Factors influencing ER subtype-mediated cell proliferation and apoptosis

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Factors influencing ER subtype-mediated cell proliferation and apoptosis

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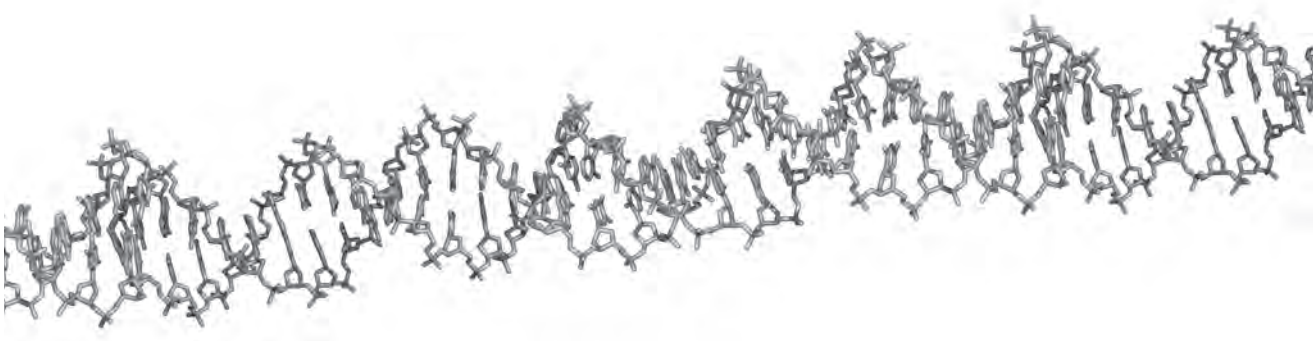
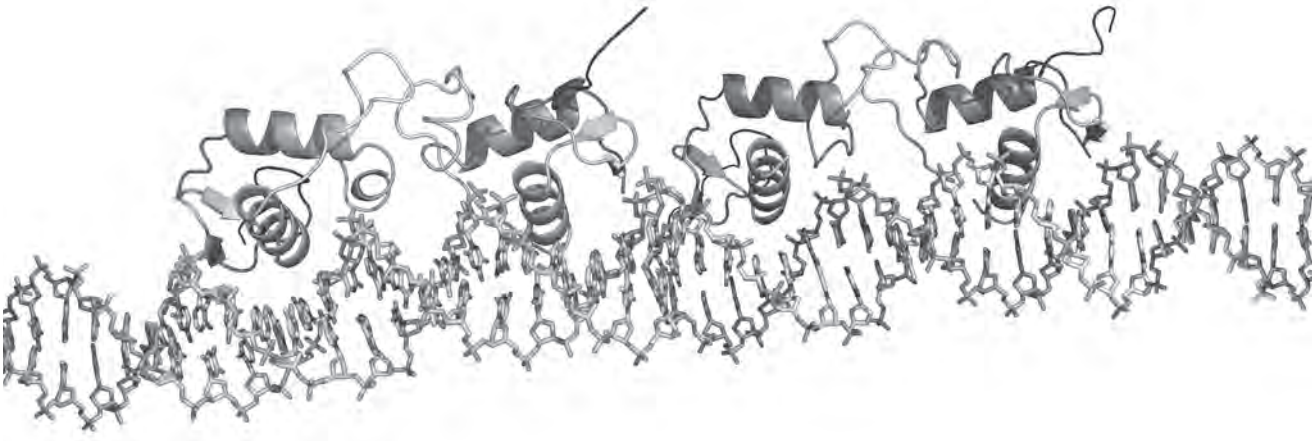


1 Introduction



Estrogenic compounds exert their effects on cells through the binding and activation of estrogen receptors (ERs) [1]. Major biological events influenced by estrogenic compounds upon activation of the ERs are related to the cell cycle and (tumor) cell growth, like cell proliferation and apoptosis [2-4]. In the present thesis it is investigated to what extent different factors involved in the mode of action of the ER-mediated cellular responses influence the cell proliferation and/or apoptosis induced upon binding of estrogenic compounds to ERs. In the following sections these factors are explained in some more detail.

Estrogenic compounds: agonists and antagonists

Estrogens are naturally occurring steroid hormones present in both men and women [5]. Levels of circulating estrogens vary markedly between individuals. 17β-Estradiol (E2) levels of 10-20 pg/ml have been reported in postmenopausal women, while in vitro fertilisation (IVF) hyperstimulated women showed E2 levels of >2000 pg/ml. Premenopausal women with a normal cycle usually show values in the range of 50-500 pg/ml [6]. E2 levels reported in men are around 20 pg/ml [7]. Three different estrogenic compounds are present in the body in significant quantity; estrone (E1) is the precursor for E2, which in turn can be converted to estriol (E3) [8] (Figure 1). E2 is the most potent estrogenic compound naturally occurring in the human body [9]. It is about 10-fold more potent than estrone [10].

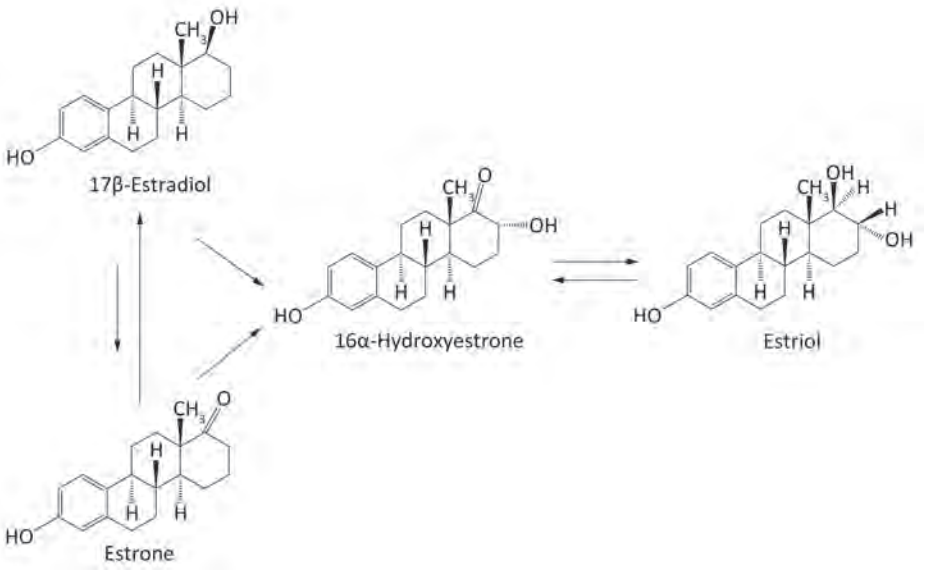


Figure 1: Schematic representation of the conversion of estrogenic compounds naturally present in the human body. Adapted from [11].

Estrogenic compounds can also be found in plants. These plant-derived estrogens are called phytoestrogens. One of the best characterized phytoestrogens is genistein, commonly found in soy beans and soy products including food supplements [12, 13].

Xenoestrogens are chemically synthesized estrogenic compounds [14]. These non-steroidal compounds can be present in for example plasticizers, food preservatives, pesticides, dyes, and chemical pollutions [15].

From this wide variety and diverse presence of compound classes it can be concluded that several types of estrogenic compounds exist, which may have different modes of action inducing different cellular responses varying from stimulation of cell proliferation [2], cancer cell migration [16], and estrogen receptor turnover [17] to stimulation of apoptosis and reduction of cell proliferation [3, 4, 18-20]. There are compounds that provoke estrogenic (E2-like) responses (agonists) and compounds that counteract estrogenic responses (antagonists), with the latter binding to the ER without inducing a physiological response [21]. Antagonistic responses can be partial or full [22] and this can even be dependent on the tissue. These tissue-specific anti-estrogens are called Selective Estrogen Receptor Modulators (SERMs) [23]. All types of estrogenic compounds exert their effect through binding to estrogen receptors [1].

Estrogen receptors

The estrogen receptor (ER) was first discovered by Jensen *et al.* in 1958 [24, 25]. In 1996 Kuipers *et al.* discovered that there are actually 2 types of ERs. The one discovered by Jensen *et al.* was renamed ERα and the one discovered by Kuipers *et al.* was named ERβ [26, 27]. Even though these two ERs are encoded by different genes localized on different chromosomes, they share a high homology in their functional regions (Figure 2). ERα and ERβ share 28% amino acid homology in the A/B domain, which contains the activation function 1 (AF-1) domain involved in ligand-independent transactivation. The C domain, also referred to as the DNA binding domain (DBD), is 96% homologous between the two ER subtypes. This region of the ER binds to the estrogen response element (ERE) in the DNA to initiate transcription [28]. Little is known about the function of the D domain other than that it contains the nuclear localization site of the ERs and post-translational modification sites [29] and that homology between ERα and ERβ for this region is only 17%. The E and F domains respectively share 58% and 18% homology between ERα and ERβ. The activation function 2 (AF-2) domain is present within the E/F region. This region also holds the ligand binding domain (LBD) [28].

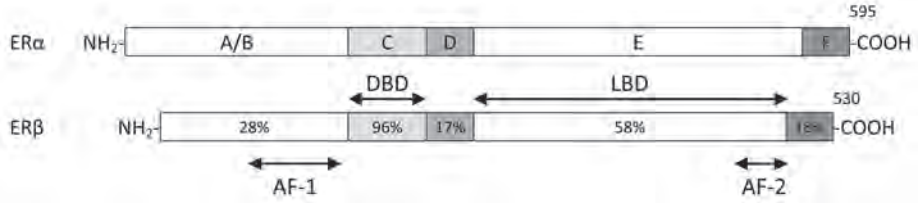


Figure 2: Schematic representation of the domain classification of the human ERα and ERβ proteins. The domains are represented in ERα, the homology percentages in ERβ. Adapted from [28].

ERs belong to the group of nuclear hormone receptors (NR) [30]. The ERs are present in the cell cytoplasm and are stabilized by heat shock protein (HSP)70 and HSP90 which specifically bind the LBD [31]. Upon binding of the ligand, a conformational change is induced and dimerization of the ERs occurs [32]. The type of dimerization (ERα-ERα or ERβ-ERβ homodimerization or ERα-ERβ heterodimerization) is dependent on the ERα/ERβ ratio [33]. It has been suggested that by ERα-ERβ heterodimerization ERβ is able to modulate ERα-mediated responses [34, 35].

Both ERs are widely distributed throughout the body. As depicted in Table 1, literature reports differential results in detected ERα and ERβ expression in various tissues. Even when ERα and ERβ are expressed in the same tissue, their expression might still vary per cell type [1].

Table 1: Messenger RNA (mRNA) ERα and ERβ expression in different tissues.

Dominant ERα expression	Dominant ERβ expression	ERα and ERβ co-expression	Species	Reference
Uterus, oviduct, cervix, vagina, mammary gland, pituitary, aorta, kidney, spleen, skeletal muscle, bone marrow	Ovary	Hypothalamus, lung, prostate, epididymis, cortex, olfactory bulb, heart, liver	Mouse	[36]
Epididymis, testis, pituitary, uterus, kidney, adrenal gland	Prostate, bladder	Ovary, uterus	Rat	[37]
Uterus, gut	Ovary, testis, adrenal gland, spleen, lung	Skin, stomach, colon	Midgestational human fetus	[38]

The G protein-coupled receptor 30 (GPR30) has proposed to be a membrane bound estrogen receptor [39, 40]. However, GPR30 agonist G1 does not stimulate estrogen-like effects in the uterus or mammary gland of mice [41], and GPR30 is incapable of mediating any actions of estrogen in cells in which ERα and ERβ are absent [42, 43]. Therefore, this receptor will not be included in the current thesis.

Coregulators

Upon binding of an estrogenic compound to the estrogen receptor and dimerization of the ERs, specific coregulators are attracted to or rejected from the dimerized ligand-ER complex [44]. Coregulators do not bind to the DNA directly, but they interact through association with the ERs [31]. There are two types of coregulators; coactivators and corepressors, which will respectively enhance or inhibit gene transcription. Some coregulators have a receptor-dependent coactivator or corepressor function. PELP1 (proline, glutamate and leucine rich protein 1) for example is a coactivator of estrogen receptor-mediated transcription and a corepressor of other nuclear hormone receptors [50]. Coactivator binding has been attributed to the AF-1 and AF-2 domain as well as the D domain of the ERs. For corepressor binding it seems that mostly AF-2 is responsible [28]. Coregulators (coactivators and corepressors) mostly interact with ERs through their LXXLL motifs (leucine - any amino acid - any amino acid - leucine - leucine) that can bind to the LBD [31]. However, interactions with the A/B domain have also been reported [51-53].

The ligand binding domain of ERs consists of 12 α-helices. Helix 12 folds differently upon agonist binding than upon antagonist binding [54]. In the presence of an agonist, coactivators are able to bind to a hydrophobic groove on the surface of the ligand binding domain. When an antagonist binds to the receptor, the folding of helix 12 blocks the coactivator binding surface and thereby influences coregulator recruitment [54].

Upon recruitment, coregulators form complexes and do not act as monomers [31, 55]. Although the role of several coregulators involved in ER signaling has been investigated (Table 2), the complexity of how modulation of the interaction of ERs with coregulators affect ER activation remains to be studied in more detail. In addition, in studies investigating modulation of the interaction of ERs with coregulators often no differentiation is made between coregulators involved in ERα signaling and coregulators involved in ERβ signaling [56, 57].

Table 2: Overview of ER-mediated coregulators, adapted from [44]. Coactivators presented in white, corepressors in grey background.

Coregulator	Full name	Function/activity	Interaction with ER
SRC-1 (p160) NCOA1	Steroid receptor coactivator-1	Histone acetyltransferase (HAT)	Binds ERs AF-2 through LXXLL motifs (highly conserved motifs that bind the LBD [45])
SRC-2 (p160) GRIP1 TIF-2 NCoA-2	Steroid receptor coactivator-2	HAT	Binds ERs AF-2 through LXXLL motifs
SRC-3 (p160) AIB-1 ACTR p/CIP RAC3 TRAM-1 NCoA-3	Steroid receptor coactivator-3	HAT	Binds ERs AF-2 through LXXLL motifs
CBP/p300	Cyclic adenosine 3', 5'-monophosphate (cAMP) response element-binding protein (CREB) [46])-binding protein	HAT	Binds ERs AF-2 through LXXLL motifs
TRAP220 TRAP/DRIP PBP			Binds ERs AF-2 through LXXLL motifs
ASC-1	Activating signal cointegrator-1	Binds HATs and NRs	Binds ERs AF-2 through LXXLL motifs
ASC-2 RAP250 TRBP AIB3	Activating signal cointegrator-2	Binds HATs and NRs	Binds ERs AF-2 through LXXLL motifs
SRA	Steroid receptor activator	Splicing	Binds ERα AF-1
P68	p68 RNA helicase	RNA helicase	Binds ERα AF-1
CARM1	Coactivator-associated arginine methyltransferase 1 [47]	Arginine histone methyltransferase	Binds ERs AF-2 indirectly through association with p160s
PRMT1	Protein methyltransferase 1	Arginine histone methyltransferase	Binds ERs AF-2 indirectly through association with p160s

Table 2: Overview of ER-mediated coregulators, adapted from [44]. Coactivators presented in white, corepressors in grey background. (*continued*)

Coregulator	Full name	Function/activity	Interaction with ER
CoCoA	Coiled-coil coactivator	Arginine histone methyltransferase	Binds ERs AF-2 indirectly through association with p160s
E6-AP	E6-associated protein	Ubiquitin ligase	Binds ERs AF-2
RPF-1	Receptor potentiating factor-1	Ubiquitin ligase	Binds ERs AF-2
PGC-1α PGC-1β	PPARγ coactivator 1	Tethering surface for other co-factors; splicing	Binds the hinge region of the ERs
CAPER-α CAPER-β CoAA	Coactivator of (AP-1) and estrogen receptors Coactivator activator	Potentiate ER activity; splicing	Associate with ER indirectly through binding ASC-2 (activating signal co-integrator 2 [48])
NCoR	Nuclear receptor corepressor	Histone deacetylase (HDAC)	Binds ERs AF-2 through CoRNR box motifs (motifs similar to LXXLL motifs [49])
SMRT	Silencing mediator for retinoid and thyroid receptors	HDAC	Binds ERs AF-2 through CoRNR box motifs
RIP140 (NRIP)	Receptor interacting protein of 140 kiloDalton (kDa)	Competes for AF-2 coactivator binding; associates with HDACs	Binds ERs AF-2 through LXXLL motifs
REA	Repressor of estrogen receptor activity	Interferes with SRC-1 access to the ERs	Indirect
RTA	Repressor of tamoxifen transcriptional activity	Interferes with SRC-1 access to the ERs RNA binding; represses tamoxifen agonist activity through ERα	Binds ER AF-1
mSiah2	Mammalian homolog of Drosophila Seven in absentia (sina)	Mediates cell-specific repression of NRs by targeting NCoR for proteasomal degradation	Associates with ER indirectly through binding NCoR

Towards gene and protein expression

When coactivators are recruited, they enhance transcriptional activity through a combination of mechanisms, including the recruitment of transcription factors. Through interaction with amongst others acetyltransferases there is a disruption of the local nucleosomal structure of the DNA [31]. According to the classical pathway of ER signaling, the ligand-ER complex with the recruited coregulators will bind to the DNA at the site of the ERE sequences in the promotor sites of target genes. In the case of an agonistic ligand, this initiates transcription and mRNA production and ultimately protein synthesis [31]. Within the DBD of the ERs, an amino acid sequence called the P-box is present. This motif is critical for receptor-DNA recognition and specificity. The P-box motif is identical between ERα and ERβ. This leads to similar affinity and specificity of ERα and ERβ in ERE binding [1].

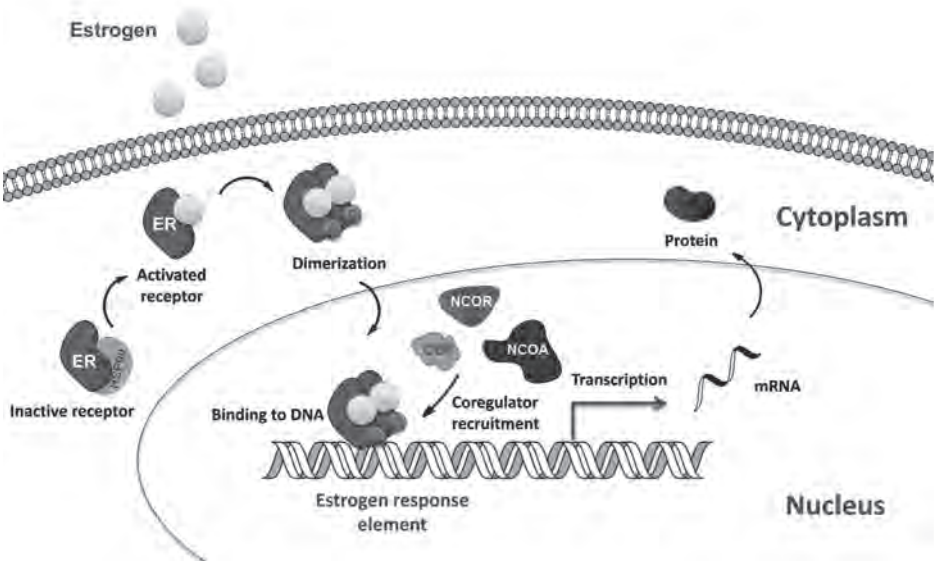


Figure 3: Schematic representation of the mechanism of action of estrogens; the classical ER signalling pathway. Figure by Wang [58], with permission.

ER agonists and ER antagonists have a different mechanism of action. As depicted in Figure 4, ER binding of agonists like E2 results in dimerization of the ligand-receptor complex and activation of both AF-1 and AF-2. Upon binding of the dimerized ligand-receptor complex to the ERE, both AF-1 and AF-2 recruit coregulators and this leads to fully activated transcription. Tamoxifen, a partial ER antagonist, only activates AF-1

upon ligand-receptor binding and dimerization. This leads to AF-1 and no AF-2-mediated coregulator recruitment, upon which transcription is partially activated. The full ER antagonist fulvestrant blocks the estrogen receptors and thereby keeps both AF-1 and AF-2 in an inactive state. As a result no coregulators are recruited and transcription is not induced. Fulvestrant is also able to induce ERα degradation [59].

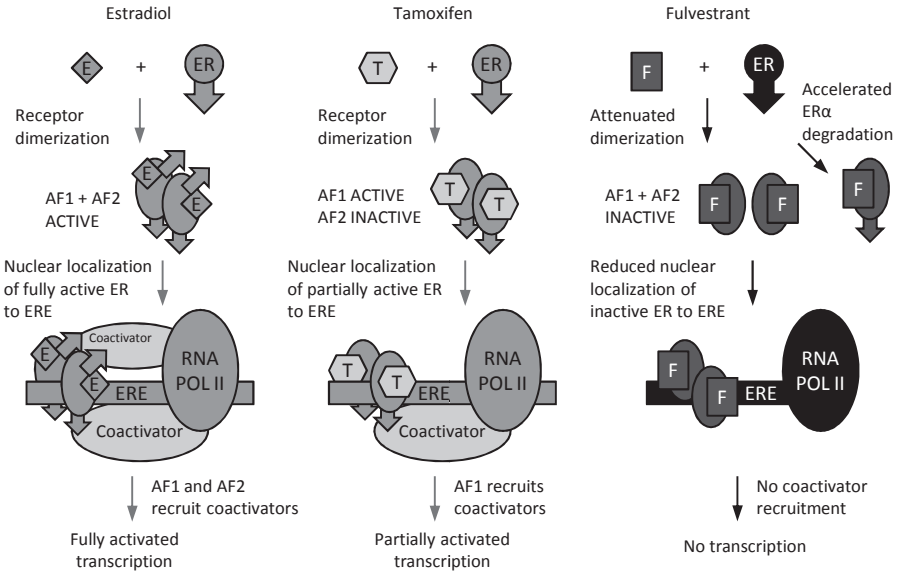


Figure 4: Mechanism of action of estradiol (ER agonist), tamoxifen (partial ER antagonist), and fulvestrant (full ER antagonist). Adapted from [59].

There are other pathways described in the literature by which ERs can induce gene expression. Growth factors like epidermal growth factor (EGF), insulin-like growth factor 1 (IGF-1), and cAMP are able to activate the ER and induce expression of ER target genes in the absence of ligand [60]. Ligand-ER complex induction of gene expression without an ERE-like sequence present in the promotor site of the target gene has also been reported [61]. Finally, ERs can be activated by phosphorylation instead of by a ligand-mediated mode of action [62].

In the current thesis the mechanisms of action behind the ER signaling pathways as described in Figure 3 and 4 were studied in more detail. More particularly, the ER signaling pathways were studied to assess differential effects of estrogenic compounds and their interaction with differentially expressed ERα and ERβ.

Biological effects

Upon gene transcription induced by estrogenic compounds, protein expression is altered which eventually leads to biological effects. Estrogenic compounds have been reported to show a variety of cellular effects. Estradiol is for example reported to stimulate cancer cell migration [16]. Anti-estrogenic compound ICI 164,384 reduces ER expression by increasing its turnover [17]. Stimulation of ERα by estrogenic compounds leads to different effects than stimulation of ERβ. ERα activation induces cell proliferation [2], while ERβ activation counteracts this and is suggested to induce apoptosis [3, 4, 18-20]. As a result the cellular ERα/ERβ ratio is essential for the ultimate effect of estrogenic compounds [63, 64].

Estrogens take part in distinct biological processes. In reproduction for example they are involved in growth and sexual differentiation [65]. Estrogens are also connected to various diseases [66] such as osteoporosis [67], cardiovascular disease [67, 68], and obesity [31].

Estrogens regulate skeletal homeostasis. They inhibit bone turnover by reducing bone resorption and enhancing bone formation. E2 deficiency is therefore linked to bone loss and osteoporosis [31, 67]. This inhibition of bone resporption and increase in bone formation is mainly regulated through ERα, while the role for ERβ in skeletal homeostasis is less clear [69].

The risk for cardiovascular disease rises in postmenopausal woman, just when circulating estrogen levels decrease. This implies that estrogens protect the female cardiovascular system [31]. This might be linked to similar types of mechanisms by which estrogens are involved in obesity. Estrogens regulate both the metabolism and the localization of white adipose tissue and are involved in adipogenesis, adipose deposition, lipogenesis, lipolysis, and adipocyte proliferation. An excess of white adipose tissue results in obesity. The lowering of circulating E2 in postmenopausal women is associated with an increase in body fat. This might be due to a decrease in E2-induced suppression of lipoprotein lipase, a lipogenic enzyme, which gene is a direct transcriptional target of estrogens. Lipoprotein lipase regulates the metabolism of plasma triglycerides to free fatty acids and increases lipid storage by adipocytes [69].

Estrogens have been associated with cancer occurring in estrogen-sensitive tissue like the breast, uterus, ovary, prostate, and testicles [70-73]. This thesis focuses mainly on the implications of a differential ERα/ERβ ratio for cell proliferation, and ultimately breast cancer. There are two hypotheses to explain the association between estrogens and breast cancer. The first hypothesis proposes binding of the estrogens to the ERs, resulting in stimulation of proliferation of breast cells and increases in the target cell

number. Due to the increased cell division and DNA synthesis the risk for replication errors is elevated, which may result in detrimental mutations which could interfere with cellular processes like apoptosis, cell proliferation, and DNA repair. In the second hypothesis the metabolism of estrogens leads to genotoxic by-products that could directly cause DNA damage, resulting in point mutations [69].

In breast tumors, ERβ expression is generally lost while ERα expression persists, resulting in a higher ERα/ERβ ratio [74]. The persistent expression of ERα in ER(α)-positive breast cancers is an interesting target for breast cancer therapy. Endocrine therapy for ER(α)-positive breast cancer with ER antagonistic compounds like tamoxifen or fulvestrant is common practice [75]. They are amongst the least toxic and most effective treatments for hormone-responsive breast cancer patients. The response to endocrine therapy for ER(α)-positive breast cancer patients (regardless of progesterone receptor and human epidermal growth factor receptor 2 (HER2) status, which also influence therapy choice and outcome) is 53%, while only 13% of the patients with an ER(α)-negative status responds to endocrine therapy [65]. Until now it is unknown what role the ERβ plays in these responses as usually its presence is not measured.

Objective, approach, and outline of this thesis

The aim of the work presented in the current thesis is to elucidate the role of differential expression of cellular ERα and ERβ levels and ERα/ERβ ratios and the impact on cell proliferation and apoptosis induced by estrogenic compounds. To this end, different types of ER agonists (with a preference for either ERα or ERβ) and ER antagonists (partial or full) were assessed in vitro for their intrinsic potency towards ERα and ERβ, the modulation of the interaction of ERs with coregulators upon binding of the estrogenic compounds to the different ER subtypes, and their ultimate biological effect on cell proliferation depending on the cellular ERα/ERβ ratio.

Chapter 1 presents an introduction to estrogenic compounds, estrogen receptors, and their mechanism of action.

Given the importance of the cellular ERα/ERβ ratio, in chapter 2 it is investigated whether the T47D-ERβ cell model, using differentially expressed ERβ levels, can be used to mimic ERα/ERβ ratios in estrogen-responsive tissues. Also the physiological relevance of the ERα/ERβ ratios in the native T47D and MCF-7 cell models commonly used in estrogen research is characterized.

Chapter 3 and 4 present the results obtained with the ERα-LBD as well as the newly developed ERβ-LBD-based Microarray Assay for Real-time Coregulator – Nuclear receptor Interaction (MARCoNI). In this assay the interaction of the ERs with several

coregulators is studied in more detail. Although the role of several coregulators involved in ER signaling has been investigated to some extent, the complexity of how modulation of the interaction of the ERs with coregulators can affect ERα and ERβ activation is not completely elucidated.

In chapter 3 first different types of ER agonists are characterized for ER subtype preference and their effect on cell proliferation. Next, using the MARCoNI assay containing 154 unique coregulator motifs derived from 66 different coregulators, coregulator modulation induced by the ER agonists upon binding to the ERα- or ERβ-LBD is assessed. This chapter should provide insight in the possible contribution of coregulators to the differences in ERα- or ERβ-induced cellular responses by ER agonists.

In chapter 4 the modulation of the interaction of ERα and ERβ with coregulators in ligand-dependent responses by the ER antagonistic compounds 4-hydroxytamoxifen (4OHT) and fulvestrant is investigated. By comparing these results to ligand-dependent modulation of the interaction of ERα and ERβ with coregulators induced by the ER agonist E2, it is investigated if and how differences in the (ant)agonist-dependent modulation profiles of the interaction of ERα and ERβ with coregulators contribute to the differences in (ant)agonist responses.

In both chapter 3 and 4 it is also investigated whether the MARCoNI assay is able to discriminate ER agonists and antagonists by means of differential profiling based on the modulation of the interaction of ERα and ERβ with coregulators. For this purpose various modulation profiles are established for every estrogenic compound tested and these profiles are compared upon clustering of the modulation profiles.

In chapter 5 a quantitative proteomics study into ER subtype-mediated effects of 4OHT, the active metabolite of tamoxifen, on cell proliferation and apoptosis of T47D-ERβ breast cancer cells is described. In this chapter it is investigated how the cellular ERα/ERβ ratio influences important biological effects like cell proliferation and apoptosis at the protein level in cells which are exposed to 4OHT.

Chapter 6 presents a general discussion on the newly obtained insights into the mode of action of ER agonists and antagonists, including the importance of ERα and ERβ levels and ratios for the ultimate biological effects of estrogen agonists or antagonists on cell cycle processes like cell proliferation and apoptosis, and the coregulators involved in ligand-dependent gene transcription. Chapter 6 also presents implications for the clinical practice and perspectives for mechanistic and clinical future research.

Chapter 7 holds a summary of the results obtained in this thesis.

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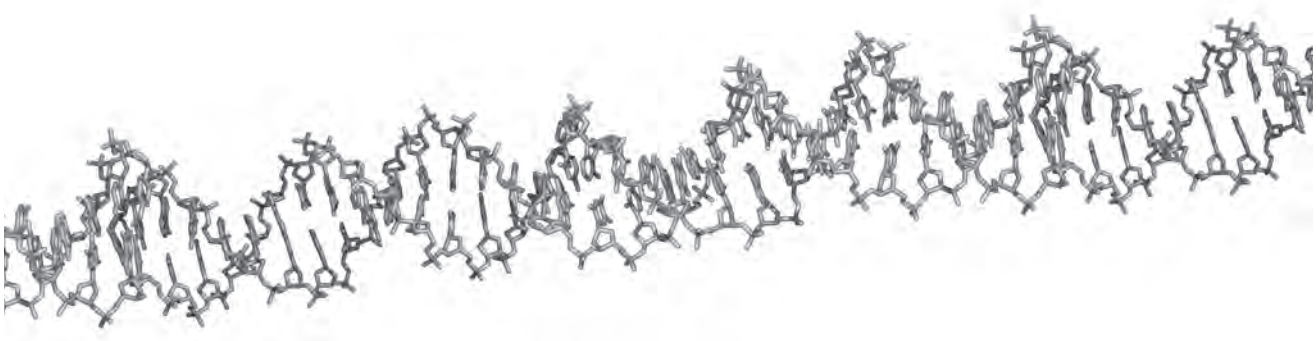
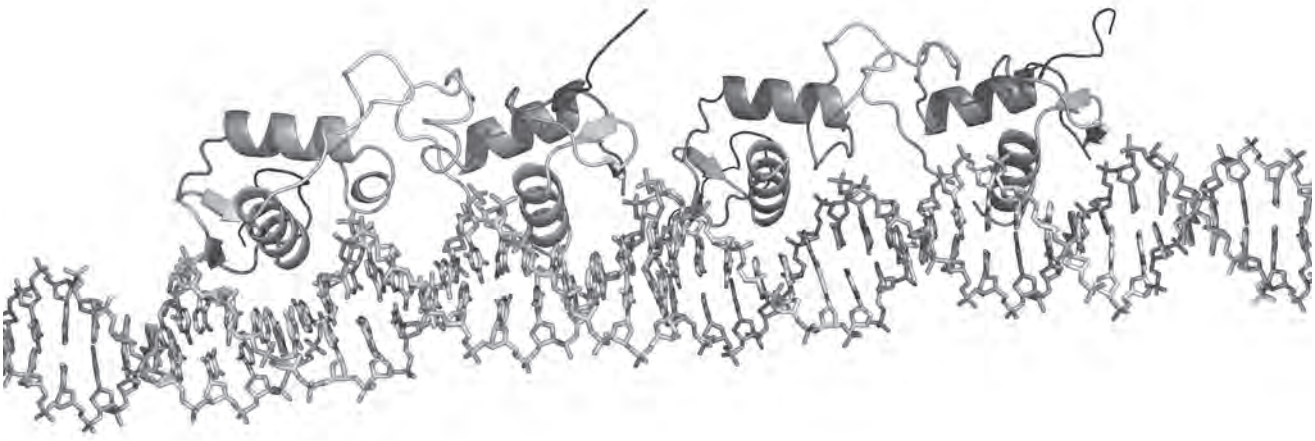


2

Human T47D breast cancer cells with tetracycline-dependent ERβ expression reflect ERα/ERβ ratios in rat and human breast tissue



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Abstract

T47D-ERβ breast cancer cells with tetracycline-dependent ERβ expression and constant ERα expression can be used to investigate effects of varying ERα/ERβ ratios on estrogen-induced cellular responses. This study defines the conditions at which ERα/ERβ ratios in T47D-ERβ cells best mimic ERα/ERβ ratios in breast and other estrogen-sensitive tissues in vivo in rat as well as in human. Protein and mRNA levels of ERα and ERβ were analyzed in T47D-ERβ cells exposed to a range of tetracycline concentrations and compared to ERα and ERβ levels found in breast, prostate, and uterus or endometrium from rat and human origin. The ERα/ERβ ratio in T47D-ERβ cells exposed to >150 ng/ml tetracycline is comparable to the ratio found in rat mammary gland and in human breast tissue. The ERα/ERβ ratio of other estrogen-sensitive rat and human tissue can also be mimicked in T47D-ERβ cells. The ERα/ERβ ratio found in MCF-7 and native T47D breast cancer cell lines did not reflect ratios in analyzed rat and human tissues, which further supports the use of T47D-ERβ cells as model for estrogen-responsive tissues. Using 17β-estradiol (E2) and the T47D-ERβ cells under the conditions defined to mimic various tissues it could be demonstrated how these different tissues vary in their proliferative response.

Introduction

Estrogen receptors (ERs) modulate the effects of estrogens on cells and tissues [1] with ERα and ERβ being the two major ERs [2]. ERα and ERβ are encoded by distinct genes [3] and have different roles in gene regulation [4]. As a consequence ERα and ERβ have differential effects on the cell cycle in various estrogen-sensitive tissues. It has been shown that ERα activation in breast and uterus enhances cell proliferation, which is necessary for growth and maintenance of tissues [5]. ERβ has been shown to counteract the ERα-mediated stimulation of cell proliferation [6-10]. Hence ERα and ERβ have different roles in gene regulation and their relative level and varying ratio within tissues may influence the response towards different estrogens. When the response to estrogens by the endocrine system is deregulated, ERα activation might eventually result in tumor formation [1, 11-14], whilst ERβ activation has been shown to stimulate apoptosis [15-17]. In certain types of cancer, the ERα/ERβ ratio is increased compared to healthy tissue. This has been shown for both ER-positive breast tumors and ovarian carcinomas [18, 19]. It was shown that the increase in ERα/ERβ ratio was due to a decreased level of ERβ [20, 21]. This observation might be related to the antagonistic effect of ERβ-mediated gene expression on cell proliferation induced by ERα activation [10].

Given the different biological effects on the cell cycle resulting from differential ERα or ERβ activation, it is of interest to note that ERα and ERβ also differ in their relative and absolute tissue distribution and tissue levels [3]. Levels of ERα and ERβ have been shown to vary in such a way that one of them is dominantly present in a specific tissue. For example, ERα is predominant in the uterus [22], while ERβ is predominant in the prostate [22, 23]. The different levels of ERα and ERβ within specific tissues are expected to determine the responses of these tissues to estrogens and to estrogenic compounds, which may have different affinities for ERα and ERβ [24] .

To investigate the potential impact of the ERα/ERβ ratio on estrogen-induced cellular effects, Ström *et al.* [7] developed the T47D-ERβ cell line. This model consists of T47D human ductal breast epithelial cancer cells with tetracycline-dependent ERβ expression (short, 485 amino acids isoform) and a constant ERα expression. When exposed to an increasing concentration of tetracycline, ERβ expression in these T47D-ERβ cells decreases and thus the ERα/ERβ ratio increases [7]. In previous studies the cell model was applied to investigate the influence of various ERβ levels on cellular proliferation in response to E2 and other estrogenic and anti-estrogenic compounds [7, 9]. In order to be able to better translate the findings reported in in vitro studies with T47D-ERβ cells with tetracycline-dependent ERβ expression towards the in vivo situation, the ERα/ERβ levels in these T47D-ERβ breast cancer cells at varying tetracycline concentrations have

to be compared to levels actually occurring in breast and other estrogen-responsive tissues in both experimental animals and humans to assess the physiological relevance compared to the levels in vivo. Therefore, the aim of the present study was to define the conditions at which the ERα/ERβ ratio in the T47D-ERβ breast cancer cells with tetracycline-dependent ERβ expression would best mimic the actual ERα/ERβ ratio in rat and human breast and other estrogen-sensitive tissues (uterus or endometrium and prostate) in vivo to further support their use as models for estrogen-responsive tissues. For comparison also the MCF-7 and native T47D cell lines were investigated for their ERα/ERβ ratios to see to what extent these cell lines provide an adequate model for mimicking physiologically relevant ERα/ERβ ratios.

Materials and Methods

Cell culture

The T47D human ductal breast epithelial cancer cell line was purchased from the American Type Tissue Culture Collection (Manassas, VA, USA). The T47D-ERβ cell line was made and provided by Ström [7]. Native T47D and T47D-ERβ cells were cultured in a 1:1 mixture of Ham's nutrient mixture F12 and Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, Scotland, #31331-038) supplemented with 5% fetal calf serum (FCS) (Invitrogen, Paisley, Scotland, #10099). For the T47D-ERβ cells, 1000 ng/ml tetracycline (Sigma, Zwijndrecht, the Netherlands, #T7660) to fully inhibit ERβ expression [9] was added to the medium. Sotoca *et al.* [9] reached maximal induction of an enhanced green fluorescent protein (EGFP) gene which is co-expressed with ERβ after 24 hours of depleting the T47D-ERβ cells from tetracycline, thus suggesting that at 24 hours also ERβ expression is maximal. ERα levels in T47D-ERβ cells are constant and quantification of the ERβ levels in the cells upon 48 hours of cultivation in the absence of tetracycline revealed similar ERβ levels as detected at 24 hours (data not shown). This supports that at the time of quantification of the ERα/ERβ ratios (24 hours) the ERβ expression reached a steady state and ERα/ERβ ratios are stable. Therefore, exposure to specific tetracycline concentrations was for 24 hours.

MCF-7 human breast adenocarcinoma cells were purchased from the American Type Tissue Culture Collection (Manassas, VA, USA). They were cultured in a 1:1 mixture of Ham's nutrient mixture F12 and DMEM (Gibco, Paisley, Scotland, #31331-038) supplemented with 5% FCS. All cells were incubated at 37°C and 5% CO_2 in a humidified atmosphere.

Because phenol red exerts estrogenic activity [25], at least 24 hours before exposure, cells were washed three times with phosphate buffered saline (PBS) (Gibco,

Paisley, Scotland, #10010-015) and transferred to phenol red free medium (Gibco, Paisley, Scotland, #21041-025) supplemented with 5% hormone-free dextran-coated charcoal-treated fetal calf serum (DCC-FCS) (Perbio Science, Waltham, MA, USA, #SH30068.05).

Tissue collection

Estrogen-responsive tissues from adult (13-16 weeks old) male and female Sprague Dawley rats were collected at the animal facility of Merck Sharp & Dohme (MSD) in Schaijk, the Netherlands. Before autopsy, animals were anesthesized and tissues were removed, placed in foil, and immediately emerged in liquid nitrogen. This experiment was approved by the animal welfare committee of MSD. Estrus cycle data as determined by histological examination of vaginal smears after haematoxylin and eosin (HE) staining of the female rats, as described by Allen and Doisy [26], are shown in Table A.

Table A: Phase in estrus cycle of sampled rats as determined by histological examination of HE stained vaginal smears.

Sample no.	Phase
1	Diestrus
2	Metestrus
3	Proestrus
4	Metestrus
5	Diestrus

Collection of human material was approved by the medical ethical committees of the respective hospitals. Human breast tissue was collected from female donors at hospital De Gelderse Vallei in Ede, the Netherlands. Human endometrium samples were obtained at Maastricht University Medical Centre in Maastricht, the Netherlands from premenopausal women undergoing hysterectomy for benign indications. Prostate tissue from male patients with benign prostatic hyperplasia (BPH) was collected at the Radboud University Nijmegen Medical Centre in Nijmegen, the Netherlands. Known relevant characteristics from the tissue donors are displayed in Table B.

Protein and mRNA isolation

For protein and messenger ribonucleic acid (mRNA) isolation, 80% confluent cells in 75 cm^2 cell culture flasks (Corning, #430641) were scraped in 1 ml TRIzol (Invitrogen, Paisley, Scotland, #15596-018) and stored at -80°C. Tissue samples were snap-frozen in liquid nitrogen and stored at -80°C. Frozen tissue (50-100 mg) was homogenized in 1 ml TRIzol using a mini-beadbeater (MBB-8 Cell Disrupter, Biospec products, Bartlesville, OK, USA) for breast and prostate tissue and by using a power homogenizer (PowerGen

GLH 220, Omni International, Kennesaw, GA, USA) for bone and uterus or endometrium. Protein and mRNA were isolated from TRIzol containing homogenized samples according to the manufacturer's instructions. Only protein samples with a concentration of more than 125 µg/ml as determined with the Pierce bicinchoninic acid (BCA) protein assay kit (Thermo Scientific, Waltham, MA, USA, #23225) were included in the analysis. The mRNA concentration was measured using a NanoDrop Spectrophotometer (ND-1000, NanoDrop Technologies, Wilmington, DE, USA). Only mRNA samples with a 260/280 absorbance ratio between 1.8 and 2.1 were included in the analysis.

Table B: Background data for human breast and endometrium samples.

	Sample no.	Age	Characteristics
Breast	1	76	No use of oral contraceptives, tissue irradiated, no tumor in sampled breast, post-menstrual
	2	52	No use of oral contraceptives, tissue not irradiated, tumor in sampled breast, menstrual cycle day 14
	3	75	No use of oral contraceptives, tissue not irradiated, tumor in sampled breast, post-menstrual
	4	37	No use of oral contraceptives, tissue not irradiated, tumor in sampled breast, menstrual cycle day 14
	5	48	No use of oral contraceptives, tissue not irradiated, no tumor in sampled breast, post-menstrual
Endometrium	1	45	No use of contraceptives, menstrual cycle day 5-6
	2	46	No use of contraceptives, menstrual cycle day 7-8
	3	46	No use of contraceptives, menstrual cycle day 8-10
	4	45	No use of contraceptives, menstrual cycle day 10
	5	49	No use of contraceptives, menstrual cycle day 13-15

Western blot

For Western blot analysis, 10 µg of the cell or tissue protein sample was mixed with 5x Laemmli sample buffer (60 mM Tris-HCl pH 6.8 (Tris: Roche, Mannheim, Germany, # 732010) (HCl: Merck, Darmstadt, Germany, #1.00317.1000), 2% sodium dodecyl sulfate (SDS) (Sigma, Zwijndrecht, the Netherlands, #L4390), 10% glycerol (Acros Organics, Pittsburg, PA, USA, #327255000), 0.01% bromophenol blue (Sigma, Zwijndrecht, the Netherlands, #B0126)) supplemented with 5% β-mercaptoethanol (Fluka, Buchs, Switzerland, # R02701). After denaturation at 95°C for 3 minutes, 30 µl sample was loaded on 10% Mini-PROTEAN® TGX™ precast gel (BioRad, Veenendaal,

the Netherlands, #456-1033). Electrophoresis was carried out at 50V for 30 minutes followed by 100V for 30 minutes using anode buffer (0.2 M Tris-HCl, pH 8.9) and cathode buffer (Biorad, Veenendaal, the Netherlands, # 161-0744, 1x buffer containing 0.1 M Tris, 0.1 M Tricine, 0.1% SDS, pH 8.3). Protein was transferred to Immobilon-FL, 0.45 µm pore size PVDF membrane (Millipore BV, Billerica, MA, USA, # IPFL00010) at 100V for 1 hour in Tris-glycine buffer (1.92 M glycine, 0.25 M Tris) (glycine: Calbiochem, Nottingham, UK, #3570) containing 20% methanol (Biosolve, Valkenswaard, the Netherlands, #13683502). Subsequently, the membranes were blocked with Odyssey blocking buffer (LI-COR, Lincoln, NE, USA, #927-40000) for 1 hour after which the membranes were probed overnight with an ERα primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA, 2Q418 # sc-71064) or an ERβ primary antibody (Cell Signaling, Danvers, MA, USA, #5513), both diluted 1:500, and with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) primary antibodies (Cell Signaling, Danvers, MA, USA, #2118) diluted 1:1000 in Tris-buffered saline (NaCl: Merck, Darmstadt, Germany, #1.06404.1000) containing 1% Tween 20 (Merck, Darmstadt, Germany, #8.221840500) (TBS-T) and 5% bovine serum albumin (BSA) (Sigma, Zwijndrecht, the Netherlands, #A6003). The following day the membranes were washed with TBS-T and incubated for 1 hour with infrared dye-labeled IRDye 800CW Donkey anti-mouse IgG (LI-COR, Lincoln, NE, USA, # 926-32212) and IRDye 680 Donkey Anti-Rabbit IgG (LI-COR, Lincoln, NE, USA, #926-32223). The final protein level was detected and quantified using an Odyssey infrared imaging system (Westburg, Leusden, the Netherlands).

cDNA synthesis

Prior to cDNA (complementary DNA) synthesis, 1 µg of mRNA sample was made DNA free by DNase treatment (Invitrogen, Paisley, Scotland, #18068-015) performed according to the manufacturer's instructions. DNase was inactivated by addition of ethylenediaminetetraacetic acid (EDTA) (J.T. Baker, Phillipsburg, NJ, USA, # 1073) and heating the sample to 65°C for 10 minutes. Additionally, 0.5 µg of oligo dT (Invitrogen, Paisley, Scotland, DNA oligo dT 15 primer) and pd(N)6 random hexamer (Amersham, Buckinghemshire, UK, # 27-2166-01) were added to the sample prior to heating. The mix was placed directly on ice and 200 units of Moloney Murine Leukemia Virus (M-MLV) reverse transcriptase, RNaseH Minus, Point Mutant (Promega, Madison, WI, USA, #M3683), 10 nmol of each deoxynucleotide triphosphate (dNTP, Invitrogen, Paisley, Scotland, #18427-088), and reverse transcriptase (RT) buffer (Promega, Madison, WI, USA, #M531A, 1x buffer containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3 mM $MgCl_2$, and 10 mM DTT) were added to a final volume of 20 µl. The mix was incubated for 10 minutes at 25°C, 50 minutes at 40°C, and finally 10 minutes at 65°C to inactivate the enzyme.

cDNA samples were stored at -20°C. cDNA was diluted 20 times and 5 µl was added to a mixture of 12.5 µl of SYBR green supermix (BioRad, Veenendaal, the Netherlands, #170-8885), 1 µl of each 10 µM primer, and 5.5 µl of milliQ. The quantitative polymerase chain reaction (qPCR) was performed on an iCycler iQ (BioRad, Veenendaal, the Netherlands) and data were analyzed with iCycler 3.1 software. Primer sequences for cell and human tissue samples were: ERα: forward 5'-CCTAACTTGCTCTTGGACAGGA-3' and reverse 5'-GCCAGCAGCATGTCGAAGAT-3' (Biolegio, Nijmegen, the Netherlands), ERβ: forward 5'-CGACAAGGAGTTGGTACACATGA-3' and reverse 5'-CCAAGAGCCGCACTTGGT-3' (Biolegio, Nijmegen, the Netherlands), heterochromatin protein 1 (HP-1): forward 5'-CC CACGTCCCAAGATGGAT-3' and reverse 5'-CTGATGCACCACTCTTCTGGAA-3' (Biolegio). Primer sequences for rat tissue samples were: ERα: forward 5'- TAAGAACCGGAGG AAGAGTTG-3' and reverse 5'- TCATGCGGAATCGACTTG-3' (Biolegio, Nijmegen, the Netherlands), ERβ: forward 5'- GAGCTCAGCCTGTTGGACC-3' and reverse 5'- GGCCTTCAC ACAGAGATACTCC-3' (Biolegio, Nijmegen, the Netherlands), rat ribosomal protein S18 (S18): forward 5'- GTCCCCCAACTTCTTCTTAGAG-3' and reverse 5'- CACCTACGGAAAC CTTGTTAC-3' (Biolegio, Nijmegen, the Netherlands).

BrdU proliferation assay

T47D-ERβ cells were seeded in 96-well view plates at a density of $1.8*10^5$ cells/ml, 100 µl/well in phenol red free medium. Twenty-four hours after seeding, cells were washed with PBS and exposed to different tetracycline concentrations (0 to 1000 ng/ml as indicated, in phenol red free medium). Forty-eight hours after seeding, keeping the tetracycline concentrations constant, cells were exposed to the test compound. After 48 hours of exposure to the test compound, cell proliferation was determined by measuring 5-bromo-2'-deoxyuridine (BrdU) incorporated into the DNA following Roche's colorimetric BrdU protocol [9, 27]. BrdU, a pyrimidine analogue, was added to the cells during the last 4 hours of exposure to the test compounds and was incorporated into the DNA of proliferating cells. This was detected by an antibody raised against BrdU, conjugated with the enzyme peroxidase, which was in turn detected by a substrate reaction. The subsequently produced blue color was quantified by measuring absorbance at 370 and 492 nm (background) using a spectrophotometer (Spectramax microplate reader M2, MSD analytical technologies) [27].

Data analysis

The intensity of the ERα and ERβ bands on the Western blot reflecting the level of ERα and ERβ respectively, was quantified using the Odyssey infrared imaging system. The band intensities were expressed relative to the level of the endogenous GAPDH control [28]. Cell and rat/human tissue ERα and ERβ levels thus obtained were expressed relative

to the intensity of the corresponding ERα and ERβ protein bands of the T47D-ERβ cells without tetracycline (T47D-ERβ calibration standard, T47D-ERβ-CS), which was set to 1. Western blot T47D-ERβ-CS ERα intensity normalized to the reference protein was 11.6 and ERβ intensity was 1.2. Although the GAPDH antibody used for the human and rat samples was the same, it cannot be excluded that it is more prone to bind either one, hampering direct comparison of absolute values for the ERα and ERβ levels found in rat and human samples or the human cell lines. For the calculation of the ERα/ERβ ratios, the preference of the GAPDH antibody to bind either to rat or human samples does not make a difference in the outcome, and therefore the protein ERα/ERβ ratios of rat and human samples and of human cell lines can be compared directly. Rat and human tissue ERα and ERβ levels represent the average result of 5 individual donor tissues ± standard deviation (SD).

QPCR data for the expression of ERα and ERβ in all cell and human samples were expressed relative to the expression of the reference gene HP-1 [29]. For more accurate normalization of ER levels and ratios it would be best to use multiple reference genes [30], but due to limited sample availability, this was not possible. QPCR data for the expression of ERα and ERβ in all rat samples were expressed relative to the expression of the reference gene S18 [31]. Although both are highly constitutive reference genes, one should keep in mind that absolute ERα and ERβ levels found in rat tissue samples cannot be directly compared to the ERα and ERβ gene expression levels found in human samples or the human cell lines. For the calculation of the ERα/ERβ ratios, the normalization to different reference genes does not make a difference in the outcome, and therefore the ERα/ERβ ratios of rat and human samples can be compared directly. Cell and tissue ERα and ERβ levels thus obtained were expressed relative to the T47D-ERβ-CS, determined as calibration sample on all plates to correct for plate-to-plate variations. The ERα and ERβ levels of the T47D-ERβ-CS were set to 1. QPCR T47D-ERβ-CS ERα level normalized to the reference gene was 0.6 and ERβ level was 2.2. Rat and human tissue ERα and ERβ levels represent the average result of 5 individual donor tissues ± SD. Levels higher than 1 represent that the level is higher than that in the T47D-ERβ-CS, while levels lower than 1 represent a lower level than that in the T47D-ERβ-CS.

Based on the results obtained, it appeared that the difference between individual rats was in the same order of magnitude as the difference between cycle points. Power analysis on the data revealed that 70 rats per cycle point would have to be used to be able to discriminate estrogen receptor levels per cycle point. Therefore it was decided to combine the data of the rats from different estrus cycles to determine the average ER levels (see results section). Also the results for the human tissues indicated that the difference between individual human tissue samples was in the same order of magnitude

as the difference between cycle points. Assuming that a similar amount of human tissue donors would be needed to be able to discriminate estrogen receptor levels per cycle point as for the rat tissue, a very large number of human tissues would have to be used per cycle point. Therefore, it was decided to combine the data of the different menstrual cycle phases as was done for the rats (see results section).

For the BrdU cell proliferation assay, an EC_{50} concentration of E2 has been tested (n=6) for calibration of the results (30 pM E2). Obtained data points were expressed relative to the response of the cells treated with the EC_{50} concentration of E2, determined as calibration sample on all plates to correct for plate-to-plate variations. Cell proliferation levels have been measured in multiple 3 biological replicates, data presented here show results of one representative experiment ± SD of 6 technical replicates. EC_{50} values were calculated using PRISM V (GraphPad, San Diego, CA, USA).

Results

ER levels and ratios in breast cancer cell models

In T47D-ERβ cells exposed to increasing tetracycline concentrations for 24 hours, both Western blot (Figure 1) and qPCR (Supplementary Figure S1) data reveal a tetracycline concentration-dependent decline in ERβ expression. As the ERα expression remained relatively constant (both Western blot and qPCR), a dose-dependent increase of the ERα/ERβ ratio was apparent.

In addition to the T47-ERβ cells, also other breast cancer cell models were analyzed for their ERα/ERβ levels (Table C). The level of ERα in native T47D and MCF-7 cell lines is higher than the ERα level in the T47D-ERβ-CS, although the difference is less than one order of magnitude. Neither the native T47D, nor the MCF-7 cell model seem to express a detectable amount of ERβ (Table C).

Table C: Level of ERα and ERβ in different breast cancer cell lines.

	Western blot		QPCR	
Celltype	**ERα level**	**ERβ level**	**ERα level**	**ERβ level**
T47D-ERβ	1	1	1	1
T47D-wt	1.03	<DL	7.6	<DL
MCF-7	1.27	<DL	5.6	<DL

Levels expressed relative to the T47D-ERβ-CS set to 1, as determined by Western blot and qPCR. <DL: below detection limit. Cellular ERα and ERβ levels have been measured in multiple biological replicates, data presented show results of one representative experiment.

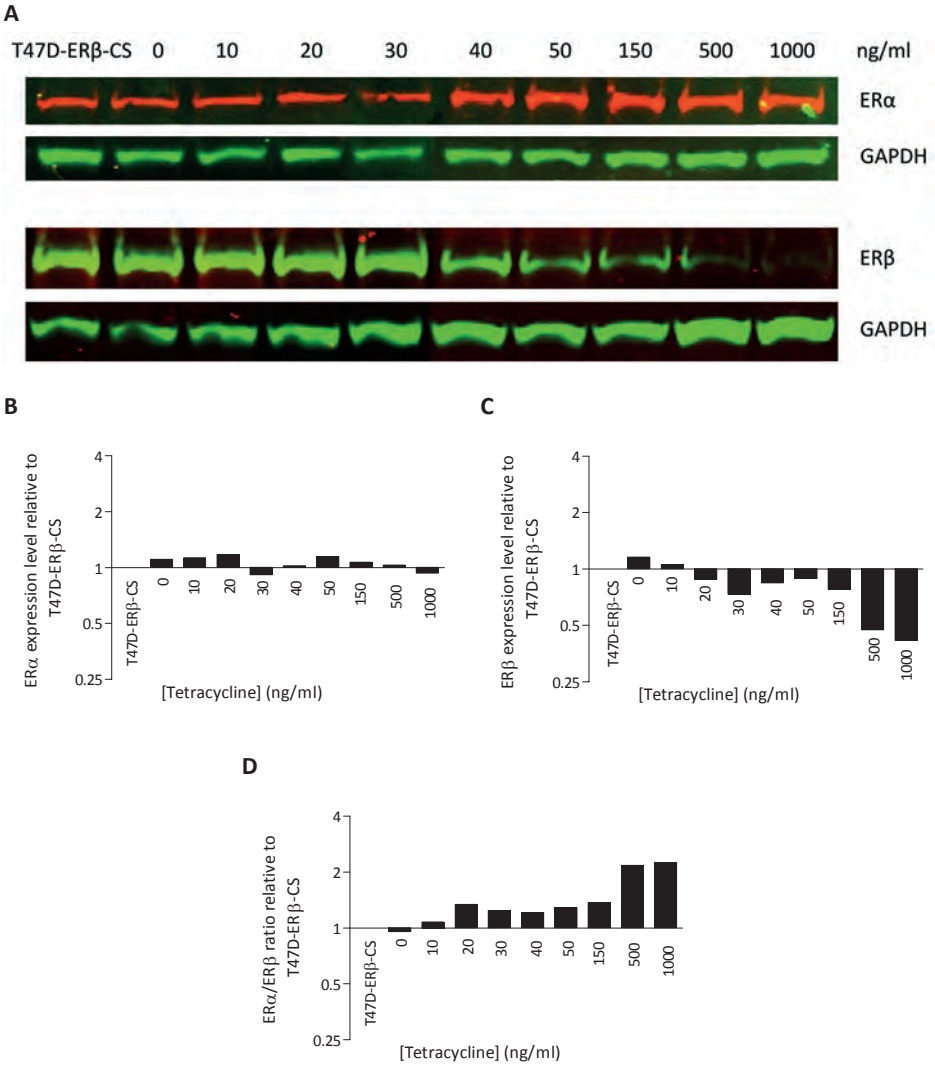

Figure 1: Western blot results T47D-ERβ cells. **(A)** Western blot image of T47D-ERβ cells exposed to an increasing concentration of tetracycline for 24 hours. Upper (red) bands show ERα expression (image compiled from 2 gels) with right below (green) the corresponding GAPDH expression used for normalization. Lower (green) bands show ERβ expression (image compiled from 2 gels) with right below (also green) the corresponding GAPDH expression used for normalization. Corresponding tetracycline concentration is stated on top. **(B)** ERα and **(C)** ERβ protein level of T47D-ERβ cells exposed to increasing concentrations of tetracycline for 24 hours, normalized for GAPDH intensity and expressed relative to the levels in T47D-ERβ-CS set to 1 (n=1). **(D)** Corresponding ERα/ERβ ratio, expressed relative to the ERα/ERβ ratio in T47D-ERβ-CS set to 1. Cellular ERα and ERβ levels have been measured in 3 biological replicates, data presented here show results of one representative experiment.

ER levels and ratios in rat mammary gland tissue

The relative ERα and ERβ gene expression and protein levels and resulting ERα/ERβ ratios were also determined in rat mammary gland tissue. The results obtained are presented in Figure 2 (Western blot) and Supplementary Figure S2 (qPCR). Western blot results indicate that all tissue samples have ERα protein levels that are similar to the ERα protein levels detected in the T47D-ERβ-CS. At protein level, the ERβ level of the rat mammary gland tissue samples is up to 2 times lower than the ERβ protein levels detected in the T47D-ERβ-CS. At protein level, the relative ERα/ERβ ratio is up to 1.9-fold higher than that of the T47D-ERβ-CS. QPCR results confirm that to mimic rat mammary gland tissue ERα/ERβ ratios both at protein and mRNA level, the T47D-ERβ cells have to be exposed to tetracycline concentrations of >150 ng/ml.

ER levels and ratios in human breast tissue

The relative ERα and ERβ levels and resulting ERα/ERβ ratios were also determined in human breast tissue. The results obtained are presented in Figure 2 (Western blot) and Supplementary Figure S2 (qPCR). Human breast tissues show ERα protein levels similar to the level of ERα protein detected in the T47D-ERβ-CS, while the ERβ protein levels

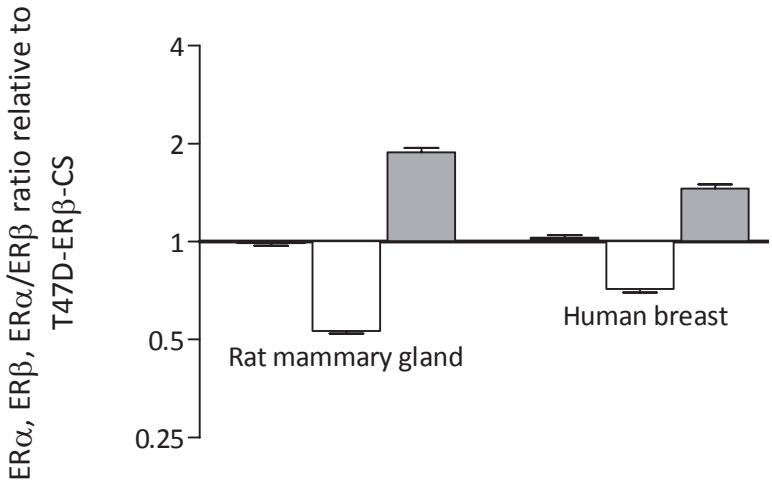

Figure 2: Western blot results rat mammary gland and human breast tissue. ERα and ERβ protein levels in rat mammary gland and human breast tissue samples, normalized for GAPDH intensity and expressed relative to the levels in T47D-ERβ-CS set to 1, and the corresponding ERα/ERβ ratio, expressed relative to the ERα/ERβ ratio in T47D-ERβ-CS set to 1. Rat and human tissue ERα and ERβ levels represent the average result of 5 individual donor tissues ± SD. Black bars: ERα level. White bars: ERβ level. Grey bars: ERα/ERβ ratio.

are 0.7 times those in the T47D-ERβ-CS. At protein level, the relative ERα/ERβ ratio is 1.5 times the ERα/ERβ ratio of the T47D-ERβ-CS. Combining these results with those of the qPCR shows that to mimic human breast tissue ERα/ERβ ratios at mRNA or protein level, the T47D-ERβ cells have to be exposed to tetracycline concentrations of >150 ng/ml.

ER levels and ratios in other estrogen-sensitive rat tissues

Relative ERα and ERβ levels and the resulting ERα/ERβ ratios determined in uterus from female, and prostate from male Sprague Dawley rats are presented in Figure 3 (Western blot) and Supplementary Figure S3 (qPCR). By applying similar reasoning as given above for the rat mammary gland and human breast tissue, the Western blot and qPCR data reveal that to best mimic uterus from female rats or prostate from male rats the T47D-ERβ cells should be exposed to respectively 500 and 0 ng/ml tetracycline (Table D).

ER levels and ratios in other estrogen-sensitive human tissues

ERα and ERβ levels and the resulting ERα/ERβ ratios were also determined in human endometrium from premenopausal women undergoing hysterectomy for benign indications and prostate from BPH patients (Figure 3 (Western blot) and Supplementary

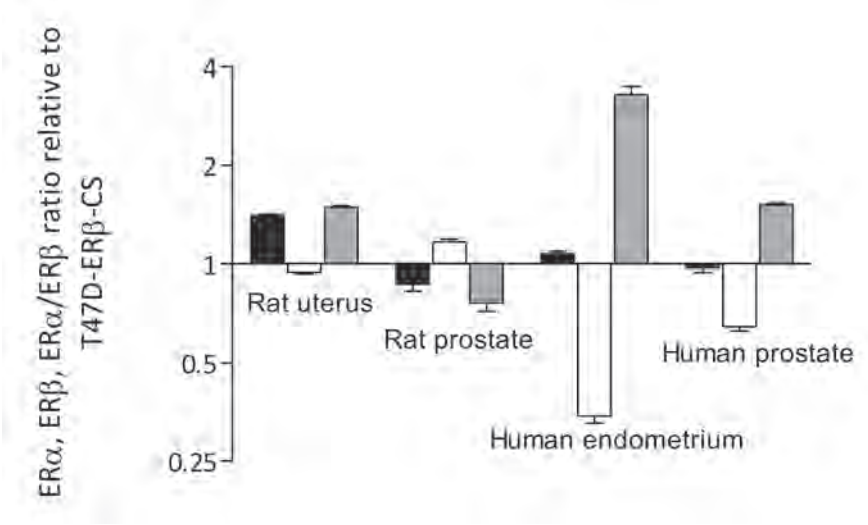

Figure 3: Western blot results rat and human uterus or endometrium and prostate tissue. ERα and ERβ protein levels in rat and human uterus or endometrium and prostate tissue, normalized for GAPDH intensity and expressed relative to the levels in T47D-ERβ-CS set to 1, and the corresponding ERα/ERβ ratio, expressed relative to the ERα/ERβ ratio in T47D-ERβ-CS set to 1. Rat and human tissue ERα and ERβ levels represent the average result of 5 individual donor tissues ± SD. Black bars: ERα level. White bars: ERβ level. Grey bars: ERα/ERβ ratio.

Figure S3 (qPCR)). By applying similar reasoning as given above for the rat mammary gland and human breast tissue, the Western blot and qPCR data reveal that to best mimic human endometrium or human prostate from BPH patients the T47D-ERβ cells should be exposed to respectively >500 and >150 ng/ml tetracycline (Table D).

Table D: Amount of tetracycline the T47D-ERβ cell line should be exposed to to best mimic ERα/ERβ levels in specific estrogen-sensitive tissues.

	Western blot	QPCR
Tissue	**# tetracycline (ng/ml)**	**# tetracycline (ng/ml)**
Rat breast	>150	>150
Human breast	>150	>150
Rat uterus	>150	±500
Rat prostate	0	0
Human endometrium	>500	±500
Human prostate	>150	>150

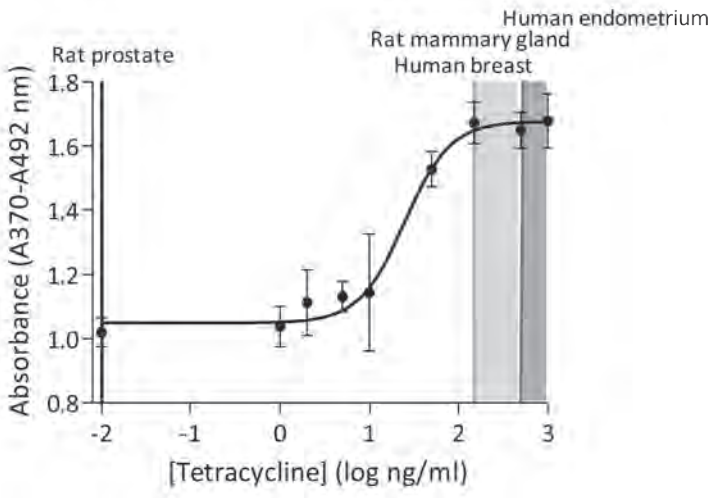

Figure 4: Cell proliferation measured as absorbance in the BrdU colorimetric method of T47D-ERβ cells exposed to 30 pM E2 at different tetracycline concentrations ranging from 0 (high ERβ expression) to 1000 (no ERβ expression) ng/ml tetracycline. Cell proliferation levels have been measured in multiple biological replicates, data presented here show results of one representative experiment ± SD of 6 technical replicates. Tetracycline exposure conditions to mimick ERα/ERβ ratios of several tissues are represented. Black: rat prostate. Light grey: rat mammary gland and human breast. Dark grey: human endometrium. At the higher tetracycline concentrations human breast (>150), rat mammary gland (>150), and human endometrium (>500) partially overlap.

The effect of E2 on cell proliferation at physiologically relevant ERα/ERβ ratios

In a final set of experiments the tetracycline-calibrated T47D-ERβ cell model was applied to characterize possible differences in tissue-specific responses to E2. To stimulate cell proliferation, cells were exposed to 30 pM E2. Figure 4 shows the tetracycline-dependent (and thus ERα/ERβ ratio-dependent) increase in cell proliferation. At 0 ng/ml tetracycline an absorbance (measure for cell proliferation) of 1 is measured, increasing up to 1.6 times at 1000 ng/ml tetracycline. In this cell proliferation curve, the ERα/ERβ ratios of relevant tissues are represented. This reflects the different cell proliferation rates at the different ERα/ERβ ratios that are found in the different tissues. At the higher tetracycline concentrations human breast, rat mammary gland, and human endometrium partially overlap, showing a relatively high cell proliferation, while rat prostate ERα/ERβ ratios are best reflected by 0 ng/ml tetracycline, at which there is relatively low cell proliferation.

Discussion

The aim of the present study was to define the conditions at which the ERα/ERβ levels in the T47D-ERβ breast cancer cells with tetracycline-dependent ERβ expression would best mimic the ERα/ERβ levels in rat and human breast and other estrogen-sensitive tissues in vivo, in order to further support their use as models for investigating the relationship of ERα and ERβ levels in estrogen-responsive tissues. To this end, the ERα/ERβ protein and mRNA levels and ratios in rat and human breast tissue and other estrogen-sensitive tissues (uterus or endometrium, prostate) were compared to the ERα/ERβ protein and mRNA levels and ratios found in the T47D-ERβ cell line exposed to increasing tetracycline concentrations.

The ERα/ERβ ratio has been shown to affect both physiology and pathology of hormone-sensitive organs [32] and a shift in the ERα/ERβ ratio could lead to adverse effects including proliferation and stimulation of tumor formation. This has been observed in breast and uterus when the ERβ level is relatively low compared to the ERα level [33, 34]. In contrast, a shift in the ERα/ERβ ratio could also lead to growth reduction (and progression of apoptosis) as observed in prostate when the ERβ level is relatively high compared to the ERα level and ERβ is stimulated with an ERβ-selective agonist [35]. It has been shown already for distinctive physiological processes that the ERα/ERβ ratios are at least as important as the levels of the two receptors [36]. However, the final cellular response to estrogenic compounds is dependent on ERα/ERβ ratios, but also on absolute ER subtype levels [37].

The T47D-ERβ cell line offers the potential to study different ERα/ERβ ratios at controlled culture conditions because in this cell line ERβ levels can be modulated.

Results from Western blot and qPCR analysis confirm that in the T47D-ERβ cell line the ERα/ERβ ratio increases with higher tetracycline concentration due to decreasing ERβ expression (Figure 1 and Supplementary Figure 1). This result is in line with data reported before by Ström *et al.* based on Western blot and qPCR analysis [7] and Sotoca *et al.* based on Western blot analysis [9]. ER levels have been measured with Western blot and qPCR. Western blot is a semi-quantitative technique, while qPCR is quantitative but does not measure absolute ERα and ERβ levels. It is important to note that the ERα/ERβ ratios are expressed on a relative scale because Western blot and qPCR analysis do not quantify absolute values but quantify the ERα and ERβ levels on a relative scale. This is due to the use of different primers and antibodies with possible differences in affinity and effectiveness. The ERα/ERβ expression levels relative to the expression levels in the T47D-ERβ-CS reveal that for protein expression levels the data can be displayed on a log 2 scale while for mRNA expression levels a log 10 scale can be used. This reflects that at mRNA level ER expression is induced more distinctive than the subsequent ER expression at protein level. Such differences between levels of expression of a gene at protein or mRNA level are observed more often [38, 39]. These differences were systematic and observed for all samples, and thus do not affect the conclusions on the tetracycline concentration needed to mimic a specific tissue.

In addition to the T47D-ERβ cell line, also other cell lines which are frequently used to characterize estrogen-mediated activity, native T47D and MCF-7 cells, were tested for their ERα/ERβ levels and ratios and were compared to those from rat and human tissues. This revealed that the ERα levels in native T47D and MCF-7 cells were comparable to the ERα level in the T47D-ERβ-CS. The ERα/ERβ ratios found in the MCF-7 and native T47D cell lines, however, do not reflect the ratios in the analyzed tissues, mainly because these cell lines do not seem to possess ERβ. This raises the question to what extent the responses of these MCF-7 and native T47D cell lines to estrogen (ant(agonists)) reflect physiological relevance. The absence of ERβ and the resulting deviating ERα/ERβ ratios in these cell lines may be due to the fact that these are cancer cell lines, not representing the situation of normal tissue.

Since the rat is one of the most used animal models, also in estrogen research, it was decided to compare the ERα/ERβ ratios in mammary gland collected from female rats to ERα/ERβ ratios which can be obtained in the T47D-ERβ cell model system measured at both protein and mRNA level. Although the results for Western blot (protein) and qPCR (mRNA) are not always directly comparable since mechanisms like post-translational regulation and (partial) degradation can play a role [37, 40], it was found that both protein and mRNA analysis in most cases show a similar trend, indicating that to mimic rat mammary gland tissue ERα/ERβ ratios the T47D-ERβ cells have to be exposed to 150 ng/ml tetracycline or more to reduce their ERβ levels.

In a next step the ERα/ERβ ratios found in human breast tissue were compared to the ERα/ERβ ratios that can be obtained in the T47D-ERβ cell model system. Human breast tissue could be obtained from patients undergoing breast reducing surgery due to tumor formation. In normal breast tissue, the ERα/ERβ ratio is low and this ratio increases when a breast tumor progresses [20]. In this study the ERα/ERβ ratio relative to the T47D-ERβ-CS in the analyzed human breast tissues revealed limited inter-individual variation in spite of differences between subject characteristics such as age, whether the analyzed tissue was irradiated, whether a tumor was present in the sampled breast, or the phase in the menstrual cycle at the moment of sampling (Table B). Our results indicate that to mimic human breast tissue levels of ERβ and ERα/ERβ ratios at both mRNA and protein level the T47D-ERβ cells have to be exposed to 150 ng/ml tetracycline or more. Rat and human samples from breast tissue showed similar results. This indicates that, at least based on ERα/ERβ levels, the rat is a valid model for human tissue in estrogen receptor research.

The ERα/ERβ ratio of other estrogen-sensitive rat and human tissues can also be mimicked in the T47D-ERβ cells, although differences in the role of coactivators and corepressors cannot be excluded. There could also be possible differential levels of ERα and ERβ expression between different cell types in these tissues [41-43]. The data of the present study further validate the T47D-ERβ cell line as an in vitro model to study the role of ERα/ERβ ratios. It is noted that this model is restricted to the T47D breast cancer cell line and if models in other cell types would be essential the use of siRNA as an efficient alternative approach to modulate ERα/ERβ ratios can be considered.

For rat uterus the ERα/ERβ ratios at both protein and mRNA level are in a similar range as the ratios in T47D-ERβ cells exposed to 500 ng/ml tetracycline or more. The phase of the estrus cycle of the sampled rats are presented in Table A. This cycle might influence the estrogen receptor levels present in the uterus. Wang *et al.* [44] described that during the proestrus phase the ERα level was most elevated, while the ERα level was a little less elevated during diestrus and even less during metestrus. ERβ levels did not differ too much between the phases of the estrus cycle of the rats, levels were only slightly elevated during metestrus and diestrus.

Rat prostate ERα/ERβ ratios are relatively low compared to the ratios in other tissue samples, due to a high ERβ level. This relatively high level of ERβ in rat prostate tissue is in line with previously reported data [45, 46]. For the rat prostate samples, the resulting ERα/ERβ ratio is comparable to the ERα/ERβ ratio seen in the T47D-ERβ-CS or even lower. This indicates that the best way to mimic the ERα/ERβ ratio in rat prostate with the T47D-ERβ cell model at both protein and mRNA level is to deprive the T47D-ERβ cells from tetracycline, although to be fully consistent one would rather have

an even slightly lower ERα/ERβ ratio. This cannot be accomplished with the T47D-ERβ cell line.

Human endometrium tissue predominantly expresses the ERα receptor [22]. The relatively high level of ERα in the human endometrium samples compared to other tissues was also confirmed in this research by both Western blot and qPCR analysis. For human endometrium tissue, ERα/ERβ ratios can be mimicked by the T47D-ERβ cell model at both protein and mRNA level after exposure of the cells to 500 ng/ml tetracycline or more.

Human prostate tissue was obtained from men with benign prostate hyperplasia. It is known that, in contrast to breast and endometrium tissue, in prostate tissue predominantly ERβ is present [22]. BPH patients have an increased level of both the ERα and the ERβ receptor in their prostate compared to healthy individuals [47]. The results for the human prostate samples indicate that to mimic human prostate tissue levels of ERβ and ERα/ERβ ratios at both protein and mRNA level the T47D-ERβ cells have to be exposed to 150 ng/ml tetracycline or more. The prostate tissue ERα/ERβ ratios varied between rat and human; rat ERα/ERβ ratios turned out to be much lower. This is in line with the finding that rat prostate has been shown to contain high levels of ERβ [46]. The deviating results for the observed ERα/ERβ ratio in rat and human prostate in our study could possibly be explained by the fact that the human subjects were diagnosed with BPH. For the human tissues it should be taken into account that, although the analyzed samples were not tumorous tissue, the presence of tumorous tissue in the patient could have altered the ERα/ERβ ratio compared to healthy tissue.

Comparing all ERα and ERβ levels of the analyzed tissues, it is striking that especially in rat and human breast tissue and human endometrium and prostate ERα levels are quite stable, while the variation in ERβ levels determines the variation in ERα/ERβ ratio. This makes the T47D-ERβ cell line particularly suitable to investigate various estrogen-responsive tissues, since in this T47D-ERβ cell line the level of ERα is stable and comparable to the level of ERα in the various tissues, while the ERβ level can be varied by the addition of tetracycline.

In a final set of experiments the tetracycline-calibrated T47D-ERβ cell model was used to reveal possible differences in tissue-specific responses to estrogens using E2 as the model compound. Rat prostate tissue (mimicked by 0 ng/ml tetracycline) is less sensitive to induction of cell proliferation by E2 than human breast tissue or rat breast (mimicked by >150 ng/ml tetracycline), which in turn is less sensitive than human endometrium (mimicked by >500 ng/ml tetracycline). It is of interest to note that proliferation of the T47D-ERβ cells but also of estrogen-sensitive cells in vivo may be influenced by other nuclear and growth receptors in addition to the ERα/ERβ ratios. Thus when interpreting

ligand-induced cell proliferation via estrogen-mediated pathways, the influence of other nuclear and growth factor receptors should also be considered, such as for example the activation of the progesterone receptor, which in certain cell lines has been shown to block estrogen-induced growth [48-50].

In summary, the results of the present study demonstrate how the T47D-ERβ cell line with tetracycline-dependent ERβ expression can mimic the ERα/ERβ ratio of rat and human breast and other estrogen-sensitive tissues, which further supports the use of this T47D-ERβ cell line as a valuable in vitro model to investigate the effect of this differential ERα and ERβ expression of tissues observed in vivo.

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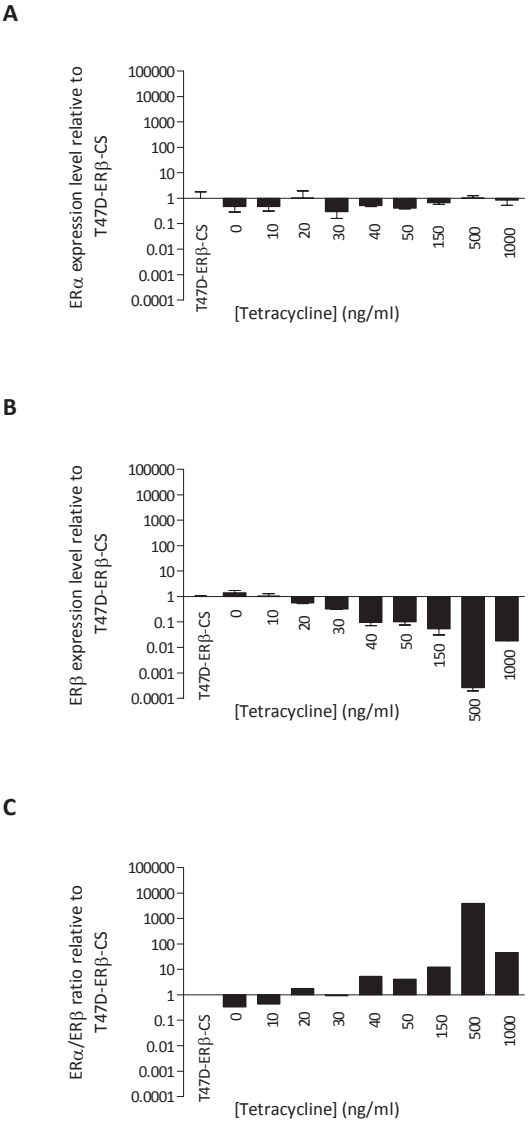
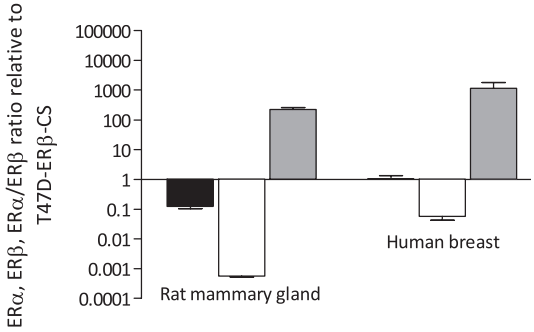
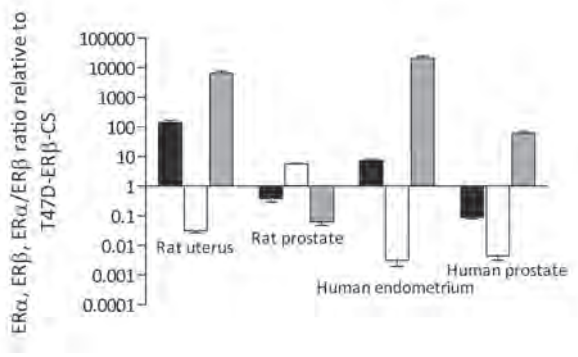
Supplementary Figures



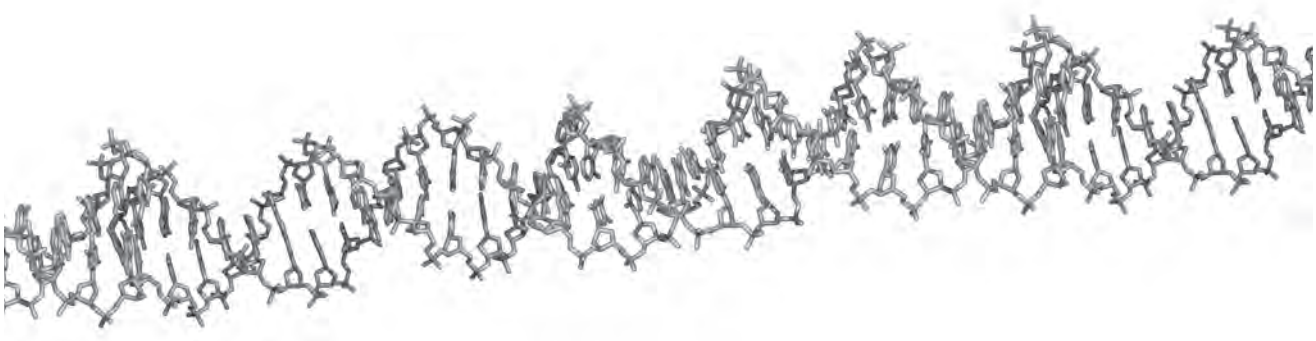
Supplementary Figure S1: QPCR results T47D-ERβ cells. **(A)** ERα and **(B)** ERβ mRNA level of T47D-ERβ cells exposed to increasing concentrations of tetracycline for 24 hours, normalized for housekeeping gene and expressed relative to the levels in T47D-ERβ-CS set to 1, and **(C)** the corresponding ERα/ERβ ratio, expressed relative to the ERα/ERβ ratio in T47D-ERβ-CS set to 1. Data presented here show results of one representative experiment ± SD of 3 technical replicates.



Supplementary Figure S2: QPCR results rat mammary gland and human breast tissue. ERα and ERβ mRNA levels in rat mammary gland and human breast tissue samples, normalized for housekeeping gene and expressed relative to the levels in T47D-ERβ-CS set to 1, and the corresponding ERα/ERβ ratio, expressed relative to the ERα/ERβ ratio in T47D-ERβ-CS set to 1. Rat and human tissue ERα and ERβ levels represent the average result of 5 individual donor tissues ± SD. For the human breast tissue, in this case there are 4 individual donor tissues, due to breakdown of the mRNA of the 5th sample. For one of those 4 samples, the ERα levels were below detection limits (<DL), and therefore also no ERα/ERβ ratio was determined for this sample, so the results of 3 samples are presented. Black bars: ERα level. White bars: ERβ level. Grey bars: ERα/ERβ ratio.

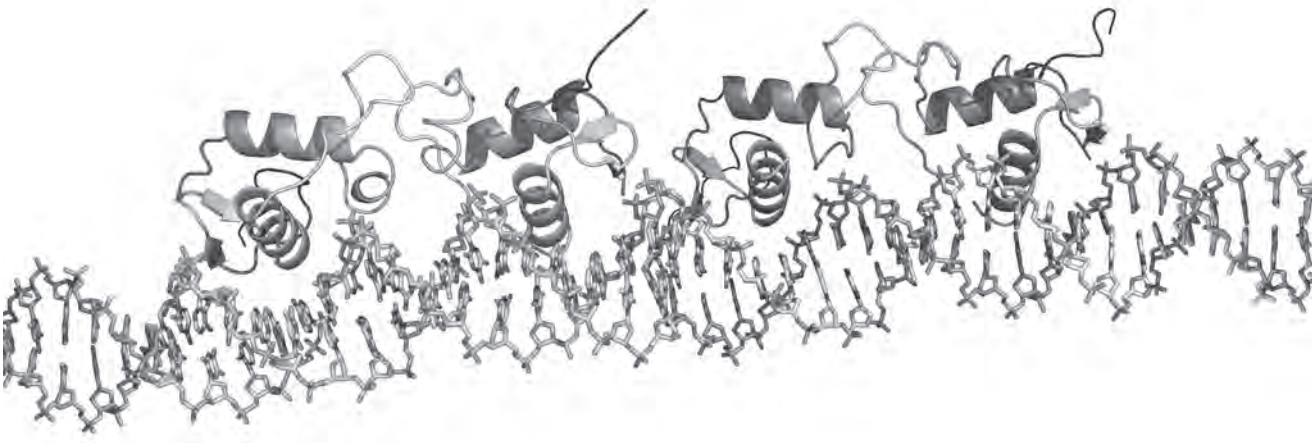


Supplementary Figure S3: QPCR results rat and human uterus or endometrium and prostate tissue. ERα and ERβ mRNA levels in rat and human uterus or endometrium and prostate tissue, normalized for housekeeping gene and expressed relative to the levels in T47D-ERβ-CS set to 1. Levels ± SD (n=3), and the corresponding ERα/ERβ ratio, expressed relative to the ERα/ERβ ratio in T47D-ERβ-CS set to 1. Rat and human tissue ERα and ERβ levels represent the average result of 5 individual donor tissues ± SD. Black bars: ERα level. White bars: ERβ level. Grey bars: ERα/ERβ ratio.



3

Cell proliferation and modulation of interaction of estrogen receptors with coregulators induced by ERα and ERβ agonists



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Abstract

The aim of the present study was to investigate modulation of the interaction of ERα and ERβ with coregulators in responses induced by estrogenic compounds. To this end, selective ERα and ERβ agonists were characterized for intrinsic relative potency reflected by EC_{50} and maximal efficacy towards ERα and ERβ in ER-selective reporter gene assays, subsequently tested for induction of cell proliferation in T47D-ERβ cells with variable ERα/ERβ ratio, and finally for ligand-dependent modulation of the interaction of ERα and ERβ with coregulators using the MARCoNI assay with 154 unique nuclear receptor coregulator peptides derived from 66 different coregulators. Results obtained reveal an important influence of the ERα/ERβ ratio and receptor selectivity of the compounds tested on induction of cell proliferation. ERα agonists activate cell proliferation, whereas ERβ suppresses ERα-mediated cell proliferation. The responses in the MARCoNI assay reveal that upon ERα or ERβ activation by a specific agonist, the modulation of the interaction of the ERs with coregulators are very similar, indicating only a limited number of differences upon ERα or ERβ activation by a specific ligand. Differences in the modulation of the interaction of the ERs with coregulators between the different agonists were more pronounced. Based on ligand-dependent differences in the modulation of the interaction of the ERs with coregulators, the MARCoNI assay showed to be able to classify the ER agonists discriminating between different agonists for the same receptor, a characteristic not defined by the ER-selective reporter gene or proliferation assays. It is concluded that the ultimate effect of the model compounds on proliferation of estrogen-responsive cells depends on the intrinsic relative potency of the agonist towards ERα and ERβ and the cellular ERα/ERβ ratio, whereas differences in the modulation of the interaction of the ERα and ERβ with coregulators contribute to the ligand-dependent responses induced by estrogenic compounds.

Introduction

Estrogens present in the human body exert different physiological effects, for example in the development of secondary sexual characteristics and regulating the menstrual cycle in women [1, 2], as well as in the reproductive system in men [2, 3]. Estrogens also influence cell growth in several tissues [3-5]. They exert their effects by binding to estrogen receptor (ER)α and/or ERβ, thereby regulating gene transcription through ligand binding and modulation of the interaction of the ERs with coregulators [1, 6, 7]. The estrogenic effects of compounds depend on their relative binding affinities for both estrogen receptors [8, 9], but also on the ERα/ERβ ratio in the cells or tissue of interest and the type of coactivators binding to the ligand-ER complex [6, 7]. ERα and ERβ have been shown to differ in relative and absolute distribution and tissue levels, with ERα being dominantly present in for example breast and uterus [3, 5, 10, 11] and ERβ for example in the prostate [10, 12]. ERα and ERβ have been reported to exert counteracting effects on cell proliferation. Whereas ERα activation enhances cell proliferation [13], ERβ activation counteracts ERα-mediated cell proliferation [9, 14-17] and has been suggested to stimulate apoptosis [18-21].

Besides the ERα/ERβ ratio [22] and the ER selectivity of the estrogenic compound, also coregulator binding is of importance to induce estrogenic responses [11]. Coregulators influence the ER-mediated activation and transcription of target genes. Coactivators and corepressors regulate the response to estrogenic compounds, chromatin condensation, and mediate transcription [6, 23]. Dependent on the type of coregulators present in the cell that can bind to the ligand-ER complex, gene expression is induced and biological effects become prominent. This aspect of coregulator interaction with the ligand-ER complex in the cell has not been studied yet in great detail.

The aim of the present study was to investigate the modulation of the interaction of ERs with coregulators in the ERα- and ERβ-mediated cellular responses induced by estrogenic compounds. To this end, selective ERα and ERβ agonists were tested for intrinsic relative potency reflected by EC_{50} and efficacy towards ERα and ERβ to characterize their ER selectivity. The T47D-ERβ cell model, T47D human ductal breast epithelial cancer cells with variable ERα/ERβ ratio, was used to investigate the role of the ERα/ERβ ratio on the induction of cell proliferation by the selected model compounds. The ligand-dependent modulation of the interaction of ERα or ERβ with coregulators by the model compounds was investigated using a newly established Microarray Assay for Real-time Coregulator – Nuclear receptor Interaction (MARCoNI) based on ERβ-ligand binding domain (LBD) together with the ERα-LBD-based MARCoNI assay [24, 25],

both with 154 unique nuclear receptor coregulator peptides derived from 66 different coregulators.

Materials and Methods

Model compounds

The model compounds selected for the study were 17β-estradiol (E2), ERA-45, ERB-041, and genistein. E2 is known to be both an ERα and ERβ agonist with an approximate 10-fold higher potency towards ERα over ERβ as shown in ligand binding experiments with solubilized *Spodoptera frugiperda 9 (Sf9)* insect cell extracts and human osteosarcoma U2OS reporter cell lines [9, 26, 27]. ERA-45 has been reported to be a selective ERα agonist with a more than 35 times higher potency towards ERα over ERβ as shown in ERα and ERβ transactivation assays with Chinese hamster ovary (CHO) cells [28]. ERB-041 has been reported to be a selective ERβ agonist with a more than 900-fold higher binding affinity for ERβ over ERα as shown in a competitive radio ligand binding assay [29]. Genistein is an ERβ-selective phytoestrogen [29] with a more than 14 times higher potency for ERβ over ERα when tested in U2OS reporter gene assays [17]. Progesterone is included in the studies as a negative control for ER activation [30, 31].

Cell culture

The human osteosarcoma (U2OS) cell lines, stably expressing ERα or ERβ in addition to a 3x estrogen response element and TATA box binding protein combined with a luciferase gene (3x ERE-TATA-luciferase gene), were kindly provided by the Hubrecht Institute, Utrecht [9]. U2OS-ERα cells were grown in DMEM:F12, a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's nutrient mixture F12 (Gibco, Paisley, Scotland, 31331-028), supplemented with 10% fetal calf serum (FCS) (Invitrogen, Paisley, Scotland, #10099), 0.5% non-essential amino acids (NEAA) (Gibco, Paisley, Scotland, 11140-035), 0.2 mg/ml geneticin G418 (PAA Laboratories GmbH, Pasching, Austria, #P02-012), and 0.05 mg/ml hygromycin (Duchefa, Haarlem, the Netherlands, # H0192.0001). U2OS-ERβ cells were grown in 1:1 DMEM:F12 culture medium supplemented with 10% FCS, 0.5% NEAA, and 0.2 mg/ml geneticin G418 [9].

T47D-ERβ cells were grown in a 1:1 DMEM:F12 culture medium (Gibco, Paisley, Scotland, #31331-038) supplemented with 5% FCS. To fully inhibit ERβ expression, 1000 ng/ml tetracycline (Sigma, Zwijndrecht, the Netherlands, #T7660) was added to the medium. Every 10 passages, the cells were reselected with 0.5 µg/ml puromycin (Gibco, Paisley, Scotland, #A1113802) [9]. To vary ERα/ERβ ratios, the cells were exposed

to either 0 (high ERβ expression) or 1000 (no ERβ expression) ng/ml tetracycline for 24 hours [9, 11]. All cells were incubated at 37°C and 5% CO_2 in a humidified atmosphere.

Because phenol red exerts estrogenic activity [32], at least 24 hours before exposure to the selected model compounds the cells were washed three times with phosphate buffered saline (PBS) (Gibco, Paisley, Scotland, #10010-015) and transferred to phenol red free medium (Gibco, Paisley, Scotland, #21041-025) supplemented with 5% hormone-free dextran-coated charcoal-treated fetal calf serum (DCC-FCS) (Perbio Science, Waltham, MA, USA, #SH30068.05). For the U2OS cell lines, the phenol red free medium was also supplemented with 0.5% NEAA.

U2OS reporter gene assay

Cells were seeded in 96-well view plates (PerkinElmer, Groningen, the Netherlands, #655180) at a density of 10^5 cells/ml for U2OS-ERα and $7.5*10^4$ cells/ml for U2OS-ERβ, 100 µl/well. Twenty-four hours after seeding, medium was changed to phenol red free medium. Forty-eight hours after seeding, cells were exposed to the test compounds in triplicate in phenol red free medium. After 24 hours of exposure, cells were washed with 0.5*PBS and lysed with 30 µl of hypotonic low-salt buffer containing (final concentrations) 10 mM Tris (Sigma-Aldrich, St. Louis, MO, USA, #T1503), 2 mM dithiothreitol (DTT) (Sigma-Aldrich, St. Louis, MO, USA, #D-0632), and 2 mM 1,2-diaminocyclohexanetetraacetic acid (CDTA) (Sigma-Aldrich, St. Louis, MO, USA, #32869); pH 7.8. Plates were kept on ice for at least 10 minutes and were subsequently stored at -80°C until analysis. One hour before measurement, plates were thawed on a plate shaker until they reached room temperature. Luciferase activity was determined using a luminometer (Labsystems, Luminoskan RS). In short, background light emission was measured for 2 seconds, after which 100 µl of flashmix (20 mM tricine (Sigma-Aldrich, St. Louis, MO, USA, #T5816), 1.07 mM $(MgCO_3)4Mg(OH)_2*5H_2O$ (Aldrich, Milwaukee, WI, USA, #22,766-8), 2.6 mM magnesium sulfate ($MgSO_4$, Sigma-Aldrich, St. Louis, MO, USA, #24.697-2), 0.1 mM ethylenedinitrilotetraacetic acid ($EDTA{\cdot}2H_2O$, Merck, Darmstadt, Germany, #1.08418.1000), 2 mM DTT, 0.47 mM D-luciferin (Duchefa Biochemie, Haarlem, the Netherlands, # L1349.0250), and 5 mM adenosine triphosphate (ATP, Duchefa Biochemie, Haarlem, the Netherlands, # A1335.0010); pH 7.8) was automatically injected, followed by measurement of light emission for another 2 seconds, after which the light emission was extinguished with 50 µl 0.2 M sodium hydroxide (NaOH, Merck, Darmstadt, Germany, # 1.06498.1000) [33].

BrdU proliferation assay

T47D-ERβ cells were seeded in 96-well view plates at a density of $1.8*10^5$ cells/ml, 100 µl/well in phenol red free medium. Twenty-four hours after seeding, cells were washed with PBS and exposed to different tetracycline concentrations (0 or 1000 ng/ml as indicated, in phenol red free medium). Forty-eight hours after seeding, cells were exposed to the test compounds which were added from 250 times concentrated stock solutions in dimethyl sulfoxide (DMSO) to result in the final concentrations indicated in the figures, keeping the tetracycline concentrations the same. After 48 hours of exposure to the test compounds, cell proliferation was determined by measuring 5-bromo-2'-deoxyuridine (BrdU) incorporated into the DNA following Roche's colorimetric BrdU protocol [9, 34]. BrdU, a pyrimidine analogue, was added to the cells during the last 4 hours of exposure to the test compounds and was incorporated into the DNA of proliferating cells. This was detected by an antibody raised against BrdU, conjugated with a peroxidase, which was in turn detected by conversion of tetramethyl-benzidine. The subsequently produced blue color was quantified by measuring absorbance at 370 and 492 nm (background) using a spectrophotometer (Spectramax microplate reader M2, MSD analytical technologies) [34].

MARCoNI assay

Ligand-modulated interaction of coregulators with ERα or ERβ was assessed using a PamChip peptide microarray with 154 unique coregulator motifs derived from 66 different coregulators (#88102 and #90012, PamGene International BV, Den Bosch, the Netherlands) as described previously [35]. In short, all incubations were performed on a PamStation®-12 or -96 (handling 12 or 96 arrays in parallel per run respectively) (PamGene) at 20°C using two cycles per minute. Polyhistidine (His) tagged ERα ligand binding domain (amino acids 302-552, partly purified from *Escherichia coli (E. coli),* final concentration between 1 and 10 nM) and His antibody penta-His Alexa Fluor 488 conjugate (Qiagen, Germantown, MD, USA,#35310, final concentration 25 nM) were diluted in time-resolved fluorescence resonance energy transfer (TR-FRET) reaction buffer containing 20 mM Tris-HCl; pH 7.5 (Tris: Sigma-Aldrich, St. Louis, MO, USA, #T1503) (HCl: Merck, Darmstadt, Germany, #1.00317.1000), 500 mM NaCl (Merck, Darmstadt, Germany, #1.06404.1000), 0.2% bovine serum albumin (BSA, Calbiochem, Merck, Darmstadt, Germany, #126609), and 0.05% Tween 20 (Bio-Rad, Veenendaal, the Netherlands, #170-6531). Glutathione S-transferase (GST) tagged ERβ subtype-specific ligand binding domain (amino acids 243-530 final concentration 10 nM) and anti-GST Alexa Fluor 488 conjugate (Molecular probes, Life Technologies Ltd, UK, #A11131, final concentration 50 nM) were diluted in reaction buffer containing

coregulator buffer E (Invitrogen, Paisley, Scotland, #PV4540) supplemented with 5 mM DTT (Sigma, Zwijndrecht, the Netherlands, #43819). All mixtures were kept on ice until they were transferred to the PamChip microarrays. Ligand was predissolved in DMSO (concentrations as indicated, final DMSO concentration 2%). A reaction mixture with only DMSO (2% final concentration) served as negative control.

Each array was blocked for 20 cycles using 25 µl of blocking buffer (Tris-buffered saline) (TBS, Bio-Rad, Veenendaal, the Netherlands, #170-6435) supplemented with 0.01% Tween 20 (Bio-Rad, Veenendaal, the Netherlands, #170-6531) and 1% BSA (Calbiochem, Merck, Darmstadt, Germany, #126609). Next, the blocking buffer was removed by aspiration and the reaction mixture was added to the PamChip microarray together with the ligand in a final volume of 25 µl. This reaction-ligand mixture was incubated (pumped up and down the porous microarray membrane containing the 154 unique coregulator peptides) for 80 cycles. Subsequently, unbound receptor was removed by washing of the arrays with 25 µL TBS and finally a tiff image of each array was acquired by the charge coupled device (CCD) camera of the PamStation.

Data analysis

For calibration of the results of all U2OS and BrdU assays, several concentrations of E2 were tested (each of the concentrations tested at least in triplicate) on each plate to correct for plate-to-plate variations. Data from the U2OS reporter gene assay were expressed in relative light units (RLU) corrected for the corresponding background signal measured before luciferase induction. EC_{50} values were calculated using PRISM V (GraphPad, San Diego, CA, USA). Potency of the compounds was determined based on the EC_{50} value and efficacy based on maximal height of induction of luciferase activity in the U2OS reporter gene assays and the maximal height of induction of cell proliferation as determined in the BrdU assay. Data from the BrdU assay were expressed as absorbance measured at 370 nm, corrected for the corresponding background signal at 492 nm.

The concentrations of the model compounds tested in the MARCoNI assay were chosen based on their relative estrogenic potencies in the U2OS reporter gene assays. To accomplish a substantial binding signal in the MARCoNI assay, a concentration of approximately 10^5 times the EC_{50} concentration in the U2OS assays was tested. The highest concentration tested in MARCoNI assay was 10^{-4} M. MARCoNI assay image analysis was performed using BioNavigator (Version 5.1, PamGene International B.V.). Per array, the fluorescent signal of each spot, representative of ER binding to that particular coregulator motif, was quantified. Each tiff image (single array) was overlaid with a synthetic grid of spot-sized circles. An algorithm was used to optimise placement

of each circle around its respective spot (actual peptide position) on the tiff image. The median fluorescence within each circle, as well as that in a defined area surrounding the circle, was quantified. For each spot, the binding signal as median fluorescent signal minus background for each peptide was calculated. The modulation index (MI) was subsequently calculated as the compound-induced log 10-fold change of binding (fluorescence) in the presence of ligand over that in the presence of solvent only. In addition, a Student's t-test on ER binding was conducted to assess the significance of the compound effect (DMSO vs. compound-stimulated). Each array contains 154 unique coregulator motifs and hence each compound is characterized by a 154-point MI signature. Compound signatures were subjected to hierarchical clustering by Euclidean distance and average linkage. Hierarchical clustering was performed using stats packages in R (version 2.15.3, copyright© 2013 The R Foundation for Statistical Computing). Per receptor, compound (dis)similarities were visualized as a dendrogram of a clustered MI heatmap in which significance of the modulation of each interaction is indicated.

For E2, concentration-response curve fitting was performed using the dose response curve (DRC) package in R, by means of a sigmoidal, 4-parameter Hill (logistic) model (response=((A-D)/(1+((concentration/C)^B)))+D, with parameters A=response minimum, B=Hill slope, C=EC_{50}, and D=response maximum).

Results

Characterization of the relative potency and efficacy of the model estrogens for ERα or ERβ

The U2OS-ERα and U2OS-ERβ reporter gene assays stably expressing ERα or ERβ were used to establish the intrinsic ability of the selected model compounds E2, ERA-45, ERB-041, genistein, and progesterone to induce ERα- or ERβ-mediated gene expression (Figure 1). Previously, E2 and genistein were characterized in these U2OS cell models, leaving the specificity of the other selective agonist in these models to be established to allow comparison. From the observed concentration-response curves, EC_{50} values were determined and these are presented in Table A. E2 showed a 9-fold lower EC_{50} value in the U2OS-ERα than in the U2OS-ERβ cell line (Figure 1A, 1C, Table A). The EC_{50} value for the selective ERα agonist ERA-45 was 200-fold lower in the U2OS-ERα than in the U2OS-ERβ cell line and its Estradiol Equivalence Factor (EEF) ERα/EEF ERβ ratio was 22 (Figure 1B, 1D, Table A), corroborating its development as a selective ERα agonist. ERB-041 showed selective ERβ properties, with an EC_{50} value of 0.5 µM in U2OS-ERβ cells, while for the U2OS-ERα cell line the EC_{50} value appeared to be higher than the highest concentration that could be experimentally tested (30 µM), resulting in an EEF ERα/EEF

ERβ ratio of <0.0017 (Figure 1B, 1D, Table A), indicating ER selectivity of >588 (EEF ERβ/EEF ERα ratio), corroborating its development as a selective ERβ agonist. EC_{50} values for genistein were 47-fold lower for ERβ compared to ERα and the EEF ERα/EEF ERβ ratio was 0.0023 (Figure 1A, 1C, Table A), which was in line with the previous identification of this phytoestrogen as a selective ERβ agonist (EEF ERβ/EEF ERα ratio of 435). The negative control progesterone showed no ER-mediated luciferase induction in either the U2OS-ERα or the U2OS-ERβ cell line. For the U2OS-ERα reporter gene assay, the order for maximal efficacy of the ligands is genistein = E2 ≈ ERA-45, while for the U2OS-ERβ reporter gene assay, the order for efficacy is genistein > E2 > ERA-45 > ERB-041.

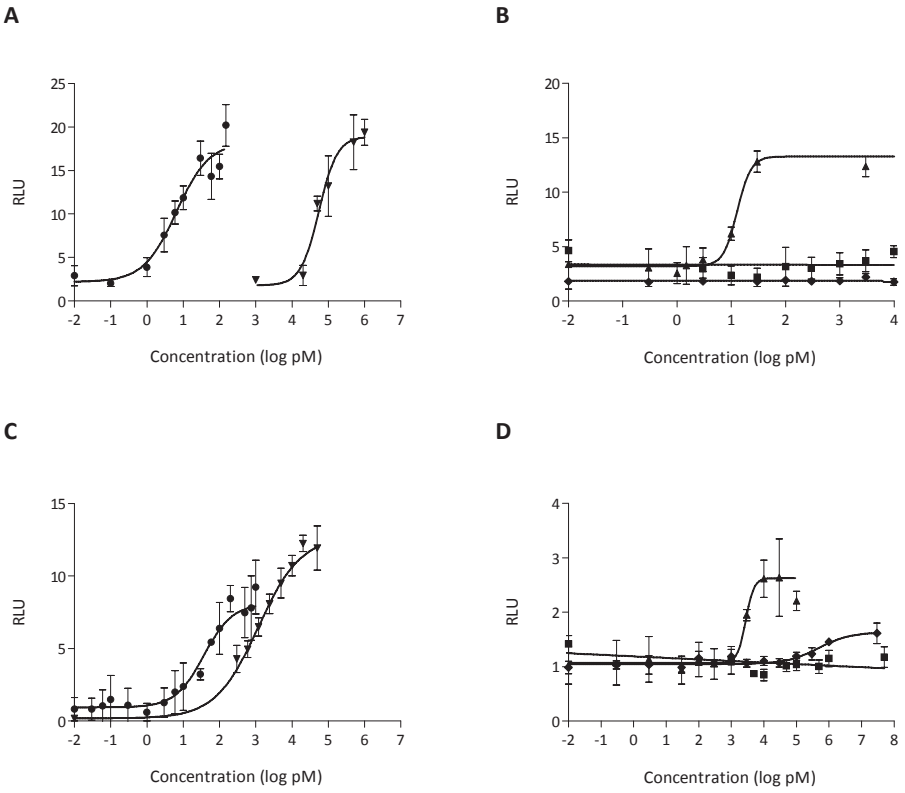

Figure 1: ERE-mediated luciferase activity in U2OS-ERα **(A** and **B)** and U2OS-ERβ **(C** and **D)** cells exposed to E2 (●), genistein (▾), ERA-45 (▴), ERB-041 (♦), and progesterone (■). Data points ± standard deviation (SD) (n=3).

Table A: EC_{50} values for E2, ERA-45, ERB-041, and genistein as determined by the U2OS reporter gene assays, the BrdU cell proliferation assay, and the MARCoNI assay (MARCoNI EC_{50} values only for E2).

Compound	U2OS EC_{50} ERα (nM)	EEF ERα	U2OS EC_{50} ERβ (nM)	EEF ERβ	EEF ERα/EEF ERβ ratio	ERα/ERβ fold potency ratio	BrdU EC_{50} T47D-ERβ with tetracycline (only ERα expression) (nM)	BrdU EC_{50} T47D-ERβ without tetracycline (ERα + high ERβ expression) (nM)	MARCoNI EC_{50} ERα (nM)	MARCoNI EC_{50} ERβ (nM)
E2	0.0065	NA	0.06	NA	NA	9.2	0.03	-	13	34
ERA-45	0.014	0.46	2.8	0.02	22	200	37	-	-	-
ERB-041	>30000	$>2.2*10^{-7}$	470	$1.3*10^{-4}$	<0.0017	<0.16	-	-	-	-
Genistein	57	$1.2*10^{-4}$	1.2	0.05	0.0023	0.02	98	-	-	-
Progesterone	-	-	-	-	-	-	-	-	-	-

EEF for ERA-45, ERB-041, and genistein: Estradiol Equivalent Factor calculated as U2OS EC_{50} E2/U2OS EC_{50} test compound.
-: Could not be determined. NA: Not applicable.

The effect of the different model estrogens on cell proliferation at varying ERα/ERβ ratios

The T47D-ERβ cell line, T47D human ductal breast epithelial cancer cells with tetracycline-dependent ERβ expression and a constant ERα expression, was used as a model to investigate estrogen-mediated cellular effects on cell proliferation induced by the selected model estrogens as a function of cellular ERα/ERβ ratios [16]. The T47D-ERβ cells depleted of tetracycline, with ERβ being highly expressed, showed no cell proliferation upon incubation with the estrogenic model compounds (Figure 2).

When suppressing ERβ by exposing the cells to 1000 ng/ml tetracycline, cell proliferation was induced by an increasing concentration of E2, ERA-45, and at relatively high concentrations also by genistein (Figure 2A-C). EC_{50} values for cell proliferation of the T47D-ERβ cells with only ERα expressed are in the same order as EC_{50} and EEF values determined for the U2OS-ERα reporter gene assay, i.e. EC_{50} E2 < EC_{50} ERA-45 < EC_{50} genistein (Table A). As for maximal efficacy, the order ERA-45 ≈ E2 > genistein differs from the U2OS-ERα reporter gene assay (genistein = E2 ≈ ERA-45), which points towards a more prominent role for the potency rather than the efficacy of a compound in the correlation between the induction of gene expression and the induction of biological effects. The ERβ agonist ERB-041 as well as the negative control progesterone did not induce cell proliferation (Figure 2D, 2E).

Estrogen receptor-specific modulation of the interaction of ERs with coregulators by selective estrogenic model compounds

To investigate differential compound-induced modulation of the interaction of ERs with coregulators upon ligand binding to either ERα or ERβ, both ERs were tested for ligand-induced coregulator binding in the MARCoNI assay [25]. As an example, Figure 3 shows the E2 concentration-dependent induction of ERα-LBD binding (Figure 3A) and ERβ-LBD binding (Figure 3B) to the coactivator-derived peptide NCOA1_677_700.

Supplementary Figure S shows the E2 concentration-response curves of ERα-LBD binding (Supplementary Figure S1) and ERβ-LBD binding (Supplementary Figure S2) to all 154 unique coregulator-derived peptides from 66 different coregulators. Of the 154 unique coregulator motifs, ERα showed concentration-dependent responses for 75 and ERβ for 52 coregulator motifs when induced with E2 (taking into account the goodness-of-fit to the sigmoidal model with a coefficient of determination (R^2) >0.9). Forty-six coregulator motifs showed a concentration-dependent effect with E2 for both ERα and ERβ. This implies that of the 52 modulated interactions of the ERβ-LBD with coregulator motifs upon E2 binding, 46 (88%) are also modulated upon E2 binding to the ERα-LBD.

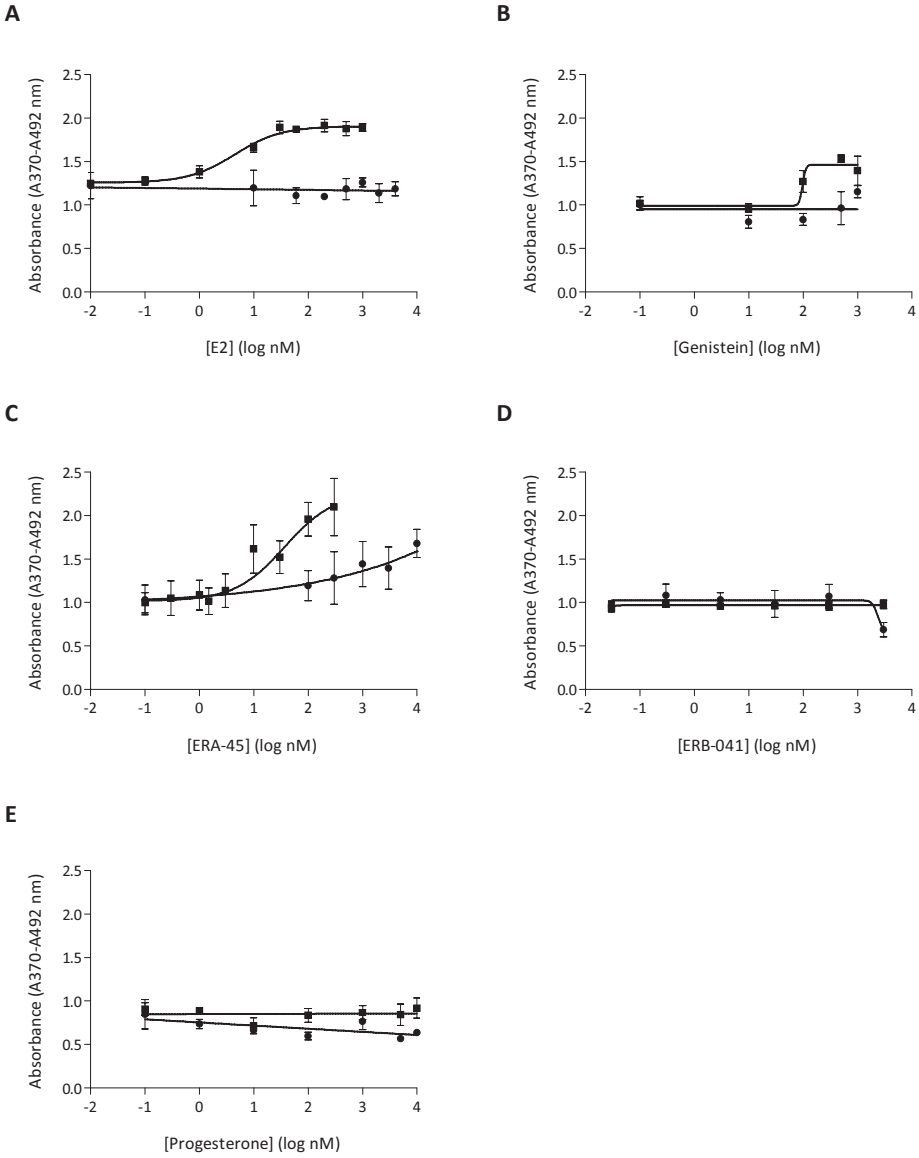

Figure 2: Effect of **(A)** E2, **(B)** genistein, **(C)** ERA-45, **(D)** ERB-041, and **(E)** progesterone on the proliferation of T47D-ERβ cells exposed to 0 (●) or 1000 (■) ng/ml tetracycline (respectively high and no ERβ expression). Data points ± standard deviation (SD) (n=6).

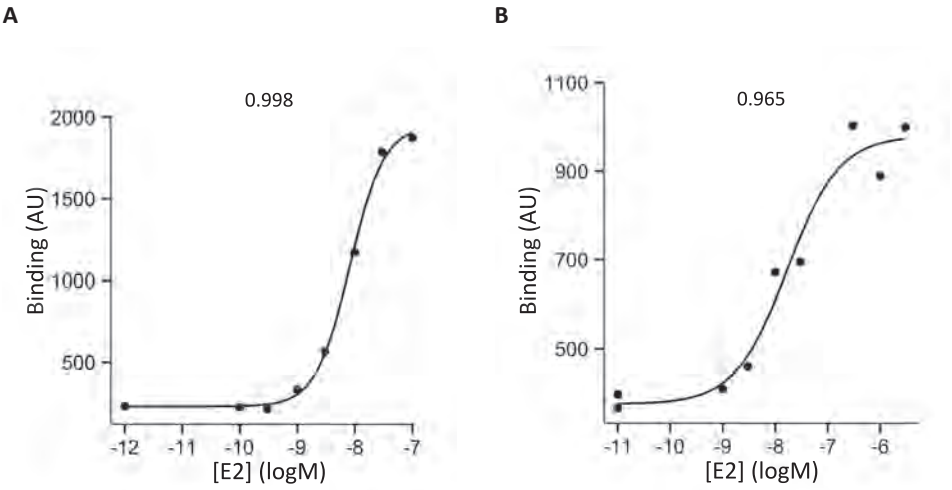

Figure 3: Concentration-response curves for **(A)** ERα-LBD and **(B)** ERβ-LBD binding to the coactivator-derived binding motif NCOA1_677_700 in the MARCoNI assay induced by E2. Binding in arbitrary units (AU). Value displayed in the graphs: R^2.

EC_{50} values for the coregulator motifs with R^2 >0.9 were generally in the same order of magnitude (Supplementary Table S). The median EC_{50} value for the coregulator motifs with an R^2 >0.9 activated upon E2 binding to the ERα-LBD was lower than that for E2 binding to the ERβ-LBD, i.e. 13 and 34 nM respectively (Supplementary Table S). This lower EC_{50} value for ERα compared to ERβ is in line with the lower EC_{50} for E2 in the U2OS-ERα than the U2OS-ERβ reporter gene assay, although the fold difference is somewhat less in the MARCoNI assay (i.e. 2.6-fold) as compared to the 9-fold difference in the reporter gene assays. Among the E2 activated coregulators showing a concentration-response with R^2 >0.9 for both ERα and ERβ were for example nuclear coactivator (NCOA) motifs NCOA1_677_700, NCOA2_628_651, and NCOA3_673_695, derived from the Sarcoma (SRC)-family of coregulators known to stimulate gene expression [36, 37]. It is also of interest to note that of the modulation of the interaction of ERs with all 154 coactivator motifs, 74 (48%) were not modulated in a well-fitting concentration-dependent manner (R^2 <0.9) upon ligand binding. For example coactivator centromere protein R (CENPR_1_18) and zinc transporter 9 (ZNT9_449_471) did not show E2-induced binding for both ERα and ERβ, even though other studies have shown them to be likely a coactivator for ERα [24, 38, 39]. In addition also some well-known nuclear corepressor (NCOR) motifs, like NCOR1_1925_1946 and NCOR2_2330_2352 [40], did not show a concentration-response upon increasing E2 concentrations.

Figure 4 shows a comparison of the modulation of the interaction of ERα with coregulators and modulation of the interaction of ERβ with coregulators for E2. From this figure it can be concluded that the MI patterns for E2 for ERα- and ERβ-mediated coregulator binding are remarkably similar with only a few subtle differences for possible ER-specific coregulator motifs. One of those possible ER-specific coregulator motifs is DEAD box (DDX)5_133_155 (indicated with an arrow in Figure 4). Figure 5 shows that this possible ER type specificity of coregulator motif DDX5_133_155 also holds for ERA-45, ERB-041, and genistein.

The model compounds were classified based on hierarchical clustering of the ER-coregulator MI profiles using Euclidian distance (Figure 6). The results reveal that ERA-45, ERB-041, and genistein resulted in MI patterns similar to the reference compound E2, but each of these compounds showed its own specific potency (the potentcy of the compounds in the U2OS reporter gene assay correlates with color intensity in the hierarchical clustering). As expected, the negative control progesterone hardly induced any modulation of the interaction of ERs with coregulators. For ERα (Figure 6A), E2 was found to cluster together with the ERα agonist ERA-45, whereas ERB-041 and genistein, which both have a preference for ERβ (based on EEF in the reporter gene assay), also cluster together. The negative control progesterone only clusters with the DMSO control. For ERβ (Figure 6B), ERα agonist ERA-45, ERB-041, and genistein cluster together, while E2 is also associated with these model compounds, and here again progesterone did not cluster with the other model compounds except for the DMSO control. As for maximal efficacy, the order ERA-45 ≈ E2 > genistein in the cell proliferation assay is different from the U2OS-ERα reporter gene assay (genistein = E2 ≈ ERA-45). For the U2OS-ERβ reporter gene assay, the order for maximal efficacy is genistein > E2 > ERA-45 > ERB-041. The hierarchical clustering of the MARCoNI assay does not explain these orders in efficacy. It seems that other factors than modulation of the interaction of ERs with coregulators are responsible for these differences.

Differences in the MI patterns for the four ER agonists tested mainly appeared in the extent of the modulation being generally higher for ERα (Figure 6A) with E2 and ERA-45 (compounds with higher potency) than with genistein and ERB-041 (compounds with lower potency). For ERβ (Figure 6B), the extent of the modulation was generally higher with E2 than with the other three agonists.

When multiple binding motifs of one coregulator are present in the MARCoNI assay, the modulation of the interaction of ERs with different motifs belonging to one coregulator are not all modulated in the same manner (for example all melanoma antigen preferentially expressed In tumors (MAPE) motifs, Figure 6). This might indicate that not all parts of the coregulator protein are equally attracted to the ERα- or ERβ-LBD.

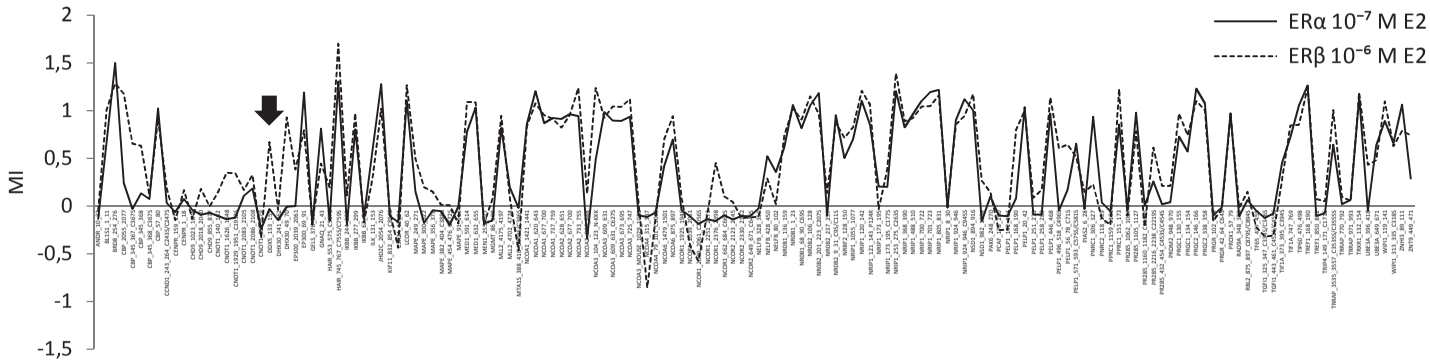

Figure 4: Modulation profiles of the interaction of ERs with coregulators induced by E2 for ERα (solid line) and ERβ (dotted line) expressed as modulation index (MI) (n=3). Arrow in Figure 4 indicates one of the most obvious differences in coregulator response between the two ERs.

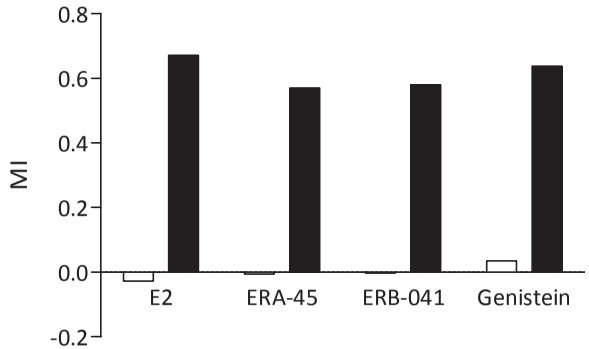

Figure 5: Modulation index of the interaction of ERα (white) and ERβ (black) with coregulator motif DDX5_133_155, one of the most obvious differences in coregulator response between the two ERs as indicated in Figure 4 with an arrow, induced by E2, ERA-45, ERB-041, and genistein.

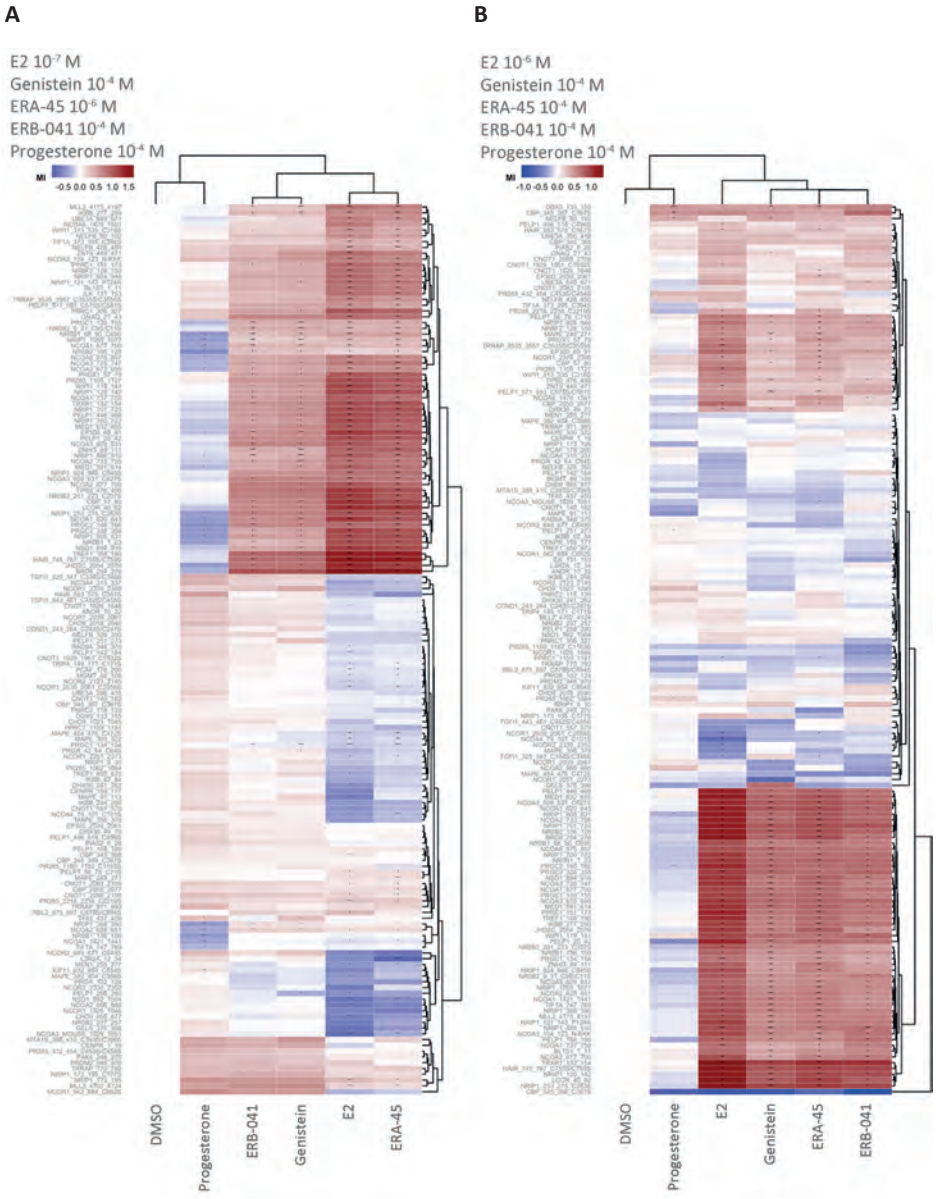

Figure 6: MI heatmap of **(A)** ERα and **(B)** ERβ interaction with coregulators across compounds and coregulators. Red represents positive MI, blue represents negative MI. In the heatmap the significance of the modulation of each interaction is indicated (*: P value <0.05; **: P value <0.01; ***: P value <0.001).

Discussion

An important factor that may influence the ultimate gene expression and biological effect induced by estrogenic compounds is the type of coregulators present in the cell that can interact with the ligand-ER complex. The aim of the present study was to investigate the modulation of the interaction of ERα and ERβ with coregulators in the ligand-dependent responses induced by estrogenic compounds. This study was conducted to better understand the differential effects and possible mechanisms of the model compounds that could explain their mode of ER subtype interactions.

E2 and the selective ERα agonist ERA-45 were able to induce ERE-mediated gene transcription in U2OS-ERα and -ERβ reporter gene assay with a higher potency towards ERα than towards ERβ. For E2, the 9-fold difference between the EC_{50} for ERα and the EC_{50} for ERβ in the U2OS reporter gene assays is in line with previous results [9]. For ERA-45, the U2OS reporter cell model shows a clear preference for ERα with EC_{50} values which are 200 times higher for ERβ than for ERα and an EEF ERα/EEF ERβ ratio of 22. Genistein was also able to activate both ERs, but with a 47-fold higher potency towards ERβ and the EEF ERα/EEF ERβ ratio of 0.0023 (Figure 1A, 1C, Table A), which is in line with the previous identification of this phytoestrogen as a selective ERβ agonist. This higher potency of genistein towards ERβ than towards ERα has been related to the inhibiting effects of genistein on tumor cell growth since ERβ activation has been shown to suppress ERα-mediated induction of cell proliferation [41-43], a phenomenon also observed in the present study. E2, the ERα agonist ERA-45, and genistein, which were able to induce ERE-mediated luciferase activity in the U2OS-ERα reporter cell model, were also able to induce cell proliferation in the T47D-ERβ cells when ERβ expression was inhibited. When ERβ was highly expressed in the T47D-ERβ cell model, these model compounds could no longer induce cell proliferation. This confirms that ERα induction leads to cell proliferation, whereas expression of ERβ counteracts cell proliferation. Together, these data in the T47D-ERβ cells corroborate the importance of the cellular ERα/ERβ ratio for the ultimate effect of an estrogenic compound on cell proliferation and indicate that the effect on a tissue of interest will vary with the cellular ERα/ERβ ratio. This is especially of importance given that ERβ levels are generally low in tumor tissue [14] facilitating estrogen-mediated cell proliferation of these cells, but also explaining the effectiveness of ER antagonists like for example tamoxifen [44] and fulvestrant [45] in cancer treatment.

The results of the present study also indicate that the selectively developed ERβ agonist ERB-041 could induce ERE-mediated luciferase activity in the U2OS-ERβ cell line, but only at very high concentrations. This is in line with literature where this ERβ

agonist has also been described as not highly potent [6, 7, 24, 46]. Given the potential for highly potent ERβ agonists to counteract ERα-mediated effects on cell proliferation, development of more selective and potent ERβ agonists seem an important challenge for future research. Genistein tested in the present study is a good example of a compound that activates ERα only at concentrations at which it also activates ERβ, possibly underlying its more favourable estrogenic activity profile compared to the estrogenic activity profile of E2 which, at lower concentrations, may activate ERα without activating ERβ, as indicated in Figure 1.

Given the fact that the reporter gene assays and the T47D-ERβ cell proliferation assays do not provide detailed mode of action on ERα- and ERβ-mediated estrogenic effects, a high-throughput in vitro assay enabling quantification of the modulation of the interaction of ERs with coregulators upon ligand binding would have the potential to add relevant information on the mode of action of the compounds under investigation. Previously it was shown that the ERα-based MARCoNI assay enables not only discrimination of ER agonists from antagonists, but could also discriminate between different agonists, a potential not provided by the U2OS or T47D cell models [25]. In the present study, a newly established MARCoNI assay based on ERβ-LBD was used together with the ERα-LBD-based MARCoNI [24, 25] to investigate modulation of the interaction of ERα and ERβ with coregulators in the ligand-dependent responses induced by estrogenic compounds.

The results of the present study revealed that the interaction of ERs with coregulators induced by either ERα- or ERβ-specific agonists are very similar. All model compounds show induction of known transcription coactivator motifs possessing intrinsic histone acetyltransferase activity NCOA1_677_700, NCOA2_628_651, and NCOA3_673_695 [47]. Only a few ERα and ERβ subtype-specific modulations of the interaction of ERs with coregulators were detected. For example, the interaction of ERs with coregulator motif DDX5_133_155 showed positive modulation with ERβ, but not with ERα, for all agonists. DDX5 is a transcriptional coactivator for the tumor suppressor protein p53 and is involved in the p53 transcriptional response to DNA damage and p53-dependent apoptosis [48]. The modulation of the interaction of ERβ with this DDX5 coactivator, but not of ERα, is in line with ERβ counteracting ERα-mediated cell proliferation by means of apoptosis.

It is of interest to note that the differences in the modulations of the interaction of ERs with coregulators between the different ERα or ERβ agonists incubated with their respective receptors are limited, although subtle differences do allow discrimination between different agonist for one specific receptor. Differences are especially observed

in the magnitude of the coregulator response resulting in variable MI values in the MARCoNI assay for different ER agonists. In this way modulation of the interaction of ERs with coregulators may affect the differences in the ultimate physiological response generated upon ER activation. However, the limited differences observed could also point towards possible competition for the ERE and for available coregulators as a mode of action explaining ERβ-mediated effects on ERα-induced responses.

The results of the present study also revealed that the ERβ agonist ERB-041 did not induce ERE-mediated luciferase activity in the U2OS-ERα reporter cell model or cell proliferation in the T47D-ER cell model at the tested concentrations, but, in contrast to this, ERB-041 was able to induce modulation of the interaction of ERα with coregulators in the MARCoNI assay. It could be that at the high concentration (10^{-4} M) of ERB-041 tested in the MARCoNI assay ERB-041 binds to the ERα as well, an effect not detected in the U2OS assay and the BrdU cell proliferation assay since in these assays only concentrations up to 30 µM could be tested due to cytotoxicity occurring at higher concentrations. The ERβ-based MARCoNI assay was performed for the first time, and therefore agonist concentrations at expected maximum efficiacy were chosen and compared to efficacy of the agonists in the U2OS reporter gene assays and cell proliferation assay. To be able to investigate the potency of the antagonistic compounds in the MARCoNI assay, full dose response curves should be conducted. It is expected that the compounds that have a higher potency towards one of the ER subtypes will show modulation of the interaction of ERs with coregulators at a lower concentration upon interaction with the high potency ER subtype compared to the low potency ER subtype. This can be a valuable hypothesis for follow-up studies.

Quantitative hierarchical clustering of the estrogenic model compounds and the negative control progesterone tested in the present study based on modulation of the interaction of ERs with coregulators (Figure 6) showed that for ERα E2 was found to cluster together with the ERα agonist ERA-45, whereas the preferential ERβ agonists ERB-041 and genistein, although able to activate ERα, could be discriminated from E2 and ERA-45. This illustrates the potential of the MARCoNI assay to discriminate between ERα and ERβ agonists. Progesterone, the negative control, stands alone and only clusters with the DMSO control. The hierarchical clustering of the model compounds in this study can be linked to their effect on cell proliferation as E2 and ERA-45, that are both able to induce cell proliferation in cells expressing ERα, and ERB-041 and genistein, ERβ agonists able to inhibit this ERα-mediated cell proliferation, cluster together in the hierarchic clustering analysis of the MARCoNI assay ERα data.

This compound comparison by using hierarchical clustering, discriminating E2 and ERA-45 from ERB-041 and genistein, could not be performed based on the U2OS or cell proliferation data. For ERβ, the ERα agonist ERA-45 turned out to be more similar to ERB-041 and genistein than to E2 as ERA-45, ERB-041, and genistein all cluster together. This clustering of the model compounds in the ERα- and ERβ-based MARCoNI assay is partially in line with what is expected based on the EC_{50} and EEF values of the model compounds in the U2OS cell models. In the U2OS-ERα cell line EC_{50} and EEF values of E2 and ERA-45 are quite close, while for the U2OS-ERβ cell line the EC_{50} and EEF value of ERA-45 is closer to that of genistein than to that of E2 (Table A). Also for the ERβ-based data progesterone did not cluster with the other model compounds.

As for maximal efficacy, the order ERA-45 ≈ E2 > genistein in the cell proliferation assay is different from the U2OS-ERα reporter gene assay (genistein = E2 ≈ ERA-45). For the U2OS-ERβ reporter gene assay, the order for maximal efficacy is genistein > E2 > ERA-45 > ERB-041. The hierarchical clustering of the MARCoNI assay does not explain these orders in efficacy. It seems that other factors than the modulation of the interaction of ERs with coregulators are responsible for the observed differences.

In the present study, although the profiles of the modulation of the interaction of ERs with coregulators between the different ERα or ERβ agonists incubated with their respective receptors are generally similar, the MARCoNI assay was shown to be able to classify the different ERα and ERβ agonists discriminating between different agonists for the same receptor, a characteristic not defined by the ER-selective reporter gene or proliferation assays. Most importantly, several coregulators showed distinct modulation upon exposure of the same ligand to ERα- and ERβ-LBDs. These coregulators have a potential to be used as markers to distinguish selective ERα agonists from ERβ agonists. The importance of the subtle differences for the ultimate biological effect remains to be established and is an important topic for future research, in which the presence of different coregulators in cells or tumors should be taken into account. It is concluded that in addition to the U2OS reporter gene assays and the T47D-ERβ cell proliferation assay, the MARCoNI-based coregulator binding assay provides valuable information in the ERα- and ERβ-dependent responses induced by estrogenic compounds.

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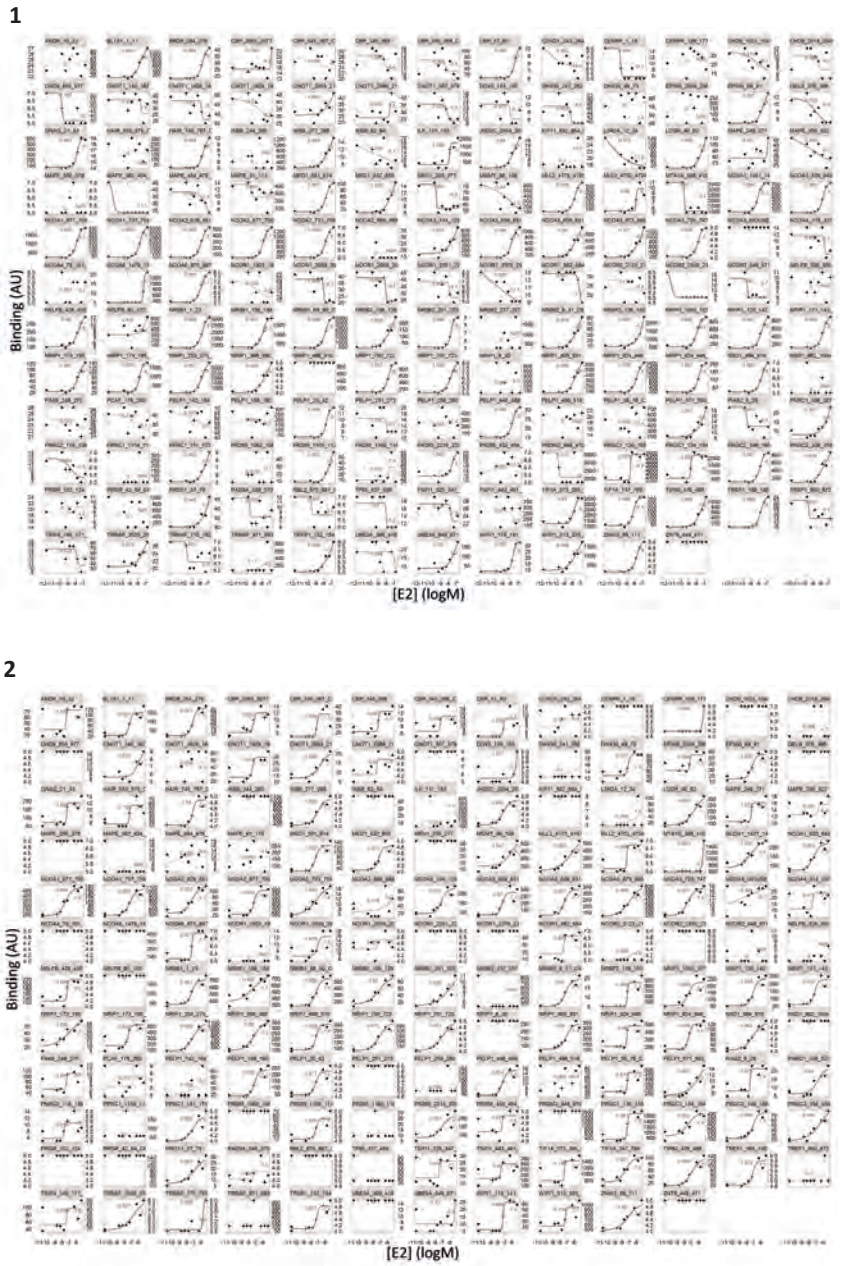
Supplementary Figure



Supplementary Figure S: Concentration-response curves for **(1)** ERα-LBD and **(2)** ERβ-LBD binding to the 154 coregulator-derived binding motifs in the MARCoNI assay induced by E2. Binding displayed in arbitrary units (AU).

Supplementary Table

Supplementary Table S: EC_{50} and IC_{50} (underlined) values for ERα-LBD and ERβ-LBD binding to coregulator-derived binding motifs in the MARCoNI assay induced by E2, only displayed for concentration-response curves with $R^2 > 0.9$.

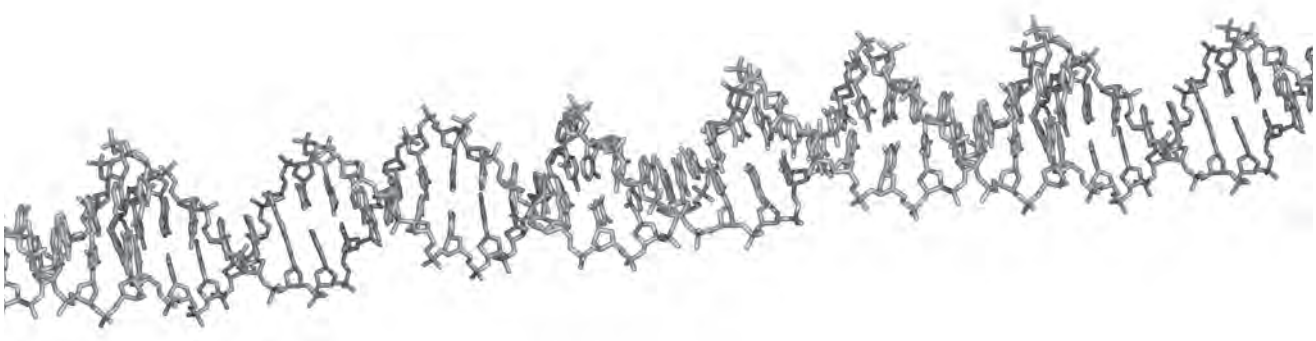
Coregulator	R^2 ERα	EC_{50} ERα (M)	R^2 ERβ	EC_{50} ERβ (M)
BL1S1_1_11	0.99	$1.38 \cdot 10^{-08}$	0.93	$2.94 \cdot 10^{-08}$
BRD8_254_276	0.10	$1.25 \cdot 10^{-08}$	0.98	$4.09 \cdot 10^{-08}$
CBP_57_80	0.10	$2.20 \cdot 10^{-08}$		
CENPR_159_177			0.99	$1.18 \cdot 10^{-05}$
CNOT1_2083_2105			0.91	$1.07 \cdot 10^{-07}$
DHX30_49_70			0.92	$3.85 \cdot 10^{-08}$
EP300_69_91	0.99	$2.55 \cdot 10^{-08}$	0.95	$3.14 \cdot 10^{-08}$
GNAQ_21_43	0.99	$9.09 \cdot 10^{-09}$		
HAIR_745_767_C755S/C759S	0.10	$1.65 \cdot 10^{-08}$	0.94	$3.75 \cdot 10^{-08}$
IKBB_277_299	0.98	$4.18 \cdot 10^{-08}$	0.96	$5.02 \cdot 10^{-08}$
ILK_131_153	0.90	$8.96 \cdot 10^{-09}$		
JHD2C_2054_2076	0.99	$2.12 \cdot 10^{-08}$	0.95	$3.01 \cdot 10^{-08}$
LCOR_40_62	0.10	$3.91 \cdot 10^{-08}$	0.96	$1.78 \cdot 10^{-07}$
MAPE_382_404_C388S	0.10	$\underline{3.47 \cdot 10^{-12}}$		
MED1_591_614	0.10	$1.08 \cdot 10^{-08}$	0.95	$2.50 \cdot 10^{-08}$
MED1_632_655	0.98	$3.16 \cdot 10^{-08}$	0.92	$3.41 \cdot 10^{-08}$
MEN1_255_277	0.95	$\underline{1.68 \cdot 10^{-10}}$		
MLL2_4175_4197	0.10	$1.26 \cdot 10^{-08}$	0.92	$6.13 \cdot 10^{-07}$
MLL2_4702_4724			0.90	$2.34 \cdot 10^{-08}$
MTA1S_388_410_C393S/C396S	0.97	$1.54 \cdot 10^{-10}$	0.99	$1.13 \cdot 10^{-05}$
NCOA1_1421_1441	0.10	$4.70 \cdot 10^{-09}$	0.93	$2.61 \cdot 10^{-08}$
NCOA1_620_643	0.10	$1.19 \cdot 10^{-08}$	0.95	$2.67 \cdot 10^{-08}$
NCOA1_677_700	0.10	$8.23 \cdot 10^{-09}$	0.97	$1.59 \cdot 10^{-08}$
NCOA1_737_759	0.10	$1.22 \cdot 10^{-08}$	0.96	$3.60 \cdot 10^{-08}$
NCOA2_628_651	0.10	$8.95 \cdot 10^{-09}$	0.96	$3.06 \cdot 10^{-08}$
NCOA2_677_700	0.10	$8.61 \cdot 10^{-09}$	0.93	$3.03 \cdot 10^{-08}$
NCOA2_733_755	0.10	$9.67 \cdot 10^{-09}$	0.95	$3.22 \cdot 10^{-08}$
NCOA3_104_123_N-KKK	0.91	$8.76 \cdot 10^{-08}$	0.93	$3.78 \cdot 10^{-08}$
NCOA3_609_631	0.99	$8.17 \cdot 10^{-09}$		
NCOA3_609_631_C627S	0.96	$3.37 \cdot 10^{-08}$	0.96	$2.07 \cdot 10^{-08}$
NCOA3_673_695	0.97	$1.51 \cdot 10^{-07}$	0.97	$8.08 \cdot 10^{-08}$
NCOA3_725_747	0.10	$9.35 \cdot 10^{-09}$	0.98	$1.72 \cdot 10^{-08}$

Supplementary Table S: EC_{50} and IC_{50} (underlined) values for ERα-LBD and ERβ-LBD binding to coregulator-derived binding motifs in the MARCoNI assay induced by E2, only displayed for concentration-response curves with $R^2 > 0.9$. (*continued*)

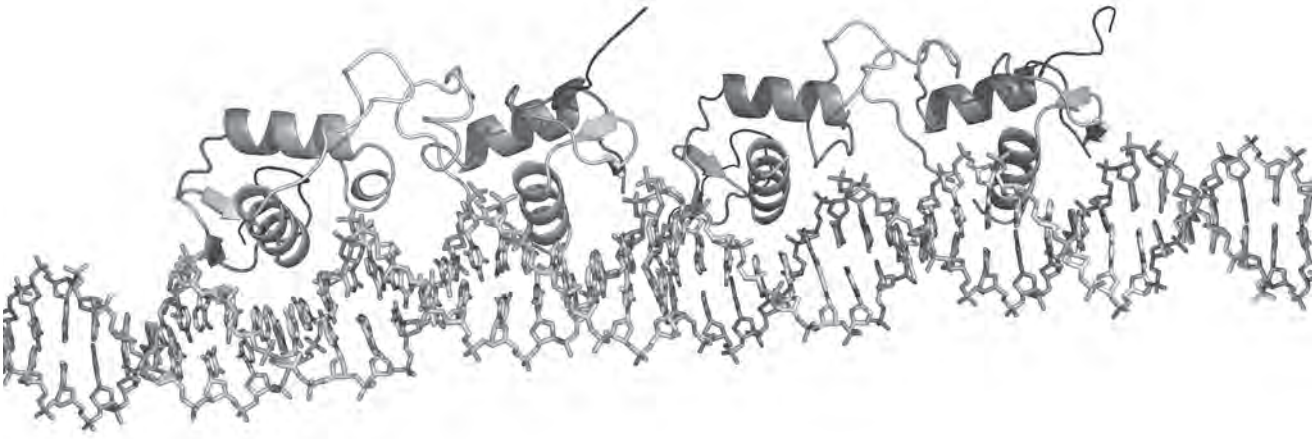
Coregulator	R^2 ERα	EC_{50} ERα (M)	R^2 ERβ	EC_{50} ERβ (M)
NCOA6_1479_1501	0.10	$3.28 \cdot 10^{-08}$		
NCOA6_875_897	0.10	$8.42 \cdot 10^{-09}$	0.95	$3.03 \cdot 10^{-08}$
NCOR1_662_684_C662S	0.94	$\underline{4.60 \cdot 10^{-08}}$		
NCOR2_2330_2352	0.10	$\underline{3.10 \cdot 10^{-12}}$		
NELFB_428_450	0.99	$1.31 \cdot 10^{-08}$		
NR0B1_1_23	0.10	$1.19 \cdot 10^{-08}$	0.96	$2.89 \cdot 10^{-08}$
NR0B1_136_159	0.99	$7.91 \cdot 10^{-09}$		
NR0B1_68_90_C69S	0.10	$8.97 \cdot 10^{-09}$	0.97	$2.78 \cdot 10^{-08}$
NR0B2_106_128	0.96	$3.05 \cdot 10^{-08}$	0.93	$3.45 \cdot 10^{-08}$
NR0B2_201_223_C207S	0.10	$1.31 \cdot 10^{-08}$		
NR0B2_9_31_C9S/C11S	0.98	$4.97 \cdot 10^{-08}$	0.97	$3.03 \cdot 10^{-08}$
NRBF2_128_150	0.99	$3.28 \cdot 10^{-07}$	0.93	$2.76 \cdot 10^{-08}$
NRIP1_1055_1077	0.10	$6.71 \cdot 10^{-09}$		
NRIP1_120_142	0.10	$1.33 \cdot 10^{-08}$	0.96	$4.42 \cdot 10^{-08}$
NRIP1_121_143_P124R	0.10	$1.17 \cdot 10^{-08}$	0.94	$3.45 \cdot 10^{-08}$
NRIP1_173_195	0.99	$1.43 \cdot 10^{-08}$		
NRIP1_173_195_C177S	0.10	$9.78 \cdot 10^{-09}$		
NRIP1_253_275_C263S	0.10	$1.13 \cdot 10^{-08}$	0.96	$4.71 \cdot 10^{-08}$
NRIP1_368_390	0.96	$3.58 \cdot 10^{-08}$	0.99	$3.75 \cdot 10^{-08}$
NRIP1_488_510	0.10	$1.09 \cdot 10^{-08}$	0.98	$2.78 \cdot 10^{-08}$
NRIP1_700_722	0.10	$1.09 \cdot 10^{-08}$	0.97	$4.19 \cdot 10^{-08}$
NRIP1_701_723	0.10	$1.49 \cdot 10^{-08}$	0.98	$6.44 \cdot 10^{-08}$
NRIP1_805_831	0.98	$1.64 \cdot 10^{-08}$	0.96	$4.21 \cdot 10^{-08}$
NRIP1_924_946	0.10	$1.25 \cdot 10^{-08}$		
NRIP1_924_946_C945S	0.10	$1.09 \cdot 10^{-08}$	0.98	$2.33 \cdot 10^{-08}$
NSD1_894_916	0.10	$1.50 \cdot 10^{-08}$	0.96	$4.23 \cdot 10^{-08}$
PELP1_168_190			0.94	$3.70 \cdot 10^{-08}$
PELP1_20_42	0.10	$1.18 \cdot 10^{-08}$	0.91	$2.61 \cdot 10^{-08}$
PELP1_446_468	0.10	$1.14 \cdot 10^{-08}$	0.93	$2.53 \cdot 10^{-08}$
PELP1_571_593_C575S/C581S	0.93	$4.25 \cdot 10^{-07}$		
PNRC1_306_327	0.93	$1.30 \cdot 10^{-07}$		
PPRC1_151_173	0.99	$4.78 \cdot 10^{-08}$		
PR285_1105_1127	0.10	$3.51 \cdot 10^{-08}$		
PR285_2216_2238_C2219S	0.91	$2.00 \cdot 10^{-08}$		

Supplementary Table S: EC_{50} and IC_{50} (underlined) values for ERα-LBD and ERβ-LBD binding to coregulator-derived binding motifs in the MARCoNI assay induced by E2, only displayed for concentration-response curves with $R^2 > 0.9$. (*continued*)

Coregulator	R^2 ERα	EC_{50} ERα (M)	R^2 ERβ	EC_{50} ERβ (M)
PRGC1_130_155	0.98	$1.74 \cdot 10^{-09}$	0.96	$2.77 \cdot 10^{-08}$
PRGC1_134_154	0.96	$1.04 \cdot 10^{-09}$		
PRGC2_146_166	0.10	$9.13 \cdot 10^{-09}$	0.96	$4.34 \cdot 10^{-08}$
PRGC2_338_358	0.93	$1.96 \cdot 10^{-08}$	0.95	$3.43 \cdot 10^{-07}$
PROX1_57_79	0.95	$1.21 \cdot 10^{-06}$		
TIF1A_373_395_C394S	0.97	$9.48 \cdot 10^{-09}$		
TIF1A_747_769	0.10	$4.51 \cdot 10^{-09}$	0.92	$1.70 \cdot 10^{-08}$
TIP60_476_498	0.99	$4.31 \cdot 10^{-08}$	0.94	$6.54 \cdot 10^{-08}$
TREF1_168_190	0.10	$1.09 \cdot 10^{-08}$		
TRXR1_132_154	0.96	$7.84 \cdot 10^{-08}$	0.92	$3.41 \cdot 10^{-08}$
UBE3A_649_671	0.93	$4.76 \cdot 10^{-08}$		
WIPI1_119_141	0.10	$1.23 \cdot 10^{-08}$	0.96	$3.03 \cdot 10^{-08}$
WIPI1_313_335_C318S	0.94	$2.75 \cdot 10^{-08}$		
ZNHI3_89_111	0.10	$1.19 \cdot 10^{-08}$		



4 Identification of coregulators involved in estrogen receptor subtype-specific binding of the ER antagonists 4-hydroxytamoxifen and fulvestrant



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Abstract

The aim of the present study was to investigate modulation of the interaction of ERα and ERβ with coregulators in the ligand-dependent responses induced by the ER antagonistic compounds 4OHT and fulvestrant. Comparison of these results to ligand-dependent interaction of ERα and ERβ with coregulators expressed in MI profiles for the ER agonist E2 will elucidate whether differences in the (ant)agonist-dependent interaction of ERα and ERβ with coregulators expressed in MI profiles contribute to the differences in (ant)agonist responses. To this end, the selected ER antagonistic compounds were first characterized for intrinsic relative potency reflected by IC_{50} and efficacy towards ERα and ERβ using ER-selective U2OS reporter gene assays, and subsequently tested for ligand-dependent modulation of the interaction of ERα and ERβ with coregulators using the MARCoNI assay with 154 different nuclear receptor coregulator peptides derived from 66 different coregulators. Results obtained with the U2OS reporter gene assays indicate a preference of 4OHT to antagonize ERβ and find fulvestrant to be less ER-specific. The responses in the MARCoNI assay reveal that ERα- and ERβ-mediated interaction with coregulators expressed in MI profiles are similar for 4OHT and fulvestrant and generally opposite to the MI profile of the ER agonist E2. Hierarchical clustering with Euclidian distance as the cluster distance metric based on the MI profiles appeared able to clearly discriminate the two compounds with ER antagonistic properties from the ER agonist E2. Taken together, the data reveal that modulation of the interaction of ERs with coregulators discriminates ER agonists from antagonists but does not discriminate between the preferential ERβ antagonistic compound 4OHT and the less specific ER antagonist fulvestrant. It is concluded that differences in modulation of the interaction of ERα and ERβ with coregulators contribute to the differences in ligand-dependent responses induced by ER agonists and ER antagonists, but the importance of the subtle differences in modulation of the interaction of ERs with coregulators between the ER antagonistic compounds 4OHT and fulvestrant for the ultimate biological effect remains to be established.

Introduction

Estrogens affect cell growth in several tissues [1-3]. These effects are modulated by binding of the estrogens to estrogen receptor (ER)α and ERβ, thereby regulating gene transcription [4-6]. Estrogenic compounds that bind to ERα and ERβ may be agonists inducing a physiological response similar to that induced by the natural ligand 17β-estradiol (E2), or antagonists, of which the binding results in inhibition of the E2-mediated activation and cellular responses [7, 8]. In addition to the nature of the ligand, being an agonist or antagonist, also the intrinsic potency and efficacy (partial or full) of the ligand for the two receptors [7, 9], and the ERα/ERβ ratio in the cells or tissue of interest is important for the ultimate biological effect [5-7, 10].

ERα and ERβ have been reported to exert counteracting effects on cell proliferation. ERα activation enhances cell proliferation [11], whereas ERβ activation counteracts ERα-mediated cell proliferation [7, 12-15]. Therefore, ERα antagonism leads to repression of cell growth [16], whereas ERβ antagonism leads to enhanced cell proliferation [17]. It has been suggested that estrogen-mediated effects on cell proliferation through the action of ERα and ERβ are involved in the development and progression of cancer of especially estrogen-responsive tissues [12, 18]. For ER-positive breast tumors as well as for other estrogen-dependent tumors, it has been shown that in tumorous tissue compared to normal tissue the ERα/ERβ ratios increase due to a decreased ERβ expression [12]. This role for ER-mediated effects on cell proliferation and cancer is also reflected in the use of ER antagonists in hormonal cancer therapy for the treatment of ERα-positive breast cancer [8]. With appropriate endocrine therapy based on ER antagonists, patients with ERα-positive breast tumors have a better prognosis than those with ERα-negative tumors [19].

ER antagonistic compounds for the treatment of breast cancer include tamoxifen [20, 21] and fulvestrant [22]. Tamoxifen is a first line breast cancer drug widely used for treatment of ERα-positive breast cancers [23]. It acts by blocking the ER (both ERα and ERβ) [24, 25]. Tamoxifen would only properly work in breast cancer cells with relatively high ERα and low ERβ expression, since then tamoxifen will block ERα and thereby reduce ERα-induced cell proliferation [17]. 4-Hydroxytamoxifen (4OHT) is the active metabolite of tamoxifen, tested in the current study in the trans isoform, which possesses stronger anti-estrogenic activity than the cis isoform [26]. It has a 100 times higher potency towards both ER isoforms than tamoxifen itself [27]. In competition binding assays with E2, the compound 4OHT has a 1.6 times higher binding affinity for ERβ over ERα [28]. However, tamoxifen and 4OHT are not full ER antagonists, but are Selective Estrogen Receptor Modulators (SERMs) displaying both ER agonistic and antagonistic properties

depending on the physiological context [21]. In contrast, fulvestrant is a full ERα and ERβ antagonist and is used as a second line breast cancer drug [29]. Fulvestrant acts by blocking both ERs and reducing cellular levels of ERα [8, 30-33]. In tests for its antagonist activity towards E2, it has a 9 times higher antagonistic preference for ERα over ERβ [34].

An important factor that may influence the ultimate gene expression and biological effect induced by estrogenic compounds is the type of coactivators that can bind to the ligand-ER complex. This aspect of interaction of ERs with coregulators has not been studied in great detail. It has been shown that these coregulators influence the ER-mediated activation or repression and transcription of target genes. Coactivators and corepressors have a role in the response to estrogenic and anti-estrogenic compounds, chromatin condensation, and mediating transcription [5, 10, 35, 36].

Recently the modulation of the interaction of ERα and ERβ with coregulators in the ligand-dependent responses induced by ER agonists was investigated [37]. The data obtained revealed that differences in modulation of the interaction of ERα and ERβ with coregulators contribute to the different ligand-dependent responses induced by different ER agonists, but do not contribute significantly to the differences between ERα- and ERβ-mediated responses by a given ER agonist. The aim of the present study was to investigate modulation of the interaction of ERs with coregulators upon exposure to the ER antagonistic compounds 4OHT and fulvestrant. To this end, 4OHT and fulvestrant were tested for intrinsic relative potency reflected by IC_{50} and efficacy towards ERα and ERβ using human osteosarcoma U2OS reporter gene assays, and the ligand-dependent modulation of the interaction of ERα and ERβ with coregulators induced by the model compounds was investigated using a Microarray Assay for Real-time Coregulator – Nuclear receptor Interaction (MARCoNI) with 154 unique nuclear receptor coregulator peptides derived from 66 different coregulators.

Materials and Methods

Cell culture

The U2OS cell lines, stably expressing ERα or ERβ in addition to a 3x estrogen response element and TATA box binding protein combined with a luciferase gene (3x ERE-TATA-luciferase gene) were kindly provided by the Hubrecht Institute, Utrecht [7]. U2OS-ERα cells were grown in DMEM:F12, a 1:1 mixture of Dulbecco's modified Eagle's medium (DMEM) and Ham's nutrient mixture F12 (Gibco, Paisley, Scotland, 31331-028) supplemented with 10% fetal calf serum (FCS, Invitrogen, Paisley, Scotland, #10099), 0.5% non-essential amino acids (NEAA, Gibco, Paisley, Scotland, 11140-035), 0.2 mg/ml geneticin G418 (PAA Laboratories GmbH, Pasching, Austria, #P02-012), and 0.05 mg/ml

hygromycin (Duchefa, Haarlem, the Netherlands, # H0192.0001). U2OS-ERβ cells were grown in 1:1 DMEM:F12 culture medium supplemented with 10% FCS, 0.5% NEAA, and 0.2 mg/ml geneticin G418 [7]. All cells were incubated at 37°C and 5% CO_2 in a humidified atmosphere.

Because phenol red exerts estrogenic activity [38], at least 24 hours before exposure to the selected model compounds, cells were washed 3 times with phosphate buffered saline (PBS, Gibco, Paisley, Scotland, #10010-015) and transferred to phenol red free medium (Gibco, Paisley, Scotland, #21041-025) supplemented with 5% hormone-free dextran-coated charcoal-treated fetal calf serum (DCC-FCS, Perbio Science, Waltham, MA, USA, #SH30068.05) and 0.5% NEAA.

U2OS reporter gene assay

Cells were seeded in 96-well view plates (PerkinElmer, Groningen, the Netherlands, #655180) at a density of 10^5 cells/ml for U2OS-ERα and $7.5*10^4$ cells/ml for U2OS-ERβ, 100 µl/well. Twenty-four hours after seeding, medium was changed to phenol red free medium. Forty-eight hours after seeding, cells were exposed to the test compounds in triplicate, in phenol red free medium. 17β-Estradiol (E2) was chosen as the positive standard estrogenic agonist and used to characterize the antagonist properties of fulvestrant and 4OHT. E2 is known to be both an ERα and ERβ agonist with an approximate 10-fold higher preference for ERα over ERβ as shown in ligand binding experiments with solubilized *Spodoptera frugiperda 9 (Sf9)* insect cell extracts and U2OS reporter cell lines [5, 39, 40]. Progesterone was included as a negative control for ER binding [41, 42].

After 24 hours of exposure, cells were washed with 0.5*PBS and lysed with 30 µl of hypotonic low-salt buffer containing (final concentrations) 10 mM Tris, 2 mM dithiothreitol (DTT, Sigma-Aldrich, St. Louis, MO, USA, # D-0632), and 2 mM 1,2-diaminocyclohexanetetraacetic acid monohydrate (CDTA, Sigma-Aldrich, St. Louis, MO, USA, #32869); pH 7.8. Plates were kept on ice for at least 10 minutes and subsequently stored at -80°C until analysis. One hour before measurement, plates were thawed on a plate shaker until they reached room temperature. Luciferase activity was determined using a luminometer (Labsystems, Luminoskan RS). In short, background light emission was measured for 2 seconds, after which 100 µl of flashmix (20 mM tricine (Sigma-Aldrich, St. Louis, MO, USA, #T5816), 1.07 mM $(MgCO_3)4Mg(OH)_2*5H_2O$ (Aldrich, Milwaukee, WI, USA, #22,766-8), 2.6 mM magnesium sulfate ($MgSO_4$, Sigma-Aldrich, St. Louis, MO, USA, #24.697-2), 0.1 mM ethylenedinitrilotetraacetic acid ($EDTA*2H_2O$, Merck, Darmstadt, Germany, #1.08418.1000), 2 mM DTT, 0.47 mM D-luciferin (Duchefa Biochemie, Haarlem, the Netherlands, # L1349.0250), and 5 mM adenosine triphosphate (ATP, Duchefa Biochemie, Haarlem, the Netherlands, # A1335.0010); pH 7.8) was

automatically injected, followed by measurement of light emission for another 2 seconds, after which the light emission was extinguished with 50 µl 0.2 M sodium hydroxide (NaOH, Merck, Darmstadt, Germany, # 1.06498.1000) [43].

For calibration of the results of all U2OS assays, 3 concentrations of E2 were tested (each of the concentrations tested at least in triplicate) on each plate to correct for plate-to-plate variations. Data from the U2OS reporter gene assay were expressed in relative light units (RLU), corrected for the corresponding background signal measured before luciferase induction. EC_{50} and IC_{50} values were calculated using PRISM V (GraphPad, San Diego, CA, USA). Potency of the compounds was determined based on the IC_{50} value and efficacy based on maximal height of the induction of luciferase activity in the U2OS reporter gene assays.

MARCoNI assay

Ligand-modulated interaction of coregulators with ERα or ERβ was assessed using a PamChip peptide microarray with 154 unique coregulator motifs derived from 66 different coregulators (#88102 and #90012, PamGene International BV, Den Bosch, the Netherlands) as described previously [44]. In short, all incubations were performed on a PamStation®-12 or -96 (handling 12 or 96 arrays in parallel per run respectively) (PamGene) at 20°C using two cycles per minute. Polyhistidine (His) tagged ERα ligand binding domain (amino acids 302-552, partly purified from *Escherichia coli (E. coli),* crude lysate, final concentration between 1 and 10 nM) and His antibody penta-His Alexa Fluor 488 conjugate (Qiagen, Germantown, MD, USA,#35310, final concentration 25 nM) were diluted in time-resolved fluorescence resonance energy transfer (TR-FRET) reaction buffer containing 20 mM Tris-HCl; pH 7.5 (Tris: Sigma-Aldrich, St. Louis, MO, USA, #T1503) (HCl: Merck, Darmstadt, Germany, #1.00317.1000), 500 mM NaCl (Merck, Darmstadt, Germany, #1.06404.1000), 0.2% bovine serum albumin (BSA, Calbiochem, Merck, Darmstadt, Germany, #126609), and 0.05% Tween 20 (Bio-Rad, Veenendaal, the Netherlands, #170-6531). Glutathione S-transferase (GST) tagged ERβ subtype-specific ligand binding domain (amino acids 243-530 final concentration 10 nM) and anti-GST Alexa Fluor 488 conjugate (Molecular probes, Life Technologies Ltd, UK, #A11131, final concentration 50 nM) were diluted in reaction buffer containing coregulator buffer E (Invitrogen, Paisley, Scotland, #PV4540) supplemented with 5 mM DTT (Sigma, Zwijndrecht, the Netherlands, #43819). All mixtures were kept on ice until they were transferred to the PamChip microarrays. The concentrations of the model compounds tested in the MARCoNI assay were approximately 10^5 times the EC_{50} or IC_{50} concentration in the U2OS assays with 10^{-4} M being the highest concentration tested in the MARCoNI assay. Ligand was predissolved in dimethyl sulfoxide (DMSO) (concentrations as

indicated, final DMSO concentration 2%). Reaction mixture with 2% DMSO served as negative control. Each array was blocked for 20 cycles using 25 µl of blocking buffer (Tris-buffered saline) (TBS, Bio-Rad, Veenendaal, the Netherlands, #170-6435) supplemented with 0.01% Tween 20 (Bio-Rad, Veenendaal, the Netherlands, #170-6531) and 1% BSA (Calbiochem, Merck, Darmstadt, Germany, #126609). Next, the blocking buffer was removed by aspiration and the reaction mixture was added to the PamChip microarray together with the ligand in a final volume of 25 µl. This reaction-ligand mixture was incubated (pumped up and down the porous microarray membrane containing the 154 different coregulator peptides) for 80 cycles. Subsequently, unbound receptor was removed by washing of the arrays with 25 µL TBS and finally a tiff image of each array was acquired by the charge coupled device (CCD) camera of the PamStation.

Data analysis

MARCoNI assay image analysis was performed using BioNavigator (Version 5.1, PamGene International B.V.). Per array, the fluorescent signal of each spot, representative of ER binding to that particular coregulator motif, was quantified. Each tiff image (single array) was overlaid with a synthetic grid of spot-sized circles. An algorithm was used to optimise placement of each circle around its respective spot (actual peptide position) on the tiff image. The median fluorescence within each circle as well as that in a defined area surrounding the circle (background) was quantified. For each spot the binding signal as median fluorescent signal minus background for each peptide was calculated. The modulation index (MI) was subsequently calculated as the compound-induced log 10-fold change of binding (fluorescence) in the presence of ligand over that in the presence of solvent only. In addition, a Student's t-test on ER binding was conducted to assess the significance of the compound effect (DMSO vs. compound-stimulated). As each array contains 154 unique coregulator motifs, each compound was characterized by a 154-point MI profile. Obtained MI profiles were subjected to hierarchical clustering by Euclidean distance and average linkage. Hierarchical clustering was performed using stats packages in R (version 2.15.3, copyright© 2013 The R Foundation for Statistical Computing). Per receptor, compound (dis)similarities were visualized as a dendrogram of a clustered MI heatmap in which statistical significance of the modulation of each interaction was indicated.

For E2, ligand dose-response curve fitting was performed using the drc package in R, by means of a sigmoidal, 4-parameter Hill (logistic) model (response=((A-D)/(1+((concentration/C)^{B})))+D, with parameters A=response minimum, B=Hill slope, C=EC_{50}, and D=response maximum).

Results

Characterization of the intrinsic relative ERα or ERβ potency and efficacy of the selected antagonistic compounds

Intrinsic relative ERα or ERβ potency and efficacy of the ER antagonistic compounds was characterized using the U2OS cell lines stably expressing ERα or ERβ. The U2OS-ERα and U2OS-ERβ reporter gene assays were used to establish the intrinsic ability of the selected antagonistic compounds 4OHT and fulvestrant to counteract E2-mediated activation of ERα- (Figure 1) or ERβ- (Figure 2) dependent gene expression. Antagonistic effects were investigated by testing the compounds in the presence of E2 at its EC_{50}; 6 pM for ERα (Figure 1) and 60 pM for ERβ (Figure 2). From the results obtained, the IC_{50} values for the antagonistic compounds were determined (Table A). 4OHT and fulvestrant show antagonistic effects towards both ERα and ERβ (Figure 1B and 2B). IC_{50} values for 4OHT are 18-fold lower for ERβ compared to ERα and the Estradiol Equivalence Factor (EEF) ERβ/EEF ERα ratio is 169, indicating a preference for ERβ (Figure 1B and 2B, Table A). IC_{50} values for fulvestrant are about 4- to 5-fold lower for ERα than for ERβ, although the EEF ERβ/EEF ERα ratio is 2.1, indicating a much lower specific preference for one of the ER subtypes (Figure 1B and 2B, Table A).

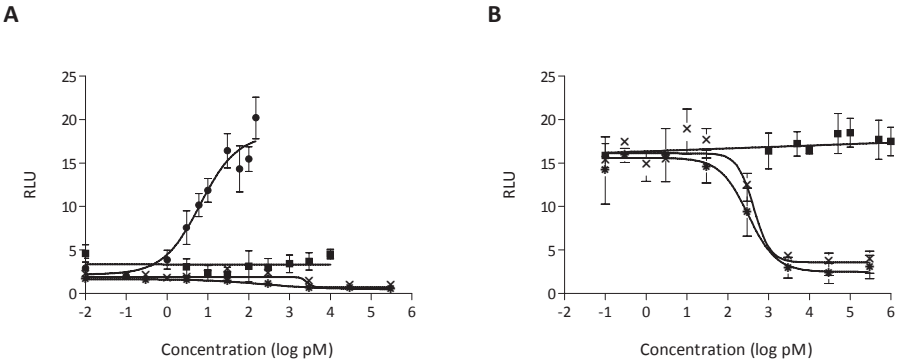

Figure 1: ERE-mediated luciferase activity in U2OS-ERα cells exposed to **(A)** E2 (●), 4OHT (✕), fulvestrant (❋), and progesterone (■) alone, or **(B)** 4OHT (✕), fulvestrant (❋), and progesterone (■) in the presence of 6 pM E2. Data points ± standard deviation (SD) (n=3).

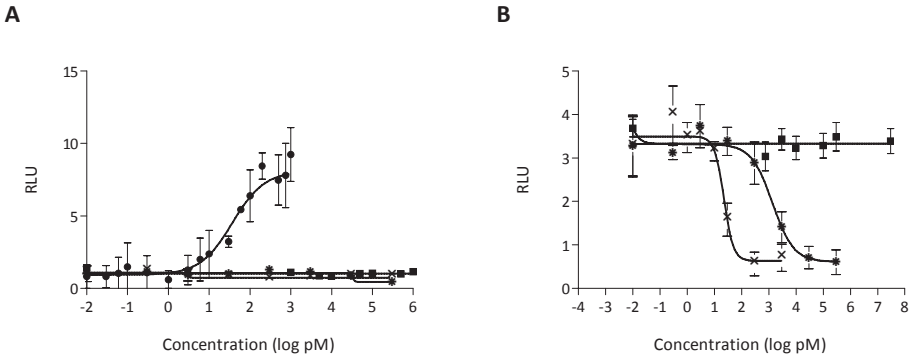

Figure 2: ERE-mediated luciferase activity in U2OS-ERβ cells exposed to **(A)** E2 (●), 4OHT (✕), fulvestrant (✱), and progesterone (■) alone, or **(B)** 4OHT (✕), fulvestrant (✱), and progesterone (■) in the presence of 60 pM E2. Data points ± SD (n=3).

The negative control progesterone shows no changes in ER-mediated luciferase activity in the U2OS-ERα or U2OS-ERβ cell line, either in the presence or absence of E2. In both the U2OS-ERα or U2OS-ERβ reporter gene assay, efficacy for 4OHT and fulvestrant appear to be similar reflected by a similar maximum reduction of luciferase activity induced by E2 (Figures 1 and 2).

Table A: EC_{50} and IC_{50} values for E2, 4OHT, and fulvestrant as determined by the U2OS reporter gene assay.

Compound	EC_{50} ERα (pM)	IC_{50} ERα (pM)	EEF ERα	EC_{50} ERβ (pM)	IC_{50} ERβ (pM)	EEF ERβ	EEF ERβ/ EEF ERα ratio	ERα/ERβ fold potency ratio
E2	6.5	-	NA	59.8	-	NA	NA	9.2 (agonist)
4OHT	-	413	0.016	-	22.6	2.65	169	0.05 (antagonist)
Fulvestrant	-	308	0.021	-	1370	0.044	2.1	4.5 (antagonist)

EEF for 4OHT and fulvestrant: Estradiol Equivalent Factor calculated as EC_{50} E2/IC_{50} test compound.
NA: Not Applicable.

Estrogen receptor-specific modulation of interaction with coregulators by 4OHT and fulvestrant

To investigate differential compound-induced modulation of the interaction of ERα or ERβ with coregulators during ligand binding, both antagonistic compounds were tested with the so called MARCoNI assay in the presence of the ligand binding domain (LBD) of either ERα or ERβ [45].

In Figure 3 the ligand-dependent interaction of ERα and ERβ with different coregulator peptides for the two model compounds is displayed, expressed as the MI profile which is the ligand-induced log fold modulation of binding, for comparison presented together with the MI profile for the agonist E2. For both ERα (Figure 3A) and ERβ (Figure 3B), the interaction with coregulators expressed as modulation patterns induced by 4OHT and fulvestrant seem to be very similar and are clearly different from the modulation patterns for E2. In fact, the MI profiles of 4OHT and fulvestrant are for most coregulator motifs opposite to that of E2. For example, for both ERα and ERβ upon incubation with 4OHT and fulvestrant, nuclear coactivator (NCOA) motifs NCOA1_677_700, NCOA2_628_651, and NCOA3_673_695 show negative modulation, whereas upon incubation of the ERs with the agonist E2, positive modulation of interaction of ERs with these coactivator motifs is shown.

For both 4OHT and fulvestrant, when multiple binding motifs of one coregulator are present in the MARCoNI assay, the binding to different motifs belonging to one coregulator are all negatively modulated, though not all equally strong (for example all nuclear receptor interacting protein 1 (NRIP1) motifs, Figure 3).

Although the MI profiles induced by 4OHT and fulvestrant appeared similar, for ERβ one distinct difference is observed between the two antagonistic model compounds. Fulvestrant shows negative and 4OHT positive modulation of interaction of ERs with coregulator motif DEAD box (DDX)5_133_155 (indicated with an arrow in Figure 3B). The negative control progesterone showed only limited modulation of interaction of ERα and ERβ with coregulators (Supplementary Figure S).

To investigate to what extent the MARCoNI assay is able to discriminate ER agonist E2 from ER antagonistic compounds based on their MI profile, the model compounds were classified based on hierarchical clustering of the MI profile using Euclidian distance (Figure 4). For clustering based on ERα (Figure 4A) or ERβ (Figure 4B), the antagonistic compounds 4OHT and fulvestrant cluster together, discriminating clearly from agonist E2, whereas the negative control progesterone did not cluster with the other model compounds, only with DMSO.

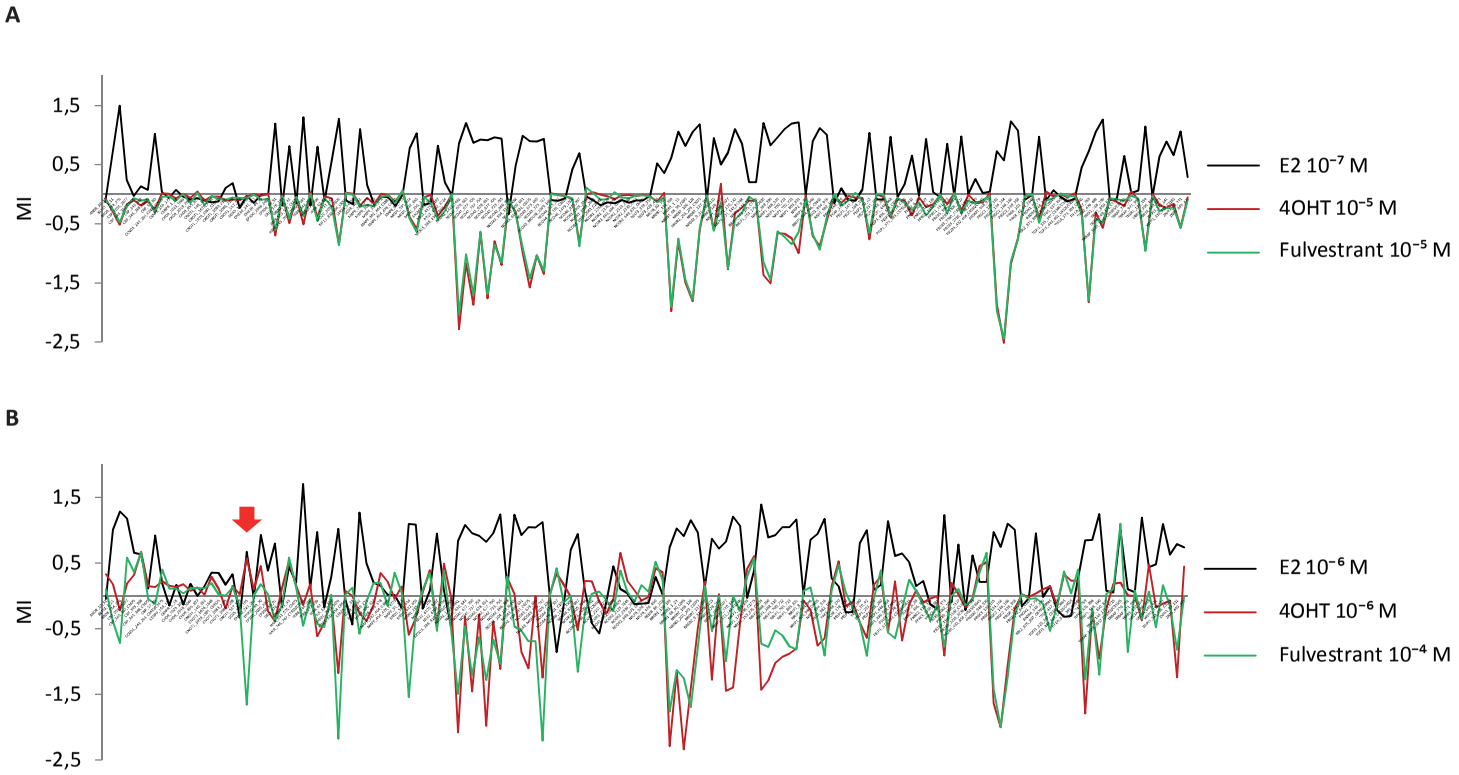

Figure 3: The interaction of ERS with coregulators expressed as MI profiles for **(A)** ERα and **(B)** ERβ for E2 (black), 4OHT (red), and fulvestrant (green) (n=3).

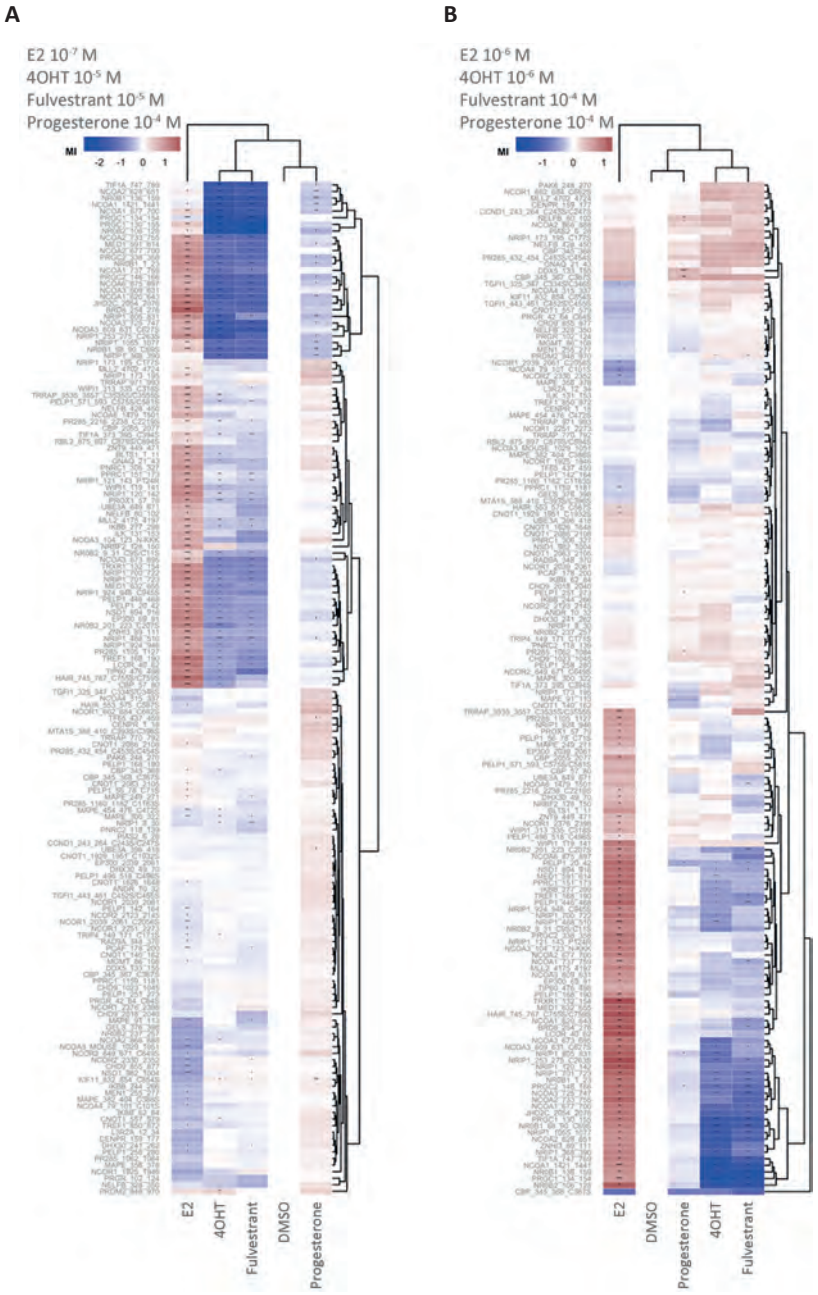

Figure 4: MI heatmap of **(A)** ERα and **(B)** ERβ, interaction with coregulators across compounds and coregulators. Red represents positive MI, blue represents negative MI. In the heatmap significance of the modulation of each interaction is indicated (*: P value <0.05; **: P value <0.01; ***: P value <0.001).

Discussion

An important factor that may influence the ultimate gene expression and biological effect induced by ER antagonists is the type of coregulators that can bind to the ligand-ER complex. The aim of the present study was to investigate coregulator interaction with ERα and ERβ upon exposure to the ER antagonistic compounds 4OHT and fulvestrant. First the antagonist activity of these two model compounds towards ERα and ERβ was characterized using the U2OS-ERα and U2OS-ERβ reporter gene assays performed in the presence of E2. E2 was able to induce ERE-mediated gene transcription in the U2OS-ERα and U2OS-ERβ reporter gene assay with a higher potency for ERα than for ERβ. The 9-fold difference between the EC_{50} for ERα and the EC_{50} for ERβ in the U2OS reporter gene assays is in line with previous results [7]. When combined with 6 or 60 pM E2 in respectively the U2OS-ERα and U2OS-ERβ reporter gene assay (Figure 1B and 2B), both 4OHT and fulvestrant show clear antagonistic effects. 4OHT is not able to bring the E2-induced response completely back to baseline in the U2OS-ERα reporter gene assay, which can be interpreted as partial agonism [46]. The IC_{50} value for the antagonist activity of 4OHT appeared to be 18-fold lower for ERβ than for ERα with an EEF ERβ/EEF ERα ratio of 169, indicating a higher antagonistic potency towards ERβ. For fulvestrant, the IC_{50} value is 4.5-fold lower for ERα than for ERβ and the EEF ERβ/EEF ERα ratio is 2.1, indicating a much lower specific preference for one of the ER subtypes (Table A). The negative control progesterone was not able to induce or inhibit E2-induced responses in the U2OS-ERα and U2OS-ERβ reporter gene assay. These data indicate a difference in ERα or ERβ preference for the two model compounds. Efficacy towards the ERs were similar for 4OHT and fulvestrant.

To investigate whether differences in modulation of the interaction of ERs with coregulators play a role in the ERα- and ERβ-dependent responses to the antagonistic compounds, ligand-dependent modulation of the interaction of ERα and ERβ with coregulators by the different model compounds was determined in the MARCoNI assay. The ERβ-based MARCoNI assay was performed for the first time with antagonistic compounds and therefore antagonist concentrations at expected maximum efficacy were chosen.

The results of the present study revealed that the MI profiles induced by 4OHT and fulvestrant appeared similar, but are clearly different from the MI profile for E2 (Figure 3). The MI profiles of 4OHT and fulvestrant are opposite to that of E2. The NCOA1, NCOA2, NCOA3, and NCOA6 coactivator motifs are negatively modulated by both ER antagonistic compounds in combination with both ER receptors, while for E2 these NCOA motifs are mostly positively modulated. Also, for E2, most of the nuclear corepressor (NCOR)1 and

NCOR2 motifs are significantly negatively modulated in combination with both ERs, while both ER antagonistic compounds do not show significant modulation of these motifs (Figure 3). It is worth noting that in the MARCoNI assay the MI profiles induced by the antagonistic compounds 4OHT and fulvestrant can be detected directly, i.e. without the requirement for addition of E2, which is an unique property of the MARCoNI assay over the U2OS reporter gene assays.

The MI profiles induced by 4OHT and fulvestrant appeared similar, which indicates that these two compounds do share, to some extent, the same mode of action, especially by blocking the interaction of the ER with coactivator peptides. However, for ERβ, one distinct difference is observed between the two antagonistic compounds. Fulvestrant shows negative and 4OHT positive modulation of the interaction of ERβ with coregulator motif DDX5_133_155 (indicated with an arrow in Figure 3B). DDX5 is a transcriptional coactivator for the tumor suppressor protein p53 and is involved in the p53 transcriptional response to DNA damage and p53-dependent apoptosis [47]. The recruitment of this DDX5 coactivator motif by ERβ as induced by tamoxifen could be beneficial for ERβ reducing ERα-mediated cell proliferation since it could stimulate p53-dependent apoptosis. It is of interest to note that previously tested agonistic compounds showed positive modulation of the interaction of ERβ with coregulator motif DDX5_133_155 as well [37]. This could possibly be linked to the partial agonistic actions tamoxifen and 4OHT have in specific tissue [21].

The differences in the type of modulation of the interaction of ERs with coregulators between the different ER antagonistic compounds incubated with ERα or ERβ are limited. The MI profiles as well as the efficacy of 4OHT and fulvestrant in the U2OS reporter gene assays are very similar.

Quantitative hierarchical clustering of the two ER antagonistic compounds 4OHT and fulvestrant, the ER agonist E2, and the negative control progesterone tested in the present study based on the interaction of ERs with coactivators (Figure 4) showed that for both ERα and ERβ, 4OHT was found to cluster together with fulvestrant and could clearly be discriminated from the negative control progesterone clustering together with DMSO, as well as from the ER agonist E2. This illustrates the potential of the MARCoNI assay to clearly discriminate between ER agonists and antagonists. A similar type of approach was previously shown for ERα coregulator modulation [45, 48].

In the present study, only subtle differences in the modulation of the interaction of ERα and ERβ with coregulators by a specific ligand were detected. The importance of the subtle differences for the ultimate biological effect remains to be established and they are an important topic for future research, in which also should be established to what extent the different coregulators are present in cells or tumors. Based on the

ligand-dependent differences in modulation of the interaction of ERs with coregulators the MARCoNI assay was shown to be able to discriminate the ER agonist E2 from ER antagonistic compounds. The similarity of the MI profiles induced by 4OHT and fulvestrant indicate that these two compounds share a similar mode of action. The differences in antagonistic activity of 4OHT and fulvestrant towards ERα and ERβ might be due to the subtle differences observed on the level of interaction of ERs with coregulators, however, further research is necessary to better understand the function of the identified coregulators in a cellular context.

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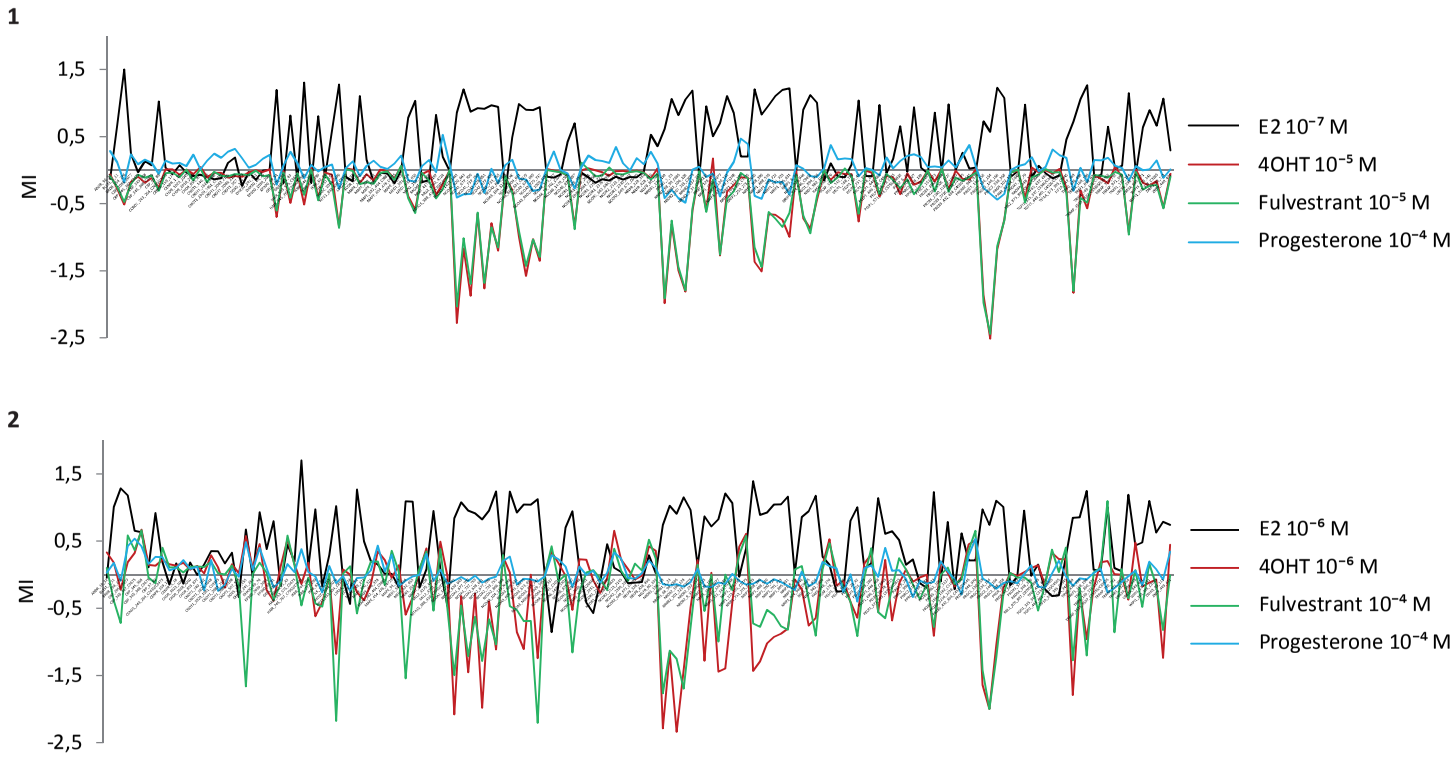
Supplementary Figure



Supplementary Figure S: MI profile for **(A)** ERα and **(B)** ERβ for E2 (black), 4OHT (red), fulvestrant (green), and progesterone (blue) (n=3).

Supplementary Table

Supplementary Table S: EC_{50} and IC_{50} (underlined) values for ERα-LBD and ERβ-LBD binding to coregulator-derived binding motifs in the MARCoNI assay induced by E2, only displayed for dose response curves with coefficient of determination R^2 >0.9.

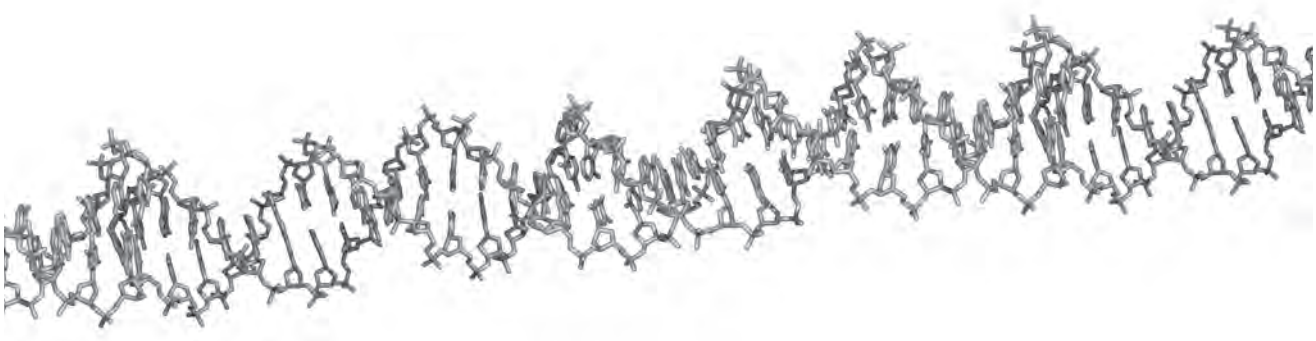
Coregulator	R^2 ERα	EC_{50} ERα	R^2 ERβ	EC_{50} ERβ
BL1S1_1_11	0.99	$1.38 \cdot 10^{-08}$	0.92	$2.94 \cdot 10^{-08}$
BRD8_254_276	1.00	$1.25 \cdot 10^{-08}$	0.98	$4.09 \cdot 10^{-08}$
CBP_57_80	1.00	$2.20 \cdot 10^{-08}$		
CENPR_159_177			0.99	$1.18 \cdot 10^{-05}$
CNOT1_2083_2105			0.91	$1.07 \cdot 10^{-07}$
DHX30_49_70			0.92	$3.85 \cdot 10^{-08}$
EP300_69_91	0.99	$2.55 \cdot 10^{-08}$	0.95	$3.14 \cdot 10^{-08}$
GNAQ_21_43	0.99	$9.09 \cdot 10^{-09}$		
HAIR_745_767_C755S/C759S	1.00	$1.65 \cdot 10^{-08}$	0.94	$3.75 \cdot 10^{-08}$
IKBB_277_299	0.98	$4.18 \cdot 10^{-08}$	0.96	$5.02 \cdot 10^{-08}$
ILK_131_153	0.90	$8.96 \cdot 10^{-09}$		
JHD2C_2054_2076	0.99	$2.12 \cdot 10^{-08}$	0.95	$3.01 \cdot 10^{-08}$
LCOR_40_62	1.00	$3.91 \cdot 10^{-08}$	0.96	$1.78 \cdot 10^{-07}$
MAPE_382_404_C388S	1.00	$\underline{3.47 \cdot 10^{-12}}$		
MED1_591_614	1.00	$1.08 \cdot 10^{-08}$	0.95	$2.50 \cdot 10^{-08}$
MED1_632_655	0.98	$3.16 \cdot 10^{-08}$	0.92	$3.41 \cdot 10^{-08}$
MEN1_255_277	0.95	$\underline{1.68 \cdot 10^{-10}}$		
MLL2_4175_4197	1.00	$1.26 \cdot 10^{-08}$	0.92	$6.13 \cdot 10^{-07}$
MLL2_4702_4724			0.90	$2.34 \cdot 10^{-08}$
MTA1S_388_410_C393S/C396S	0.97	$1.54 \cdot 10^{-10}$	0.99	$1.13 \cdot 10^{-05}$
NCOA1_1421_1441	1.00	$4.70 \cdot 10^{-09}$	0.93	$2.61 \cdot 10^{-08}$
NCOA1_620_643	1.00	$1.19 \cdot 10^{-08}$	0.95	$2.67 \cdot 10^{-08}$
NCOA1_677_700	1.00	$8.23 \cdot 10^{-09}$	0.97	$1.59 \cdot 10^{-08}$
NCOA1_737_759	1.00	$1.22 \cdot 10^{-08}$	0.96	$3.60 \cdot 10^{-08}$
NCOA2_628_651	1.00	$8.95 \cdot 10^{-09}$	0.96	$3.06 \cdot 10^{-08}$
NCOA2_677_700	1.00	$8.61 \cdot 10^{-09}$	0.93	$3.03 \cdot 10^{-08}$
NCOA2_733_755	1.00	$9.67 \cdot 10^{-09}$	0.95	$3.22 \cdot 10^{-08}$
NCOA3_104_123_N-KKK	0.91	$8.76 \cdot 10^{-08}$	0.93	$3.78 \cdot 10^{-08}$
NCOA3_609_631	0.99	$8.17 \cdot 10^{-09}$		
NCOA3_609_631_C627S	0.96	$3.37 \cdot 10^{-08}$	0.96	$2.07 \cdot 10^{-08}$
NCOA3_673_695	0.97	$1.51 \cdot 10^{-07}$	0.97	$8.08 \cdot 10^{-08}$
NCOA3_725_747	1.00	$9.35 \cdot 10^{-09}$	0.98	$1.72 \cdot 10^{-08}$

Supplementary Table S: EC_{50} and IC_{50} (underlined) values for ERα-LBD and ERβ-LBD binding to coregulator-derived binding motifs in the MARCoNI assay induced by E2, only displayed for dose response curves with coefficient of determination R^2 >0.9. (*continued*)

Coregulator	R^2 ERα	EC_{50} ERα	R^2 ERβ	EC_{50} ERβ
NCOA6_1479_1501	1.00	$3.28 \cdot 10^{-08}$		
NCOA6_875_897	1.00	$8.42 \cdot 10^{-09}$	0.95	$3.03 \cdot 10^{-08}$
NCOR1_662_684_C662S	0.94	$\underline{4.60 \cdot 10^{-08}}$		
NCOR2_2330_2352	1.00	$\underline{3.10 \cdot 10^{-12}}$		
NELFB_428_450	0.99	$1.31 \cdot 10^{-08}$		
NR0B1_1_23	1.00	$1.19 \cdot 10^{-08}$	0.96	$2.89 \cdot 10^{-08}$
NR0B1_136_159	0.99	$7.91 \cdot 10^{-09}$		
NR0B1_68_90_C69S	1.00	$8.97 \cdot 10^{-09}$	0.97	$2.78 \cdot 10^{-08}$
NR0B2_106_128	0.96	$3.05 \cdot 10^{-08}$	0.93	$3.45 \cdot 10^{-08}$
NR0B2_201_223_C207S	1.00	$1.31 \cdot 10^{-08}$		
NR0B2_9_31_C9S/C11S	0.98	$4.97 \cdot 10^{-08}$	0.97	$3.03 \cdot 10^{-08}$
NRBF2_128_150	0.99	$3.28 \cdot 10^{-07}$	0.93	$2.76 \cdot 10^{-08}$
NRIP1_1055_1077	1.00	$6.71 \cdot 10^{-09}$		
NRIP1_120_142	1.00	$1.33 \cdot 10^{-08}$	0.96	$4.42 \cdot 10^{-08}$
NRIP1_121_143_P124R	1.00	$1.17 \cdot 10^{-08}$	0.94	$3.45 \cdot 10^{-08}$
NRIP1_173_195	0.99	$1.43 \cdot 10^{-08}$		
NRIP1_173_195_C177S	1.00	$9.78 \cdot 10^{-09}$		
NRIP1_253_275_C263S	1.00	$1.13 \cdot 10^{-08}$	0.96	$4.71 \cdot 10^{-08}$
NRIP1_368_390	0.96	$3.58 \cdot 10^{-08}$	0.99	$3.75 \cdot 10^{-08}$
NRIP1_488_510	1.00	$1.09 \cdot 10^{-08}$	0.98	$2.78 \cdot 10^{-08}$
NRIP1_700_722	1.00	$1.09 \cdot 10^{-08}$	0.97	$4.19 \cdot 10^{-08}$
NRIP1_701_723	1.00	$1.49 \cdot 10^{-08}$	0.98	$6.44 \cdot 10^{-08}$
NRIP1_805_831	0.98	$1.64 \cdot 10^{-08}$	0.96	$4.21 \cdot 10^{-08}$
NRIP1_924_946	1.00	$1.25 \cdot 10^{-08}$		
NRIP1_924_946_C945S	1.00	$1.09 \cdot 10^{-08}$	0.98	$2.33 \cdot 10^{-08}$
NSD1_894_916	1.00	$1.50 \cdot 10^{-08}$	0.96	$4.23 \cdot 10^{-08}$
PELP1_168_190			0.94	$3.70 \cdot 10^{-08}$
PELP1_20_42	1.00	$1.18 \cdot 10^{-08}$	0.91	$2.61 \cdot 10^{-08}$
PELP1_446_468	1.00	$1.14 \cdot 10^{-08}$	0.93	$2.53 \cdot 10^{-08}$
PELP1_571_593_C575S/C581S	0.93	$4.25 \cdot 10^{-07}$		
PNRC1_306_327	0.93	$1.30 \cdot 10^{-07}$		
PPRC1_151_173	0.99	$4.78 \cdot 10^{-08}$		
PR285_1105_1127	1.00	$3.51 \cdot 10^{-08}$		
PR285_2216_2238_C2219S	0.91	$2.00 \cdot 10^{-08}$		

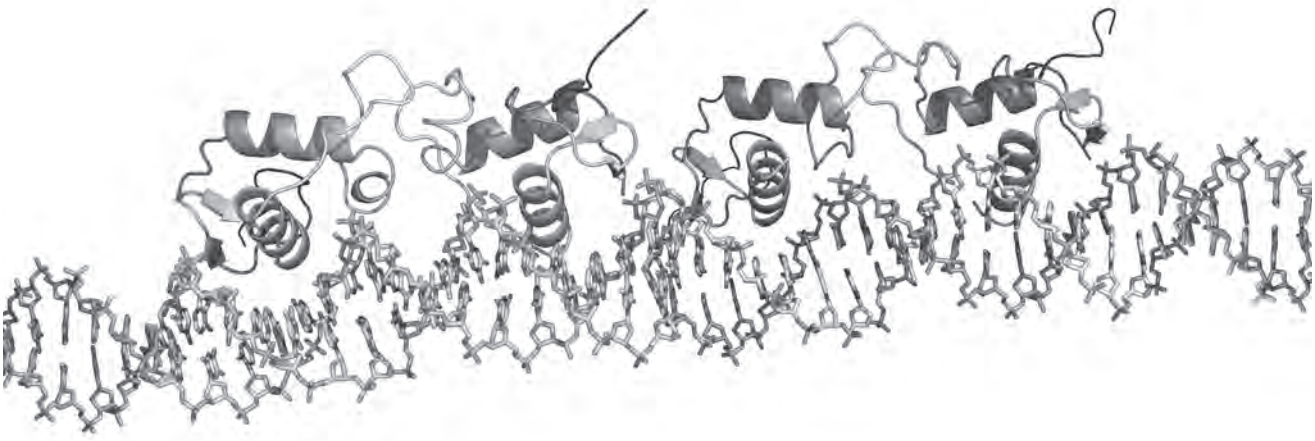
Supplementary Table S: EC_{50} and IC_{50} (underlined) values for ERα-LBD and ERβ-LBD binding to coregulator-derived binding motifs in the MARCoNI assay induced by E2, only displayed for dose response curves with coefficient of determination R^2 >0.9. (*continued*)

Coregulator	R^2 ERα	EC_{50} ERα	R^2 ERβ	EC_{50} ERβ
PRGC1_130_155	0.98	$1.74 \cdot 10^{-09}$	0.96	$2.77 \cdot 10^{-08}$
PRGC1_134_154	0.96	$1.04 \cdot 10^{-09}$		
PRGC2_146_166	1.00	$9.13 \cdot 10^{-09}$	0.96	$4.34 \cdot 10^{-08}$
PRGC2_338_358	0.93	$1.96 \cdot 10^{-08}$	0.95	$3.43 \cdot 10^{-07}$
PROX1_57_79	0.95	$1.21 \cdot 10^{-06}$		
TIF1A_373_395_C394S	0.97	$9.48 \cdot 10^{-09}$		
TIF1A_747_769	1.00	$4.51 \cdot 10^{-09}$	0.92	$1.70 \cdot 10^{-08}$
TIP60_476_498	0.99	$4.31 \cdot 10^{-08}$	0.94	$6.54 \cdot 10^{-08}$
TREF1_168_190	1.00	$1.09 \cdot 10^{-08}$		
TRXR1_132_154	0.96	$7.84 \cdot 10^{-08}$	0.92	$3.41 \cdot 10^{-08}$
UBE3A_649_671	0.93	$4.76 \cdot 10^{-08}$		
WIPI1_119_141	1.00	$1.23 \cdot 10^{-08}$	0.96	$3.03 \cdot 10^{-08}$
WIPI1_313_335_C318S	0.94	$2.75 \cdot 10^{-08}$		
ZNHI3_89_111	1.00	$1.19 \cdot 10^{-08}$		



5

Quantitative proteomics addressing the estrogen receptor subtype-mediated effects in T47D breast cancer cells exposed to 4-hydroxytamoxifen



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Submitted for publication

Abstract

The aim of the present study was to investigate by quantitative proteomics whether 4OHT, active metabolite of the breast cancer drug tamoxifen, exerts ERα/ERβ ratio-dependent effects on cell proliferation and apoptosis. This is of importance given that the ERα/ERβ ratio usually increases in tumorous tissue compared to normal tissue due to decreased ERβ expression. ERα/ERβ ratio-dependent effects of 4OHT on protein expression were characterized in the T47D-ERβ human breast cancer cell line with tetracycline-dependent ERβ expression. Results reveal ERα/ERβ ratio-dependent effects of 4OHT on cell proliferation and apoptosis. Proteomics data are in line with decreased cell proliferation and increased apoptosis induced by 4OHT in the cells expressing only ERα. In cells expressing ERα and ERβ, proteomics data point at increased cell proliferation and decreased apoptosis upon 4OHT exposure. 4OHT also induced ERα/ERβ ratio-dependent post-translational modifications, i.e. acetylation, methylation, and phosphorylation of several ribosomal and mitochondrial protein groups. Most post-translational modifications were observed upon 4OHT exposure in T47D-ERβ cells with both ERα and ERβ expressed. It is concluded that 4OHT affects major biological functions in T47D-ERβ cells including cell proliferation and apoptosis, with ultimate effects being dependent on the cellular ERα/ERβ ratio. There may be opposite effects regarding cell proliferation and apoptosis of 4OHT in tissue depending on the level of ERβ expression, with 4OHT being more effective in reducing cell proliferation and increasing apoptosis if ERα dominates and ERβ expression levels are low since 4OHT then antagonizes ERα. Based on the proteomics data of the present study it is concluded that ERα and ERβ levels and the ERα/ERβ ratio could be used as predictors of tamoxifen therapy responsiveness, with presence of ERα and absence of ERβ expression being optimal for inducing a 4OHT-mediated decrease in cell proliferation and increase in apoptosis.

Introduction

The two major estrogen receptors (ER), ERα and ERβ, influence cell proliferation and are suggested to be involved in the development and progression of certain forms of cancer of especially estrogen-responsive tissues, like breast and uterus [1-3]. Whereas ERα activation enhances cell proliferation [4], ERβ activation counteracts this effect [2, 5-8]. Several studies associate ERβ activation with apoptosis [9-11]. It has been shown for ER-positive breast tumors (tumors that express ERs) as well as for other estrogen-dependent tissue tumors at both messenger RNA (mRNA) and protein level that in tumorous tissue compared to normal tissue ERα/ERβ ratios increase due to a decreased ERβ expression [2].

Tamoxifen is used as a breast cancer drug in ER-positive breast cancer [12, 13]. 4-Hydroxytamoxifen (4OHT) is the active metabolite of tamoxifen displaying a 100 times higher binding affinity than tamoxifen for both ERα and ERβ [14]. In this study, 4OHT is tested in its trans isoform, which is a stronger anti-estrogenic molecule than the cis isoform [15]. 4OHT is considered a partial ERα agonist and an antagonist for both ERα and ERβ [16]. The partial ERα agonist classification is due to the anti-estrogenic properties attributed to 4OHT in breast tissue, but pro-estrogenic behavior of 4OHT in the endometrium [17]. Given the counteracting influence of ERβ on ERα-mediated effects [7], it is of importance to take the ER specificity of 4OHT into account. Relative binding assays with recombinant ERα and ERβ in the presence of 17β-estradiol (E2) to show antagonism activity confirm this preference of 4OHT for ERβ, resulting in a 2.3 times higher binding affinity for ERβ over ERα [18], while competition binding assays with E2 show a 1.6 times higher binding affinity for ERβ over ERα [19].

Furthermore, when studying the effects of 4OHT it is of importance to take into account the ERα/ERβ ratio of the cells or tissues under investigation. ERα and ERβ levels and the resulting ERα/ERβ ratios are known to be different in different estrogen-responsive tissues [20-22]. For example human endometrium expresses mainly ERα, while in human prostate tissue ERβ is more prominently expressed. Human breast tissue normally expresses both receptors [20, 22].

Another mechanism which might be involved in the ERα/ERβ ratio-dependent effects of 4OHT could be the induction of post-translational modifications like methylation and acetylation, which can repress or activate gene transcription [23-25], and ubiquitination, which may affect protein degradation [26].

The aim of the present study was to investigate by quantitiative proteomics the 4OHT-induced ERα/ERβ ratio-dependent proteins that affect cell proliferation and apoptosis. To this end, the effect of 4OHT on cellular protein levels was characterized in

the T47D-ERβ human breast cancer cell line with tetracycline-dependent ERβ expression by a mass spectrometry (MS)-based proteomics approach.

Materials and methods

Cell culture

The T47D-ERβ cell line, consisting of T47D human ductal breast epithelial cancer cells with tetracycline-dependent ERβ expression, was made and provided by Ström *et al.* [6]. T47D-ERβ cells were cultured in a 1:1 mixture of Ham's nutrient mixture F12 and Dulbecco's modified Eagle's medium (DMEM) (Gibco, Paisley, Scotland, #31331-038) supplemented with 5% fetal calf serum (FCS) (Invitrogen, Paisley, Scotland, #10099). To fully inhibit ERβ expression, 1000 ng/ml tetracycline (Sigma, Zwijndrecht, the Netherlands, #T7660) was added to the medium [7]. To vary the cellular ERα/ERβ ratio, 24 hours before exposure to 4OHT, the cells were exposed to specific tetracycline concentrations (either maintained at 1000 ng/ml (for full suppression of ERβ expression, only ERα expression) or brought to 0 ng/ml (ERα and ERβ expression)) [20]. The cells were incubated at 37°C and 5% CO_2 in a humidified atmosphere.

Because phenol red exerts estrogenic activity [27], at least 24 hours before exposure, cells were washed three times with phosphate buffered saline (PBS) (Gibco, Paisley, Scotland, #10010-015) and transferred to phenol red free medium (Gibco, Paisley, Scotland, #21041-025) supplemented with 0.5% hormone-free dextran-coated charcoal-treated fetal calf serum (DCC-FCS) (Perbio Science, Waltham, MA, USA, #SH30068.05).

BrdU proliferation assay

T47D-ERβ cells were seeded in 96-well view plates at a density of $1.8*10^5$ cells/ml, 100 µl/well in phenol red free medium. Twenty-four hours after seeding, cells were washed with PBS and exposed to 0 or 1000 ng/ml tetracycline (as indicated, in phenol red free medium). Forty-eight hours after seeding, keeping the tetracycline concentrations the same, cells were exposed to a concentration range of 4OHT, as well as to 30 pM E2 to induce cell proliferation. 30 pM E2 was determined to be the EC_{50} concentration for E2 induction of cell proliferation in T47D-ERβ cells expressing only ERα [20]. After 48 hours of co-exposure to E2 and 4OHT, cell proliferation was determined by measuring 5-bromo-2'-deoxyuridine (BrdU) incorporated into the DNA following Roche's colorimetric BrdU protocol [7, 28]. BrdU, a pyrimidine analogue, was added to the cells during the last 4 hours of exposure to the test compounds and was incorporated into the DNA of proliferating cells. This was detected by an antibody raised against BrdU, conjugated

with a peroxidase, which was in turn detected by conversion of tetramethyl-benzidine. The subsequently produced blue color was quantified by measuring absorbance at 370 and 492 nm (background) using a spectrophotometer (Spectramax microplate reader M2, MSD analytical technologies) [28].

Protein sample preparation for proteomics analysis

T47D-ERβ cells were exposed to 0 or 300 nM 4OHT for 24 hours. Cells were washed twice in PBS and lysed in 1 ml of modified radio immunoprecipitation assay (RIPA) buffer consisting of 50 mM Tris-HCl; pH 7.4 (Tris (tris(hydroxymethyl)aminomethane): Roche, Mannheim, Germany, #732010) (HCl (hydrochloric acid): Merck, Darmstadt, Germany, #1.00317.1000), 1% Triton X-100 (BioRad, Veenendaal, the Netherlands, #161-0407), 0.25% sodium deoxycholate (Merck, Darmstadt, Germany, #6504), 150 mM sodium chloride (NaCl, Merck, Darmstadt, Germany, #1.06404.1000), 1 mM ethylenediaminetetraacetic acid (EDTA, Merck, Darmstadt, Germany, #1.08418.1000), and protease inhibitor cocktail (Complete, Mini) (Roche, Basel, Switzerland, #11836153001) at 4°C for 20 minutes. Samples were sonicated for 30 minutes and centrifuged at 8000 rounds per minute (RPM), at 4°C for 15 minutes. The protein concentration of the supernatant was determined using a bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Thermo Scientific, Waltham, MA, USA, #23225).

Then 20 µg of the protein sample was mixed with 5x concentrated Laemmli sample buffer (250 mM Tris-HCl; pH 6.8, 10% sodium dodecyl sulfate (SDS, Sigma, Zwijndrecht, the Netherlands, #L4390), 50% glycerol (Acros Organics, Pittsburg, PA, USA, #327255000), 0.1% bromophenol blue (Sigma, Zwijndrecht, the Netherlands, #B0126)). Subsequently, 48 µl sample was loaded on a 12% Mini-PROTEAN_ TGX™ (Tris-glycine extended) precast gel (BioRad, Veenendaal, the Netherlands, #456-1044). Electrophoresis was carried out at 50 V for 30 minutes followed by 100 V for 30 minutes using 0.2 M Tris-HCl, pH 8.9 as anode buffer and 0.1 M Tris, 0.1 M Tricine, 0.1% SDS, pH 8.3 as cathode buffer. The gel was stained using colloidal Coomassie blue (NOVEX® Colloidal blue staining kit, Invitrogen, Paisley, Scotland, #LC6025). Excess colloidal Coomassie blue was washed off with water (performed twice). Each lane was cut into 5 equal sections. Then 100 µl 50 mM dithiotreitol (DTT, Sigma, Zwijndrecht, the Netherlands, #D9163) in 50 mM ammonium bicarbonate (NH_4HCO_3, Fluka, Sigma Aldrich, Zwijndrecht, the Netherlands, #09830) was added to each sample for cysteine reduction. The samples were sonicated for 1 minute and then incubated for 1 hour at 60°C. DTT was removed and 100 µl 50 mM iodoacetamide (Sigma, Zwijndrecht, the Netherlands, #I1149) in 50 mM NH_4HCO_3 was added for alkylation. The samples were sonicated for 1 minute and then incubated for 1 hour at room temperature in the dark. The gel pieces were washed 3 times with

100 µl 50 mM NH_4HCO_3 at pH 8. Subsequently, 100 µl 10 ng/µl trypsin (sequencing grade, Roche, Basel, Switzerland, #11047841001) in 50 mM NH_4HCO_3 was added to the gel pieces to perform proteolytic digestion at room temperature overnight. Samples were centrifuged and 25 µl of the supernatant containing the digested peptides was recovered and transferred to a new eppendorf tube. The pH was brought to 2-4 with 10% trifluoracetic acid (TFA, Merck, Whitehouse Station, NJ, USA, #1.08262.0100). All samples were measured one time by nano liquid chromatography (LC)-LTQ (Linear Trap Quadropole)-OrbitrapXL-MS as described by Lu *et al*. [29].

Data analysis

For calibration of the results of the BrdU assays, on all plates, several concentrations of E2 were tested (each concentration at least in n=3) to correct for plate-to-plate variations. Data from the BrdU assay were expressed as absorbance measured at 370 nm, corrected for the corresponding background signal at 492 nm.

LCMS runs with all MSMS spectra obtained were analyzed with MaxQuant 1.3.0.5 [30] using default settings for the Andromeda search engine [31], except that extra variable modifications were set for de-amidation of asparagine (N) and glutamine (Q). In a second search, also phosphorylation of serine (S),threonine (T), and/or tyrosine (Y), methylation of lysine (K) or arginine (R), and ubiquitination (GlyGly) of K were included as variable modifications up to a maximum of 3 modifications per peptide.

A homo sapiens database downloaded from Uniprot (http://www.uniprot.org, 117448 entries, at November 30th 2011) was used together with a contaminants database that contains sequences of common contaminants as for instance: BSA (P02769, bovin serum albumin precursor), Trypsin (P00760, bovin), Trypsin (P00761, porcin), Keratin K22E (P35908, human), Keratin K1C9 (P35527, human), Keratin K2C1 (P04264, human), and Keratin K1CI (P35527, human). The “label-free quantification” as well as the “match between runs” (set to 2 minutes) options were enabled.

The MS proteomics data have been deposited to the ProteomeXchange Consortium (http://proteomecentral.proteomexchange.org) via the PRIDE partner repository [32] with the dataset identifier PXD000615 (PRIDE: PXD000615).

Filtering and further bioinformatic analysis of the MaxQuant/Andromeda workflow output and the analysis of the abundances of the identified protein groups were performed with the Perseus 1.3.0.4 module (available at the MaxQuant suite). Accepted were peptides and proteins with a false discovery rate (FDR) of less than 0.1 and proteins with at least 2 identified and quantified peptides of which at least 1 should be unique and at least 1 should be unmodified. Reversed hits were deleted from the MaxQuant result table as well as all results showing a label free quantitation protein

MS1 intensitiy (LFQ) value of 0 for both sample and control. The normal logarithm was taken from normalised LFQ as obtained from MaxQuant. Zero Log LFQ values (NaN) were replaced by 4.5. All samples were measured one time by nanoLC-LTQ-OrbitrapXL-MS as described by Lu et.al. [29]. The 3 biological replicates for each test condition were grouped. Only proteins with 3 valid values in at least 1 group of triplicate samples were included to make sure the protein was clearly measured in all 3 samples. A two sample t-test was performed using the "LFQ intensity" columns obtained with FDR threshold 0.1 and S0=1. 4OHT treated samples were tested against untreated samples with the same tetracycline concentrations.

Results

The effect of 4OHT on T47D-ERβ cell proliferation

The T47D-ERβ cell line, consisting of T47D human ductal breast epithelial cancer cells with tetracycline-dependent ERβ expression and a constant ERα expression, was used as the model to investigate ER-mediated cellular effects of 4OHT on E2-induced cell proliferation. When only ERα is expressed in the T47D-ERβ cells, E2-induced cell proliferation is decreased by an increasing concentration of 4OHT (Figure 1). The maximum decrease of cell proliferation is observed at 300 nM 4OHT. To be able to analyse differences in protein expression at an effective, non-cytotoxic 4OHT concentration, a

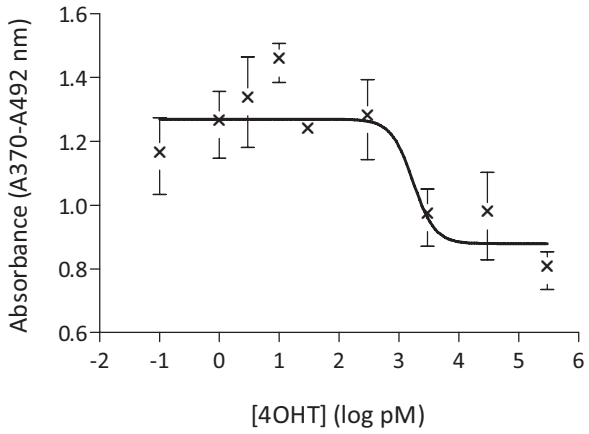


Figure 1: Effect of 4OHT on the E2-induced proliferation of T47D-ERβ cells exposed to 1000 ng/ml tetracycline (only ERα expression). Data points ± standard deviation (SD) (n=6).

concentration of 300 nM 4OHT was then chosen as the exposure concentration for the T47D-ERβ cells in the proteomics experiment.

Protein groups and networks influenced by 4OHT exposure in T47D-ERβ cells with varying ERα/ERβ ratios

In the total data set obtained from the 4OHT-exposed and unexposed T47D-ERβ cells with varying ERα/ERβ ratios 2856 proteins were detected, of which 1739 proteins remained after very strict filtering (protein groups identified by at least 2 peptides of which at least 1 peptide had to be unique in the database and at least 1 peptide had to be unmodified, and only proteins with 3 valid values in at least 1 group of triplicate samples were included). At an FDR of 0.1 protein samples of 4OHT-exposed T47D-ERβ cells expressing ERα and ERβ show 45 significantly different expressed protein groups due to the 4OHT exposure when compared to unexposed T47D-ERβ cells expressing ERα and ERβ (Figure 2A). In Figure 3A the top ten affected molecular and functional classes are shown, including protein synthesis, gene expression, RNA post-transcriptional processing, and post-translational modifications. Table A shows the significantly up- and down-regulated protein groups of 4OHT-exposed T47D-ERβ cells expressing ERα and ERβ when compared to unexposed T47D-ERβ cells expressing ERα and ERβ and their biological functions. Table B reveals 18 networks that were affected in 4OHT-exposed T47D-ERβ cells expressing ERα and ERβ compared to unexposed T47D-ERβ cells expressing ERα and ERβ, including top networks involved in RNA post-transcriptional modification, protein synthesis, gene expression, cellular assembly and organisation, free radical scavenging, cell cycle, and DNA replication, recombination, and repair.

For bioinformatics network analysis, Ingenuity Pathway Analysis (Ingenuity Systems Inc.) was used. For significantly modified protein groups, protein ID's were used to manually search the UniProt database for biological pathways and functions on www.uniprot.org.

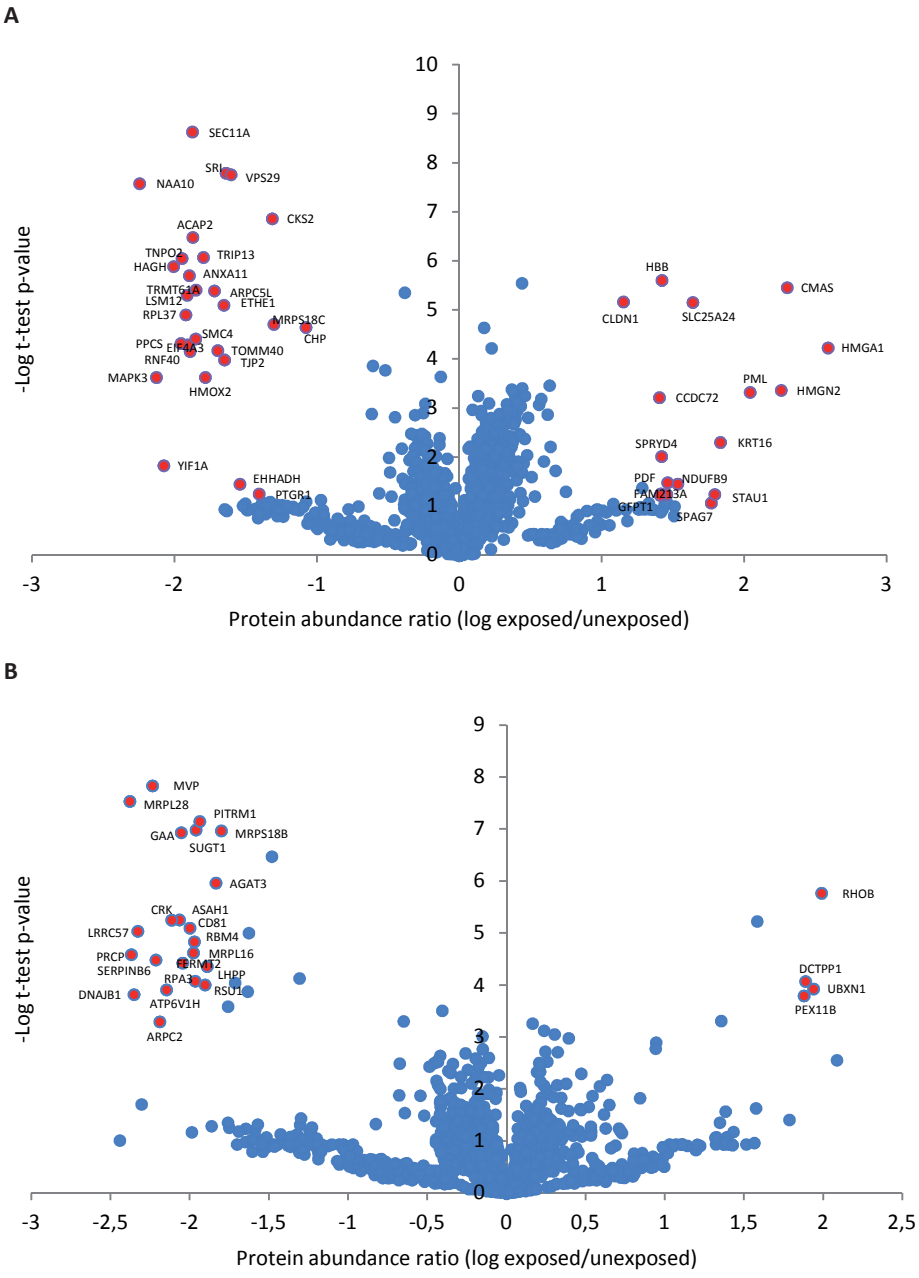


Figure 2: Vulcano plot of protein groups influenced by 4OHT exposure in T47D-ERβ cells when **(A)** ERα and ERβ are both expressed and **(B)** only ERα is expressed. Blue dots: not significantly changed proteins after 4OHT treatment. Red dots: significantly changed proteins after 4OHT treatment at FDR ≤ 0.1 (indicated with gene names of Table A + C).

Table A: Protein groups most influenced (significantly different at FDR ≤0.1) by 4OHT exposure in T47D-ERβ cells expressing ERα and ERβ. Affected biological pathway/function derived from www.uniprot.org.

Protein group names	Gene names	Protein IDs	Up-/down-regulated after 4OHT treatment	Biological pathway/function
Claudin-1	CLDN1	O95832	Up	Calcium-independent cell adhesion; cell-cell junction organization.
Double-stranded RNA-binding protein Staufen homolog 1	STAU1	O95793	Up	Intracellular mRNA localization; Binds double-stranded RNA (regardless of the sequence) and tubulin. May play a role in specific positioning of mRNAs at given sites in the cell by cross-linking cytoskeletal and RNA components, and in stimulating their translation at the site.
Glutamine synthetase	PIG59; GLUL	P15104	Up	It catalyzes the production of glutamine and 4-aminobutanoate (gamma-aminobutyric acid, GABA), the latter in a pyridoxal phosphate-independent manner. Essential for proliferation of fetal skin fibroblasts.
Hemoglobin subunit beta; LVV-hemorphin-7	HBB	P68871	Up	Involved in oxygen transport from the lung to the various peripheral tissues. LVV-hemorphin-7 potentiates the activity of bradykinin, causing a decrease in blood pressure.
Calcium-binding mitochondrial carrier protein SCaMC-1	SLC25A24	Q6NUK1	Up	Transmembrane transport; Calcium-dependent mitochondrial solute carrier. Mitochondrial solute carriers shuttle metabolites, nucleotides, and cofactors through the mitochondrial inner membrane. May act as a ATP (adenosine triphosphate)-Mg/Pi exchanger that mediates the transport of Mg-ATP in exchange for phosphate, catalyzing the net uptake or efflux of adenine nucleotides into or from the mitochondria.
Keratin, type I cytoskeletal 16	KRT16	P08779	Up	Heterodimer of a type I and a type II keratin. KRT16 associates with KRT6 isomers. Interacts with TCHP. Interacts with TRADD. Expressed in the hair follicle, nail bed and in mucosal stratified squamous epithelia and, suprabasally, in oral epithelium and palmoplantar epidermis. Also found in luminal cells of sweat and mammary gland ducts.

Protein PML	PML; PML-RAR; promyelocytic leukemia protein	P29590	Up	Key component of PML nuclear bodies that regulate a large number of cellular processes by facilitating post-translational modification of target proteins, promoting protein-protein contacts, or by sequestering proteins. Functions as tumor suppressor. Required for normal, caspase-dependent apoptosis in response to DNA damage, FAS, TNF, or interferons. Plays a role in transcription regulation, DNA damage response, DNA repair and chromatin organization. Plays a role in processes regulated by retinoic acid, regulation of cell division, terminal differentiation of myeloid precursor cells and differentiation of neural progenitor cells. Required for normal immunity to microbial infections. Plays a role in antiviral response. In the cytoplasm, plays a role in TGFB1-dependent processes. Regulates p53/TP53 levels by inhibiting its ubiquitination and proteasomal degradation. Regulates activation of p53/TP53 via phosphorylation at 'Ser-20'. Sequesters MDM2 in the nucleolus after DNA damage, and thereby inhibits ubiquitination and degradation of p53/TP53. Regulates translation of HIF1A by sequestering MTOR, and thereby plays a role in neoangiogenesis and tumor vascularization. Regulates RB1 phosphorylation and activity. Required for normal development of the brain cortex during embryogenesis. Can sequester herpes virus and varicella virus proteins inside PML bodies, and thereby inhibit the formation of infectious viral particles. Regulates phosphorylation of ITPR3 and plays a role in the regulation of calcium homeostasis at the endoplasmic reticulum. Regulates transcription activity of ELF4. Inhibits specifically the activity of the tetrameric form of PKM. Together with SATB1, involved in local chromatin-loop remodeling and gene expression regulation at the MHC-I locus. Regulates PTEN compartmentalization through the inhibition of USP7-mediated deubiquitination.

Table A: Protein groups most influenced (significantly different at FDR ≤0.1) by 4OHT exposure in T47D-ERβ cells expressing ERα and ERβ. Affected biological pathway/function derived from www.uniprot.org. (*continued*)

Protein group names	Gene names	Protein IDs	Up-/down-regulated after 4OHT treatment	Biological pathway/function
NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 9	NDUFB9; DKFZp566O173	Q9Y6M9	Up	Accessory subunit of the mitochondrial membrane respiratory chain NADH dehydrogenase (Complex I), that is believed to be not involved in catalysis. Complex I functions in the transfer of electrons from NADH to the respiratory chain. The immediate electron acceptor for the enzyme is believed to be ubiquinone.
Non-histone chromosomal protein HMG-17	HMGN2	P05204	Up	Binds to the inner side of the nucleosomal DNA thus altering the interaction between the DNA and the histone octamer. May be involved in the process which maintains transcribable genes in an unique chromatin conformation. Binds to nucleosomes, regulating chromatin structure and consequently, chromatin-dependent processes such as transcription, DNA replication and DNA repair. Affects both insulin and glucagon levels and modulates the expression of pancreatic genes involved in insulin secretion. Regulates the expression of the glucose transporter SLC2A2 by binding specifically to its promoter region and recruiting PDX1 and additional transcription factors. Regulates the expression of SLC6A9, a glycine transporter which regulates the glycine concentration in synaptic junctions in the central nervous system, by binding to its transcription start site. May play a role in ocular development and astrocyte function.
High mobility group protein HMG-I/HMG-Y	HMGA1	P17096	Up	HMG-I/Y bind preferentially to the minor groove of A+T rich regions in double stranded DNA. It is suggested that these proteins could function in nucleosome phasing and in the 3'-end processing of mRNA transcripts. They are also involved in the transcription regulation of genes containing, or in close proximity to A+T-rich regions.

N-acylneuraminate cytidylyltransferase	CMAS	Q8NFW8	Up	Catalyzes the activation of N-acetylneuraminic acid (NeuNAc) to cytidine 5'-monophosphate N-acetylneuraminic acid (CMP-NeuNAc), a substrate required for the addition of sialic acid. Has some activity toward NeuNAc, N-glycolylneuraminic acid (Neu5Gc) or 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid (KDN).
SPRY domain-containing protein 4	SPRYD4	Q8WW59	Up	-
Peptide deformylase, mitochondrial	PDF	Q9HBH1	Up	Removes the formyl group from the N-terminal Met of newly synthesized proteins.
Coiled-coil domain-containing protein 72	CCDC72	Q9Y2S6	Up	-
Peroxisomal bifunctional enzyme; Enoyl-CoA hydratase/3,2-trans-enoyl-CoA isomerase; 3-hydroxyacyl-CoA dehydrogenase	EHHADH	Q08426	Down	Lipid metabolism; fatty acid beta-oxidation
Protein YIF1A	YIF1A	O95070	Down	Possible role in transport between endoplasmic reticulum and Golgi.
E3 ubiquitin-protein ligase BRE1B	RNF40	O75150	Down	Protein modification; Component of the RNF20/40 E3 ubiquitin-protein ligase complex that mediates monoubiquitination of 'Lys-120' of histone H2B (H2BK120UB1). H2BK120UB1 gives a specific tag for epigenetic transcriptional activation and is also prerequisite for histone H3 'Lys-4' and 'Lys-79' methylation (H3K4me and H3K79me, respectively). It thereby plays a central role in histone code and gene regulation. The RNF20/40 complex forms a H2B ubiquitin ligase complex in cooperation with the E2 enzyme UBE2A or UBE2B; reports about the cooperation with UBE2E1/UBCH are contradictory. Required for transcriptional activation of Hox genes; ligase.

Table A: Protein groups most influenced (significantly different at FDR ≤0.1) by 4OHT exposure in T47D-ERβ cells expressing ERα and ERβ. Affected biological pathway/function derived from www.uniprot.org. (*continued*)

Protein group names	Gene names	Protein IDs	Up-/down-regulated after 4OHT treatment	Biological pathway/function
Sorcin	SRI	P30626	Down	Calcium ion binding; Calcium-binding protein that modulates excitation-contraction coupling in the heart. Contributes to calcium homeostasis in the heart sarcoplasmic reticulum. Modulates the activity of RYR2 calcium channels.
Actin-related protein 2/3 complex subunit 5;Actin-related protein 2/3 complex subunit 5-like protein	ARPC5L	Q9BPX5	Down	Functions as component of the Arp2/3 complex which is involved in regulation of actin polymerization and together with an activating nucleation-promoting factor (NPF) mediates the formation of branched actin networks.
Heme oxygenase 2	HMOX2	P30519	Down	Heme oxygenase cleaves the heme ring at the alpha methene bridge to form biliverdin. Biliverdin is subsequently converted to bilirubin by biliverdin reductase. Under physiological conditions, the activity of heme oxygenase is highest in the spleen, where senescent erythrocytes are sequestrated and destroyed. Heme oxygenase 2 could be implicated in the production of carbon monoxide in brain where it could act as a neurotransmitter.
Structural maintenance of chromosomes protein 4;Structural maintenance of chromosomes protein	SMC4	Q9NTJ3	Down	Central component of the condensin complex, a complex required for conversion of interphase chromatin into mitotic-like condense chromosomes. The condensin complex probably introduces positive supercoils into relaxed DNA in the presence of type I topoisomerases and converts nicked DNA into positive knotted forms in the presence of type II topoisomerases.
Annexin;Annexin A11	ANXA11	P50995	Down	Binds specifically to calcyclin in a calcium-dependent manner. Required for midbody formation and completion of the terminal phase of cytokinesis.

Transportin-2	TNPO2 variant protein; TNPO2	Q6IN77	Down	Intracellular protein transport; probably functions in nuclear protein import as nuclear transport receptor. Serves as receptor for nuclear localization signals (NLS) in cargo substrates. Is thought to mediate docking of the importin/substrate complex to the nuclear pore complex (NPC) through binding to nucleoporin and the complex is subsequently translocated through the pore by an energy requiring, RAN-dependent mechanism. At the nucleoplasmic side of the NPC, RAN binds to the importin, the importin/substrate complex dissociates and importin is re-exported from the nucleus to the cytoplasm where GTP hydrolysis releases RAN. The directionality of nuclear import is thought to be conferred by an asymmetric distribution of the GTP- and GDP-bound forms of RAN between the cytoplasm and nucleus.
Calcium-binding protein p22	CHP	Q99653	Down	Calcium-binding protein involved in different processes such as regulation of vesicular trafficking, plasma membrane Na^+/H^+ exchanger and gene transcription. Involved in the constitutive exocytic membrane traffic. Mediates the association between microtubules and membrane-bound organelles of the endoplasmic reticulum and Golgi apparatus and is also required for the targeting and fusion of transcytotic vesicles (TCV) with the plasma membrane. Functions as an integral cofactor in cell pH regulation by controlling plasma membrane-type Na^+/H^+ exchange activity. Affects the pH sensitivity of SLC9A1/NHE1 by increasing its sensitivity at acidic pH. Required for the stabilization and localization of SLC9A1/NHE1 at the plasma membrane. Inhibits serum- and GTPase-stimulated Na^+/H^+ exchange. Plays a role as an inhibitor of ribosomal RNA transcription by repressing the nucleolar UBF1 transcriptional activity. May sequester UBF1 in the nucleoplasm and limit its translocation to the nucleolus. Associates to the ribosomal gene promoter. Acts as a negative regulator of the calcineurin/NFAT signaling pathway. Inhibits NFAT nuclear translocation and transcriptional activity by suppressing the calcium-dependent calcineurin phosphatase activity.

Table A: Protein groups most influenced (significantly different at FDR ≤0.1) by 4OHT exposure in T47D-ERβ cells expressing ERα and ERβ. Affected biological pathway/function derived from www.uniprot.org. (*continued*)

Protein group names	Gene names	Protein IDs	Up-/down-regulated after 4OHT treatment	Biological pathway/function
				Also negatively regulates the kinase activity of the apoptosis-induced kinase STK17B. Inhibits both STK17B auto- and substrate-phosphorylations in a calcium-dependent manner.
Hydroxyacylglutathione hydrolase, mitochondrial	HAGH	Q16775	Down	Thiolesterase that catalyzes the hydrolysis of S-D-lactoyl-glutathione to form glutathione and D-lactic acid.
Protein LSM12 homolog	LSM12	Q3MHD2	Down	-
Tight junction protein ZO-2	TJP2	B7Z2R8	Down	Plays a role in tight junctions and adherens junctions.
60S ribosomal protein L37; Ribosomal protein L37	RPL37	P61927	Down	Binds to the 23S rRNA. Ribosomal protein.
Protein ETHE1, mitochondrial	ETHE1	O95571	Down	Probably plays an important role in metabolic homeostasis in mitochondria. May function as a nuclear-cytoplasmic shuttling protein that binds transcription factor RELA/NFkB3 in the nucleus and exports it to the cytoplasm. Suppresses p53-induced apoptosis by preventing nuclear localization of RELA.
Mitochondrial import receptor subunit TOM40 homolog	TOMM40	O96008	Down	Channel-forming protein essential for import of protein precursors into mitochondria.

Mitogen-activated protein kinase 3	MAPK3; DKFZp686O0215	P27361	Down	Serine/threonine kinase which acts as an essential component of the MAP kinase signal transduction pathway. MAPK1/ERK2 and MAPK3/ERK1 are the 2 MAPKs which play an important role in the MAPK/ERK cascade. They participate also in a signaling cascade initiated by activated KIT and KITLG/SCF. Depending on the cellular context, the MAPK/ERK cascade mediates diverse biological functions such as cell growth, adhesion, survival and differentiation through the regulation of transcription, translation, cytoskeletal rearrangements. The MAPK/ERK cascade plays also a role in initiation and regulation of meiosis, mitosis, and postmitotic functions in differentiated cells by phosphorylating a number of transcription factors. About 160 substrates have already been discovered for ERKs. Many of these substrates are localized in the nucleus, and seem to participate in the regulation of transcription upon stimulation. However, other substrates are found in the cytosol as well as in other cellular organelles, and those are responsible for processes such as translation, mitosis and apoptosis. Moreover, the MAPK/ERK cascade is also involved in the regulation of the endosomal dynamics, including lysosome processing and endosome cycling through the perinuclear recycling compartment (PNRC); as well as in the fragmentation of the Golgi apparatus during mitosis. The substrates include transcription factors (such as ATF2, BCL6, ELK1, ERF, FOS, HSF4 or SPZ1), cytoskeletal elements (such as CANX, CTTN, GJA1, MAP2, MAPT, PXN, SORBS3 or STMN1), regulators of apoptosis (such as BAD, BTG2, CASP9, DAPK1, IER3, MCL1 or PPARG), regulators of translation (such as EIF4EBP1) and a variety of other signaling-related molecules (like ARHGEF2, FRS2 or GRB10). Protein kinases (such as RAF1, RPS6KA1/RSK1, RPS6KA3/RSK2, RPS6KA2/RSK3, RPS6KA6/RSK4, SYK, MKNK1/MNK1, MKNK2/MNK2, RPS6KA5/MSK1, RPS6KA4/MSK2, MAPKAPK3 or MAPKAPK5) and phosphatases (such as DUSP1, DUSP4, DUSP6 or DUSP16) are other substrates which enable the propagation the MAPK/ERK signal to additional cytosolic and nuclear targets, thereby extending the specificity of the cascade.

Table A: Protein groups most influenced (significantly different at FDR ≤0.1) by 4OHT exposure in T47D-ERβ cells expressing ERα and ERβ. Affected biological pathway/function derived from www.uniprot.org. (*continued*)

Protein group names	Gene names	Protein IDs	Up-/down-regulated after 4OHT treatment	Biological pathway/function
Cyclin-dependent kinases regulatory subunit 2	CKS2	P33552	Down	Cell cycle; binds to the catalytic subunit of the cyclin-dependent kinases and is essential for their biological function.
Eukaryotic initiation factor 4A-III	EIF4A3	P38919	Down	ATP-dependent RNA helicase. Component of a splicing-dependent multiprotein exon junction complex (EJC) deposited at splice junction on mRNAs. The EJC is a dynamic structure consisting of a few core proteins and several more peripheral nuclear and cytoplasmic associated factors that join the complex only transiently either during EJC assembly or during subsequent mRNA metabolism. Core components of the EJC, that remains bound to spliced mRNAs throughout all stages of mRNA metabolism, functions to mark the position of the exon-exon junction in the mature mRNA and thereby influences downstream processes of gene expression including mRNA splicing, nuclear mRNA export, subcellular mRNA localization, translation efficiency and nonsense-mediated mRNA decay (NMD). Constitutes at least part of the platform anchoring other EJC proteins to spliced mRNAs. Its RNA-dependent ATPase and RNA-helicase activities are induced by CASC3, but abolished in presence of the MAGOH/RBM8A heterodimer, thereby trapping the ATP-bound EJC core onto spliced mRNA in a stable conformation. The inhibition of ATPase activity by the MAGOH/RBM8A heterodimer increases the RNA-binding affinity of the EJC. Involved in translational enhancement of spliced mRNAs after formation of the 80S ribosome complex. Binds spliced mRNA in sequence-independent manner, 20-24 nucleotides upstream of mRNA exon-exon junctions. Shows higher affinity for single-stranded RNA in an ATP-bound core EJC complex than after the ATP is hydrolyzed.

N-alpha-acetyltransferase 10	NAA10	P41227	Down	In complex with NAA15, displays alpha (N-terminal) acetyltransferase activity. Without NAA15, displays epsilon (internal) acetyltransferase activity towards HIF1A, thereby promoting its degradation. Represses MYLK kinase activity by acetylation, and thus represses tumor cell migration.
Signal peptidase complex catalytic subunit SEC11A;Putative signal peptidase complex catalytic subunit SEC11B	SEC11A; SPC18; SEC11B; SEC11L1	P67812	Down	Component of the microsomal signal peptidase complex which removes signal peptides from nascent proteins as they are translocated into the lumen of the endoplasmic reticulum
Arf-GAP with coiled-coil, ANK repeat and PH domain-containing protein 2	ACAP2	Q15057	Down	GTPase-activating protein (GAP) for ADP ribosylation factor 6 (Arf6).
Pachytene checkpoint protein 2 homolog	TRIP13	Q15645	Down	Plays a key role in chromosome recombination and chromosome structure development during meiosis. Required at early steps in meiotic recombination that leads to non-crossovers pathways. Also needed for efficient completion of homologous synapsis by influencing crossover distribution along the chromosomes affecting both crossovers and non-crossovers pathways. Also required for development of higher-order chromosome structures and is needed for synaptonemal-complex formation. In males, required for efficient synapsis of the sex chromosomes and for sex body formation. Promotes early steps of the DNA double-strand breaks (DSBs) repair process upstream of the assembly of RAD51 complexes. Required for depletion of HORMAD1 and HORMAD2 from synapsed chromosomes.
Phosphopantothenate-cysteine ligase	PPCS	Q9HAB8	Down	Catalyzes the first step in the biosynthesis of coenzyme A from vitamin B5, where cysteine is conjugated to 4'-phosphopantothenate to form 4-phosphopantothenoylcysteine.

Table A: Protein groups most influenced (significantly different at FDR ≤0.1) by 4OHT exposure in T47D-ERβ cells expressing ERα and ERβ. Affected biological pathway/function derived from www.uniprot.org. (*continued*)

Protein group names	Gene names	Protein IDs	Up-/down-regulated after 4OHT treatment	Biological pathway/function
tRNA (adenine(58)-N(1))-methyltransferase catalytic subunit TRMT61A	TRMT61A	Q96FX7	Down	Catalytic subunit of tRNA (adenine-N(1)-)-methyltransferase, which catalyzes the formation of N(1)-methyladenine at position 58 (m1A58) in initiator methionyl-tRNA.
Vacuolar protein sorting-associated protein 29	VPS29; DKFZp667O202	Q9UBQ0	Down	Essential component of the retromer complex, a complex required to retrieve lysosomal enzyme receptors (IGF2R and M6PR) from endosomes to the trans-Golgi network. Also required to regulate transcytosis of the polymeric immunoglobulin receptor (pIgR-pIgA). Has low protein phosphatase activity towards a serine-phosphorylated peptide derived from IGF2R (in vitro).
28S ribosomal protein S18c, mitochondrial	MRPS18C	Q9Y3D5	Down	Translation; structural constituent of ribosome
Redox-regulatory protein FAM213A	FAM213A	Q9BRX8	Up	Involved in redox regulation of the cell. Acts as an antioxidant. Inhibits TNFSF11-induced NFkB1 and JUN activation and osteoclast differentiation. May affect bone resorption and help to maintain bone mass.
Sperm-associated antigen 7	SPAG7	O75391	Up	Nucleic acid binding.
Glucosamine--fructose-6-phosphate aminotransferase [isomerizing] 1	GFPT1	Q06210	Up	Controls the flux of glucose into the hexosamine pathway. Most likely involved in regulating the availability of precursors for N- and O-linked glycosylation of proteins.

Prostaglandin reductase 1	PTGR1	Q14914	Down	Functions as 15-oxo-prostaglandin 13-reductase and acts on 15-oxo-PGE1, 15-oxo-PGE2 and 15-oxo-PGE2-alpha. Has no activity towards PGE1, PGE2 and PGE2-alpha. Catalyzes the conversion of leukotriene B4 into its biologically less active metabolite, 12-oxo-leukotriene B4. This is an initial and key step of metabolic inactivation of leukotriene B4.

Table B: Networks that were identified in T47D-ERβ cells expressing ERα and ERβ exposed to 4OHT. Bold protein names: up-regulated expression. Underlined protein names: down-regulated expression. Non-formatted protein names: proteins incorporated through relationships with other proteins.

ID	Proteins in network	Score	Focus proteins	Top functions
1	**ANP32A**, **BTF3L4**, **CCS**, **CPSF6**, **DDX5**, **DYNC1LI1**, **DYNLRB1**, EIF4A3, FARSB, **GTF2F1**, HNRNPA0, **HNRNPF**, Holo RNA polymerase II, **MATR3**, **PAICS**, **PARK7**, **PCBP1**, **PHGDH**, **PRKCSH**, RNA polymerase II, RPL8, RPL10, **RPL12**, **RPL17**, RPL19, RPL35, **RPLP1**, **SFPQ**, **SRSF1**, **SRSF3**, SUCLG1, **TAF15**, TNPO2, trypsin, Vegf	54	31	RNA Post-Transcriptional Modification, Protein Synthesis, Gene Expression
2	Akt, ANXA4, CAND1, **CCDC124**, Collagen type VI, **EIF2A**, GNB2L1, HDLBP, **MRPL43**, **MRPS7**, **MRPS22**, **MRPS23**, OTUB1, **PIN4**, Ribosomal 40s subunit, RNF40, Rnr, **RPS7**, **RPS9**, RPS15, RPS19, **RPS21**, RPS29, RPS4X, **RPSA**, **SRSF10**, **TOLLIP**, TRMT61A, UBE2, **UBE2L6**, **UBE2M**, **UBE2V1**, Ubiquitin, UFD1L, **UTRN**	50	29	Gene Expression, Protein Synthesis, RNA Post-Transcriptional Modification
3	adenylate kinase, **AK1**, Ant, **CAPNS1**, CLUH, **COX5A**, **COX6C**, **COX7A2L**, CYFIP1, Cytochrome bc1, cytochrome C, cytochrome-c oxidase, **DIABLO**, DNM1L, ECH1, **HMGB3**, Jnk, Mitochondrial complex 1, **MRPL11**, **NDUFB9**, **NDUFB10**, **NDUFV2**, **NHP2L1**, **PAFAH1B2**, **PDIA4**, PGAM1, PGAM5, **PGK1**, **RNH1**, SRI, **TFAM**, **UQCRB**, **UQCRC2**, **UQCRFS1**, **YWHAH**	48	28	Cell Morphology, Cellular Assembly and Organization, Neurological Disease
4	3-hydroxyacyl-CoA dehydrogenase, ACTN1, **ALYREF**, **CCDC6**, CD9, **CPNE1**, **CSRP1**, **CSTB**, **DUT**, Dynamin, EEA1, EHHADH, Eotaxin, **ERH**, ERK1/2, Erm, EZR, HSD17B4, **HSD17B10**, MVP, NAA10, NNT, **PLEKHF2**, RAB5, **RAB5B**, **RAB5C**, **RAB7A**, RDX, Rho gdi, **SARNP**, **SCP2**, **SH3GLB2**, Tap, **VAT1**, VPS29	45	27	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
5	ADH5, **Arf3**, Arf, BCAP31, Calcineurin protein(s), CHP1, COPA, COPB1, CYB5R3, **DAD1**, Fcer1, GBF1, **HINT1**, **HMGA1**, Ikb, Integrin alpha 3 beta 1, **KPNA4**, MAP2K1/2, NFAT (complex), NFkB (complex), **NOL3**, peroxidase (miscellaneous), PI3K (family), **PRDX1**, **PRDX2**, **PRDX3**, **PRDX4**, **PRDX6**, **RNF7**, **SRSF9**, **SRXN1**, **STIP1**, **TCP1**, TIP60, **TMED10**	38	24	Free Radical Scavenging, Small Molecule Biochemistry, Infectious Disease

6	20s proteasome, 26s Proteasome, **ACP1**, **ANXA7**, **BAG2**, **BAX**, **C11orf54**, calpain, **CCT7**, **CRYZ**, **DDAH2**, DFF, **ERP29**, **ETHE1**, Hsp27, Immunoproteasome Pa28/20s, MAPK, MHC CLASS I (family), myosin-light-chain kinase, **PML**, Proteasome Pa700/20s, **PSMA2**, **PSMA5**, **PSMB1**, **PSMB2**, **PSMB3**, PSMB, **PSMD6**, **PSME2**, PTP, **PYCRL**, **SELENBP1**, **SPTAN1**, **TAGLN2**, **TRIP13**	35	23	Infectious Disease, Renal and Urological Disease, Dermatological Diseases and Conditions
7	**ASS1**, caspase, **CBX5**, **CCDC53**, **CRIP2**, Cyclin A, DCTN1, E2f, **EEF1B2, EEF1D**, **ENSA**, **FEN1**, Hdac, Histone h3, Histone h4, **HMGB1**, HMOX2, Hsp70, IFN gamma, IL12 (complex), **LUC7L2**, MCM3, MCM4, NADPH oxidase, **NASP**, Nos, P38 MAPK, Pro-inflammatory Cytokine, RAD50, RB, **RBBP4**, **RPA3**, SMC4, VARS, ZW10	30	21	Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair
8	Actin, ACTN4, **ACTR1A**, Alpha Actinin, Alpha catenin, ANXA6, Arp2/3, ARPC5L, Beta Tubulin, Cadherin, CKS2, **CORO1B**, **DUSP3**, Ephb, ERK, F Actin, **HEXB**, HIP1R, Integrin alpha 5 beta 1, Laminin1, **MYL6**, MYO6, **OSTF1**, **P4HB**, **PDCD10**, Pdgf (family), **PEBP1**, **PFN1**, **PFN2**, Profilin, Rap1, Rock, Talin, TARS, VCL	27	19	Cell-To-Cell Signaling and Interaction, Cell Cycle, Cell Morphology
9	60S ribosomal subunit, ANXA11, ATP2B1, CBR1, CDH1, **CLDN1**, **CLDN3**, CLDN, Collagen type I, Collagen type III, Collagen type IV, Collagen(s), Growth hormone, **HMGN2**, HNRNPUL2, HYOU1, IARS, **KHSRP**, Ldh, LDL, **NPC2**, **PCMT1**, Pdgf (complex), Pdgf BB, Pkc(s), Pkg, PP2A, RPL13, RPL15, RPL29, RPL37, RPL37A, STAT5a/b, TJP2, Wnt	27	19	Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization, Cellular Function and Maintenance
10	AHSG, **ALDOC**, **ATP5D**, **ATP5L**, **ATP5O**, **ATP6V1E1**, **ATP6V1G1**, Cbp/p300, CD3, estrogen receptor, **FKBP3**, Focal adhesion kinase, H+-exporting ATPase, **HBB**, hemoglobin, **HIGD1A**, HISTONE, Hsp90, **HspA5**, **IDH2**, Insulin, NCAM2, p85 (pik3r), Pdgfr, PHB2, **RHOA**, RPL30, Secretase gamma, Shc, Sos, SRC (family), TGF beta, **TMPO**, **TPP1**, **TSTA3**	26	19	Molecular Transport, Nucleic Acid Metabolism, Small Molecule Biochemistry
11	**AK2**, **AP1S1**, AP1S2, ATP6V1F, BCL7A, CHMP2A, **CMPK1**, **DAD1**, **EIF3J**, **EIF4B**, **EIF4H**, GOLM1, GPRC5C, HARS2, HPD, **HRSP12**, **HspB1**, **ITP**, let-7a-5p (and other miRNAs w/seed GAGGUAG), **LRBA**, LSM12, **OLA1**, PLSCR3, **SLC25A24**, SNRNP40, **SPRYD4**, SUCLG1, **SUCLG2**, TRAPPC1, **TRAPPC3**, TRAPPC5, TRAPPC13, TRAPPC6B, UBC, WDR83	25	18	Lipid Metabolism, Nucleic Acid Metabolism, Small Molecule Biochemistry

Table B: Networks that were identified in T47D-ERβ cells expressing ERα and ERβ exposed to 4OHT. Bold protein names: up-regulated expression. Underlined protein names: down-regulated expression. Non-formatted protein names: proteins incorporated through relationships with other proteins. (*continued*)

ID	Proteins in network	Score	Focus proteins	Top functions
12	**ABHD14B**, ACTN1, **AGR3**, **AGRN**, **APOA1BP**, C5, **C1orf123**, **C9orf142**, CAV2, **CCDC58**, **COA3**, CSK, CYB5R1, DAG1, DNM1, FURIN, **GGCT**, **GSDMD**, ITGA7, LAMA1, **LAMB1**, LAMC1, MAP2, **MRPS36**, MRPS18C, **NDUFAF2**, OPTN, **PLEC**, RYR1, SGCB, SLC44A2, **TMA7**, UBC, **UTRN**, **VPS13C**	25	18	Hereditary Disorder, Skeletal and Muscular Disorders, Developmental Disorder
13	ACAD9, ADRM1, ATP13A2, C20orf24, **C21orf33**, CSNK1D, **DNPH1**, **EFHD1**, ELF4, **FAHD1**, FBXO11, FXN, **GNPNAT1**, **HN1L**, IARS2, IARS, KRT33B, **MANF**, MT-ND1, **NUDCD2**, **PGRMC2**, **PMPCB**, PPAP2A, PPCS, QPRT, **RAB1B**, RASA4, **SEPT2**, SLC25A20, SUFU, TCHH, TST, UBC, **USMG5**, YIF1A	23	17	Cellular Assembly and Organization, Cellular Compromise, Hereditary Disorder
14	Alpha tubulin, **ANP32B**, atypical protein kinase C, BCR (complex), **CALR**, EphA/B, FGF, GLG1, **IFIT1**, IL8r, Integrin, ITGA2, JINK1/2, Laminin, LASP1, MHC Class I (complex), MTCH2, MTORC1, NCK, p70 S6k, Pak, PARP, **PDIA3**, PI3K (complex), PI3K p85, PLd, Ptk, **RAB21**, **RAB2A**, **RALA**, **STMN1**, **STMN2**, TCR, **TPD52L2**, TWF1	19	15	Cell Signaling, DNA Replication, Recombination, and Repair, Nucleic Acid Metabolism
15	**ABAT**, ACAP2, APP, **APRT**, **BTF3**, **CLIC3**, **CMAS**, ER1, ESYT1, HADH, HAGH, HBZ, HDL-cholesterol, HIST1H2BB, IKKG, INA, **MACROD1**, MAPK15, **MMAB**, **MRPL53**, ODAM, PEX2, **PHF5A**, SEC11A, SULF1, **TIMM44**, TIMM17B, TIMM23B, TOMM5, **TOMM6**, TOMM7, TOMM40, TOMM20L, TOMM40L, VAPA	18	15	Metabolic Disease, Renal and Urological Disease, Behavior
16	14-3-3, ADRB, Alp, AMPK, Ap1, **APRT**, **ARL6IP5**, CaMKII, Cg, Creb, **CS**, DDX6, FSH, GNRH, Gsk3, **IFIT3**, **IFITM1**, IFN, IFN Beta, IgG, IL1, Interferon alpha, ITPR, Lh, MAPK3, Mek, NFAT (family), **NPM3**, Pka, **RAB14**, Ras, TCF, Tnf (family), **TPR**, **UBR5**	11	11	Molecular Transport, RNA Trafficking, Developmental Disorder
17	**ALDOC**, **ANP32A**, **CMC1**, CX3CL1, DHTKD1, DUSP5, DUSP6, ECH1, ENO2, ERCC6L, ETV1, FGF1, GCG, HAP1, HBEGF, **HTT**, IRS2, **KIAA1324**, MAP2, **MDH1**, MEIS2, MMP10, **NDUFB9**, NUPR1, PC, PDX1, **PEA15**, PPP1R1A, pyruvate kinase, RAB17, RCN2, **SDHA**, SST, **TFAM**, **ZNF706**	9	9	Small Molecule Biochemistry, Hereditary Disorder, Neurological Disease

18	Adaptor protein 2, AP2S1, AVPR2, **BLVRB**, **BTF3**, Calmodulin, Ck2, CTNNB1, FPR1, G protein alphai, G-protein beta, GPSM1, **GRB2**, **HMGN2**, Ige, Immunoglobulin, KCNN1, **LIN7C**, **LYPLA2**, Mmp, MT-ATP8, P2RY2, PDE6G, PLA2, PLC, **PLXNB2**, Rac, Ras homolog, RASGRF2, **RPL9**, **RPL18A**, RPL39L, S1PR5, TRHR, Tubulin	9	9	Carbohydrate Metabolism, Small Molecule Biochemistry, Cell Signaling

The biological functions corresponding with modified protein expression were identified using Fisher's exact test at a cutoff P value of 0.05. The network significance score was calculated by Ingenuity Pathway Analysis with the right-tailed Fisher's exact test. This score indicates the likelihood that the assembly of a set of proteins in a network could be explained by random chance.

Ingenuity Pathway Analysis predicted that in T47D-ERβ cells expressing ERα and ERβ, cell proliferation is increased upon 4OHT exposure when compared to unexposed T47D-ERβ cells expressing ERα and ERβ, with a P value of $8.95*10^{-3}$ (P value obtained from Ingenuity Pathway Analysis, diseases and biofunctions). Apoptosis is predicted to be decreased by Ingenuity Pathway Analysis in T47D-ERβ cells expressing ERα and ERβ upon 4OHT exposure when compared to unexposed T47D-ERβ cells expressing ERα and ERβ, with a P value of $2.56*10^{-4}$ (P value obtained from Ingenuity Pathway Analysis, diseases and biofunctions).

Using an FDR of 0.1, protein samples of T47D-ERβ cells expressing only ERα show 26 significantly modified protein groups due to the 4OHT exposure when compared to unexposed T47D-ERβ cells expressing only ERα (Figure 2B). In Figure 3B the top ten affected molecular and functional classes are shown, that including cell death and survival, DNA replication, recombination and repair, and protein synthesis. In Table C the significantly up- and down-regulated protein groups and their biological functions are shown.

Table D reveals 11 networks that were affected in 4OHT-exposed T47D-ERβ cells expressing only ERα compared to unexposed T47D-ERβ cells expressing only ERα, including top networks involved in protein degradation and synthesis, DNA replication, recombination and repair, cell cycle, and RNA post-transcriptional modification.

Ingenuity Pathway Analysis predicted that in T47D-ERβ cells only expressing ERα, cell proliferation is decreased upon 4OHT exposure when compared to unexposed T47D-ERβ cells expressing only ERα, with a P value of $3.09*10^{-3}$ (P value obtained from Ingenuity Pathway Analysis, diseases and biofunctions). Apoptosis is predicted to be increased by Ingenuity Pathway Analysis in T47D-ERβ cells only expressing ERα upon 4OHT exposure when compared to unexposed T47D-ERβ cells, with a P value of $3.58*10^{-6}$ (P value obtained from Ingenuity Pathway Analysis, diseases and biofunctions).

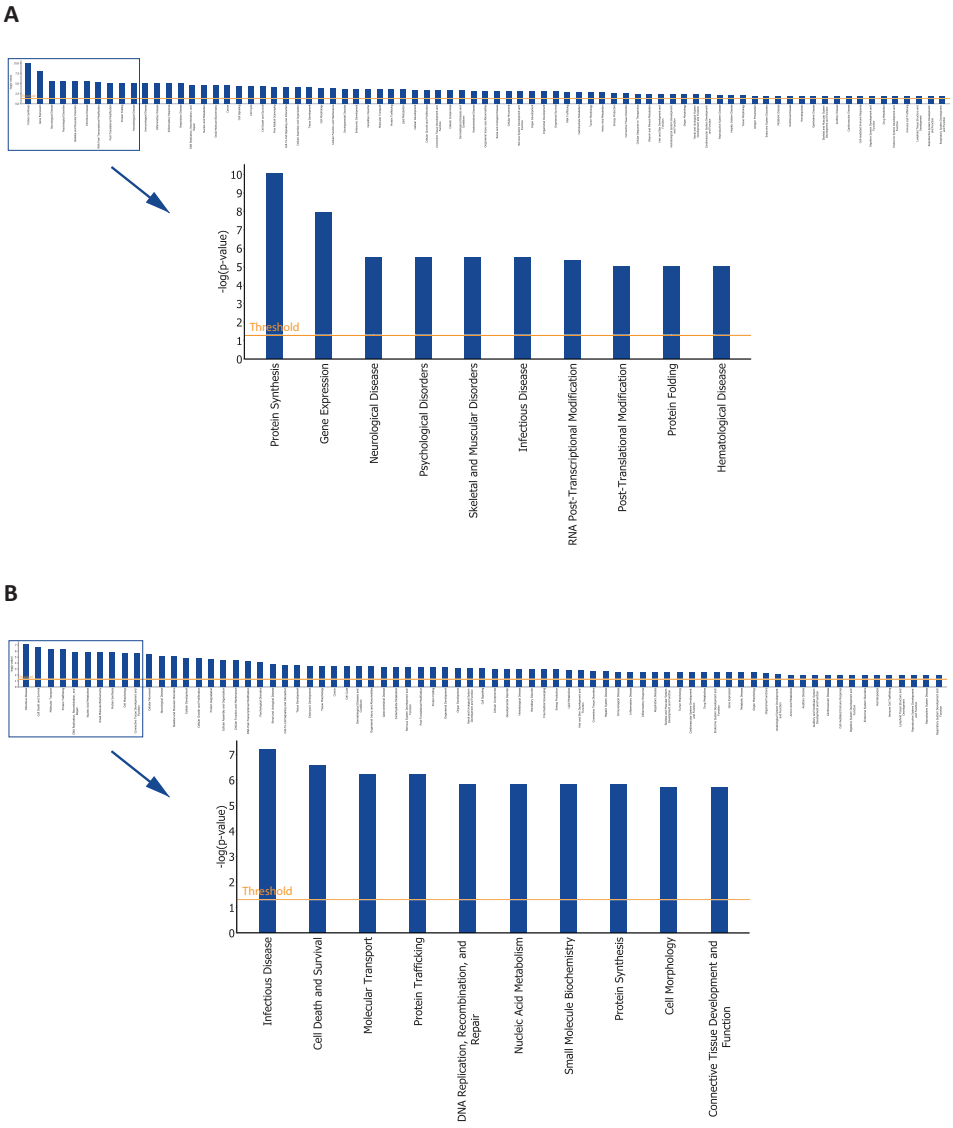


Figure 3: Top ten molecular and cellular functional classes influenced by 4OHT exposure in T47D-ERβ cells when **(A)** ERα and ERβ are expressed and **(B)** only ERα is expressed. The functional categories most involved are displayed along the x-axis in decreasing order of significance. Yellow line: cutoff for significance (P value 0.05).

Table C: Protein groups most influenced (significantly different at FDR ≤0.1) by 4OHT exposure in T47D-ERβ cells expressing only ERα. Affected biological pathway/function derived from www.uniprot.org.

Protein group names	Gene names	Protein IDs	Up-/down-regulated after 4OHT treatment	Biological pathway/function
39S ribosomal protein L28, mitochondrial	MRPL28	Q13084	Down	Structural constituent of ribosome
Major vault protein	MVP	Q14764	Down	Required for normal vault structure. Vaults are multi-subunit structures that may act as scaffolds for proteins involved in signal transduction. Vaults may also play a role in nucleo-cytoplasmic transport. Down-regulates INFG-mediated STAT1 signaling and subsequent activation of JAK. Down-regulates SRC activity and signaling through MAP kinases.
Lysosomal Pro-X carboxypeptidase	PRCP	A8MU24	Down	Cleaves C-terminal amino acids linked to proline in peptides such as angiotensin II, III and des-Arg9-bradykinin. This cleavage occurs at acidic pH, but enzymatic activity is retained with some substrates at neutral pH.
Leucine-rich repeat-containing protein 57	LRRC57	Q8N9N7	Down	-
Rho-related GTP-binding protein RhoB	RHOB	B2R692	Up	Mediates apoptosis in neoplastically transformed cells after DNA damage. Not essential for development but affects cell adhesion and growth factor signaling in transformed cells. Plays a negative role in tumorigenesis as deletion causes tumor formation. Involved in intracellular protein trafficking of a number of proteins. Targets PKN1 to endosomes and is involved in trafficking of the EGF receptor from late endosomes to lysosomes. Also required for stability and nuclear trafficking of AKT1/AKT which promotes endothelial cell survival during vascular development. Serves as a microtubule-dependent signal that is required for the myosin contractile ring formation during cell cycle cytokinesis. Required for genotoxic stress-induced cell death in breast cancer cells.

UBX domain-containing protein 1	UBXN1	Q04323	Up	Ubiquitin-binding protein that interacts with the BRCA1-BARD1 heterodimer and regulates its activity. Specifically binds 'Lys-6'-linked polyubiquitin chains. Interaction with autoubiquitinated BRCA1, leads to inhibit the E3 ubiquitin-protein ligase activity of the BRCA1-BARD1 heterodimer. Component of a complex required to couple deglycosylation and proteasome-mediated degradation of misfolded proteins in the endoplasmic reticulum that are retrotranslocated in the cytosol.
Peroxisomal membrane protein 11B	PEX11B	O96011	Up	Involved in peroxisomal proliferation. May regulate peroxisomes division by recruiting the dynamin-related GTPase DNM1L to the peroxisomal membrane.
dCTP pyrophosphatase 1	DCTPP1	Q9H773	Up	Hydrolyzes deoxynucleoside triphosphates (dNTPs) to the corresponding nucleoside monophosphates. Has a strong preference for modified dCTP. Activity is highest with 5-iodo-dCTP, followed by 5-bromo-dCTP, unmodified dCTP, 5-methyl-dCTP and 5-chloro-dCTP. Hydrolyzes 2-chloro-dATP and 2-hydroxy-dATP with lower efficiency, and has even lower activity with unmodified dATP, dTTP and dUTP (in vitro). Does not hydrolyze ATP, UTP, ITP, GTP, dADP, dCDP or dGTP. May protect DNA or RNA against the incorporation of non-canonical nucleotide triphosphates. May protect cells against inappropriate methylation of CpG islands by DNA methyltransferases.
Replication protein A 14 kDa subunit	RPA3	A4D105	Down	Required for DNA recombination, repair and replication. The activity of RP-A is mediated by single-stranded DNA binding and protein interactions. Functions as component of the alternative replication protein A complex (aRPA). aRPA binds single-stranded DNA and probably plays a role in DNA repair; it does not support chromosomal DNA replication and cell cycle progression through S-phase. In vitro, aRPA cannot promote efficient priming by DNA polymerase alpha but supports DNA polymerase delta synthesis in the presence of PCNA and replication factor C (RFC), the dual incision/excision reaction of nucleotide excision repair and RAD51-dependent strand exchange.

Table C: Protein groups most influenced (significantly different at FDR ≤0.1) by 4OHT exposure in T47D-ERβ cells expressing only ERα. Affected biological pathway/function derived from www.uniprot.org. (*continued*)

Protein group names	Gene names	Protein IDs	Up-/down-regulated after 4OHT treatment	Biological pathway/function
CD81 antigen	CD81	A6NMH8	Down	May play an important role in the regulation of lymphoma cell growth. Interacts with a 16-kDa Leu-13 protein to form a complex possibly involved in signal transduction. May act as the viral receptor for HCV.
Acid ceramidase; Acid ceramidase subunit alpha; Acid ceramidase subunit beta	ASAH1	D3DSQ1	Down	Hydrolyzes the sphingolipid ceramide into sphingosine and free fatty acid.
Suppressor of G2 allele of SKP1 homolog	SUGT1	Q9Y2Z0	Down	May play a role in ubiquitination and subsequent proteasomal degradation of target proteins.
Fermitin family homolog 2	FERMT2	B5TJY2	Down	Participates in the connection between ECM adhesion sites and the actin cytoskeleton and also in the orchestration of actin assembly and cell shape modulation. Recruits migfilin (FBLP1) protein to cell-ECM focal adhesion sites.
RNA-binding protein 4	RBM4; RBM14/ RBM4 fusion	Q9BWF3	Down	RNA-binding factor involved in multiple aspects of cellular processes like alternative splicing of pre-mRNA and translation regulation. Modulates alternative 5'-splice site and exon selection. Acts as a muscle cell differentiation-promoting factor. Activates exon skipping of the PTBP pre-mRNA during muscle cell differentiation. Antagonizes the activity of the splicing factor PTBP1 to modulate muscle cell-specific exon selection of alpha tropomyosin. Binds to intronic pyrimidine-rich sequence of the TPM1 and MAPT pre-mRNAs. Required for the translational activation of PER1 mRNA in response to circadian clock. Binds directly to the 3'-UTR of the PER1 mRNA.

				Exerts a suppressive activity on Cap-dependent translation via binding to CU-rich responsive elements within the 3'UTR of mRNAs, a process increased under stress conditions or during myocytes differentiation. Recruits EIF4A1 to stimulate IRES-dependent translation initiation in response to cellular stress. Associates to internal ribosome entry segment (IRES) in target mRNA species under stress conditions. Plays a role for miRNA-guided RNA cleavage and translation suppression by promoting association of EIF2C2-containing miRNPs with their cognate target mRNAs. Associates with miRNAs during muscle cell differentiation. Binds preferentially to 5'-CGCGCG[GCA]-3' motif in vitro.; Required for the translational activation of PER1 mRNA in response to circadian clock. Binds directly to the 3'-UTR of the PER1 mRNA
28S ribosomal protein S18b, mitochondrial	MRPS18B	B0S7P4	Down	Structural constituent of ribosome
V-type proton ATPase subunit H	ATP6V1H	Q9UI12	Down	Subunit of the peripheral V1 complex of vacuolar ATPase. Subunit H activates the ATPase activity of the enzyme and couples ATPase activity to proton flow. Vacuolar ATPase is responsible for acidifying a variety of intracellular compartments in eukaryotic cells, thus providing most of the energy required for transport processes in the vacuolar system. Involved in the endocytosis mediated by clathrin-coated pits, required for the formation of endosomes.
Presequence protease, mitochondrial	PITRM1	C9JSL2	Down	ATP-independent protease that degrades mitochondrial transit peptides after their cleavage. Also degrades other unstructured peptides. Specific for peptides in the range of 10 to 65 residues. Able to degrade amyloid beta A4 (APP) protein when it accumulates in mitochondrion, suggesting a link with Alzheimer disease. Shows a preference for cleavage after small polar residues and before basic residues, but without any positional preference.
39S ribosomal protein L16, mitochondrial	MRPL16	Q9NX20	Down	Component of the large subunit of mitochondrial ribosome.

Table C: Protein groups most influenced (significantly different at FDR ≤0.1) by 4OHT exposure in T47D-ERβ cells expressing only ERα. Affected biological pathway/function derived from www.uniprot.org. (*continued*)

Protein group names	Gene names	Protein IDs	Up-/down-regulated after 4OHT treatment	Biological pathway/function
Actin-related protein 2/3 complex subunit 2	ARPC2	O15144	Down	Functions as actin-binding component of the Arp2/3 complex which is involved in regulation of actin polymerization and together with an activating nucleation-promoting factor (NPF) mediates the formation of branched actin networks. Seems to contact the mother actin filament.
Lysosomal alpha-glucosidase; 76 kDa lysosomal alpha-glucosidase; 70 kDa lysosomal alpha-glucosidase	GAA	P10253	Down	Essential for the degradation of glygogen to glucose in lysosomes.
DnaJ homolog subfamily B member 1	DNAJB1	P25685	Down	Interacts with Hsp70 and can stimulate its ATPase activity. Stimulates the association between HSC70 and HIP.
Serpin B6	SERPINB6	P35237	Down	May be involved in the regulation of serine proteinases present in the brain or extravasated from the blood. Inhibitor of cathepsin G, kallikrein-8 and thrombin. May play an important role in the inner ear in the protection against leakage of lysosomal content during stress and loss of this protection results in cell death and sensorineural hearing loss.
Adapter molecule crk	CRK	P46108	Down	The Crk-I and Crk-II forms differ in their biological activities. Crk-II has less transforming activity than Crk-I. Crk-II mediates attachment-induced MAPK8 activation, membrane ruffling and cell motility in a Rac-dependent manner. Involved in phagocytosis of apoptotic cells and cell motility via its interaction with DOCK1 and DOCK4. May regulate the EFNA5-EphA3 signaling.

Ubiquitin-like-conjugating enzyme ATG3	ATG3	Q9NT62	Down	E2-like enzyme involved in autophagy and mitochondrial homeostasis. Catalyzes the conjugation of ATG8-like proteins (GABARAP, GABARAPL1, GABARAPL2 or MAP1LC3A) to phosphatidylethanolamine (PE). PE-conjugation to ATG8-like proteins is essential for autophagy. Preferred substrate is MAP1LC3A. Also acts as an autocatalytic E2-like enzyme, catalyzing the conjugation of ATG12 to itself, ATG12 conjugation to ATG3 playing a role in mitochondrial homeostasis but not in autophagy. ATG7 (E1-like enzyme) facilitates this reaction by forming an E1-E2 complex with ATG3.
Ras suppressor protein 1	RSU1	Q32Q10	Down	Potentially plays a role in the Ras signal transduction pathway. Capable of suppressing v-Ras transformation in vitro.
Phospholysine phosphohistidine inorganic pyrophosphate phosphatase	LHPP	Q9H008	Down	Phosphatase that hydrolyzes imidodiphosphate, 3-phosphohistidine and 6-phospholysine. Has broad substrate specificity and can also hydrolyze inorganic diphosphate, but with lower efficiency

Table D: Networks that were identified in T47D-ERβ cells with only ERα expression exposed to 4OHT. Bold protein names: up-regulated expression. Underlined protein names: down-regulated expression. Non-formatted protein names: proteins incorporated through relationships with other proteins.

ID	Proteins in network	Score	Focus proteins	Top functions
1	26s Proteasome, **ArfGAP2**, ATPase, **ATPIF1**, CAMK2G, COPE, CYB5R3, DNAJA1, DNAJB1, ECHS1, EPCAM, **FDXR**, FERMT2, Hdac, Hsp, **HspD1**, Ikb, MHC CLASS I (family), **MYH9**, NFkB (complex), PSMD2, PSMD14, RSU1, SEC22B, **SEC61B**, **TFG**, UBA1, UBE2, **UBE2C**, UBE2M, UBE2V1, Ubiquitin, **UBXN1**, UFD1L, VCP	48	26	Protein Degradation, Protein Synthesis, DNA Replication, Recombination, and Repair
2	60S ribosomal subunit, **AIFM1**, ANXA2, BLVRA, calpain, Cyclin A, Cyclin E, Cytochrome bc1, E2f, **IMMT**, Jnk, MCM4, **MCM6**, Mitochondrial complex 1, NDUFA2, NDUFB9, **PA2G4**, PHB, PHF5A, RB, RPA3, **RPL15**, **RPL35A**, **RPL37A**, **RPL7A**, RPLP2, SLC25A6, **SUMO2**, TAGLN2, **TCOF1**, thymidine kinase, TOMM6, UQCRB, **UQCRQ**, VDAC3	46	25	Cell Cycle, DNA Replication, Recombination, and Repair, Cardiovascular Disease
3	ADSS, Akt, ALYREF, **CHCHD3**, CSE1L, DPY30, EIF3C/EIF3CL, **EIF4A2**, ERH, FDPS, Histone h4, IFN gamma, Importin alpha, Importin beta, IPO9, KPNB1, KYNU, MUC1, NFAT (family), OAS3, **PSPH**, Ribosomal 40s subunit, Rnr, RPL5, RPS6, RPS16, RPS18, **RPS23**, **RPS29**, **SDC4**, **SRRM1**, **SSB**, TNPO1, **WIBG**, XPO1	45	27	Infectious Disease, RNA Post-Transcriptional Modification, Protein Synthesis
4	ANXA3, APP, APRT, ATP6V1G1, BDH1, C14orf93, **C19orf43**, **DCTPP1**, DECR1, DPCD, ECHDC1, ESD, **ETHE1**, GAA, GNPTG, HIGD1A, **INIP**, LACRT, LYRM7, MRPL28, **NAP1L4**, **NUCKS1**, PARP3, PITRM1, PRCP, PYGB, **RAB3D**, SSR4, STX8, SURF4, TRAPPC3, TRMT112, UBC, WDR45, ZW10	41	24	Organismal Functions, Carbohydrate Metabolism, Developmental Disorder
5	Alpha tubulin, ATP6V1H, C14orf166, caspase, **CAST**, **CCT8**, CLIC4, **CRABP2**, Cyclin B, Cyclin D, cytochrome C, cytochrome-c oxidase, **DAP**, DNM1L, DUB, EIF2S1, ERK, HSD17B4, HSD17B10, HSD17B12, HSD17B, KRT18, **LAMP1**, **MAP4**, MAP2K1/2, Mek, **PEX11B**, PRDX5, RAF, **SFN**, Sos, TCF, **UCHL3**, USP47, USP9X	36	21	Cell Cycle, Cellular Assembly and Organization, Hepatocellular Peroxisome Proliferation
6	Actin, AGRN, ALDOA, Alpha catenin, AP2A1, Arp2/3, ARPC2, ARPC4, ASAH1, BCR (complex), Cadherin, CD81, **CLINT1**, **COTL1**, Crk, ERK1/2, Erm, F Actin, Fascin, **GAPVD1**, Gef, MVP, PCBP2, PRAF2, Ptk, RAB5, RAB6A, RAB7A, Rho gdi, **RHOB**, RHOG, SERPINB6, Troponin t, VAV, VPS35	34	20	Cellular Function and Maintenance, Molecular Transport, Protein Trafficking

7	AP2S1, ASAP2, **C21orf33**, CCND1, CENPH, CHCHD1, CTNNBIP1, D2HGDH, **DCTPP1**, DNAJB4, DPY30, GNPDA1, HERC4, HIBADH, **HN1L**, **INIP**, ITP, KRT4, MRPL28, MRPL43, MRPS16, MRPS23, MRPS18B, NABP1, NQO2, NUBP1, NUBP2, OXA1L, **RAB25**, RAB11FIP1, RAB11FIP5, RMI2, **SERF2**, SPR, UBC	27	17	Developmental Disorder, Hereditary Disorder, Metabolic Disease
8	ABHD11, ACAA1, ACADL, ACOT2, **ACOX1**, ATP13A2, **C19orf43**, CARKD, **CCDC124**, CD63, CSTB, CYP4F12, DPCD, **HSDL2**, IMPACT, inorganic diphosphatase, LHPP, LONP1, LPCAT3, LRRC57, **LYRM4**, LYRM7, MRPL16, NFS1, OSTC, PHLDA1, PITRM1, PLSCR1, PPA2, PVRL2, **RNPEP**, SERTAD2, **SGTA**, TAGLN2, UBC	25	16	Lipid Metabolism, Small Molecule Biochemistry, Hereditary Disorder
9	Ap1, ASS1, ATG3, **B2M**, BANF1, CaMKII, chemokine, COX5B, **DLD**, **HMGB1**, **HMGB2**, Hsp70, IFN, IFN Beta, Ige, IgG, Igm, IKK (complex), IL1, IL12 (complex), Immunoglobulin, Interferon alpha, mediator, MHC Class I (complex), Pkc(s), Pro-inflammatory Cytokine, **S100A13**, Sapk, **SNRNP70**, SNRNP, SNRPD1, SNRPD3, SNRPF, STAT3, Tnf (family)	20	14	Increased Levels of Albumin, Cell Death and Survival, Nervous System Development and Function
10	AK1, AMPK, Collagen type I, Collagen(s), creatine kinase, FHL2, Fibrinogen, Focal adhesion kinase, HK1, Integrin, JINK1/2, Laminin, LAMTOR1, LDL, LRRC59, Mlc, MTORC1, NADPH oxidase, PARK7, PARP, **PDAP1**, Pdgf (complex), Pdgf BB, PGD, PI3K (complex), PLd, PP2A, Rap1, RAP1B, RHOA, SEC13, **SEPT9**, STAT5a/b, TCR, TGF beta	15	12	Post-Translational Modification, Protein Degradation, Cell Cycle
11	Calmodulin, Cbp/p300, CD3, CDC42, Ck2, Creb, CTNNB1, **EPRS**, estrogen receptor, FSH, G protein beta gamma, Gsk3, HISTONE, Histone h3, Hsp90, Insulin, Lh, MAPK, OSBP, P38 MAPK,p85 (pik3r), Pdgfr, Pka, **PTBP1**, Rac, Ras, Ras homolog, RNA polymerase II, SRC (family), SRPRB, SUGT1, **SUPT16H**, **TMEM109**, **VAPA**, Vegf	12	10	Cellular Development, Embryonic Development, Hair and Skin Development and Function

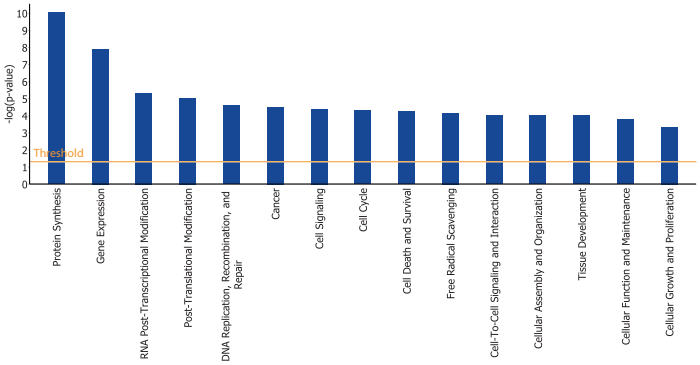
Molecular and functional classes of interest influenced by 4OHT exposure

Because the aim of this study is to investigate in what way quantitative proteomics reflect 4OHT-induced ERα/ERβ ratio-dependent effects on cell proliferation and apoptosis, further functional analysis of the protein groups influenced by 4OHT exposure is conducted to investigate molecular and functional classes related to cell proliferation and apoptosis. The following Ingenuity Pathway classes are included as molecular and functional classes of interest: cancer, cell cycle, cell death and survival, cell signalling, cell-to-cell signalling and interaction, cellular assembly and organisation, cellular function and maintenance cellular growth and proliferation, DNA replication, recombination, and repair, free radical scavenging, gene expression, post-translational modification, protein synthesis, RNA post-transcriptional modification, and tissue development. Figure 4A shows the molecular and functional classes of interest affected in T47D-ERβ cells expressing ERα and ERβ, comparing 4OHT-exposed samples to unexposed samples. Especially protein synthesis and gene expression are modified by the 4OHT exposure. Protein relationships involved in cell proliferation and apoptosis are visualised in a network (Figure 4B). Cathenin, mitogen-activated protein kinase 3 (MAPK3), and growth factor receptor-bound protein 2 (GRB2) are central proteins in the network of protein groups influenced by 4OHT exposure in T47D-ERβ cells expressing ERα and ERβ.

In T47D-ERβ cells only expressing ERα, the affected molecular and functional classes show differences compared to the T47D-ERβ cells expressing ERα and ERβ after 4OHT exposure (Figure 5A). The modifications in cell death and survival and DNA replication, recombination, and repair are more significant after 4OHT exposure when only ERα is present compared to when both ERα and ERβ are expressed. Protein relationships involved in cell proliferation and apoptosis are visualised in a network (Figure 5B). Cathenin and small ubiquitin-like modifier 2 (SUMO2) are central proteins in the network of protein groups influenced by 4OHT exposure in T47D-ERβ cells with only ERα expression.

Most proteins listed in functions like cell cycle, DNA replication, recombination, and repair, and protein synthesis, show opposite expression profiles upon 4OHT exposure when only ERα is expressed or when ERα and ERβ are present (Table B and D). This holds for example for ubiquitin-conjugating enzyme E2M (UBE2M, an ubiquitin-protein ligase involved in protein binding), ubiquitin-conjugating enzyme E2 variant 1 (UBE2V1, protein binding and transcription regulator), and replication protein A3 (RPA3, involved in protein binding and single-stranded DNA binding).

A



B

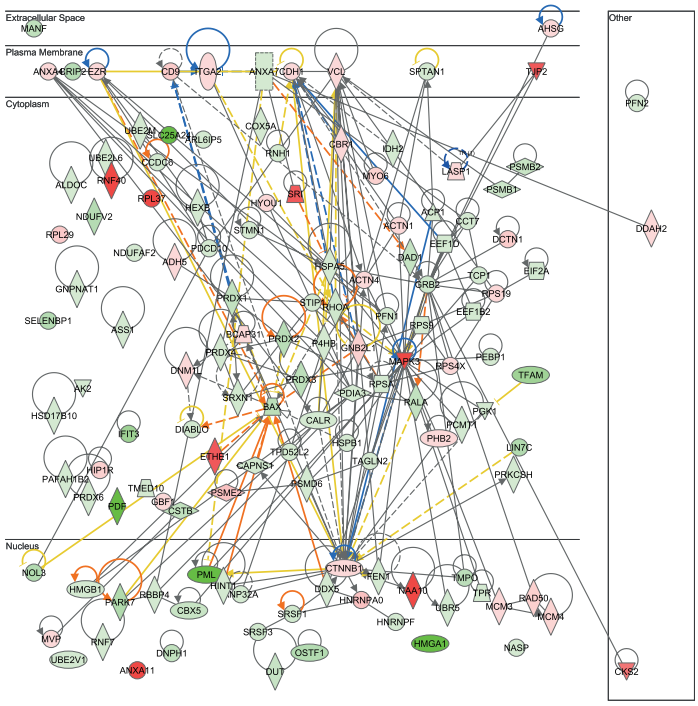
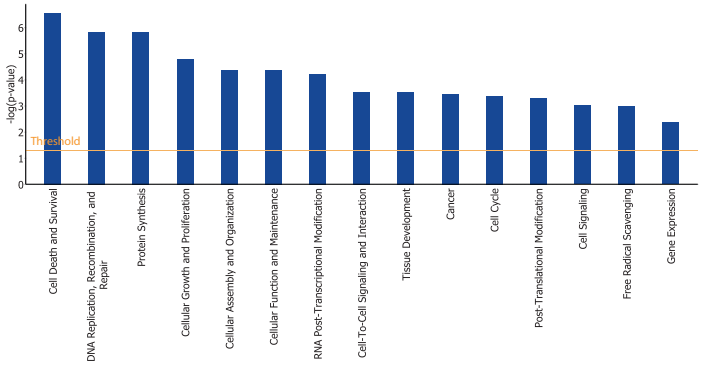


Figure 4: (A) Molecular and cellular functional classes of interest influenced by 4OHT exposure in T47D-ERβ cells when ERα and ERβ are expressed. The functional categories involved in this analysis are displayed along the x-axis in decreasing order of significance. The yellow line indicates the cutoff for significance (P value 0.05). **(B)** Network in subcellular view of protein group interactions based on cell proliferation and apoptosis that were identified in T47D-ERβ cells with ERα and ERβ expression influenced by the exposure to 4OHT. Red colored proteins: up-regulated. Green colored proteins: down-regulated. A more detailed legend to the protein interaction network can be found in Supplementary Figure S1, explaining the symbols and colors.

A



B

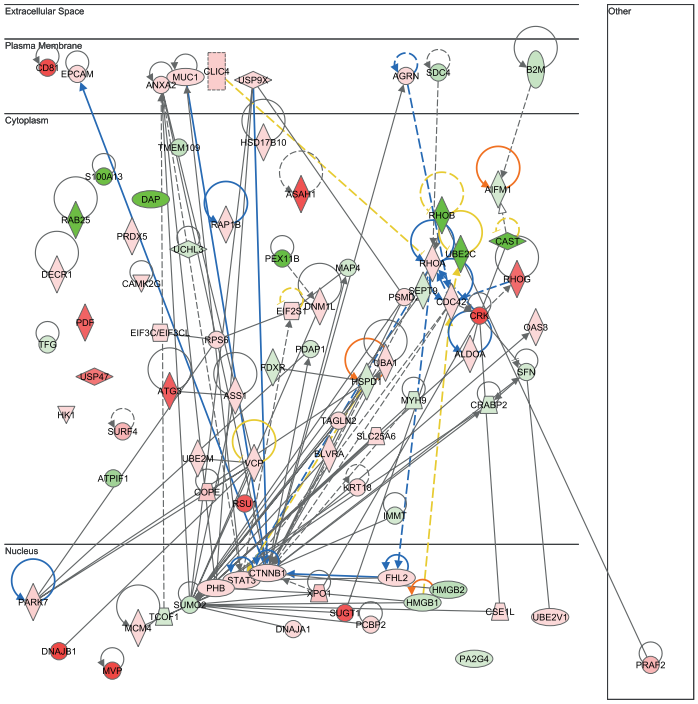


Figure 5: (A) Molecular and cellular functional classes of interest influenced by 4OHT exposure in T47D-ERβ cells when only ERα is expressed. The functional categories involved in this analysis are displayed along the x-axis in decreasing order of significance. The yellow line indicates the cutoff for significance (P value 0.05). **(B)** Network in subcellular view of protein group interactions based on cell proliferation and apoptosis that were identified in T47D-ERβ cells expressing only ERα influenced by the exposure to 4OHT. Red colored proteins: up-regulated. Green colored proteins: down-regulated. A more detailed legend to the protein interaction network can be found in Supplementary Figure S1, explaining the symbols and colors.

Post-translational and epigenetic modifications

Since it is known that post-translational modifications can influence gene transcription [23-25] and protein degradation [26], and this might influence the ERα/ERβ ratio-dependent effects of 4OHT, a MaxQuant analysis including acetylation, methylation, phosphorylation, and ubiquination modifications, allowing at most 3 modifications per peptide, was conducted. In this analysis 4297 proteins were identified, of which 1667 remained after very strict filtering (only protein groups identified by at least 2 peptides of which at least 1 peptide had to be unique in the database and at least 1 peptide had to be unmodified, and only proteins with 3 valid values in at least 1 group of triplicate samples were included).

Table E shows the detected post-translational and epigenetic modifications of protein groups which are significantly different in the samples. In T47D-ERβ cells expressing ERα and ERβ, 4OHT exposure resulted in seven significantly differently expressed protein groups with post-translational modifications. Ubiquitination and methylation of dynactin subunit 1 was detected at respectively lysine 277 and arginine 274. Dynactin is required for the cytoplasmic dynein-driven retrograde movement of vesicles and organelles along microtubules. Dynein-dynactin interaction is a key component of the mechanism of axonal transport of vesicles and organelles [33, 34]. Cytoplasmic leucine transfer RNA (tRNA) ligase, which catalyzes the specific attachment of an amino acid to its cognate tRNA and exhibits a post-transfer editing activity to hydrolyze mischarged tRNAs [35], showed acetylation and methylation at lysine 23 and arginine 28 respectively. Cytoplasmic fragile X mental retardation 1 (FMR1)-interacting protein 1 was observed to be phosphorylated at threonine 139 and methylated at lysine 149. FMR1-interacting protein 1 is a component of the cytoplasmic FMR1 interacting protein 1-eukaryotic translation initiation factor-fragile X mental retardation 1 (CYFIP1-EIF4E-FMR1) complex which binds to the mRNA cap and mediates translational repression. FMR1-interacting protein 1 may modulate invasion in cancers [36]. A type II cytoskeletal keratin protein, which may regulate the activity of kinases such as protein kinase C (PKC) and sarcoma (SRC) via binding to integrin beta-1 (ITB1) and the receptor of activated protein kinase C (receptor for activated C kinase 1/guanine nucleotide binding protein, beta polypeptide 2-like 1, RACK1/GNB2L1), was found to be methylated at arginine 602 [37, 38]. Phosphorylation of cytochrome c oxidase subunit 4 isoform 1 was detected at serine 158. This cytochrome c oxidase subunit 4 isoform 1 protein is one of the nuclear-coded polypeptide chains of cytochrome c oxidase, the terminal oxidase in mitochondrial electron transport [39]. The mitochondrial deoxyuridine 5'-triphosphate nucleotido hydrolase was ubiquitinated at lysine 251 upon exposure to 4OHT. This enzyme is involved in nucleotide metabolism: it

Table E: Significant post-translational modified proteins showing acetylation, methylation, or phosphorylation upon 4OHT exposure.

Post-translational modification	Condition	Site position	Protein name	Gene name	Amino acid	Up-/down-regulated protein expression
Ubiquitination	ERα and ERβ expression	277	Dynactin subunit 1	DCTN1	Lysine	Down
Methylation	ERα and ERβ expression	274	Dynactin subunit 1	DCTN1	Arginine	Down
Acetylation	ERα and ERβ expression	23	Cytoplasmic leucine tRNA ligase	LARS	Lysine	Down
Methylation	ERα and ERβ expression	28	Cytoplasmic leucine tRNA ligase	LARS	Arginine	Down
Methylation	ERα and ERβ expression	149	Cytoplasmic FMR1-interacting protein 1	CYFIP1		Down
Phosphorylation	ERα and ERβ expression	139	Cytoplasmic FMR1-interacting protein 1	CYFIP1		Down
Methylation	ERα and ERβ expression	602	Type II cytoskeletal keratin protein	KRT1	Arginine	Up
Phosphorylation	ERα and ERβ expression	158	Cytochrome c oxidase subunit 4 isoform 1	COX4I1	Serine	Down
Ubiquitination	ERα and ERβ expression	251	Deoxyuridine 5'-triphosphate nucleotidohydrolase	DUT	Lysine	Up
Methylation	ERα and ERβ expression	80	Histone H3.2	HIST2H3A	Lysine	Down
Acetylation	Only ERα expression	9	Lupus La protein	SSB	Lysine	Up
Phosphorylation	Only ERα expression	366	Lupus La protein	SSB	Serine	Up

produces deoxyuridine monophosphate (dUMP), the immediate precursor of thymidine nucleotides and it decreases the intracellular concentration of dUTP deoxyuridine triphosphate so that uracil cannot be incorporated into DNA [40]. Histone H3.2 was methylated at lysine 80. Histones are core components of the nucleosome. Nucleosomes wrap and compact DNA into chromatin, limiting DNA accessibility to the cellular machineries which require DNA as a template. Histones thereby play a central role in transcription regulation, DNA repair, DNA replication, and chromosomal stability. DNA accessibility is regulated via a complex set of post-translational modifications of histones, also called histone code, and nucleosome remodeling [41, 42].

In T47D-ERβ cells expressing only ERα, 4OHT exposure resulted in only two post-translational modifications on the same protein, i.e. acetylation at lysine 9 and phosphorylation at serine 366 of the Lupus La protein. The Lupus La protein binds to the 3' poly(U) termini of nascent RNA polymerase III transcripts, protecting them from exonuclease digestion and facilitating their folding and maturation [43, 44].

Discussion

So far, several studies into proteomic analysis of breast cancer cells in relation to 4OHT have been conducted in MCF-7 and T47D cells [45, 46]. However, these studies did not take different ERα/ERβ ratios into account. Both MCF-7 and T47D cells express no or only very little ERβ [20]. Taking different ERα/ERβ ratios into account is essential for a better understanding of the mechanism by which 4OHT exerts its effects, since 4OHT acts upon binding to ERα or ERβ and ERβ counteracts the cell proliferative effect of ERα [2, 5-8]. Therefore, in the present study a quantitative analysis of differences in protein expression to address the effects of ERα and ERβ in the T47D-ERβ breast cancer cells exposed to 4OHT was conducted by means of nanoLC-LTQ-OrbitrapXL-MS. These T47D-ERβ cells possess tetracycline-dependent ERβ expression in contrast to native T47D cells [6, 20].

To be able to analyse differences in protein expression at an effective, non-cytotoxic 4OHT concentration, a BrdU cell proliferation assay was conducted. The effect of the ER agonist E2 on cell proliferation of T47D-ERβ cells was characterized and used to detect the ER antagonist activity of 4OHT and select the concentration to be tested in the proteomic analysis. In the T47D-ERβ cells expressing only ERα, E2 induces cell proliferation [20], which is dose-dependently decreased by 4OHT. The maximum decrease of cell proliferation is observed at 300 nM 4OHT, which was then chosen as the exposure concentration for the T47D-ERβ cells in the proteomics experiment.

Molecular and functional classes as well as network analysis of the proteomics data reveal several functions significantly influenced by 4OHT exposure that can be linked to cell proliferation or apoptosis, including DNA replication, recombination, and repair, cancer, cellular function and maintenance, and protein synthesis. Most proteins listed in these functions show opposite expression profiles in 4OHT-exposed T47D-ERβ cells when only ERα is expressed compared to 4OHT-exposed T47D-ERβ cells expressing both ERα and ERβ (Table B and D).

In samples expressing ERα and ERβ, cell proliferation was predicted to be increased and apoptosis to be decreased upon 4OHT exposure compared to unexposed cells. In the samples expressing only ERα, cell proliferation was predicted to be decreased upon 4OHT exposure when compared to unexposed cells. This is in line with BrdU cell proliferation results which reflected decreased proliferation of T47D-ERβ cells with only ERα expression upon exposure to 4OHT (Figure 1). Ingenuity Pathway Analysis predicted apoptosis to be increased upon 4OHT exposure of T47D-ERβ cells expressing only ERα. These ERα/ERβ ratio-dependent effects of 4OHT on cell proliferation and apoptosis are in line with 4OHT being an antagonist for both ERα and ERβ, with a preference for ERβ. The antagonist activity towards ERβ reduces the ERβ-dependent inhibition of ERα-mediated cell proliferation, thereby facilitating increased cell proliferation.

It is also of interest to note that the proteins listed in Table B and D show opposite expression profiles upon 4OHT exposure when ERα and ERβ are expressed or when only ERα is expressed. The proteins showing this opposite expression pattern upon 4OHT exposure of cells with different ERα/ERβ ratio are involved in functions like cell cycle, DNA replication, recombination, and repair, and protein synthesis. These results corroborate that the cellular effects of 4OHT are different depending on the ERα/ERβ ratio and are in line with the different cellular response with respect to cell poliferation. The observation that 4OHT-induced inhibition of cell proliferation is mainly observed for cells with low levels of ERβ is especially of interest when considering that in tumor tissues ERβ expression can be lost [2], whereas in non-tumorous tissue ERα and ERβ expression is detected [20].

Network analysis of protein groups involved in cell proliferation and apoptosis revealed that cathenin, a transcription factor known to be involved in cell proliferation and apoptosis [47], is connected to a lot of other proteins in the cell proliferation and apoptosis network for both 4OHT-exposed T47D-ERβ cells expressing only ERα and 4OHT-exposed T47D-ERβ cells expressing ERα and ERβ. Other central proteins in the cell proliferation and apoptosis network are MAPK3 which is down-regulated upon 4OHT exposure in T47D-ERβ cells expressing ERα and ERβ, and GRB2 which is up-regulated

in T47D-ERβ cells expressing ERα and ERβ. MAPK3 is involved in the regulation of proliferation, differentiation, and cell cycle progression [48]. GRB2 is involved in cellular growth and proliferation [49].

In T47D-ERβ cells expressing only ERα, next to cathenin also SUMO2, which upon 4OHT exposure is up-regulated, has a central role in the protein interaction network. SUMO2 is involved in protein stability and apoptosis [50]. Both cathenin and SUMO2 have a role in transcription regulation [51, 52]. This network analysis underlines the clear ERα/ERβ ratio-dependent differences in the response towards 4OHT exposure in T47D-ERβ cells.

Several studies have proposed that epigenetic mechanisms might be involved in ERα- and ERβ-mediated physiological effects [53-55]. The present study was able to define some post-translational and epigenetic modifications induced by 4OHT exposure. With ERβ being expressed in the T47D-ERβ cells, 4OHT exposure resulted in acetylation, methylation, and phosphorylation of seven ribosomal and mitochondrial protein groups, of which several are involved in transcription regulation. Most observed post-translational modifications have not been described in literature before and their physiological consequences remain to be elucidated. However, one of the most well studied modifications is histone methylation, which was induced by 4OHT exposure in T47D-ERβ cells expressing ERα and ERβ. Garcia *et al.* linked histone H3.2 methylation at lysine 80 to gene activation [56].

When only ERα was expressed in the T47D-ERβ cells, 4OHT exposure resulted in 2 modifications of the Lupus La protein. Acetylation at lysine 9 of the Lupus La protein has not been described before. Fan *et al.* described that phosphorylation of the Lupus La protein at serine 366 makes the protein transcriptionally inactive [57].

It is concluded that 4OHT affects molecular and cellular functional classes in T47D-ERβ cells including cell proliferation and apoptosis and induces post-translational modifications, with the ultimate effect being dependent on the ERα and ERβ levels and the ERα/ERβ ratio of the cells. There may be opposite effects regarding cell proliferation and apoptosis of 4OHT in cells with ERα but with or without ERβ expression. Given that the data presented indicate that in cells with ERα but without ERβ 4OHT will reduce cell proliferation, whereas in cells with ERβ in addition to ERα, 4OHT may stimulate cell proliferation, it could be of interest to monitor the ERα and ERβ levels and the ERα/ERβ ratio in breast tumor tissue to enable a more personalised medical use of 4OHT. Based on a randomized neo-adjuvant trial for the treatment of postmenopausal occuring breast cancer Madeira *et al.* [58] concluded that the ERα/ERβ ratio and ERβ levels could be used as predictors of endocrine therapy responsiveness. However, in contrast to our data, these authors concluded that especially in ERβ-positive groups tamoxifen

produced a significant reduction in post-treatment Ki67 scores, a marker for cell proliferation. It should be noted however that the post-treatment Ki67 scores in their ERβ-positive group upon tamoxifen treatment amounted to 76.3% ($P < 0.014$) (n=18) of the pretreatment scores whereas the values for the ERβ-negative group were 73.9% (not significant) (n=5) and thus actually similar probably being not statistically significant due to the lower group size. Furthermore, in both the ERβ-positive and ERβ-negative patient groups ERα-positive and ERα-negative cases were combined. Given the important role for ERα observed in the present study for 4OHT-induced effects on cell proliferation and apoptosis, this combining of ERα-positive and ERα-negative cases hampers comparison of the data of the trial with the proteomics data of the present study. Based on the proteomics data of the present study it is concluded that ERβ but also ERα levels and the ERα/ERβ ratio should be used as predictors of tamoxifen therapy responsiveness, with the presence of ERα and the absence of ERβ expression being optimal for inducing a 4OHT-mediated decrease in cell proliferation and increase in apoptosis.

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Supplementary Figure

Supplementary Figure S1: Legends to the protein interaction network from Ingenuity Pathway Analysis explaining the symbols and colors.

Supplementary Data

Additional abbreviations used in tables.

2-5A, 2'-5'-oligoadenylates
A, adenine
AAK, adaptor-associated protein kinase
ABAT, 4-aminobutyrate aminotransferase
ABC, ATP-binding cassette
ABHD, abhydrolase domain-containing protein
ACAA, acetyl-CoA acyltransferase
ACAD, acyl-CoA dehydrogenase
ACADL, acyl-CoA dehydrogenase, long chain
ACAP, ArfGAP with coiled-coil, ankyrin repeat and PH domains
ACAT, acetyl-CoA acetyltransferase
AChE, acetylcholinesterase
AChR, acetylcholine receptor
ACOT, acyl-CoA thioesterase
ACOX, acyl-CoA oxidase
ACP, acid phosphatase
ACSF, acyl-CoA synthetase family
ACTN, alpha-actinin
ACTR, alpha-centractin
ADAM, a disintegrin and metalloproteinase domain
ADH, alcohol dehydrogenase
ADP, adenosine diphosphate
ADR, adrenoceptor
ADRM, adhesion regulating molecule
ADSS, adenylosuccinate synthase
AGL, amylo-alpha-1, 6-glucosidase, 4-alpha-glucanotransferase
AGR, anterior gradient protein
AGRN, agrin
AHNAK, neuroblast differentiation-associated protein
AHSA, activator of heat shock 90kDa protein ATPase homolog
AHSG, alpha-2-HS-glycoprotein
AIFM, apoptosis-inducing factor, mitochondrion-associated
AK, adenylate kinase
AKR, aldo-keto reductase family
AKT, a serine/threonine-specific protein kinase
ALDH, aldehyde dehydrogenase
ALDO, aldolase
Alp, alkaline phosphatase
ALYREF, alymphoplasia/RNA export factor
AMPK, adenosine monophosphate-activated protein kinase
ANKRD, ankyrin repeat domain
ANLN, anillin
ANP, acidic leucine-rich nuclear phosphoprotein
Ant, adenine nucleotide translocator
ANX, annexin
AP, adaptor protein
APC, adenomatous polyposis coli
APEH, N-acylaminoacyl-peptide hydrolase
APK, adenylylsulfate kinase
APO, apolipoprotein
APOA1BP, apolipoprotein A-I binding protein
APOBEC, apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like
APP, amyloid precursor protein
APRT, adenine phosphoribosyltransferase
Arf, ADP-ribosylation factor
ArfGAP, ADP-ribosylation factor GTPase activating protein

ARHGEF, Rho guanine nucleotide exchange factor 2
ARL6IP, ADP-ribosylation-like factor 6 interacting protein
ARHGAP, Rho GTPase activating protein
ARID, AT rich interactive domain
ARL6IP, ADP-ribosylation-like factor 6 interacting protein
Arp, actin-related protein
aRPA, alternative replication protein A
ARPC, actin related protein 2/3 complex
ARPC5L, actin-related protein 2/3 complex subunit 5-like
ASAH, N-acylsphingosine amidohydrolase
ASAP, ArfGAP with SH3 domain, ankyrin repeat and PH domain
ASS, argininosuccinate synthase
ATF, activating transcription factor
ATG, autophagy-related protein
ATIC, 5-aminoimidazole-4-carboxamide ribonucleotide formyltransferase/IMP cyclohydrolase
ATL, atlastin GTPase
ATP, adenosine triphosphate
ATP6V1, V-type proton ATPase subunit, ATPase, adenosinetriphosphatase
ATPIF, ATPase inhibitory factor
AVPR, arginine vasopressin receptor
ATXN, ataxin
AZI, 5-azacytidine-induced
B2M, beta-2-microglobulin
BAD, BCL-2-associated death promoter
BACE, Beta-secretase
BAG, BCL2-associated athanogene
BANF, barrier to autointegration factor
BARD1, BRCA1-associated RING domain protein
BAX, Bcl-2–associated X protein
BCAP, B-cell receptor-associated protein
BCAT, branched chain amino-acid transaminase
BCL, B-cell lymphoma
BCR, breakpoint cluster region protein
BDH, 3-hydroxybutyrate dehydrogenase
BET1, blocked early in transport 1 homolog
BET1L, blocked early in transport 1 homolog-like
BLVR, biliverdin reductase
BP, binding protein
BRCA, breast cancer susceptibility protein
BRK, BRICK
BTF, basic transcription factor
BTF3L, basic transcription factor 3-like
BTG, B-cell translocation gene
C...orf, chromosome ... open reading frame
CALR, calreticulin
CaMK, calcium/calmodulin-dependent protein kinase
cAMP, cyclic adenosine monophosphate
CAND, cullin-associated NEDD8-dissociated protein
CANX, calnexin
Cap, cyclase associated protein
CAPN, calpain
CAPNS, calpain small subunit
CAPRIN, cell cycle associated protein
CARKD, carbohydrate kinase domain containing
CASC, cancer susceptibility candidate
CASP, caspase
CAST, calpastatin
CAV, caveolin
Cbp, Creb-binding protein
CBR, carbonyl reductase
CBX, chromobox homolog
CCDC, coiled-coil domain containing

CCN, cyclin

CCS, copper chaperone for superoxide dismutase

CCT, chaperonin containing T-complex protein

CCVs, clathrin-coated vesicles

CD, cluster of differentiation

CDC, cell division cycle

CDH, cadherin

CDKN, cyclin-dependent kinase inhibitor

CDKN2AIPNL, cyclin-dependent kinase inhibitor 2A interacting protein N-terminal like

CECR, cat eye syndrome chromosome region

CENP, centromere protein

CFL, cofilin

Cg, chorionic gonadotropin

CHCHD, coiled-coil-helix-coiled-coil-helix domain containing

Chi3l, chitinase 3-like

CHIKV, Chikungunya virus

CHMP, charged multivesicular body protein

CHP, calcium binding protein

CHRAC, chromatin accessibility complex

CHTOP, chromatin target of protein arginine methyltransferase

CIRBP, cold inducible RNA binding protein

CISD, CDGSH iron sulfur domain

Ck, casein kinase

CKAP, cytoskeleton associated protein

CKS, cyclin-dependent kinases regulatory subunit

CLDN, claudin

CLIC, chloride intracellular channel

CLINT, clathrin interactor

CLK, CDC-like kinase

CLUH, clustered mitochondria homolog

CMAS, cytidine monophosphate N-acetylneuraminic acid synthetase

CMC, cytochrome C oxidase assembly mitochondrial protein homolog

CMPK, cytidine monophosphate kinase

CMP-NeuNAc, cytidine 5'-monophosphate N-acetylneuraminic acid

CNN, calponin

CoA, coenzyme A

COA, cytochrome c oxidase assembly factor

COASY, CoA synthase

COMMD, copper metabolism domain containing

COP, coatomer protein

COPS, constitutive photomorphogenic homolog subunit

CORO, coronin, actin binding protein

COTL, coactosin-like

COX, cyclooxygenase

CpG islands, genomic regions where a cytosine nucleotide occurs next to a guanine nucleotide in the linear sequence of bases

CPNE, copine

CPSF, cleavage and polyadenylation specificity factor subunit

CPT, carnitine palmitoyltransferase

CRABP, cellular retinoic acid binding protein

Creb, cAMP response element-binding protein

CRIP, cysteine-rich protein

Crk, CT10 regulator of kinase, where CT10 is the avian virus from which was isolated a protein, lacking kinase domains, but capable of stimulating phosphorylation of tyrosines in cells

CRYZ, crystallin, zeta

CS, citrate synthase

CSE1L, chromosome segregation 1-like

CSK, cellular SRC tyrosine kinase

CSNK, casein kinase

CSRP, cysteine and glycine-rich protein

CST, cystatin
CSTF, cleavage stimulation factor
CTNN, catenin
CTNNBIP, catenin, beta interacting protein
CTTN, cortactin
CTNN, catenin
CU-rich, cytosine uracil rich
CUL, cullin
CX3CL, chemokine (C-X3-C motif) ligand
CYB5R, cytochrome b5 reductase
CYC, cytochrome c
CYFIP, cytoplasmic fragile X mental retardation protein interacting protein
cytochrome bc1, coenzyme Q : cytochrome c – oxidoreductase
CYCS, cytochrome c somatic
CYFIP, cytoplasmic fragile X mental retardation 1 interacting protein
CYP, cytochrome P450
D, dextrorotatory
D2HGDH, D-2-hydroxyglutarate dehydrogenase
DAD, defender against cell death
dADP, deoxyadenosine diphosphate
DAG, dystrophin-associated glycoprotein
DAK, dihydroxyacetone kinase 2 homolog
DAP, death-associated protein
DAPK, death-associated protein kinase
dATP, deoxyadenosine triphosphate
DBI, diazepam binding inhibitor
dCDP, deoxycytidine diphosphate
DCPS, decapping enzyme, scavenger
DCTN, dynactin
dCTP, deoxycytidine triphosphate
DCTPP, dCTP pyrophosphatase
DDAH, dimethylarginine dimethylaminohydrolase
DDX, DEAD (aspartic acid-glutamic acid-alanine-aspartic acid) box
DECR, 2,4-dienoyl CoA reductase
DEGS, delta(4)-desaturase, sphingolipid
DENR, density-regulated protein
DENV, dengue virus
dGTP, deoxyguanosine triphosphate
DHCR24, 24-dehydrocholesterol reductase
DHFR, dihydrofolate reductase
DHTKD, dehydrogenase E1 and transketolase domain containing
DFF, DNA fragmentation factor
DIABLO, direct inhibitors of apoptosis proteins binding protein with low isoelectric point
DLD, dihydrolipoamide dehydrogenase
DNAJ, DnaJ (Hsp40) homolog
DNM, dynamin
DNM1L, dynamin 1-like, receptor on the surface of cells that initiates apoptosis
DNPH, 2'-deoxynucleoside 5'-phosphate N-hydrolase
dNTPs, deoxynucleoside triphosphates
DOCK, dedicator of cytokinesis
DPCD, deleted in primary ciliary dyskinesia homolog
DPY, dosage compensation protein
DSBs, double-strand breaks
DSP, desmoplakin
dsRNA, double-stranded RNA
DSTN, destrin
dTTP, deoxythymidine triphosphate
DTYMK, deoxythymidylate kinase
DUB, deubiquitinating enzyme
DUSP, dual specificity protein phosphatase
DUT, dUTP pyrophosphatase
dUTP, deoxyuridine triphosphatase

DYNC1LI1, cytoplasmic dynein 1 light intermediate chain 1

DYNLRB, dynein light chain roadblock

E1, branched-chain alpha-keto acid decarboxylase

E2, lipoamide acyltransferase

E2f, a group of genes that codifies a family of transcription factors in higher eukaryotes

E3, lipoamide dehydrogenase

EB, end-binding protein

EBERs, Epstein-Barr virus-encoded RNAs

EBV, Epstein-Barr virus

EBP, emopamil binding protein

ECH, enoyl coenzyme A hydratase

ECHDC, enoyl CoA hydratase domain containing

ECHS, enoyl coenzyme A hydratase, short chain

ECM, extracellular matrix

EDF, endothelial differentiation-related factor

EEA, early endosome antigen

EEF, eukaryotic translation elongation factor

EFH, EF-hand domain

EFNA, ephrin-A

EGF, epidermal growth factor

EHHADH, enoyl-CoA, hydratase/3-hydroxyacyl CoA dehydrogenase

EIF, eukaryotic initiation factor

EJC, exon junction complex

ELAVL, embryonic lethal, abnormal vision-like

ELF, ETS-related transcription factor

ELK, ETS domain-containing protein

ENO, enolase

ENSA, endosulfine alpha

EPCAM, epithelial cell adhesion molecule

Eph, ephrin (receptor)

EPPK, epiplakin

EPRS, glutamyl-prolyl-tRNA synthetase

ER, endoplasmatic reticulum

ERCC6L, excision repair cross-complementing rodent repair deficiency, complementation group 6-like

ERF, ETS domain-containing transcription factor

ESYT, extended synaptotagmin-like protein

ETV, ets variant

ERH, enhancer of rudimentary homolog

ERK, extracellular signal-regulated kinase

Erm, family of three closely related proteins, ezrin, radixin and moesin

ERP, endoplasmic reticulum protein

ES, esterase

ESYT, extended synaptotagmin-like protein

ETHE, ethylmalonic encephalopathy

ETS, E-twenty six

EZR, ezrin

FAHD, fumarylacetoacetate hydrolase domain

FAM, family with sequence similarity

FARS, phenylalanyl-tRNA synthetase

FARSB, phenylalanyl-tRNA synthetase beta chain

FBLP, filamin-binding LIM protein

FBXO, F-box only protein

Fc, fragment, crystallizable

FCGR, Fc fragment of IgG receptor

FDPS, farnesyl diphosphate synthase

FDXR, ferredoxin reductase

FEN, flap endonuclease

FERMT, fermitin

FGF, fibroblast growth factor

FGFR, fibroblast growth factor receptors

FHL, four and a half LIM domains

FIS, fission

FKBP3, FK506 (tracolimus) binding protein

FLNB, filamin B
FOS, feline osteosarcoma virus
FPR, formyl peptide receptor
FRS, fibroblast growth factor receptor substrate
FSH, follicle stimulating hormone
FUBP, far upstream element (FUSE) binding protein
FURIN, feline sarcoma upstream region protein
FUS, fused in sarcoma
FXN, frataxin
G, guanine
G1 phase, first growth period of the cell cycle
GAA, glucosidase, alpha, acid
GABA, gamma-aminobutyric acid
GABARAP, gamma-aminobutyric acid receptor-associated protein
GABARAPL, gamma-aminobutyric acid receptor-associated protein-like
G-actin, actin with at first sight globular structure
GAF, gamma-activated factor
GALK, galactokinase
GAMT, guanidinoacetate N-methyltransferase
GAP, GTPase-Activating Protein
GAPDHS, glyceraldehyde-3-phosphate dehydrogenase, spermatogenic
GAPVD, GTPase activating protein and vacuolar sorting protein 9 domains
GAS, gamma activated sequence
GBF, golgi-specific brefeldin A-resistance guanine nucleotide exchange factor
GCG, glucagon
gdi, GDP-dissociation inhibitor
GDP, guanosine diphosphate
Gef, guanine nucleotide exchange factor
GFPT, glutamine-fructose-6-phosphate transaminase
GGCT, gamma-glutamylcyclotransferase
GJA, Gap junction alpha
GLG, Golgi glycoprotein
GMPS, guanine monphosphate synthetase
GNB2L1, guanine nucleotide- binding protein subunit beta-2-like 1
GNPDA, glucosamine-6-phosphate deaminase
GNPNAT, glucosamine-phosphate N-acetyltransferase
GNPTG, N-acetylglucosamine-1-phosphate transferase
GNRH, gonadotropin-releasing hormone
GOLM, Golgi membrane protein
GOLPH3L, golgi phosphoprotein 3-like
GOSR, Golgi SNARE complex
GP, glycoprotein
G protein, guanosine nucleotide-binding protein
GPRC, G-protein coupled receptor family C
GPSM, G-protein signaling modulator
GRB, growth factor receptor-bound protein
GRPEL, GrpE-like
GSDMD, gasdermin domain-containing protein
Gsk, glycogen synthase kinase
GSN, gelsolin
GSPT, G1 to S phase transition
GST, glutathione S-transferase
GTPBP, GTP binding protein
GTF, general transcription factor
GTP, guanosine-5'-triphosphate
GTPase, enzyme that hydrolyses GTP
H2AF, H2A histone family
H2BK120UB1, histone H2B 'Lys-120' monoubiquitination

H3K4me, histone H3 'Lys-4' methylation
H3K79me, histone H3 'Lys-79' methylation
HADH, hydroxyacyl-CoA dehydrogenase
HAGH, hydroxyacylglutathione hydrolase
HAP, huntingtin-associated protein
HARS, histidyl-tRNA synthetase
HB, hemoglobin
HBEGF, heparin-binding EGF-like growth factor
HBV, hepatitis B virus
HBZ, hemoglobin zeta
HCV, hepatitis C virus
Hdac, histone deacetylase
HDL, high density lipoprotein
HDLBP, high density lipoprotein-binding protein
HECTD, HECT domain containing E3 ubiquitin protein ligase
HELZ, helicase with zinc finger
HERC, HECT and RLD domain containing E3 ubiquitin protein ligase
HEX, hexosaminidase
HIBADH, 3-hydroxyisobutyrate dehydrogenase
HIBCH, 3-hydroxyisobutyryl-CoA hydrolase
HID, high-temperature-induced dauer-formation protein
HIF, hypoxia-inducible factor
HIGD, hypoxia inducible domain
HINT, histidine triad nucleotide-binding protein
HIP, HSC70 Interacting Protein
HIP1R, HSC70 Interacting Protein 1 related
HIST, histone
HIV, human immunodeficiency virus
HK, hexokinase
HMG, high-mobility group protein
HMGN, high mobility group nucleosomal binding domain
HMOX, heme oxygenase
HN, hematological and neurological expressed
HN1L, hematological and neurological expressed 1-like protein
HNF, hepatocyte nuclear factor
HNRNP, heterogeneous nuclear ribonucleoprotein particle
HORMAD, HORMA domain (named after the Hop1p, Rev7p and MAD2 proteins)
HPD, 4-hydroxyphenylpyruvate dioxygenase
HRAS, Harvey rat sarcoma viral oncogene homolog
HRSP, heat-responsive protein
HSC, heat shock protein
HSD17B, hydroxysteroid (17-beta) dehydrogenase
HSDL, hydroxysteroid dehydrogenase like
HSF, heat shock factor
Hsp, heat shock protein
HTT, huntingtin
HYOU, hypoxia up-regulated protein
HYPK, huntingtin interacting protein K
IARS, isoleucyl-tRNA synthetase
IAV, influenza A virus
ICT, immature colon carcinoma transcript
IDH, isocitrate dehydrogenase
IER, immediate early response
IFIT, interferon-induced protein with tetratricopeptide repeats
IFITM, interferon-induced transmembrane protein
IFN, interferon
IFRD, interferon-related developmental regulator
Ig, immunoglobulin
IGF, insulin-like growth factor
IGSF, immunoglobulin superfamily
IGF2R, Insulin-like growth factor 2 receptor

Ikb, inhibitor of kappa B
IKK, inhibitor of kappa B kinase
IL, interleukin
INSR, insulin receptor
IMMT, inner membrane protein, mitochondrial
IMPACT, imprinted and ancient gene
IN, internexin neuronal intermediate filament protein
INFG, interferon gamma
INIP, INTS3 (integrator complex subunit 3) and NABP (nucleic acid binding protein) interacting protein
IPO, importin
IPS, interferon-beta promoter stimulator
IQC, IQ motif containing
IRF, interferon regulatory factor
IRS, insulin receptor substrate
ISG15, interferon -stimulated 15 kDa gene
ISGF, interferon-stimulated transcription factor
ISOC, isochorismatase domain containing
ISRE, IFN stimulated response element
ITAP, inhibitor of T cell apoptosis protein
ITG, integrin
ITP, inosine triphosphate
ITPR, inositol trisphosphate receptor
ITPRIP, inositol 1,4,5-trisphosphate receptor interacting protein
JAK, Janus kinase
JEV, Japanese encephalitis virus
Jnk, c-Jun N-terminal kinases
JUN, oncoprotein
k, kinase
KA, kinase alpha
KARS, lysyl-tRNA synthetase
KCN, potassium intermediate/small conductance calcium-activated channel
kDa, kilo dalton
KDN, 2-keto-3-deoxy-D-glycero-D-galacto-nononic acid
KHSRP, KH-type splicing regulatory protein
KIT, gene encoding mast/stem cell growth factor receptor or CD117
KITLG, KIT ligand
KPN, karyopherin
KLHL, kelch-like
KRT, keratin
KYNU, kynureninase
LACRT, lacritin
LACV, La Crosse virus
LAM, laminin
LAMP, lysosomal-associated membrane protein
LAMTOR1, late endosomal/lysosomal adaptor, MAPK and MTOR activator 1, guanyl-nucleotide exchange factor, protein binding
LAP, leucine aminopeptidase
LASP, LIM and SH3 domain protein
LC, light chain
Ldh, lactate dehydrogenase
LDL, low-density lipoprotein
let, lethal
Lfa, lymphocyte function-associated antigen
LGALS, lectin, galactoside-binding, soluble
Lh, luteinizing hormone
LHPP, phospholysine phosphohistidine inorganic pyrophosphate phosphatase
LIM domain, protein structural domains, composed of two contiguous zinc finger domains, separated by a two-amino acid residue hydrophobic linker, initialy discovered in the proteins Lin11, Isl-1 & Mec-3
LMCD, LIM and cysteine-rich domains
LMN, lamin
LONP, lon peptidase

LPCAT, lysophosphatidylcholine acyltransferase
LRBA, lipopolysaccharide-responsive vesicle trafficking, beach and anchor containing
LRRC, leucine rich repeat containing
LSM, like smith antigen
LSS, lanosterol synthase
LUC7L, Luc7-like protein
LVV, leucine-valine-valine
LYPLA, lysophospholipase
LYRM, LYR motif containing
m1A58, N(1)-methyladenine at position 58
M6PR, mannose-6-phosphate receptor
Mac1, macrophage-1 antigen
MACROD, MACRO domain containing
MAGE, melanoma antigen
MAGOH, protein mago nashi homolog
MANF, mesencephalic astrocyte-derived neurotrophic factor
MAP, mitogen-activated protein
MAPK, mitogen-activated protein kinase
MAPKAPK, MAP kinase activated protein kinase
MAPRE, microtubule-associated protein, RP/EB family
MAPT, microtubule-associated protein tau
MATR, matrin
MAVS, mitochondria antiviral signaling protein
MBP, myelin basic protein
MCCC, methylcrotonoyl-CoA carboxylase
MCL, induced myeloid leukemia cell differentiation protein
MCM, mini-chromosome maintenance
MDH, malate dehydrogenase
MDM2, mouse double minute 2 homolog
MEIS, myeloid ecotropic viral integration site
Mek, mitogen-activated protein kinase
MESDC, mesoderm development candidate
Met, methionine
MeV, measles virus
MGST, microsomal glutathione S-transferase
MHC, major histocompatibility complex
MIA, melanoma inhibitory activity
miRNA, microRNA
miRNPs, microribonucleoproteins
MKNK/MNK, MAP kinase-interacting serine/threonine-protein kinase
Mlc, megalencephalic leukoencephalopathy with subcortical cysts
MMAB, methylmalonic aciduria (cobalamin deficiency) cblB type
MMP, matrix metallopeptidase
MNF, mitochondrial nucleoid factor
MPST, mercaptopyruvate sulfurtransferase
mRNA, messenger RNA
MRP, mitochondrial ribosomal protein
MSH, MutS homolog
MSK, mitogen- and stress- activated protein kinase
MTAP, methylthioadenosine phosphorylase
MTCH, mitochondrial carrier homolog
MTFR, mitochondrial fission regulator
MT-ND, mitochondrially encoded NADH dehydrogenase
MTOR, mammalian target of rapamycin
MT-P2RY, mitochondrially encoded purinergic receptor P2Y
MTX, metaxin
MUC, mucin
MutS, mutator S
MVP, major vault protein
MX1/MxA, myxovirus (influenza virus) resistance
MYC, myelocytomatosis oncogene
MYH, myosin, heavy chain

MYL6, myosin light polypeptide
MYLK, myosin light chain kinase
MYO, myosin
NAA, N(alpha)-acetyltransferase
MYOF, myoferlin
NABP, nucleic acid binding protein
NADH, reduced nicotinamide adenine dinucleotide
NADPH, reduced form of nicotinamide adenine dinucleotide phosphate
NANS, N-acetylneuraminic acid synthase
NAP1L, nucleosome assembly protein 1-like
NASP, nuclear autoantigenic sperm protein
NCAM, neural cell adhesion molecule
NCDN, neurochondrin
NCK, non-catalytic region of tyrosine kinase
NDRG, N-myc downstream regulated gene
NDUF, NADH dehydrogenase ubiquinone
NDUFV, NADH dehydrogenase ubiquinone flavoprotein
NEDD, neural precursor cell expressed, developmentally down-regulated
Neu5Gc, N-glycolylneuraminic acid
NeuNAc, N-acetylneuraminic acid
NFAT, nuclear factor of activated T-cells
NFS, nitrogen fixation homolog
NHP, non-histone protein
NFkB, nuclear factor kappa-light-chain-enhancer of activated B cells, lymphocytes derived from the bone marrow
NFS, nitrogen fixation
NHE, sodium-hydrogen antiporter
NHP2L1, non-histone chromosome protein 2-like
NIP, 4-nitrophenylphosphatase
NLS, nuclear localization signals
NMD, nonsense-mediated mRNA decay
NNT, nicotinamide nucleotide transhydrogenase
NOL, nucleolar protein
NOLC, nucleolar and coiled-body phosphoprotein
Nos, nitric oxide synthases
NP, nucleoproteins
NPC, nuclear pore complex
NPF, nucleation-promoting factor
NPM, nucleophosmin/nucleoplasmin
NQO, NAD(P)H dehydrogenase, quinone
NSF, N-ethylmaleimide-sensitive factor
NUBP, nucleotide binding protein
NUCKS, nuclear casein kinase and cyclin-dependent kinase substrate
NUDCD, nuclear distribution C domain containing
NUDT, nudix (nucleoside diphosphate linked moiety X)-type motif
NUPR, nuclear protein
OAS, 2'-5'-oligoadenylate synthetase
ODAM, odontogenic, ameloblast asssociated
OGFR, opioid growth factor receptor
OLA, Obg-like ATPase
OPTN, optineurin
ORMDL, orosomucoid 1-like
OSBP, oxysterol binding protein
OST, oligosaccharyltransferase
OSTC, oligosaccharyltransferase complex subunit
OSTF, osteoclast-stimulating factor
OTUB, otubain
p, protein
OXA1L, oxidase (cytochrome c) assembly 1-like
P4HB, prolyl 4-hydroxylase
Pa, protein activator
PA, proliferation-associated
PABPC, poly(A) binding protein cytoplasmic
PAFAH, platelet-activating factor acetylhydrolase

PAICS, phosphoribosylaminoimidazole carboxylase
Pak, p21 activated kinase
PALLD, palladin
PARK, parkinson disease (autosomal recessive, early onset)
PARP, poly (ADP-ribose) polymerase
PC, pyruvate carboxylase
PCBP, poly(rC)-binding protein
PCMT, protein-L-isoaspartate(D-aspartate) O-methyltransferase
PCNA, proliferating cell nuclear antigen
PDAP, PdgfA associated protein
PDCD, programmed cell death protein
PDE, phosphodiesterase
Pdgf, platelet-derived growth factor
Pdgfr, platelet-derived growth factor receptor
PDI, protein disulfide isomerase
PDK, pyruvate dehydrogenase kinase
PDX, pancreatic and duodenal homeobox
PE, phosphatidylethanolamine
PEA, phosphoprotein enriched in astrocytes
PEBP, phosphatidylethanolamine-binding protein
PER, period circadian protein homolog
PES, pescadillo ribosomal biogenesis factor
PEX, peroxisomal biogenesis factor
PFN, profilin
PGAM, phosphoglycerate mutase
PGD, phosphogluconate dehydrogenase
PGE, prostaglandin E
PGF, prostaglandin F
PGK, phosphoglycerate kinase
PGM, phosphoglucomutase
PGP, phosphoglycolate phosphatase
PGRMC, progesterone receptor membrane component
PHB, prohibitin
PHF, plant homeo domain finger protein
PHB, prohibitin
PHGDH, phosphoglycerate dehydrogenase
PHLD, pleckstrin homology-like domain
PICALM, phosphatidylinositol binding clathrin assembly protein
pik3/PI3K, phosphatidylinositide 3-kinase
pIgR, polymeric immunoglobulin receptor
PIN, peptidyl-prolyl cis-trans isomerase NIMA-interacting
PITRM, pitrilysin metallopeptidase
Pk, protein kinase
Pkg, cGMP-dependent protein kinase
PKM, pyruvate kinase muscle isozyme
PKN, protein kinase
PKR, protein kinase RNA-activated
PL, phospholipase
PLCG, phospholipase C gamma
PLEC, plectin
PLEKH, pleckstrin homology domain-containing
PLIN, perilipin
PLS, plastin
PLSCR, phospholipid scramblase
PLXN, plexin
PML, promyelocytic leukemia protein
PMPCB, peptidase (mitochondrial processing) beta
PNMA, paraneoplastic Ma antigen
PNRC, proline-rich nuclear receptor coregulatory protein
POR, P450 (cytochrome) oxidoreductase
PP, protein phosphatase
PPA, pyrophosphatase
PPAP, phosphatidic acid phosphatase
PPARG, peroxisome proliferator-activated receptor gamma

PPCS, phosphopantothenoylcysteine synthetase
PPM, protein phosphatase, Mg2+/Mn2+ dependent
PPP1R, protein phosphatase 1, regulatory (inhibitor) subunit
PPP6C, protein phosphatase 6, catalytic subunit
PPP-RNA, single-stranded RNA bearing a 5'-triphosphate group
PRAF, PRA1 domain family
PRCP, prolylcarboxypeptidase
PRDX, peroxiredoxin
PREX, phosphatidylinositol-3,4,5-trisphosphate-dependent Rac exchange factor
PRKAR, protein kinase, cAMP-dependent, regulatory
PRKCSH, human protein kinase C substrate
PRPF, pre-mRNA processing factor homolog
PRPH, peripherin
PSM, proteasome (prosome, macropain) subunit
PSPH, phosphoserine phosphatase
PTBP, polypyrimidine-tract binding protein
PTEN, phosphatase and tensin homolog
Ptk, protein tyrosine kinase
PTM, prothymosin
PTP, protein tyrosine phosphatase
PTPN, protein tyrosine phosphatase, non-receptor
PUS, pseudouridylate synthase
PVRL, poliovirus receptor-related
PXN, paxillin
PYCR, pyrroline-5-carboxylate reductase
PYCRL, pyrroline-5-carboxylate reductase-like
PYGB, phosphorylase, glycogen, brain
QPRT, quinolinate phosphoribosyltransferase
R/r, receptor
RA, receptor alpha chain
RAB, Ras-related protein
RAB11FIP, RAB11 family interacting protein
Rac, Ras-related C3 botulinum toxin substrate
RAD, Ras associated with diabetes
RAE, RNA export homolog
RAF, rapidly accelerated fibrosarcoma
RAL, Ras-related protein
RAN, Ras-related nuclear protein
RANBP, RAN binding protein
Ras, rat sarcoma, small GTPase protein
RASA, RAS p21 protein activator
RASGRF, Ras protein-specific guanine nucleotide-releasing factor
Rap, Ras-proximate
RB, retinoblastoma protein
RBBP, retinoblastoma binding protein
RBM, RNA-binding motif
RCN, reticulocalbin
RDX, radixin
RELA, transcription factor p65
RHO, Ras homolog
RIG, retinoid-inducible gene
RIMKL, ribosomal modification protein rimK-like
RMI, RecQ-mediated genome instability
RNase, ribonuclease
RNF, ring finger protein
RNH, ribonuclease inhibitor
RNPEP, arginyl aminopeptidase
Rnr, ribonucleotide reductase
Rock, Rho-associated, coiled-coil containing protein kinase
RP, ribosomal protein/replication protein
RPL, ribosomal protein, large
RPS, ribosomal protein S
rRNA, ribosomal RNA

RRP, ribosomal RNA processing homolog
RSK, ribosomal protein S kinase
RSU, Ras suppressor protein
RYR, ryanodine receptor
S, sinister (left)
S (concerning ribosome), Svedberg
S (concerning amino acid), serine
S1PR, sphingosine-1-phosphate receptor
SAMHD, sterile alpha motif and histidine-aspartic domain
Sapk, stress-activated protein kinase
SARNP, SAP (SAF-A/B, Acinus and PIAS) domain containing ribonucleoprotein
SARNP, SAP domain containing ribonucleoprotein
SART, squamous cell carcinoma antigen recognized by T cells
SATB, special AT-rich sequence-binding protein
SCF, stem cell factor
SCGB, secretoglobin
SCP, sterol carrier protein
SDC, syndecan
SDH, succinate dehydrogenase complex
SEC, signal peptidase complex
SELENBP, selenium-binding protein
SEPT, septin
SERF, small EDRK-rich factor
SERPIN, serine protease inhibitor
SERTAD, SERTA domain containing
SF, splicing factor
SFN, stratifin
SFPQ, splicing factor, proline- and glutamine-rich
SFV, Semliki forest virus
SGCB, sarcoglycan beta
SGT, small glutamine-rich tetratricopeptide repeat (TPR)-containing
SH, SRC homology
SH3GL, SH3-domain growth factor receptor-bound protein 2-like endophilin
Shc, SRC homology 2 domain containing
SINV, Sindbis virus
SKI, Sloan–Kettering institute
SKIL, SKI-like oncogene
SLC, solute carrier
SMC, structural maintenance of chromosomes protein
SNAP, SNARE associated protein
SNARE, soluble NSF attachment protein receptor
SNC, synuclein
SNRNP, small nuclear ribonucleoprotein polypeptide
SNX, sorting nexin
SORBS, sorbin and SH3 domain-containing protein
Sos, Son of Sevenless
SPA, sperm autoantigenic protein
SPCS, signal peptidase complex subunit homolog
S-phase, synthesis phase
SPI2A, serine protease inhibitor A
SPR, sepiapterin reductase
SPRYD, sprouty homolog domain
SPTAN, spectrin, alpha
SPTBN, spectrin, beta
SPZ, spermatogenic leucine zipper protein
SRC, sarcoma
SRI, sorcin
SRP, signal recognition particle
SRPR, signal recognition particle receptor
SRRM, serine/arginine repetitive matrix
SRSF, serine/arginine-rich splicing factor
SRXN, sulfiredoxin
SS, Sjogren syndrome

ssRNA, single-stranded RNA
SSR, signal sequence receptor
SST, somatostatin
ST, suppression of tumorigenicity
StAR, steroidogenic acute regulatory protein
STARD, StAR-related lipid transfer (START) domain containing
STAT, signal transducers and activators of transcription
STIP, stress-induced-phosphoprotein
STK, serine/threonine-protein kinase
STMN, stathmin
STOM, stomatin
STOML, stomatin (EPB72)-like
STX, syntaxin
SUCLG, succinyl-coenzyme A ligase
SUFU, suppressor of fused homolog
SUGT, suppressor of G2 allele of S-phase kinase-associated protein
SULF, sulfatase
SULT, sulfotransferase
SUMO, small ubiquitin-related modifier
SUPT16H, suppressor of Ty 16 homolog
SUPV3L, suppressor of var1, 3-like
SURF, surfeit
SYK, spleen tyrosine kinase
SYN, synapsin
SYNCRIP, synaptotagmin binding, cytoplasmic RNA interacting protein
SYNJ2BP, synaptojanin 2 binding protein
T, thymine
TAF, TATA-binding protein-associated factor, 150 kDa
TAGLN, transgelin
Tap, transporter associated with antigen processing
TARDBP, transactive response DNA binding protein
TARS, threonyl-tRNA synthetase
Tat, trans-activator of transcription
TAX1BP, Tax1 (human T-cell leukemia virus type) binding protein
TBK, serine/threonine-protein kinase
T-cells, thymus-derived lymphocytes
TCF, T-cell-specific transcription factor
TCHH, trichohyalin
TCHP, trichoplein keratin filament-binding protein
TCOF, Treacher Collins-Franceschetti syndrome
TCP, T-complex protein
TCR, T-cell receptor
TCV, transcytotic vesicles
TEFM, transcription elongation factor, mitochondrial
TERF, telomeric repeat binding factor
TERT, telomerase reverse transcriptase
TFAM, mitochondrial transcription factor A
TFG, tyrosine kinase-fused gene
TFRC, transferrin receptor
TGF, transforming growth factor
TGM, transglutaminase
TGN, trans-Golgi network
THOV, thogoto virus
TIMM, translocase of inner mitochondrial membrane homolog
TIP, Tat interactive protein
TJP, tight junction protein
TK, thymidine kinase
TLN, talin
Tlr, toll-like receptor
TMA, translation machinery associated homolog
TMED, transmembrane emp24 domain-containing protein
TMEM, transmembrane protein

TMPO, thymopoietin

TMX, thioredoxin-related transmembrane protein

TNF, tumor necrosis factor

TNFSF, tumor necrosis factor ligand superfamily member

TNPO, transportin

TOLLIP, Toll interacting protein

TOMM, translocase of outer mitochondrial membrane homolog

TP, tumor protein

TPD52L, tumor protein D52-like

TPP, tripeptidyl-peptidase

TPR, translocated promoter region

TPT, tumor protein translationally-controlled

TRADD, tumor necrosis factor receptor type 1-associated DEATH domain protein

TRA, transformer homolog

TRAP, TNF receptor-associated protein

TRAPP, transport protein particle

TRHR, thyrotropin-releasing hormone receptor

TRIM, tripartite motif containing

TRIP, thyroid receptor-interacting protein

TRMT, tRNA methyltransferase

tRNA, transfer RNA

troponin t, tropomyosin-binding subunit of troponin

TRPC, transient receptor potential canonical channels

TRPS, trichorhinophalangeal syndrome

TSC22D, TGF-beta-stimulated clone 22 domain family

TST, thiosulfate sulfurtransferase

TSTA, tissue-specific transplantation antigen

TUB, tubulin

TWF, twinfilin

TXNDC, thioredoxin domain containing

TYK, non-receptor tyrosine-protein kinase

TYMP, thymidine phosphorylase

U, uracil

U2AF, U2 small nuclear RNA auxiliary factor

UB, ubiquitin

UBCH, human polyubiquitin C

UBE, ubiquitin-conjugating enzyme

UBF, upstream binding factor

UBP43, same as USP18 in standard nomenclature

UBR, ubiquitin protein ligase E3 component n-recognin

UBXN, UBX domain containing protein

UCH, ubiquitin carboxyl-terminal esterase

UFD, ubiquitin fusion degradation protein

UFD1L, ubiquitin fusion degradation 1 like

UNC, uncoordinated protein

UQCR, ubiquinol-cytochrome c reductase

UQCRB, ubiquinol cytochrome c reductase binding protein

UQCRC, ubiquinol cytochrome c reductase complex

UQCRFS1, ubiquinol-cytochrome c reductase, Rieske iron-sulfur polypeptide 1

USMG, up-regulated during skeletal muscle growth homolog

USP, ubiquitin-specific peptidase

UTP, uridine-triphosphatase

UTR, untranslated region

UTRN, utrophin

V1 complex, VAMP, vesicle-associated membrane protein

VAP, (vesicle-associated membrane protein)-associated protein

VAT, vesicle amine transport protein

v-ATPase domain responsible for hydrolysis

VARS, valyl-tRNA synthetase

v-ATPase, vacuolar ATPase

VCAM, vascular cell adhesion molecule

VCL, vinculin

VCP, valosin containing protein

VDAC, voltage-dependent anion channel

Vegf, vascular endothelial growth factor

VHL, von Hippel-Lindau tumor suppressor, E3 ubiquitin protein ligase

VPS, vacuolar protein sorting protein

v-Ras, virus encoded Ras gene

v-SNARE, vesicle soluble NSF attachment protein receptor

VSV, vesicular stomatitis virus

VTA, vacuolar protein sorting 20-associated homolog

VTI, vesicle transport through interaction with t-SNAREs

WAC, WW domain containing adaptor with coiled-coil

WDR, WD (tryptophan-aspartic acid) repeat containing

WIBG, within benign gonial cell neoplasm homolog

Wnt, wingless/integration protein family

WNV, west Nile virus

XPO, exportin

YARS, tyrosyl-tRNA synthetase

YBX, Y box binding protein

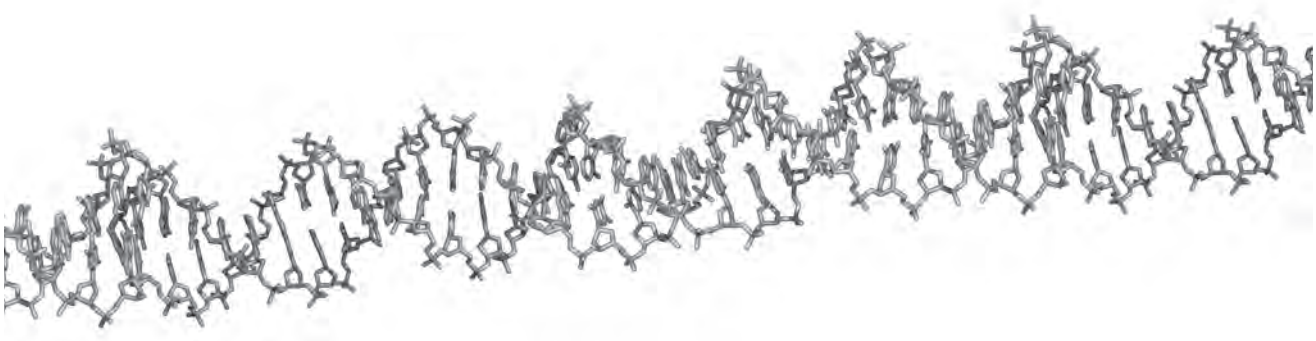
YIF1, YPT-interacting protein 1 interacting factor homolog

YTHDF, YTH domain family

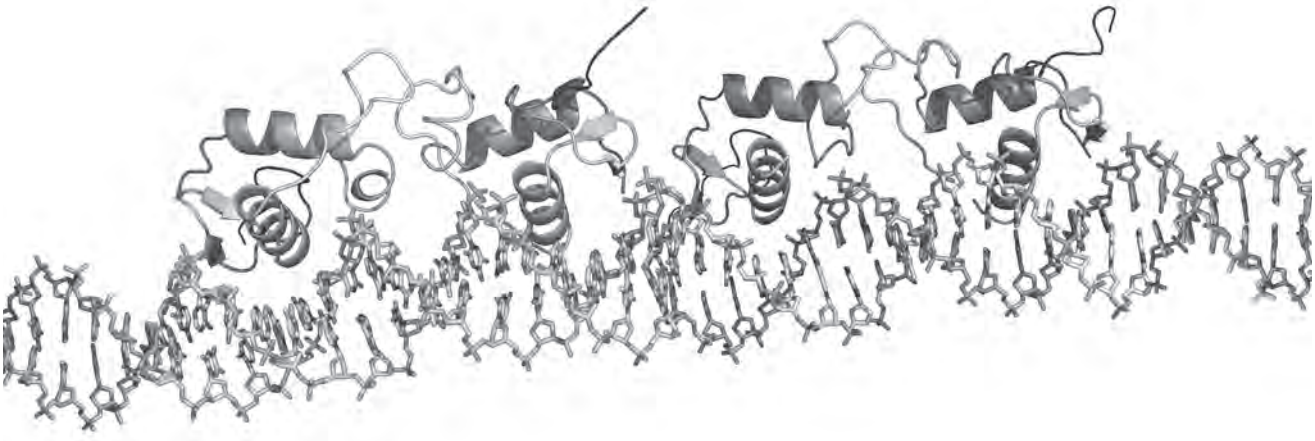
YWHAH, tyrosine 3-monooxygenase/ tryptophan 5-monooxygenase activation protein, eta polypeptide

ZNF, zinc finger protein

ZW, zeste white



6 Discussion



The aim of the present thesis was to elucidate the role of estrogen receptor (ER)α and ERβ in cell proliferation and apoptosis induced by estrogenic compounds. Special attention was paid to the importance of the receptor preference of the estrogenic compounds, the cellular ERα/ERβ ratio, the role of coregulators, and ER-mediated induction of protein expression.

Estrogenic compounds: agonists and antagonists

To cover a wide range of potential estrogenic actions, in addition to the reference compound 17β-estradiol (E2) [1-3], an ERα agonist (ERA-45) [4], an ERβ agonist (ERB-041) [5], a partial ERα antagonist (4-hydroxytamoxifen, 4OHT) [6-8], and a full ER antagonist (fulvestrant) [9-13] were investigated. For the coregulator research (chapter 3 and 4) also genistein, a phytoestrogen which was already well characterized for its effects on cell proliferation by Sotoca *et al.* [14], was included. The intrinsic relative potency (reflected by EC_{50}) and maximal efficacy of the selected ERα and ERβ agonists towards ERα and ERβ were determined in chapter 3 using ER-selective reporter gene assays. E2 showed a 9-fold lower EC_{50} value in the U2OS-ERα than in the U2OS-ERβ cell line. The EC_{50} and EEF (Estradiol Equivalence Factor) values corroborated selective ERα agonist activity for ERA-45, and selective ERβ agonist activity for ERB-041 and genistein. For the U2OS-ERα reporter gene assay, the order for efficacy of the ligands as derived from the height of the dose response curve induced by the ligand in the U2OS-ERα reporter gene assay was genistein = E2 ≈ ERA-45, while for the U2OS-ERβ reporter gene assay the order for efficacy was genistein > E2 > ERA-45 > ERB-041. Intrinsic relative potency and maximal efficacy of the selected antagonists towards ERα and ERβ were characterized in chapter 4 using ER-selective U2OS reporter gene assays performed in the presence of E2 at its EC_{50}. These studies indicated a preference of 4OHT to inhibit ERβ and found fulvestrant to be less ER-specific in its antagonizing activity. Efficacy of both antagonistic compounds towards both ERα and ERβ as derived from the lowest point of the dose response curve induced by the ligand in the U2OS reporter gene assays appeared similar.

Estrogen receptors

In previous studies it has been demonstrated that stimulation of ERα and ERβ may have opposite effects on cell proliferation [15-20]. Therefore it is of importance to use experimental models able to mimic physiologically relevant levels of ERα and ERβ. A model of interest consists of the T47D cell model with tetracycline-dependent ERβ expression and constant ERα expression developed by Ström *et al.* [19]. Chapter 2 describes under which conditions these T47D-ERβ breast cancer cells with tetracycline-dependent ERβ closely mimic ERα/ERβ ratios in breast and other estrogen-sensitive

tissues in vivo in rat as well as in human. At protein and messenger RNA (mRNA) level, ERα and ERβ levels and ratios were determined in T47D-ERβ cells exposed to a range of tetracycline concentrations and these levels were compared to levels found in breast, prostate, and uterus or endometrium tissue samples from both rat and human. The ERα/ERβ ratio found in rat mammary gland and in human breast tissue can be mimicked by exposing the T47D-ERβ cells to >150 ng/ml tetracycline, but the ERα/ERβ ratio of other estrogen-sensitive rat and human tissues can also be mimicked. Given the limited number of human samples available, it was not possible to also investigate factors underlying possible subtle differences in the ERα/ERβ levels and ratio between different subjects. One could expect that factors like (but not limited to) age [21], menstrual cycle [22], and consumption of supplements and food, especially for Asian countries where a lot of soy is consumed containing phytoestrogens such as genistein [23] influence ERα and ERβ levels and ratios. To be able to take these factors into account while analyzing data from different subjects, thereby enabling mimicking more personalized ERα and ERβ levels and ratios, tissue samples of a larger population should be studied.

In addition to the T47D-ERβ cells, ERα/ERβ ratios were also determined in MCF-7 and native T47D breast cancer cell lines and compared to ERα/ERβ ratios in rat and human tissues. The ERα/ERβ ratios in MCF-7 and native T47D cells were relatively high due to a lack of ERβ expression and therefore did not reflect ratios in analyzed rat and human tissues. This may indicate that these cell lines may not represent adequate models to study the effects of estrogens in healthy tissues. Given that these cell lines are derived from breast adenocarcinoma (MCF-7) [24] and breast ductal carcinoma (T47D) [25], and that tumor tissue is known to often express low levels of ERβ [26], the effects observed in the ERα-positive cell lines may rather represent responses of tumorous tissue.

In this context, it is of interest to note that at present no absolute quantitative techniques are available to determine ERα and ERβ levels and ratios. The qPCR and Western Blot analysis used in chapter 2 of the present thesis for determination of ER levels and ratios are semi-quantitative techniques [27, 28]. By using these two complementary techniques measuring mRNA and protein levels and making relative comparisons to T47D-ERβ cells not exposed to tetracycline for which levels were quantified in the same manner as for the test samples, the effect of antibody or probe specificity was eliminated. To be able to investigate ER levels in a more absolute quantitative manner, one might consider proteomics techniques. In that case, known concentrations of synthetic, isotope labeled peptides similar to the target peptides should be added to the sample under investigation, which subsequently should be analyzed by LC-MS/MS. The isotope labeled peptides and the unlabeled target peptides are analyzed simultaneously

and the abundance of the target peptide in the sample is compared to the abundance of the labeled peptide. By means of a standard curve, the initial concentration of the target peptide can then be determined. To be able to use this technique, isotope labeled peptides to determine ERα and ERβ levels would have to be developed, which is costly and time-consuming [29].

Coregulators

It is known that upon binding of an estrogenic compound to the estrogen receptor and dimerization of the ER, specific coregulators in the cell can play a key role in the gene transcription induced by ER binding. These coregulators are attracted to or rejected from the dimerized ligand-ER complex to enhance or oppose gene transcription by which the estrogenic compound exerts its effects [30]. In chapter 3 the modulation of the interaction of ERα and ERβ with various coregulators induced by estrogenic compounds was investigated. In this chapter the ligand-dependent modulation of the interaction of ERα and ERβ with coregulators was studied by using the Microarray Assay for Real-time Coregulator – Nuclear receptor Interaction (MARCoNI), with 154 unique nuclear receptor coregulator peptides derived from 66 different coregulators. The responses in the MARCoNI assay reveal that the modulation of the interaction of ERα or ERβ with coregulators induced by the different agonists upon ERα or ERβ activation are similar and imply only a limited number of differences upon ERα or ERβ activation by a specific ligand. For example modulation of the interaction of ERs with coregulator motif DDX5_133_155 showed positive modulation with ERβ, but not with ERα, and this effect was consistently seen for all ER agonists. DDX5 is a transcriptional coactivator for the tumor suppressor protein p53 and is involved in the p53 transcriptional response to DNA damage and p53-dependent apoptosis [31]. The modulation of the interaction of ERβ with this DDX5 coactivator, but not ERα, is in line with ERβ counteracting ERα-mediated cell proliferation by means of apoptosis. Based on the observed differences in type and extent of the modulation of the interaction of the ERs with these coregulators between the different agonists, the compounds could be hierarchically clustered, which is not possible based on the outcomes of ER-selective reporter gene or proliferation assays.

Upon characterizing the modulation of the interaction of ER subtypes with coregulators upon binding of selected ER agonists in chapter 3, chapter 4 describes the modulation of the interaction of ERα and ERβ with coregulators induced by the ER antagonistic compounds 4OHT and fulvestrant. The MARCoNI assay shows similar ERα- and ERβ-mediated modulation index (MI) profiles for 4OHT and fulvestrant, which are generally opposite to the coregulator MI profile of the ER agonist E2. One distinct

difference was observed between the MI profiles of 4OHT and fulvestrant. Fulvestrant shows negative and 4OHT positive modulation of the interaction of ERβ with coregulator motif DDX5_133_155. The recruitment of this DDX5 coactivator motif by ERβ as induced by tamoxifen could be beneficial for ERβ reducing ERα-mediated cell proliferation since it could stimulate p53-dependent apoptosis. It is of interest to note that previously tested agonistic compounds showed positive modulation of the interaction of ERβ with coregulator motif DDX5_133_155 as well. This could possibly be linked to the partial agonistic actions that tamoxifen and 4OHT have in specific tissues [32]. Hierarchical clustering based on the MI profiles could clearly discriminate the two ER antagonists from the ER agonist E2. However, the MARCoNI assay was unable to discriminate between the preferential ERβ antagonistic compound 4OHT and the less specific ER antagonist fulvestrant based on the modulation of the interaction of ERα and ERβ with coregulators. For both the ER agonists described in chapter 3 and the ER antagonists described in chapter 4 differences in the modulation of the interaction of ERα and ERβ with coregulators contribute to the ligand-dependent responses, but do not fully explain the differences in pharmacology between ER-mediated responses by the different ligands. Even though the functions of coregulators are known to some extent (Table 1), the importance of the subtle differences in modulation of the interaction of ERs with coregulators between the ER agonistic compounds E2, ERA-45, ERB-041, and genistein, and the ER antagonistic compounds 4OHT and fulvestrant for the ultimate biological effect remains to be established.

Such a comparative analysis of the modulation of the interaction of ERα and ERβ with various coregulators induced by different estrogenic compounds as shown in chapter 3 and 4 reveals a new research strategy. Although MI profiles by the different ERs for a given ligand show only relatively small differences, these differences might contribute to the ultimate effect on the compound's specific pharmacology. The data as described in chapter 3 and 4 are quite exploratory and the first results are not yet fully conclusive. However, the approach shown here by studying the differential interaction of ERα and ERβ with coregulators is promising and it is a potentially important aspect of future research into the interaction of ERα and ERβ with various coregulators, ER agonists and antagonists, and ultimate physiological effects of differential ERα and ERβ expression.

Table 1: Overview of coregulators of the MARCoNI assay and short transcriptional function description if known (main database www.uniprot.org and www.nursa.org).

Motif	Name	Function
ANDR_10_32	Androgen receptor-related coregulator	Unknown
BL1S1_1_11	Biogenesis of lysosome-related organelles complex 1 subunit 1	Unknown
BRD8_254_276	Bromodomain-containing protein 8	May act as a coactivator during transcriptional activation by hormone-activated nuclear receptors (NR). At least isoform 1 and isoform 2 are components of the NuA4 histone acetyltransferase (HAT) complex which is involved in transcriptional activation of select genes principally by acetylation of nucleosomal histones H4 and H2A. This modification may both alter nucleosome - DNA interactions and promote interaction of the modified histones with other proteins which positively regulate transcription. This complex may be required for the activation of transcriptional programs associated with oncogene and proto-oncogene-mediated growth induction, tumor suppressor-mediated growth arrest and replicative senescence, apoptosis, and DNA repair. NuA4 may also play a direct role in DNA repair when recruited to sites of DNA damage [33, 34].
CBP_2055_2077 CBP_345_367_C367S CBP_345_368 CBP_345_368_C367S CBP_57_80	Nuclear cap-binding protein	The CBP protein is known to acytelate histones as well as non-histone proteins, thereby enhancing transcriptional activity [35-38].

Table 1: Overview of coregulators of the MARCoNI assay and short transcriptional function description if known (main database www.uniprot.org and www.nursa.org). (*continued*)

Motif	Name	Function
CCND1_243_264_C243S/ C247S	Cyclin D1	Regulatory component of the cyclin D1-CDK4 (DC) complex that phosphorylates and inhibits members of the retinoblastoma (RB) protein family and regulates the cell-cycle during G_1/S transition. Phosphorylation of RB1 allows dissociation of the transcription factor E2F from the RB/ E2F complex and the subsequent transcription of E2F target genes which are responsible for the progression through the G_1 phase [39].
CENPR_159_177 CENPR_1_18	Centromere protein R	Transcription coregulator that can have both coactivator and corepressor functions. Acts as a coactivator for estrogen receptor alpha. Induces apoptosis in breast cancer cells, but not in other cancer cells, via a caspase-2-mediated pathway that does not require other caspases [40-42].
CHD9_1023_1045 CHD9_2018_2040 CHD9_855_877	Chromodomain-helicase-DNA-binding protein 9	Acts as a transcriptional coactivator for PPARA and possibly other nuclear receptors. Proposed to be a ATP-dependent chromatin remodeling protein. Has DNA-dependent ATPase activity and binds to A/T-rich DNA. Associates with A/T-rich regulatory regions in promoters of genes that participate in the differentiation of progenitors during osteogenesis [43, 44].

Table 1: Overview of coregulators of the MARCoNI assay and short transcriptional function description if known (main database www.uniprot.org and www.nursa.org). (*continued*)

Motif	Name	Function
CNOT1_140_162 CNOT1_1626_1648 CNOT1_1929_1951_ C1932S CNOT1_2083_2105 CNOT1_2086_2108 CNOT1_557_579	CCR4-NOT transcription complex subunit 1	Scaffolding component of the CCR4-NOT complex which is one of the major cellular mRNA deadenylases and is linked to various cellular processes including translational repression during translational initiation and general transcription regulation. Represses the ligand-dependent transcriptional activation by nuclear receptors [45, 46].
DDX5_133_155	DEAD box protein 5	DDX5 is a transcriptional coactivator for the tumor suppressor protein p53 and is involved in the p53 transcriptional response to DNA damage and p53-dependent apoptosis [31].
DHX30_241_262 DHX30_49_70	DEAH box protein 30	Unknown
EP300_2039_2061 EP300_69_91	E1A-associated protein p300	Functions as histone acetyltransferase and regulates transcription via chromatin remodeling. Acetylates all four core histones in nucleosomes. Histone acetylation gives an epigenetic tag for transcriptional activation. Also functions as acetyltransferase for nonhistone targets. Is proposed to indirectly increase the transcriptional activity of TP53 through acetylation. Can also mediate transcriptional repression [47-49].
GELS_376_398	Gelsolin	Unknown
GNAQ_21_43	Guanine nucleotide-binding protein alpha-q	Unknown
HAIR_553_575_C567S HAIR_745_767_C755S/ C759S	Hairless	Has been characterized as a corepressor for several members of the nuclear receptor superfamily [50, 51].

Table 1: Overview of coregulators of the MARCoNI assay and short transcriptional function description if known (main database www.uniprot.org and www.nursa.org). (*continued*)

Motif	Name	Function
IKBB_244_266 IKBB_277_299 IKBB_62_84	I-kappa-B-beta	Unknown
ILK_131_153	Integrin-linked protein kinase	Unknown
JHD2C_2054_2076	Probable JmjC domain-containing histone demethylation protein 2C	May be involved in hormone-dependent transcriptional activation [52].
KIF11_832_854_C854S	Kinesin-like protein KIF11	Component of a large chromatin remodeling complex [53].
L3R2A_12_34	Loss of heterozygosity 3 chromosomal region 2 gene A protein	Unknown
LCOR_40_62	Ligand-dependent corepressor	Repressor of ligand-dependent transcription activation by ERα and ERβ [54].
MAPE_249_271 MAPE_300_322 MAPE_356_378 MAPE_382_404_C388S MAPE_454_476_C472S MAPE_91_113	Melanoma antigen preferentially expressed in tumors	Functions as a transcriptional repressor, inhibiting the signaling of retinoic acid through the retinoic acid receptors RARA, RARB and RARG. Prevents retinoic acid-induced cell proliferation arrest, differentiation and apoptosis [55].
MED1_591_614 MED1_632_655	Mediator of RNA polymerase II transcription subunit 1	Component of the Mediator complex, a coactivator involved in the regulated transcription of nearly all RNA polymerase II-dependent genes. Mediator functions as a bridge to convey information from gene-specific regulatory proteins to the basal RNA polymerase II transcription machinery. Mediator is recruited to promoters by direct interactions with regulatory proteins and serves as a scaffold for the assembly of a functional preinitiation complex with RNA polymerase II and the general transcription factors [56-66].

Table 1: Overview of coregulators of the MARCoNI assay and short transcriptional function description if known (main database www.uniprot.org and www.nursa.org). (*continued*)

Motif	Name	Function
MEN1_255_277	Menin	Functions as a transcriptional regulator [67].
MGMT_86_108	Methylated-DNA--protein-cysteine methyltransferase	MGMT is a human DNA repair enzyme which carries out suicidal repair of mutagenic O(6)-alkylguanine lesions caused by alkylating carcinogens, undergoing conversion to R-MGMT in the process. The R-MGMT form has been characterized as an estrogen receptor corepressor [68, 69].
MLL2_4175_4197 MLL2_4702_4724	Myeloid/lymphoid or mixed-lineage leukemia protein 2	MLL2 is part of a complex shown to be a transcriptional regulator of beta-globin and estrogen receptor genes [70].
MTA1S_388_410_C393S/C396S	Metastasis-associated protein MTA1	May be involved in the regulation of gene expression by covalent modification of histone proteins. Isoform Long is a corepressor of estrogen receptor (ER). Isoform Short binds to ER and sequesters it in the cytoplasm and enhances non-genomic responses of ER [71, 72].
NCOA1_1421_1441 NCOA1_620_643 NCOA1_677_700 NCOA1_737_759	Nuclear receptor coactivator 1	Nuclear receptor coactivator that directly binds nuclear receptors and stimulates the transcriptional activities in a hormone-dependent fashion. Involved in the coactivation of different nuclear receptors, such as for steroids (PGR, GR and ER). Displays histone acetyltransferase activity toward H3 and H4. Plays a central role in creating multisubunit coactivator complexes that act via remodeling of chromatin, and possibly acts by participating in both chromatin remodeling and recruitment of general transcription factors [73-76].

Table 1: Overview of coregulators of the MARCoNI assay and short transcriptional function description if known (main database www.uniprot.org and www.nursa.org). (*continued*)

Motif	Name	Function
NCOA2_628_651 NCOA2_677_700 NCOA2_733_755 NCOA2_866_888	Nuclear receptor coactivator 2	Transcriptional coactivator for steroid receptors and nuclear receptors. Coactivator of the steroid binding domain (AF-2) but not of the modulating N-terminal domain (AF-1) [77].
NCOA3_104_123_N-KKK NCOA3_609_631 NCOA3_609_631_C627S NCOA3_673_695 NCOA3_725_747 NCOA3_ MOUSE_1029_1051	Nuclear receptor coactivator 3	Nuclear receptor coactivator that directly binds nuclear receptors and stimulates the transcriptional activities in a hormone-dependent fashion. Plays a central role in creating a multisubunit coactivator complex, which probably acts via remodeling of chromatin. Involved in the coactivation of different nuclear receptors, such as for steroids (GR and ER) [78].
NCOA4_315_337 NCOA4_79_101_C101S	Nuclear receptor coactivator 4	Enhances the androgen receptor transcriptional activity in prostate cancer cells. Ligand-independent coactivator of the peroxisome proliferator-activated receptor (PPAR) gamma [79].
NCOA6_1479_1501 NCOA6_875_897	Nuclear receptor coactivator 6	Nuclear receptor coactivator that directly binds nuclear receptors and stimulates the transcriptional activities in a hormone-dependent fashion. Coactivates expression in an agonist- and AF2-dependent manner. Involved in the coactivation of different nuclear receptors, such as for steroids (GR and ERs). Probably functions as a general coactivator, rather than just a nuclear receptor coactivator. May coactivate expression via a remodeling of chromatin and its interaction with histone acetyltransferase proteins [80-83].

Table 1: Overview of coregulators of the MARCoNI assay and short transcriptional function description if known (main database www.uniprot.org and www.nursa.org). (*continued*)

Motif	Name	Function
NCOR1_1925_1946 NCOR1_2039_2061 NCOR1_2039_2061_ C2056S NCOR1_2251_2273 NCOR1_2376_2398 NCOR1_662_684_C662S	Nuclear receptor corepressor 1	NCOR1 mediates transcriptional repression. It is part of a complex which promotes histone deacetylation and the formation of repressive chromatin structures which may hamper the access of transcription factors [84].
NCOR2_2123_2145 NCOR2_2330_2352 NCOR2_649_671_C649S	Nuclear receptor corepressor 2	Transcriptional corepressor. Mediates the transcriptional repression activity of some nuclear receptors by promoting chromatin condensation, thus preventing access of the basal transcription. Isoform 1 and isoform 5 have different affinities for different nuclear receptors [85, 86].
NELFB_328_350 NELFB_428_450 NELFB_80_102	Negative elongation factor B	NELFB in complex negatively regulates transcription elongation and causes transcriptional pausing [87, 88].
NR0B1_136_159 NR0B1_1_23 NR0B1_68_90_C69S	Nuclear receptor subfamily 0 group B member 1	Acts as a coregulatory protein that inhibits the transcriptional activity of nuclear receptors through heterodimeric interactions [89].
NR0B2_106_128 NR0B2_201_223_C207S NR0B2_237_257 NR0B2_9_31_C9S/C11S	Nuclear receptor subfamily 0 group B member 2	Unknown
NRBF2_128_150	Nuclear receptor-binding factor 2	May modulate transcriptional activation by target nuclear receptors. Can act as transcriptional activator (in vitro) [90].

Table 1: Overview of coregulators of the MARCoNI assay and short transcriptional function description if known (main database www.uniprot.org and www.nursa.org). (*continued*)

Motif	Name	Function
NRIP1_1055_1077 NRIP1_120_142 NRIP1_121_143_P124R NRIP1_173_195 NRIP1_173_195_C177S NRIP1_253_275_C263S NRIP1_368_390 NRIP1_488_510 NRIP1_700_722 NRIP1_701_723 NRIP1_805_831 NRIP1_8_30 NRIP1_924_946 NRIP1_924_946_C945S	Nuclear receptor-interacting protein 1	NRIP1 can both co-activate and co-repress transcription [91, 92].
NSD1_894_916 NSD1_982_1004	Nuclear receptor-binding SET domain-containing protein 1	Histone methyltransferase. Transcriptional intermediary factor capable of both negatively or positively influencing transcription, depending on the cellular context [93].
PAK6_248_270	p21-activated kinase 6	PAK6 kinase plays a role in the regulation of gene transcription. It inhibits androgen receptor and ERα-mediated transcription and may protect cells from apoptosis [94, 95].
PCAF_178_200	P300/CBP-associated factor	Functions as a histone acetyltransferase (HAT) to promote transcriptional activation [35].

Table 1: Overview of coregulators of the MARCoNI assay and short transcriptional function description if known (main database www.uniprot.org and www.nursa.org). (*continued*)

Motif	Name	Function
PELP1_142_164 PELP1_168_190 PELP1_20_42 PELP1_251_273 PELP1_258_280 PELP1_446_468 PELP1_496_518_C496S PELP1_56_78_C71S PELP1_571_593_C575S/C581S	Proline-, glutamic acid- and leucine-rich protein 1	Coactivator of estrogen receptor-mediated transcription and a corepressor of other nuclear hormone receptors and sequence-specific transcription factors. Plays a role in estrogen receptor (ER) genomic activity when present in the nuclear compartment by activating the ER target genes in a hormonal stimulation dependent manner. Plays a role in E2-mediated cell cycle progression. May have important functional implications in ER/growth factor cross-talk. May promote tumorigenesis via its interaction with and modulation of several oncogenes. Plays a role in cancer cell metastasis via its ability to modulate E2-mediated cytoskeleton changes and cell migration [96-101].
PIAS2_6_28	Protein inhibitor of activated STAT2	Plays a crucial role as a transcriptional coregulator in various cellular pathways, including the STAT pathway, the p53 pathway and the steroid hormone signaling pathway. The effects of this transcriptional coregulation, transactivation or silencing may vary depending upon the biological context and the PIAS2 isoform studied. However, it seems to be mostly involved in gene silencing. Binds to sumoylated ELK1 and enhances its transcriptional activity by preventing recruitment of HDAC2 by ELK1, thus reversing SUMO-mediated repression of ELK1 transactivation activity [102, 103].
PNRC1_306_327	Proline-rich nuclear receptor coactivator 1	Nuclear receptor coactivator. May play a role in signal transduction [104].

Table 1: Overview of coregulators of the MARCoNI assay and short transcriptional function description if known (main database www.uniprot.org and www.nursa.org). (*continued*)

Motif	Name	Function
PNRC2_118_139	Proline-rich nuclear receptor coactivator 2	Acts as a nuclear receptor coactivator [105].
PPRC1_1159_1181 PPRC1_151_173	Peroxisome proliferator-activated receptor gamma coactivator-related protein 1	PPRC1 acts as a coactivator during transcriptional activation of nuclear genes related to mitochondrial biogenesis and cell growth [106, 107].
PR285_1062_1084 PR285_1105_1127 PR285_1160_1182_C1163S PR285_2216_2238_C2219S PR285_432_454_C453S/C454S	Peroxisomal proliferator-activated receptor A-interacting complex 285 kDa protein	Unknown
PRDM2_948_970	PR domain-containing protein 2	PRDM2 is a tumor suppressor protein [108].
PRGC1_130_155 PRGC1_134_154	Peroxisome proliferator-activated receptor gamma coactivator 1-alpha	Transcriptional coactivator for steroid receptors and nuclear receptors [109].
PRGC2_146_166 PRGC2_338_358	Peroxisome proliferator-activated receptor gamma coactivator 1-beta	Plays a role of stimulator of transcription factors and nuclear receptors activities [110].
PRGR_102_124 PRGR_42_64_C64S	Progestrerone receptor	The steroid hormones and their receptors are involved in the regulation of eukaryotic gene expression and affect cellular proliferation and differentiation in target tissues. Progesterone receptor isoform B (PRB) is involved activation of c-SRC/MAPK signaling on hormone stimulation [111-116].
PROX1_57_79	Prospero homeobox protein 1	Prox1 is a prospero-related homeobox transcription factor that was originally characterized as a coregulator through its ability to suppress LRH-1-mediated transcription of the cholesterol 7-α-hydroxylase gene [117].

Table 1: Overview of coregulators of the MARCoNI assay and short transcriptional function description if known (main database www.uniprot.org and www.nursa.org). (*continued*)

Motif	Name	Function
RAD9A_348_370	Cell cycle checkpoint control protein RAD9A	A negative coregulator in the repression of androgen receptor transactivation in prostate cancer cells [118].
RBL2_875_897_C879S/ C894S	Retinoblastoma-like protein 2	Directly involved in heterochromatin formation by maintaining overall chromatin structure and, in particular, that of constitutive heterochromatin by stabilizing histone methylation. Recruits and targets histone methyltransferases, leading to epigenetic transcriptional repression. Probably acts as a transcription repressor by recruiting chromatin-modifying enzymes to promoters. May act as a tumor suppressor [119].
TF65_437_459	Transcription factor p65	Transcription factor present in almost all cell types and is the endpoint of a series of signal transduction events that are initiated by a vast array of stimuli related to many biological processes such as inflammation, immunity, differentiation, cell growth, tumorigenesis and apoptosis. Different dimer combinations act as transcriptional activators or repressors [120, 121].
TGFI1_325_347_C334S/ C346S TGFI1_443_461_C452S/ C455S	Transforming growth factor beta-1-induced transcript 1 protein	In the nucleus, functions as a nuclear receptor coactivator regulating glucocorticoid, androgen, mineralocorticoid and progesterone receptor transcriptional activity. May play a role in the processes of cell growth, proliferation, migration, differentiation and senescence. May have a zinc-dependent DNA-binding activity [122-127].

Table 1: Overview of coregulators of the MARCoNI assay and short transcriptional function description if known (main database www.uniprot.org and www.nursa.org). (*continued*)

Motif	Name	Function
TIF1A_373_395_C394S TIF1A_747_769	Transcription intermediary factor 1-alpha	TIF1A is a transcriptional coactivator that plays a role in the regulation of cell proliferation and apoptosis, at least in part via its effect on TP53 levels [128].
TIP60_476_498	60 kDa Tat-interactive protein	Catalytic subunit of the NuA4 histone acetyltransferase complex which is involved in transcriptional activation of select genes principally by acetylation of nucleosomal histones H4 and H2A. This modification may both alter nucleosome-DNA interactions and promote interaction of the modified histones with other proteins which positively regulate transcription. This complex may be required for the activation of transcriptional programs associated with oncogene and proto-oncogene-mediated growth induction, tumor suppressor-mediated growth arrest and replicative senescence, apoptosis, and DNA repair [34, 129-132].
TREF1_168_190 TREF1_850_872	Transcriptional-regulating factor 1	Activates transcription of CYP11A1. Interaction with CREBBP and EP300 results in a synergistic transcriptional activation of CYP11A1 [133].
TRIP4_149_171_C171S	Thyroid receptor-interacting protein 4	Transcription coactivator of nuclear receptors which functions in conjunction with CBP-p300 and SRC-1 and may play an important role in establishing distinct coactivator complexes under different cellular conditions. Plays a pivotal role in the transactivation of NF-kappa-B, SRF and AP1. Acts as a mediator of transrepression between nuclear receptor and either AP1 or NF-kappa-B. Plays a role in androgen receptor transactivation and in testicular function [134].

Table 1: Overview of coregulators of the MARCoNI assay and short transcriptional function description if known (main database www.uniprot.org and www.nursa.org). (*continued*)

Motif	Name	Function
TRRAP_3535_3557_ C3535S/C3555S TRRAP_770_792 TRRAP_971_993	Transformation / transcription domain-associated protein	Coactivator TRRAP is found in protein complexes possessing histone acetyltransferase activity. It is involved in transcription activation of for example proto-oncogene MYC but also of tumor suppressor gene p53 [135-137].
TRXR1_132_154	Thioredoxin reductase TR1	The selenoprotein thioredoxin reductase (TrxR1) is a modulator of estrogen signaling by binding to the estrogen receptors α and β by modulating the estrogen receptor-coactivator complex assembly on non-classical estrogen response elements such as AP-1 [138].
UBE3A_396_418 UBE3A_649_671	Ubiquitin-protein ligase E3A	Coactivator for the nuclear hormone receptor superfamily [139].
WIPI1_119_141 WIPI1_313_335_C318S	WD repeat domain phosphoinositide-interacting protein 1	Plays a distinct role in controlling the transcription of melanogenic enzymes and melanosome maturation, a process that is distinct from starvation-induced autophagy [140-142].
ZNHI3_89_111	Zinc finger HIT domain-containing protein 3	Unknown
ZNT9_449_471	Zinc transporter 9	Plays a role in the p160 coactivator signaling pathway that mediates transcriptional activation by nuclear receptors. Plays a role in transcriptional activation of Wnt-responsive genes [143].

Towards gene and protein expression

As described in the introduction of the current thesis, upon binding of an estrogenic compound to the estrogen receptor and dimerization of the ERs, attraction or rejection of specific coregulators, and binding to the estrogen response element (ERE), gene transcription is initiated and protein expression is induced [144]. This process will eventually lead to biological effects in the cells and organs. Cell proliferation and apoptosis are major biological events in every aspect of cell cycle control, but especially of interest in studying cancer progression [145, 146]. These events are known to be influenced by estrogenic compounds, exerting their effects through binding ERα and/or ERβ [145, 146]. ERα activation induces cell proliferation [15], while ERβ activation counteracts this and is suggested to induce apoptosis [16-20]. This latter aspect is of importance given that the ERα/ERβ ratio usually increases in tumorous tissue compared to normal tissue due to decreased ERβ expression [26]. One of the compounds in our test panel, 4OHT, is an active metabolite of the widely used breast cancer drug tamoxifen [6, 147]. To investigate whether 4OHT exerts ERα/ERβ ratio-dependent effects on cell proliferation and apoptosis, in chapter 5 a quantitative proteomics study is presented. ERα/ERβ ratio-dependent effects of 4OHT on cell proliferation and apoptosis of the T47D-ERβ human breast cancer cell line with tetracycline-dependent ERβ expression were characterized. In the cells expressing only ERα decreased cell proliferation and increased apoptosis was induced by 4OHT, which was opposite to the cells expressing both ERα and ERβ, for which increased cell proliferation and decreased apoptosis upon 4OHT exposure was found. Post-translational modifications like acetylation, methylation, and phosphorylation of several ribosomal and mitochondrial protein groups were induced by 4OHT, mostly in T47D-ERβ cells with both ERα and ERβ expressed. Altogether the results suggested that ERα/ERβ ratio-dependent major biological functions like cell proliferation and apoptosis are affected by 4OHT in T47D-ERβ cells. 4OHT may have differential effects in tissue, being more effective in reducing cell proliferation and increasing apoptosis if ERα dominates and ERβ expression levels are low since 4OHT then antagonizes ERα.

Biological effects

The current thesis focused on cell proliferation and apoptosis as major biological effects known to be influenced by estrogenic compounds and playing an important role in cancer progression [145, 146]. In chapter 2 it was demonstrated how different estrogen-responsive tissues may vary in their proliferative response by exposing T47D-ERβ cells exposed to defined tetracycline concentrations to mimic various tissues to E2. It is known that ERα and ERβ levels and ratios vary between tissues as well as between cell types [148]. This variability in ERα and ERβ levels and ratios will likely contribute to

the different effects estrogenic compounds can have on different tissues (for example tamoxifen which has anti-proliferative action in breast tissue but stimulates proliferation in the endometrium) [32]. However, also the difference in metabolizing capacity of the different tissues has been suggested to contribute to differential effects of estrogenic compounds. In different tissues, different cytochrome P450 (CYP) enzymes could be present [149-154]. In human endometrium for instance, formation of the DNA reactive α-hydroxytamoxifen metabolite of tamoxifen, a different metabolite than 4OHT, was shown [155, 156]. If the CYP enzymes present in the endometrium are more prone towards the formation of the DNA reactive α-hydroxytamoxifen metabolite than to 4OHT, this could be a (partial) explanation for the proliferative action of tamoxifen in the endometrium despite the relatively high ERα/ERβ ratio in this tissue. Furthermore, it was shown that after treatment with tamoxifen, tamoxifen-associated malignant endometrial tumors have more frequent ERβ expression compared to non-tamoxifen-associated endometrial cancers [157]. As shown in chapter 5 of the current thesis, 4OHT may be less effective in reducing cell proliferation and increasing apoptosis if ERβ expression levels are high since 4OHT then antagonizes ERβ. This might also be a (partial) explanation for the proliferative action of tamoxifen in the endometrium.

In chapter 3 it was demonstrated that ERα agonists activate cell proliferation, whereas ERβ seems to suppress ERα-mediated cell proliferation in the T47D-ERβ cells. These results corroborate that effects on cell proliferation depend on the intrinsic relative potency of the agonist towards ERα and ERβ and the cellular ERα/ERβ ratio. These ERα/ERβ ratio-dependent effects are especially of importance for anti-estrogenic compounds used in the hormonal treatment of ERα-positive breast cancer since the ERα/ERβ ratio usually increases in tumorous tissue compared to normal tissue due to decreased ERβ expression [26]. To obtain more insight in the 4OHT-induced ERα/ERβ ratio-dependent proteins that are able to affect cell proliferation and apoptosis, in chapter 5 a quantitative proteomics study was conducted, investigating ERα/ERβ ratio-dependent effects induced by 4OHT, the active metabolite of the breast cancer drug tamoxifen. One of the main conclusions from this proteomics study presented in chapter 5 is that 4OHT may have differential effects in tissues depending on the ERα and ERβ levels and ratios, being more effective in reducing cell proliferation and increasing apoptosis if ERα dominates and ERβ expression levels are low since 4OHT then antagonizes ERα. This conclusion suggests that it may be of value to monitor ERα and ERβ levels and ratios throughout breast cancer treatment with tamoxifen enabling more effective and personalized treatment.

Madeira *et al.* conducted a randomized neo-adjuvant trial for the treatment of postmenopausal occurring breast cancer, from which it was concluded that the ERα/ERβ ratio and ERβ levels could be used as predictors of endocrine therapy responsiveness [158]. However, in contrast to the data presented in chapter 5, these authors concluded that especially in ERβ-positive groups tamoxifen produced a significant reduction in post-treatment Ki67 scores, a marker for cell proliferation. The post-treatment Ki67 scores in their ERβ-positive group upon tamoxifen treatment and the ERβ-negative group were actually similar in percentage, and most probably not statistically significant due to the small group size. Furthermore, in both patient groups ERα-positive and ERα-negative cases were combined. Given the important role for ERα for 4OHT-mediated effects on cell proliferation and apoptosis, this combining of ERα-positive and ERα-negative cases hampers comparison of the data of the trial with the proteomics data presented in chapter 5.

It is also of interest to note that tamoxifen is commonly used in breast cancer treatment at an oral dose of 20 mg per day for 5 years [159]. This has shown to be an effective dose, but it is not known exactly how much of this tamoxifen and its active metabolite are available to the tumor cells. Plasma levels of 4OHT have been reported to be around 1495 pg/ml [160]. In the proteomics study presented in chapter 5, the cells were exposed to 300 nM 4OHT, the dose where a maximum inhibition of cell proliferation induced by 30 pM E2 was observed in the T47D-ERβ cell model to ascertain that the dose of 4OHT would be effective. This concentration of 300 nM 4OHT would (MW=387.51 [161]) correspond to 116 ng/ml, which is about 78 times the reported plasma levels. This is thus a relatively high dose compared to in vivo concentrations. However, it was shown in the BrdU cell proliferation assay that approximately 30 times more 4OHT is necessary than the amount of E2 to abolish the effect of E2 on cell proliferation (EC_{50} E2 is 30 pM, IC_{50} 4OHT is approximately 30x higher). The reported plasma levels of 4OHT of 1495 pg/ml would thus be able to abolish the effect of 130 pM E2. Plasma levels of E2 of postmenopausal tamoxifen treated women drop with around 12% [162]. Taking into account that normal E2 levels in plasma in postmenopausal woman are around 15 pg/ml [163], with a drop of 12% corresponding to 50 pM E2 (MW = 272.38 [164]), the amount of 4OHT relative to the amount of E2 in plasma is also high. To investigate if the changes detected by quantitative proteomics in the present thesis would be observed at lower 4OHT concentrations remains a topic of interest for future research.

Another point that should be taken into account when addressing the results described in the current thesis is the fact that ER-positive breast cancer treatment sometimes consists of a combination of therapeutic agents (for example a combination of tamoxifen and the aromatase inhibitor anastrozole) [165]. These compounds may

interact with cell proliferation or estrogen levels as well [166]. Although this thesis describes the interaction of different kinds of (anti-)estrogenic compounds with ERα and ERβ and eventually their effects on cell proliferation and apoptosis, interaction between those compounds and other therapeutics can enhance or attenuate the effects [167]. How the ERα and ERβ levels and ratios influence possible combination effects resulting from combined exposure remains a topic for future investigations. Each combination of therapeutic compounds might then be carefully investigated for its ultimate biological effects within a cellular background of different ERα and ERβ levels and ratios. This thesis described and validated possible approaches for such research into ERα and ERβ level- and ratio-dependent combination therapy in treatment of breast cancer.

Altogether, the present thesis describes how a selection of estrogenic compounds with different modes of action interact with ERα and ERβ and subsequently recruit or reject coregulators, and how this eventually leads to a shift in protein expression and affects major biological events like cell proliferation and apoptosis. The results obtained clearly corroborate the importance of ERα and ERβ levels and ratios, especially in tamoxifen treatment in breast cancer.

Future perspectives

The research in the present thesis focuses on the role of differential expression of ERα and ERβ and their ratios and how this affects cell proliferation and apoptosis induced by estrogenic compounds and the interaction of the ERs with coregulators upon stimulation by ligands. Although especially the importance of the receptor preference of the estrogenic compounds, the cellular ERα/ERβ ratio, the interaction of the ERs with coregulators upon stimulation by ligands, and ER subtype-mediated induction of protein expression is elucidated, there are some additional topics to be considered in future research. These are discussed in some more detail in the next sections.

Estrogenic compounds: agonists and antagonists

The compounds used in the present thesis were characterized for their potency and efficacy towards ERα and ERβ. From these results it was demonstrated that the selective ERβ agonist ERB-041 shows low potency and efficacy. Furthermore, it was demonstrated that 4OHT preferentially inhibits ERβ, whereas fulvestrant appears to be less ER subtype-specific. ER antagonistic compounds like tamoxifen and fulvestrant are used as breast cancer drugs for their ability to reduce ERα-mediated cell proliferation [13]. The downside of these compounds are their severe side effects [168, 169]. ERβ agonists are thought to have similar effects as the antagonistic compounds 4OHT and

fulvestrant as indicated in the current thesis, showing no induction of cell proliferation even when ERβ was fully suppressed and only ERα was expressed. In cell proliferation assays as described in chapter 3 of the current thesis, no effect on cell proliferation was observed upon exposure of the T47D-ERβ cells to ERB-041, although with the T47D-ERβ cells expressing ERβ, a suppression of cell proliferation was expected with this ERβ agonist since ERβ is known to suppress cell proliferation [16-20]. Suppression of cell proliferation could probably not be seen in the cell proliferation assay due to the low potency and efficacy of the ERB-041 compound.

Several of the reported side effects of tamoxifen and fulvestrant are related to the endometrium. Since ERβ agonists might have less or less severe side effects, at least on endometrium as shown for genistein [170], it is a challenge for pharmaceutical companies to develop a more potent and efficient ERβ agonist than ERB-041. Genistein might seem an interesting candidate given that genistein has a preference for ERβ with high potency and efficacy as shown in the U2OS reporter gene assays in chapter 3. However, in vitro and rodent data have also reported that genistein can stimulate tumor cell proliferation and growth [171]. The dual behaviour of genistein (and any newly developed ERβ agonist) as drugs or food supplements for postmenopausal women should therefore be carefully considered.

Estrogen receptors

In the current thesis the importance of ERα and ERβ levels and ratio for the ultimate biological effect of estrogenic compounds has been demonstrated by characterizing the effects of varying ERα and ERβ levels and ratio on estrogenic compound-induced cell proliferation and apoptosis. One aspect of estrogen receptors that has not been taken into account in the current thesis are splice variants. Splice variants are truncated (or sometimes elongated) forms of the ERs [172]. For ERα over 20 different splice variants have been reported in tumors detected on mRNA level [173]. Two of these isoforms, of which also protein expression could be detected, were reported to have opposing genomic actions compared to the full length ERα [174, 175]. Both of these isoforms have a truncated A/B domain and are missing their AF-1 region, involved in ligand-independent transactivation [174-176]. One of the two also has a shortened E and F domain and is missing its AF-2 region, affecting the ligand binding domain [175, 176]. For ERβ at least 5 different splice variants are known on mRNA level [177]. They are mostly truncated in the E and F domain, affecting the AF-2 region and thereby the activity of ERβ [178, 179]. It has been shown that different splice variants occur in breast tissue and breast cancer cell lines [172]. Since multiple splice variants have been reported to occur in a specific

cell line or in breast tissue, it is of interest to further elucidate the implications of the occurrence of these splice variants on their biological action.

Coregulators

The by the different agonists and antagonistic compounds induced interaction of ERα or ERβ with coregulators reveals that the modulation of the interaction of ERα or ERβ with coregulators upon stimulation of a specific (ant)agonist show similar MI profiles. It was evident that MI profiles for the antagonistic compounds were generally opposite to that of the agonistic compounds, and the MI profiles allowed hierarchical clustering and clear discrimination between agonists and antagonists and even between different agonists. This characteristic of the MARCoNI assay to discriminate and cluster estrogenic compounds is not defined by the conventional ER-selective reporter gene or proliferation assays. It is of importance to note that these interactions of ERα or ERβ with coregulators were investigated using the ligand binding domain (LBD) of ERα and ERβ. This LBD is known to be responsible for the interaction of the ER with ligands and coregulators in the cells [30]. Currently, this MARCoNI assay has not been used in combination with full length receptors. Given the previously described opposite effects that splice variants of estrogen receptors might have [174, 175], it could be of interest to use for example cell extract containing full length receptors in the MARCoNI assay. If the use of cell extract in the MARCoNI assay will prove to be possible it would also be of interest to use T47D-ERβ cells with tetracycline-dependent ERβ expression to be able to investigate differential effects occurring at varying ERα/ERβ ratios.

Furthermore, although for many of the coregulators present on the MARCoNI assay the function is more or less known (Table 1), the eventual biological effect of the complete set of modulated interactions of the ER with coregulators is not yet specified. The same holds true for the differential expression of coregulators in the different cells or organs and how coregulators interact with each other [180]. Since more and more proteins are identified as coregulators [181], it will be a great challenge in the near future to further elucidate their biological effects. In the current thesis, modulation profiles induced by the different ligands were compared to the compound-specific pharmacology. The MARCoNI assay based on the ERβ-LBD was conducted for the first time, and therefore the ligands were tested at concentrations expected to show maximum efficacy in the MARCoNI assay and compared to the efficacy of the ligands in the U2OS reporter gene assay and the cell proliferation assay. From the comparison between efficacy order of the ligands in the MARCoNI and the U2OS reporter gene assay it turned out that the efficacy of the ligands could not be explained by the induced MI profiles in the MARCoNI assay alone, suggesting that mechanisms other than coregulator recruitment to the LBD

are also involved in efficacy of the ligands. Next to the efficacy of the ligands, to study the effect of the interaction of ERα and ERβ with coregulators on potency reflected by EC_{50} as well, full dose response curves should be conducted to broaden the knowledge on ligand-dependent interactions with coregulators.

Towards gene and protein expression

The proteomics study described in chapter 5 indicates that in the cells expressing only ERα decreased cell proliferation and increased apoptosis was induced by 4OHT, which was opposite to the cells expressing ERα and ERβ, where increased cell proliferation and decreased apoptosis upon 4OHT exposure was indicated by protein expression. It was concluded that 4OHT may have differential effects in tissues, being more effective in reducing cell proliferation and increasing apoptosis if ERα dominates and ERβ expression levels are low since 4OHT then antagonizes ERα. This study was conducted for 4OHT, the active metabolite of tamoxifen, since cell proliferation and apoptosis are major biological events of interest in cancer progression [145, 146]. Tamoxifen is a first line breast cancer drug. If treatment with tamoxifen does not succeed, fulvestrant is commonly used as a second line breast cancer drug [182]. Given the ER subtype-dependent effects of 4OHT on protein level, it is recommended to conduct a similar proteomics study with fulvestrant to investigate ER subtype-dependent effects at protein level.

Biological effects

The importance of ERα and ERβ levels and ratios is especially illustrated in this thesis, as ultimate biological effects of estrogenic compounds like cell proliferation and apoptosis seem largely dependent on the intrinsic potency towards one of the ER subtypes and the cellular ERα and ERβ levels and ratios present. Especially the striking differences in ERα- and ERβ-mediated responses to 4OHT related to cell proliferation and apoptosis indicated in the proteomics study described in chapter 5 underline the importance of determination of both ERα and ERβ levels and their ratios in breast cancer treatment. It was concluded that 4OHT may have differential effects in different tissues, being more effective in reducing cell proliferation and increasing apoptosis if ERα dominates and ERβ expression levels are low since 4OHT then antagonizes ERα. It would be interesting to validate these results in a clinical study and monitor ER levels in patients receiving tamoxifen treatment. Based on the results of the present thesis it may be hypothesized that fulvestrant may be more effective than tamoxifen/4OHT as a breast cancer drug in tumors with relatively high ERβ expression levels, since in chapter 4 of the current thesis it was shown that fulvestrant is less potent in antagonizing ERβ than 4OHT is in the U2OS reporter gene assays. With relatively high ERβ levels and a low ERα/ERβ

ratio fulvestrant will be less prone to suppress the ERα inhibiting activity of ERβ thereby increasing proliferation instead of decreasing it as observed for 4OHT.

Often after about 15 months of breast cancer treatment with tamoxifen, tamoxifen resistance occurs [183]. One could speculate that the tumor cells adapt to the tamoxifen treatment by increasing ERβ expression, the action of which is then antagonized by tamoxifen overruling the effect of tamoxifen-mediated ERα antagonism, and facilitating cell proliferation. Speirs *et al.* already observed, by means of RT-PCR, that median ERβ mRNA levels were approximately 2-fold higher than ERα levels in tamoxifen-resistant tumors compared to tamoxifen-sensitive tumors [184]. It would be of interest to test this hypothesis by monitoring ERα and ERβ levels and ratios continuously during tamoxifen treatment, and possibly adjust treatment to for example fulvestrant upon detection of changing ERα and ERβ levels and ratios in the tumor tissue. However, this might be too invasive for the patient, since it would require regular biopsies.

The current thesis focuses on cell proliferation and apoptosis, major biological events known to be influenced by estrogenic compounds and playing an important role in cancer progression [145, 146]. Estrogenic compounds are involved in other biological effects as well, for example previously shown for immune responses [23]. It is important to keep this in mind when planning future research into the effects of estrogenic compounds. Also dose and absorption, distribution, metabolism, and excretion of the estrogenic compounds should be taken into account [185].

Concluding remarks

Results of the present thesis have elucidated the action of different estrogenic compounds, their interaction with the two ER subtypes, and the subsequent recruitment or rejection of coregulators, as well as the resulting effects on cell proliferation and apoptosis. The results illustrate the importance of ERα and ERβ levels and their ratios, especially in tamoxifen treatment in breast cancer. The action of estrogenic compounds and their interaction with ERs as described in the present thesis should be taken into account and can be used as a starting point for future research into mechanisms of action of estrogenic compounds, the role of coregulators, and endocrine treatment in breast cancer therapies.

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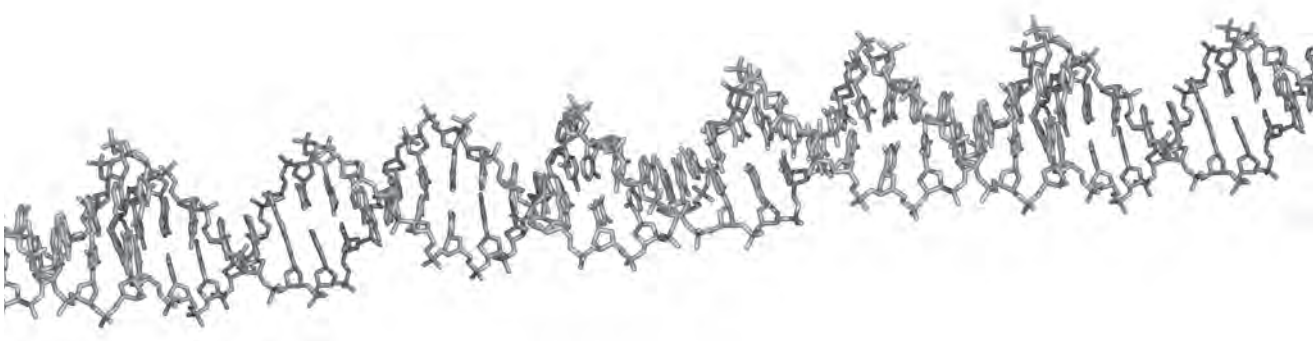
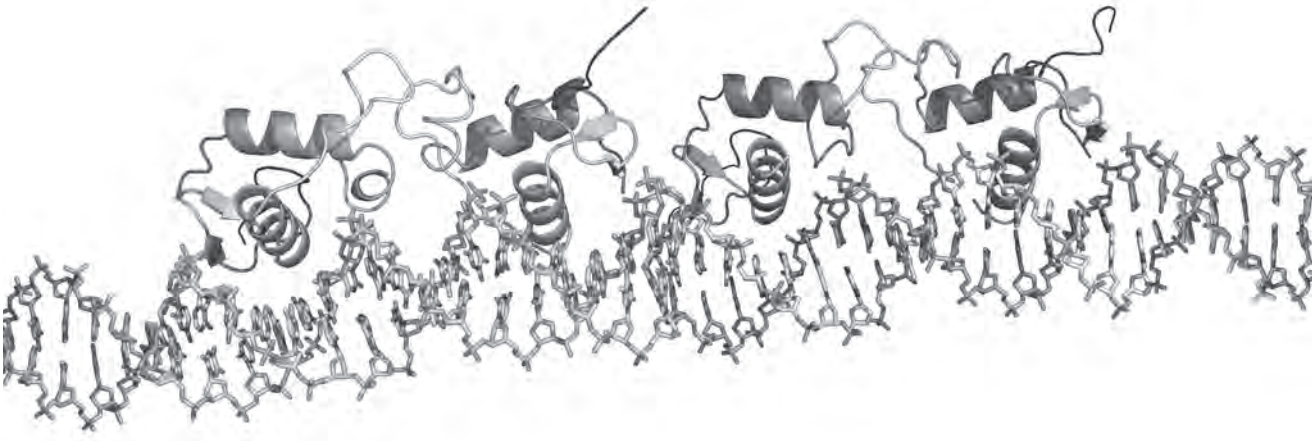


7

Summary



The aim of the current thesis is to elucidate the role of estrogen receptor (ER)α and ERβ in cell proliferation and apoptosis induced by estrogenic compounds. Special attention is paid to the importance of the receptor preference of the estrogenic compounds, the cellular ERα/ERβ ratio, the role of coregulators, and ER-mediated induction of protein expression. In **chapter 1** estrogenic compounds and their interaction with estrogen receptors are described and the two different estrogen receptors, ERα and ERβ, are introduced. It is described how estrogenic compounds eventually exert biological effects through coregulator recruitment upon ER binding, transcription initiation, and protein expression.

Chapter 2 describes under which conditions T47D-ERβ breast cancer cells with tetracycline-dependent ERβ expression and constant ERα expression best mimic ERα/ERβ ratios in breast and other estrogen-sensitive tissues in vivo in rat as well as in human. At protein and mRNA level, ERα and ERβ levels and ratios are determined in T47D-ERβ cells exposed to a range of tetracycline concentrations and in rat and human breast, prostate, and uterus or endometrium. The ERα/ERβ ratio in rat mammary gland and in human breast tissue can be mimicked by exposing the T47D-ERβ cells to >150 ng/ml tetracycline, but the ERα/ERβ ratio of other estrogen-sensitive rat and human tissues can also be mimicked. The ERα/ERβ ratios in MCF-7 and native T47D cells are high due to a lack of ERβ expression and therefore do not reflect ratios in rat and human tissues. It is demonstrated how these different tissues might vary in their proliferative response towards 17β-estradiol (E2) by exposing T47D-ERβ cells to E2 under defined tetracycline concentrations.

In **chapter 3** the modulation of the interaction of ERα and ERβ with coregulators in the ligand-dependent responses induced by estrogenic compounds is investigated. To this end, selective ERα and ERβ agonists are characterized for intrinsic relative potency reflected by EC_{50} and maximal efficacy towards ERα and ERβ in ER-selective reporter gene assays, and subsequently tested for stimulation of cell proliferation in T47D-ERβ cells with variable ERα/ERβ ratio and for ligand-dependent modulation of the interaction of ERα and ERβ with coregulators using the Microarray Assay for Real-time Coregulator – Nuclear receptor Interaction (MARCoNI) with 154 unique nuclear receptor coregulator peptides derived from 66 different coregulators. Results obtained reveal an important influence of the ERα/ERβ ratio and receptor selectivity of the compounds on stimulation of cell proliferation. ERα agonists activate cell proliferation whereas ERβ seems to suppress ERα-mediated cell proliferation. The responses in the MARCoNI assay reveal that the modulation of the interaction of ERα or ERβ with coregulators by a specific agonist are very similar indicating only a limited number of differences upon ERα or ERβ activation by a specific ligand. Differences in the modulation of the

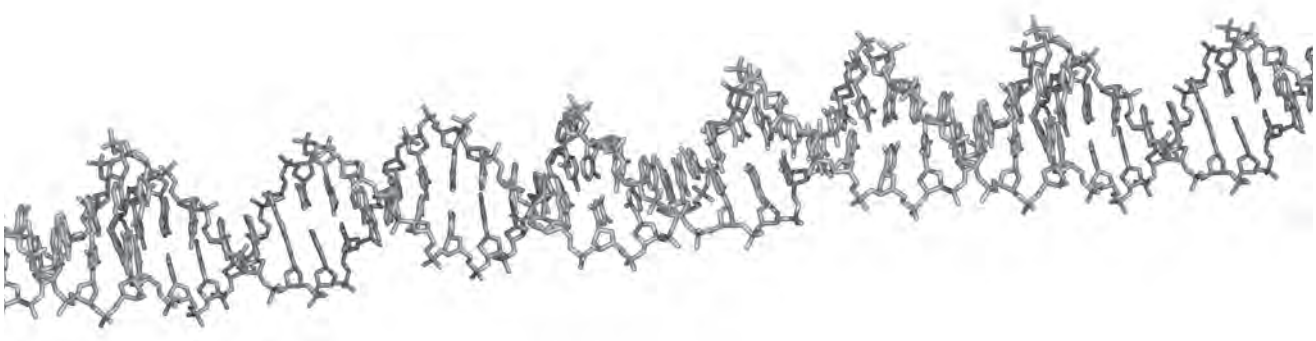
interaction of the ERs with coregulators between the different agonists are more substantial and can be used to classify the different agonists by hierarchical clustering. The results obtained corroborate that the ultimate effect of the model compounds on proliferation of estrogen-responsive cells depends on the intrinsic relative potency of the agonist towards ERα and ERβ and the cellular ERα/ERβ ratio whereas differences in the modulation of the interaction of the different ERs with coregulators for a given ligand might also contribute to the compound-specific pharmacology. Based on ligand-dependent differences in the modulation of the interaction of the ERs with coregulators, the MARCoNI assay is able to classify the different ERα and ERβ agonists discriminating between different agonists for the same receptor, a characteristic not defined by the ER-selective reporter gene or proliferation assays. It is concluded that differences in the modulation of the interaction of ERα and ERβ with coregulators contribute to the ligand-dependent responses but do not fully explain the differences in pharmacology between ER-mediated responses by the different estrogenic compounds.

To investigate if this is also the case for ER antagonists, **chapter 4** handles the modulation of the interaction of ERα and ERβ with coregulators in the ligand-dependent responses induced by the ER antagonistic compounds 4-hydroxytamoxifen (4OHT) and fulvestrant. Comparison of these results to ligand-dependent interaction of ERα and ERβ with coregulators expressed in modulation index (MI) profiles for the ER agonist E2 elucidates whether differences in the (ant)agonist-dependent interaction of ERα and ERβ with coregulators contribute to the differences in (ant)agonist responses. To this end, the selected ER antagonistic compounds are first characterized for intrinsic relative potency reflected by IC_{50} and maximal efficacy towards ERα and ERβ using ER-selective U2OS reporter gene assays, and subsequently tested for ligand-dependent modulation of the interaction of ERα and ERβ with coregulators using the MARCoNI assay. Results obtained with the U2OS reporter gene assays indicate a preference of 4OHT to bind ERβ and find fulvestrant to be less ER-specific. The responses in the MARCoNI assay reveal that ERα- and ERβ-mediated interaction with coregulators expressed in MI profiles are similar for 4OHT and fulvestrant and generally opposite to the MI profile of the ER agonist E2. Hierarchical clustering with Euclidian distance as the cluster distance metric, based on the MI profiles, is able to clearly discriminate the two compounds with ER antagonistic properties from the ER agonist E2. Taken together the data reveal that modulation of the interaction of ERs with coregulators discriminates ER agonists from antagonists but does not discriminate between the preferential ERβ antagonistic compound 4OHT and the less specific ER antagonist fulvestrant. It is concluded that differences in modulation of the interaction of ERα and ERβ with coregulators contribute to the differences in ligand-

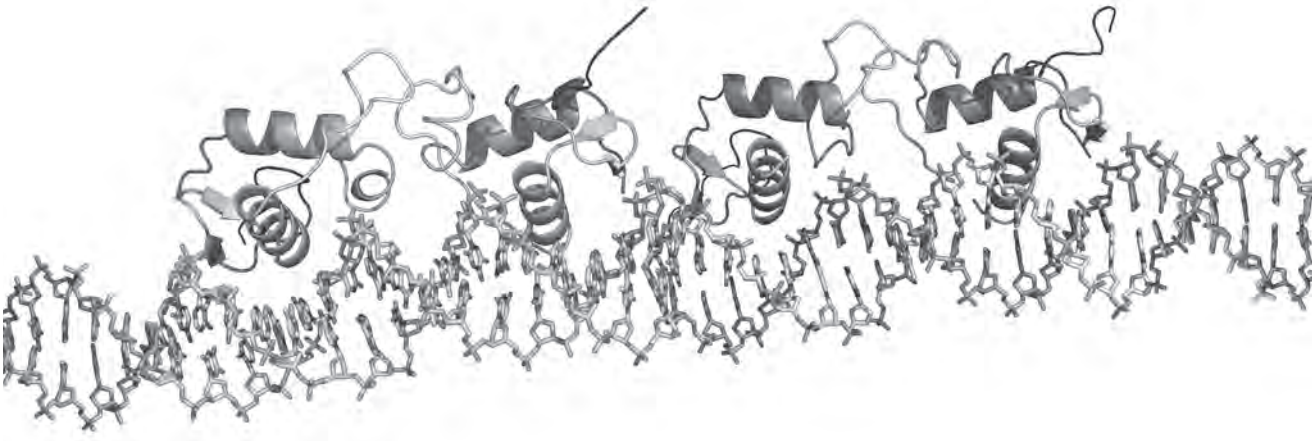
dependent responses induced by ER agonists and ER antagonists, but the importance of the subtle differences in modulation of the interaction of ERs with coregulators between the ER antagonistic compounds 4OHT and fulvestrant for the ultimate biological effect remains to be established.

To further investigate whether 4OHT, the active metabolite of the breast cancer drug tamoxifen, exerts ERα/ERβ ratio-dependent effects on cell proliferation and apoptosis, in **chapter 5** the results of a quantitative proteomics study are described. This is of importance given that the ERα/ERβ ratio usually increases in tumorous tissue compared to normal tissue due to decreased ERβ expression. ERα/ERβ ratio-dependent effects of 4OHT on cell proliferation and apoptosis of the T47D-ERβ human breast cancer cell line with tetracycline-dependent ERβ expression are detected. In the cells expressing only ERα decreased cell proliferation and increased apoptosis is induced by 4OHT, which is opposite to the effects detected in cells expressing ERα and ERβ, where increased cell proliferation and decreased apoptosis upon 4OHT exposure is found. Post-translational modifications like acetylation, methylation, and phosphorylation of several ribosomal and mitochondrial protein groups are induced by 4OHT, mostly in T47D-ERβ cells with both ERα and ERβ expressed. Altogether the results suggest that effects of 4OHT on major biological functions like cell proliferation and apoptosis in the T47D-ERβ cells are affected by the ERα/ERβ ratio. 4OHT may have differential cellular effects, being more effective in reducing cell proliferation and increasing apoptosis if ERα dominates and ERβ expression levels are low since 4OHT then antagonizes ERα.

Chapter 6 presents a discussion on the implications of the mechanisms of action of several estrogenic compounds discussed in this thesis. Altogether the results of the present thesis have elucidated the action of different estrogenic compounds, their interaction with the two ER subtypes, and the subsequent recruitment or rejection of coregulators, as well as the resulting effects on cell proliferation and apoptosis, and these results emphasize the importance of the ERα/ERβ ratio for the ultimate effects of estrogenic compounds on cell proliferation and apoptosis.



8 Samenvatting



Het doel van dit proefschrift is om te verhelderen wat de rol van oestrogeenreceptor (ER)α en ERβ is in door oestrogene stoffen geïnduceerde celproliferatie en apoptose. In het bijzonder is gekeken naar de invloed van de receptorvoorkeur van de oestrogene stoffen, de cellulaire ERα/ERβ-ratio, de rol van coregulatoren en ER-gemedieerde inductie van eiwitexpressie. In **hoofdstuk 1** worden oestrogene stoffen en hun interactie met oestrogeenreceptoren beschreven waarbij de twee verschillende oestrogeenreceptoren, ERα en ERβ, worden geïntroduceerd. Beschreven wordt hoe oestrogene stoffen uiteindelijk hun biologische effect uitoefenen door middel van coregulatorrekrutering die optreedt bij binding aan de ER, gevolgd door het initiëren van transcriptie en eiwitexpressie.

Hoofdstuk 2 beschrijft onder welke condities T47D-ERβ-borstkankercellen met tetracycline-afhankelijke ERβ-expressie en constante ERα-expressie het beste de ERα/ERβ-ratio's in borstweefsel en andere oestrogeengevoelige weefsels in vivo van zowel ratten als mensen imiteren. Op eiwit- en mRNA-niveau zijn de hoeveelheden ERα en ERβ en ERα/ERβ-ratio's bepaald in T47D-ERβ-cellen die blootgesteld zijn aan een serie tetracyclineconcentraties en in borst-, prostaat- en uterus- of endometriumweefsel van ratten en mensen. De ERα/ERβ-ratio die aanwezig is in borstweefsel van de mens en de rat kan worden geïmiteerd door de T47D-ERβ-cellen bloot te stellen aan >150 ng/ml tetracycline; de ERα/ERβ-ratio van andere oestrogeengevoelige weefsels kan ook worden geïmiteerd. De ERα/ERβ-ratio's van MCF-7- en oorspronkelijke T47D-cellen zijn hoog vanwege een gebrek aan ERβ-expressie en daarom reflecteren ze niet de ratio's die zijn gevonden in weefsel van ratten en mensen. Door T47D-ERβ-cellen bloot te stellen aan E2 onder gedefinieerde tetracyclineconcentraties is aangetoond hoe deze cellen en mogelijk ook de verschillende weefsels kunnen variëren in hun door 17β-oestradiol (E2) geïnduceerde proliferatieve reactie.

In **hoofdstuk 3** wordt de modulatie van de interactie van ERα en ERβ met coregulatoren in door oestrogene stoffen geïnduceerde ligandafhankelijke reacties onderzocht. De intrinsieke relatieve potentie van selectieve ERα- en ERβ-agonisten is gekarakteriseerd door de EC_{50} en maximale effectiviteit ten opzichte van ERα en ERβ in ER-selectieve U2OS-reportergenassays te bepalen. Vervolgens zijn deze agonisten getest op de mate waarin zij celproliferatie stimuleren in de T47D-ERβ-cellen met een variabele ERα/ERβ-ratio. Daarnaast zijn de agonisten getest op ligandafhankelijke modulatie van de interactie van ERα en ERβ met coregulatoren met behulp van de MicroarrayTest voor Real-time Coregulator – Nucleaire receptor Interactie (Microarray Assay for Real-time Coregulator – Nuclear receptor Interaction, MARCoNI) met 154 unieke nucleaire receptor coregulator peptiden verkregen vanuit 66 verschillende coregulatoren. De verkregen resultaten laten een belangrijke invloed van de ERα/

ERβ-ratio en receptorselectiviteit van de stoffen op de stimulatie van celproliferatie zien. ERα-agonisten activeren celproliferatie terwijl actievering van ERβ de ERα-gemedieerde celproliferatie lijkt te remmen. De reacties in de MARCoNI-assay onthullen dat de door een specifieke agonist-gemoduleerde interactie van ERα en ERβ met coregulatoren sterk op elkaar lijken en slechts een beperkt aantal verschillen laten zien na ERα- of ERβ-activering door een specifiek ligand. Verschillen in de modulatie van de interactie van de ERs met coregulatoren tussen de verschillende agonisten is substantiëler en is gebruikt om de verschillende agonisten te classificeren door middel van hiërarchische clustering. De verkregen resultaten bevestigen dat het ultieme effect van de modelstoffen op de proliferatie van oestrogeen-responsieve cellen afhankelijk is van de intrinsieke relatieve potentie van de agonist ten opzichte van ERα en ERβ en de cellulaire ERα/ERβ-ratio, terwijl verschillen in de modulatie van de interactie van de verschillende ERs met coregulatoren voor een gegeven ligand ook kunnen bijdragen aan de stofspecifieke farmacologie. De MARCoNI-assay blijkt, gebaseerd op ligandafhankelijke verschillen in de modulatie van de interactie van de ERs met coregulatoren, de verschillende ER-agonisten te kunnen classificeren en te kunnen discrimineren tussen verschillende agonisten voor dezelfde receptor. Over deze eigenschappen beschikken de ER-selectieve reportergen- en de proliferatie-assays niet. Uit deze resultaten is geconcludeerd dat de verschillen in modulatie van de interactie van ERα en ERβ met coregulatoren bijdragen aan de ligandafhankelijke reacties, maar dat deze niet geheel de verschillen in farmacologie verklaren tussen ER-gemedieerde reacties door de verschillende oestrogene stoffen.

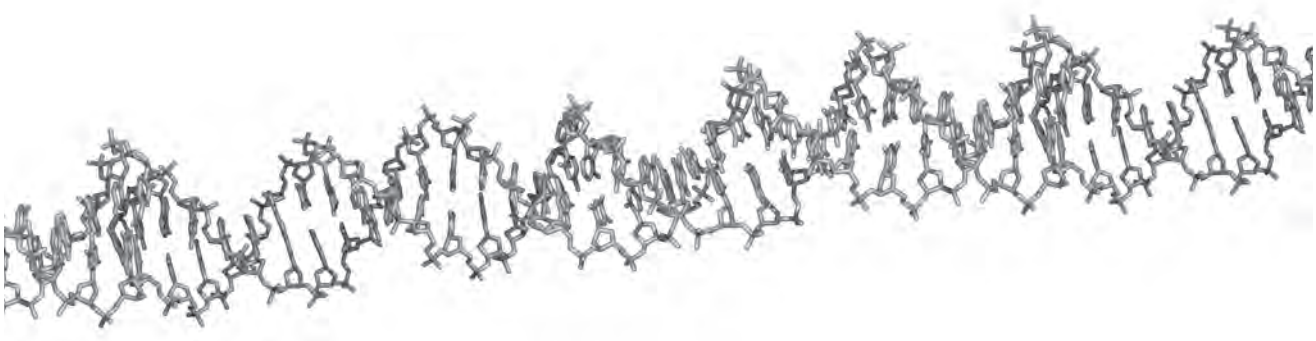
Om te onderzoeken of dit ook het geval zou zijn voor ER-antagonisten, wordt in **hoofdstuk 4** de modulatie van de interactie van ERα en ERβ met coregulatoren in de door de ER-antagonistische stoffen 4-hydroxytamoxifen (4OHT) en fulvestrant geïnduceerde ligandafhankelijke reacties behandeld. Vergelijking van deze resultaten met de ligandafhankelijke interactie van ERα en ERβ met coregulatoren voor de ER-agonist E2, uitgedrukt in modulatie-index (MI)-profielen, heeft verhelderd of verschillen in de (ant)agonistafhankelijke interactie van ERα en ERβ met coregulatoren bijdragen aan de verschillen in (ant)agonistische reacties. Om dit te bewerkstelligen zijn de geselecteerde ER-antagonistische stoffen eerst gekarakteriseerd op hun intrinsieke relatieve potentie gereflecteerd door IC_{50} en maximale effectiviteit ten opzichte van ERα en ERβ met behulp van ER selectieve U2OS reportergenassays. Vervolgens zijn ze getest op ligandafhankelijke modulatie van de interactie van ERα en ERβ met coregulatoren met behulp van de MARCoNI-assay. De resultaten verkregen met de U2OS reportergenassays wijzen op een voorkeur van 4OHT om aan ERβ te binden en op minder ER-specificiteit van fulvestrant. De reacties in de MARCoNI-assay onthullen

dat ERα- en ERβ-gemedieerde interactie met coregulatoren, uitgedrukt in MI-profielen, gelijk zijn voor 4OHT en fulvestrant en over het algemeen tegenovergesteld aan het MI-profiel van de ER-agonist E2. Hiërarchische clustering, met de Euclidische afstand als de clusterafstandmetriek, gebaseerd op de MI-profielen, blijkt duidelijk de twee ER-antagonistische stoffen te kunnen onderscheiden van de ER-agonist E2. Al met al onthullen de data dat de modulatie van de interactie van ERs met coregulatoren ER-agonisten van -antagonisten kan onderscheiden, maar geen onderscheid kan maken tussen de antagonistische stof 4OHT met een voorkeur voor ERβ en de minder specifieke ER-antagonist fulvestrant. Hieruit is geconcludeerd dat de verschillen in modulatie van de interactie van ERα en ERβ met coregulatoren bijdragen aan de verschillen in door ER-agonisten en ER-antagonisten geïnduceerde ligandafhankelijke reacties, maar het belang van de subtiele verschillen in de modulatie van de interactie van de ERs met coregulatoren tussen de ER-antagonistische stoffen 4OHT en fulvestrant voor het uiteindelijke biologische effect moet nog worden vastgesteld.

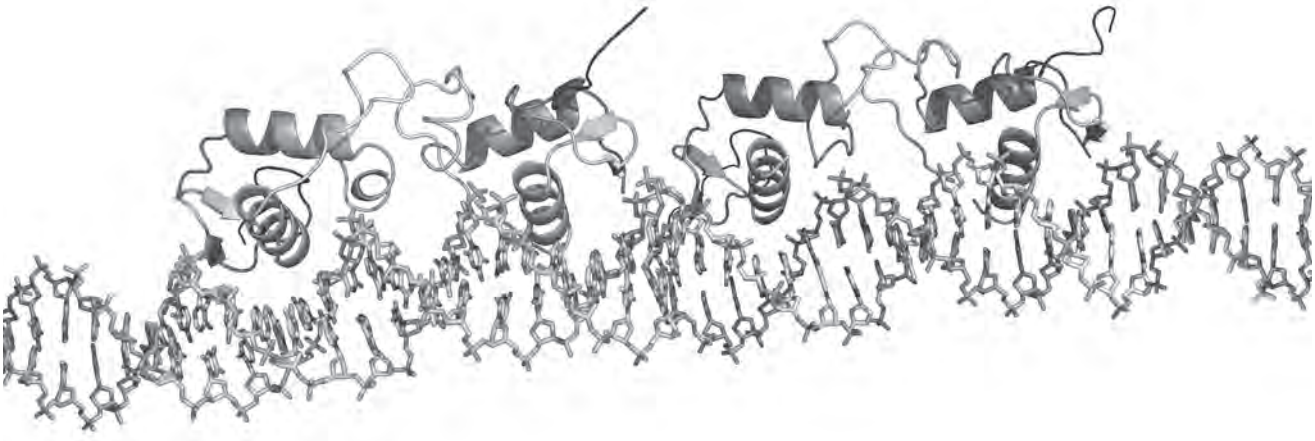
Om verder te onderzoeken of 4OHT, de actieve metaboliet van het borstkankermedicijn tamoxifen, ERα/ERβ-ratio-afhankelijke effecten vertoont op celproliferatie en apoptose, worden in **hoofdstuk 5** de resultaten van een kwantitatieve proteomics-studie beschreven. Dit is van belang aangezien de ERα/ERβ-ratio over het algemeen stijgt in tumorweefsel in vergelijking met normaal weefsel door een verminderde ERβ-expressie. Er zijn ERα/ERβ-ratio-afhankelijke effecten van 4OHT op celproliferatie en apoptose van de T47D-ERβ humane borstkankercellijn met tetracycline-afhankelijke ERβ-expressie gedetecteerd. In de cellen met enkel ERα-expressie induceert 4OHT verminderde celproliferatie en meer apoptose, wat omgekeerd is ten opzichte van de effecten gezien in cellen met zowel ERα- als ERβ-expressie, waar meer celproliferatie en verminderde apoptose wordt waargenomen na blootstelling aan 4OHT. Door blootstelling aan 4OHT worden post-translationele modificaties zoals acetylatie, methylatie en fosforylatie van verschillende ribosomale en mitochondriale eiwitgroepen geïnduceerd, vooral in T47D-ERβ-cellen met zowel ERα- als ERβ-expressie. Samengevat suggereren de resultaten dat effecten van 4OHT op belangrijke biologische functies zoals celproliferatie en apoptose in de T47D-ERβ-cellen worden beïnvloed door de ERα/ERβ-ratio. 4OHT kan verschillende cellulaire effecten hebben, waarbij het effectiever is in het reduceren van celproliferatie en het verhogen van apoptose als ERα dominant is en ERβ-expressieniveaus laag zijn omdat 4OHT dan ERα tegenwerkt.

In **hoofdstuk 6** worden de implicaties van de werkingsmechanismen van verschillende oestrogene stoffen in dit proefschrift bediscussieerd. Samengevat hebben de resultaten van dit proefschrift zowel de acties van verschillende oestrogene

stoffen, als hun interactie met de twee ER-subtypen en de daaropvolgende rekrutering of afstoting van coregulatoren verhelderd, evenals de resulterende effecten op celproliferatie en apoptose. Hierbij ligt de nadruk op het belang van de ERα-/ERβ-ratio voor de uiteindelijke effecten van oestrogene stoffen op celproliferatie en apoptose.



Appendix



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Curriculum vitae

Nynke Evers was born on October 3rd, 1984 in Amersfoort and grew up in Scherpenzeel. After her graduation from secondary school at Rembrandt college in Veenendaal in 2003, she started her study Nutrition and Health at Wageningen University. She obtained her BSc degree in 2008. During her master study, Nynke completed two MSc theses; one about the effect of processing on peanut allergenicity at the department of Cell Biology and Immunology and one about the role of surface charge and oxidative stress on the cytotoxicity of nanoparticles towards macrophages at the division of Toxicology, both at Wageningen University. She conducted her internship at TNO Quality of Life in Zeist, on the subject of metabolic activation and genotoxic potential of benzo[a]pyrene in the skin. Nynke obtained her MSc degree in Nutrition and Health in 2009. From September 2009 until August 2013, she worked as a PhD student on the project presented in the current thesis, which was a collaboration between the division of Toxicology and MSD, Oss. During her PhD study, she completed several postgraduate courses in toxicology, which will enable her to register as a European toxicologist. Nynke is currently working as a regulatory affairs officer at VSM Geneesmiddelen bv in Alkmaar.

List of publications and abstracts

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S. Bhattacharjee, L.J.H. de Haan, N.M. Evers, X. Jiang, A.T.M. Marcelis, H. Zuilhof, I.M.C.M. Rietjens, G.M. Alink. Role of surface charge and oxidative stress in cytotoxicity of organic monolayer-coated silicon nanoparticles towards macrophage NR8383 cells. *Particle and Fibre Toxicology 7 (2010) 1-12.*

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N.M. Evers, T.M.C. van de Klundert, Y.M. van Aesch, S. Wang, W.K. de Roos, A. Romano, L.H.J. de Haan, A.J. Murk, A.G.H. Ederveen, I.M.C.M. Rietjens, J.P. Groten. Human T47D breast cancer cells with tetracycline-dependent ER expression reflect ERα/ERβ ratios in rat and human breast tissue. *Toxicology in Vitro 27 (2013) 1753-1761.*

N.M. Evers, J.H.J. van den Berg, S. Wang, R. Houtman, D. Melchers, L.H.J. de Haan, A.G.H. Ederveen, J.P. Groten, I.M.C.M. Rietjens. Cell proliferation and modulation of interaction of estrogen receptors with coregulators induced by ERα and ERβ agonists. *Submitted for publication.*

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N.M. Evers, S. Boeren, J.H.J. van den Berg, J.P. Groten, I.M.C.M. Rietjens, J.J.M. Vervoort. Quantitative proteomics addressing the estrogen receptor subtype-mediated effects in T47D breast cancer cells exposed to 4-hydroxytamoxifen. *Submitted for publication.*

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L.H.J. de Haan, N.M. Evers, S. Bhattacharjee, Ton Marcelis, Han Zuilhof, I.M.C.M. Rietjens, G.M. Alink. Effect of silicon nanoparticles with differently charged attached organic monolayers on viability, phagocytosis index, intracellular ROS formation and MIP-2 production in the rat macrophage cell line NR8383. *Poster presentation at the*

Anniversary Meeting of the Dutch Society of Toxicology (NVT, Nederlandse Vereniging voor Toxicologie), 18-19 June 2009, Veldhoven, the Netherlands.

N.M. Evers, J.H. J. van den Berg, A.M. Sotoca Covaleda, L. Bastos Sales, L.M. Kuiper, A.G.H. Ederveen, A.J. Murk, I.M.C.M. Rietjens, J.P. Groten. Linking differential ERα/ERβ expression of human T47D breast cancer cells to ERα/ERβ ratio in rat and human tissues. *Poster presentation at the Annual Meeting of the NVT, 1-2 June 2010, Zeist, the Netherlands.*

N.M. Evers, A.G.H. Ederveen, A.J. Murk, I.M.C.M. Rietjens, J.P. Groten. The role of ERα and ERβ in the risk benefit analysis of mixtures of estrogens; effect of differential ER expression on cell proliferation and apoptosis. *Abstract in 'Current research 2010' by VLAG graduate school.*

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N.M. Evers, A.J. Murk, A.G.H. Ederveen, I.M.C.M. Rietjens, J.P. Groten. Human T47D breast cancer cells with tetracycline-dependent ERβ expression as an in vitro model for ERα/ERβ ratio-dependent responses in breast tissue. *Poster presentation at the Society of Toxicology (SOT) 51st Annual Meeting and ToxExpo, 11-15 March 2012, San Francisco, USA.*

N.M. Evers, S. Boeren, J.P. Groten, J. Vervoort, I.M.C.M. Rietjens. Protein expression and epigenetic changes in human T47D breast cancer cells with varying intracellular ERα/ERβ ratio upon exposure to 4OH-tamoxifen.*Poster presentation at the SOT 52nd Annual Meeting and ToxExpo, 10-14 March 2013, San Antonio, USA.*

Overview of completed training activities

Discipline-specific courses

Toxicogenomics, PET (Postgraduate Education in Toxicology), 2010
Reproductive toxicology, PET, 2010
Ecotoxicologie, PET, 2011
Risk assessment, PET, 2011
Organ toxicology, PET, 2011
Proteomics, VLAG, 2011
Mutagenesis & carcinogenesis, PET, 2012

Attended conferences

Benelux nuclear receptor meeting, Oegstgeest, the Netherlands, 2009
Annual meeting of the NVT, Zeist, the Netherlands, 2010
Annual Meeting of the NVT, Zeist, the Netherlands, 2011
SOT annual meeting and ToxExpo, San Francisco, USA, 2012
SOT annual meeting and ToxExpo, San Antonio, USA, 2013

General courses

VLAG PhD week, VLAG, 2010
Medical, forensic and regulatory toxicology**,** PET, 2010
Coaching in effectiveness, Meijer and Meijaard, 2011
Mobilising your -scientific- network, Wageningen Graduate Schools (WGS), 2013

Optional activities

Preparing PhD research proposal, 2009
Attending scientific presentations at the division of Toxicology, 2009-2013
MSc course proefdierkunde, WUR, 2010
Toxicology PhD trip to Switzerland and Italy, 2011

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