatment response with polymorphisms of CYP1A1 and CYP1B1 that lead to changes in the ability of these enzymes to metabolize E2, or by correlating response to the relative ratio of expression of CYP1A1 to CYP1B1. It may also be feasible to measure the intra-tumoral changes in concentration of E2 and E2 metabolites before and after treatment with TAM, and track the ability to alter E2 levels with response. With these experiments we can delineate additional mechanisms by which TAM achieves clinical efficacy, with the expectation that future therapeutic development can better exploit these particular mechanisms.

### 7.2.2 4OHT regulation of AHR in the skeleton

The bone is a clinically important ER target tissue, which led us to question whether there could be a role for ER/AHR cross-talk here as well. Both receptors are thought to be expressed and to have an impact on many cell types in the bone, including T cells, OBs, and OCs. Starting with OCs, whose differentiation is clearly inhibited by estrogen, we determined that the ability of 4OHT to also inhibit OC differentiation was not wholly dependent on ER, but instead relied in a significant manner on AHR expression. Conversely, the ability of another SERM, RAL, to inhibit OC differentiation did depend entirely on ER expression, highlighting another difference in mechanism of
action between these two SERMs. Whether 4OHT also functions to modulate OB and T cell function through either ER or AHR remains to be determined. In the end, it will be important to show that the ability of 4OHT to preserve post-menopausal bone density is dependent on AHR expression and activity; this study is currently underway in our laboratory. The sum of the actions of 4OHT in regulating both ER and AHR in breast and bone suggests that selective co-modulation of ER and AHR could be therapeutically useful.

### 7.2.3 Regulation of AHR in other ER target tissues

Herbal medicines have become quite popular, especially among women looking for an alternative to ET/HT. However, there is much concern about the use of these alternative remedies without prior consultation with a physician since there are many side effects of herbal remedies, and often serious negative interactions with traditional medicines (272). Given proper studies, alternative medicine can complement traditional Western medicine, particularly in the area of reproductive medicine. One herb of particular interest for the treatment of endometriosis is PV, which is anti-estrogenic in both *in vitro* and *in vivo* models. Similar to many other herbs and natural products, we found that PV also modulated AHR by increasing AHR-dependent transcription in cellular models of endometriosis. Therefore, the biological effects of PV may be in part mediated by regulation of AHR signaling in the endometrium, which action may also contribute to its anti-estrogenic functions. It is clear that AHR and ER both play an important role in the endometrium, and teasing out their individual actions and interplay will allow for the development of therapeutics that are not associated with the toxicity and carcinogenesis associated with either AHR or ER. This can be accomplished using mouse models of endometriosis in which expression of AHR or ER is eliminated or
in which panels of selective ligands are employed, and development or progression of the
disease is monitored. Further, our data highlights the need for a thorough
understanding of the mechanism of action of alternative medicines to appreciate their
risks and utilities, and to understand how they may interact with other therapies.

Evidence is accumulating that TAM has utility in the treatment of glioblastoma
multiforme and anaplastic astrocytoma, tumor types with no detectable expression of ER
(280-287). The ability of TAM to induce apoptosis in cultured gliomas, but not in non-
neoplastic cells, may be due to inhibition of PKC or modulation of the NFκB signaling
pathway (280, 288, 289). However, another hypothesis pertains to the demonstrated
ability of 4OHT and TAM to regulate AHR, a functionality that could also be relevant in
tumors of the brain. Expression of AHR has been found in the brain, particularly in
brain microvessels, and in glioma and medulloblastoma cell lines (290-292). Treatment
in vitro with AHR agonists perturbs proper differentiation and cell
proliferation/apoptosis under certain contexts, indicating a role for this signaling
pathway in development or progression of such tumors (291, 293). Studies thus must be
undertaken to ascertain whether any portion of the efficacy of TAM and 4OHT in
gliomas pertains to their ability to modulate AHR, or perhaps whether this functionality
attenuates their efficacy in any manner. Understanding these mechanisms will allow for
the development of better treatments for these aggressive diseases, which as of now have
abysmal survival rates (median survival ~ 12 months) (294).

7.3 Towards a better understanding of ER action

There are many aspects of ER signaling that remain to be fully elucidated.
Although there has been success with therapeutic compounds that displace agonist
binding, a new area of research in the oncology field in general is focused on targeting
protein-protein interactions as a way to develop more process-specific antagonists. To move this new way of thinking into the field of ER biology, a better understanding of the unique protein-protein interaction surfaces on ER and how they are used for distinct processes is necessary. Combinatorial peptide phage display allowed for the preliminary delineation of such surfaces on the receptor, and the nature of the peptides that bound in a ligand-dependent manner to ER offered information as to potential proteins that interact in a similar manner. Specifically, this analysis demonstrated that 27HC-bound ER adopts a conformation distinct from both E2-bound and 4OHT-bound ER, but with some similarities as well. The sequence analysis of these peptides indicated that 27HC-bound ER may signal from the actin cytoskeleton or the microtubule network, and in many ways may influence cell proliferation, migration, or differentiation. However, confirmation of these interactions in the context of cell signaling is necessary, and it will be important to ascertain their role in pathologies associated with ER using, for example, \textit{in vivo} and cellular models of breast cancer, endometriosis, or osteoporosis to manipulate the levels of these identified proteins and then assessing their impact on ER signaling.

The discovery that some anti-estrogens have utility as second-line therapies in patients that have failed at least one previous endocrine therapy supports the notion that not all anti-estrogens utilize a similar mechanism of action to disrupt ER signaling. In particular, the SERDs, which lead to rapid degradation of ER\(\alpha\) protein, are useful in some patients that have failed on TAM. However, all SERD-mediated degradation of ER\(\alpha\) is not equal as shown by the differential utilization of distinct protein-protein interaction surfaces during the degradation process, but as of now the mechanisms of action of compounds in this class are not fully elucidated. Our efforts to understand the
mechanism by which one SERD, ICI, induces ERα degradation led to the preliminary identification of residues and surfaces on ERα that are necessary for this action. From this, proteins can be identified that utilize these surfaces to bind to ERα and bridge interactions with degradation machinery. As the precise mechanisms of action are determined for each anti-estrogen, therapeutics can be combined to increase efficacy against particular pathologies while sparing the positive effects of ER biology.

The wide-spread clinical use of anti-estrogens, cholesterol-lowering statins, and alternative medicines necessitates a better understanding of how these compounds impact biological processes, both individually and in concert. We have identified a link between cholesterol metabolism and ER signaling, with demonstrated implications in breast cancer and bone homeostasis, and the potential for involvement in other ER target organs such as lung, brain, and the uterus. Specifically, interactions between ER and AHR signaling were uncovered in the finding that the SERM 4OHT activates AHR, and that this action potentially contributes to the efficacy of 4OHT in preserving BMD. Another intersection was identified in studies of the endometrium, where the natural medicine PV modulates both ER and AHR activity, through perhaps direct and indirect mechanisms, to influence the growth and progression of endometriosis. The preliminary identification of novel ER-interacting proteins such as LAMA3, SH3B2, and LRRTM4 supports the idea that ER is not just a transcription factor, but can conceivably interact with cytoplasmic signaling pathways and modulate cytoskeletal networks in classic ER target tissues as well as newer ones like the brain. Our use of chimeras of the two genetic subtypes of ER, ERα and ERβ, allowed us to identify regions that are required for anti-estrogen-induced receptor degradation, opening the way to the identification of the proteins that mediate this process.
Taken together, these studies advance our understanding of both ER and AHR biology, particularly in the context of breast cancer and the bone, and lead the way to many *in vitro* and *in vivo* studies as to how these findings and intersections play out in more complex models of biology and disease.
8 Materials and Methods

8.1 Chemicals

17β-estradiol, 4-hydroxy-Tamoxifen, β-naphthoflavone, and α-naphthoflavone, were purchased from Sigma (St. Louis, MO). 27-hydroxycholesterol was purchased from Research Plus, Inc (Manasquan, NJ). Actinomycin D was purchased from Calbiochem (San Diego, CA). Methyltrienolone (R1881) was purchased from Perkin-Elmer (Waltham, MA).

PCR and qRT-PCR reagents were obtained from Bio-Rad (Hercules, CA), Qiagen (Valencia, CA), Integrated DNA Technologies (Coralville, IA), and Sigma (St. Louis, MO).

ICI 182,780 was a gift from Dr. A. Wakeling (Zeneca Pharmaceuticals, Macclesfield, UK). Raloxifene was a gift from Dr. E. Larson (Pfizer, Inc, Groton, CT). 3-methoxy-4-nitroflavone was a gift from Dr. Stephen Safe (Texas A&M University). RU58668 was a gift from Michel Renoir (UMR Centre National de la Recherche Scientifique, France). Mifepristone (RU486) was a gift from Ligand Pharmaceuticals, Inc. (San Diego, CA). Bicalutamide (casodex) was a gift from P. Turnbull (GlaxoSmithKline, Research Triangle Park, NC). RTI-001 and RTI-018 were gifts from RTI International (Research Triangle Park, NC).

8.2 Plasmids

8.2.1 Expression plasmids

pcDNA3.1nv5-ERα is a CMV-driven expression plasmid containing amino acids 1-595 of human ERα with an N-terminal v5 tag (24). pcDNA3.1nv5-hERβ(530aa) contains amino acids 2-530 of human ERβ and was created by PCR subcloning ERβ from pRST7-hERβ(530aa) into the SalI and XbaI sites of pENTR1A (Invitrogen). An LR
reaction was performed to transfer hERβ(530aa) into pcDNA3.1nv5-DEST (Invitrogen). pcDNA3.1nv5-ERβ-N496D and –V487L were made using the QuickChange Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) with primers specific for the desired mutations. pcDNA3.1nv5-ERβ-LBDα contains base-pairs 904-1656 of ERα in place of 763 – 1512 of ERβ. This region of ERα was amplified from VP16-ERα, then was subcloned into pENTR1A-ERβ using NgoMI and PmlI, and then by LR reaction transferred into pcDNA3.1nv5-DEST. pCDNA3.1nv5-AHR containing full length human AHR was subcloned from pSPORT-AHR (a gift from Dr. Chris Bradfield, University of Wisconsin-Madison) with KpnI and NotI. pCDNA3.1nv5-ARNT containing full length human ARNT was subcloned from pSPORT-ARNT (a gift from Dr. Chris Bradfield, University of Wisconsin-Madison) with SalI and EcoRV (details available upon request). SG5-AR is from GlaxoSmithKline. The longer splice variant of CCDC137 (305 residues) was cloned from MCF7 cDNA using a high GC kit (BD Biosciences), transferred into pENTR1A using KpnI and XbaI, and by LR reaction pMGb-CCDC137 and pCDNA3.1nv5-CCDC137 were created. CCDC137 N, dL, and L mutants were created using pCDNA3.1nv5-CCDC137 as a template with unique 3’ PCR primers, cloned into pENTR1A using KpnI and XbaI, and by LR reaction the mutants were introduced into pMGb and pCDNA3.1nv5-DEST. The NAAIRS mutation in CCDC137 was made using PCR mutagenesis of pENTR1A-CCDC137, and by LR reaction was introduced into pMGb and pCDNA3.1nv5-DEST. pCMV-SPORT6/PBXIP1 (also known as HPIP) was purchased from Origene (Rockville, MD), cloned into pENTR1A using KpnI and XbaI, and by LR reaction pMGb-HPIP and pCDNA3.1nv5-HPIP were created.
8.2.2 Reporter Constructs

The 3XERE-TATA-Luc \((8)\) and the 5XGal4Luc3 \((106)\) reporters have both been previously described. pAHRE-TK-luc3 was a gift from Dr. Alvaro Puga (University of Cincinnati). 5XGal4Luc3 \((106)\), PB-Luc (from Jorma J. Palvimo (University of Helsinki, Finland)), and MMTV-Luc \((295)\) reporters have been previously described.

8.2.3 Plasmids for M2H Assays

A plasmid expressing \(\text{ER}\alpha\) as a fusion to the yeast VP16 transactivation domain was used in mammalian two-hybrid assays \((\text{pVP16-ER}\alpha)\) and has been previously described \((106)\). VP16-ER\(\alpha\) 3X, LL, and stop have been described \((106)\). VP16-AR was a gift from K. Marschke (Ligand Pharmaceuticals, San Diego, CA), from which VP16-AR T877A and VP16-AR E893stop were created by PCR mutagenesis.

The bait peptides are expressed as fusions to the Gal4-DNA binding domain (Gal4DBD). GRIP1-NR, SRC1-NR, and D30 have been previously described \((106)\). bI2 and bT1 were described elsewhere \((107)\). AIB1-NR contains amino acids 621 – 821 of human AIB1. ASC2-NR contains amino acids 746 – 917 of human ASC2. These fragments were identified in an unrelated screen performed in our laboratory.

8.3 Mammalian Cell Culture

All cell lines were obtained from American Type Culture Collection (Manassa, VA) and cultured in media from Invitrogen (Carlsbad, CA). HeLa and HepG2 cells were maintained in MEM, MCF7 and ECC-1 cells in DMEM/F12, and T47D and RAW264.7 cells in DMEM, LNCaP and SKBR3 cells in RPMI 1640, each supplemented with 8% fetal bovine serum (FBS) (Hyclone Laboratories), 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids (Invitrogen). All cell lines were propagated in a 37°C incubator with 5% \(\text{CO}_2\).
8.4 Transient Transfection Assays

For transactivation assays with HeLa cells, cells were plated in phenol red-free media containing 8% charcoal-stripped serum (CFS) (HyClone Laboratories, Logan, UT), 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids 24 hours prior to transfection. Lipofectin (Invitrogen)-mediated transfection has been described in detail previously (111). Each triplicate sample contained 0.1 µg pCMV-βgal, 1.5 µg luciferase reporter, 5 ng pCDNA3.1nV5-ERα, and 0.395 µg pCDNA3.1nV5-DEST filler vector. Ligands were added to the cells 24 hours after transfection and cells were assayed following overnight treatment. Luminescence and β-galactosidase (β-gal) activity were measured on a Fusion luminometer (Perkin Elmer, Waltham, MA). Results are expressed as average luciferase activity (normalized to β-gal for transfection efficiency) ± SEM relative to vehicle-treated samples for three independent experiments performed in triplicate.

For transient transfections with SKBR3 cells, cells were plated in phenol red-free media containing 8% CFS, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids 24 hours prior to transfection with pCMV-βgal and AHRE-TK-luc3. Ligands were added 24 hours after transfection and cells were assayed following overnight treatment. Luminescence and β-gal activity were measured as above. Results are expressed as average luciferase activity (normalized to β-gal for transfection efficiency) ± SEM relative to vehicle-treated samples for three independent experiments performed in triplicate.

For mammalian 2-hybrid assays, HepG2 cells were plated 24 hours prior to transfection in phenol red-free media containing 8% CFS, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids. A DNA-lipofectin mixture containing a total of 3 µg of plasmid for each triplicate sample was added to the cells, where each triplicate sample
contained 0.1 µg pCMV-βgal, 1.5 µg 5XGal4Luc3, 0.4 µg VP16-NR, and 1 µg pM-peptide. Ligands were added to the cells 24 hours after transfection and cells were assayed following overnight treatment. Luminescence and β-gal activity were measured as above.

8.5 RNA Isolation and qRT-PCR

Most cells were seeded in 6-well plates in phenol-red free media containing 8% CFS, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids. For studies with primary osteoblasts (OBs), cells were seeded in phenol-red free αMEM containing 8% CFS and penicillin/streptomycin (pen/strep). For studies with RAW264.7, cells were seeded and grown in differentiating media [αMEM with 8% FBS and supplemented with 30 ng/mL RANKL (R&D Systems) and 20 ng/mL M-CSF (R&D Systems)] for 8 days, then treated for 6 hours with the indicated ligand. For all other cells, 48 hours after plating they were treated with the appropriate ligand. Importantly, the media was not changed at the time of ligand treatment to avoid activation of AHR by tryptophan derivatives in culture media (296). After the indicated time period, cells were harvested and total RNA was isolated using the Aurum Total RNA Mini Kit (Bio-Rad). One µg of RNA was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad). The Bio-Rad iCycler Realtime PCR System was used to amplify and quantitate levels of target gene cDNA. qPCR reactions were performed with 1 µl cDNA, 10 µM specific primers, and iQ SYBRGreen supermix (Bio-Rad). Data are normalized to the 36B4 (human) or Cyclophilin (mouse) housekeeping genes and presented as fold induction over vehicle or normalized expression. Data are the mean±SEM for triplicate amplification reactions from one representative experiment. Human qRT-PCR primer sequences are in Table 8.1.
Table 8.1: Human qRT-PCR primer sequences

<table>
<thead>
<tr>
<th>Human qRT-PCR</th>
<th>Primer sequence</th>
</tr>
</thead>
</table>
| SDF-1         | F GTGGTCGTGCTGGTGCTCTC  
R GATGCTTGACCTGCTCTG |
| PR            | F GCATCGTTGATAAAAATCCGAG  
R AATCTCTGCGTCTGCTCTG |
| pS2           | F TCCCGCTGTTGTTTCTACTCTACTAC  
R GCAGTCACACTGCTGCTGAG |
| E2F1          | F ACCTGACCTGCTGAGGACCT  
R GATCGGGCCCTTGTGCTCT |
| WISP2         | F TGAAGGCAACACGAGAC  
R ACAGCCATCCAGAGACAC |
| ERBB4         | F GAAAGATTCCTTGGAAACAGAG  
R GGATGATCCATACCTTGCCAT |
| CYCLIN D1     | F CAACTTCTCTCCTACTACC  
R CCTCCTCCCTCTTCTCC |
| 36B4          | F GGACATGTTGCTGGCAGAATA  
R GGGCCGAGACGCTCTGTT |
| CYP1A1        | F TGCGAGATGCTGCAAGGAG  
R AGCTCAGAGAGTGGTCAATA |
| CYP1B1        | F CTTGATCCATACCTTGCAAC  
R TGCTCAGAGAGTGGTCAATA |
| AHRR          | F AGCTGTCAGGGATTCATGCT  
R TTCTGTCAGGGATTCATGCT |
| AHR           | F TCCAGCTGTCGCTGCTGCTCT  
R TCGTGCAACAGCTCTGCTCAGTAT |
| ERα           | F GAAAGGTGGGATACGAAAAGACC  
R GTTCAGCTCAGCTCTGCTC |
| 14-3-3n       | F GACATGGGCTCGCTGATGAG  
R CAATGCTGCTAATGACCTCC |
| CCDC137       | F GAAGAAAGTGAAGCTGCAAGC  
R GTCTTCTGAGGGTCACTG |
| TGFβ2         | F ACAAAGAGCAGGAAGGCGAAT  
R TGGCCATCAATACCTGCAAAAT |
| FKBP51        | F CGGAAGACCAACGGAAGG  
R CTTTGCCCACTGTAATGC |

For analysis of gene expression in calvaria, this tissue was collected, snap-frozen in liquid N2, then RNA was harvested by Trizol extraction using glycogen. Contaminating DNA was removed from isolated RNA using TURBO DNase (Ambion,
Austin, TX). cDNA preparation and gene expression analysis by qRT-PCR was performed as described above. Mouse qRT-PCT primer sequences are in Table 8.2.

**Table 8.2: Mouse qRT-PCR primer sequences**

<table>
<thead>
<tr>
<th>Mouse qRT-PCR</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>mCYP1A1</td>
<td>F ATCTGTGCCTGCTTCCTACT G</td>
</tr>
<tr>
<td>mActinB</td>
<td>F CCTTCCCTTCTTGGTATGGA</td>
</tr>
<tr>
<td>mCyclophilin</td>
<td>F GAGCTGTTTCAGACAAAGTTTC</td>
</tr>
<tr>
<td>mERα</td>
<td>F TGAGGCGTGTAGGGTGTTGATT</td>
</tr>
<tr>
<td>mSDF-1</td>
<td>F CCAACGTCAAGCATCTGAAA</td>
</tr>
<tr>
<td>mAHR</td>
<td>F CCAATGCACGCTGTTTACC</td>
</tr>
<tr>
<td>mAlkaline</td>
<td>F GAGGACGAAATCTCTAGGTA</td>
</tr>
<tr>
<td>Phosphatase</td>
<td></td>
</tr>
<tr>
<td>mCalcitonin</td>
<td>F TAGACACCCCTGACAGCAAC</td>
</tr>
<tr>
<td>Receptor</td>
<td></td>
</tr>
<tr>
<td>mCathepsin K</td>
<td>F CATGGTGAGCTTTGCTTGT</td>
</tr>
<tr>
<td>mRANKL</td>
<td>F CAGCTATGATGGAAGAGTCA</td>
</tr>
<tr>
<td>mOsteocalcin</td>
<td>F AGACAAGTCCACAGACACAG</td>
</tr>
<tr>
<td>mOsteoprotegrin</td>
<td>F TGCCAGAAGTGTAAGAGAGGA</td>
</tr>
<tr>
<td>mIL-7</td>
<td>F CACATCATCTGAGGCCACA</td>
</tr>
<tr>
<td>mTNFα</td>
<td>F CCAAGGGAGTGGAAAGTTC</td>
</tr>
</tbody>
</table>

**8.5.1 siRNA Studies**

For siRNA experiments, cells were plated in the presence of 40 nM siRNA or siRNA control (Stealth siRNA, Invitrogen, or Dharmacon, Lafayette, CO) using DharmaFECT 1 (Dharmacon, Lafayette, CO) as a transfection reagent. After 48 hours,
cells were treated with the indicated ligand for the indicated time, then harvested and assayed for RNA expression as detailed above.

8.6 Cell Viability

8.6.1 Cell Proliferation

For MCF-7 cell proliferation and BrdU-incorporation assays, cells were seeded on day 0 in phenol red-free DMEM/F12 media containing 8% CFS, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids. On day 2, the media was replaced with serum- and phenol red-free media for 24 hours. For proliferation assays, cells were treated with ligands on day 3 and 5 in media containing 8% charcoal-stripped serum. On day 6, cell proliferation was measured as follows. Media was aspirated and plates were frozen at -80°C. After thawing completely, 100 µl H₂O was added to each well, incubated at 37°C for one hour, re-frozen at -80°C, then thawed again. 100µl assay buffer [10 mM Tris, 2 M NaCl, 1 mM EDTA, 2 mM sodium azide, and 2.5 µg/ml Hoechst 33258 (Sigma, St. Louis, MO)] was added to each well and fluorescence was measured at 360 nm / 460 nm. For BrdU assays, the cells were treated on day 3 with ligand for 22 hours, at which time the BrdU labeling reagent was added for 3 hours. Cells were then assayed according to manufacturer’s protocol (Cell Proliferation ELISA BrdU kit, Roche Applied Science, Indianapolis, IN). For both assays, data is presented as the mean±SEM for triplicate wells in one representative experiment.

ECC-1 cells were grown in phenol-red free media containing 8% CFS in the presence of 10 nM DES with or without increasing concentrations of PV or 1 µM ICI. Cell number was determined according to manufacturers instructions (CellTiter96 Aqueous One Solution Cell Proliferation Kit, Promega, Madison, WI). For details, see (245).
Primary osteoblasts (OBs) were plated at 2000 cells/well in phenol-red free αMEM supplemented with 8% CFS and pen/strep in a 96-well plate (CellBIND, Corning Inc., Corning, NY). Cells were allowed to attach overnight, then treated with the indicated ligand; ligand treatment was refreshed on day 2, 4, 6, and 8. On the appropriate day, media was aspirated and proliferation was assessed as described above for MCF7 cell proliferation assays.

8.6.2 Cell Viability

ECC-1 cells were grown in phenol-red free media containing 8% CFS for 24 hours prior to treatment with PV, DES, or ICI. Treatments were replaced daily. Viability was assessed with the Cell Titer-Blue TM Cell Viability Assay (Promega, Madison, WI).

8.7 Alkaline Phosphatase Activity

Primary OBs were plated at 20,000 cells/well in phenol-red free media containing 8% CFS and pen/strep in a 48-well plate. After overnight incubation, the cells were treated with the indicated ligand for 36 hours, at which time the cells were lysed with 0.1% TritonX-100. Lysate was incubated with substrate solution [0.1 M glycine, 1 mM MgCl$_2$, 1 mM ZnCl$_2$, 1 mg/ml 4-nitrophenol phosphate (Sigma, St. Louis MO)] for 30 minutes at 37°C. The reaction was stopped by addition of NaOH, and absorbance was read at 405 nm. Total protein was determined by the Bradford method. Absorbance values were normalized to total protein in each sample.

ECC-1 cells were assayed for alkaline phosphatase activity as described previously (297). Cells were treated daily with extracts, DES, or DES + ICI for a total of 72 hours, then harvested as described.
8.8 Western Blotting

MCF7 cells were seeded in phenol red-free media containing 8% charcoal-stripped serum, 1 mM sodium pyruvate, and 0.1 mM non-essential amino acids. For ERα degradation and protein expression studies, cells were treated after 48 hours for the indicated time with the appropriate ligand. For chimera studies, cells were transiently transfected using lipofectin for 6 hours, allowed to recover for 2 days, then treated for 4 hours with vehicle or 100 nM ligand. For siRNA experiments, cells were plated in the presence of 40nM siAIB1 or siRNA control (Stealth siRNA, Invitrogen) using DharmaFECT-1 (Dharmacon, Lafayette, CO) as a transfection reagent. After 48 hours of protein knockdown, the cells were treated for 8 hours with vehicle, 1 nM E2, 10 μM 27HC, 100 nM 4OHT, or 100 nM ICI. In both experiments, whole cell extracts were isolated using RIPA Buffer [50 mM Tris-HCl pH=7.5, 150 mM NaCl, 0.1% NP40, 0.5% sodium-deoxycholate, 0.05%SDS, 1 mM EDTA, and 1X protease inhibitor mixture (EMD Chemicals, Inc)]. 50 μg of whole cell lysate was resolved by SDS-PAGE and transferred to a nitrocellulose membrane. ERα was detected using monoclonal mouse antibody D12, Cytokeratin 18 with the mouse monoclonal antibody DC-10, and AIB1 with the goat polyclonal antibody C-20 (all from Santa Cruz Biotechnology). EGFP antibody is from Clontech (Mountain View, CA) and the V5 antibody is from Invitrogen (Carlsbad, CA). PR was detected using the mouse monoclonal antibody PR1294 (a gift from Dr. D. P. Edwards, Baylor College of Medicine, Houston, TX). Secondary antibodies were purchased from Bio-Rad. The scanned images of chemiluminescence were quantitated using Image J software.
8.9 **M13 Phage Screen**

The M13 phage panning protocol has been previously described \(^{106}\). Modifications to the original protocol are as follows. We used 4 pmol of recombinant ER\(\alpha\) (Affinity BioReagents, Golden, CO). Approximately \(10^7\) plaque-forming units of phage libraries were added to each well for the panning process. Four rounds of panning were performed, with PCR being used to recover peptide inserts from the fourth round, which showed significant enrichment of target binding phage. The PCR products were digested with \(Xho\)1 and \(Xba\)1 for ligation into the expression vector pM5.1 for mammalian-two hybrid assays.

8.10 **Chromatin Immunoprecipitation**

ChIP of ER\(\alpha\) in MCF7 cells has been described previously \(^{24}\). Cells were treated with vehicle, 100 nM E2, or 10 \(\mu\)M 27HC for 45 minutes. IP was performed with 10 \(\mu\)g ER\(\alpha\) antibody (H-184, Santa Cruz Biotechnology) or 10 \(\mu\)g rabbit IgG control. DNA was isolated with a QIAquick PCR Purification kit (Qiagen, Valencia, CA). qPCR reactions were performed with 1 \(\mu\)l immunoprecipitated DNA, 10 \(\mu\)M specific primers, and iQ SYBRGreen supermix (Bio-Rad, Hercules, CA). Data is normalized to the input for the IP. ChIP primer sequences are in Table 8.3.

**Table 8.3: Primers used for ChIP analysis**

<table>
<thead>
<tr>
<th>ChIP</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CYP1A1 - AHRE</td>
<td>F  CCTGGGCGCGGTCTTTC</td>
</tr>
<tr>
<td></td>
<td>R  AGTGCTTTGATGGCCAGAGC</td>
</tr>
<tr>
<td>CYP1A1 - Control</td>
<td>F  CAAGGTGGGTGATGGAAATGAG</td>
</tr>
<tr>
<td></td>
<td>R  TTACCTCCACCTAGTTCCC</td>
</tr>
<tr>
<td>pS2 - ERE</td>
<td>F  CTAAGCGGAATGGGCTTCATGAG</td>
</tr>
<tr>
<td></td>
<td>R  CAGAAAGTCCCTCTTTCCATGGAG</td>
</tr>
<tr>
<td>pS2 - Control</td>
<td>F  CCAGAGGCCTGGCAGGAAAC</td>
</tr>
<tr>
<td></td>
<td>R  CGTCTCTCCACACACCATCTTC</td>
</tr>
</tbody>
</table>
For ChIP of AHR in SKBR3 or MCF7 cells, cells were grown to 90% confluence in 15-cm dishes in phenol red-free media supplemented with 8% charcoal-stripped serum, 1 mM sodium pyruvate, and 0.1 mM nonessential amino acids. Cells were then treated with vehicle, 100 nM BNF, or 100 nM 4OHT for 75 minutes. The remainder of the procedure has been described previously (24). The antibodies used are as follows: AHR SA-210 (BioMol, Plymouth Meeting, PA), anti-Acetyl Histone H4 06-866 (Millipore, Billerica, MA), RNA Polymerase II (Pol II) sc-899 (Santa Cruz Biotechnology, Santa Cruz, CA). ChIP primer sequences are in Table 8.3.

### 8.11 EMSA

In vitro transcription and translation was performed using the Promega TNT Quick Coupled Transcription/Translation T7 System per the manufacturer's directions. Briefly, 40 μl TNT Quick Master Mix was combined with 3 μl PCR Enhancer solution, 5 μl plasmid DNA (25 ng pCDNA3.1v5-AHR or 50 ng pCDNA3.1v5-ARNT), and 2 μl 1mM methionine. After 90 min at 30°C, AHR and ARNT were mixed 1:1, diluted 1:2 with 2X HEGD (1X: 25 mM HEPES, 1.5 mM EDTA, 10% glycerol, and 1 mM DTT), and allowed to incubate for 15 minutes at RT with vehicle, BNF, or 4OHT (approximately 33 μM each ligand). Protein-ligand mixtures were then incubated with binding buffer (0.2 μg/μl BSA, 2 mM DTT, 100mM NaCl, and 0.02 μg/μl sheared salmon sperm DNA in HEG) for 15 minutes at RT. 32P-labeled oligo (for sequence, see (298)) was then added to the mixture and allowed to incubate for 20 minutes at RT. Samples were resolved on a 5% non-denaturing acrylamide gel, dried, and analyzed by autoradiography.
8.12 Osteoclast Differentiation Assay

RAW264.7 cells were seeded in differentiating media [αMEM with 8% FBS and supplemented with 30 ng/mL RANKL and 20 ng/mL M-CSF (both from R&D Systems Minneapolis, MN)] in a 48 well plate at 2000 cells/well for staining and 20,000 cells/well for activity along with ligands at the indicated concentrations. Media was changed and new ligands added every two days. After eight days, cells were stained for TRAcP according to the manufacturer’s instructions (Sigma, St. Louis, MO). TRAcP-positive cells with ≥3 nuclei were counted. Data represent the average percent suppression from a representative experiment. TRAcP activity was measured by lysing the cells with 0.1% TritonX-100, then incubating the cells with p-nitrophenyl as a substrate (in citrate buffer at pH=5.5 with 0.4 M sodium tartrate), and reading absorbance at 405 nm (modified from (226)). Absorbance values are normalized to total protein determined by the Bradford method.

8.13 Yeast 2-hybrid Screen

The yeast 2-hybrid screen was performed according to the Clontech protocol using pGBT9-ERLBD3X as bait and a brain cDNA library as prey in HF7c cells using SD/-trp/-leu/-his media including 0.5 mM 3-AT. The screen was performed with or without 1 µM E2, and clones were isolated that were unique in the presence of E2.

8.14 Breeding, Care, and Use of Animals

All studies involving animals were approved by the Institutional Animal Care and Use Committee at Duke University. Animals were housed in a temperature controlled room with a daily 12 hour light/12 hour dark cycle and free access to water and chow (Laboratory Rodent Diet #5001). The Cyp27ar/- and the Cyp7br/- strains, and their respective wild-type strains, were obtained with permission from Dr. E. Leitersdorf
(Hadassah University Hospital, Jerusalem, Israel) and Dr. D. Russell (University of Texas Southwestern Medical Center, Dallas, TX), respectively. Although maintained by homozygous matings, pups were genotyped at 10 days of age by PCR using the primers in Table 8.4.

**Table 8.4: Genotyping primers**

<table>
<thead>
<tr>
<th>Genotyping</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyp7b1</td>
<td>3UTRas ACAGGAAGCCTATAGGCTAAATCACAGTC</td>
</tr>
<tr>
<td>SI-75</td>
<td>AACAAGAGTAGCGGTGGATTTTGGGGAGT</td>
</tr>
<tr>
<td>6S</td>
<td>GATGGGAAGACAAATAGCAGGCGATGC</td>
</tr>
<tr>
<td>Cyp27a1</td>
<td>A CCACAAACTCCGGATCAT</td>
</tr>
<tr>
<td></td>
<td>B CATAGCCAAAGGCCAACAGA</td>
</tr>
<tr>
<td></td>
<td>C CCATCAGAAGCTGACCTAGA</td>
</tr>
</tbody>
</table>

### 8.14.1 Baseline Study of Bone Phenotype

Adult female mice (9 – 10 per group) of each genotype were sham-operated at 6 weeks of age, then harvested at 10 weeks of age for ex vivo analysis. Lumbar spine and femur were analyzed by DEXA to obtain BMD (g/cm²). Femur was analyzed by μCT at the mid-femur (cortical) and diaphysis (trabecular) to obtain cortical thickness, percent bone fraction (bone volume / total volume), trabecular number, trabecular thickness, and trabecular separation.

### 8.14.2 Ovariectomy and Estrogen Replacement

Adult female mice (9 – 10 per group) of the Cyp7b1−/− and Cyp7b1+/+ genotypes were assigned to one of three groups: sham-operated, ovariectomy (OVX) plus placebo, or OVX plus 10 µg/kg 17β-estradiol. Mice were treated by daily subcutaneous injection using corn oil as a vehicle for 28 days. Mice also received two calcein (1 mg/ml) injections, 7 days apart, at the end of the study. Urine was collected over 10 hours using
metabolic cages within 3 days of the end of the study. Blood was collected by cardiac puncture following CO\textsubscript{2} euthanasia, after which tissues were harvested.

8.14.3 Estrogen Supplementation

Adult female Cyp\textit{7b1}\textsuperscript{-/-} mice (4 - 5 per group) were assigned to receive placebo (corn oil), 10 µg/kg or 20 µg/kg 17β-estradiol by daily subcutaneous injection for 28 days, starting at 6 weeks of age. Urine, blood, and tissues were harvested as described above.

8.14.4 Serum and Urine Measurements

Serum osteocalcin was measured by EIA according to the manufacturer's instructions (Biomedical Technologies Inc., Stoughton, MA). Urine deoxypyridinoline (DPD) crosslinks were measured by EIA according to the manufacturer's instructions (Quidel, San Diego, CA).

8.14.5 Histology

Tibias were fixed for 24 hours in 10% buffered formalin, then transferred to 70% ethanol. After decalcification, embedding, and sectioning, the slides were stained with hematoxylin and eosin (H&E). Pictures were taken on an Axio Imager at 5X magnification. Representative images are shown.

8.14.6 Isolation of Primary Osteoblasts

Calvaria from 3 - 5 day old pups (Cyp\textit{7b1}\textsuperscript{+/+}) were removed, cut into pieces, and placed in HBSS + pen/strep. Calvaria were washed (HBSS + 4 mM EDTA and pen/strep) three times at 37°C for 10 minutes each, then digested with collagenase (5 mg/ml in HBSS + pen/strep for 20 minutes at 37°C. The first digest was discarded, and the subsequent 4 digests were pooled and filtered (70µm). Filtrate was pelleted,
followed by re-suspension in phenol red-free αMEM supplemented with 8% FBS. Cells were washed in media twice more, then introduced into culture.

**8.14.7 FACS analysis**

Marrow was extracted by flushing the femur and tibia with HNSS + pen/strep and re-suspended with a 10 ml syringe. Spleens were removed and mashed in RPMI + β-mercaptoethanol, L-glutamine, and pen/strep. Marrow cells and splenocytes were filtered (70µm), pelleted, and re-suspended in ACK lysis buffer (to lyse erythrocytes). Cells were then washed in RPMI + β-mercaptoethanol, L-glutamine, and pen/strep and plated in a round-bottom 96-well culture plate. Cells were incubated with GolgiPLug to block TNFα secretion. Cells were then stained for CD4 (APC CD4 anti-mouse, BD Biosciences, San Diego, CA), washed 2X (PBS + 2% FBS), fixed, washed 3X (PermWash, BD Biosciences, San Diego, CA), then stained for intracellular TNFα (PE TNFα anti-mouse, BD Biosciences, San Diego, CA). Cells were washed 3X with PermWash, 2X with PBS + 2 % FBS, then data was acquired by FACS.
9 Appendix: Evaluation of novel proteins that modulate the ER and AR signaling pathways

9.1 Introduction

The yeast 2-hybrid screen described in this chapter was performed by Dr. Ching-yi Chang in our laboratory. Most of the data presented here is preliminary, and perhaps when time and personnel permit will be pursued.

9.2 The identification of novel ER binding proteins

ER plays a well-documented role in regulating the transcription of target genes either directly through EREs or indirectly through interaction with AP1-element binding proteins and Sp1 proteins. For both, recruitment of histone acetyl transferases, such as the p160 class of coactivators, plays a major role in activating transcription of ER target genes. However, potential roles exist for non-classical transcriptional regulatory proteins in regulating both the genomic and non-genomic actions of ER. Thus, we undertook a study to identify proteins that interact with and modulate ER activity using the yeast 2-hybrid assay. To avoid the re-identification of the classic p160 coactivators, we used as bait a mutant form of the LBD of ERα (ERLBD3X) that does not interact with this class of coactivators due to mutation of three charged residues (D538N, E542Q, and D545N) within H12 of ERα. However, this form of ERα maintains some transcriptional activity (299). Many proteins were identified that interacted with ERLBD3X in the presence of E2, a select few of which are detailed below.
9.2.1 Coiled-coil domain containing protein 137 (CCDC137)

9.2.1.1 CCDC137 interacts with ERα in the presence of anti-estrogens

To evaluate the interaction between ERα and the identified peptides, and subsequently the corresponding full-length proteins, we used a M2H assay in which ERα (or mutants thereof) is fused to the VP16 transactivation domain and the unknown peptides and full-length proteins are expressed as fusions to the yeast Gal4-DNA-binding domain (Gal4DBD). Upon interaction between ERα and the peptides or full-length proteins, luciferase expression from a 5XGal4-Luc3 reporter is increased.

One peptide identified was HB199, which interacted with ERLBD3X and ERα in the presence of E2, but not the SERM 4OHT or the pure ER antagonist ICI (Figure 9.1 and data not shown). Near the C-terminus of HB199 is an LxxLL sequence, which is the canonical NR coactivator binding motif, and therefore we hypothesized that this motif would mediate the interaction between HB199 and ERα. As transcriptional activation is an integral part of E2 action, we examined whether over-expression of HB199 impacted ERα transcriptional activity. As shown in Figure 9.1B, there was a significant decrease in E2-mediated induction of ERα transcriptional activity when HB199 was over-expressed, suggesting that HB199 interacted with ERα through a protein-protein interaction surface that was important for transcriptional activation of target genes by ERα, thus blocking this activity.
Figure 9.1: HB199 interacts with E2-ERα and inhibits its transcriptional activity

(A) HepG2 cells were transfected overnight with VP16 or VP16-ERα, pM or pM-HB199, a 5XGal4Luc3 reporter, and the CMV-βgal transfection control. Cells were treated overnight with vehicle, 100 nM E2, 100 nM 4OHT, or 100 nM ICI. A representative experiment is shown. (B) Hela cells were transfected overnight with pcDNA3.1nv5-ERα, pM-HB199, a 3XERE-TATA-luc reporter, and the CMV-βgal transfection control. Cells were treated overnight with vehicle, 100 nM E2, 100 nM 4OHT, or 100 nM ICI. Data is the mean±SEM for three independent triplicate experiments.

By sequence analysis, we determined that peptide HB199 corresponds to coiled-coil domain containing 137 (CCDC137), which is located on chromosome 17 at 17q25.3 and encodes a protein of 289 amino acids. The biological significance of this protein is unknown, but coiled-coil domains in general are important for protein-protein interactions and have been implicated in diverse processes such as protein degradation and cell growth, proliferation, and differentiation.

Interestingly, when we analyzed the interaction of the full length protein CCDC137 with ERα, we found that it interacted most robustly with ERα in the presence of ICI, not E2 (Figure 9.2A). Further, there was a small but significant interaction between ERα and CCDC137 in the presence of 4OHT. Therefore, it appeared that we had identified a protein that preferentially interacted with anti-estrogen-bound ERα. A further sequence analysis of CCDC137 identified two CoRNR box-like motifs, which in general are important for corepressor binding to ERα. These motifs, [IPFRL]REI in the
N-terminus and LTRARQRI in the C-terminus, may mediate the interaction between CCDC137 and ER in the presence of anti-estrogens. Mutation of the specific residues within these interaction motifs will determine whether they contribute to CCDC137 binding to ERα.

To map the interaction between CCD137 and ERα, we used previously described mutants of ERα: ER3X, ERαLL, and ERα stop, which terminates the protein after residue 535. We evaluated by M2H their interaction with CCDC137 in the presence of E2 or the ER antagonists RU58668 and ICI (Figure 9.2B). From this, we found that the interaction between CCDC137 and ERα mediated by ER antagonists required the C-terminal region of ERα, from H12 onward (residues 535 – 595). Further, mutation of the charged or hydrophobic residues within H12 attenuated the interaction between ERα and CCDC137.
Figure 9.2: CCDC137 interaction with anti-estrogen-bound ERα requires H12 through the F domain of ERα

(A) HepG2 cells were transfected overnight with VP16, VP16-ERα, or -ERα3X, pMGB or pMGB-CCDC137, a 5XGal4Luc3 reporter, and the CMV-βgal transfection control. Cells were treated overnight with vehicle, 100 nM E2, 100 nM 4OHT, or 100 nM ICI as indicated, then harvested and assayed for activity. (B) HepG2 cells were transfected overnight with VP16, VP16-ERα, -ERα3X, -ERαLL, or -ERαstop, pMGB or pMGB-CCDC137, a 5XGal4Luc3 reporter, and the CMV-βgal transfection control. Cells were treated overnight with vehicle, 100 nM E2, 100 nM RU58668, or 100 nM ICI, then harvested and assayed for activity. For A and B, a representative experiment is shown.

Given the previously established role of the LxxLL motif in mediating ER-coactivator interactions, we wanted to evaluate its role in the ER-CCDC137 interaction. Therefore we made a series of C-terminal truncations of CCDC137 and tested their interaction with ERα in the presence of either E2 or ICI. Interestingly, there was no dependence on the LxxLL motif for interaction between CCDC137 and ERα (Figure 9.3). Further, the ability of the short N-terminal mutant of CCDC137 to interact with ERα suggests that there is a motif, perhaps the CoRNR box-like one, contained in this region instead that mediates this anti-estrogen-induced interaction. Although the LxxLL motif likely mediated the interaction between the peptide HB199 and E2-bound ERα, a distinct motif in the N-terminus was responsible for interaction of CCDC137 and ERα in the presence of the anti-estrogens ICI and RU58668.
Figure 9.3: LxxLL motifs are not required for the interaction between ERα and CCDC137

(A) HepG2 cells were transfected overnight with VP16 or VP16-ERα, pMGb, pMGb-CCDC137, -CCDC137 N, -CCDC137 dL, or –CCDC137 L, a 5XGal4Luc3 reporter, and the CMV-βgal transfection control. Cells were treated overnight with vehicle, 100 nM E2, 100 nM RU58668, or 100 nM ICI. A representative experiment is shown. (B) A schematic of the constructs of CCDC137 that are used in A.

A recent study of the mechanisms underlying anti-estrogen-mediated degradation of ERα led to the identification of a motif that may be important for mediating this activity. Expression of a small peptide, AEIP, that contained this motif blocked the ability of SERDs to induce degradation of ERα, presumably by competitively interacting with an important protein-protein interaction surface presented on ERα in the presence of SERDs and thereby blocking the recruitment of the protein(s) necessary for degradation (186). By mutational analysis, it was determined that an SPM motif
within AEIP was necessary for interaction with ERα. Therefore, we examined the sequence of CCDC137 and found a similar motif, SPR, near the N-terminus of the protein. We have already shown that this region is necessary for anti-estrogen-mediated interaction between CCDC137 and ERα. Therefore, we used mutagenesis to replace this motif and the surrounding residues with the NAAIRS sequence, which has been used successfully to remove protein motifs without significantly impacting the overall structure of a protein. However, when we mutated the GPGSPR sequence within CCDC137 to NAAIRS, there was no significant effect on the ability of CCDC137 to interact with ERα in an anti-estrogen-dependent manner (Figure 9.4).

![Graph](image)

**Figure 9.4: The AEIP-like motif in CCDC137 is not required for interaction with ERα**

(A) HepG2 cells were transfected overnight with VP16 or VP16-ERα, pMgB, pMgB-CCDC137, or -CCDC137 NAAIRS, a 5XGal4Luc3 reporter, and the CMV-βgal transfection control. Cells were treated overnight with vehicle, 100 nM E2, 100 nM RU58668, or 100 nM ICI. A representative experiment is shown. (B) The sequence of CCDC137 where the NAAIRS mutation was introduced aligned with that AEIP.

Since increased expression of HB199 negatively impacted E2-mediated transcriptional activation of ERα, we were interested in determining if the corresponding protein CCDC137 similarly affected the transcriptional activity of ERα. However, CCDC137 did not impact the activity of ERα in the presence of E2, RU58668, or ICI (Figure 9.5). The basal transcriptional activity of ERα increased upon over-expression of CCDC137, but more detailed studies are necessary to determine the mechanism behind
this activity. Unfortunately, antibodies are not available to CCDC137, so we were unable to confirm endogenous protein expression. With the use of an N-terminal V5 tag, we tested the transfection efficiency and resulting expression level, but could not reliably detect this tagged protein.

[Graph: Normalized Response vs. Concentration]

**Figure 9.5: CCDC137 may not impact the transcriptional activity of ERα**

MCF7 cells were transfected overnight with pcDNA3.1v5-DEST or –CCDC137, a 3XERE-TATA-luc reporter, and the CMV-βgal transfection control. Cells were treated overnight with vehicle, 100 nM E2, 100 nM RU58668 (RU), or 100 nM ICI. Data is the mean±SEM from a representative triplicate experiment.

Lacking appropriate tools to analyze protein expression, we evaluated the mRNA expression of *CCDC137* in breast cancer cell lines. We confirmed that *CCDC137* is expressed in ER-positive breast cancer cells (Figure 9.6A). After 4 hours of ligand treatment, there is down-regulation of *CCDC137* by both 4OHT and ICI in MCF7 and T47D cells (Figure 9.6B and C). In contrast, E2 up-regulated *CCDC137* expression in MCF7 cells, but repressed its expression in T47D cells. This cell-type specific regulation of *CCDC137* expression could be due to differential cofactor protein expression in the two cellular models. Despite the contrast, the data suggest that *CCDC137* expression may be controlled in part by ER.
Figure 9.6: CCDC137 is expressed in ER-positive breast cancer cells

(A) MCF7 cells were treated with vehicle, 100 nM E2, or 100 nM ICI for 4, 8, 16, or 24 hours. (B) MCF7 or (C) T47D cells were treated for 4 hours with vehicle, 100 nM E2, 100 nM 4OHT, or 100 nM ICI. For all, cells were harvested and cDNA prepared from isolated RNA was subjected to qRT-PCR analysis. Data are normalized to 36B4 and expressed as mean fold change over vehicle±SEM.

As an alternative, a functionality can be assigned to a protein based on the biological response that occurs when that particular protein is removed from the cell using siRNA technology. Therefore, we evaluated the impact of siRNA targeting CCDC137 (siCCDC137) on the E2-mediated induction of ERα transcriptional activity in MCF7 cells by analyzing endogenous ER target gene expression by qRT-PCR. siCCDC137 achieved greater than 90% knockdown of CCDC137 at the mRNA level (Figure 9.7). In control transfected cells, treatment with E2 led to down-regulation of TGFβ2 mRNA, whereas ICI had no effect. In the presence of siCCDC137, inhibition of TGFβ2 expression by E2 was lost, and further there was a modest increase in basal TGFβ2 expression. Since this gene is up-regulated by 4OHT, and CCDC137 may be involved in anti-
estrogen-mediated actions of ER, it would be interesting to see if siCCDC137 impacts the ability of 4OHT to regulate ER target gene expression. Together, these data suggest that CCDC137 is involved in E2-mediated repression of target gene expression, although further analysis of other down-regulated genes is necessary. This is intriguing considering that CCDC137 did not robustly interact with ER in the presence of E2, but perhaps a transient interaction occurs that has a robust biological consequence. It is difficult to examine the effect of CCDC137 on genes regulated by anti-estrogen-bound ER as none have been reliably identified.

**Figure 9.7: CCDC137 may have a role in E2-mediated gene repression**

MCF7 cells were transfected with siRNA to CCDC137 (siCCDC137), high GC content control (HGC), or mock for 72 hours. Cells were then treated for 8 hours with vehicle, 100 nM E2, or 100 nM ICI. Cells were harvested and cDNA prepared from isolated RNA was subjected to qRT-PCR analysis. Data are normalized to 36B4 and expressed as mean fold change over vehicle±SEM.

In summary, we identified a novel protein, CCDC137, that interacts with ERα primarily in the presence of anti-estrogens. H12, and perhaps the F domain of ERα, are required for appropriate interaction with CCDC137. The N-terminus of CCDC137, which contains a putative CoRNR box motif, is required for interaction with anti-estrogen-bound ERα, whereas the C-terminal fragment isolated from the yeast 2-hybrid screen interacts with E2-bound ERα, likely through an LxxLL motif. The implications of this interaction have yet to be fully elucidated, but our data hints at a role for CCDC137 in
ER-mediated transcription. Given the complex and important roles of other coiled-coil domain proteins in cell proliferation and differentiation, it is possible that CCDC137 plays a fundamental role in these aspects of ER biology.

9.2.1.2 CCDC137 modulates the transcriptional activity of AR

In a cross-screen with numerous NR family members, we found that CCDC137 also interacted in a ligand-dependent manner with the androgen receptor (AR). AR is a critical regulator of processes involved in the development of male characteristics and the male reproductive system, and as such controls prostate growth, skeletal growth and maintenance, and muscle mass. However, aberrant AR signaling can lead to the development of prostate cancer and thus therapeutics targeting the AR pathway have been developed for the treatment of this disease (300). On the other hand, AR agonists are clinically useful for treating osteoporosis and muscle wasting disease, which often occurs in patients with cancer (301). Selective AR modulators (SARMs) are thus in development to capitalize on tissue- and promoter-selective actions of AR to harness the anabolic activities of AR without impacting prostate function. These selective properties stem from differential protein-protein interactions, similar to SERM action, and these distinct interaction surfaces engage particular signaling pathways that culminate in specific biological outcomes. Thus, it is possible to correlate protein-protein interactions with biological functions. However, the exact proteins that bind to AR and transduce signals to downstream elements have yet to be fully elucidated.

We found that CCDC137 and AR interacted most robustly in the presence of either AR agonists (R1881) or a particular class of SARMs (RU486, RTI-001, and RTI-018) (Figure 9.8). These SARMs fall into the same class in terms of their cofactor binding profile, effect on AR transcriptional activity, and androgen-mediated prostate
cancer cell proliferation. CCDC137 might mediate activities that this class of ligands shares with full AR agonists such as R1881.

**Figure 9.8: CCDC137 interacts with AR**

HepG2 cells were transfected overnight with VP16 or VP16-AR, pMGb or pMGb-CCDC137, a 5XGal4Luc3 reporter, and the CMV-βgal transfection control. Cells were treated overnight with ligand as indicated. Mean±SEM from a representative experiment is shown.

To better understand the interaction between CCDC137 and AR, we analyzed their interaction when particular residues and regions had been mutated in each protein. The T877A mutation within AR promotes increased responsiveness to agonist, and allows AR to perceive some antagonists as agonists. AR E893stop does not contain H12 of the LBD or the C-terminus past H12. The pattern of interaction between CCDC137 and AR T877A was similar to that with wild-type AR; however, the E893stop mutation disrupted the interaction between AR and CCDC137 in the presence of the agonist R1881 and one SARM (RTI-018), but not the other two SARMs (Figure 9.9). It is known that R1881 does not bind with high enough affinity to AR E893stop to allow for DNA binding; perhaps the same is true for RTI-018, and further that DNA binding is required for interaction of AR and CCDC137. The different interaction patterns suggest that either these ligands do not bind with enough stability to AR E893stop to elicit protein-protein interactions, or that there exists more than one surface on AR with which CCDC137 can interact. The interaction between CCDC137 and AR was mediated primarily by the N-
terminus of CCDC137; however, the presence of the C-terminal end stabilized the interaction, leading to a more robust signal (Figure 9.9). Not surprisingly, the LxLL motif found in CCDC137 was not required for interaction with AR. With AR, often an FxxLF, FxxFF, or FxxMF motif mediates the interaction with known coactivators (302); however, there is no such a motif in CCDC137. Therefore, the interaction between AR and CCDC137 required two unidentified motifs, one within the N-terminus and a second at the C-terminal end.

**Figure 9.9: The C-terminus of CCDC137 is not required for interaction with agonist- and SARM-bound AR**

HepG2 cells were transfected overnight with (A) VP16, VP16-AR T877A, or −AR E893stop, pMGb or pMGb-CCDC137, a 5XGal4Luc3 reporter, and the CMV-βgal transfection control, or (B) VP16 or VP16-AR, pMGb, pMGb-CCDC137, −CCDC137 N, −CCDC137 dL, or −−CCDC137 L, a 5XGal4Luc3 reporter, and the CMV-βgal transfection control. For A and B, cells were treated overnight with vehicle or 100 nM ligand as indicated. Mean±SEM from a representative experiment is shown.

AR mediates many biological responses through direct changes in target gene expression, so we assessed the effect of overexpressing CCDC137 on the transcriptional activity of AR. In the presence of R1881 and RU486, AR transcriptional activity is increased on both a mouse mammary tumor virus (MMTV)-luc and a probasin (PB)-luc reporter (Figure 9.10). Over-expression of CCDC137 led to a small but significant reduction in AR transcriptional activity on both reporter constructs, indicating that CCDC137 may play a role in repressing AR activity at the level of transcription.
HepG2 cells were transfected overnight with SG5 or SG5-AR, pcDNA3.1nv5-DEST or pcDNA3.1nv5-CCDC137, either an MMTV-luc (A) or a PB-luc (B) reporter, and the CMV-βgal transfection control. Cells were treated overnight with vehicle, 100 nM R1881, 100 nM RU486, or 100 nM casodex. Mean±SEM from a representative experiment is shown.

However, when we used siRNA to decrease endogenous expression of CCDC137 and then analyzed the expression of the AR target gene FKBP51 (FK506-binding immunophilin 51), the results were reversed. siCCDC137 inhibited the induction of FKBP51 by R1881 and RU486 in LNCaP prostate cancer cells (Figure 9.11), suggesting that CCDC137 is a positive regulator of AR transcriptional activity. The contradiction between the over-expression and knockdown studies may stem from a promoter-specific effect (PB-luc, MMTV-luc, and FKBP51), a cell-type specific effect (HepG2 versus LNCaP), or perhaps CCDC137 affects AR activity outside the context of transcriptional activity. However, given the ligand-specific interaction of CCDC137 with AR, and the
need for a better understanding of process-specific regulation of AR, further studies are warranted to elucidate the exact role of CCDC137 in AR signaling pathways in both prostate and muscle.

![Figure 9.11](image)

**Figure 9.11: siCCDC137 attenuates the ligand-induced expression of FKBP51**

LNCaP cells were transfected with siRNA to CCDC137 (siCCDC137), high GC content control (HGC), or mock for 72 hours. Cells were then treated for 8 hours with vehicle, 100 nM R1881, or 100 nM RU486. Cells were harvested and cDNA prepared from isolated RNA was subjected to qRT-PCR analysis. Data are normalized to 36B4 and expressed as mean fold change over vehicle±SEM.

### 9.2.2 PBXIP1/HPIP

Numerous other proteins were identified from the yeast 2-hybrid screen, including hematopoetic pre-B cell leukemia transcription factor (PBX)-interacting protein (PBXIP1/HPIP). HPIP has largely been characterized for its ability to inhibit PBX activity by interfering with its DNA binding ability (303). It also interacts with cytoskeletal fibers and microtubules and shuttles between the cytoplasm and nucleus (304, 305), suggesting that it impacts protein trafficking and signaling in both cellular compartments. There is evidence that ER can initiate rapid signaling events through the MAPK and PI3K/AKT pathways, but the mechanism(s) by which this occurs is not entirely clear. A recent study provided the framework for one potential mechanism by showing that E2-bound ERα interacts with microtubules via a direct protein-protein interaction with HPIP (305). This HPIP-ERα interaction was important for recruitment
of Src kinase and PI3K to ERα, and subsequently signaling was initiated through the MAPK and PI3K/AKT pathways, which regulate cell proliferation, growth, and survival (305). When HPIP provided a scaffold for ERα to interact with components of the MAPK and PI3K pathways, flux through these pathways was increased (305). These kinases phosphorylate ERα primarily in the AF-1 domain, which increases the output of this transcriptional domain (306). Thus, the interaction of HPIP with E2-bound ERα has direct effects on ERα phosphorylation that result in increased transcriptional activity and breast cancer proliferation (305).

We showed that HPIP interacted with ERα in the presence of 4OHT and ICI in addition to the previously published interaction in the presence of E2 (Figure 9.12) (305). However, the significance of this interaction in the presence of 4OHT and ICI has yet to be described. 4OHT modulates signaling through the calcium/calmodulin pathway (193), and HPIP could be involved in bringing 4OHT-ERα together with components of the calcium/calmodulin pathway. Nevertheless, some data suggest that cytoplasmic signaling by 4OHT is ER-independent. It will be interesting to dissect the role of HPIP in 4OHT- and ICI-mediated actions of ERα, and to compare these to the actions initiated by HPIP-ERα in the presence of E2.
HepG2 cells were transfected overnight with VP16 or VP16-ERα, pMGb or pMGb-HPIP, a 5XGal4Luc3 reporter, and the CMV-βgal transfection control. Cells were treated overnight with vehicle, 100 nM E2, 100 nM 4OHT, or 100 nM ICI. Mean±SEM from a representative experiment is shown.

9.2.3 14-3-3η

14-3-3 family members are scaffolding proteins that influence such diverse processes as cell cycle, cell proliferation, differentiation, and apoptosis through the regulation of numerous signal transduction pathways. In their scaffolding function, they regulate protein localization and stability. An involvement in NR signaling was suggested in 2005, when 14-3-3η was shown to interact with GR and enhance its transcriptional activity by inhibiting ligand-induced GR degradation (307). Additionally, 14-3-3η increases gene expression of certain AR-regulated genes, again presumably through its ability to increase AR protein stability (308). Thus, it is likely that 14-3-3η can modulate signaling by other NRs, such as ER. In the case of ERα, the impact of 14-3-3η on protein stability may have a contrasting effect on transcriptional activity since agonist-dependent degradation of ERα has been positively correlated with efficient transcriptional activation, although this idea is still controversial. It is intriguing that 14-3-3η is highly expressed in the brain, where it is involved in hypothalamic-pituitary-adrenal (HPA) axis regulation (309), neuroprotection (310), and damage in Parkinson’s disease (311). There are numerous hypothesized roles of estrogen signaling in the brain,
and 14-3-3η is a strong candidate for involvement in ER action in the brain as well as other sites throughout the body.

14-3-3η was isolated from the yeast-2 hybrid screen and found to interact with ERα in the presence of agonists (E2), SERMs (4OHT), and antagonists (ICI), and may interact with ERα in the absence of ligand, which would fit with its role in protecting receptors from degradation (Figure 9.13A). The most robust interaction between ERα and 14-3-3η occurred in the presence of ICI, where 14-3-3η could sequester ERα outside the nucleus and thereby block transcriptional activation. 14-3-3η is expressed in ER-positive breast cancer cells (Figure 9.13B), but its expression level was not regulated by ER ligands and the exact role it plays in ER biology in the breast and the brain has yet to be elucidated.

![Diagram](image_url)

**Figure 9.13:** The interaction between 14-3-3η and ERα occurs most robustly in the presence of ICI

(A) HepG2 cells were transfected overnight with VP16 or VP16-ERα, pM or pM-14-3-3η, a 5XGal4Luc3 reporter, and the CMV-βgal transfection control. Cells were treated overnight with vehicle, 100 nM E2, 100 nM 4OHT, or 100 nM ICI. A representative experiment is shown. (B) MCF7 cells were treated for 8 hours with vehicle, 100 nM E2, 100 nM 4OHT, or 100 nM ICI. Cells were harvested and cDNA prepared from isolated RNA was subjected to qRT-PCR analysis. Data are normalized to 36B4 and expressed as mean fold change over vehicle±SEM.
9.3 Screening of random peptide libraries for novel protein-protein interaction motifs

Ligand-induced conformational changes are critical in dictating the biological response to a particular ER ligand by allowing for the presentation of unique protein-protein interaction surfaces. From the discovery that 27HC is an endogenous SERM, it followed that 27HC likely induced a unique conformation of ER and therefore may allow for the presentation of unique interaction surfaces that mediate specific biological processes. Using small peptides as conformational probes, we utilized two approaches to confirm that 27HC indeed induced a unique structural change in ER upon binding that likely leads to unique array of protein-protein interactions: first, by testing the interaction of known ER binding peptides with 27HC-bound ER, and second, by performing an M13 phage display screen to identify unique peptides that bound with high affinity to 27HC-ER.

We performed the M13 screen using both an LxxLL and a CoRNR box library, and used as bait either 27HC-ERα or 27HC-ERβ (for more details, see Chapter 2). By M2H, we characterized the ligand-dependent interaction of these identified peptides with ERα or ERβ. Although this combinatorial phage display study was initiated to survey the conformation of 27HC-bound ER and to compare this conformation to E2- and 4OHT-bound ER, the high affinity interacting peptides identified in this screen represent motifs found in potential ER-interacting proteins and thus can be used to identify proteins that bind to 27HC-ER. The peptides probe unique interaction surfaces that are presented on the surface of ER, and many of these sites represent true protein-protein interaction surfaces that are differentially displayed in the presence of ER ligands. Therefore, we performed a BLAST analysis to identify proteins with similarity to...
the unique peptide sequences, particularly the LxxLL or CoRNR box motifs that are known to be important for coregulatory protein interaction with ER.

**9.3.1 Identification of peptides that exhibit unique ER-binding characteristics**

For a list of selected peptides and their similarity to identified proteins, see Table 9.1. The CDD.29 peptide interacts specifically with ERα in the presence of 27HC and 4OHT, but not E2. The most significant alignment of this peptide with the human genome is with laminin 3α (LAMA3, NO_001121189). LAMA3 is part of the Laminin 5 heterotrimer, an important component of the extracellular matrix component in breast epithelium (312). An interaction between LAMA3 and ERα suggests either a role for LAMA3 outside of matrix composition, or that 27HC and 4OHT regulate novel actions of ERα in regulating the extracellular matrix or cytoskeleton. To support the second hypothesis, ERα interacts with α- and β-tubulin through its AF-1 domain in breast cancer cells (313), and this may contribute to rapid signaling through kinase cascades. It is likely that just as there are ligand-specific effects on ER transcriptional activity, there are ligand-specific effects on the rapid actions of ERα, perhaps by controlling which cytoplasmic or membrane associated proteins ER interacts with, or dictating the downstream signaling molecules with which it can couple.
Table 9.1: Select peptide sequences and the proteins with which they share similarity

<table>
<thead>
<tr>
<th>Peptide</th>
<th>Sequence</th>
<th>Similar Protein</th>
<th>Protein Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDD.64</td>
<td>LQDHPILGLQLQKQGT</td>
<td>SH3BP2, CD2L5, RA114</td>
<td>QDHIPQSEEAPHVVLGLRLGGAQGDW ILLNLQSK and QQPPLLPLQ QOD---LLSLLQAK</td>
</tr>
<tr>
<td>CDD.30</td>
<td>LEADFFRLFNLTDDGW</td>
<td>LRRTM4, FSD1</td>
<td>AHFFRLFNLEAEFQSLFSLL</td>
</tr>
<tr>
<td>CDD.14</td>
<td>LGESHPILMLLTTENVTH</td>
<td>TRAP220, Exportin 7</td>
<td>HPMLMNLLKDN ESQHHLMQLL</td>
</tr>
<tr>
<td>CDD.21</td>
<td>DLRDFAILGLGLQTRAEL</td>
<td>F8, CXCL12</td>
<td>LKDFPFLPG, GLLGPIQAE PILSPHPFRAGQARA</td>
</tr>
<tr>
<td>CDD.35</td>
<td>VQMEYPILTGKSLQAM</td>
<td>SLC7A1, PREX2</td>
<td>ILTGLLTVKES-AM YPLILKEK and EALQAM</td>
</tr>
<tr>
<td>CDD.51</td>
<td>FNIDPYILVGLLTSDKVSM</td>
<td>TMC3, KIAA0195, PANK3</td>
<td>YSLIIALL-DKVNMS NIDNVPLVPLPTD and YPLLSISLL FNDoPPLLY</td>
</tr>
<tr>
<td>CDD.11</td>
<td>ASDVTCKLPIICSLLMAKEAES</td>
<td>F-box protein 15 SH3TC2, Kelch-like 36</td>
<td>CSLLMAKQ LEPLLCSL KPAVCSSL-FKEA</td>
</tr>
<tr>
<td>CDD.29</td>
<td>ITSSRDSLRLHIAAQLLTTSVNAA</td>
<td>KIAA1524, LAMA3</td>
<td>DTLRMHIAKILTTS ISSSRQSLRL</td>
</tr>
<tr>
<td>CDD.6</td>
<td>SGWDLQLHFTACTGYNHCVK</td>
<td>PTOV1, CNOT1</td>
<td>LFPHISFC, FPQYDFRLHITACL and LAGLAPHIT</td>
</tr>
</tbody>
</table>

CDD.11 interacted with both ERα and ERβ in the presence of E2 and 27HC, and this sequence was pulled out with high frequency. This class of peptides represents the fact that the conformation of ER induced by E2 and 27HC is quite similar. It is interesting that this peptide contains a CoRNR box motif, which mediates the interaction between antagonist-bound ER and corepressor proteins (107). However, many diverse motifs are likely involved in making interactions with proteins that repress ER activity, and further, E2-bound ER actively represses transcription of some target genes, indicating that this complex can recruit transcriptional repressors (314). ERα, and to a lesser extent ERβ, undergoes ligand-mediated degradation through the 26S proteosome.
pathway, but the exact ubiquitin conjugating enzyme(s) and ligase(s) that mediate this have not been conclusively identified. Interestingly, peptide CDD.11, which interacts with both ERα and ERβ in the presence of E2 or 27HC, contains a similar sequence as that found in F-box protein 15, which is part of protein-ubiquitin ligase complexes. This potentially implicates F-box protein 15 in agonist-mediated degradation of ER.

Peptide CDD.14 showed similarity to the known ER-interacting protein mediator complex subunit 1 (also known as TRAP220). This peptide interacted with ERα in the presence of E2 or 27HC, and given the involvement of TRAP220 in ER transcriptional activity, this interaction highlights a common surface presented on ER by 27HC and E2 that regulates agonist activity. Another peptide with a similar interaction profile is CDD.64, which shares amino acid similarity to SH3-domain binding protein 2 (SH3BP2), a protein that regulates TNFα production by macrophages, thereby influencing processes such as bone homeostasis (315). It is clear that E2 plays an important role in regulating bone turnover, perhaps through interactions with SH3BP2, but the mechanisms by which it accomplishes this are not fully elucidated. CDD.64 is also similar to CDC2-related protein kinase (CDC2L5) which is involved in processes already linked to E2 signaling such as cell proliferation, differentiation, or apoptosis. Another indication that ER is actively involved in cytoskeletal organization and signaling is the determination that CDD.64 is also similar to the cytoskeleton-associated protein retinoic-acid-induced protein 14 (RAI14).

9.3.2 Specific peptides that are selective for 27HC-bound ER

There were many other peptides with similarity to known proteins that could interact with ER and be involved in aspects of ER biology. CDD.30, which interacted with both ERα and ERβ specifically in the presence of 27HC, shows similarity to FSD1
(fibronectin type 3 and SPRY domain containing 1), a protein thought to be important in microtubule organization and stability. This supports our earlier finding that 27HC-bound ER could have signaling functions at microtubule and cytoskeletal networks. CDD.30 is also similar to LRRTM4, a leucine rich repeat neuronal protein that is important for the development of the nervous system. Recent data posit that 27HC has a crucial role in the etiology of neurodegenerative disease, stemming from its ability to down-regulate amyloid beta secretion from primary human neurons (316).

Other proteins that may be involved specifically in 27HC-mediated signaling through ERβ include:

- CDD.6, similar to prostate tumor overexpressed 1 (PTOV1) which promotes proliferation and may be involved in transcription, and subunit 1 of the CCR4-NOT transcription complex (CNOT1), a homeobox protein.
- CDD.21, similar to stromal cell derived factor 1 (CXCL12), an important mediator of chemotaxis and homing to the bone microenvironment, and coagulation factor VIII (F8), a protein that binds to von Willebrand factor and is therefore critical for clot formation evidenced by the fact that mutations cause hemophilia.
- CDD.35, similar to solute carrier family 7 member 1 (SLC7A1), a permease involved in the transport of cationic amino acids outside the liver, and phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor 2 (PREX2).
- CDD.51, similar to transmembrane channel-like 3 (TMC3), a protein though to be involved in calcium transport, and pantothenate kinase 3 (PANK3), which has been implicated in neurodegeneration.

9.4 Discussion

The yeast 2-hybrid screen performed with ERLBD3X and the combinatorial peptide screen with ERα and ERβ in the presence of 27HC together yielded many potential ER interacting proteins with a diverse set of biological functions. The diversity of these identified proteins highlights the fact that ER is not solely an activator of transcription, but can also repress transcription, signal rapidly through kinase pathways, modulate protein trafficking or signaling on cytoskeletal/microtubule networks, and impact cytoskeletal reorganization and cell motility, among other roles certain to be
ascertained in future studies. How these diverse functionalities are differentially regulated by ER ligands will be an important aspect of our understanding of how to treat pathologies associated with aberrant ER signaling.
References


28. R. S. Pollenz, C. A. Sattler, A. Poland. The aryl hydrocarbon receptor and aryl hydrocarbon receptor nuclear translocator protein show distinct subcellular localizations in Hepa 1c1c7 cells by immunofluorescence microscopy. *Mol Pharmacol* 45, 428 (Mar, 1994).


40. L. J. Hushka, J. S. Williams, W. F. Greenlee. Characterization of 2,3,7,8-
tetrachlorodibenzo-p-dioxin-dependent suppression and AH receptor pathway gene
expression in the developing mouse mammary gland. *Toxicol Appl Pharmacol*
**152**, 200 (Sep, 1998).

Hennighausen. The aryl hydrocarbon receptor (AhR) and its nuclear translocator
(Arnt) are dispensable for normal mammary gland development but are required

42. M. S. Denison, S. R. Nagy. Activation of the aryl hydrocarbon receptor by
structurally diverse exogenous and endogenous chemicals. *Annu Rev Pharmacol

43. S. Khan, R. Barhoumi, R. Burghardt, S. Liu, K. Kim, S. Safe. Molecular
mechanism of inhibitory aryl hydrocarbon receptor-estrogen receptor/Sp1 cross

44. J. K. Hockings, P. A. Thorne, M. Q. Kemp, S. S. Morgan, O. Selmin, D. F.
Romagnolo. The ligand status of the aromatic hydrocarbon receptor modulates
transcriptional activation of BRCA-1 promoter by estrogen. *Cancer Res* **66**, 2224
(Feb 15, 2006).

45. F. Ohtake, A. Baba, I. Takada, M. Okada, K. Iwasaki, H. Miki, S. Takahashi, A.
Kouzmenko, K. Nohara, T. Chiba, Y. Fujii-Kuriyama, S. Kato. Dioxin receptor is a

46. J. Ruegg, E. Swedenborg, D. Wahlstrom, A. Escande, P. Balaguer, K. Pettersson,
I. Pongratz. The transcription factor aryl hydrocarbon receptor nuclear
translocator functions as an estrogen receptor beta-selective coactivator, and its
recruitment to alternative pathways mediates antiestrogenic effects of dioxin.

47. M. Abdelrahim, E. Ariazi, K. Kim, S. Khan, R. Barhoumi, R. Burghardt, S. Liu, D.
Hill, R. Finnell, B. Wlodarczyk, V. C. Jordan, S. Safe. 3-Methylcholanthrene and
other aryl hydrocarbon receptor agonists directly activate estrogen receptor

The major cyclic trimeric product of indole-3-carbinol is a strong agonist of the


154. H. A. Tanriverdi, A. Barut, S. Sarikaya. Statins have additive effects to vertebral bone mineral density in combination with risedronate in hypercholesterolemic


173. K. M. Robertson, M. Norgard, S. H. Windahl, K. Hultenby, C. Ohlsson, G. Andersson, J. A. Gustafsson. Cholesterol-sensing receptors, liver X receptor


202. V. Lecureur, E. L. Ferrec, M. N'Diaye, M. L. Vee, C. Gardyn, D. Gilot, O. Fardel. ERK-dependent induction of TNFalpha expression by the environmental


213. Q. Ma, K. T. Baldwin. 2,3,7,8-tetrachlorodibenzo-p-dioxin-induced degradation of aryl hydrocarbon receptor (AhR) by the ubiquitin-proteasome pathway. Role of the transcription activaton and DNA binding of AhR. *J Biol Chem* 275, 8432 (Mar 24, 2000).


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