

Unravelling the mechanism of
differential biological responses
induced by food-borne xeno- and
phyto-estrogenic compounds

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Unravelling the mechanism of differential biological responses induced by food-borne xeno- and phyto-estrogenic compounds

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“Caminante no hay camino, se hace camino al andar.
Al andar se hace camino, y al volver la vista atrás
se ve la senda que nunca se ha de volver a pisar”

- *Antonio Machado* -

A mi madre.

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General Introduction

Estrogen receptors and cancer: A Ying-Yang relationship

Estrogens are a family of steroid hormones that are needed for normal developmental, physiological, and reproductive processes in vertebrates (1). In addition to the natural estrogens including 17β -estradiol (E2), there are many estrogen-like chemicals that can mimic the function of E2. For many decades, exposure to estrogen-like compounds has been related to adverse health effects including increased cancer risks. Increased exposure to estrogens has been linked to increased risk of developing breast and uterus cancer in women (2) and increased occurrence of prostate and testicular cancer in men (3, 4).

In contrast to these adverse effects also beneficial health effects and even reduced instead of increased cancer incidences have been related to estrogen exposure. Estrogen deficiency, as occurring in menopausal women, is the main cause of osteoporosis and heart diseases (5). A reduction of cancer risks (6), cardiovascular diseases (7) and osteoporosis (8) have been reported especially upon high intake of soy-based phyto-estrogens.

The exact mechanisms underlying the differential biological responses towards estrogen-like compounds have not been elucidated yet, but may be related to a differential biological response of the two estrogen receptors, ER α and ER β , and their mutual interaction. Pettersson and Gustafsson (9) hypothesised that ER β may act as an inhibitor of the ER α mediated transcription activation. Interestingly, ER β activation does not only result in a dominant suppression of all ER α inducible genes, it also inhibits proliferation of ER α -negative breast cancer cells (10, 11). Most studies have shown decreased ER β expression in cancer tissues as compared to benign tumours or normal tissues, whereas ER α expression persists (10, 12). Hence ER α and ER β may have different roles in gene regulation and their relative levels or ratios within the tissues may influence cellular responses to estrogens. It is known that the ratio of ER α /ER β expression is higher in breast tumors than in normal tissues due to lower expression of ER β (10) and that ER α and ER β are antagonistic to each other; for example, ER β appears to reduce the cell proliferation induced by ER α activation (13). Such modulating interactions provide support for the hypothesis of the present project that estrogen-like compounds that will activate the ER α may lead to adverse effects including stimulation of cell proliferation, whereas compounds stimulating the ER β may be beneficial through their direct or indirect interaction with the ER α protein and down-regulation of the ER α response thereby reducing cell proliferation and stimulating apoptosis (Figure 1).

The overall aim of the work presented in this thesis was to obtain insight in the role of ER α , ER β and the ratio of ER α /ER β present within a cell, in the cellular response to estrogen-like compounds. To this end, the thesis addresses the transcriptional activity and effects on

cell proliferation under the influence of specifically-acting estrogen-like molecules when varying the ratio of ER α /ER β present in the cells under study and links these results on cell proliferation as the biological end-point to the genomics and proteomics data. The results obtained should provide better insight in the mechanisms underlying the differential effects of estrogen-like compounds on cell proliferation and, ultimately, cancer incidences, and the role of the two estrogen receptors in these mechanisms.

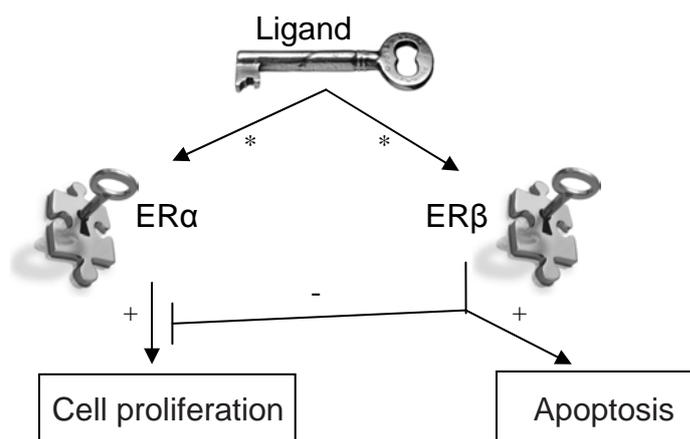


Figure 1: Illustration of the working hypothesis. Because estrogen-induced activation of ER α or ER β mediated gene expression activates different genes and pathways the ultimate biological outcome may depend on the ratio of ER α /ER β present in the cells. (* indicates binding, + indicates stimulation, - indicates inhibition).

Estrogen receptor and signalling function

Estrogen receptors are members of the nuclear receptor subfamily and they are involved in a broad range of physiological effects, such as growth, differentiation and physiology of the reproductive processes (14). The role of ERs in non-reproductive tissues is at least as important as in reproductive ones, since they are affecting bones, the cardiovascular system, brain and liver (15). Until 1996, only one human ER was known. At that time Kuiper et al. revealed a novel nuclear estrogen receptor cloned from rat prostate. The known ER was renamed and called ER α to differentiate it from the novel ER, ER β (16). The complete human ER β cDNA was sequenced in 1998 by Ogawa et al. (17).

Estrogen receptors are products of distinct genes localized on different chromosomes, ER α is encoded on chromosome 6q24-q27 (18), while the gene encoding ER β is localized on chromosome 14q22-q24 (19). Despite their distinct localization, gene organization of the two receptors is well conserved. ESR1 (ER α) and ESR2 (ER β) genes contain eight exons, separated by seven long intronic sequences. As members of the nuclear receptor superfamily, ERs contain 6 regions in their protein structure common for all nuclear receptors, namely: A, B, C, D, E and F which form functionally different but interacting domains (Figure 2). Exon

1 encodes the A/B region in ER α and ER β , exons 2 and 3 encode part of the C region. Exon 4 encodes the remaining part of region C, all region D and part of the region E. Exons 4 to 8 contain the rest of the region E sequence and region F is encoded by part of exon 8 [reviewed in (20)].

Although ER α and ER β are encoded separately they share a high degree of homology. The most conserved domain among ERs is the DNA binding domain (DBD) corresponding to the C region, with 96% homology between α and β ER subtypes. The DBD is responsible for binding to the specific DNA sequences (Estrogen Responsive Elements or ERE) in target gene promoter regions. The A/B region located in the N-terminal protein site encompasses the AF-1 domain responsible for ligand independent transactivation. The AF-1 domain is the least conserved part among the two ERs with only 30% homology and it is functional only in the ER α subtype (21). The C-terminal protein part encloses the ligand dependent transactivation domain AF-2 together with the ligand binding domain (LBD) and homo-heterodimerization site. Identity between the E/F regions of both proteins is 53%, which results in differences in ligand binding affinities between both receptors. The hinge region localized in the D domain contains the nuclear localization site of the ERs as well as post translational modification sites (22). Information on the structure/function relationship of this region is very limited. The domain appears to be a variable and not well conserved part of the ERs (only 30% homology).

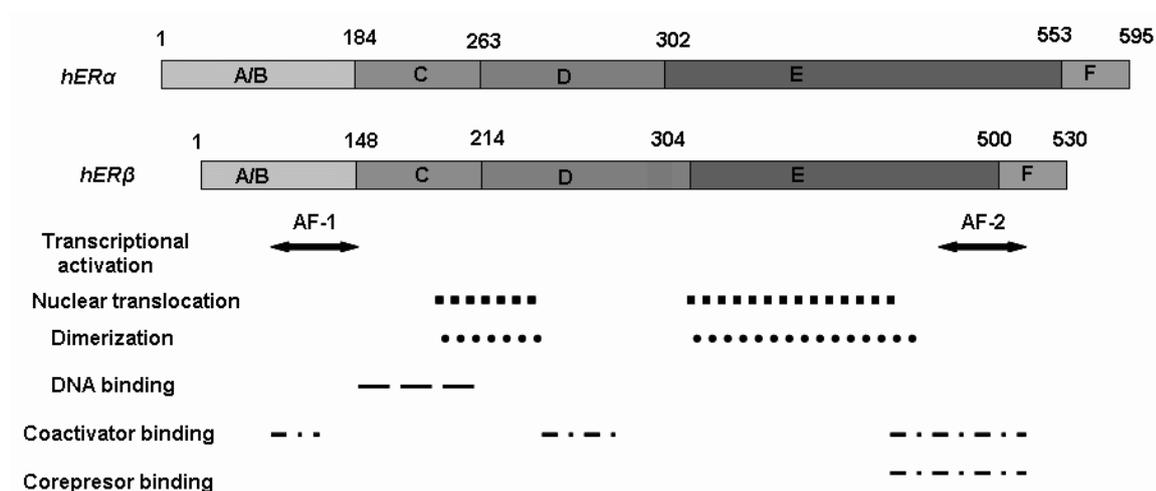


Figure 2: The domain structure of human the ER α and ER β proteins. Based on Matthews and Gustafsson (23).

Estrogen binding to the receptor induces the LBD to undergo a conformational change, upon which the receptor dimerizes, binds to DNA, and consequently stimulates gene expression (24, 25).

Estrogen receptor distribution

The distribution of ERs varies both between and within human tissues (see Table 1). The cardiovascular system, brain, and bones express both receptors. ER β is predominant in the male reproductive system. Expression of both ER α and ER β has been found in all major human uterine cell types at every menstrual stage. However, expression varies from cell-type to cell-type with expression of ER α mRNA being generally greater than that of ER β (26). Changes in expression of estrogen receptors has been found in certain tumour types. Normal mammary tissue in man has been shown to express predominantly ER β mRNA, whereas most ER-positive breast tumours appear to exhibit increased ratios of ER α /ER β (27). Likewise, an increased ratio in ER α /ER β mRNA has been demonstrated in ovarian carcinoma compared with normal tissue or cysts (12). High concentrations of ER β have also been found within the human gut (19).

Table 1: Tissue distribution of ER subtypes in humans.

Organ/Tissue	Human ER subtype		Organ/Tissue	Human ER subtype	
	ER α	ER β		ER α	ER β
Heart	✓	✓	Adrenal	✓	-
Lung	-	✓	Kidney	✓	✓
Vascular	✓	✓	Prostate	-	✓
Bladder	-	✓	Testes	-	✓
Epididymus	-	✓	Brain	✓	✓
Pituitary	-	✓	Thymus	-	✓
Liver	✓	-	Breast	✓	✓
Muscle	-	-	Uterus	✓	✓
Fat	-	-	Endometrium	✓	✓
Gastrointestinal tract	-	✓	Vagina	✓	-
Colon	-	✓	Fallopian tube	-	✓
Small intestine	-	✓	Ovary	✓	✓
Bone	✓	✓			

Therefore, the ultimate estrogenic effect of a certain compound on cells or tissues will be dependent on the receptor phenotype of these cells or tissues.

Mechanism of estrogen action

Estrogens act on target tissues by binding to ERs. These proteins function as transcription factors when they are bound to a ligand. Biological action of ERs involves

complex and broad mechanisms. For the ERs two main mechanisms of action have been described, including a genomic and a non-genomic pathway (Figure 3).

The *genomic action* of ERs occurs in the nucleus of the cell, when the receptor binds specific DNA sequences directly (“direct activation” or classical pathway) or indirectly (“indirect activation” or non-classical pathway). In the absence of ligand, ERs are associated with heat-shock proteins. The Hsp90 and Hsp70 associated chaperone machinery stabilizes the ligand binding domain (LBD) and makes it accessible to the ligand. Ligand-ER is activated through phosphorylation and dissociates from the heat-shock proteins. Then, the ligand-bound activated ER changes its conformation, dimerizes, and binds to specific DNA sequences called estrogen responsive elements (ERE) in order to regulate transcription (28). In the presence of the natural ligand 17 β -estradiol (E2), ER induces chromatin remodelling and increased transcription of estrogen regulated genes (29).

In the non-classical pathway, AP-1 (30) and SP-1 (31) are alternative regulatory DNA sequences used by both isoforms of the receptor, ER α and ER β , to modulate gene expression. In this case, ER does not interact directly with DNA but interacts with other DNA-bound transcription factors such as c-Jun or c-Fos, or with other coactivator proteins (32). Both AF-1 and AF-2 domains of ER are required for the interaction with Fos/Jun complex and both receptors differentially affect AP-1 dependent genes. In the presence of ER α , E2 works as AP-1 agonist by enhancing activity of the coactivators at AP-1 sites (33), while in the presence of ER β it antagonizes AP-1 activity (28). When both receptors are present, ER β inhibits the action of ER α on AP-1 promoters (34). Interactions of ERs with other transcription factors might be also selectively modulated by different ligands, such as genistein and quercetin, which are not able to stimulate AP-1 dependent transcription (35, 36).

Even though ERs are considered transcription factors they can act through *non-genomic* mechanisms. Rapid ER effects were first observed in 1960s when administration of a physiological dose of E2 was reported to increase uterine cAMP levels in ovariectomized rats within 15 seconds (37), a time scale that is considered too fast for a genomic action. There is still no agreement if receptors responsible for rapid actions of estrogens are the same proteins as nuclear ERs or distinct G-protein coupled steroid receptors (38-41). However, a broad range of other rapid pathways induced by E2 has been identified so far. Some of these pathways include MAPK/ERK pathway, activation of endothelial nitric oxide synthase (eNOS) PLC stimulated IP₃ production, calcium influx and PI3K/Akt pathway activation (42, 43) (20). Similarly to non-classic mechanisms of activation, phytoestrogens might affect rapid pathways in a different way than E2. Quercetin for example has been shown to fail to phosphorylate ERK-2 kinase (opposite to E2) nor did it stimulate transcription of Cyclin D1,

the transcription of which depends on rapid ER pathways (42). The stimulation of eNOS, which plays a positive role in cardiovascular health effects induced by E2 also seems to be regulated differently by phytoestrogens. Rapid activation of eNOS in the presence of E2 is dependent on ER α (44), while both receptors are required for prolonged effects. However phytoestrogens do not activate eNOS in a rapid manner but seem to activate it through a prolonged, ER β dependent transcriptional mechanism (44).

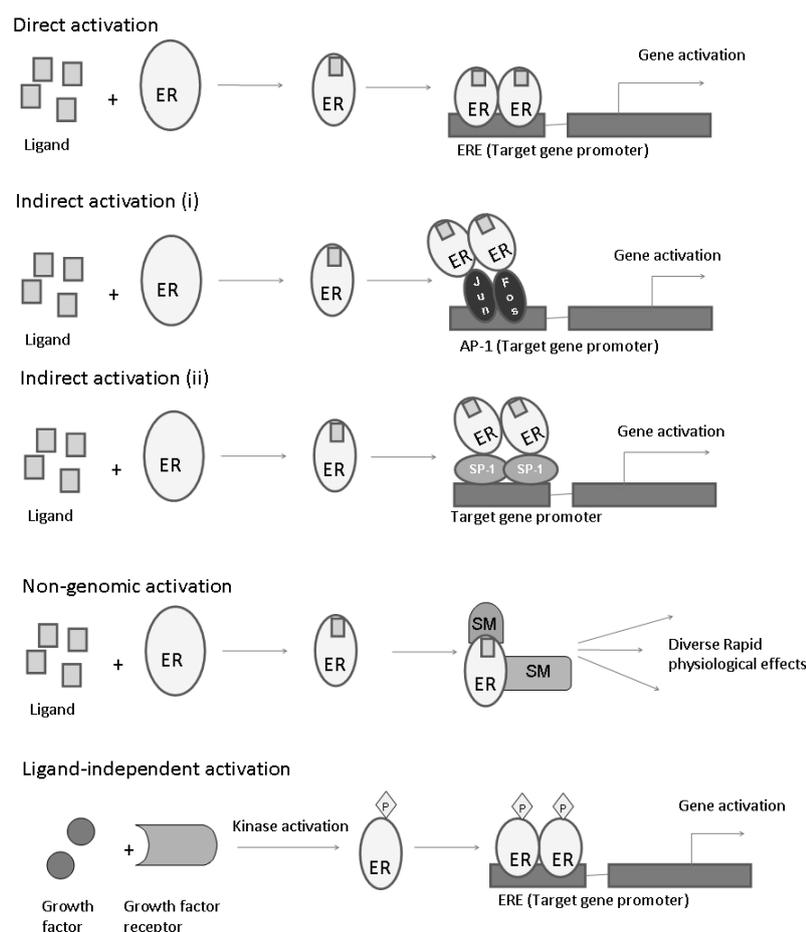


Figure 3: Mechanisms of estrogen receptor (ER) action. In the *direct activation*, ERs dimerize after ligand binding and attach to the ERE in the promoter of target genes. In the *indirect activation* manner, ligand-bound ER dimers might activate transcription of non-ERE containing genes, by binding to other transcription factors (e.g. AP1 or SP1). In the *non-genomic pathway*, ligand-bound ERs interact directly with and change the function of proteins some of which function as 'second messengers' (SM). ERs can also be activated by phosphorylation in the absence of ER ligands (*ligand-independent activation*). Based on Morani *et al.* 2008 (45).

In addition to ligand dependent mechanisms, ER α has ligand independent activity mediated through AF-1, which has been shown to be associated with stimulation of MAPK through growth factors such as Insulin like Growth Factor – 1 (IGF-1) and Epidermal Growth Factor (EGF). Activity of AF-1 is dependent on phosphorylation of Ser 118. A good example

of the cross-talk between ER and growth factor signalling is phosphorylation of Ser 118 by MAPK in response to growth factors, such as IGF-1 and EGF (46). The importance of growth factors in ER signalling is well illustrated by the fact that EGF can mimic effects of E2 in the mouse reproductive tract (28).

Ligand dependent effects

The overall biological effects of E2 and other estrogenic compounds are the result of complex interplay between various mechanisms, which largely depend on cellular context, ratio between ER subtypes, expression of coactivators in the cell, sequences of target EREs but also cross-talk with growth factor pathways and activity of kinases and phosphatases. All these factors together enable a precise and targeted response to the natural hormone. However a broad range of pathways involved in ER signaling provides many points of possible signal modulation by estrogens and estrogen-like compounds and small structural changes between different ligands might result in significantly different responses.

Structural differences in the LBD underlie differences in affinity and transcriptional activity induced, for certain ligands and provide one of the mechanisms for selective modulation of ER responses. ER β has an impaired AF-1 domain compared with ER α and the necessary synergy with AF-2 is dramatically reduced (47). These differences suggest that it is possible to develop ligands with different affinities, potencies, and agonist vs antagonist behavior for the two ER subtypes.

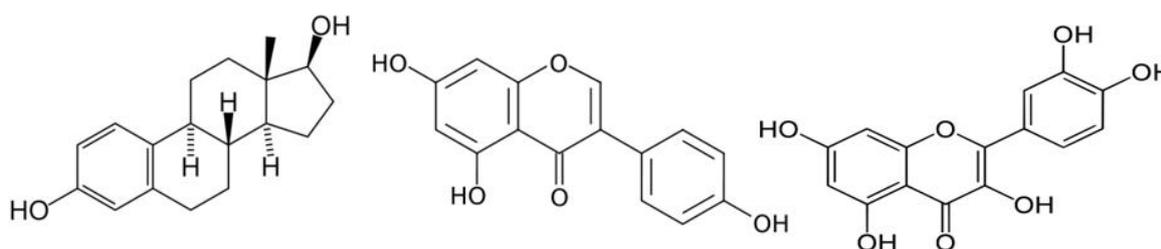


Figure 4: Chemical structure of estradiol, genistein and quercetin.

It has been demonstrated that E2 has higher affinity towards ER α than to ER β (48, 49), and certain selective estrogen receptor modulators (SERMs) might exhibit a preference towards one of the receptors (50). Plant derived phytoestrogens, which are structurally similar to E2 (Figure 4) are a good example of ligand selectivity (51). Genistein, is the major isoflavone present in soy and fava beans whereas quercetin is present in red onions, apples, cappers or red grapes among others (51). *In vitro* studies with reporter gene assays proved that phytoestrogens are able to stimulate ERE-dependent genes at high concentrations. Therefore they are considered weak ER agonists with the majority of them preferentially

binding to ER β (52, 53). The main hypothesis on the positive role of phytoestrogens in modulation of ER signaling is their higher affinity towards the ER β subtype, which can silence ER α dependent signaling and decrease overall cell sensitivity to E2 (21), which is thought to be significant in cancer prevention and will be further discussed in this thesis.

Objective, approach and outline of this thesis

The overall aim of the work presented in this thesis was to obtain insight in the role of ER α , ER β and the ratio of ER α /ER β present within a cell, in the cellular response to estrogen-like compounds. To this end, the thesis addresses the transcriptional activity and effects on cell proliferation under the influence of specifically-acting estrogen-like molecules when varying the ratio of ER α /ER β present in the cells under study. The ultimate aim was to link the resulting cell proliferation as the biological end-point to the genomics and proteomics data.

Chapter 1 presents the background and aim of this thesis. Chapter 2 presents studies investigating how variable cellular expression ratios of the estrogen receptors ER α and ER β , modulate the effects on cell growth induced by ER α or ER β agonists, respectively. Using human osteosarcoma (U2OS) ER α or ER β reporter cells, and T47D human breast cancer cells with tetracycline dependent expression of ER β (T47D-ER β), effects on ER α and ER β mediated transcriptional activation as well as on cell proliferation was characterised for E2 and two selected selective estrogen receptor modulators (SERMs). In chapter 3, based on the interest in the potential health effects of dietary phytoestrogens, two plant derived compound, genistein and quercetin were tested in the same *in vitro* model systems.

Chapter 4 of the thesis presents results from studies investigating the possible mechanisms and biological relevance underlying the phenomenon referred to as superinduction of estrogen receptor mediated gene expression in luciferase based reporter gene assays. Using *in vitro* luciferase-based reporter-gene bioassays for testing estrogenicity, several estrogenic model compounds including the isoflavonoid model compound of the present thesis, genistein, have been reported to induce a higher maximal response than E2. The phenomenon has been referred to as superinduction. So far, the mechanism underlying this effect and thus also its biological relevance remain to be elucidated. Chapter 4 reports results from studies investigating several hypotheses for the possible mechanisms underlying this superinduction using genistein as the model compound known to induce the effect.

In chapter 5, and 6 the consequences of the intracellular ER α /ER β ratio for the effects induced by genistein was investigated in more detail using “omics” technologies,

characterizing both gene and protein expression patterns and comparing the results obtained to those previously reported for estradiol in the same model system (54). To this end, state-of-the-art high-throughput methods for systems-wide gene and protein expression analysis were applied. These methods included DNA microarrays, commonly used for global analysis of gene expression changes. In addition we applied stable isotope labeling by amino acids in cell culture (SILAC) which is a differential and quantitative proteomics technique based on mass spectrometry (MS) analysis (55). Since the metabolic incorporation of the labels does not affect the integrity of genes or proteins, the transcriptomics and SILAC proteomics experiments could be performed on the same cell samples allowing comparison of the data sets.

In Chapter 5 data analysis focussed on the functional analysis of the differential behaviour of ER-mediated activation of gene and protein expression towards cell proliferation and cell apoptosis, whereas protein data analysis in Chapter 6 pointed at the immune modulation by genistein. These mechanisms of action were related to the difference in presence and relative amount of the two estrogen receptors ER α and ER β .

Finally, chapter 7 presents a summary of the results obtained in this thesis, a discussion of the new physiologically based, functional mechanistic insights obtained as well as some future perspectives.

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Influence of Cellular ER α /ER β Ratio on the
ER α -Agonist Induced Proliferation of Human
T47D Breast Cancer Cells

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Abstract

Breast cancer cells show overexpression of estrogen receptor (ER) α relative to ER β compared to normal breast tissues. This observation has led to the hypothesis that ER β may modulate the proliferative effect of ER α . This study investigated how variable cellular expression ratios of the ER α and ER β modulate the effects on cell proliferation induced by ER α or ER β agonists, respectively. Using human osteosarcoma (U2OS) ER α or ER β reporter cells, propyl-pyrazole-triol (PPT) was shown to be a selective ER α and diarylpropionitrile (DPN) a preferential ER β modulator. The effects of these selective estrogen receptor modulators (SERMs) and of the model compound E2 on the proliferation of T47D human breast cancer cells with tetracycline-dependent expression of ER β (T47D-ER β) were characterized. E2-induced cell proliferation of cells in which ER β expression was inhibited was similar to that of the T47D wild-type cells, whereas this E2-induced cell proliferation was no longer observed when ER β expression in the T47D-ER β cells was increased. In the T47D-ER β cell line, DPN also appeared to be able to suppress cell proliferation when levels of ER β expression were high. In the T47D-ER β cell line, PPT was unable to suppress cell proliferation at all ratios of ER α /ER β expression, reflecting its ability to activate only ER α and not ER β . It is concluded that effects of estrogen-like compounds on cell proliferation are dependent on the actual ER α /ER β expression levels in these cells or tissues and the potential of the estrogen agonists to activate ER α and/or ER β .

Introduction

Steroid hormones such as estrogens are needed for normal developmental, physiological, and reproductive processes in vertebrates (1). Many of these events are modulated by the activity of estrogen receptor α (ER α) and estrogen receptor β (ER β) (2, 3). These two receptors are encoded by distinct genes and differ in their relative and absolute tissue distribution (4). In the absence of estrogen, ERs are sequestered within the nucleus and preserved in an inactive state by association with heat-shock proteins. Binding of estrogen or estrogen-like compounds induces a conformational change in the receptor, an event that promotes ER homo- or heterodimerization (5). Once the estrogen receptor protein complex is bound to the DNA, it regulates the expression of estrogen-responsive genes. The ER homo- and heterodimers activate different signalling pathways and, therefore, different sets of genes (6-8).

During the last few years, an increasing number of studies have reported that xenobiotic compounds from different sources are able to mimic the natural estrogens, thus exerting comparable effects by activating gene transcription through ER α and/or ER β (9-14). Estrogens stimulate cell proliferation in normal developing breast tissues and may prevent osteoporosis by increasing bone mineral density (15). However, several studies also suggest that estrogens may stimulate the growth of a large proportion of ER α -positive breast cancers (16-20). It has been shown that the ratio of ER α /ER β expression is higher in breast tumors than in normal tissues due to lower expression of ER β (21) and that ER α and ER β are antagonistic to each other; for example, ER β appears to reduce the cell proliferation induced by ER α activation, as shown in *in vitro* cell transfection studies (22-24). Different breast cancer cell lines have been used for these studies, mainly MCF-7 cells, which all have a high ER α /ER β ratio (25-27). It is proposed that differential responses and tissue-specific effects induced by food-born endocrine disrupters, including selective estrogen receptor modulators (SERMs), might be influenced by their relative affinity for the two ERs and the interactive effects of the estrogen-ER complex with the regulating proteins.

The overall objective of the present study was to quantitatively determine the proliferative/antiproliferative effect of two model-selective ER agonists in T47D human breast cancer cells in the presence of increasing amounts of intracellular ER β . The model compounds studied were propyl-pyrazole-triol (PPT), a selective ER α agonist, and diarylpropionitrile (DPN), a preferential ER β agonist. For comparison and validation of the different cellular model systems, estradiol (E2) was included in the studies as well.

The natural ligand E2 is known to stimulate both ERs, with an approximately 10-fold higher affinity for ER α than for ER β (28, 29). DPN was reported to have a 70-fold higher relative binding affinity for ER β than for ER α , and PPT has a reported 40-fold higher binding affinity for ER α than for ER β (30-32). In the present study, the relative isoform-specific activity of the three model compounds was characterised using the human osteosarcoma (U2OS) reporter cell lines, stably transfected with ER α or ER β and a luciferase reporter gene with an 3xestrogen responsive element(ERE)-TATA containing minimal promoter region (29).

In subsequent experiments, the effect of the three compounds on proliferation of T47D-ER β cells with varying ratios of ER α /ER β expression was quantified. In wild-type T47D cells, ER α /ER β mRNA levels were found to be present in a ratio of 9:1 (23). The T47D-ER β cells are T47D cells stably transfected with a tetracycline-inducible ER β which allows studying the influence of SERMs on cell proliferation in cells with varying ratio of ER α /ER β expression, by altering expression of ER β . Inhibition of the expression of the exogenous ER β is expected to make the T47D-ER β cell line function as a “pseudo”-wild-type T47D cell. Since in concurrence with the expression of ER β , an enhanced green fluorescent protein (EGFP) from a bidirectional tetracycline-responsive promoter is coexpressed in the T47D-ER β cells, the levels of ER β expression can be monitored on the basis of EGFP fluorescence. To better quantify the relative levels of ER β expression in the T47D-ER β cells, a method to quantify the EGFP fluorescence in the cell lysate was developed in the present study.

With the newly developed method to quantify the relative ER β expression, the effects of E2, DPN, and PPT on the T47D-ER β cell proliferation were studied at different levels of ER β expression to determine to what extent the estrogen-induced cell proliferation depends on the balance between the two major ER subtypes. In addition, it was investigated whether the effects observed match those that would have been predicted based on the U2OS reporter gene test results for these compounds and the hypothesis that stimulation of ER α activates and of ER β reduces estrogen mediated cell proliferation.

Materials and Methods

Materials: 17 β -Estradiol (E2)(>98 %) and ANTI-FLAG M2[®] Monoclonal antibody peroxidase conjugate was purchased from Sigma (Zwijndrecht, The Netherlands). 2,3-Bis(4-hydroxyphenyl)-propionitrile (DPN) and 4,4',4''-(4-propyl-[1H]pyrazole-1,3,5-triyl) trisphenol (PPT) were purchased from Tocris Cookson Ltd (Bristol, UK). Dimethyl sulfoxide (DMSO)(>99%) was obtained from Acros Organics (Pittsburgh, PA, USA). Tetracycline, streptomycin, penicillin and puromycin were acquired from Gibco (Paisley, Scotland). Fetal

calf serum (FCS)(Australian origin, 10099), resazurin and geneticin were provided by Invitrogen Life Technologies (Paisley, Scotland). Hyclone dextran-charcoal-treated FCS (DCC-FCS, #SH30068.05) obtained from Perbio Science NV (Etten-Leur, The Netherlands) was heat inactivated (30 min at 56°C) followed by two 45-min DCC-treatment at 45°C (33). Phosphate-buffered saline (PBS)(without Ca^{2+} and Mg^{2+}), nonessential amino acids (100×, 11140-035), growth medium 1:1 mixture of Ham's nutrient mixture F12 and Dulbecco's modified Eagle's medium (DMEM)(31331-038 and 31331-028), phenol red-free exposure medium (21041-025) were supplied by Gibco (Paisley, Scotland). Trypsin 0.25 g/100 ml in PBS was obtained from Difco (Detroit, USA). Sodium bicarbonate (NaHCO_3 >99.5%), sodium hydroxide (NaOH) ethylenedinitrotetraacetic acid ($\text{EDTA} \cdot 2\text{H}_2\text{O}$; Titriplex), magnesium sulphate ($\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$), and 1,4-dithiothreitol (DTT) were obtained from Merck (Darmstadt, Germany). Magnesium carbonate ($(\text{MgCO}_3)_4\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$) was obtained from Aldrich (Saint Louis, MO, USA). *trans*-1,2-diaminocyclohexane-N,N,N,'N'-tetraacetic acid monohydrate (CDTA) was obtained from Fluka (Buchs, Switzerland). Hygromycin and D-luciferin were obtained from Duchefa (Haarlem, The Netherlands). ATP and the BrdU kit (colorimetric, 11647229001) were obtained from Roche Diagnostics (Mannheim, Germany). BSA Protein Assay Kit was purchased from Pierce (Germany). Tween 20 was obtained from Merck (Bonn, Germany). SDS (sodium dodecyl sulphate) was obtained from BDH (United Kingdom). Acrylamide (30% acrylamide/bis solution 29:1), N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulphate (APS), and the precision plus dual colour protein marker were obtained from BioRad (The Netherlands). Milk solution was provided by Campina (The Netherlands). Nitrocellulose membrane was purchased from Whatman (The Netherlands). ER β specific primary (Ab288/14C8) and secondary antibody (rabbit anti mouse) were provided by Abcam (Cambridge, MA, USA). Chemiluminescent detection ECL kit and photographic hyperfilm were provided by Amersham (United Kingdom).

Cell lines: T47D human breast cancer cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The stably transfected T47D tetracycline-inducible cell line (T47D-ER β) was made and provided by Ström (23). The human osteosarcoma (U2OS) cell lines stably expressing ER α or ER β , in addition to 3xERE-tata-luciferase were used as described before (29).

Cell culture conditions: The T47D wild-type cell line was cultured in a 1:1 mixture of Ham's nutrient mixture F12 and DMEM (31331-038) supplemented with 5 % FCS. The cells were incubated at 37° C and 5% CO₂ in a humidified atmosphere. T47D-ER β cells were cultured at the same conditions but in the presence of 1000 ng/ml tetracycline to fully inhibit ER β expression. Every 10 passages (about 3 weeks) the cells were reselected with 0.5 μg

puromycin/ml as a selection marker to prevent loss of ER β and EGFP expression and a concurrent change in phenotype.

ER α - and ER β -U2OS cells were cultured in a 1:1 mixture of DMEM and F12 (31331-028) buffered with 1260 mg/L NaHCO₃, supplemented with 7.5% Australian FCS, and 0.5% nonessential amino acids. ER α -U2OS-Luc growth medium was supplemented with geneticin (200 μ g/ml) and hygromycin (50 μ g/ml) as selection markers. ER β -U2OS-Luc growth medium was supplemented with geneticin (200 μ g/ml) as selection marker. Cells were cultured at 37°C at 7.5% CO₂ in a humidified atmosphere.

Behaviour of T47D-wt and T47D-ER β cells during culturing and exposure: The T47D-wt cells were growing well and nicely attached to the bottom when cultured in flasks at no more than 85% confluency. Higher cell densities resulted in cluster formation and reduction of cell size. The T47D-ER β cells, however, were much more difficult to grow. Especially when ER β -expression was present, the cells often started to round up and detach. This loss of cell attachment hampered the application of methods to quantify cell proliferation in the cases where ER β was expressed. Both the resazurin and BrdU method gave good results when compared to protein measurement assay and cell counting, and we chose to mostly apply the resazurin method as this method requires less cell handling than the BrdU method.

Exposure conditions for T47D and T47D-ER β cells: Because of estrogenic activity of phenol red (34), experiments were performed in phenol red-free exposure medium supplemented with 5% DCC-FCS. Cells were seeded in 96-well plates (100 μ L/well; Costar, Cat. Nr. 3548) at densities of 10⁵ cells/ml for proliferation and 1.8x10⁵ cells/ml for fluorescence assays in the presence of different concentrations of tetracycline (0-1000 ng/ml) as indicated. The starting percentage of coverage for fluorescence experiments was higher than for proliferation experiments because wells had to be fully confluent for optimal sensitivity in the fluorescence measurements, whereas less confluent wells were needed for proliferation assays. Plates were incubated overnight at 37°C and 5% CO₂. After 24 h, cells were washed with PBS to remove any trace of tetracycline and exposed to different concentrations of tetracycline and/or the test compounds as indicated.

Cell proliferation measurements: After 24 h of exposure, proliferation was determined by measuring 5-bromo-2'-deoxy-uridine (BrdU) incorporated into DNA following BrdU Roche's colorimetric protocol and/or after 96 h of exposure by measuring mitochondrial activity of viable cells on the basis of chemical reduction of resazurin to resorufin as previously described (35). Measurement of incorporated BrdU was performed in a spectrophotometer at 370 nm excitation wavelength and 492 nm emission wavelength, and

resorufin was measured with a fluorometer at an excitation wavelength of 530 nm and emission wavelength of 590 nm. Other methods for studying cell proliferation included cell counting and measurement of protein content, the latter by Bicinchoninic Acid (BCA) Protein Assay.

Measurement of ER β expression-related fluorescence: To quantify ER β expression-related EGFP fluorescence after 24 h of exposure, medium was removed from the wells and the cells were washed with 100 μ L of diluted PBS (0.5x PBS in demiwater). To each well, 110 μ L of low-salt buffer, consisting of 10 mM tris-HCl pH 7.8 containing 2 mM DTT and 2 mM CDTA, was added, and the cells were allowed to swell while the plates were kept on ice for 20 min. The plates were then frozen at -80° C for at least 1 h, and before analysis, they were thawed on ice and shaken briefly until reaching room temperature. Then, 100- μ L aliquots of cell lysates from each well were transferred to a 96-well transparent plate with rounded bottom (Greiner, Frickenhausen, Germany) to allow fluorescence measurement in the Millipore Cytofluor 2350 fluorometer. Excitation was at 485 nm (band width of 20 nm) and emission at 530 nm (band width of 25 nm).

Protein Isolation and SDS-PAGE: For the analysis of the ER β protein expression levels, T47D-ER β cells were grown in growth medium with 1000 ng/ml of tetracycline in small cell culture flasks until 80-90% confluence. Cells were seeded in exposure medium with tetracycline for 24 h. Medium was removed, and cells were incubated for 24 h in new exposure medium with different tetracycline concentrations (0, 10 and 1000 ng/ml of tetracycline). Cells were collected with a scraper and suspended in PBS. After centrifugation (13000g, 5 min), PBS was removed and cells were suspended in lysis buffer supplemented with protease inhibitors. Lysis was done by three cycles of freezing in -80° C and thawing. Total protein content was determined using a bicinchoninic acid protein assay kit and a total protein amount of 20 μ g/lane plus ¼ of 4x sample buffer (8% w/v SDS, 40% w/v glycerol, 0.2 M Tris/HCl pH 6.8, 0.02% bromophenol blue, 25% v/v mercaptoethanol) was loaded onto the gel. Running gel (12% acrylamide) was prepared by mixing 9.9 ml of deionized water, 12 ml 30% acrylamide plus 0.8% bisacrylamide, 7.5 ml 1.5 M Tris-HCl pH 8.8, 300 μ l 10% SDS. Polymerization was started by addition of 150 μ l 10% APS and 12 μ l of TEMED. For the preparation of stacking gel (5% acrylamide) reagents were mixed in the following proportions: 6.66 ml water, 1.66 ml 30% acrylamide plus 0.8% bisacrylamide, 1.26 ml 1.5 M Tris pH 8.8, 100 μ l 10% SDS and 100 μ l 10% APS together with 10 μ l TEMED. Electrophoresis was run at 100V for approximately 2 h. After electrophoresis, gels were stained with Coomassie or used for Western blotting.

Western blotting: Blotting was performed at 100V for 1 h. After the transfer, unspecific binding sites on the membrane were blocked with 5% milk solution in tris-buffered saline

(TBS) 0.05% Tween 20 for 1-2 h. The membrane was washed in TBS with 0.05% Tween 20 twice for 5 min. For detection of the exogenous FLAG-ER β , the monoclonal ANTI-FLAG M2[®] antibody was diluted in TBS with 0.05% Tween 20. For detection of ER β in the control T47D cell line, ER β mouse monoclonal 14C8 antibody was used. After incubation for 1 h at room temperature with ANTI-FLAG M2[®] antibody the membrane was washed with TBS with 0.05% Tween 20 six times for 5 min each time before ECL treatment. Incubation with the 14C8 antibody was performed overnight at 4°C. After incubation, the membrane was washed with TBS 0.1% Tween 20 three times for 10 min. Secondary antibodies was diluted 5000 three times in TBS 0.1% Tween 20, and incubation was run for 45 min at room temperature. Rabbit anti-mouse antibody conjugated with peroxidase was used for ER β . Final washing steps were done two times with TBS 0.1% Tween and one time with TBS only. Finally, the membrane was treated with peroxidase substrate (ECL kit) for protein detection. The reaction was run for 5-7 min, and bands were visualized using photographic film. As a final step, membranes were stained with coomassie blue.

ER α - and ER β -specific U2OS reporter gene assay: Cultured U2OS cells were washed with PBS, trypsinized, and seeded in transparent 96-well plates (Greiner, Frickenhausen, Germany) at 100 μ L/well at a density of 10×10^4 cells/ml (U2OS-ER α) or 7.5×10^4 cells/ml (U2OS-ER β) in a 1:1 mixture of DMEM and Ham's F12 medium without phenol red, buffered with 1260 mg/L NaHCO₃, and supplemented with 5% DCC-FCS and 0.5% nonessential amino acids. Culture medium was refreshed after 24 h. Forty-eight hours after seeding, the cells were exposed in triplicate to E2, DPN or PPT at the indicated concentrations (final DMSO concentration 0.2%) for 24 h at 37°C and 7.5% CO₂ in a humidified atmosphere. On each plate, the cells were exposed to different concentrations of test compounds and calibration points for E2 (EC₁₀, EC₅₀, E₁₀₀) to be able to correct for plate to plate variations. After 24 h, the medium was removed, and cells were washed with 100 μ L diluted PBS (0.5x in demiwater) per well. Cells were lysed with 30 μ L of a hypotonic low-salt buffer, consisting of 10 mM tris-HCl pH 7.8 containing 2 mM DTT and 2 mM CDTA. Plates were put on ice for 10 min and subsequently frozen at -80°C. Before analysis, plates were thawed on ice for 20 min and shaken briefly until reaching room temperature. Analyses were performed in a Luminoskan (RS, Labsystems) at room temperature as follows: background light emission of each well was measured for 2 sec, then, 100 μ L of flashmix was added (20 mM tricine buffer, pH 7.8, supplemented with 1.07 mM (MgCO₃)₄Mg(OH)₂·5H₂O, 2.67 mM MgSO₄, 0.1 mM EDTA ·2H₂O, 2 mM DTT, 0.47 mM D-luciferin, and 5 mM ATP), light emission was immediately measured for 2 sec and extinguished with 50 μ L 0.2 M NaOH to prevent cross-talk to the neighbouring wells.

Data analysis: Relative light units (RLUs) in every well were corrected for the corresponding background signal, measured before luciferin addition. The response of the

solvent control was taken as 0% induction. The maximum induction of luciferase obtained at 30 pM E2 for ER α -U2OS cells and at 300 pM E2 for ER β -U2OS cells was set at 100%. The exposure concentration of the compound at which 50% of the maximum luciferase activity is reached (EC₅₀) was determined using Slidewrite 6.10 for Windows. The estradiol equivalency factors (EEF) were calculated as EC₅₀ estradiol/EC₅₀ compound. The concentration of tetracycline at which 50% of the EGFP fluorescence is inhibited (IC₅₀) was determined using Slidewrite 6.10 for Windows as well. EGFP fluorescence reflecting the level of ER β induction was expressed relative to the fluorescence of cells exposed to the solvent control (PBS 0.2%) set at 100%. In addition, in each experiment calibration points for E2 were included to be able to correct for plate to plate variations. The obtained data from proliferation quantified by the resazurin method was plotted after subtraction of background signal (obtained from a well containing all components except for the cells), as % proliferation. Results from BrdU were calculated as percentage of proliferation after background subtraction. The response of cells exposed to the solvent control (DMSO 0.2%) was set at 100%.

Results

Characterisation of the selected selective estrogen receptor modulators (SERMs)

The selectivity of PPT and DPN for ER α and ER β was studied in the ER α -U2OS-Luc and ER β -U2OS-Luc cells. Typical dose-response curves for the natural ligand E2 as well for the SERMs are shown in Figure 1.

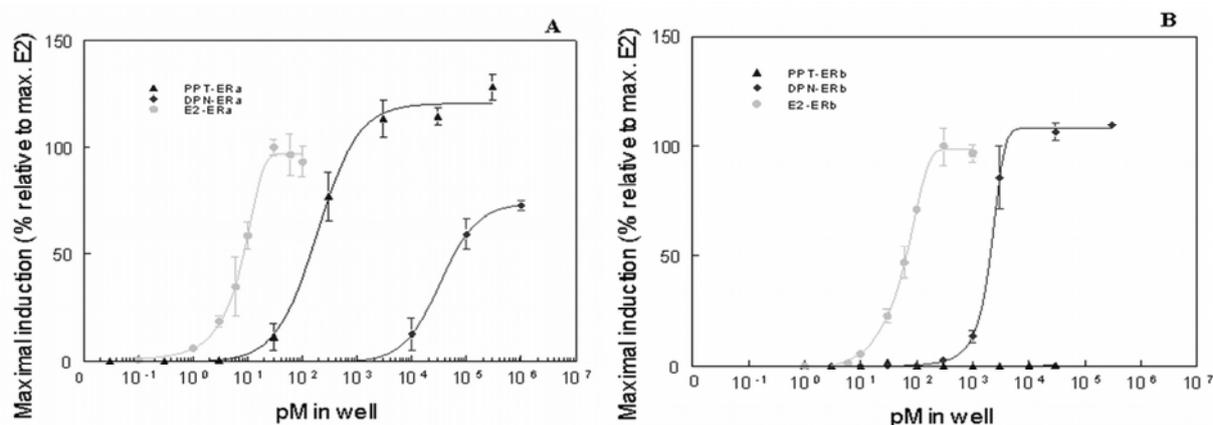


Figure 1: ERE-mediated luciferase activity in U2OS-ER α (A) and U2OS-ER β (B) cells exposed to E2 (●), the ER α -selective PPT (▲) and the ER β -selective DPN (◆). Induction was expressed relative to maximal estradiol response, set at 100%. Data points represent the mean of triplicates exposure \pm standard deviation.

As previously demonstrated, E2 showed higher binding affinity for ER α than for ER β , with EC₅₀-values of 8 and 65 pM respectively, but E2 is clearly both an ER α and ER β

agonist. The ER α -selective PPT was unable to induce any transcription of the reporter gene in the ER β -U2OS-Luc cell line, confirming its nature as a selective ER α modulator. The EC₅₀ for the ER α -dependent response was 140 pM (Figure 1A) resulting in an EEF compared to E₂ of 0.057, and the maximal induction was 120%. As expected, DPN showed ER β selectivity with EC₅₀ values of 2 nM and 59 nM for ER β and ER α , respectively. At present, a more selective ER β agonist could not be identified, and the ER β specificity of DPN in the U2OS cells was at least higher than that of E₂ since the ratios of the EC₅₀ for ER α and the EC₅₀ for ER β activation are respectively 0.12 and 29.5 for E₂ and DPN. In the ER α -U2OS cells, DPN did not reach the maximal E₂ induction level, but in the ER β -U2OS the maximal induction level of DPN was 110% of the value obtained for E₂ (Figure 1B). The EEFs for DPN were $1.3 \cdot 10^{-4}$ in the ER α -U2OS and 0.03 in the ER β -U2OS cells. Table 1 shows an overview of the EC₅₀, EEF, and maximum effect of PPT and DPN compared to E₂ using the U2OS cell system.

Table 1: Overview of the EC₅₀, EEF values and maximum effect of E₂, PPT and DPN tested using the U2OS cell system.

	ERα EC₅₀	Max effect as % relative to E₂-max	ERα EEF	ERβ EC₅₀	Max effect as % relative to E₂-max	ERβ EEF
E₂	8 pM	100	1	65 pM	100	1
PPT	140 pM	120	0.057	--	--	--
DPN	59 nM	73	1.3×10^{-4}	2 nM	110	0.03

Tetracycline-dependent expression of ER β in the T47D-ER β cell line quantified by measuring EGFP-Fluorescence

T47D cells were stably transfected with the ER β expression plasmid under tetracycline-responsive promoter regulation and with an EGFP gene as a coexpressed reporter also under regulation of the same tetracycline-responsive promoter. This allows qualitative/semiquantitative confirmation of ER β expression by fluorescence microscopy. Maximal levels of fluorescence were reached after 24 h of cultivation of the cells in the absence of tetracycline. A simple method for quantitative measurements of the EGFP as sensitive reporter molecule in cell lysate of the T47D-ER β was developed. Wells seeded with high density number of the T47D-ER β cells were exposed to different concentrations of tetracycline (Figure 2A). EGFP fluorescence was measured in the cell lysate. Tetracycline treatment suppressed EGFP fluorescence in T47D-ER β cells, with concentrations above 150 ng/ml, resulting in total fluorescence suppression. Values above 2000 ng/ml of tetracycline

not only completely depleted EGFP expression but also caused cytotoxicity (data not shown). The concentration of tetracycline at which 50% of the fluorescence, and thus ER β expression, was inhibited (IC₅₀) was determined to be 9.6 ng/ml tetracycline. Since the expression of EGFP is linked to the expression of recombinant ER β , the presence of ER β at protein level was confirmed using western blot (Figure 2B). No detectable FLAG-ER β protein was expressed in the presence of 1000 ng tetracycline/ml.

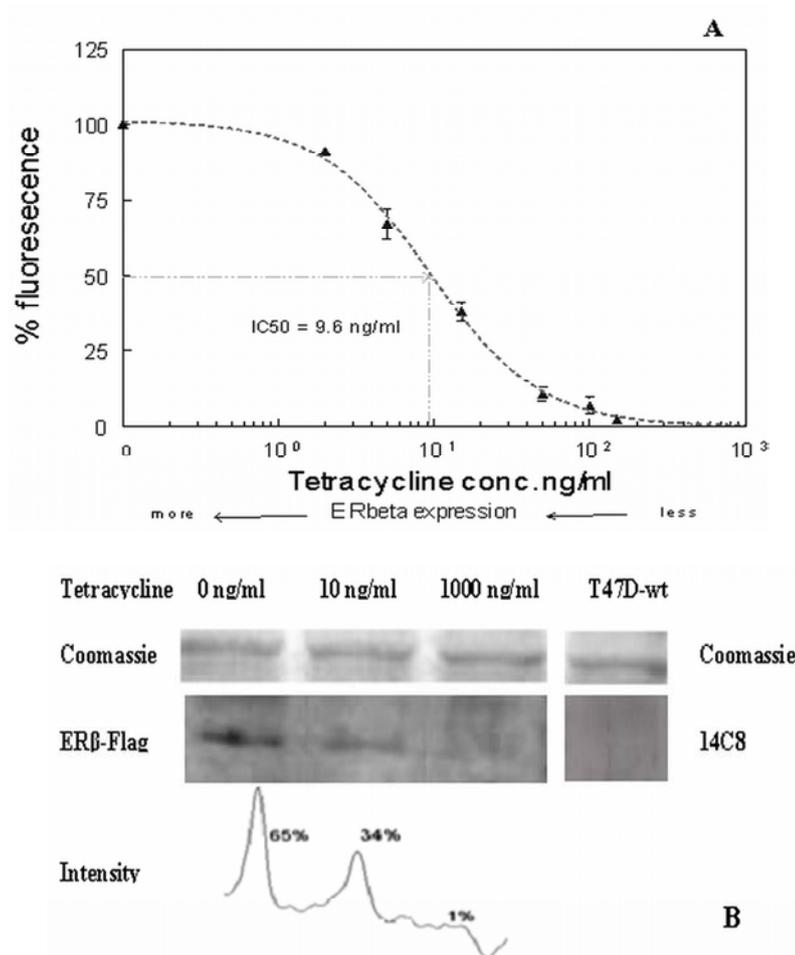


Figure 2: (A) Tetracycline-induced inhibition of ER β expression in T47D-ER β cells measured via the concurrent expression of EGFP after 24 h of exposure at 100% cell density. Fluorescence is expressed relative to maximum expression at 0 ng/ml of tetracycline set at 100%. No fluorescence is observed above 150 ng of tetracycline per ml. Each data point represents the mean of triplicate exposure \pm standard deviation. (B) T47D-ER β tet-off FLAG-ER β cell were cultured in the presence (1000ng/ml and 10 ng/ml) or absence of tetracycline for 24 h, 14C8 antibody was used to detect ER β in the T47D wild-type, coomassie staining was used as loading control. Quantification of the intensity of the ER β -FLAG bands was measured by ImageJ.

E2-induced proliferation of T47D-wt cells and of T47D-ER β cells with inhibited ER β expression

T47D-wt cells showed a clear E2-dependent cell proliferation with a maximum of 131% maximal induction of proliferation at 100 pM E2 (Figure 3). T47D-ER β cells in which ER β expression was completely inhibited by incubating them in the presence of 1000 ng tetracycline/ml showed an E2-dependent cell proliferation that was comparable with the response obtained in wild-type cells (Figure 3). The T47D-ER β transfected cells with no ER β expression reached the same maximum proliferation of 131% at 1 nM of E2 as the wild-type cells. In both cell lines, the shape of the dose-response curves was similar and at concentrations above 1 nM E2, cell proliferation decreased when increasing the concentration of E2. The dose-response curves for E2-induced proliferation obtained by measuring BrdU incorporation during the last 4 h of the 24-h period exposure were comparable to those obtained when measuring mitochondrial activity during the last 4 h of 96-h exposure using the resazurin method with both cell lines.

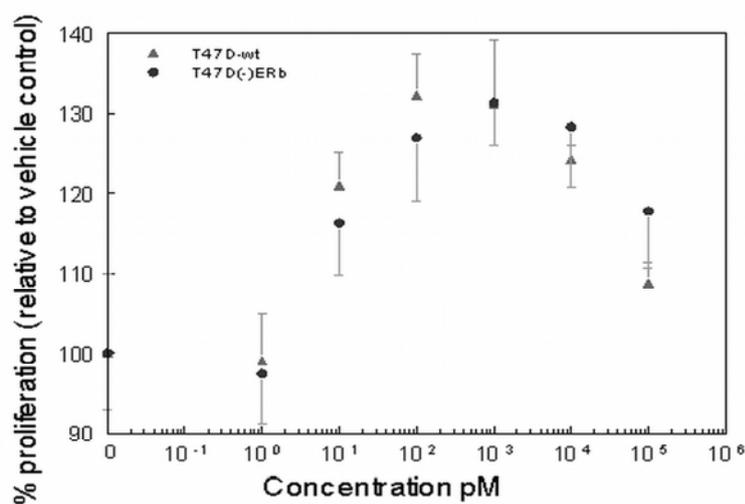


Figure 3: The effect of estradiol (E2) on cell proliferation of T47D-wt and T47D-ER β cells the latter with maximum inhibition of ER β expression by 1000 ng tetracycline/ml. Cell proliferation after 24 h of exposure is expressed relative to vehicle control (DMSO) set at 100%. Each data point represents the mean of triplicates exposure \pm standard deviation.

Expression of ER β inhibits E2-induced cell proliferation of T47D-ER β cells

Mitochondrial activity of the T47D-ER β cells treated with 1 nM E2 and an increasing concentration of tetracycline, causing decreasing cellular expression levels of ER β , showed a tetracycline-related increase in proliferation (Figure 4). In absence of E2, the cells did not proliferate. The EC₅₀ for tetracycline-dependent stimulation of E2-mediated cell proliferation was 41 ng tetracycline/ml. At tetracycline concentrations lower than 10 ng/ml, where ER β expression levels were high, no E2 induced proliferation was observed.

Exposure of T47D-ER β (Figure 5) cells to increasing E2 concentrations in combination with 41 ng/ml (EC₅₀) and 1000 ng/ml of tetracycline resulted in a dose-dependent cell proliferation. E2-induced proliferation was almost absent in the presence of 0 and 9.6 ng (IC₅₀ for fluorescence) tetracycline/ml. Altogether these data demonstrate the validity of the test system and support that E2 cannot induce cell proliferation under conditions where ER β is expressed to relatively higher levels and able to suppress ER α -mediated induction of cell proliferation.

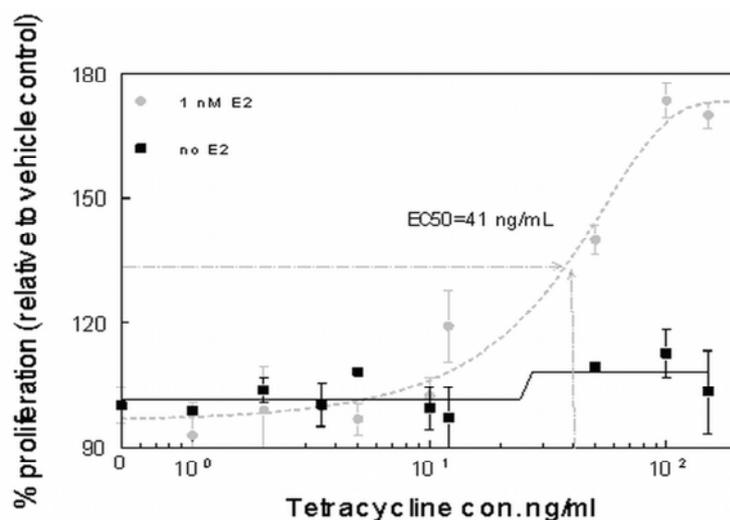


Figure 4: Cell proliferation of T47D-ER β cells in absence (■) or presence (●) of 1 nM E2 at different concentrations of tetracycline. Exposure was performed during 96 h, and proliferation was quantified as mitochondrial activity measured by the resazurin method with fluorescence as endpoint. Each data point represents the mean of triplicate exposure \pm standard deviation.

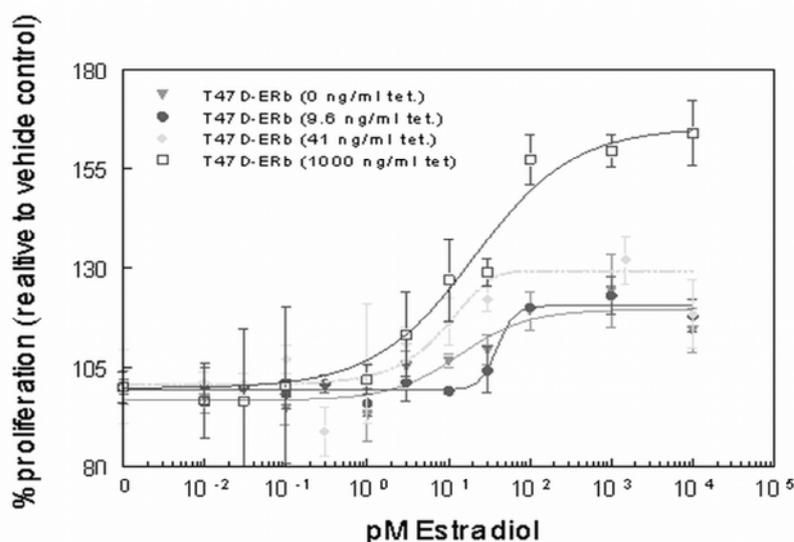


Figure 5: E2-dependent cell proliferation in the T47D-ER β cells after 96 h of exposure in combination with 0, 9.6, 41, and 1000 ng tetracycline/ml to inhibit ER β expression. Each data point represents the mean of triplicate exposure \pm standard deviation.

The proliferative effect of two SERMs

Figure 6 presents the results from experiments in which the proliferative effect of PPT (Figure 6A) and DPN (Figure 6B) was studied in the T47D-ER β cells at the same four tetracycline concentration as used in the E2 study (Figure 4). The straight line drawn at 164% cell proliferation represents the maximum of T47D-ER β (ER β absent) and T47D cell proliferation at 1 nM of estradiol.

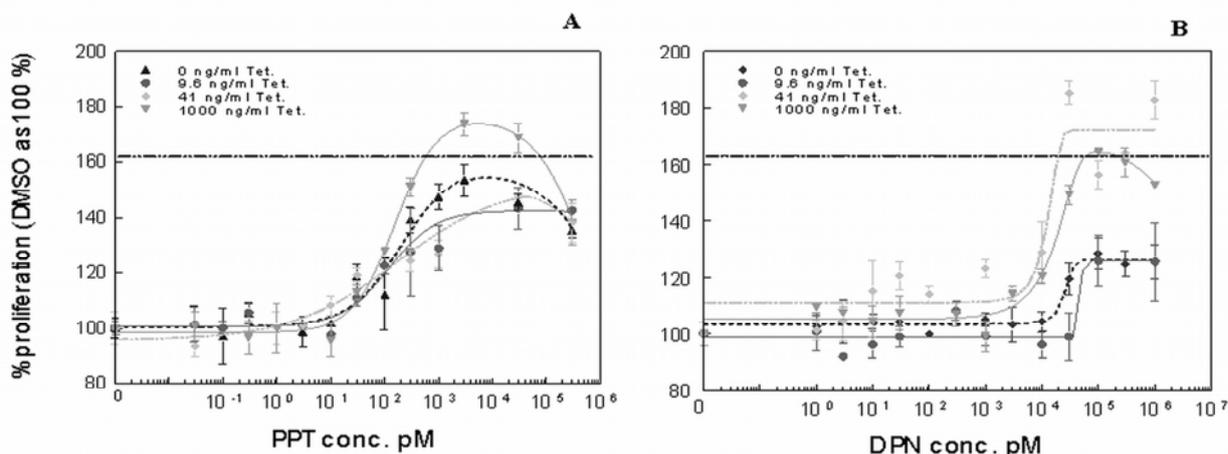


Figure 6: The effect of PPT (A) and DPN (B) on T47D-ER β cell proliferation after 96 h of exposure in combination with 0, 9.6, 41, and 1000 ng tetracycline/ml, quantified as fluorescence using the resazurin method. The line indicates the proliferation induced by 1 nM of E2 when ER β expression was completely inhibited. Each data point represents the mean of triplicate exposure \pm standard deviation.

At 1000 ng/ml of tetracycline, when expression of ER β is suppressed, PPT was able to induce cell proliferation to a level of 173%, an induction level that was slightly higher than the maximal induction of cell proliferation by E2 (164%) under these conditions. This is in spite of the fact that the affinity of ER α for PPT was lower than for E2. Although the E2-induced proliferation with full expression of ER β was reached at 3 nM of PPT (153%), no considerable reduction of proliferation compared with E2 (124%) was observed under all ER α /ER β ratios studied due to the inability of PPT to activate ER β .

At the same tetracycline concentration (1000 ng/ml), DPN induced similar proliferation maximums as E2 (164%) although at a higher concentration than required for maximal induction by E2. This can be due to the fact that ER α has a lower affinity for DPN than for E2. However, DPN appeared able to suppress cell proliferation when levels of ER β expression were high. No differences in cell proliferation were observed at the two lowest tetracycline concentrations (0 and 9.6 ng tetracycline/ml) either for PPT, DPN, or E2.

E2-induced proliferation with full expression of ER β was lower than proliferation induced with PPT and DPN. The maximum induced proliferation in the absence of

tetracycline was 153% with 3 nM PPT, 128% with 100 nM DPN, and 124% with 1 nM E2. In the presence of high levels of ER β , E2- and DPN-induced proliferation was 40% (from 164% to 124%) lower compared to the induced proliferation in absence of ER β .

Discussion

Invasion, uncontrolled proliferation and metastasis are the most important properties of a malignant cancer. Thus, proliferation is not the only hallmark of malignant transformation, and proliferation and invasion may under certain conditions even be contrasting events (36). In the present study, proliferation was selected as the endpoint to characterize the influence of ER α /ER β ratios and not the invasiveness of the tumor cells since the T47D cell line in which the variable ER α /ER β ratios can be generated is a non- or poorly-invasive cell line (37).

The ratio of ER α /ER β expression in breast tumors is higher than in normal breast tissues due to a lower expression of ER β . This has led to the hypothesis that low levels of ER β may result in high proliferation rates because of the absence of ER β -mediated modulation of the proliferative effect of ER α . This would imply that high levels of ER β stimulation lead to decreased cell proliferation whereas high levels of ER α stimulation lead to increased cell proliferation. Therefore, the objective of the present study was to quantify the differential effect of a selective ER α and a selective ER β agonist on cell proliferation of human breast cancer cells with varying but well-defined ratios of ER α /ER β expression. To this end, the T47D-ER β cell model was applied in which the levels of the ER β receptor could be reduced by adding tetracycline. In addition to the E2-induced cell proliferation under different levels of ER β expression, also the effect of two pseudo-estrogens reported to be specific ER α or ER β agonists was determined.

Using human osteosarcoma (U2OS) ER α or ER β reporter cell lines, it could be demonstrated that, compared to E2, PPT is a selective ER α modulator and DPN a preferential ER β modulator. In the ER α - and ER β -specific U2OS-Luc cells, E2 induced ER/ERE-mediated luciferase activity with 8 times higher affinity for ER α than for ER β . DPN was able to induce luciferase activity through both receptors with a 30 times higher potency through ER β than ER α . PPT was found to be a fully ER α -specific inducer ($EEF_{\alpha} = 0.057$) while DPN only reasonably specifically induced ER β ($EEF_{\alpha} = 1.3 \cdot 10^{-4}$, $EEF_{\beta} = 0.03$). This is in accordance with the results previously reported (38). The fact that PPT was not able to activate the transcription of the reporter gene in the ER β -U2OS-Luc is in accordance with earlier observations (39). The maximum induction by the partial ER α agonists DPN did not reach the maximum induction induced by E2 in the ER α -U2OS system, but DPN induced an even slightly higher maximum response than E2 in the ER β -U2OS system.

In normal breast tissues, the ER β to ER α ratio is high, and decreases when breast tumour progresses (21). Earlier studies have suggested that when both receptors are expressed in the cell at the same mRNA levels, E2-induced proliferation of T47D cells is reduced compared to the E2-induced proliferation of cells in which only ER α is expressed (23). Our results in the T47D showed that cells proliferated in the absence of ER β and presence of the natural ligand, E2, indicating that proliferation is E2-ER α mediated. Therefore, to study the role of ER β in cell proliferation, we used the T47D-ER β cell line with inducible ER β expression to directly compare the effects of ER β levels in the same cellular background. As a validation of the cell system, it could be demonstrated that the complete inhibition of ER β expression with 1000 ng/ml of tetracycline resulted in a “pseudo”-wild-type T47D cell with similar E2-induced proliferation responses whereas this E2-induced cell proliferation was no longer observed when ER β expression in the T47D-ER β cells was increased. Furthermore, given the fact that the T47D-ER β cell line is derived from human breast cancer tissue, the expression levels of ER α and ER β in the cells when grown in the presence of 1000 ng tetracycline/ml (no additional ER β expression) can be expected to be physiologically relevant. Furthermore, previous data reported by Ström et al. (23) revealed that when the cells were grown in the absence of tetracycline (full ER β expression), the level of ER β , as judged from mRNA expression levels, appears to be 4-fold higher than that of ER α . Given the fact that physiological levels of ER α to ER β may vary in such a way that either one of the two receptors is dominant (3, 40, 41) it can be concluded that the range of ER α to ER β ratios in the T47D-ER β line with increasing concentrations of tetracycline reflects physiologically relevant variations in the receptor ratio.

The T47D-ER β cell line was engineered to coexpress the EGFP in concurrence with ER β , which allows indirect quantification of ER β by measuring the fluorescence of EGFP. In the present study, a simple microtiter plate method was developed to be able to detect the expression levels of ER β by measuring in the cell lysate the EGFP fluorescence. The IC₅₀ for the EGFP expression after 24 h of exposure was 9.6 ng tetracycline/ml (Figure 2). After 96 h, the EC₅₀ for tetracycline-mediated stimulation of E2-induced cell proliferation was 41 ng tetracycline/ml (Figure 4). The difference between the IC₅₀ value for tetracycline-mediated suppression of EGFP an ER β expression and the EC₅₀ value for tetracycline-mediated stimulation of E2-mediated cell proliferation reflects that for 50% stimulation of E2-mediated cell proliferation, ER β expression needs to be inhibited by more than by 50%.

Our results clearly show an important role of the ER α /ER β ratio in E2-induced cell proliferation. To better understand the interaction between ER α and ER β , the quantification of the exact levels of expression of these receptors is crucial. Our findings also show that the ER subtype ratio determines the functional response to SERMs. Our results were consistent

with the hypothesis that ER β opposes ER α proliferative effects in response to E2. Herein, we show that the proliferative actions in the T47D-ER β cells were mediated by the ER α , whereas ER β played an important role in inhibiting the ER α effectiveness. It cannot yet be concluded whether the inhibition via ER β results in a reduced transcription of genes involved in cell division or that possibly nongenomic signal transduction pathways are induced as well. It has been demonstrated that ER α /ER β heterodimers and ER α homodimers are preferentially formed in intact cells and heterodimers bind to the ERE onto the DNA with similar affinity to that of ER α homodimers and higher affinity than that of ER β homodimers (42).

The ER α -selective agonist PPT was unable to induce luciferase activity through ER β (U2OS cells) (Figure 1B), which implies that PPT does not activate ER β homodimer-mediated gene transcription. Moreover, it has been shown that ER α /ER β heterodimers are only effective in coactivator interaction when both ER α and ER β are doubly occupied with agonists (43). DPN and PPT are as effective in stimulation of cell proliferation as E2 in the absence of ER β (Figure 6). In the presence of ER β , cell proliferation is decreased. DPN is more potent than PPT in inhibition of cell proliferation when both ER α and ER β are present as in contrast to PPT DPN can activate ER β .

In contrast to exposure to E2 and DPN, exposure to PPT in the presence of high levels of ER β expression did not give rise to visible cell death. This corroborates a role of the activated ER β in the induction of cell death as previously reported (44, 45). Therefore, it is important to explain the specific roles of the ER α and ER β when both receptors are present and link this to the proliferation outcome.

The current results and developed method show that activation of ER β can result in a reduction of ER α -mediated cell proliferation. In the T47D-ER β cell line, PPT was unable to suppress cell proliferation at all ratios of ER α /ER β expression, indicating its ability to activate only ER α . Whereas DPN appeared to be able to suppress cell proliferation when levels of ER β expression were high since it was able to bind preferentially to ER β . It is concluded that effects of estrogen-like compounds on cell proliferation are dependent on the actual ER α /ER β expression levels in these cells or tissues, and the potential of the estrogen agonists to activate ER α and/or ER β .

Thus, the use of ER β protein expression levels as a biomarker in tumour screening, in addition to protein expression levels of ER α , has the potential of more successful indication of therapeutic responses and course/outcome of the disease in ER-positive tumors. Future studies at a molecular level will be performed to further elucidate how ER β exerts these effects.

Acknowledgments

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Phytoestrogen-mediated Inhibition of
Proliferation of the Human T47D Breast
Cancer Cells Depends on the ER α /ER β Ratio

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Abstract

This study investigates the importance of the intracellular ratio of the two estrogen receptors ER α and ER β for the ultimate potential of the phytoestrogens genistein and quercetin to stimulate or inhibit cancer cell proliferation. This is of importance because i) ER β has been postulated to play a role in modulating ER α -mediated cell proliferation, ii) genistein and quercetin may be agonists for both receptor types and iii) the ratio of ER α to ER β is known to vary between tissues. Using human osteosarcoma (U2OS) ER α or ER β reporter cells it was shown that compared to estradiol (E2), genistein and quercetin have not only a relatively greater preference for ER β but also a higher maximal potential for activating ER β -mediated gene expression. Using the human T47D breast cancer cell line with tetracycline-dependent ER β expression (T47D-ER β), the effect of a varying intracellular ER α /ER β ratio on E2- or phytoestrogen-induced cell proliferation was characterised. E2-induced cell proliferation of cells in which ER β expression was inhibited was similar to that of the T47D wild type cells, whereas this E2-induced cell proliferation was no longer observed when ER β expression was increased. With increased expression of ER β the phytoestrogen-induced cell proliferation was also reduced. These results point at the importance of the cellular ER α /ER β ratio for the ultimate effect of (phyto)estrogens on cell proliferation.

Introduction

Phytoestrogens are a group of plant-derived compounds with estrogenic properties (1, 2). The major types of phytoestrogen are isoflavones, flavones, coumestans, lignans and stilbenes. Among the most studied flavonoids with respect to anti-tumour functions are genistein and quercetin. Phytoestrogens have been considered a natural alternative to hormone replacement therapy (HRT) since these chemicals are found in the regular diet (3). Genistein is mostly present in soybeans whereas quercetin is part of the colouring found in the skins of apples and red onions. High consumption of phytoestrogen-rich food has been reported to correlate with reduced incidence of breast cancer (4) and especially genistein and quercetin have been shown to exert potent anti-proliferative effects on tumour cells *in vitro* by halting the cell cycle and inducing apoptosis (5-7).

Two main estrogen receptors (ERs), ER α and ER β , have been identified in rats, mice, primates and humans (8). Different biological responses may occur when a phytoestrogen binds to the different ERs. Compared to ER α subtype, ER β may mediate different biological effects and display different intracellular and tissue distribution patterns (9). ER α and ER β subtypes can interact with a wide variety of different compounds although some ligands appear to have different relative affinities for the subtypes (10-13). The affinity of phytoestrogens for the ERs is related to their chemical structure. Indeed, although the phytoestrogens can bind to the ER α , they appear to prefer binding to the ER β (2, 14-16).

The protective role of ER β in cancer research is now being studied widely. Most studies have shown decreased ER β expression in cancer tissues as compared to benign tumours or normal tissues, whereas ER α expression persists (17, 18). Hence ER α and ER β may have different roles in gene regulation and their relative levels or ratios within the tissues may influence cellular responses to estrogens.

The aim of the present study was to identify the importance of the intracellular ratio of the two receptors ER α and ER β for the ultimate potential of two model phytoestrogens, genistein and quercetin, to stimulate or inhibit cancer cell proliferation. To this end the activity of the two model compounds to activate either ER α - or ER β -mediated gene transcription was characterised and compared to that of E2 using human osteosarcoma cells (U2OS) stably transfected with ER α or ER β and a luciferase reporter gene with a 3xERE-containing promoter region. This assay provided a quantitative indication of the estrogenic activity of the phytoestrogens relative to that of E2 at the receptor level. In addition, phytoestrogen-dependent cell proliferation was investigated in a T47D breast cancer cell line

with a tetracycline-inducible ER β (19) to characterize the effect of phytoestrogens on cell proliferation when altering the intracellular ER α /ER β ratio.

Materials and methods

Materials: 17 β -estradiol (E2)(purity>98%), genistein and ANTI-FLAG M2[®] Monoclonal-antibody Peroxidase Conjugate were purchased from Sigma (Zwijndrecht, The Netherlands). Dimethyl sulfoxide (DMSO)(purity>99%) and quercetin dehydrate were purchased from Acros Organics (Pittsburgh, PA, USA). Tetracycline, streptomycin, penicillin and puromycin were acquired from Gibco (Paisley, Scotland). Fetal calf serum (FCS), and geneticin were provided by Invitrogen Life Technologies (Paisley, Scotland). Phosphate-buffered saline (PBS), non-essential amino acids, growth medium 1:1 mixture of Ham's nutrient mixture F12 and DMEM, and exposure medium phenol-free were supplied by Gibco (Paisley, Scotland). Hyclone dextran-charcoal-treated FCS (DCC-FCS) obtained from Perbio Science NV (Etten-Leur, The Netherlands) was heat inactivated (20). Trypsin was obtained from Difco (Detroit, USA). Ascorbic acid, sodium bicarbonate, sodium hydroxide, ethylenedinitrotetraacetic acid (EDTA), magnesium sulphate, and 1,4-dithiothreitol (DTT) were obtained from Merck (Darmstadt, Germany). Magnesium carbonate was obtained from Aldrich (St. Louis, MO, USA). *trans*-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid monohydrate (CDTA) was obtained from Fluka (Buchs, Switzerland). Hygromycin and diferin were obtained from Duchefa (Haarlem, The Netherlands). ATP, (BrdU)-Roche Cell proliferation ELISA, BrdU (colorimetric) kit and protease inhibitors mix were provided by Roche Diagnostics (Mannheim, Germany). BSA Protein Assay Kit was purchased from Pierce (Germany). Tween 20 was obtained from Merck (Germany). Sodium dodecyl sulphate (SDS) was obtained from BDH (United Kingdom). Acrylamide (30% acrylamide/bis), N,N,N',N'-tetramethylethylenediamine (TEMED), ammonium persulphate (APS) and the precision plus dual colour protein marker were obtained from BioRad (The Netherlands). Milk solution ELK was provided by Campina (The Netherlands). Nitrocellulose membrane was purchased from Whatman (The Netherlands). Chemiluminescent detection ECL kit and photographic hyperfilm were provided by Amersham (United Kingdom).

Cell lines: T47D human breast cancer wild type cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The stably transfected T47D-ER β tetracycline-inducible cell line were used as described before (19). The human osteosarcoma (U2OS) cell lines ER α -U2OS-Luc and ER β -U2OS-Luc stably expressing either ER α or ER β , respectively, in addition to 3xERE (Estrogen Responsive Element)-tata-luciferase were used as described before (21).

Cell culture conditions: ER α - and ER β -U2OS-Luc, T47D wild type and T47D-ER β cells were cultured as described before (22).

Measurement of ER β expression-related fluorescence: Quantification of ER β expression-related EGFP fluorescence after 48 h of exposure was performed following the methods previously described (22).

Exposure conditions for T47D and T47D-ER β cells: Experiments were performed in phenol red-free exposure medium supplemented with 5% dextran-charcoal-treated FCS (DCC-FCS). Confluent cells were seeded in 96-well plates (100 μ l/well) at 10^5 cells/ml for the proliferation assays in the presence of tetracycline and at 1.8×10^5 cells/ml for fluorescence assays, in the presence of different concentrations of tetracycline (0 - 1000 ng/ml) as indicated. Plates were incubated overnight at 37°C with 5% CO₂. Three different tetracycline concentrations were used to obtain three different ER β expression levels (for details see Results section); 1000 ng/ml tetracycline for full inhibition of ER β expression, 40 ng/ml tetracycline for 50% inhibition of ER β expression, and 0 ng/ml tetracycline for full expression of ER β . After 24 h, cells were washed with phosphate-buffered saline and exposed to different concentrations of tetracycline as indicated (1000, 40 or 0 ng/ml) and/or the test compound at the concentrations indicated. For incubations with quercetin, 600 μ M ascorbic acid was added for compound stabilization (23).

Cell proliferation measurements: After 48 h of exposure, proliferation was determined by measuring 5-bromo-2'-deoxy-uridine (BrdU) incorporated into DNA following BrdU Roche's colorimetric protocol. Measurements were performed in a spectrophotometer at 370 nm excitation and 492 nm emission wavelengths.

ER α - and ER β -specific U2OS reporter gene assay: Cultured ER-U2OS-Luc cells were washed with PBS, trypsinized and seeded in transparent 96-well plates at 100 μ l/well at a density of 10×10^4 cells/ml (ER α -U2OS-Luc) or 7.5×10^4 cells/ml (ER β -U2OS-Luc) in a 1:1 mixture of DMEM and Ham's F12 medium without phenol red, buffered with 1260 mg/l NaHCO₃ and supplemented with 5% dextran-charcoal-treated FCS (DCC-FCS) and 0.5% nonessential amino acids. Culture medium was refreshed after 24 h. Forty-eight hours after seeding cells were exposed in triplicate to E2, genistein or quercetin at the indicated concentrations (final DMSO concentration 0.2%) for 24 h at 37°C and 7.5% CO₂ in a humidified atmosphere. On each plate, cells were exposed to a full dose of test compound and calibration points for E2 were also included. After 24 h, the medium was removed and cells were washed with 100 μ l of diluted PBS (0.5x PBS in demineralised water). Cells were lysed with 30 μ l of a hypotonic low-salt buffer, consisting of 10 mM Tris-HCl pH 7.8 containing 2 mM DTT and 2 mM CDTA. Plates were put on ice for 10 min and subsequently

frozen at -80°C . Before analysis, plates were thawed on ice for 20 min and shaken briefly until reaching room temperature. Analyses were performed in a Luminoskan (RS, Labsystems) at room temperature as follows: background light emission of each well was measured for 2 sec, then 100 μl of flashmix was added (20 mM tricine buffer, pH 7.8, supplemented with 1.07 mM $(\text{MgCO}_3)_4\text{Mg}(\text{OH})_2 \cdot 5\text{H}_2\text{O}$, 2.67 mM MgSO_4 , 0.1 mM EDTA $\cdot 2\text{H}_2\text{O}$, 2 mM DTT, 0.47 mM diferin, and 5 mM ATP), light emission was immediately measured for 2 sec and extinguished with 50 μl 0.2 M NaOH.

Protein Isolation and SDS-PAGE: For the analysis of the ER β protein expression levels, T47D-ER β cells were grown in growth medium with 1000 ng/ml of tetracycline in small cell culture flasks until 80-90% confluence. Cells were seeded in exposure medium with tetracycline for 24 h. Medium was removed and cells were incubated for 48 h in new exposure medium with different tetracycline concentrations (0, 40 and 1000 ng/ml of tetracycline). Proteins samples were obtained as described previously (22).

Western blotting: Blotting was performed at 100V for 1 h. After the transfer unspecific binding sites on the membrane were blocked with 5% milk solution ELK in TBS 0.05% Tween 20 for 1-2 h. The membrane was washed in TBS with 0.05% Tween 20 twice for 5 min. Monoclonal ANTI-FLAG M2[®] antibody was diluted in TBS with 0.05% Tween 20 and after incubation for 1 h at room temperature the membrane was washed with TBS with 0.05% Tween 20 six times for 5 min each time. Next, the membrane was treated with peroxidase substrate (ECL kit) to detect the FLAG fusion protein. The reaction was run for 5-7 min and bands were visualized using photographic film.

Calculations and statistics: Luciferase induction by quercetin and genistein in the U2OS cells was compared with the luciferase induction by the natural ligand E2. The background luciferase induction by the solvent control was set at 0% induction. The maximum induction of luciferase obtained at 12 pM E2 for ER α cells and at 300 pM E2 for ER β cells was set at 100%. The EC₅₀ values, concentration of the compound at which 50% of the maximum luciferase activity is reached, were derived from the dose-response curves using R. The average EC₅₀ values and their S.E. were calculated from taking the average of the EC₅₀ values of 3 independent experiments. Estradiol equivalency factors (EEF) were calculated as $\text{EC}_{50 \text{ estradiol}}/\text{EC}_{50 \text{ compound}}$.

EGFP fluorescence, reflecting the level of ER β induction, was expressed relative to the fluorescence of cells exposed to the solvent control without tetracycline (PBS 0.2%) set at 100%.

Normalized data from breast cancer cell results were plotted as absorbance at A_{492} minus A_{370} nm where the maximal induction in the control cells cultured in the presence of 1000 ng tetracycline/ml was set as 1. Statistical analysis of the data from T47D-ER β proliferation assays was done with the Tukey HSD test (ANOVA) at 95% confidence level. For each compound concentration results obtained from cells with inhibited expression of recombinant ER β (1000 ng/ml tetracycline) were compared to data obtained from cells expressing half (in the presence of 40 ng/ml tetracycline) and full (in the absence of tetracycline) recombinant ER β levels to test whether the intracellular expression of recombinant ER β has a statistically significant effect on E2- or phytoestrogen-induced cell proliferation.

Results

Activity of E2 and the model phytoestrogens in the ER-U2OS-Luc reporter gene cells

The estrogenic potency of genistein and quercetin was measured using the human osteosarcoma (U2OS) ER α or ER β reporter cell line, and compared to the activity of the natural ER ligand E2 in these cells. These cells were stably transfected with either ER α or ER β and luciferase expression plasmid with 3x ERE. Initially treatment of the cells with quercetin resulted in no response (data not shown), except for the highest compound concentration. Addition of ascorbic acid at a concentration of 600 μ M was previously shown to increase the stability of the quercetin and to have no toxic or inducing effects in the U2OS cell lines (12, 23). Our studies confirmed that ascorbic acid itself does not have an effect on ER-mediated gene expression (data not shown). Treatment of the ER α -U2OS-Luc and ER β -U2OS-Luc cells with E2, genistein and ascorbate-stabilized quercetin resulted in a concentration-dependent expression of luciferase (Figure 1). Table 1 presents the half maximal effective concentration (EC_{50}) values derived from these curves for E2-, genistein- and quercetin-mediated induction of ERE-Luc activity in the U2OS cell lines. For E2 the EC_{50} value for ER α activation was lower (3.8 pM) than for ER β activation (76 pM) suggesting an about 20-fold higher preference of E2 for ER α - than for ER β -mediated induction of gene expression. Activation of both ER α - and ER β -mediated induction of gene expression by genistein as well as quercetin required much higher agonist concentrations. Quercetin EC_{50} values were 6.5 μ M and 9 μ M for ER α and ER β , respectively. Calculated EEFs were 5.85×10^{-7} for ER α and 8.44×10^{-6} for ER β . The affinity of genistein, towards both ERs was higher than that of quercetin, and genistein preferentially activated ER β as indicated by the EC_{50} values, which amounted to 100 nM for ER α and 7 nM for ER β . Calculated EEFs for ER α and ER β based on these EC_{50} values were 3.8×10^{-5} and 0.01, respectively.

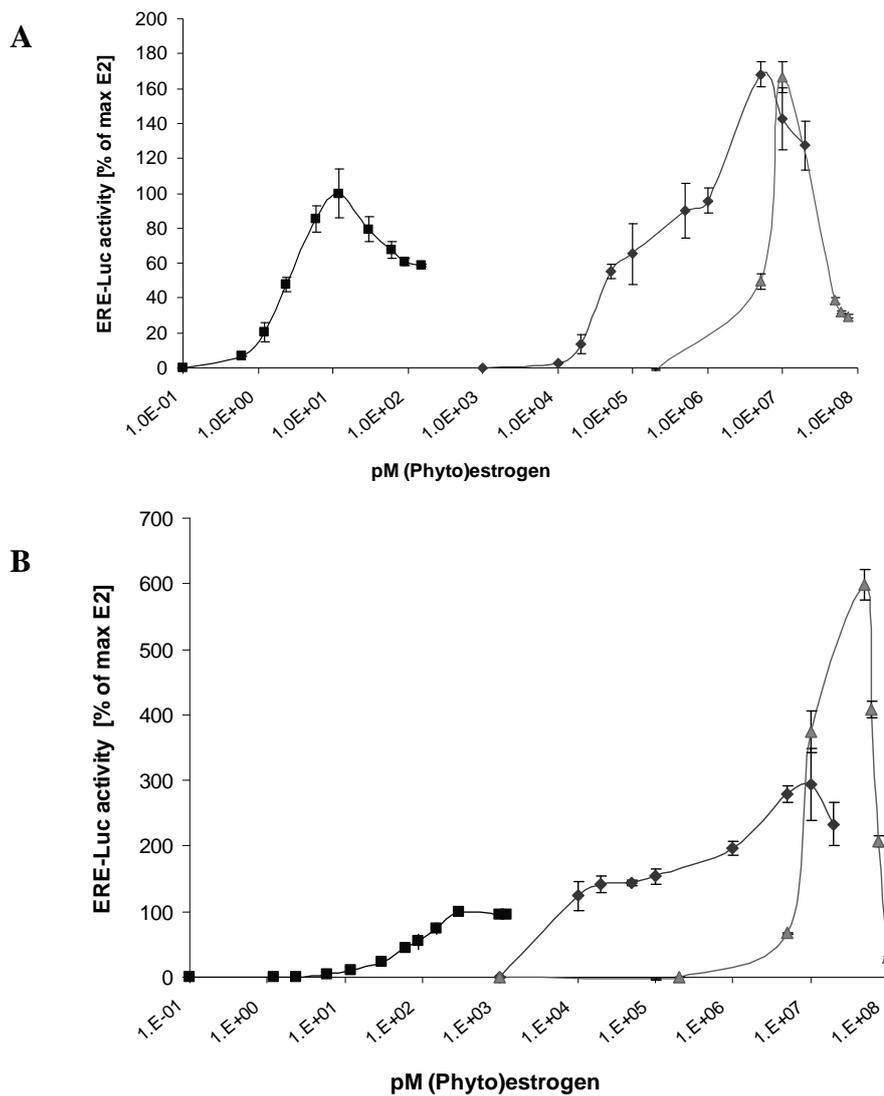


Figure 1: Induction of ERE-mediated luciferase activity in the U2OS-ER α (A) and U2OS-ER β (B) cells upon exposure to E2 (■), genistein (◆) and ascorbate stabilized quercetin (▲). Induction was expressed relative to maximal E2 response set at 100%. Data points represent the mean of triplicates exposure \pm standard deviation.

Even though both phytoestrogens were shown to bind to both ERs with much lower affinity (higher EC₅₀) than the natural ligand E2, the maximum level of Luc activity induced was significantly higher. The maximal ERE-Luc activity induced by genistein treatment was 168% and 293% of the maximal ERE Luc activity induced by E2 in the ER α - and ER β -U2OS-Luc cells, respectively (Figure 1). This effect was even more pronounced with quercetin, which resulted in a maximal ERE-Luc activity that amounted to 166% that induced by E2 in ER α -U2OS cells and to 598% that induced by E2 in ER β -U2OS cells (Figure 1).

Table 1: Overview of the EC₅₀, EEF and maximum effect of genistein and quercetin tested using the U2OS cell system compared to E2.

	ERα EC ₅₀	Max effect % relative E2-max	ERα EEF	ERβ EC ₅₀	Max effect % relative E2-max	ERβ EEF	ERα EEF/ ERβ EEF ratio
E2	3.8 ± 1.2 pM	100	1	76 ± 4 pM	100	1	1
Genistein	100 ± 8 nM	168	3.8 × 10 ⁻⁵	7 ± 4 nM	293	0.01	0.038
Quercetin	6.5 μM	166	5.85 × 10 ⁻⁷	9 ± 0.02 μM	598	8.44 × 10 ⁻⁶	0.069

(EC₅₀: concentration of the compound at which 50% of the maximum luciferase activity is reached; EEF: estradiol equivalent factor calculated as EC₅₀ (E2)/EC₅₀ (test compound)).

Tetracycline-dependent expression of ER β in the T47D-ER β cell line quantified by measuring EGFP Fluorescence and Western blot analysis

T47D cells were stably transfected with the tagged ER β and an Enhanced Green Fluorescence Protein (EGFP) gene as a co-expressed reporter under regulation of the same tetracycline-responsive promoter. Wells seeded at high-density number of the T47D-ER β cells were exposed to different concentrations of tetracycline and after 48 h cultivation EGFP fluorescence was measured in the cell lysate. Figure 2A presents the EGFP fluorescence thus detected as a function of the tetracycline concentration. Maximal levels of fluorescence were reached after cultivation of the cells at the lowest tetracycline concentrations. Tetracycline treatment suppressed EGFP fluorescence in T47D-ER β cells, resulting in total fluorescence suppression at concentrations above 150 ng/ml. The concentration of tetracycline at which 50% of the fluorescence was inhibited (IC₅₀) was 40 ng/ml.

EGFP fluorescence measured in the cell lysate is linked to the expression of recombinant ER β since both are co-expressed. Based on these fluorescence results three different tetracycline concentrations were defined that would result in three ER β expression levels: (i) 1000 ng/ml tetracycline where no expression of EGFP was detected and thus no expression of recombinant ER β is expected; (ii) 40 ng/ml tetracycline resulting in 50% expression of EGFP and thus of ER β and (iii) 0 ng/ml tetracycline, leading to full expression of EGFP and ER β . To further confirm that these tetracycline-concentrations result in maximal, 50% and no ER β expression levels the tagged ER β protein levels at the three tetracycline concentrations were characterised using Western blot analysis (Figure 2B). Detection of the tagged ER β was confirmed at 0 ng/ml and 40 ng/ml of tetracycline, whereas no ER β protein was detected at 1000 ng/ml. Quantifications of the intensity of the bands by ImageJ corroborated that at 40

ng/ml tetracycline expression levels were about 50% of those detected at 0 mg/ml tetracycline (Figure 2B).

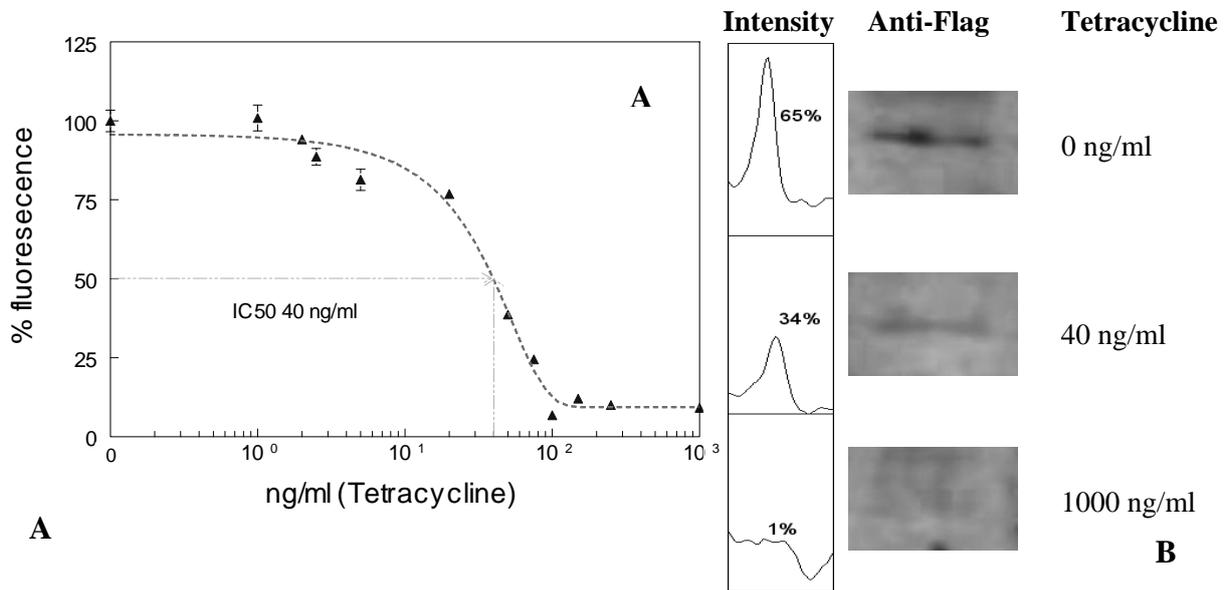


Figure 2: (A) Tetracycline-induced inhibition of ER β expression in T47D-ER β cells measured via the concurrent expression of Enhanced Green Fluorescence Protein (EGFP) after 48 h of exposure. Fluorescence is expressed relative to maximum expression at 0 ng/ml of tetracycline set at 100%. No fluorescence is observed above 150 ng of tetracycline per mL. Each data point represents the mean of triplicate exposure \pm standard deviation. (B) Western blot of T47D-ER β cells treated with three different concentrations of tetracycline (0, 40 and 1000 ng/ml) for 48 h. The blotting was done with protein FLAG antibody to detect tagged ER β . Band intensities were determined using ImageJ gel analyzer, available at <<http://rsb.info.nih.gov/ij/>>.

Proliferation of the T47D wild type cells

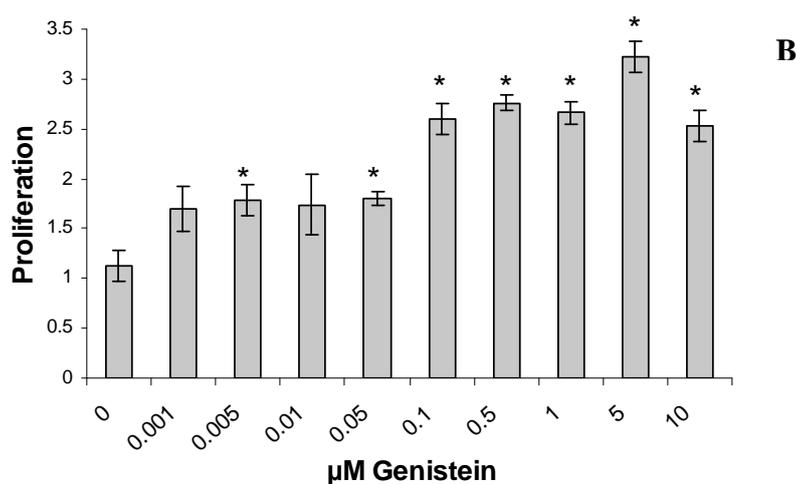
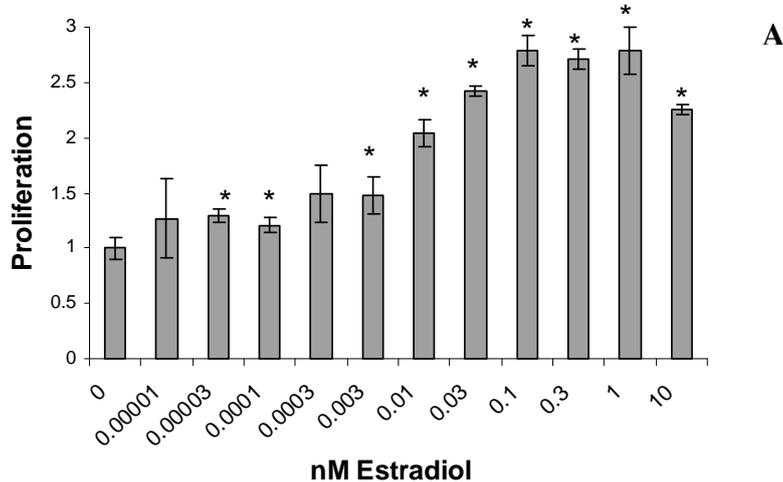
Treatment of T47D wild type cells during 48 h with E2, genistein or ascorbate stabilized-quercetin resulted in a dose-dependent increase in cell proliferation (Figure 3). All proliferation data have been normalized to the same reference point, being proliferation levels obtained at 1 nM E2, and therefore it can be derived from the data presented in Figure 3 that genistein and E2 reached an almost similar maximum proliferation level but at different concentrations. The maximum level of proliferation, induced by quercetin was slightly lower than that induced by E2 and genistein. The maximum levels of proliferation were reached at about 100 pM E2, 500 nM genistein and 1 μ M quercetin. The EC₅₀'s for proliferation amounted to 8.2 pM E2, 78 nM genistein and 81 nM quercetin (Table 2).

No toxic effects or cell death were observed using optical microscopy at any concentration of (phyto)estrogens tested (data not shown).

Table 2: Overview of the EC₅₀ and EEF of estradiol, genistein and quercetin using the T47D wild type and T47D-ER β cell lines (np= no proliferation, therefore no EC₅₀ could be calculated).

	T47D wild type				T47D-ER β			
	ER α	ER α	1000 ng/ml	Tet.	40 ng/ml	Tet.	0 ng/ml	Tet.
	EC ₅₀	EEF	EC ₅₀	EEF	EC ₅₀	EEF	EC ₅₀	EEF
E2	8.2 \pm 2.5 pM	1	30 \pm 20 pM	1	28 \pm 10 pM	1	np	np
Genistein	78 \pm 7 nM	1.05 \times 10 ⁻⁴	98 \pm 21 nM	3.0 \times 10 ⁻⁴	94 \pm 18 nM	2.9 \times 10 ⁻⁴	927 \pm 346 nM	3 \times 10 ⁻⁵
Quercetin	81 \pm 5 nM	1.01 \times 10 ⁻⁴	90 nM	3.3 \times 10 ⁻⁴	np	np	np	np

(EC₅₀: concentration of the compound at which 50% of the maximum luciferase activity is reached; EEF: estradiol equivalent factor calculated as EC₅₀ (E2)/EC₅₀ (test compound)).



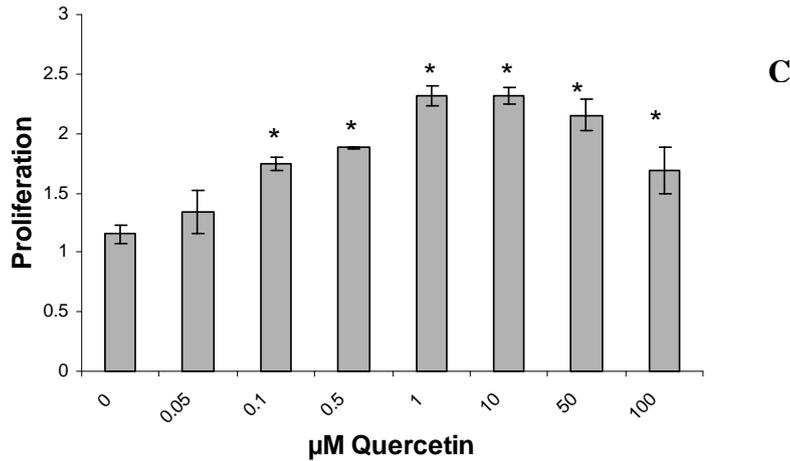
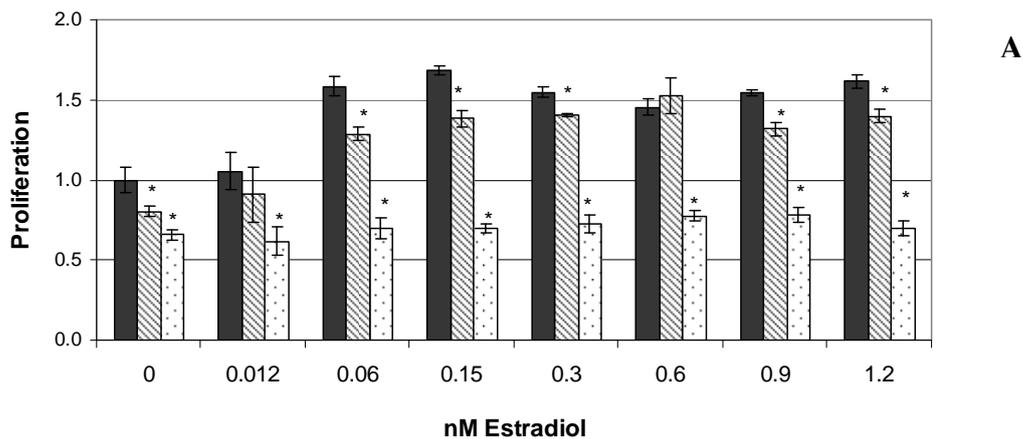


Figure 3: (A-C) Effect of E2, genistein and ascorbate stabilized quercetin on the proliferation of T47D wild type cells. Data are presented normalized with respect to the proliferation of control cells set at 1. Each normalized data point represents the mean of triplicate exposure \pm standard deviation. * Significantly different from the control (DMSO) at $p < 0.05$.

Proliferation of the T47D-ER β cells at three different ER α /ER β ratios

The effect of the intracellular ratio of ER α /ER β on E2 and phytoestrogen-induced cell proliferation was tested using the T47D-ER β recombinant cell line in which the intracellular levels of ER β can be modified. Expression levels of ER β in the T47D-ER β cell line are tetracycline-dependent, and full expression of the ER β receptor is observed upon tetracycline removal from the medium.



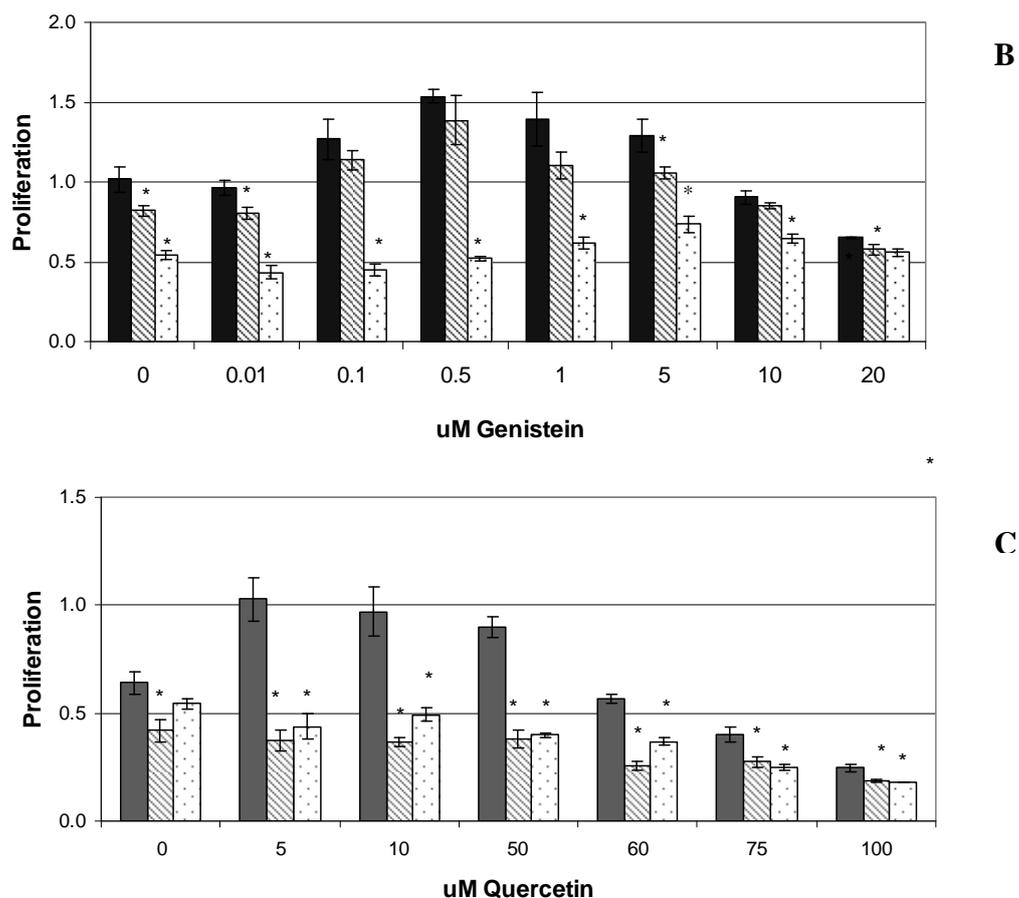


Figure 4. Effect of estradiol (A), genistein (B) and ascorbate stabilized quercetin (C), at three different levels of ER β expression, on the proliferation of T47D-ER β cells. Fully coloured columns represent exposure to the test compounds in combination with 1000 ng/ml tetracycline, the shaded columns with 40 ng/ml tetracycline and the dotted columns with 0 ng/ml tetracycline. Proliferation was quantified measuring BrdU incorporation after 48 hours exposure. Data are presented normalized with respect to the proliferation of control cells in the presence of 1000 ng tetracycline/ml set at 1. Each normalized data point represents the mean of triplicate exposure \pm standard deviation. * Significantly different from the control (proliferation at 1000 ng/ml Tet.) at $p < 0.05$.

Figure 4 shows the concentration-dependent effect of respectively E2 (Figure 4A), genistein (Figure 4B) and ascorbate-stabilized quercetin (Figure 4C) on proliferation of T47D cells with varying levels of ER β expression.

Exposure of the cells to the test compounds in the presence of 1000 ng/ml tetracycline resulted in stimulation of cell proliferation. In the presence of 1000 ng/ml tetracycline the recombinant T47D cells do not express ER β and their response to exposure to the test compounds can be compared to that observed for the wild type T47D cells (Figure 3). Comparison of the EC₅₀ values obtained in T47D wild type and in T47D-ER β cells grown in the presence of 1000 ng/ml tetracycline inhibiting recombinant ER β expression (Table 2) reveals that in both cell lines genistein and quercetin induce ER α -mediated cell proliferation

with lower potency than E2, and that their effect on cell proliferation is similar in both cell lines. In E2-treated T47D-ER β cells proliferation increased until 1.2 nM (Figure 4A) and decreased above that concentration (data not shown). Genistein (Figure 4B) was used in a concentration range from 10 nM up to 20 μ M, concentrations that were effective for both ER α - and ER β -mediated induction of gene expression in the U2OS-Luc cells. Genistein-stimulated proliferation reached its maximum at 0.5 μ M and decreased in a dose-dependent manner at higher concentrations until showing complete inhibition of cell proliferation at concentrations above 10 μ M. Ascorbate stabilized-quercetin was used in concentrations between 5 and 100 μ M (Figure 4C). Growth stimulation was observed within a concentration range from 5 to 50 μ M quercetin. Above 60 μ M quercetin cell growth decreased to values that were even lower than that of the control cells incubated without quercetin.

In the presence of half of the maximal expression of ER β , a decrease in the levels of cell proliferation induced by E2 and genistein was observed as compared to the cell proliferation induced by these compounds in cells with no ER β expression (Figure 4A and 4B). Moreover, the quercetin-induced cell proliferation was completely inhibited when the cellular ER β expression level was at 50%. As a result no EC₅₀ could be derived for quercetin-induced cell proliferation of T47D cells with 50% ER β expression levels. Together these data obtained with T47D cells with 50% ER β expression levels reveal that the growth inhibitory effect of ER β was stronger with quercetin (full inhibition of cell proliferation, no EC₅₀) than with E2 (EC₅₀ 28 pM) and genistein (EC₅₀ 94 nM).

Upon complete withdrawal of tetracycline from the medium, resulting in full expression of ER β , E2-, genistein- and quercetin-induced cell proliferation was significantly reduced. From the data obtained only those for genistein still allowed definition of an EC₅₀ value for genistein-induced cell proliferation of T47D cells with full expression of ER β amounting to 927 nM.

In addition, T47D-ER β cells incubated in the absence of tetracycline and expressing full ER β levels showed a reduced proliferation rate as compared to the cells when incubated in the presence of tetracycline, even when not exposed to an estrogen agonist. Furthermore, for these cells, cell death was observed based on microscopic examination. Cell death upon exposure to phytoestrogens was more pronounced for cells with full expression of ER β (data not shown).

Discussion

The present study has investigated the importance of the intracellular ratio of the two receptors ER α and ER β for the ultimate potential of the model phytoestrogens genistein and

quercetin to inhibit cancer cell proliferation. This is of importance because (i) ER β has been postulated to play a role in modulating ER α -mediated cell proliferation (24), (ii) the phytoestrogen model compounds of the present study, genistein and quercetin, may be agonists for both receptor types (1) and (iii) the ratio of ER α to ER β is known to vary between tissues (19, 25).

In this study, it was demonstrated that the binding affinity of phytoestrogens for ER α and ER β is one to three orders of magnitude lower than that of E2. The affinity in favour of ER β is reflected in the ER α EEF/ER β EEF ratio which is lower for quercetin and genistein (0.069 and 0.038, respectively) than for E2. The higher transcriptional activity of the phytoestrogens compared to E2 resulted in maximal ER α -mediated induction of gene expression by genistein and quercetin that was 1.7 times higher than that induced by E2. For ER β this difference was even more pronounced, with a maximal induction that was three and six times higher, respectively, for genistein and quercetin than for E2. The relatively higher ER β affinity and the relatively higher level of ER β receptor-mediated induction of gene expression for the phytoestrogen model compounds are in accordance with previously results in other reporter gene cell lines (12, 26). Legler *et al.* suggested that this "superagonism", might be due to stimulated receptor and/or cofactor renewal. An enhanced ability of ER β to recruit coactivators in the presence of xenoestrogens has been suggested before. There are differences in the positioning of the helix-12 in the ER β -genistein-bound complex as compared with E2-bound ER β complex which may allow the cofactor SRC-3 NR box I to bind with a 3-fold greater amount to genistein-bound than E2-bound ER β (27, 28). It could be speculated that this effect might be even more pronounced with quercetin.

The ratio of endogenous ER α to ER β mRNA in the T47D wild type cell line is 9 to 1 (19). To investigate whether ER β plays an important role in inhibiting ER α -mediated induction of cell proliferation by the phytoestrogens we used the T47D-ER β cell line with inducible recombinant ER β expression. E2, genistein and quercetin all stimulated cell proliferation in the absence of ER β expression. The bell-shaped dose-response proliferation curve is similar to those previously obtained for genistein-mediated effects on proliferation of wild type T47D cells (29, 30). Quercetin exposure of cells with ER β -inhibited expression also resulted in a bell-shaped dose-response curve for cell proliferation. Quercetin was able to stimulate cell proliferation in a concentration range between 5 and 50 μ M, while inhibition was observed at concentrations above 50 μ M. These results were in accordance to those obtained previously with the wild type T47D cells (12).

Quercetin showed a significantly higher maximum level of activation of gene expression in the U2OS-ER β reporter cells than E2 (i.e. six times higher) and also a relatively higher

affinity for ER β than for ER α . This may explain why the increase in the intracellular level of recombinant ER β had a more pronounced effect on quercetin-induced cell proliferation than on E2-induced cell proliferation. With both half and full expression of recombinant ER β , quercetin inhibited cell growth to levels that were even lower than that observed for non-treated controls. Similar to E2, genistein inhibited growth-stimulatory effects more efficiently in cells with full expression of ER β than in cells with no expression of recombinant ER β exposed to genistein. Surprisingly however, at 20 μ M genistein, the highest concentration tested, growth of cells at all levels of ER β expression tested was comparable to that of non-treated control cells with full expression of ER β . This suggests that in the cells exposed to 20 μ M genistein the ER β -mediated induction of gene expression by genistein is only in part responsible for the observed antiproliferative effects.

In contrast to E2 and genistein, with quercetin exposure maximal inhibition of cell proliferation was observed already with half expression of the intracellular recombinant ER β . Moreover, in cells with high levels of ER β expression E2- and flavonoid-induced cell death was observed. Since this was not observed upon exposure of the wild type T47D cells and also not in the T47D-ER β cells in which ER β expression was suppressed by 1000 ng/ml tetracycline, cytotoxic effects, as suggested by other authors (31), can be disregarded as the cause of this observation. The findings rather suggest, that agonist-mediated activation of ER β not only reduces ER α -mediated gene expression that results in cell proliferation but also inhibits cell proliferation by other mechanisms. This would be in line with previous studies suggesting an ER β -mediated induction of apoptosis (32-34). Noteworthy is the fact that significant inhibition of cell proliferation was also observed in vehicle treated cells when ER β levels were significantly expressed, suggesting that ER β can inhibit cell growth in a ligand-independent manner, which is consistent with results obtained in MCF7 cells (35). Moreover, it has been demonstrated that ER α /ER β heterodimers and ER α homodimers are preferentially formed in intact cells (36). Increasing levels of ER β expression might enhance the ratio of heterodimer formation thereby leading to reduced ER α -homodimer-mediated growth stimulation even in the absence of ligand.

Recently Williams et al. reported transcriptome data for E2-exposed T47D-ER β cells with varying levels of recombinant ER β (37), and concluded that E2-induced ER α -regulated genes were involved in cell cycle progression whereas E2-induced ER β -regulated genes were involved in suppression of cell proliferation. The up-regulation of e.g. Cyclin A (CCNA2), CDC20 cell division cycle 20 homolog (CDC20), BUB1 budding uninhibited by benzimidazoles 1 homolog (BUB1) and down-regulation of the tumour protein p53 inducible nuclear protein (TP53INP1) in T47-ER β cells in which ER β expression was inhibited, implicates a role of ER α in proliferative processes. In contrast, in T47D cells in which ER β

was expressed, E2 upregulated genes such as quiescin Q6 (QSCN6) and septin (SEPT9), involved in negative regulation of cell proliferation, as well as serine/threonine kinase 3 (STK3), connected to apoptotic processes.

These transcriptomic results are in accordance with our E2-proliferation data and corroborate the modulating effect of ER β reducing the E2-induced ER α -mediated proliferation. Similar transcriptional analysis of T47D-ER β cells with different levels of ER β expression exposed to phytoestrogens should reveal whether the effects induced by the phytoestrogens proceed by similar differential gene expression pathways and mechanisms. These studies are presently in progress in our laboratory.

In summary, our study shows that activation of ER β results in a reduction of ER α -mediated cell proliferation. The results obtained point at the importance of the cellular ER α /ER β ratio for the ultimate effect of (phyto)estrogens on cell proliferation, and this may provide a basis to explain the differential effects reported for the influence of (phyto)estrogens on cancer incidence.

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Superinduction of Estrogen Receptor Mediated
Gene expression in Luciferase Based Reporter
Gene Assays is Mediated by a Post-Transcriptional
Mechanism

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Abstract

Several estrogenic compounds including the isoflavonoid genistein, have been reported to induce a higher maximal response than the natural estrogen 17 β -estradiol in *in vitro* luciferase-based reporter-gene bioassays for testing estrogenicity. The phenomenon has been referred to as superinduction. The mechanism underlying this effect and thus also its biological relevance remain to be elucidated. In the present study several hypotheses for the possible mechanisms underlying this superinduction were investigated using genistein as the model compound. These hypotheses included i) a non estrogen receptor (ER)-mediated mechanism, ii) a role for an ER activating genistein metabolite with higher ER inducing activity than genistein itself, and iii) a post-transcriptional mechanism that is not biologically relevant but specific for the luciferase based reporter gene assays. The data presented in this study indicate that induction and also superinduction of the reporter gene is ER-mediated, and that superinduction by genistein could be ascribed to stabilization of the firefly luciferase reporter enzyme increasing the bioluminescent signal during the cell-based assay. This indicates that the phenomenon of superinduction may not be biologically relevant but may rather represent a post-transcriptional effect on enzyme stability.

Introduction

Isoflavones represent the most important group of phytoestrogens and have structural similarities to endogenous estrogens, suggesting that these compounds might exert their estrogenic effects via the estrogen receptor (ER) (1). Several *in vitro* assays have been used over the past years to investigate and define the hormonal, including also estrogenic, activity of isoflavones. Indeed, many of these studies have shown that isoflavones exert estrogenic and/or anti-estrogenic activities (2). An important model isoflavonoid tested in these studies is genistein, which is the main isoflavone present in legumes, particularly soy beans. Diverse biological activities have been associated with genistein including its estrogenicity and chemopreventive and/or antioxidant potential (3, 4). *In vitro*, genistein showed a wide range of cellular activities including inhibition of tyrosine kinase, inhibition of topoisomerase, inhibition of autophosphorylation of the epidermal growth factor receptor, mutagenesis but also antimutagenesis, induction of DNA damaging oxidation but also its prevention, and the promotion of cell death by apoptosis (5). Some of the estrogenic characteristics of genistein may contribute to protective roles in osteoporosis and menopause symptoms (6), but also to have positive effects against heart diseases, diabetes or even cancer (7). These proposed beneficial health effects of phytoestrogens in general, and of genistein in particular, have led to a wide range of isoflavonoid based food supplements.

Numerous *in vitro* ER assays have been developed to test the agonism or antagonism of a given substance. Reporter gene assays have become the most popular technique for measuring short-term screening of estrogenic activity. However, a response in an *in vitro* ER agonist or antagonist assay is not sufficient to predict biological or *in vivo* effects.

Genistein and other isoflavones have been shown to be weakly estrogenic and to have relatively low receptor affinity as compared to the natural estrogen receptor ligand 17 β -estradiol (E2). However, in some mammalian cell-based assays, using human U2OS bone or Chinese hamster ovarian (CHO) cells, stably transfected to express estrogen receptor (ER) to make them responsive to estrogens, or using MCF7 breast cancer cells containing endogenous ER, the maximal induction of the luciferase reporter gene by genistein has been shown to be substantially higher than the maximal induction by estradiol (E2) (8-12). In contrast, during cell-based estrogen stimulated proliferation assays, cell proliferation rates induced by genistein are similar to those induced by E2, although occurring at higher concentrations (11, 13, 14). No “superproliferation” effect by genistein or other isoflavones has been reported so far.

The mechanism underlying the so-called superinduction of estrogen receptor mediated gene expression in luciferase based reporter gene assays and also its biological relevance remain to be elucidated. The aim of the present study was to investigate possible mechanisms underlying this superinduction in reporter gene assays using genistein as the model compound known. Using the ER antagonists ICI 182,780 and RU58668 it was investigated whether the superinduction phenomenon was dependent on ER activation. In a T47D cell proliferation assay it was investigated whether superinduction was also reflected in superproliferation (and thus biologically relevant). Furthermore the possible role for a genistein metabolite with higher induction potency than E2 was investigated. And finally, using RT-PCR, it was quantified whether the increased luciferase activity was a result of increased gene expression and thus mRNA levels or rather a post-transcriptional effect on enzyme stability. Together these experiments provided insight in the mechanism underlying the observed superinduction, pointing at a post-transcriptional effect on enzyme stability and thus an assay artefact rather than at a biologically relevant effect.

Materials and methods:

Materials: 17 β -Estradiol (E2; purity>98%) and genistein (purity>98%), acetonitrile and trifluoroacetic acid were purchased from Sigma (Zwijndrecht, The Netherlands). RU58668 was a gift from N.V. Organon (Oss, The Netherlands). Orobol was purchased from APIN Chemicals LTD (Oxon, UK). ICI 182,780 was provided by Zeneca Pharmaceuticals (Cheshire, UK). Dimethyl sulfoxide (DMSO, purity>99%) was purchased from Acros Organics (Pittsburgh, PA, USA). Fetal calf serum (FCS, Australian origin, 10099), geneticin, G418 and Trizol Reagent were provided by Gibco Invitrogen Life Technologies (Paisley, UK). Phosphate-buffered saline (PBS, without Ca²⁺ and Mg²⁺), Hank's balanced salt solution (HBSS), nonessential amino acids (100 \times , 11140-035), growth medium 1:1 mixture of Ham's nutrient mixture F12 and DMEM (31331-028), Alpha-Modified Eagle's Medium (22561-021), and exposure medium phenol-free (21041-025) were supplied by Gibco Invitrogen (Paisley, UK). Dextran-charcoal-treated FCS (DCC-FCS) was heat inactivated (30 min at 56 $^{\circ}$ C) followed by two 45 min DCC-treatments at 45 $^{\circ}$ C (15). Trypsin was obtained from Difco (Detroit, USA; 0.25 g/100 ml in phosphate-buffered saline, PBS). Sodium bicarbonate (NaHCO₃ >99.5%), sodium hydroxide (NaOH) ethylenedinitrotetraacetic acid (EDTA \cdot 2H₂O; Titrplex), magnesium sulfate (MgSO₄ \cdot 7H₂O), and 1,4-dithiothreitol (DTT) were obtained from Merck (Darmstadt, Germany). Magnesium carbonate ((MgCO₃)₄Mg(OH)₂ \cdot 5H₂O) was obtained from Aldrich (St. Louis, MO, USA). *trans*-1,2-diaminocyclohexane-N,N,N',N'-tetraacetic acid monohydrate (CDTA) was obtained from Fluka (Buchs, Switzerland). Hygromycin and Diferin were obtained from Duchefa (Haarlem, The Netherlands). ATP was provided by Roche Diagnostics (Mannheim, Germany) and the BCA Protein Assay Kit by

Pierce (Bonn, Germany). RNeasy mini kit and SYBR green were provided by Quiagen (Hilden, Germany).

Cell lines: The stably transfected human osteosarcoma (U2OS) cell line (ER α -U2OS-Luc) expressing the human ER α in addition to 3xERE(GAGCTTAGGTCACCTGTGACCT)-tata-luciferase reporter construct was used as described before (16). T47D human breast cancer wild type cells were purchased from the American Type Culture Collection (Manassas, VA, USA). The T47D-Luc cell line stably transfected with an estrogen receptor mediated luciferase reporter gene construct was described before (17).

Cell culture conditions: ER α -U2OS-Luc cells were cultured in a 1:1 mixture of DMEM and F12 (31331-028) buffered with 1260 mg/L NaHCO₃, supplemented with 7.5% fetal calf serum (FCS) and 0.5% nonessential amino acids. ER α -U2OS-Luc growth medium was supplemented with geneticin (200 μ g/mL) and hygromycin (50 μ g/mL) as selection markers. The cells were incubated at 37° C and 7.5% CO₂ in a humidified atmosphere.

The T47D wild type and T47D-Luc cell lines were cultured in a 1:1 mixture of Ham's nutrient mixture F12 and DMEM (31331-038) supplemented with 5% FCS. The cells were incubated at 37° C and 5% CO₂ in a humidified atmosphere.

ER α -U2OS-Luc, T47D-Luc assay: hER α -specific U2OS and T47D luciferase reporter gene assays expressing endogenous ER α were carried out as described previously (11).

REA bioassay: The yeast estrogen bioassay, is based on a yeast cell stably expressing hER α and stably transfected with a yeast enhanced green fluorescent protein (yEGFP) as a reporter gene in response to estrogens. Estrogenic responses using the REA assay were measured as described before (18).

YES assay: The yeast estrogen screen (YES) was created by expressing human estrogen receptor (hER) and two estrogen response elements (ERE) linked to the β -galactosidase (lacZ) reporter gene. The assay was performed according to methods described before (19).

Cell proliferation measurements: After 48 h of exposure, proliferation in the ER α -U2OS-Luc and T47D cells was determined by measuring incorporation of 5-bromo-2'-deoxy-uridine (BrdU) into DNA following BrdU Roche's colorimetric protocol. Measurements were performed in a spectrophotometer at 370 nm excitation and 492 nm emission wavelengths.

HPLC-DAD analysis: Samples from medium in which T47D and ER α -U2OS-Luc cells were exposed for 24 hours under the same conditions described above to different

concentrations of genistein, however without DCC treated serum in the medium, were collected for HPLC-DAD analysis. The HPLC-DAD system consisted of a Waters (Milford, MA, USA) Alliance 2695 separation module connected to a Waters 2996 photodiode array detector (DAD), equipped with an Alltech (Breda, The Netherlands) Alltima C18 5- μ m 150- x 4.6-mm reverse phase column with a 7.5- x 4.6-mm guard column. Before injection, samples were centrifuged at 16,000g for 4 min, and 50 μ l was injected and eluted at a flow rate of 1 ml/min starting at 0% acetonitrile in nanopure water containing 0.1% trifluoroacetic acid (TFA), increasing to 10% acetonitrile in 5 min, to 15% in the following 16 min, and to 50% in the next 16 min all in nanopure with 0.1% TFA. Thereafter, the percentage acetonitrile was increased to 80% in 1 min. This condition was kept for 1 min as a cleaning step, followed by a decrease to 0% acetonitrile in 1 min, keeping this condition for 10 min, allowing the column to re-equilibrate at the initial conditions (total run time, 50 min). DAD spectra were detected between 200 and 420 nm, and HPLC chromatograms acquired at 280 nm were used for quantification and presentation.

LC-MS/MS analysis: Medium of ER α -U2OS-Luc cells exposed to 5 μ M genistein under the same conditions as described above but without DCC treated serum in the medium was collected after 24 h of exposure and analyzed by LC-MS/MS. The medium samples were analyzed by injecting 18 μ l sample over a 32 x 0.10 mm Prontosil 300-3-C18H pre-concentration column (Bischoff, Germany) at a flow of 6 μ l/min for 10 min. Compounds were eluted from the pre-concentration column onto a 200 x 0.10 mm Prontosil 300-3-C18H analytical column with an acetonitrile gradient at a flow of 0.5 μ l/min. The gradient consisted of an increase from 15 to 50% acetonitrile in water containing 1 ml/L formic acid in 16 min. Thereafter the percentage acetonitrile was increased to 80% in 3 min as a column cleaning step. Downstream of the column, an electrospray potential of 1.8 kV was applied directly to the eluent via a solid 0.5 mm platina electrode fitted into a P875 Upchurch microT. Full scan positive mode FTMS spectra were measured between an m/z of 200 and 600 at a resolution of 60.000 on a Thermo electron LTQ-Orbitrap (San Jose, CA, USA). MS/MS scans of the four most abundant singly, doubly or triply charged peaks in the FTMS scan were recorded in data dependent mode in the linear trap (MS/MS threshold = 10.000).

RT-PCR: RNA was extracted after 6 and 24 h exposure using the TRIzol precipitation method and purified using an RNeasy mini kit protocol for second RNA clean up according to the manufacturer's protocol. Integrity and quantity of the extracted RNA was then assessed by using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc., DE, USA). Approximately 1-2 μ g of total RNA was collected per sample. Samples were stored at -80° C until reverse transcription (RT-)PCR using 1 μ g of total RNA. First-strand cDNA synthesis was carried out with an oligo(dT)15 primer and Moloney murine leukemia virus reverse transcriptase; during synthesis the recombinant ribonuclease inhibitor RNaseOUT™

was present. The amplification reaction was carried out on a LightCycler (Roche Diagnostics) with gene-specific primers and used the SYBR Green 1 protocol. The following LightCycler protocol was used: 15 min heat start at 95° C; 45 cycles of denaturation at 95° C for 30 s, annealing at the optimal annealing temperature for the primer set for 30 s, and extension at 72° C for 45 s; and a terminal extension at 72° C for 5 min. Fluorescence detection was carried out at 72° C. *Luc* mRNA levels were expressed as the *Luc*: β -*actin* ratio. The oligonucleotide sequences for firefly luciferase were reverse primer: 5'-GCCTCACCTACCTCCTTGCT-3' and forward primer 5'-CTTCGTGACTTCCCATTGTC-3'; for β -actine the reverse primer was 5'-CCAGAGGCGTACAGGGATAG-3' and the forward primer was 5'-CACCCCGTGCTGCTGAC-3'.

Calculations and statistics: Luciferase induction by genistein in the ER α -U2OS-Luc, T47D-Luc cells, and yEGFP and β -galactosidase induction in yeast cells were compared with the induction of the respective activities by the natural ligand E2. The background induction by the solvent control was set at 0% induction. The maximum induction of luciferase obtained at 12 pM E2 for ER α -U2OS-Luc cells, at 20 pM E2 for the T47D-Luc cells and at 3 nM and 150 pM E2 for the yEGFP and β -galactosidase induction in yeast cells were set as 100%. The data obtained for proliferation as quantified by BrdU incorporation were plotted as induction of proliferation compared to the solvent control (DMSO).

Results

Activation of ERE-mediated gene expression in the ER α -U2OS-Luc reporter gene assay:

Treatment of the human ER α -U2OS-Luc cells with E2 and genistein resulted in dose-dependent expression of luciferase (Figure 1). Induction of ER α -mediated luciferase expression by E2 occurs at concentrations between 1 and 20 pM. Induction by genistein appears to be biphasic. Exposure to genistein shows a first phase of dose-related luciferase induction at concentrations from 10 to 200 nM and a second phase with a further dose-related increased of luciferase induction at concentrations from 1 to 5 μ M. The maximum level of luciferase induction in the first phase of the genistein induction curve is similar to the maximum level of induction by 12 pM E2 and amounts to 104% of the maximum E2 induction at 100 nM genistein. In the second phase, genistein exposure of the cells resulted in a so-called superinduction of luciferase activity amounting to 188% of the maximum level of induction by E2 at 5 μ M genistein. When co-exposing cells to E2 with 5 nM of the anti-estrogen ICI 182,780, the luciferase induction by E2 is completely inhibited (Figure 1). When genistein is co-exposed with 5 nM of the anti-estrogen ICI 182,780, luciferase induction in the high affinity first phase is inhibited and only the low affinity luciferase induction in the second phase remains, as the shape of the curve suggests that only the first part of the

genistein dose-response curve was inhibited by ICI 182,780 (Figure 1). However, the luciferase induced by genistein was totally inhibited by co-exposure with 100 nM ICI 182,780 and 180 nM of another pure ER antagonist, RU58668.

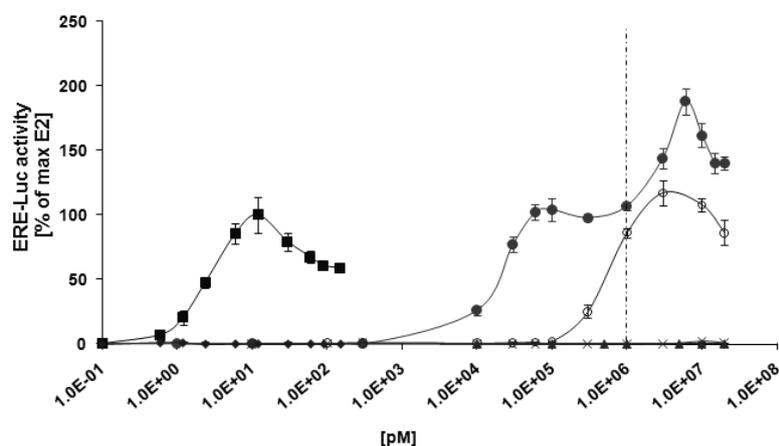


Figure 1: Induction of ER α -mediated luciferase activity in the ER α -U2OS-Luc cells upon exposure to various (anti)estrogens. (Anti)estrogens tested include E2 (■), E2 + 5 nM ICI 182,780 (◆), genistein (●), genistein + 5 nM ICI 182,780 (○), genistein + 100 nM ICI 182,780 (×), genistein + 180 nM RU58668 (▲). Induction is expressed relative to maximal E2 response set at 100%. Data points represent the mean of triplicate exposure \pm standard deviation. The dashed line indicates from what concentration onwards genistein concentrations superinduction is observed.

Activation of ERE-mediated gene expression in the T47D-Luc reporter gene assay:

Similar experiments were performed with the human breast cancer cell line T47D-Luc reporter gene assay in order to investigate whether the above described effects of genistein were cell-type specific. The breast cancer derived T47D-Luc cells use the same reporter gene construct that is used in the human ER α -U2OS-Luc cell line. The only difference is that the T47D cells make use of the endogenous ER α , while the ER α -U2OS-Luc cells contain an extra construct to make them express the hER α . The results shown in figure 2 reveal that the responses of E2 and genistein are in the same concentration range and similar to those obtained with the ER α -U2OS-Luc cells. Also the treatment of the T47D-Luc cells with genistein resulted in a biphasic concentration-dependent increase in luciferase activity. Genistein shows a first dose-related luciferase induction at concentrations from 10 to 200 nM (first curve) and a second dose-related increase in luciferase induction at concentrations from 1 to 10 μ M (Figure 2). The maximum level of luciferase activity reached in the first phase at 100 nM genistein amounts to 108% of the maximum induction level reached at 20 pM E2. Genistein-mediated superinduction of luciferase in the second phase amounts to 227% of the maximum level of induction by E2 at 10 μ M genistein. When cells were co-exposed to E2 and 18 nM of the anti-estrogen RU58668, the E2 induced luciferase induction was completely inhibited. When cells were co-exposed to genistein and 18 nM of this anti-estrogen, the luciferase induction was only partly inhibited. Again, the shape of the curve

shows that low concentrations of the anti-estrogen inhibit mainly the first part of the induction curve. Again, luciferase induction by genistein was completely inhibited by a high concentration of RU58668 (180 nM).

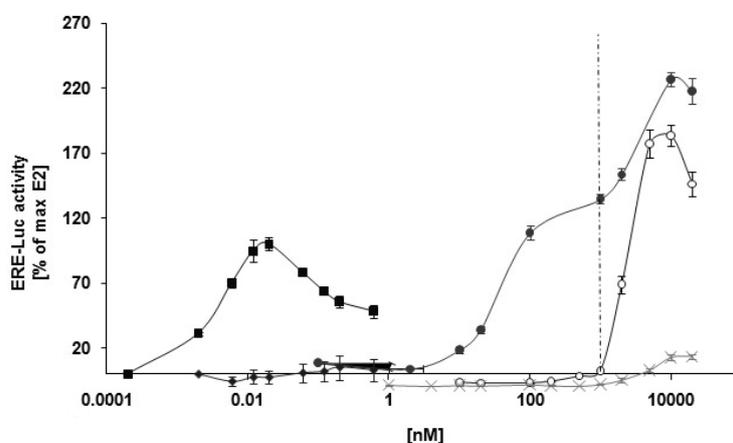


Figure 2: Induction of ER α -mediated luciferase activity in the ER α -T47D-Luc cells upon exposure to various (anti)estrogens. (Anti)estrogens tested include E2 (■), E2+ 18 nM RU58668 (◆), genistein (●), genistein + 18 nM RU58668 (○) and genistein + 180 nM RU58668 (×). Induction is expressed relative to maximal E2 response set at 100%. Data points represent the mean of triplicate exposure \pm standard deviation. The dashed line indicates from what concentration onwards genistein concentrations superinduction is observed.

T47D cell proliferation

In a next series of experiments, it was investigated whether the observed genistein-mediated superinduction of luciferase activity in reporter gene assays would also be reflected in genistein induced ER α -mediated superinduction of cell proliferation, which is a biologically relevant endpoint of estrogenic activity. Since the T47D cell line is known to express endogenous ER α , and to show estrogen induced ER α mediated cell proliferation, cells from the wild type T47D cell line were selected as the model system to investigate the possible occurrence of genistein induced ER α -mediated superinduction of cell proliferation.

As shown in figure 3, treatment of T47D wild type cells for 48 h with genistein resulted in a dose-dependent increase in cell proliferation. Proliferation data have been normalized to the proliferation levels obtained at 1 nM E2, the maximum of E2 induced T47D cell proliferation. Figure 3 shows that the maximal proliferative effect elicited by genistein is similar to that of 1 nM E2 and is reached at a concentration of 1 μ M genistein, which is also the genistein concentration inducing the maximum response in the first phase of the luciferase induction in the two reporter cell assays. At concentrations of 3-20 μ M genistein, the range where in both reporter gene assays genistein-mediated superinduction was observed, no genistein induced “superproliferation” of the T47D cells was observed.

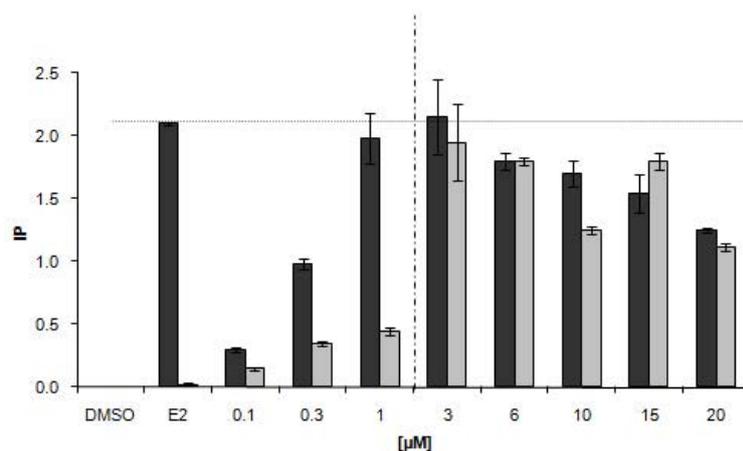
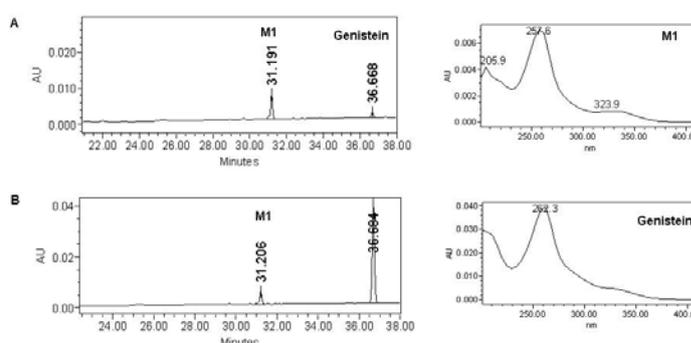


Figure 3: T47D cell proliferation. Effect of DMSO, estradiol and genistein (black) and DMSO, estradiol and genistein + 10 nM ICI 182,780 (grey) on induction of T47D cell proliferation (IP). The horizontal dotted line indicates the maximal proliferation by E2 at 1 nM. Proliferation was quantified measuring BrdU incorporation after 48 hours exposure. The dashed line indicates from what concentration onwards genistein concentrations superinduction is observed.

When cells were co-exposed with the antagonist ICI 182.780 (10 nM), the E2-induced proliferation was completely inhibited, and this concentration was able to reduce genistein induced cell proliferation at genistein concentrations of 0.1-1 μ M, but was not able to inhibit the proliferation caused by genistein at 3-20 μ M (Figure 3).

Genistein metabolite formation

One possible explanation for the observed superinduction by genistein, would be the formation of a metabolite that is also an active ER agonist by itself. Above 1 μ M genistein, at which the superinduction occurs, HPLC analysis of the culture medium revealed the formation of a genistein metabolite (M1) (Figure 4). The medium from ER α -U2OS-Luc cells incubated with 1 and 5 μ M genistein was collected upon 24 h exposure.



Figures 4: HPLC analysis. Representative sections of the HPLC chromatograms of ER α -U2OS-Luc medium samples after 24 h of exposure to A) 1 and B) 5 μ M genistein, and the UV spectra belonging to the peaks of M1 (UVmax 257.6 nm) and genistein (UVmax 262.3 nm).

HPLC-DAD chromatograms obtained from medium of genistein-treated cells showed the presence of genistein at a retention time of 36.7 min and another compound (M1) at a retention time of 31.2 min (Figure 4). Based on the shorter retention time and similarities in the UV spectra, M1 is likely to be a polar metabolite of genistein. The formation of this unknown metabolite (M1) by the ER α -U2OS-Luc cells was significant at concentrations of 0.5 μ M genistein and higher.

The samples used for the HPLC-DAD analysis, were subsequently analyzed by LC-FT-MS/MS in positive ion mode. The protonated genistein corresponds to the most abundant ion at m/z 271. The MS/MS spectrum (with fragments formed at m/z 271, 253, 243, 225, 215, 187 and 153) confirmed that the molecular ion $[M+H]^+$ at m/z 271 was indeed genistein (data not shown). The M1 metabolite ($[M + H]^+$ ion at m/z 287) was subsequently analysed by LC-MS/MS. Fragmentation of the m/z 287 ion yielded product ions at m/z 269, 259, 241, 231 and 161. The presence of the ion at m/z 161 had only been observed earlier with isoflavones as a rearrangement product for a dihydroxylated B-ring fragment (20). The losses at $MH^+ - 18$ (269), $MH^+ - 28$ (259) and $MH^+ 28 - 28$ (231) are typical for hydroxyisoflavones. Taken together, the MS/MS data are consistent with a hydroxylation on the ring B for product M1 identifying the metabolite as 5,7,3',4'-tetrahydroxyisoflavone, better known as orobol. Subsequently, this metabolite, identified as orobol, was also tested in the ER α -U2OS-Luc reporter gene assay (Figure 5). Orobol is able to induce reporter gene expression at concentrations above 1 μ M reaching a superinduction of 187% at 40 μ M.

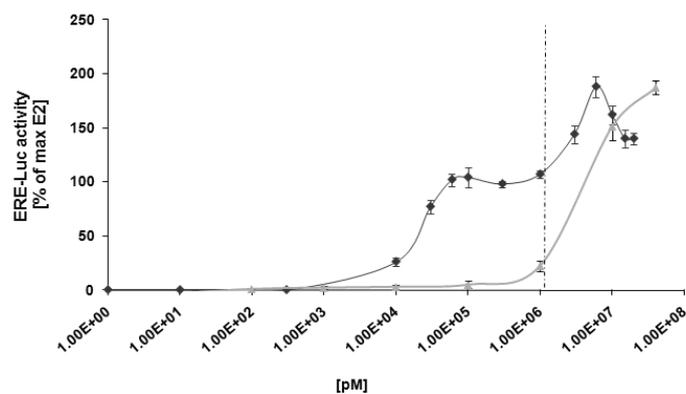


Figure 5: Induction of ER α -mediated luciferase activity in the ER α -U2OS-Luc cells upon exposure to genistein (●) and orobol (▲). Induction is expressed relative to maximal E2 response set at 100%. Data points represent the mean of triplicate exposure \pm standard deviation. The dashed line indicates from what concentration onwards genistein concentrations superinduction is observed.

Yeast estrogen bioassay

Two more reporter gene assays were used to test whether the superinduction caused by genistein and orobol could also be observed using different reporter genes, namely enhanced green fluorescent protein (yEGFP) and β -galactosidase (β -gal). Both of these reporter gene

assays are based on yeast cells that have been previously shown to be metabolically inactive with regard to natural and synthetic estrogens (21, 22). The yeast cells expressing hER α were exposed to genistein and orobol. Figure 6A and figure 6B show that both genistein and orobol resulted in the same maximal response as E2. Thus, no superinduction was observed in bioassays based on yeast cells and using different reporter genes than firefly luciferase.

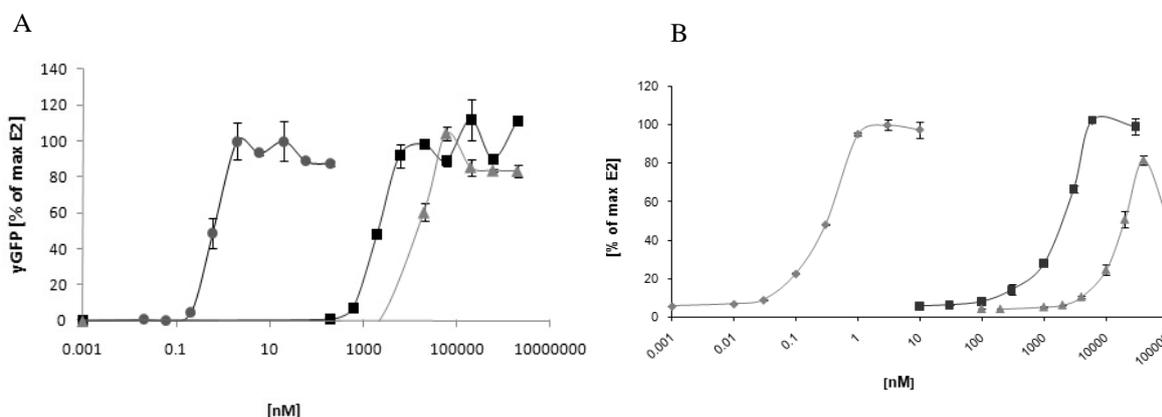


Figure 6: Induction of ERE-mediated reporter gene in yeast estrogen bioassays. (A) Induction of ERE-mediated yEGFP expression in a yeast estrogen bioassay using yEGFP as reporter gene upon exposure to E2 (●), genistein (■) and orobol (▲). Data points represent the mean of triplicate exposure \pm standard deviation. (B) Induction of ERE-mediated β -galactosidase expression in a yeast estrogen bioassay upon exposure to E2 (●), genistein (■) and orobol (▲). Data points represent the mean of triplicate exposure \pm standard deviation. (†) Represents cytotoxicity.

Genistein and E2 induced luciferase gene expression as detected by RT-PCR

Additional experiments were performed aiming at studying the effect of genistein on luciferase mRNA and/or protein activity and stability. Post-transcriptional activation and stabilisation of luciferase mRNA and/or protein might be an alternative explanation for the observed superinduction at high concentrations of genistein ($>1\mu\text{M}$) explaining increased luciferase activity without the need for increased transcriptional activity. To actually verify whether or not the increased luciferase activity is a true reflection of increased transcriptional activity, RT-PCR based quantification of luciferase mRNA formation as a function of increasing genistein concentrations was performed and results obtained were compared to the level of mRNA formation induced at optimal levels of E2. To this end, ER α -U2OS-Luc cells were treated with E2 and genistein during 6 and 24 h. RNA from all the samples was extracted, and expression of firefly luciferase gene mRNA was compared with that of the housekeeping gene β -actin by RT-PCR. RT-PCR analysis revealed that the firefly luciferase gene expression was 23-fold up-regulated by 12 pM E2 (Figure 7). Increasing concentrations of genistein resulted in increased expression of luciferase mRNA, but the maximum level of mRNA induction reached was 9-fold, and never reached the induction factor observed for E2. Moreover, no superinduction of the mRNA level due to genistein treatment at concentration above 1 μM genistein was observed.

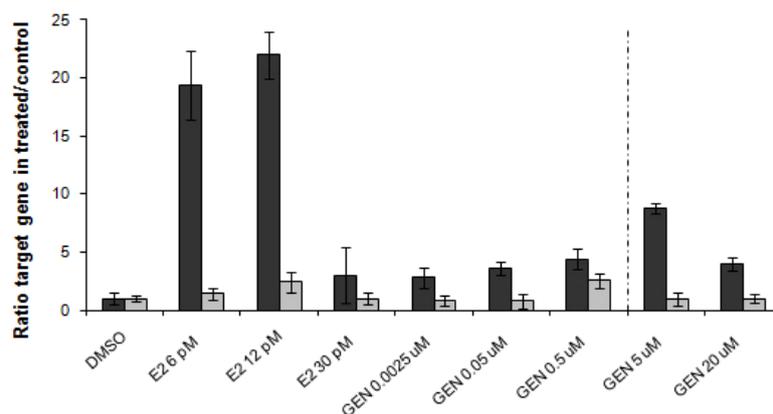


Figure 7: Relative firefly luciferase mRNA expression levels in ER α -U2OS-Luc cells normalized to the expression of β -actin after 6 h (black column) and 24 h (grey column). The dashed line indicates from what concentration onwards genistein concentrations superinduction is observed.

Genistein at concentrations above 1 μ M increased the luciferase activity. Therefore, in subsequent experiments we used a cell-free biochemical assay to measure the interaction between genistein and the luciferase protein. In order to investigate whether genistein and other compounds causing superinduction may act through interacting with the luciferase enzyme thereby ultimately stabilising the enzyme, increasing concentrations of genistein, estradiol and resveratrol, another phytoestrogen that induces superinduction, were incubated with a known and constant concentration of pure firefly luciferase protein.

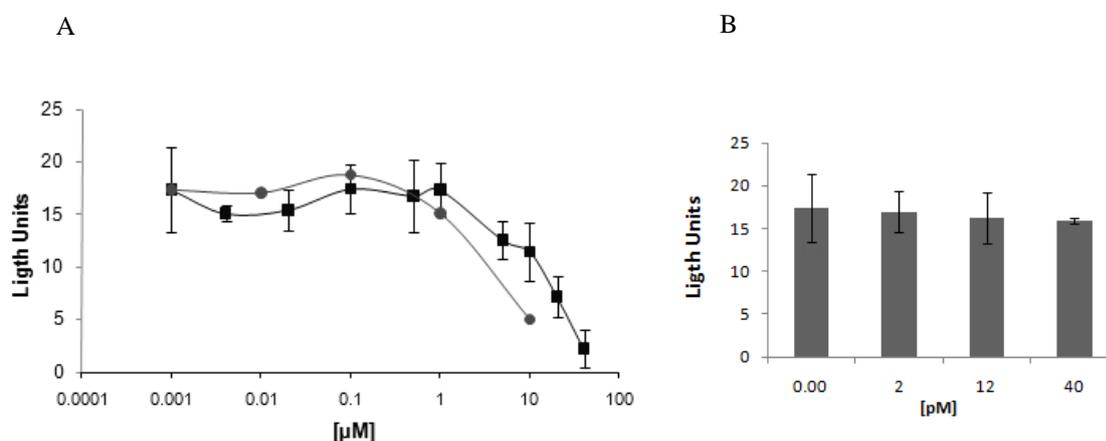


Figure 8: Genistein and resveratrol inhibit firefly luciferase activity. A) Firefly luciferase activity after co-incubation with increasing concentrations of genistein (■) or resveratrol (●) or B) with estradiol. Data points represent the mean of triplicate exposure \pm standard deviation. The dashed line indicates from what concentration onwards genistein concentrations superinduction is observed.

The firefly luminescence read-out was dose-dependent with an inhibition of the enzymatic reaction at concentrations above 1 μ M for genistein and resveratrol (Figure 8A), whereas bioluminescence was unaffected by estradiol (Figure 8B).

Discussion

In recent years, several *in vitro* assays have been developed to assess estrogenic activity of chemicals and naturally occurring compounds. From those, several reporter gene assays showed higher transactivation activities, or induction of the reporter gene, for genistein compared to E2. This effect was often called superagonism or superinduction, and, although the mechanism underlying the phenomenon is still unrevealed, it is often suggested that it might be biologically relevant (9). The present study investigated possible mechanisms behind this superinduction thereby at the same time addressing its biological importance.

The first hypothesis tested the possibility that the induction of the reporter gene was not ER-mediated. Hence, using the ER α -U2OS-Luc cell line, cells were exposed to E2 and genistein in the absence and presence of potent ER α antagonists (ICI 182,780 or RU58668). Using a concentration of the ER α antagonist that fully blocks luciferase induction mediated by E2-ER α (5 nM), the induction of luciferase was only blocked at low genistein concentration while at high concentrations, this was only possible with increased antagonist concentration (100 nM). The same effect was obtained when using the T47D-Luc cell line, another luciferase reporter gene assay based on a cell line that expresses endogenous ER α . Therefore, it is concluded that induction of luciferase is ER-mediated. The different sensitivity of the two phases of luciferase induction by genistein suggests that both processes, although apparently both dependent on ER α mediated luciferase induction, proceed by mechanisms that are at least in part dissimilar and displaying different affinities for genistein.

On the other hand, genistein induces superinduction of the reporter gene in the U2OS-Luc and T47D-Luc cell lines that is not reflected at cell proliferation level. No “superproliferation” was observed in the T47D cell line. This shows that the phenomenon of superinduction is not reflected in this biologically relevant endpoint.

The next hypothesis tested, was whether the superinduction was not due to genistein but to an *in vitro* formed metabolite. This hypothesis originated from the observation that a metabolite was formed at the genistein concentration where superinduction is maximal. The biological activities observed in *in vitro* or *in vivo* studies are often assumed to originate from the parent compounds that are examined, although these may have been subject to biotransformation into one or more structurally different compounds (23, 24). A number of genistein metabolites have been identified (25-27) but most of them have been shown to be less estrogenic than genistein (28, 29). The data presented in this study showed metabolite formation at high genistein concentrations, starting at 0.5 μ M and strongly increasing at concentrations up to 5 μ M, in the *in vitro* tests using U2OS and T47D cells. Orobol, or 5,7,3',4'-tetrahydroxyisoflavone, was identified as a main oxidative metabolite, which is in

agreement with previously reported data that hydroxylated metabolites of genistein are the most abundant metabolites *in vitro* (30) and that the primary oxidative metabolite of genistein is orobol (20, 25).

Therefore, genistein and orobol were tested in the ER α -U2OS-Luc cells, but also in a metabolically inactive yeast estrogen bioassay which uses another reporter gene than luciferase, namely enhanced green fluorescence protein. Additionally, genistein and orobol were measured in another yeast estrogen bioassay using β -galactosidase as a reporter gene. Both compounds were able to show a dose-response curve in the yeast based estrogen bioassays, but neither genistein nor orobol was able to show the superinduction as observed in the ER α -U2OS-Luc and T47D-Luc assays. In addition, it was shown that also orobol induced superinduction in the ER α -U2OS-Luc. As orobol does not give rise to higher maximal induction of the reporter genes than genistein, and especially because orobol is less potent than genistein, it can be concluded that orobol is not responsible for the observed superinduction by genistein.

Overall, superinduction by genistein and orobol is especially observed in the luciferase reporter gene assays, and more importantly reporter gene assays using luciferase from firefly as the reporter protein (8-12).

The data obtained can also be used to disregard another hypothesis for the superinduction. We show that the superinduction is not likely to be due to the up-regulation of the ER, as there was no difference between the ER α -U2OS-Luc and T47D-Luc. The T47D-Luc assay uses the endogenous expressed ER which could in theory be upregulated by normal physiological mechanisms, while the yeast and U2OS cells use a receptor construct that induces a strong and constitutive expression of the human ER α .

Therefore, the next step was to actually verify whether or not the increased firefly luciferase activity was a true reflection of increased transcriptional activity. Therefore, mRNA expression of the firefly luciferase gene was measured in ER α -U2OS-Luc cells after 6 and 24 hours exposures to E2 and genistein. After 6 hours exposure, the maximum induction of firefly luciferase at the transcript level was significantly higher for estradiol than for genistein-treated cells. At 12 pM E2 luciferase mRNA levels were about 3-fold higher than at 5 μ M genistein, a concentration shown before to result in clear superinduction when quantified on the basis of luciferase reporter gene activity. At 24 hours, the time of bioluminescent luciferase activity measurements in the ER α -U2OS-Luc reporter gene assay, very low induction levels of luciferase transcription were remaining. These data indicated that the superinduction detected by measuring luciferase activity is not a reflection of real mRNA induction and should more likely be ascribed to a phenomenon that leads to

prolonged enzyme activity without the need for increased transcription levels. Therefore, we tested the potential of genistein to act as a stabilizer of the luciferase reporter enzyme.

We evaluated the possible direct interaction of genistein with the reporter enzyme leading to stabilisation (decreased degradation) of the enzyme. It has been proposed that certain compounds directly bind to, and stabilize, the firefly luciferase reporter enzyme thereby increasing its half-life (31, 32). The interaction between the bioactive chemicals and the luciferase results in inhibition of the enzyme activity at the same time resulting in stabilisation of the enzyme (33, 34). These luciferase-stabilizing compounds are referred to in the literature as luciferase inhibitors (34). An accumulation of stabilized luciferase reporter enzyme will enhance the observed bioluminescence activity. Superinduction effects have been reported for other estrogenic compounds, e.g. the flavonoid resveratrol, and in all cases, this was specifically reported in firefly luciferase cell-based assays (9, 35, 36). Thus, in a cell-free biochemical assay, the possible interaction between genistein, E2 and resveratrol with firefly luciferase was investigated. We observed that resveratrol and genistein but not estradiol can specifically inhibit the bioluminescent enzymatic reaction of the firefly luciferase. Inhibition of the bioluminescence has been suggested to result from direct competitive inhibition of the enzymatic reaction (31). It can be expected that upon rupture of the cells the inhibitor will dissociate leading to increased activity of the luciferase enzyme due to this stabilising effect. Thus, genistein at concentrations above 1 μM may interact with luciferase thereby stabilising the enzyme so that upon lysis of the cells and dissociation of genistein from the enzyme due to dilution, increased activity can be measured as compared to the situation without an added stabiliser/inhibitor. It has been recently shown, that a series of bioactive compounds, that inhibit and thereby stabilise the firefly luciferase enzyme will result in an increased luminescence signal (33).

In conclusion, the data presented in this study strongly indicate that the superinduction caused by genistein can be ascribed to stabilization of the firefly luciferase reporter enzyme increasing the bioluminescent signal during the cell-based assay. This indicates that the phenomenon may not be biologically relevant but may rather represent a post-transcriptional effect on enzyme stability.

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Quantitative Proteomics and Transcriptomics
Addressing the Estrogen Receptor Subtype-
Mediated Effects in T47D Breast Cancer Cells
Exposed to the Phytoestrogen Genistein

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Abstract

The present study addresses, by transcriptomics and quantitative SILAC-based proteomics, the estrogen receptor alpha (ER α) and beta (ER β)-mediated effects on gene and protein expression in T47D breast cancer cells exposed to the phytoestrogen genistein. Using the T47D human breast cancer cell line with tetracycline-dependent ER β expression (T47D-ER β), the effect of a varying intracellular ER α /ER β ratio on genistein-induced gene and protein expression was characterised. Results obtained reveal that in ER α -expressing T47D-ER β -expression cells genistein induces transcriptomics and proteomics signatures pointing at rapid cell growth and migration by dynamic activation of cytoskeleton remodeling. The data reveal an interplay between integrins, focal adhesion kinase (FAK), CDC42 and actin cytoskeleton signaling cascades, occurring upon genistein treatment, in the T47D-ER β breast cancer cells with low levels of ER α and no expression of ER β . In addition, data from our study indicate that ER β -mediated gene and protein expression counteracts ER α -mediated effects, because in T47D-ER β cells expressing ER β and exposed to genistein transcriptomics and proteomics signatures pointing at a clear downregulation of cell growth and induction of cell cycle arrest and apoptosis was demonstrated. These results suggest that ER β decreases cell motility and metastatic potential as well as cell survival of the breast cancer cell line. It is concluded that the effects of genistein on proteomics and transcriptomics endpoints in the T47D-ER β cell model are comparable to those reported previously for estradiol, with the ultimate estrogenic effect being dependent on the relative affinity for both receptors and on the receptor phenotype (ER α /ER β ratio) in the cells or tissue of interest.

Introduction

At present two main estrogen receptors, ER α and ER β , have been identified in rats, mice, primates and humans (1). Different biological responses may occur when an estrogen binds to the different estrogen receptors (ERs). Several studies have shown decreased ER β expression in malignant cancer tissues as compared to benign tumours or normal tissues, where ER α expression persists (2-6). Hence ER α and ER β may have different roles in gene regulation and their relative levels or ratios within the tissues may influence cellular responses to estrogens. To understand the critical role of estrogens in the regulatory cascade involved in the progression of breast cancer, several studies focused on the evaluation of global transcriptomics and showed the association of ER α activation with cell proliferation and the opposing effects for activation of ER β (7-9). So far, most of these “omics” studies focused on estrogens like estradiol but did not yet widely include so-called phytoestrogens.

Phytoestrogens are a group of plant-derived compounds with estrogenic properties (10). The major types of phytoestrogen are isoflavones, flavones, coumestans, lignans and stilbenes. Among the most studied flavonoids with respect to anti-tumour functions are genistein and quercetin. Phytoestrogens have been considered a natural alternative to the hormone replacement therapy since these chemicals are found in the regular diet (11). Genistein is mostly present in soybeans. High consumption of phytoestrogen-rich food correlates with reduced incidence of breast cancer (12). These two flavonoids, genistein and quercetin, have been shown to have potent anti-proliferative effects on tumour cells by halting the cell cycle and inducing apoptosis (13-15). However, what effects are induced by phytoestrogens and which mechanisms are activated in cells remains to be elucidated. Therefore the aim of the present study was to provide “omics” data on the effects of the phytoestrogen genistein in cells with variable intracellular ratio of the two estrogen receptors ER α and ER β and to compare these outcomes qualitatively to those reported earlier for estradiol (8).

The importance of the intracellular ratio of the two estrogen receptors ER α and ER β for the ultimate potential of estradiol and of the phytoestrogen genistein to stimulate or inhibit cell proliferation was demonstrated previously (16, 17). Using the human T47D breast cancer cell line with tetracycline-dependent ER β expression (T47D-ER β), the effect of a varying intracellular ER α /ER β ratio on estradiol- or genistein-induced cell proliferation was characterized (17). With increased expression of ER β , estrogen-induced ER α -mediated cell proliferation was reduced. These results point at the importance of the cellular ER α /ER β ratio for the ultimate effect of (phyto)estrogens on cell proliferation. The results of our previous studies also revealed that in this T47D-ER β model system the effects of genistein on cell

proliferation with varying cellular ER α /ER β ratios were comparable to the effects induced by estradiol itself (17, 18).

In the present study we investigated the consequences of the intracellular ER α /ER β ratio for the effects induced by genistein in more detail using quantitative “omics” technologies, characterizing both gene and protein expression patterns and compared the results obtained to those previously reported for estradiol in the same model system (8). To this end, state-of-the-art high-throughput methods for systems-wide gene and protein expression analysis were applied. These methods included DNA microarrays, commonly used for global analysis of gene expression changes. In addition we applied stable isotope labeling by amino acids in cell culture (SILAC) which is a differential and quantitative proteomics technique that uses mass spectrometric (MS) analysis (19). The SILAC strategy is based on the metabolic incorporation of “light” (normal) and “heavy” (isotope-labeled) amino acids into the cells, which is a process shown to occur without adverse effects on cellular physiology (20). Full metabolic incorporation of the labeled amino acids into the proteins results in a mass shift of the corresponding peptides. This mass shift can be detected by MS. When two samples are combined, the ratio of peak intensities in the MS reflects the relative protein abundance. Since the metabolic incorporation of the labels does not affect the integrity of genes or proteins, the transcriptomics and SILAC proteomics experiments can be performed on the same cell samples.

The data obtained from our proteomics and transcriptomics studies indicate that genistein induces rapid cell proliferation and migration by dynamic activation of cytoskeleton remodeling in the T47D-ER β cells expressing low levels of ER α and no ER β . Interaction between integrins, focal adhesion kinase (FAK), CDC42 and actin cytoskeleton signaling cascades occurs upon genistein treatment supporting the observed cell proliferation. Our results also strengthen the concept that ER β mediated gene and protein expression counteracts ER α -mediated effects, because in T47D-ER β cells expressing ER β and exposed to genistein a clear downregulation of genes and proteins involved in cell growth and induction of cell cycle arrest and apoptosis was demonstrated.

Materials and methods

Cell culture: The stably transfected T47D tetracycline-inducible cell line (T47D-ER β) and the control cell line (T47D-PBI) were made and provided by Ström (16). DMEM SILAC medium lacking arginine (Arg) and lysine (Lys), supplemented with 5% of dialyzed fetal bovine serum and 1000 ng/ml tetracycline (Tet) was used. Arg and Lys, either “light” or

“heavy” depending on the experimental design, were incorporated in the DMEM SILAC medium. The final concentrations of Arg and Lys were 21 and 48 mg/L respectively (21).

During the adaptation phase, cells were grown in “light” or “heavy” SILAC media, containing respectively 12C614N4-arginine and 12C614N2-lysine or 13C6-15N2-lysine and 13C6-15N4-arginine, until the cells grown in “heavy” medium had fully incorporated the labeled amino acids. Full incorporation of labels into the cells was checked by MS analysis (22) of cell samples detecting the time point at which there was no further increase in the amount of label incorporated. Full incorporation of labels for the T47D-ER β cells without ER β expression (Tet containing) was observed after 5 doubling times or 10 days of cultivation (data not shown). Preparation of the samples after this adaptation phase, was performed as follows. Cells in (SILAC) DMEM medium supplemented with 5% dialyzed-fetal bovine serum (DFBS) and 1000 ng/ml Tet were seeded in plates for 24 h. After 24 h DMEM SILAC medium was replaced by the same medium without phenol red, containing the required concentrations of Tet, heavy or light amino acids, and a serum concentration that was reduced to 0.5% DFBS. To this, 10 nM ICI 182780 was added for cell synchronization (8) and Tet was withdrawn in half of the plates all 12 h before the start of the treatment to allow expression of ER β . The Tet+ and Tet- cultures were incubated with either 10 nM of the ER α and ER β antagonist ICI 182780 (23) or 500 nM of genistein for 24 h according to the experimental design (Table 1A) after which all cells were collected simultaneously for RNA and protein extraction. Three replicates of each sample were prepared in parallel to minimize experimentally induced variability.

Table 1: Experimental design of the present study based on five cellular samples prepared as depicted in Table 1A) and their pairwise comparison carried out as presented in Table 1B.

1A)

	Strain	Tetracycline Treatment	ER β expression	Compound treatment	No. of samples Transcriptomics /proteomics	Label
A	T47D-ER β	yes	no	ICI 182780	3 / 3	Light
B	T47D-ER β	no	yes	ICI 182780	3 / 3	Heavy
C	T47D-ER β	yes	no	genistein	3 / 3	Heavy
D	T47D-ER β	no	yes	genistein	3 / 3	Light
E	T47D control	no	no	genistein	3 / 3	Heavy

1B)

Sample	Targets
CA	Genistein modulated ER α mediated gene and protein expression
DB	Genistein modulated ER β mediated gene and protein expression
ED	Genistein modulated ER β mediated gene and protein expression (second approach)

Table 1A summarizes the experimental design applied in order to obtain information on the genistein induced levels of gene and protein expression in T47D cells with variable ER α /ER β ratios. Table 1A presents the five different samples prepared, indicating the cell line used, the Tet treatment and resulting ER β expression, exposure to ICI 182780 or genistein and the amino acid label (light or heavy) added to the growth medium. In addition to genistein, ICI 182780 was used in order to block the receptors thereby suppressing receptor-mediated transcription. A control cell line (T47D-PBI) was used to define genistein induced non-ER β mediated gene expression inherent to the cell system used. Table 1B presents the pairwise comparisons made using these five samples in order to analyze i) genistein induced ER α mediated gene and protein expression (C versus A, referred to as sample CA); ii) genistein induced ER β regulated gene and protein expression in the presence of endogenous levels of ER α (D versus B, referred to as sample DB) and iii) a second approach to detect genistein induced ER β mediated gene and protein expression in the presence of endogenous levels of ER α (E versus D, referred to as sample ED).

Protein and RNA preparation: Each sample was split in two, one for RNA and one for protein preparation. RNA was extracted using the TRIzol precipitation method and purified using an RNeasy mini kit protocol for second RNA clean up (RNeasy mini kit, Qiagen). Approximately 4 μ g of total RNA was collected per replicate.

For protein extraction, cells were washed twice with PBS and lysed in modified RIPA buffer [50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate] containing a protease inhibitor cocktail (Complete, Mini, Roche) at 4 $^{\circ}$ C for 15 min. Samples were sonicated for 1 min and centrifuged at 14000 g for 15 min at 4 $^{\circ}$ C, and the supernatant was collected. Equal amounts of protein (BSA Protein Assay Kit, Pierce) from each sample were mixed in a ratio of 1:1 according to the experimental design presented in table 1A and 1B, and 1 μ g of the protein sample thus obtained was separated on a 12% Tris-glycine sodium dodecyl sulfate-polyacrylamide gel by electrophoresis (SDS-PAGE), followed by staining of the SDS gel using colloidal Coomassie blue (Colloidal blue staining kit, Invitrogen). Then the resulting 3 gel lanes per replicate (CA, DB and ED) were excised and cut horizontally into 8 equal sections per lane. In-gel digestion was performed as follows: The SDS gel was destained by two washes with water. Cysteine reduction was performed by adding 100 μ l 50 mM DTT (dithiothreitol) in 50 mM NH $_4$ HCO $_3$. Samples were sonicated for 1 min and incubated at 60 $^{\circ}$ C without shaking for 1 h. Alkylation was performed when samples reached room temperature, replacing DTT by 100 μ l of 50 mM iodoacetamide in 50 mM NH $_4$ HCO $_3$. Samples were sonicated for 1 min and alkylated at room temperature in the dark for 1 h. The gel pieces were washed three times with 100 μ l 50 mM NH $_4$ HCO $_3$ at pH 8. For proteolytic digestion, samples were treated overnight with 100

µl trypsin (10 ng/µl in 50 mM NH₄HCO₃, sequencing grade, Boehringer Mannheim) at room temperature. Gel fragments were removed by centrifugation and the proteolytic peptides were recovered in the supernatant fraction (25 µl). 10% Trifluoroacetic acid was added to correct the pH up to 2 to 4. Finally all extracts were measured by LC-MS/MS.

Data acquisition, Mass Spectrometry: The protein samples were analyzed by injecting 18 µl sample over a 0.10 * 32 mm Prontosil 300-5-C18H (Bischoff, Germany) pre-concentration column (prepared in house) at a flow of 6 µl/min for 5 min with a Proxeon EASY nLC system. Compounds were eluted from the pre-concentration column onto a 0.10 * 250 mm Prontosil 300-3-C18H analytical column (prepared in house) with an acetonitril gradient at a flow of 0.5 µl/min. The gradient consisted of an increase from 9 to 34% acetonitril in water with 1ml/l formic acid in 50 min followed by a fast increase in the percentage acetonitril to 80% (with 20% water and 1 ml/l formic acid in both the acetonitril and the water) in 3 min as a column cleaning step. In between the pre-concentration and the analytical column, an electrospray potential of 3.5 kV was applied directly to the eluent via a solid 0.5 mm platina electrode fitted into a P777 Upchurch microCross. Full scan positive mode FT-MS spectra were measured between m/z 380 and 1400 at resolution 60.000 on an LTQ-Orbitrap (Thermo electron, San Jose, CA, USA). MSMS scans of the four most abundant doubly or triply charged peaks in the FTMS scan were recorded in data dependent mode in the linear trap (MSMS threshold = 10.000). Data were acquired using Xcalibur software.

Protein identification and quantitation: Mass spectra were analyzed using MaxQuant (version 1.0.13.8), which performs e.g. list generation, ratio H/L significance A, ratio H/L significance B, SILAC- and extracted ion current (XIC)-based quantification, false positive rate and data filtration (24). Data were searched against the human international protein index (IPI) database supplemented with frequently observed contaminants and concatenated with reversed copies of all sequences (total 148380 sequences) using Mascot v2.2 (Matrix Sciences). Spectra determined to result from heavy labeled peptides by pre-search Maxquant analysis were searched with the additional fixed modifications Arg10 and Lys8, whereas spectra with a SILAC state not defined a priori were searched with Arg10 and Lys8 as additional variable modifications. Precursor mass tolerance was set at 10 ppm for the complete peptides and 0.5 Da for peptide fragments as observed in the MS2 spectra. Trypsin/P cleavage specificity with up to 2 missed cleavage and three labeled amino acids (Arg and Lys) were allowed. The required false discovery rate was set to 1% at the protein level, and the minimum required peptide length was set to 6 amino acids. Carbamidomethyl cysteine was set as a fixed modification and methionine oxidation, deamidation (NQ), and acetylation of the N-terminus (protein) were allowed as variable modifications. For protein identification, at least two peptides were required, among which at least one peptide was

required to be unique in the database. Identified proteins were quantified. Protein ratios calculated by MaxQuant were subjected to manual inspection and results obtained compared with those from MSQuant software (version 1.5). Further analysis and plotting were performed using the R statistical and graphic environments.

Microarray: Integrity and quantity of the extracted RNA was assessed by using an Agilent 2100 Bioanalyzer (Agilent, Palo Alto, CA) and the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies Inc., DE, USA). cRNA was prepared according to the manufacturer's protocol and hybridized to HG-U133 plus 2.0 GeneChip arrays (Affymetrix, Santa Clara, CA, USA). HG-U133 plus 2.0 arrays contain 54,675 sets of oligonucleotide probes or probesets. Data were analyzed based on a mapping to ~17500 unique human genes with Entrez Gene annotation.

Calculations and statistics: Microarray results were processed in R (<http://www.r-project.org>) using gcRMA and filtered using MAS5 calls. Ratios were calculated using limma in R, applying moderated t-tests, and were adjusted for multiple testing. The reported ratios describing biological effects of interest were calculated as indicated in table 1A and 1B.

Bioinformatics Network Analysis: A cutoff P value ≤ 0.001 (Ratio H/L or L/H) was selected for analysis of differentially expressed proteins and a multiple testing adjusted p-value ≤ 0.05 for differentially regulated genes was used. Ingenuity Pathway Analysis 8.5 (Ingenuity Systems Inc.) was used to conduct a knowledge-based network analysis, a molecular and cellular functions analysis, and a canonical pathway analysis of the proteomics and transcriptomics data. Ingenuity Pathway Analysis tools rely on curated functional and regulatory interactions extracted from literature. The biological functions across ER-responsive genes and proteins were identified. Fischer's exact test was used to calculate a p-value determining the probability that each biological function assigned to that dataset of ER-responsive genes/proteins is due to chance alone.

Results

Optimization of the experimental protocol and receptor expression.

T47D cells were previously stably transfected with the ER β expression plasmid under Tet-responsive promoter regulation (16). Cells were grown in medium containing light or heavy amino acid labels according to the experimental design presented in table 1. After 10 days pre-incubation, the MS analysis of cell samples revealed that isotope incorporation did not further increase reflecting optimal full metabolic incorporation and indicating that the cells were ready for the exposure phase. Cell treatment was performed as indicated by Williams and co-workers to be able to compare genistein effects with their results for estradiol in the

same cell system and under the same conditions (8). Consequently, after cell synchronization by exposure to ICI 182780 cells were exposed to either ICI 182780 or genistein.

ER β expression in the T47D control cell lines and the T47D-ER β cell samples pretreated in the presence or absence of Tet was checked by fluorescence microscopy (Enhanced Green Fluorescence Protein co-expression under control of the same Tet-responsive promoter (16) and mRNA quantification (Figure 1). ER β mRNA levels were undetectable in Tet+ samples or the control cell line (no endogenous or exogenous ER β). LC-MS/MS measurements detected high levels of ER β protein in all Tet- samples (see below).

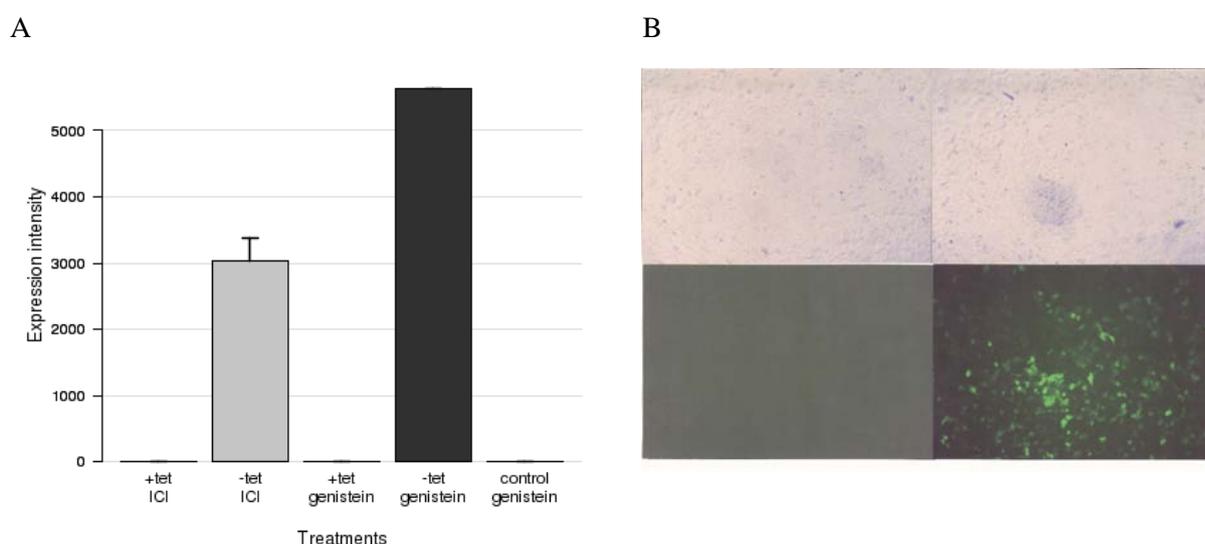


Figure 1: Estrogen receptor beta (ER β) expression. A) Mean expression intensities of ER β at mRNA level based on microarray normalized results under five different conditions, Tet+ and ICI (sample A, Table 1A), Tet- and ICI (sample B, Table 1A), Tet+ and genistein (sample C, Table 1A), Tet- and genistein (sample D, Table 1A), and Tet- in control cell line (T47D-PBI) and genistein (sample E, Table 1A). B) Picture of cells (upper) and green fluorescent protein (EGFP) detection (lower) of Tet+ (left) and Tet- (right) cultured cells treated with genistein.

Expression of ER α was not found at protein level using LC-MS/MS. However using an ER α ELISA (ActiveMotif sandwich ELISA), ER α could be detected in all the experimental samples (Figure 2) at expression levels of 6-10% of the levels observed in the wild-type T47D cells (not treated with ICI 182780).

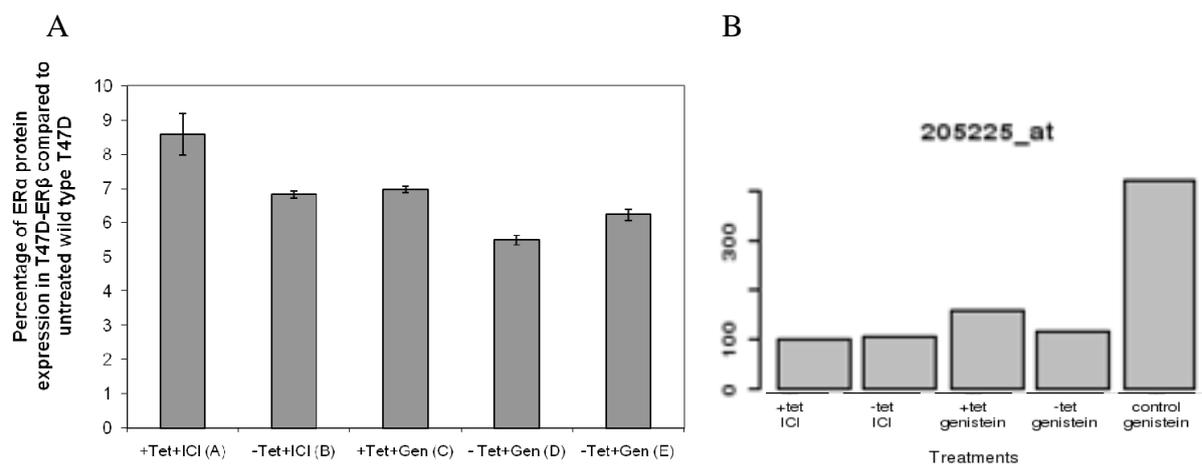


Figure 2: A) ActiveMotif sandwich ER α ELISA. Amounts of intact ER α from SILAC samples were compared with the ER α from untreated wild type T47D. Percentage of expression of three measurements of ER α protein under five different conditions, Tet+ and ICI (sample A, Table 1A), Tet- and ICI (sample B, Table 1A), Tet+ and genistein (sample C, Table 1A), Tet- and genistein (sample D, Table 1A), and Tet- in control cell line (T47D-PBI) and genistein (sample E, Table 1A). B) Mean expression intensities of ER α at mRNA probe level based on microarray normalized results under the five different conditions.

Protein identification and quantitation.

Corresponding light and heavy samples were mixed in a 1 to 1 ratio, according to the experimental design providing three data sets (CA, DB and ED, Table 1B). On average, 2600 proteins were identified per data set (2616 in sample CA, 2543 in DB and 2627 in ED). From these, for about 1950 proteins, relative quantification data with at least 2 identified peptides per experiment were obtained (1994 in sample CA, 1905 in DB and 1995 in ED). For proteins for which corresponding peptides were only detected in the heavy data set but not in the light data set, hampering calculation of an H/L ratio, the H/L ratio was set to the largest H/L ratio determined in the whole data set. Likewise, for proteins for which corresponding peptides were only detected in the light data set but not in the heavy data set, also hampering calculation of an H/L ratio, the H/L ratio was set to the smallest H/L ratio in the whole data set. This enabled comparison of the proteomic results to results obtained with the microarray or to find *de novo* regulated proteins.

The ratio distributions obtained from each experiment (CA, DB and ED) are displayed in figures 3A, 3B and 3C. The spread of the cloud is lower at high protein abundance (higher intensity on Y-axis), which indicates higher precision in the quantification. Notably, higher spread of the cloud in the X-direction (ratio (log₂)) was observed during ER β expression indicating bigger fold changes in protein expression levels.

Differentially expressed proteins were selected using a cut-off confidence level of ratio H/L or L/H Significance $B \leq 0.001$. In a next step we used canonical pathways, molecular

and cellular function classification from Ingenuity pathway analysis (IPA) to identify overrepresented biological themes among these differentially expressed genes and proteins.

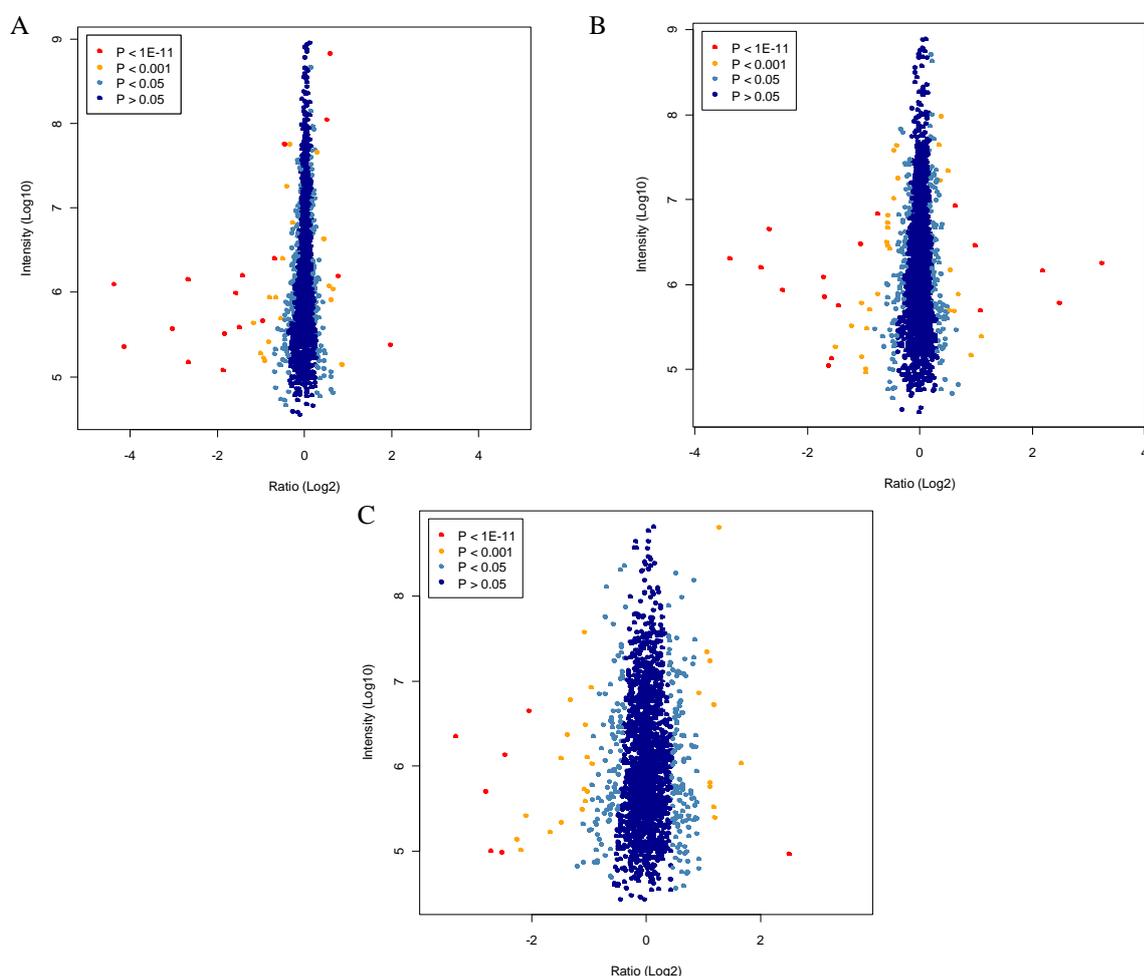


Figure 3: Proteome-wide accurate quantification significance in **A**) $ER\alpha$ -mediated proteins expressed as a result of genistein treatment (sample CA, table 1B). Normalized protein ratios (H/L) are plotted against summed peptide intensities. Left side of the plot shows proteins upregulated in the sample cultured in Light (L) amino isotope conditions: $ER\alpha(+)$ $ER\beta(-)$ and ICI 182780; Right side of the plot shows proteins upregulated in the sample cultured in Heavy (H) amino acid isotope conditions: $ER\alpha(+)$ $ER\beta(-)$ and genistein. **B**) $ER\beta$ -mediated proteins expressed as a result of genistein treatment (sample DB, Table 1B). Normalized protein ratios (H/L) are plotted against summed peptide intensities. Left side of the plot shows proteins upregulated in the sample culture in Light (L) amino isotope conditions: $ER\alpha(+)$ $ER\beta(+)$ and genistein; Right side of the plot shows proteins upregulated in the sample culture in Heavy (H) amino isotope conditions: $ER\alpha(+)$ $ER\beta(+)$ and ICI 182780. **C**) Second approach to detect $ER\beta$ -mediated proteins expressed as a result of genistein treatment (sample ED, table 1B). Normalized protein ratios (H/L) are plotted against summed peptide intensities. Left side of the plot shows proteins upregulated in the sample culture in Light (L) amino isotope conditions: $ER\alpha(+)$ $ER\beta(+)$ and genistein; Right side of the plot shows proteins upregulated in the sample culture in Heavy (H) amino isotope conditions: $ER\alpha(+)$ $ER\beta(-)$ and genistein in the T47D-PBI cell line. The spread cloud is lower at high abundance, indicating that quantification is more precise. The data points are colored by their “significance B” (intensity-dependent p-value), with dark blue circles having values > 0.05 , light blue circles between 0.05 and 0.001, yellow circles between 0.001 and $1E-11$ and red circles $< 1E-11$.

Genistein modulated protein expression in the absence of ER β (sample CA).

To determine the genistein induced ER α -mediated effects on protein expression levels, cells grown in heavy amino acid isotope containing medium were treated for 24 h with 500 nM genistein whereas cells grown in light amino acid isotope containing medium were treated for 24 h with 10 nM of the ER antagonist ICI 182780, in both cases in the presence of Tet therefore without the expression of ER β (sample CA, table 1A-B). This process resulted in the identification of 1994 proteins with at least two unique peptides. The results obtained showed small changes for most proteins (Figure 3A). Based on the statistical selection criteria (Significance $B \leq 0.001$) the data revealed 59 SILAC proteins to be significantly regulated (Table 2).

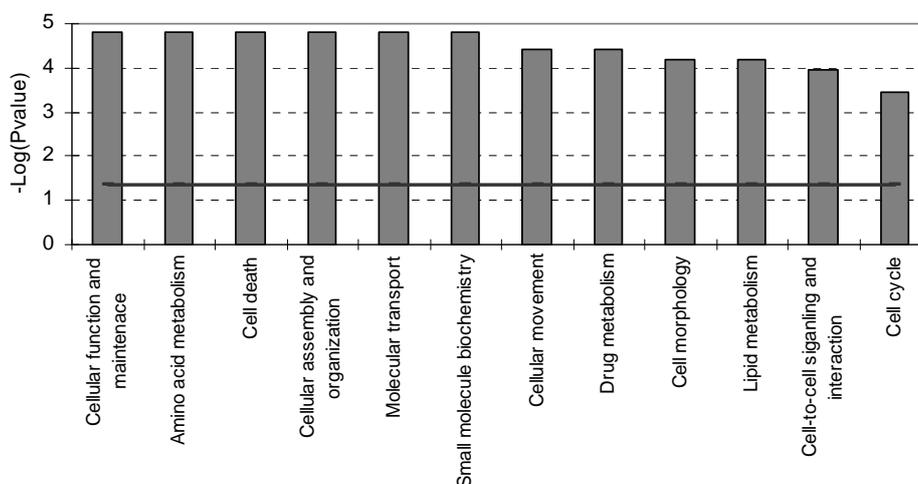


Figure 4: Molecular and cellular functional classes affected by genistein induced ER α -mediated effects on protein expression (sample CA, Table 1B). IPA was based on molecular and cellular functions of the H/L proteins with a P value <0.001 . Selected scoring method was based on Fisher's exact test p-value. The high-level functional categories that are involved in this analysis are displayed along the x-axis in decreasing order of significance. The y-axis displays the $-\log$ significance. The horizontal line denotes the cutoff for significance (p-value of 0.05).

Functional analysis (see materials and methods) of the proteins in this data set revealed that the major biological functions that were affected (increased or decreased) by genistein included cellular function and maintenance, cell death, cellular assembly and organization, cell movement, cell morphology, lipid metabolism or cell cycle (Figure 4). Most of the proteins belonging to these functional classes were upregulated, whereas downregulated proteins were categorized in the classes representing cell death and lipid metabolism (Table 2). Notably, five of the most upregulated proteins upon genistein-induced ER α -mediated protein expression were myosins (MYH10, MYH14, MYL12B, MYH9 and MYL6). The majority of these myosins are actively involved in cell assembly and organization, or cytoskeleton reorganization. Among the several significantly downregulated proteins we found at least three proteins, S100A8, S100A9 and PIP, of specific interest since their

expression is associated with a decrease of cell proliferation and induction of apoptosis (25, 26). The two S100 EF-hand calcium-binding proteins S100A8/A9 induce apoptosis in various cells, especially tumor cells like MCF7 (25).

Table 2: The most significant ER α -mediated proteins as a result of genistein treatment (sample CA, Table 1B), only H/L SILAC pairs protein list.

Gene Names	Protein Names	Ratio H/L Normalized	Ratio Significance	Ratio H/L Count	Sequence Coverage [%]
NFAT5	NFAT5 protein	3.9151	8.32E-28	2	1.5
NBN	Nibrin	1.7846	1.02E-06	2	5.3
TTBK2	Tau-tubulin kinase	1.7012	1.26E-16	2	2.9
MYH10	Myosin-10	1.5769	2.11E-07	14	15.5
CCDC25	Coiled-coil domain-containing protein 25	1.5499	0.00013085	2	11.1
MGP	Matrix Gla protein	1.521	1.53E-06	5	23.3
TMEM120A	Transmembrane protein 120A	1.5178	0.00024373	2	3.2
MYH14	Myosin-14	1.5152	0.00025613	3	4.6
MRPL20	39S ribosomal protein L20, mitochondrial	1.5095	0.0002857	2	12.8
KRT18	Keratin, type I cytoskeletal 18	1.4982	2.90E-18	230	91.9
MYL12B	Myosin regulatory light chain MRCL3 variant	1.4819	5.81E-06	8	37.3
KRT8	Keratin, type II cytoskeletal 8	1.4635	2.30E-16	545	86.1
KRT19	Keratin, type I cytoskeletal 19	1.4508	1.10E-15	246	95.5
MYH9	Myosin-9	1.4135	9.37E-14	269	63.9
MYL6	Myosin light polypeptide 6	1.3405	3.34E-06	22	61.6
ASNS	Asparagine synthetase [glutamine-hydrolyzing]	1.3256	0.00074994	4	8.9
SLC7A5	Large neutral amino acids transporter small subunit 1	1.2556	0.00024142	6	6.7
FAM129A	Protein Niban	1.2546	0.00025301	6	9.2
RCN1	Reticulocalbin-1	1.2251	0.00093813	7	18.4
NPM1	Nucleophosmin	1.2138	3.04E-05	71	64.6
LOC130773	similar to 60S ribosomal protein L23a	1.1925	0.00014684	5	30.6
SLC3A2	4F2 cell-surface antigen heavy chain	1.1855	0.00024133	54	28.7
HIST1H1C	Histone H1.2	1.1649	0.00095077	48	39.9
RPS25	40S ribosomal protein S25	0.85955	0.0001805	24	55.2
RPS16	40S ribosomal protein S16	0.81669	1.37E-06	41	61
TPR	Nucleoprotein TPR	0.81623	0.00058563	22	9.7
NPC2	Epididymal secretory protein E1	0.80913	0.00035735	16	35.3
VAT1	Synaptic vesicle membrane protein VAT-1 homolog	0.80802	0.00032994	16	35.1
BCKDHA	cDNA FLJ45695 fis, clone FEBRA2013570, highly similar to 2-oxoisovalerate dehydrogenase alpha subunit, mitochondrial (EC 1.2.4.4)	0.80718	0.00031066	8	16.1
ACTBL2	Beta-actin-like protein 2	0.78565	1.53E-08	9	21.8
RPS18	40S ribosomal protein S18	0.75035	2.95E-11	40	67.1
NFS1	Cysteine desulfurase, mitochondrial	0.73481	0.00073737	6	9.4
-	Putative uncharacterized protein ENSP00000384045 (Fragment)	0.73352	0.00069096	2	13.5
NASP	Nuclear autoantigenic sperm protein isoform 2 variant	0.73307	0.00067556	3	7.5
LMNA	Lamin-A/C	0.71605	1.83E-14	102	54.8
RPS29	40S ribosomal protein S29	0.70426	1.21E-08	5	47.4
LPP	Lipoma-preferred partner	0.6822	3.54E-05	3	8.2
MAP4	Microtubule-associated protein 4	0.63442	1.02E-06	9	12.7
THOC2	THO complex subunit 2	0.62342	0.00051663	2	1.4
NLN	Neurolysin, mitochondrial	0.62126	0.00047125	2	3
CHCHD1	Coiled-coil-helix-coiled-coil-helix domain-containing protein 1	0.61855	0.0004196	2	25.4

Gene Names	Protein Names	Ratio H/L Normalized	Ratio Significance	Ratio H/L Count	Sequence Coverage [%]
CHMP2B	Charged multivesicular body protein 2b	0.61376	0.00034025	2	4.7
TOMM40	Mitochondrial import receptor subunit TOM40 homolog	0.61264	3.56E-15	4	10.5
LMNB1	Lamin-B1	0.56768	1.45E-09	4	12.1
APLP2	Amyloid-like protein 2	0.56042	2.33E-05	2	4.7
PIP	Prolactin-inducible protein	0.52576	2.73E-06	4	26
HMGN	Non-histone chromosomal protein HMG-14	0.51956	1.79E-06	3	14
SYAP1	Synapse-associated protein 1	0.51352	1.20E-12	2	7.4
MOGS	Mannosyl-oligosaccharide glucosidase	0.49431	2.80E-07	2	3.8
HNRNPC	Heterogeneous nuclear ribonucleoproteins C1/C2	0.44009	2.22E-09	2	13.4
SEPHS1	Selenide, water dikinase 1	0.37038	4.08E-56	4	13.3
POFUT1	GDP-fucose protein O-fucosyltransferase 1	0.35029	1.99E-14	2	4.1
MYO15A	Myosin-XV	0.33393	1.87E-31	2	0.9
S100A8	Protein S100-A8	0.28004	1.44E-20	2	20.4
KPRP	Keratinocyte proline-rich protein	0.2706	1.29E-21	2	1.6
FXC1	Mitochondrial import inner membrane translocase subunit Tim9 B	0.15658	4.51E-42	2	24.3
S100A9	Protein S100-A9	0.15405	5.78E-193	5	26.3
LGALS7	Galectin-7	0.12058	2.35E-54	2	19.9
PNKD	Probable hydrolase PNKD	0.05602	8.55E-100	3	30.3

Genistein modulated protein expression in the presence of ER β (sample DB).

Proteins induced by genistein in ER α and ER β expressing cells could be identified comparing cells grown in light amino acid isotope containing medium treated with 500 nM genistein to cells grown in heavy isotope containing medium exposed to 10 nM of the pure ER antagonist ICI 182780, both grown in the absence of Tet thus expressing ER β (sample DB, table 1A-B). This resulted in the identification of 1905 proteins with at least two unique peptides. Significant changes observed in protein level after 24 h treatment are depicted in Figure 3B. Based on the statistical selection criteria (Significance $B \leq 0.001$) the data revealed 66 SILAC proteins to be significantly regulated (Table 3). For IPA and further analysis inverse ratios were used to allow additional inter-experiment comparisons.

As reported previously (17), in T47D-ER β cells grown in the absence of Tet and thus expressing both ER α and ER β , genistein induced cell proliferation was notably reduced as compared to genistein induced cell proliferation in T47D cells expressing only ER α . The SILAC data of the sample reflect genistein induced upregulation of nine histone related proteins (HIST1H4A, HIST1H2AG, H1F0, HIST1H2AB, HIST1H2BN, HIST1H1E, HIST1H1B, HIST1H1C and NUMA1) which points at a general downregulation of gene transcription (27, 28). In addition, downregulation of TOP2B (Topoisomerase II β) was significant. Topoisomerases are enzymes that play important roles in transcription, DNA synthesis, and chromosome segregation (29). This result is in line with a previously published inhibition of Topoisomerase II β by genistein influencing cell growth (30). Important proteins belonging to the same signaling network, i.e. interferon signaling

pathways (31), were STAT1, MX1, ANXA1, UBE2L6 and ISG15. Also ANXA3, ANXA1 and VCL, proteins important for actin cytoskeleton signaling, cell migration and anti-inflammatory processes (32, 33), were found to be downregulated by genistein in T47D cells expressing both ER α and ER β .

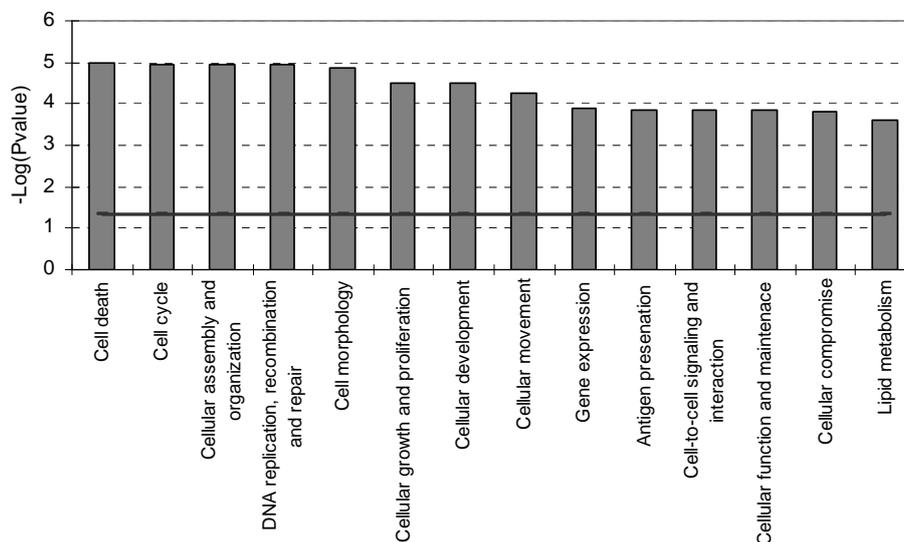


Figure 5: Molecular and cellular functional classes affected by genistein induced ER β -mediated effects on protein expression (sample DB, Table 1B). IPA molecular and cellular functions of the L/H proteins with a P value <0.001. Selected scoring method was based on Fisher's exact test p-value. The high-level functional categories that are involved in this analysis are displayed along the x-axis in decreasing order of significance. The y-axis displays the $-\log$ significance. The horizontal line denotes the cutoff for significance (p-value of 0.05).

Molecular and cellular function profiling of these data (Figure 5) points at significant effects on pathways involved in cell death, cell cycle, cellular assembly and organization, DNA replication, recombination and repair, cell growth and proliferation or cellular movement. Upregulated proteins by genistein were directly involved in the first three categories whereas the downregulated proteins were part of the categories cell movement or cell death (Table 3).

Table 3: The most significant ER β -mediated proteins as a result of genistein treatment (sample DB, Table 1B), only L/H SILAC pair protein list.

Gene Names	Protein Names	Ratio L/H Normalized	Ratio Significance	Ratio H/L Count	Sequence Coverage [%]
FGFR2	Fibroblast growth factor receptor 2	10.47724	1.33E-124	2	2.1
HIST1H4A	Histone H4	7.141837	5.69E-88	7	51.5
HIST1H2AG	Histone H2A	5.420936	8.26E-47	2	27.2
AD-001	TRM112-like protein	3.275038	5.13E-24	4	32
SEPHS1	Selenide, water dikinase 1	3.242227	1.22E-23	2	9.2
ELOVL1	Elongation of very long chain fatty acids protein 1	3.09272	1.72E-12	2	4.3
PIP	Prolactin-inducible protein	2.97274	9.65E-12	4	26
ARMC10	Armadillo repeat-containing protein 10	2.851521	5.55E-11	2	4.7
KIAA0753	Uncharacterized protein KIAA0753	2.74198	7.31E-18	2	3.1
HSD17B8	Estradiol 17-beta-dehydrogenase 8	2.345051	8.49E-08	2	10.7
HNRNPC	Heterogeneous nuclear ribonucleoproteins C1/C2	2.083811	5.49E-14	11	21.9
PRMT1	Protein arginine N-methyltransferase 1	2.078829	3.24E-10	2	10.8
MATR3	Putative uncharacterized protein MATR3	2.051366	5.57E-06	2	3
RNMTL1	RNA methyltransferase-like protein 1	1.955225	2.12E-05	2	2.9
S100A9	Protein S100-A9	1.939827	2.62E-05	2	13.2
ESR2	Estrogen receptor beta	1.93304	2.88E-05	3	7.9
EPHX2	Epoxide hydrolase 2	1.871608	6.24E-08	2	5.2
RNPEP	Putative uncharacterized protein RNPEP	1.750271	0.0003341	2	36.5
H1F0	Histone H1.0	1.687564	5.23E-06	11	19.6
HIST1H2AB	Histone H2A type 1-B/E	1.686369	1.54E-12	13	35.4
LAMP2	Lysosomal-associated membrane protein 2C	1.520751	0.0002132	2	1.9
HIST1H2BN	Histone H2B type 1-N	1.518603	1.13E-05	10	25.3
LMNA	Lamin-A/C	1.511624	0.0002583	2	51.2
ISYNA1	Inositol-3-phosphate synthase 1	1.504959	1.68E-05	14	24.2
HNRNPUL2	Heterogeneous nuclear ribonucleoprotein U-like protein 2	1.49997	0.0003293	3	3.1
SLC9A3R1	Na(+)/H(+) exchange regulatory cofactor NHE-RF1	1.497253	2.11E-05	18	34.9
MUC1	cDNA FLJ60927, highly similar to Mucin-1	1.496468	2.16E-05	16	19.1
CD9	CD9 antigen	1.494098	0.0003718	3	14.5
NUMA1	Nuclear mitotic apparatus protein 1	1.491402	3.76E-08	71	35.5
HIST1H1E	Histone H1.4	1.45588	6.87E-05	11	39.7
TFRC	Transferrin receptor protein 1	1.386155	4.75E-06	62	37.2
HIST1H1B	Histone H1.5	1.385003	5.00E-06	40	38.1
HIST1H1C	Histone H1.2	1.314389	9.28E-05	45	45.1
PYGB	Glycogen phosphorylase, brain form	1.298937	0.0001691	24	21.9
HNRNPA2B1	Heterogeneous nuclear ribonucleoproteins A2/B1	1.272961	0.000447	97	74.5
ACLY	ATP-citrate synthase	0.820075	0.0007347	65	35.6
COTL1	Coactosin-like protein	0.818666	0.0006644	22	55.6
EIF4G1	Eukaryotic translation initiation factor 4 gamma 1	0.811886	0.0003983	60	29.8
CNN2	cDNA FLJ52765, highly similar to Calponin-2	0.808211	0.0002993	27	36.7
TNKS1BP1	Tankyrase 1 binding protein 1, 182kDa	0.798276	0.0001335	63	31.9
VCL	Vinculin	0.792896	8.39E-05	98	61
REEP5	Receptor expression-enhancing protein 5	0.786225	4.62E-05	25	34.4
CYB5A	Cytochrome b5	0.770594	0.0006788	10	47
FLNA	Filamin-A	0.768285	8.22E-06	239	53.9
PPL	Periplakin	0.761325	0.0003987	34	17.3
NFKB2	Nuclear factor NF-kappa-B p100 subunit	0.753977	0.0009489	5	5.1
MAP4	Microtubule-associated protein 4	0.742666	0.0005389	4	6

Gene Names	Protein Names	Ratio L/H Normalized	Ratio Significance	Ratio H/L Count	Sequence Coverage [%]
ADSL	Adenylosuccinate lyase	0.720305	0.0001592	2	5.6
UBTF	Nucleolar transcription factor 1	0.717051	0.0001318	4	4.6
CSRP1	Cysteine and glycine-rich protein 1	0.709673	6.73E-09	30	67.9
DDRGK1	DDRGK domain-containing protein 1	0.701361	5.04E-05	2	8.6
ANXA1	Annexin A1	0.691324	2.67E-06	9	20.2
GPS1	COP9 signalosome complex subunit 1	0.654407	1.75E-06	2	6.8
ANXA3	Annexin A3	0.647249	1.76E-13	22	37.8
TOP2B	DNA topoisomerase 2-beta	0.643791	0.0004078	4	2.1
SLK	STE20-like serine/threonine-protein kinase	0.62692	0.0001979	2	1.9
DNPEP	Aspartyl aminopeptidase	0.621157	9.79E-08	2	4.9
UBE2L6	Ubiquitin/ISG15-conjugating enzyme E2 L6	0.534416	1.20E-06	2	11.8
STAT1	Signal transducer and activator of transcription 1-alpha/beta	0.511326	5.44E-17	9	15.1
TTC9C	Tetratricopeptide repeat protein 9C (TPR repeat protein 9C)	0.474518	2.35E-16	3	6.5
BAG5	cDNA FLJ77290, highly similar to Homo sapiens BCL2-associated athanogene 5 (BAG5), mRNA	0.470389	7.84E-09	2	6.6
MX1	Interferon-induced GTP-binding protein Mx1	0.221029	2.22E-78	4	11.9
ISG15	Interferon-induced 17 kDa protein	0.181045	3.92E-77	4	29.7
PRAF2	PRA1 family protein 2	0.106702	9.82E-170	2	16.9

Genistein modulated protein expression in the presence of ER β (sample ED).

Genistein modulated ER β -regulated proteins could also be identified comparing T47D-ER β cells grown in light amino acid isotope containing medium treated with 500 nM genistein to mock transfected T47D-control cells grown in heavy isotope containing medium treated with 500 nM genistein, both grown in the absence of Tet with the T47D-ER β cells expressing ER β (sample ED, table 1A-B). Significant changes observed in protein levels after 24 h treatment are depicted in Figure 3C. The mock control cell line do not express ER β . This comparison resulted in the identification of 1995 proteins with at least two unique peptides that were differentially expressed upon genistein exposure in the presence of ER β .

Based on the statistical selection criteria (Significance $B \leq 0.001$) the data revealed 58 SILAC proteins that appeared to be significantly regulated (Figure 3C and table 4). Genistein induced ER β -mediated protein expression causes the most significant changes in proteins involved in processes like cell death (ER β , FHL2, LMNA or CYB5A), lipid metabolism (ER β , ACOX1, COLT1, CYB5A), gene expression (ER β , HIST1H2AG, HIST1H4A, H2AFZ or HNRNCP), and cell cycle (LMNA, ITGAV or ER β) (Figure 6, Table 4).

Both ER β -target approaches (samples DB and ED) found comparable results. In both experiments, genistein-mediated ER β activation regulated molecular functions including cell death, cell cycle, cell movements and lipid metabolism. Among the most significant ER β -targets identified were proteins mediating gene transcription, like HNRNCP and Histones related proteins.

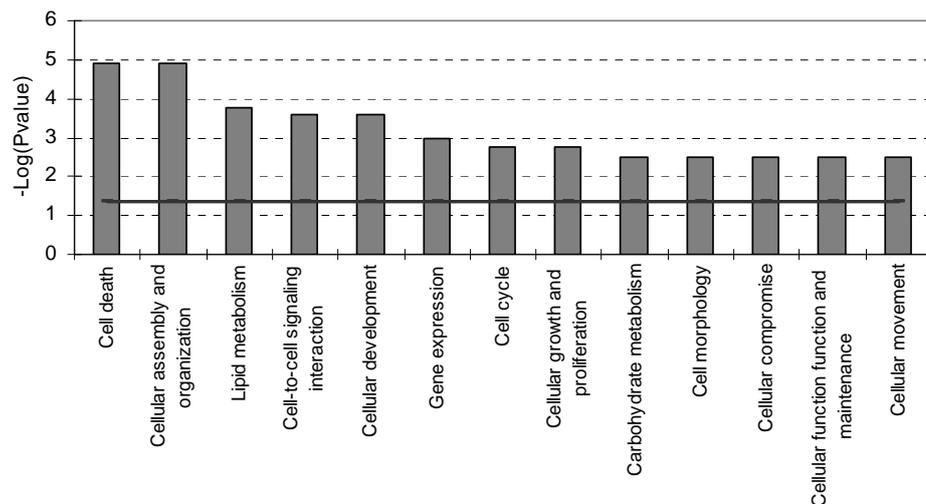


Figure 6: Molecular and cellular functional classes affected in a second approach to detect genistein induced ER β -mediated effects on protein expression (sample ED, Table 1B). IPA molecular and cellular functions of the H/L proteins with a P value <0.001. Selected scoring method was based on Fisher's exact test p-value. The high-level functional categories that are involved in this analysis are displayed along the x-axis in decreasing order of significance. The y-axis displays the $-\log$ significance. The horizontal line denotes the cutoff for significance (p-value of 0.05).

Table 4: The most significant ER β -mediated proteins as a result of genistein treatment (sample HD, Table 1B), only L/H SILAC pair protein list.

Gene Names	Protein Names	Ratio H/L Normalized	Ratio Significance	Ratio H/L Count	Sequence Coverage [%]
ANKRD36B	UPF0634 protein C	5.594	1.04E-17	2	1.5
MGP	Matrix Gla protein	3.1457	1.26E-09	7	23.3
KRT18	Keratin, type I cytoskeletal 18	2.4136	8.26E-09	215	94.4
GIPC1	PDZ domain-containing protein GIPC1	2.2777	1.76E-05	2	9.3
TP53I3	Putative quinone oxidoreductase	2.245	1.11E-07	21	40.4
CLDN1	Claudin-1	2.2442	1.38E-06	5	19
EFHD1	EF-hand domain-containing protein D1	2.1675	3.63E-06	8	18.4
KIAA1107	Uncharacterized protein KIAA1107	2.1623	3.87E-06	2	2.1
REEP5	Receptor expression-enhancing protein 5	2.1537	4.50E-07	32	34.9
GOT2	Aspartate aminotransferase, mitochondrial	2.0696	1.61E-06	36	41.6
KRT8	Keratin, type II cytoskeletal 8	2.0237	3.21E-06	535	85.7
COMTD1	cDNA FLJ52132, highly similar to Catechol-O-methyltransferasedomain-containing protein 1 (EC 2.1.1.-)	1.89	0.00061374	2	2.8
CRIP1	Cysteine-rich protein 1	1.8892	2.35E-05	7	79.2
VPS24	cDNA FLJ58988, highly similar to Charged multivesicular body protein 3	1.8736	0.00071107	2	4.8
METTL7A	Methyltransferase-like protein 7A	1.8288	0.00023505	6	17.2
RAB18	Ras-related protein Rab-18	1.8235	0.00025018	12	34.5
CNPY2	Protein canopy homolog 2	1.8106	0.00094353	14	37.9
KRT19	Keratin, type I cytoskeletal 19	1.8021	8.29E-05	240	93.8
LYRM7	Putative uncharacterized protein LYRM7	1.7869	0.00038649	4	24
NPM1	Nucleophosmin	1.7869	0.00010282	68	71.4
APRT	Adenine phosphoribosyltransferase	1.7725	0.00012622	36	78.3

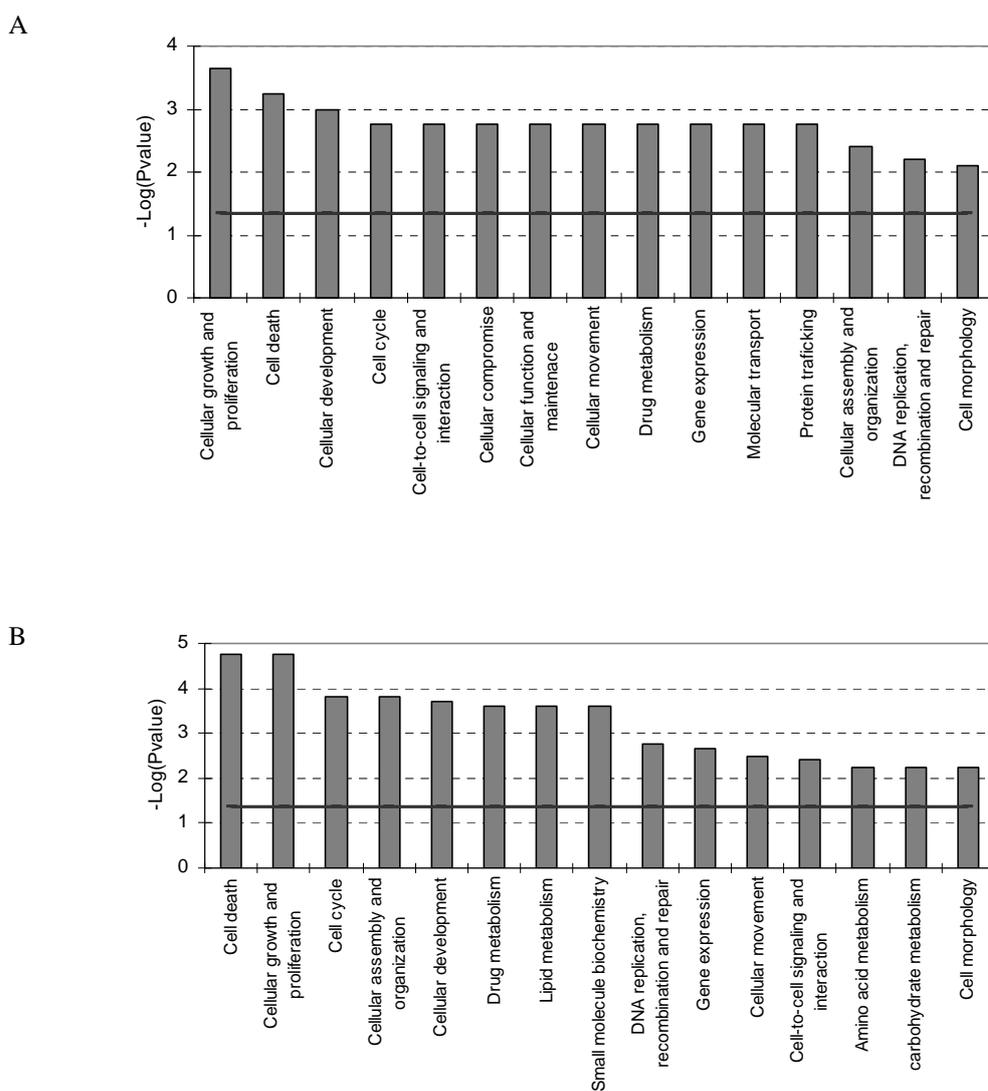
Gene Names	Protein Names	Ratio H/L Normalized	Ratio Significance	Ratio H/L Count	Sequence Coverage [%]
HSPA9	Stress-70 protein, mitochondrial	1.7722	0.00012673	142	60.8
PTGR2	Prostaglandin reductase 2	1.7713	0.00046415	3	15.7
CANX	Calnexin	1.7616	0.0001473	31	28.2
CBX5	Chromobox protein homolog 5	1.7157	0.00088702	5	19.4
ACY1	Aminoacylase-1	1.7126	0.00091913	6	10.6
FLNA	Filamin-A	0.60289	0.00070352	269	51.8
ISYNA1	Inositol-3-phosphate synthase 1	0.60177	0.00067576	17	23.8
COTL1	Coactosin-like protein	0.56046	0.00013071	22	54.2
PRSS8	Prostasin	0.54848	0.00087159	2	12.8
CYB5A	Cytochrome b5	0.52474	0.00012112	13	56
COX7A2L	Putative uncharacterized protein COX7A2L	0.51931	0.00031952	3	34.8
S100A16	Protein S100-A16	0.5155	8.08E-05	9	68
RPL38	60S ribosomal protein L38	0.51546	0.00027718	5	50
HIST1H2AG	Histone H2A	0.5063	9.01E-06	15	27.2
FHL2	Four and a half LIM-domain protein 2	0.48592	8.52E-05	6	26
ACOX1	Peroxisomal acyl-coenzyme A oxidase 1	0.48194	1.59E-05	14	20
SRPR	Signal recognition particle receptor subunit alpha	0.47482	5.24E-05	2	3.3
ANXA3	Annexin A3	0.47299	9.83E-06	14	33.7
LMNA	Lamin-A/C	0.47133	1.09E-06	113	61.1
ITGAV	Integrin alpha-V	0.46812	3.86E-05	5	4.8
S100A8	Protein S100-A8	0.46418	0.00078094	2	32.3
ARMCX3	Armadillo repeat-containing X-linked protein 3	0.45982	0.00067904	3	12.1
PREX1	Phosphatidylinositol 3,4,5-trisphosphate-dependent Rac exchanger 1 protein	0.45533	2.10E-05	11	7.1
SDHB	Succinate dehydrogenase [ubiquinone] iron-sulfur subunit, mitochondrial	0.42786	0.00022185	2	7.1
HIST1H4A	Histone H4	0.39614	2.83E-09	9	44.7
HNRNPC	Heterogeneous nuclear ribonucleoproteins C1/C2	0.37985	1.58E-08	11	25.2
H2AFZ	Histone H2A.Z	0.35593	8.43E-06	2	20.3
ABAT	4-aminobutyrate aminotransferase, mitochondrial	0.35076	1.04E-09	9	13
LMNB2	Lamin-B2	0.31103	5.26E-07	2	4.5
ISG15	Interferon-induced 17 kDa protein	0.22984	3.20E-10	5	29.7
UGDH	UDP-glucose 6-dehydrogenase	0.21775	7.17E-11	2	6.1
MX1	Interferon-induced GTP-binding protein Mx1	0.17993	3.85E-23	12	17.1
MATR3	Putative uncharacterized protein MATR3	0.17222	5.90E-14	2	3.6
ESR2	Estrogen receptor beta	0.15132	7.65E-16	2	5.7
DMD	Dystrophin	0.14177	1.63E-24	4	0.3
LDHB	L-lactate dehydrogenase B chain	0.099128	1.64E-40	6	33.2

Transcriptomics data.

In addition to the proteomics also transcriptomics was performed. Using the same samples as for the SILAC protein analysis, differentially expressed genes for each experiment (CA, DB and ED) were obtained. The comparison of gene expression levels between genistein and ICI induced T47D-ER β cells in the absence of ER β (sample CA) did not identify significant differentially expressed genes. This is different from the study of Williams et al. (8). It is however in agreement with the relatively low levels of ER α present. The presence of more subtle gene regulations which could indeed be ER α -mediated was investigated. A functional analysis of the top 30 genes as ranked by p-value (Figure 7A) showed that these genes are involved in biological processes that match ER α -mediated

effects. Cellular function and maintenance, cell-to-cell signaling and interaction, cellular movement, cell death, cellular assembly and organization, were enhanced at the mRNA level as already seen at the protein level. The majority of the top 30 geneset were upregulated as a result of genistein treatment, resulting in stimulation of pathways for cellular growth and proliferation, cell death and cellular development.

The results of the genistein treatment effects in the presence of ER β (sample DB), contained a set of 73 differentially regulated genes ($p \leq 0.05$). Functional analysis of this gene set showed upregulation of cell death and downregulation of cellular growth and proliferation, and cell cycle regulation. These effects (Figure 7B) were similar to those observed at protein level, pointing at increases in expression of genes and proteins involved in for example: cellular growth and proliferation, cell cycle, cellular assembly and organisation.



C

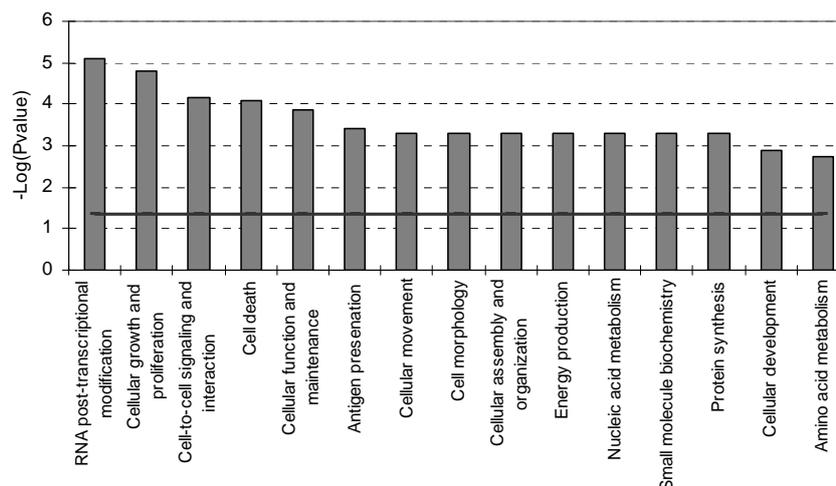


Figure 7: Most significant molecular and cellular functional classes affected as a result of A) genistein induced ER α -mediated effects on gene expression (sample CA, Table 1B), B) genistein induced ER β -mediated effects on gene expression (sample DB, Table 1B), C) a second approach to detect genistein induced ER β -mediated effects on gene expression (sample ED, Table 1B). IPA molecular and cellular functions of the genes with a P value <0.05. Selected scoring method was based on Fisher's exact test p-value. The high-level functional categories that are involved in this analysis are displayed along the x-axis in decreasing order of significance. The y-axis displays the $-\log$ significance. The horizontal line denotes the cutoff for significance (p-value of 0.05).

The second approach detecting genistein modulated ER β mediated gene expression (sample ED) revealed 1011 differentially regulated genes ($p \leq 0.05$). Genistein modulated ER β -mediated effects (Figure 7C) were related to RNA post-transcriptional modification, cellular growth and proliferation, cell-to-cell signaling and interaction, cell death, cellular function and maintenance.

Furthermore, hierarchical cluster analysis of the gene expression of the 73 differentially regulated genes by ER β was used to identify three main clusters in this gene set (Figure 8). Genes present in the upper part of the dendrogram correspond to those genes downregulated in genistein exposed T47D-ER β cells with ER β expression. Pathway and functional analysis shows the involvement of these genes with cellular growth, proliferation and cell cycle. Genes clustered in the lower part of the dendrogram correspond to those genes upregulated in genistein exposed T47D-ER β cells expressing ER β , and belong to the functional group of cell death. APOD, RBP4, IL29, NRIP3 and LRRC15 genes were found to be clearly upregulated by genistein-ER β (Figure 9). It is also observed that negative regulators of ER α transcription are clustered together with ER β and progesterone receptor (PGR). In addition, downregulation of genes involved in cellular growth and proliferation was seen in the last cluster of genes, in the center of the dendrogram. Most of the genes belonging to this group are negative regulators of colony formation of cancer cell lines.

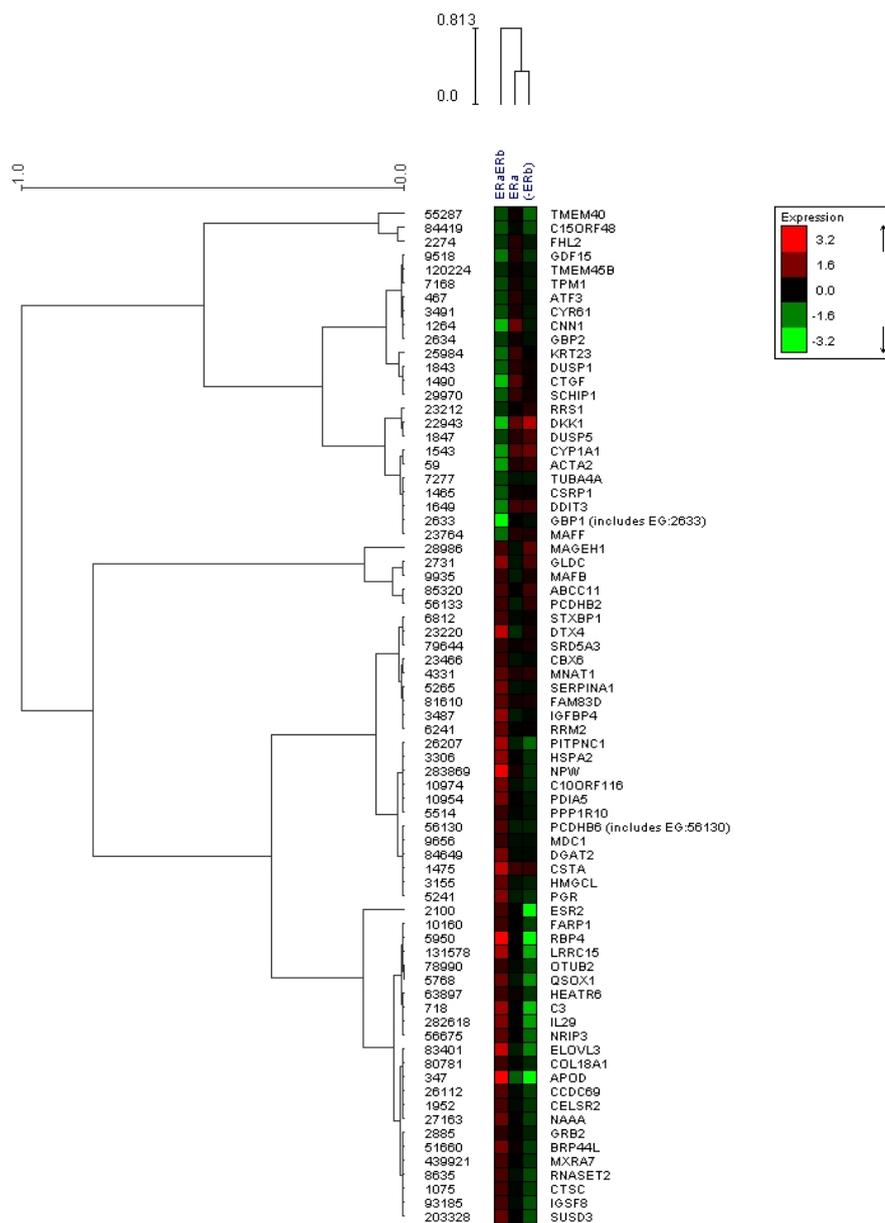
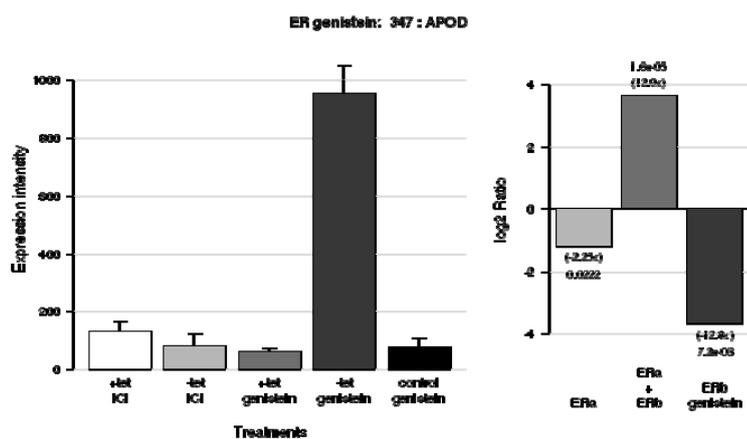
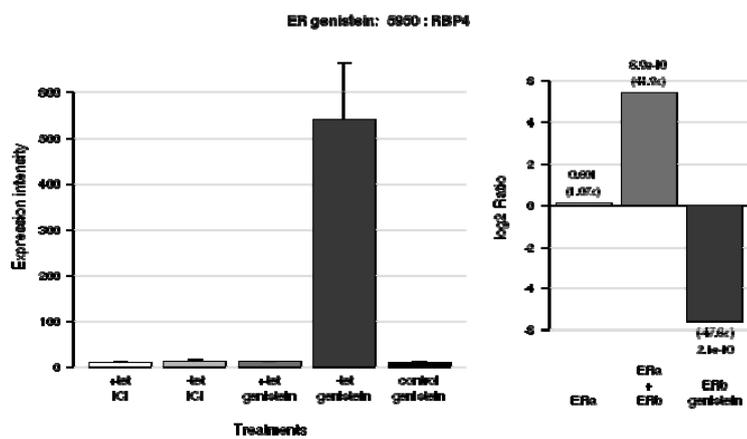


Figure 8: Hierarchical cluster analysis of the 73 differentially regulated genes. Using complete linkage and Pearson correlation similarity. Gene symbols and Entrez gene ID are shown.

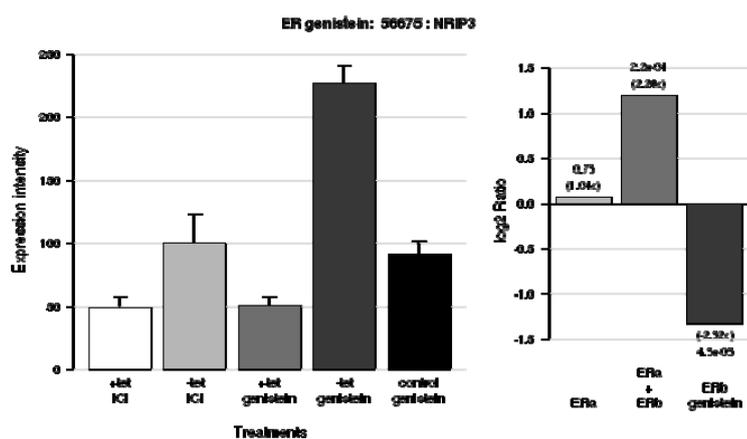
A



B



C



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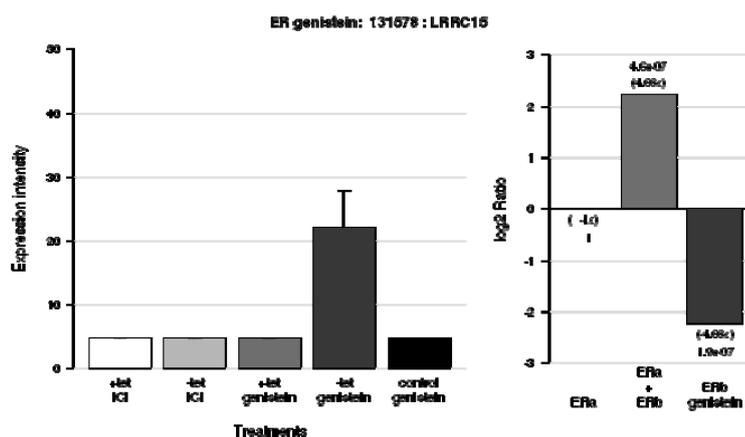


Figure 9: Mean expression intensities of A) APOD, B) RBP4, C) NRIP and D) LRRRC15 at mRNA level based on microarray normalized results under five different conditions, Tet+ and ICI (sample A, Table 1A), Tet- and ICI (sample B, Table 1A), Tet+ and genistein (sample C, Table 1A), Tet- and genistein (sample D, Table 1A), and Tet- in control cell line (T47D-PBI) and genistein (sample E, Table 1A) and their corresponding fold changes.

In summary, both at the gene and protein level, genistein induced signatures of cell proliferation and negative regulation of cell death in the absence of ER β (sample CA), whereas signatures of upregulation of cell death, negative regulation of cell cycle and cell proliferation was observed during genistein treatment in the presence of ER β (contrast DB and ED).

Discussion

Various molecular profiling technologies have been developed recently in order to identify and quantify proteins and/or genes in biological samples. From these studies it was observed that mRNA levels are only a partial reflection of the functional state of an organism and that a comprehensive understanding of the genomic information will require means of analyzing quantitative differences in protein expression on a proteome-wide scale (34-37). In general, an overall positive correlation between protein and mRNA abundance has been observed in many organisms, but simple correlations are insufficient to assess regulatory patterns of gene and protein expression (37). Therefore, in this paper, we combined SILAC-based proteomics and microarray analysis of identical samples to address the signaling effects of estrogen receptor subtypes in the T47D-ER β breast cancer cells exposed to the phytoestrogen genistein.

In ER α -positive breast cancer cells, growth and proliferation is preserved by active transcription of ER targets (38, 39), whereas ER β expression results in a significant decrease in cell proliferation (16, 18). In previous studies we observed that genistein, a phytoestrogen with relatively higher affinity for ER β (10, 40) than for ER α , increased cell-proliferation in ER α -positive cells at physiologically relevant concentrations (17). Furthermore, genistein did not induce cell-proliferation in ER-negative breast cancer cells like MDA-MB-231 (15, 41).

In the present study, ER α expression levels in the ICI synchronized T47D-ER β cell line were 6-10% compared to the wild type T47D cells as measured by ELISA (Figure 2A). However, ER α mRNA was detected in all samples (Figure 2B) albeit at levels 30 fold lower than the maximum ER β mRNA levels detected in the T47D-ER β cells in absence of tetracycline. The low levels of ER α protein observed are in accordance with previous studies where it was shown that i) the ER α protein displays a shorter half-life after ligand binding (42), ii) that ER α is subject to degradation induced by its antagonist ICI 182780 (43) and, iii) that ER α may be downregulated by the genistein treatment. It has been proposed that proteasomal degradation of ER α protein is increased in the presence of the co-regulated proteins KRT18 and KRT8 (44, 45). Upregulation of these cytokeratins (Table 2, Table 4) attract the receptor into close proximity to nuclear matrix-associated proteasomes for degradation as has been described after treatment with ICI 182780 (46). In the samples treated with genistein expression of ER α , after ICI withdrawal, could return to low basal levels (42, 44, 45), because genistein influences the expression of ER α . This observation is supported by the co-regulation of ER α and c-MYC proteins. The proto-oncogene c-MYC is normally upregulated by ER α in response to the presence of estradiol (47, 48). On the other hand ER β is able to repress c-MYC expression (4, 8, 46). In our proteome dataset, the differential regulation of most of the ER α -regulated proteins appeared to be similar to that of c-MYC (49), including upregulation of KRT18, MYH10, HNRPC, MYH9 or MYL6.

It has been proposed that epigenetic mechanisms including DNA methylation and histone modifications might contribute to ER β -mediated ER α silencing (50, 51)(44, 45). Furthermore, genistein has been shown to alter DNA methylation patterns in mice (52). A possible increase of DNA and protein methylation is supported by regulation of methyltransferases, histones and histone deacetylases as observed in our SILAC studies. Additional studies investigating epigenetic mechanisms in the presence of genistein would be of interest.

Our transcriptomics results showed some overlap with those reported by Williams and coworkers (8) for the effect of estradiol on ER β regulated genes. We used the same experimental conditions but with a different ligand (genistein instead of estradiol) and a different technology platform (affymetrix gene-chip versus operon's oligomer spotted array).

Comparing expression data, similar ER β targets are induced in both studies, e.g. LRRC15, APOD, HMGCL and NRIP3. Expression of APOD is absent in proliferating cells and induced in cells that undergo growth arrest and senescence (53). APOD protein levels were only detected in one SILAC experiment, namely in cells co-expressing both receptors during genistein treatment. Both studies confirmed that estradiol and genistein induce changes at gene and protein expression level that point at stimulation of cell proliferation in the absence of ER β expression, whereas expression of ER β drives the cell to protein and gene expression leading to negative regulation of cell cycle and induction of apoptosis.

Functional analysis of the genistein induced ER α -activated proteome data revealed that genistein was able to induce growth of the breast epithelial cells as indicated by the activation of cellular reorganization and maintenance. Cytoskeletal rearrangement, which is organized by microtubules, actin containing microfilaments and mechanochemical molecules, is a sensitive indicator of cell growth. Expression of myosins is important for proliferation and migration of breast cancer cells controlled by Rho and ERK signaling (54, 55).

ER β decreases the cell motility and cell proliferation, two of the most common components of breast cancer progression. This was observed during functional analysis of the genistein-induced ER β activated proteome (samples DB and ED). Cellular organization and cell movement (migration) were downregulated processes by ER β expression. More importantly and in accordance with gene expression experiments, ER β activity induces negative regulation of cell proliferation and induction of cell death. An increase of histone related proteins is thought to result in a general repression of ER α transcription (27, 28) and is associated with breast cancer disease progression (48). Repression of transcription was also observed by the downregulation of TOP2B after genistein treatment (30, 45).

Finally, enrichment of canonical pathways (Figure 10) comparing genistein exposed cells expressing ER β to those not containing ER β , agreed with similar findings in genistein exposed epithelial cells (55) indicating that cells not containing ER β lose their cell–cell interactions and cell polarity, and undergo a major change in their actin cytoskeleton, enabling them to acquire an increased motility and invasiveness. Expression of ER β halts genistein induced cell migration and cytoskeleton reorganization by inactivating the same signaling pathways.

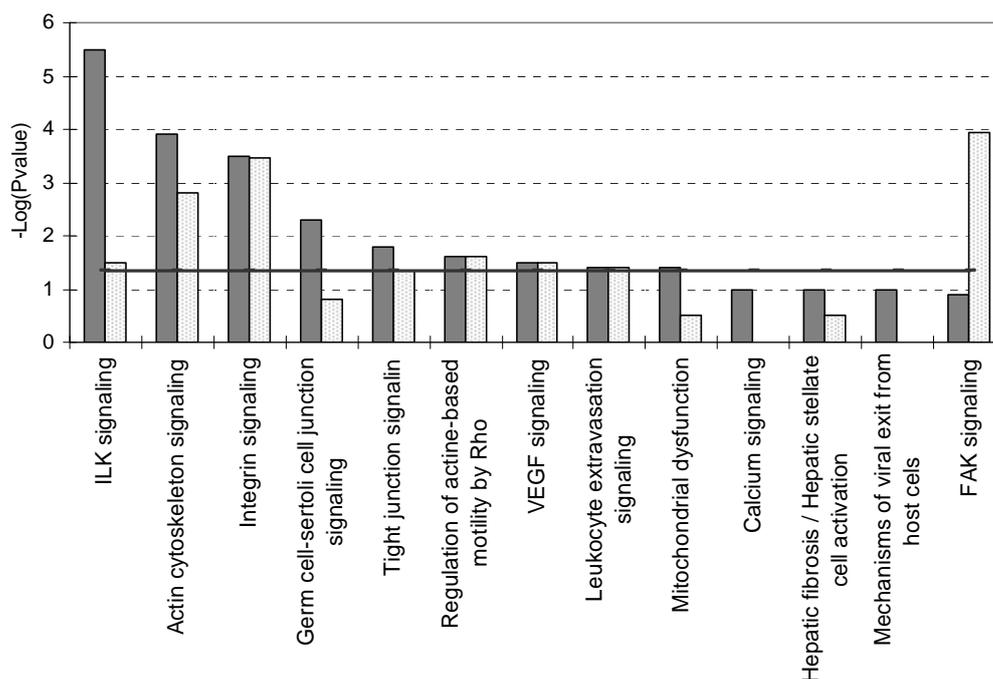


Figure 10: The top IPA canonical pathways regulated by ER α -mediated effects (dark columns) and ER β -mediated effects (light columns) as based on Fisher's exact test p-value using bigger protein data set ($P < 0.05$). The horizontal line denotes the cutoff for significance (p-value of 0.05).

In summary, we have demonstrated by using proteomics and transcriptomics that even in presence of low ER α levels genistein induces effects on gene and protein expression in the T47D-ER β cells comparable to those previously reported for estradiol. It is concluded that genistein can act as an estrogen and that its ultimate estrogenic effect on cells and tissues are dependent on the receptor phenotype and the ratio between the receptor subtypes within these cells or tissues a phenotype that may potentially be modified upon genistein exposure.

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Genistein Induced ER β -Mediated Downregulation
of Immune Signaling Networks as Revealed by
Quantitative Proteome Analysis of Genistein
Exposed Breast Cancer Cells *in vitro*

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Article in preparation

Abstract

The present study presents a protein-network based analysis of the quantitative SILAC-based proteome of T47D-ER β breast cancer cells with tetracycline-dependent varying intracellular ER α /ER β ratio exposed to the isoflavone genistein. Genistein is a phytoestrogen found in high levels in soy products, the intake of which has been frequently associated with various beneficial health effects including reduced risk on breast cancer and osteoporosis in menopausal women. In our study protein networks involved in cell proliferation and apoptosis appear to be affected upon genistein exposure of the T47D-ER β breast cancer cells. In addition the data point at genistein-induced ER β mediated immune suppression. Genistein induced ER β -mediated downregulation of the expression of the transcription factors NF- κ B and STAT3 as well as down regulation of a variety of cytokines including IL-1 β , IL-18, IFN- γ , and TNF- α . On the other hand, a considerable increase of TGF- β induction was found in genistein treated T47D-ER β cells expressing ER β compared with cells with no expression of ER β , further corroborating the genistein induced ER β mediated downregulation of immune signaling. Altogether, the results of the present study reveal that in addition to cell proliferation and apoptosis, immune signaling appears to be another major estrogen target influenced by phytoestrogen exposure.

Introduction

Epidemiological studies have concluded that Asian women consuming a diet rich in soy products have a low incidence of breast cancer (1, 2). This finding has initiated numerous studies carried out in *in vivo* and *in vitro* models trying to unravel the underlying mechanisms (3, 4). Genistein, a major isoflavone in soy and soy-based products, has been reported to exert its effects mainly through the two estrogen receptors (ERs), ER α and ER β , and also by non ER mediated-mechanisms (5). Preferential binding of genistein to ER β relative to ER α has led to the suggestion that genistein signaling via ER β is of important for its biological effects (6-8).

In our previous studies using the human T47D breast cancer cell line with tetracycline-dependent ER β expression (T47D-ER β), the effect of a varying intracellular ER α /ER β ratio on genistein-induced cell proliferation was characterized. Genistein-induced cell proliferation of cells in which ER β expression was inhibited, whereas this genistein-induced cell proliferation was no longer observed when ER β expression was increased (8). These results pointed at the importance of the cellular ER α /ER β ratio for the ultimate effect of genistein on cell proliferation. In a subsequent study this ultimate biological end point, cell proliferation, was linked to both transcriptomics and proteomics analysis data in order to unravel the mechanisms underlying observed effect (Chapter 5). Based on molecular function analysis, in the genistein exposed T47D-ER β cells with no ER β expression, changes in gene and protein levels pointed at genistein induced cell proliferation, growth and migration by dynamic activation of cytoskeleton remodeling. Moreover, in the genistein exposed T47D-ER β cells expressing high levels of ER β , a clear downregulation of genes and proteins involved in cell growth, and an upregulation of genes and proteins involved in cell cycle arrest and apoptosis was demonstrated. These effects of genistein on gene and protein expression were in agreement with our previous *in vitro* results on cell proliferation obtained using the same T47D-ER β cell model with variable ER β expression levels (8).

The aim of the present study was to investigate a global analysis at the protein level of genistein-induced ER β dependent biological effects in addition to its dualistic, ER β expression-dependent effects on cell proliferation and apoptosis. To this end, we applied a protein-network-based approach to identify subnetworks instead of individual proteins that were affected upon genistein exposure of the T47D-ER β cells expressing variable levels of ER β . This protein-network-based approach proved able to provide new insights into cellular processes affected upon genistein exposure.

Materials and methods:

Cell culture: The stably transfected T47D tetracycline-inducible cell line (T47D-ER β) and the mock control cell line (T47D-PBI) were made and provided by Ström (9, 10). DMEM SILAC medium lacking arginine (Arg) and lysine (Lys), supplemented with 5% of dialyzed fetal bovine serum and 1000 ng/ml tetracycline (Tet) was used. Arg and Lys, either “light” or “heavy” depending on the experimental design, were incorporated in the DMEM SILAC medium. The final concentrations of Arg and Lys were 21 and 48 mg/L respectively (11).

During the adaptation phase, cells were grown in “light” or “heavy” SILAC media, containing respectively 12C614N4-arginine and 12C614N2-lysine or 13C6-15N2-lysine and 13C6-15N4-arginine, until the cells grown in “heavy” medium had fully incorporated the labeled amino acids. Full incorporation of labels into the cells was checked by MS analysis (12) of cell samples detecting the time point at which there was no further increase in the amount of label incorporated. Full incorporation of labels for the T47D-ER β cells (Tet containing) was observed after 5 doubling times or 10 days of cultivation (data not shown). Preparation of the samples after the adaptation phase, was performed as follows. Cells in DMEM SILAC medium supplemented with 5% dialyzed-fetal bovine serum (DFBS) and 1000 ng/ml Tet were seeded at 10^5 cells/ml in plates for 24 h. After 24 h DMEM SILAC medium was replaced by the same medium without phenol red, containing the required concentrations of Tet, heavy or light amino acids, and a serum concentration that was reduced to 0.5% DFBS. To this, 10 nM ICI 182780 was added for cell synchronization (10). Tet was withdrawn in half of the plates 12 h before the start of the treatment to allow expression of ER β . The Tet+ and Tet- cultures were incubated with either 10 nM of the ER α and ER β antagonist ICI 182780 (13) or 500 nM of the ER ligand genistein for 24 h according to the experimental design (Table 1) after which all cells were collected simultaneously for RNA and protein extraction. Three replicates of each sample were prepared in parallel to minimize experimentally induced variability.

Table 1 summarizes the experimental design applied in order to obtain information on the genistein induced levels of protein expression in T47D- ER β cells with variable ER α /ER β ratios. Table 1 presents the five different samples prepared, indicating the cell lines used, the Tet treatment and the resulting ER β expression as well as the compound and the amino acid label (light or heavy) added to the growth medium. In addition to genistein the ER antagonist ICI 182780 was used in order to block the receptors thereby suppressing receptor-mediated transcription. A mock control cell line (T47D-PBI) was used to define genistein mediated non-ER β protein expression inherent to the cell system used. Pairwise comparisons made using these five samples in order to analyze i) genistein induced ER α mediated protein

expression in the absence of ER β (C versus A, referred to as sample CA); ii) genistein induced ER β regulated protein expression in the presence of low endogenous amounts of ER α (D versus B, referred to as sample DB) and iii) a second approach to detect genistein induced ER β mediated protein expression in the presence of low endogenous levels of ER α (E versus D, referred to as sample ED).

Table 1: Experimental design of the present study based on four cellular samples prepared.

	Strain	Tetracycline Treatment	ER β expression	Compound treatment	No. of samples	Label
A	T47D-ER β	yes	no	ICI 182780	3 / 3	Light
B	T47D-ER β	no	yes	ICI 182780	3 / 3	Heavy
C	T47D-ER β	yes	no	genistein	3 / 3	Heavy
D	T47D-ER β	no	yes	genistein	3 / 3	Light
E	T47D control	no	no	genistein	3 / 3	Heavy

Protein preparation: For protein extraction, cells were washed twice with PBS and lysed in modified RIPA buffer [50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 150 mM NaCl, 1% Triton X-100, 0.25% sodium deoxycholate] containing a protease inhibitor cocktail (Complete, Mini, Roche) at 4° C for 15 min. Samples were sonicated for 1 min and centrifuged at 14000 g for 15 min at 4° C, and the supernatant was collected. Equal amounts of protein (BSA Protein Assay Kit, Pierce) from each sample were mixed in a ratio of 1:1 according to the experimental design presented in table 1, and 1 μ g of the protein sample thus obtained was separated on a 12% Tris-glycine sodium dodecyl sulfate-polyacrylamide (SDS-PAGE) gel by electrophoresis, followed by staining of the SDS gel using colloidal Coomassie blue (Colloidal blue staining kit, Invitrogen). Then the resulting 3 gel lanes per replicate (CA, DB and ED) were excised and cut horizontally into 8 equal sections per lane. In-gel digestion was performed as follows: The SDS gel was destained by two washes with water. Cysteine reduction was performed by adding 100 μ l 50 mM DTT (dithiotreitol) in 50 mM NH₄HCO₃. Samples were sonicated for 1 min and incubated at 60°C without shaking for 1 h. Alkylation was performed when samples reached room temperature, replacing DDT by 100 μ l of 50 mM iodoacetamide in 50 mM NH₄HCO₃. Samples were sonicated for 1 min and alkylated at room temperature in the dark for 1 h. The gel pieces were washed three times with 100 μ l 50 mM NH₄HCO₃ at pH 8. For proteolytic digestion, samples were treated overnight with 100 μ l trypsin (10 ng/ μ l in 50 mM NH₄HCO₃, sequencing grade, Boehringer Mannheim) at room temperature. Gel fragments were removed by centrifugation and the proteolytic peptides were recovered in the supernatant fraction (25 μ l). 10% TFA was added to correct the pH up to 2 to 4. Finally all extracts were measured by LC-MS/MS.

Data acquisition, Mass Spectrometry: The protein samples were analyzed by injecting 18 μ l sample over a 0.10 * 32 mm Prontosil 300-5-C18H (Bischoff, Germany) pre-concentration column (prepared in house) at a flow of 6 μ l/min for 5 min with a Proxeon EASY nLC system. Compounds were eluted from the pre-concentration column onto a 0.10 * 250 mm Prontosil 300-3-C18H analytical column (prepared in house) with an acetonitril gradient at a flow of 0.5 μ l/min. The gradient consisted of an increase from 9 to 34% acetonitril in water with 1ml/l formic acid in 50 min followed by a fast increase in the percentage acetonitril to 80% (with 20% water and 1 ml/l formic acid in both the acetonitril and the water) in 3 min as a column cleaning step. In between the pre-concentration and the analytical column, an electrospray potential of 3.5 kV was applied directly to the eluent via a solid 0.5 mm platina electrode fitted into a P777 Upchurch microCross. Full scan positive mode FTMS spectra were measured between m/z 380 and 1400 at resolution 60.000 on an LTQ-Orbitrap (Thermo electron, San Jose, CA, USA). MSMS scans of the four most abundant doubly or triply charged peaks in the FTMS scan were recorded in data dependent mode in the linear trap (MSMS threshold = 10.000). Data were acquired using Xcalibur software.

Protein identification and quantitation: Mass spectra were analyzed using MaxQuant (version 1.0.13.8), which performs e.g. list generation, ratio H/L significance A, ratio H/L significance B, SILAC- and XIC-based quantification, false positive rate and data filtration (14). Data were searched against the human IPI human protein database supplemented with frequently observed contaminants and concatenated with reversed copies of all sequences (total 148380 sequences) using Mascot v2.2 (Matrix Sciences). Spectra determined to result from heavy labeled peptides by pre-search Maxquant analysis were searched with the additional fixed modifications Arg10 and Lys8, whereas spectra with a SILAC state not defined a priori were searched with Arg10 and Lys8 as additional variable modifications. Precursor mass tolerance was set at 10 ppm for the complete peptides and 0.5 Da for peptide fragments as observed in the MS2 spectra. Trypsin/P cleavage specificity with up to 2 missed cleavage and three labeled amino acids (Arg and Lys) were allowed. The required false discovery rate was set to 1% at the protein level, and the minimum required peptide length was set to 6 amino acids. Carbamidomethyl cysteine was set as a fixed modification and methionine oxidation, deamidation (NQ), and acetylation of the N-terminus (protein) were allowed as variable modifications. For protein identification, at least two peptides were required, among which at least one peptide was required to be unique in the database. Identified proteins were quantified. Protein ratios calculated by MaxQuant were subjected to manual inspection.

Bioinformatics Network Analysis: A cutoff P value ≤ 0.05 (Ratio H/L Significance B) was selected for analysis of differentially expressed proteins. Ingenuity Pathway Analysis

(IPA) version 8.5 (Ingenuity Systems Inc.) was used to conduct a knowledge-based network analysis. IPA tool relies on curated functional and regulatory interactions extracted from literature. The biological functions across ER-responsive proteins networks were identified. IPA calculates a significance score for each network. The network score is based on the hypergeometric distribution and is calculated with the right-tailed Fisher's Exact Test. This score indicates the likelihood that the assembly of a set of focus ER-genes or gene products in a network could be explained by random chance alone. Networks were ranked according to their degree of relevance to the network eligible molecules in our dataset.

Cytokine detection: The exposure medium from treated cells was used for detection and quantification of selected cytokines, including interleukins 1 β , 6, 8, 10 and 12 (IL-1 β , IL-6, IL-8, IL-10 and IL-12), interferon- γ (IFN- γ), tumor necrosis factor α (TNF- α) and tumor growth factor β (TGF- β). Detection and quantification of these cytokines was performed by Cytometric Bead Array Flex sets (BD Biosciences, San Diego, USA) as previously described (15).

Results

Human breast T47D cancer cells were previously stably transfected with the ER β expression plasmid under Tet-responsive promoter regulation (9). This cell line expresses endogenous ER α , and variable levels of ER β depending on the concentration of Tet in the culture medium, with ER β expression being maximal after complete removal of Tet from the medium (8, 16) (chapter 5).

Based on the presented SILAC experimental design (Table 1), five different proteomes were obtained. Following our SILAC comparisons, three protein datasets were obtained: one dataset that determined genistein-mediated proteins in cells not expressing ER β (sample CA), a second dataset that determined genistein-mediated protein induction in cells expressing high levels of ER β (sample DB) and a third data set also able to detect genistein-induced ER β mediated protein expression (sample ED). Differential protein expression in the three datasets was defined using a cutoff P value ≤ 0.05 , and the protein lists thus obtained were uploaded into Ingenuity pathway analysis (IPA) and contained respectively 219 proteins (sample CA), 229 proteins (sample DB) and 253 proteins (sample ED).

We applied a protein-network-based approach to analyze the expression profiles of the three different datasets. In addition, IPA software included in the SILAC derived protein-networks additional proteins that are associated with the related functions. The resulting networks were in turn ranked by the software according to their significance (scores). Table

2, 3 and 4 present the results of the IPA network analysis of the three different samples. The tables reveal a total of 18, 20 and 22 networks that were identified in respectively genistein exposed cells expressing no ER β (sample CA), genistein exposed cells expressing high levels of ER β (samples BD and ED). Protein networks presented in Table 2,3 and 4 were found significantly enriched with scores ranging from 58 to 2, reflecting high respectively low significance.

Table 2: IPA network analysis in cells expressing endogenous ER α and inhibited ER β . Bold protein names and underlined italic indicate up- and down-regulated expression, respectively. Regular black colour indicates intermediate molecules incorporated through relationships with other molecules.

ID	Molecules in Network	Score	Focus Molecules	Top Functions Genistein-ER α
1	Actin, <u>ACTR1A</u> , <u>AKR1B1</u> , <u>BANF1</u> , Caspase, <u>DHX9</u> , <u>DLAT</u> , <u>DSP</u> , <u>HIST1H1B</u> , <u>HIST1H1C</u> , <u>HNRNPC</u> , <u>ILF3</u> , <u>ILF2</u> (includes EG:3608), <u>KIAA1967</u> , KRT8 , KRT18 , KRT19 , Lamin b, <u>LMNA</u> , <u>LMNB1</u> , <u>NASP</u> , NFkB (complex), NLRP2 , <u>PCM1</u> , PKP3 , PLEC1 , <u>PNKD</u> (includes EG:25953), <u>PUF60</u> , Raf, <u>RPS25</u> , <u>SF3A3</u> , SUGT1 , <u>TFAM</u> , <u>TMPO</u> , <u>UGP2</u>	58	30	Infection Mechanism, Cellular Assembly and Organization, Hair and Skin Development and Function
2	ACTN1 , Akt, ANXA1 , ANXA2 , CaMKII, CAPN2 , CBX5 , Ck2, <u>DHCRZ</u> , E2f, <u>EBP</u> , FSH, <u>HMGN1</u> , IgG, <u>JUP</u> , LDL, Mlc, <u>MUC1</u> , MYH9 , MYH10 , MYH14 , MYL6 , MYL12B , Myosin, NBN , <u>NPC2</u> , <u>PAFAH1B1</u> , PPL , RNA polymerase II, <u>RPL22</u> , <u>RPL34</u> , <u>RPS16</u> , <u>RPS18</u> , <u>TUBB2A</u> , Vegf	38	24	Drug Metabolism, Small Molecule Biochemistry, Cellular Function and Maintenance
3	ACTN4 , <u>ALCAM</u> , Alpha Actinin, Alpha catenin, Calpain, CAPZB , CNN2 , Collagen(s), CSRP1 , DIAPH1 , ERK, F Actin, FAK, Fibrinogen, <u>HADHB</u> , Integrin, Integrinβ, ITGAV , <u>LAMP1</u> , <u>LGALS1</u> , <u>LPP</u> , MRPL20 , Pkg, PSAT1 , PTK2 , Rac, Ras homolog, RHOG , SLC3A2 , SLC7A5 , <u>SLC9A3R1</u> , TAF15 , TCR, <u>VASP</u> , VCL	35	21	Cell Morphology, Connective Tissue Development and Function, Amino Acid Metabolism
4	Ant, ASNS , <u>C20ORF72</u> , <u>CECR5</u> , CHORDC1 , CHTF18, FAM129A , <u>FBXO2</u> , <u>FXC1</u> , GDI1 , HSP90AA1 , MAP4, MARS , <u>MMS19</u> , MYC, NOC2L, NOP58, NPM1 (includes EG:18148), NPM1 (includes EG:4869), PPID , PPP1CA , PPP1R13B, <u>PTGES2</u> , <u>RPL22</u> , RPL5 (includes EG:6125), <u>SF3B3</u> , <u>SFXN1</u> , <u>SLC25A13</u> , SMG5, <u>SNCG</u> , SNRPN , TERT, TP53I3 , TSPO, XPOT	28	18	Molecular Transport, Cell Cycle, Connective Tissue Development and Function
5	ACAT1 , <u>AKR1A1</u> , <u>BLZF1</u> , <u>CIRBP</u> , <u>DAD1</u> , DDX42 , DHPS, EEF1B2 , GCDH, GH1, <u>GNMT</u> , <u>GOT1</u> , GSTM2 , GSTM3 (includes EG:2947), HEBP1 , <u>HNRNPK</u> , <u>IVD</u> , MGST1 , MGST3 , <u>MOGS</u> , NIF3L1 , <u>NPEPPS</u> , <u>PRDX3</u> , PSPH , <u>RALB</u> , <u>RBMX</u> , retinoic acid, <u>RPS19</u> , <u>RPS20</u> , <u>RRBP1</u> , <u>SDF2L1</u> , <u>SEPHS1</u> , TMEM120A , <u>TPR</u> , XBP1	22	15	Skeletal and Muscular System Development and Function, Digestive System Development and Function, Hepatic System Development and Function
6	<u>ACTBL2</u> , ADAM23, ALDH16A1 , <u>ALDH6A1</u> , ARRB2, ATP5I , <u>ATP6V1B2</u> , ATP6V1D , <u>ATP6V1F</u> , C2CD2L , CCNY, <u>CLINT1</u> , <u>COX4I1</u> , EIF4E , <u>ERI3</u> , <u>FAM82A2</u> , GARS , <u>GGCT</u> , <u>LARP1</u> , MIR205 (includes EG:406988), <u>MRPL44</u> , NOP10, <u>PDS5B</u> , <u>PFKFB2</u> , PRNP, progesterone, <u>RAB11FIP1</u> , RASSF8, <u>RPS17</u> (includes EG:6218), <u>SLC2A4</u> , <u>SRGAP2</u> , TBC1D1, YWHAD, YWHAG , YWHAH	20	14	Reproductive System Development and Function, Amino Acid Metabolism, Cardiovascular System Development and Function
7	Ap1, <u>APLP2</u> , ASB3, BAG2 , ERK1/2, FLNA , gamma-linolenic acid, GSS , Histone h3, Ikb, IL18 , Insulin, Jnk, Mapk, MAT2A , MSK1/2, Nrp-PlexinA, P38 MAPK, PDGF BB, PI3K, Pkc(s), PSMD4 , Ras, RASAL1, <u>S100A8</u> , <u>S100A9</u> , SFRS7 , SHC4, SHP2-PI3K-GAB2, <u>SLC25A5</u> , <u>SLC25A11</u> , TNFRSF4, TXN , <u>UGDH</u> , WDR5	20	14	Inflammatory Response, Lipid Metabolism, Molecular Transport
8	ABCC6, <u>AGA</u> , AGT, <u>ANK3</u> , <u>APEH</u> , BAT1 , CLPX, DDX1 , DDX39 , HNF4A, <u>HNRNPA0</u> , <u>MIPEP</u> , MME, <u>MRPL44</u> , <u>MRPS25</u> , NDUFB1, NDUFB3, <u>NDUFB4</u> , <u>NDUFB5</u> , <u>NLN</u> , <u>PEPD</u> , <u>S100A9</u> , SARNP , SPAST, <u>SUCLA2</u> , SYNJ2BP , <u>THOC2</u> , THOC3, UBA52 , UCHL3, <u>USP5</u> , <u>USP15</u> , VDAC2 , <u>VPS29</u> , <u>XPNPEP3</u>	19	14	Cancer, Cell Death, Genetic Disorder

9	ARL1 , C11ORF73 , C6ORF211 , CCDC25 , CDK5RAP3 (includes EG:80279), CYTH2 , DDRGK1 , EED , FLAD1 (includes EG:80308), FZD1 , GABPA , GSTK1 , HNFA4 , INTS3 , KIAA0664 , MAT2A , MIR135B (includes EG:442891), MRPS28 , MTHFS , PCNP , PDK2 , PPCS , PPIL2 , PPP1R12B , PRDM5 , PTGES3 (includes EG:10728), PTPN13 , RASSF5 , SAP18 , SGSH , SRPRB , TRIM23 , UBE2D3 , UBL7 , UFM1	18	13	Post-Translational Modification, Carbohydrate Metabolism, Genetic Disorder
10	ALAD , B4GALT1 , Bvr , FGF18 , GPD2 , HIPK1 , HNRNPD , HNRNPM , HSP90AB1 , HYOU1 , IARS2 , INSR , KHSRP , LDHA , LDHAL6B , LDHB , MGP , NFAT5 , NFS1 , NLRP12 , OGN , PCBP2 , PIP , PPAP2A , PSPC1 , RPSA , SAMD4A , SCYL1 , SF1 , SFPQ , SGK1 , TIAL1 , TNF , TOMM40 , TUB	18	13	Molecular Transport, Cell Death, Hematological Disease
11	ADI1 , AIFM3 , AKR1C14 , ALDH16A1 , ANXA9 , COX6B1 , COX6C , CPNE3 , CYB5R1 , DHCR24 , DNASE2 , ETFDH , F7 , GNMB , HSDL1 , IL13 , IL15 , IL1B , MIR124 , MYO15A (includes EG:51168), NAGLU , NARG1 , NDOR1 , NDUFB10 , Oxidoreductase , POFUT1 , PTGR2 , RDH12 , RDH13 , SMPDL3B , Sos , SYAP1 , TRPS1 , YARS , ZAP70	18	13	Gene Expression, Carbohydrate Metabolism, Drug Metabolism
12	ACER1 , AK3L1 , BAG3 , Ca2+ , CACNA2D2 , Calcineurin B , CARS , CASP3 , CLIC4 , CNN3 , D-sphingosine , DIDO1 , EIF4H , ETFA , FNTA , HEXB , HSD17B10 , IDI1 , KLF16 , LSS , MDH2 , MIA3 , MRPL23 , PITPNM1 , PLS3 , PPT1 , PRSS3 (includes EG:5646), RCN1 , RDH11 , S100A9 , S100A11 , SREBF1 , TGFB1 , VAT1 , VEGFA	16	12	Lipid Metabolism, Small Molecule Biochemistry, Carbohydrate Metabolism
13	ANXA3 , ASNS , ASS1 , BCKDHA , COPS5 , dihydrotestosterone , GLRX , GPI , GSR , HINT1 , HUWE1 , IGH@ , MCAM , NEBL , PADI4 , PARK7 , PHGDH , PLAC8 , PLXNB2 , PMEPA1 , PPOX , PPP1R13L , PSIP1 , RECQL4 , RPS29 , SEL1L , SHISA5 , SLC19A2 , SP1 , SUB1 , TMED7 , TP53 , TXNRD1 , UBA6 , ZYX	16	12	Cell Death, Gene Expression, Cancer
14	ARF6 , CHMP2B , CISH , CUL1 , CUL2 , CUL5 , E3 RING , EEF1A1 , EIF5 , EIF3G , EIF3K , EPAS1 , EPO , GSPT1 , HBB (includes EG:3043), MRPL14 , NEDD8 , PCBP1 (includes EG:5093), PRKCI , QPRT , RBX1 (includes EG:56438), RBX1 (includes EG:9978), RCC2 , RPS6 , SART1 , SOCS2 , TARS , TCEB1 , TCEB2 , UBXN7 , USP33 , VHL , VHL-Cul2-Elongin-RBX1 , XIAP	8	7	Post-Translational Modification, Protein Synthesis, Hematological Disease
15	CCDC56 , RYR1 (includes EG:6261)	2	1	Genetic Disorder, Skeletal and Muscular Disorders, Cell Signaling
16	ERGIC1 , ERGIC3	2	1	Molecular Transport, Protein Trafficking
17	AARS2 , Alanine-tRNA ligase	2	1	
18	DNAJC , DNAJC9 , Hsp22/Hsp40/Hsp90	2	1	

Networks identified as a result of genistein treatment of cells not expressing ER β (sample CA) included (Table 2) functions associated with infection mechanism, cellular assembly and organization, cellular function and maintenance, cell cycle, cancer, cell death, lipid and drug metabolism, inflammatory response, molecular transport and connective tissue development and function. The most significant networks in genistein exposed T47D-ER β cells expressing ER β (sample DB)(Table 3), included functional categories such as infection mechanism, lipid metabolism, gene expression, cell cycle, cell-mediated immune response, cell death and immunological diseases. The results obtained for the second sample analyzing genistein induced ER β -mediated effects (sample ED) are presented in table 4 and indicate networks affected to be involved in infection mechanism, lipid metabolism, vitamin and mineral metabolism, antigen presentation, cancer, cellular growth and proliferation, and cell death.

Table 3: IPA network analysis in cells expressing ER α and ER β . Bold protein names and underlined italic indicate up- and down-regulated expression, respectively. Regular black colour indicates intermediate molecules incorporated through relationships with other molecules.

ID	Molecules in Network	Score	Focus Molecules	Top Functions Genistein-ER α /ER β
1	AK2 , Caspase, <u><i>CLIC4</i></u> , Cytochrome c, DHX9 , DLAT , DLD , ELOVL1 , <u><i>GSTM2</i></u> , <u><i>GSTM3</i></u> (includes EG:2947), HNRNPC , HNRNPM , HNRNPU , Hsp90, ILF3 , ILF2 (includes EG:3608), <u><i>IQGAP1</i></u> , KIAA1967 , <u><i>KTN1</i></u> , MIR124, <u><i>MYL12B</i></u> , <u><i>NARG1</i></u> , <u><i>NFKB2</i></u> , NUMA1 , P38 MAPK, <u><i>PAK2</i></u> , <u><i>PGRMC2</i></u> , PRMT1 , <u><i>ROCK1</i></u> , S100A9 , SERPINB6 , <u><i>STAU1</i></u> , TRIP11 , TWF2 , Vegf	53	29	Infection Mechanism, Lipid Metabolism, Nucleic Acid Metabolism
2	Actin, <u><i>ACTN4</i></u> , ALCAM , Alpha actin, Alpha Actinin, Alpha catenin, <u><i>ANXA1</i></u> , <u><i>ANXA2</i></u> , Cadherin, Calpain, <u><i>CAPN2</i></u> , <u><i>CAPNS1</i></u> , <u><i>CFL2</i></u> , <u><i>CNN2</i></u> , Cofilin, <u><i>CSR1</i></u> , <u><i>DCTN2</i></u> , <u><i>DSTN</i></u> , ERK, F Actin, FAK, <u><i>FAU</i></u> , <u><i>FLNA</i></u> , <u><i>MAEA</i></u> , <u><i>MAP4</i></u> , <u><i>MAPK13</i></u> , MARCKSL1 , MATR3 , PCYT1A , <u><i>RPS18</i></u> , <u><i>S100A11</i></u> , SFPQ , <u><i>SSH3</i></u> , <u><i>TLN1</i></u> , <u><i>VCL</i></u>	44	25	Cellular Assembly and Organization, Antigen Presentation, Cell Morphology
3	<u><i>ACAT2</i></u> , <u><i>ACLY</i></u> , Akt, <u><i>APOE</i></u> , <u><i>BAG5</i></u> , BCAM , BCR, CD3, CD9 , CRK , <u><i>DNAJA2</i></u> , EIF4A, <u><i>EIF4G1</i></u> , <u><i>EIF4G2</i></u> , FGR , G6PD , HNRNPR , Hsp70, Jnk, LAMA5 , Laminin, LMNA , MUC1 , NAT1 , Pdgf, PDGF BB, Pdgfr, PGR , PI3K, Ras homolog, <u><i>S100A6</i></u> , SRC, TCR, Tgf beta, <u><i>UNC45A</i></u>	32	20	Gene Expression, Cell-To-Cell Signaling and Interaction, Renal and Urological System Development and Function
4	<u><i>B2M</i></u> , <u><i>CYB5R3</i></u> , DSP , ECSIT , ESR2 , <u><i>EDFT1</i></u> , Growth hormone, <u><i>HSP90B1</i></u> , lfn, IFN Beta, lfn gamma, IFN TYPE 1, Ige, IgG, <u><i>IL18</i></u> , IL12 (complex), Interferon alpha, <u><i>ISG15</i></u> , LDL, MHC Class I (complex), <u><i>MX1</i></u> , Nfkb (complex), NPC2 , <u><i>PLS3</i></u> , <u><i>PPL</i></u> , <u><i>PPP4C</i></u> , PRSS8 , Rab11, RAB11A , <u><i>STAT1</i></u> , <u><i>STAT3</i></u> , STAT5a/b, TFRC , <u><i>TMEM43</i></u> , Tnf receptor	32	20	Cell-To-Cell Signaling and Interaction, Cell-mediated Immune Response, Hematological System Development and Function
5	26s Proteasome, ACADM , ADRM1, Ap1, ASNS , <u><i>CDK5RAP3</i></u> (includes EG:80279), Ck2, CTSD , Cyclin A, <u><i>DDR1</i></u> , E2f, HIST1H1B , HIST1H2AB , Histone h3, HK1 , HNRNPA2B1 , HNRNPL , IL1, Insulin, JUP , <u><i>KIAA0776</i></u> , <u><i>LAP3</i></u> , Mapk, <u><i>MGMT</i></u> , peptidase, Pkc(s), PP2A, Rac, Ras, Sapk, TIMM13 , <u><i>TOP2B</i></u> , Ubiquitin, <u><i>UBTF</i></u> , <u><i>UFD1L</i></u>	30	19	Cancer, Cell Cycle, Immunological Disease
6	<u><i>ADSL</i></u> , C11ORF79 , C17ORF81, CCDC45, <u><i>CLDN1</i></u> , CLDN3 , DNAJB4, <u><i>DUSP3</i></u> , <u><i>EEF1A1</i></u> , FGF5, FGF6, FGF7, FGF9, FGF16, FGF17, FGF18, FGF20, FGF21, FGF22, FGF23, FGFR2 , GFM1 , HNF4A, HSPC152 , ISOC1 , <u><i>LRSAM1</i></u> , LSM3 , MRPL32 , MUTYH, PABPN1 , <u><i>PRAF2</i></u> , <u><i>RAB1B</i></u> (includes EG:81876), RMND1 , RNMTL1 , SULT1A1	22	15	Cell-To-Cell Signaling and Interaction, Carbohydrate Metabolism, Lipid Metabolism
7	<u><i>ALDH1B1</i></u> , ATAD3A , BCL2L1, <u><i>COMMD3</i></u> , <u><i>CRIP2</i></u> , <u><i>CYTSB</i></u> , <u><i>DDX1</i></u> , <u><i>EDC4</i></u> , <u><i>EPCAM</i></u> , EPHX2 , FOXJ1, GAA , GCDH, GMFB , <u><i>GOSR1</i></u> , GRB2 , H1FO , HMG2 , LEP, <u><i>LRRC47</i></u> , <u><i>MRPL12</i></u> , MYC, NFKB1, OPLAH , <u><i>RPL27</i></u> , RPL38 (includes EG:6169), <u><i>RPS18</i></u> , <u><i>RPS23</i></u> , <u><i>RPS15A</i></u> , <u><i>SHMT2</i></u> , <u><i>SLK</i></u> , SNCA, UGP2 , ZFP36, ZNF274	20	14	Cellular Compromise, Cancer, Skeletal and Muscular Disorders
8	<u><i>ABCF1</i></u> , ADI1, AIFM3, AKR1C14, ARMC10 , beta-estradiol, BLVRB , <u><i>CBR3</i></u> , CDKN2C, <u><i>CNN2</i></u> , COX6B1 , CYB5R1 , <u><i>DNPEP</i></u> , <u><i>ENO1</i></u> , <u><i>ERO1L</i></u> , <u><i>GALE</i></u> , Histone h4, HSDL1, <u><i>IDH3B</i></u> , IL6, MME, NAGLU , NDOR1, NDUFA12 , NDUFB10 , NHP2L1 , OSTF1 , Oxidoreductase, PMM2 , RBL1, <u><i>RDH11</i></u> , RDH12, RDH13 , SLC9A3R1 , TGFB1	20	14	Cell Cycle, Cancer, Cardiovascular System Development and Function
9	<u><i>AARS</i></u> , ACOT2 , <u><i>ANXA3</i></u> , <u><i>C14ORF166</i></u> , COX411 , <u><i>DDX1</i></u> , <u><i>EEF1B2</i></u> , <u><i>EIF3M</i></u> , <u><i>EIF4A2</i></u> , <u><i>EIF4E</i></u> , <u><i>EIF4G3</i></u> , <u><i>FAM62A</i></u> , GH1, <u><i>GIGYF2</i></u> , <u><i>GLG1</i></u> , GLUD1 , <u><i>GPS1</i></u> (includes EG:2873), HNRNPU , HSD17B8 , HSD17B10 , <u><i>HSP90AB1</i></u> , <u><i>MAN2B1</i></u> , <u><i>MDH1</i></u> , NANS , NPEPPS , <u><i>PPT1</i></u> , PRDX3 , <u><i>REEP6</i></u> , RPL19 (includes EG:6143), <u><i>RPS29</i></u> , <u><i>SLC2A4</i></u> , <u><i>SSBP1</i></u> , TKT , UBA7, <u><i>YWHAZ</i></u>	18	13	RNA Post-Transcriptional Modification, Gene Expression, RNA Trafficking
10	<u><i>ARF3</i></u> , <u><i>ARF4</i></u> , CECR5 , <u><i>COTL1</i></u> , CPSF1 , CTSC, <u><i>CUGBP1</i></u> , <u><i>EHD1</i></u> , <u><i>EIF2A</i></u> , FOS, GLB1 , HAS1, HIPK1, HNRNPC , HOXB6, KHSRP , LAMP2 , <u><i>LSS</i></u> , MFG8, <u><i>MGP</i></u> , MIR1, MKL1, MMD, OPTN, PIP , RASA3, RBM7, RBP1, RPS6KA4, Rxr, SF3B2 , SGK1, <u><i>TAGLN2</i></u> , TERT, TNF	18	13	Molecular Transport, Cellular Assembly and Organization, Cell Death

11	AK1, AK3L1 , BANP, <i>BZW2</i> , <i>C19ORF10</i> , <i>CYB5A</i> , DNASE2 , <i>DUT</i> (includes EG: 1854), <i>EI24</i> , ETFA, FDXR, GART , GBP1 (includes EG:2633), <i>GSDMD</i> , HEXB , HLX, HNRNPA3 , hydrogen peroxide, IFNG, IL13, LETMD1, MIR298 (includes EG:723832), <i>MOBK1A</i> , OSGIN1, <i>PIP4K2C</i> , <i>PPA1</i> , <i>RCN1</i> , <i>REEP5</i> , RNF144B, SCGB3A1, SESN1, TP53, <i>UBE2L6</i> , UBE2V1 , VEGFA	17	13	Lipid Metabolism, Small Molecule Biochemistry, Skeletal and Muscular Disorders
12	ABCC6, ACTBL2 , AFG3L1, <i>AFG3L2</i> , ARRB2, ATP6, ATP8, ATP5H (includes EG:10476), <i>ATP6V1G1</i> , ATP6V1G2, ATPase, BAT1, CA12, CGN, CLPX, DDX1, DDX39 , DHX16, ENTPD2, FN3K , HBB (includes EG:3043), HIST1H1C , KIF20B, MYO9A, RNA polymerase II, <i>S100A16</i> , <i>SFN</i> , SMARCA4, SPAST, TCOF1, <i>TNKS1BP1</i> , TOB1, <i>TOMM34</i> , <i>VCP</i> , VPS4B	16	12	Carbohydrate Metabolism, Small Molecule Biochemistry, DNA Replication, Recombination, and Repair
13	APBB2, <i>ATXN10</i> , BLZF1, CRAT, DNAJC3 , EGFR, Egfr dimer, <i>EIF5B</i> , <i>FKBP2</i> , FKBP7, HIST1H1E, HM13 , HTT, <i>LDHB</i> , NDUFA8, NDUFA12, NDUFA13, NDUFB10 , NDUFV1, NDUFV2, PDXK, PPP1CC, RAB3D , retinoic acid, RNF40, <i>SEC22B</i> , SEPHS1, SLC25A3 , SP110, SRP9 , STX1A, <i>STXBP2</i> , TPR, <i>VAPB</i> , XBP1	16	12	Skeletal and Muscular System Development and Function, Cellular Compromise, Cancer
14	ACPP, AKAP11, Androgen-AR, Calmodulin, Cbp/p300, CRB3, DLG1, EDF1 , ERK1/2, FSH, FZD4, GLYCOGEN PHOSPHORYLASE, hCG, HIST1H1D (includes EG:3007), HNRNPAB, ISYNA1 , KCNK3, KIDINS220, LIN7C, MGMT, MOCS2 , MPP2, MPP5, MPP7 , PPP1R3C, <i>PPP2CA</i> , PPP2R3A (includes EG:5523), PRKCE, Proteasome, PTPN7, PYGB, PYGM, S100A10, STK25 , UPP1	9	8	Carbohydrate Metabolism, Cell-To-Cell Signaling and Interaction, Cellular Assembly and Organization
15	MIRN349, <i>TTC9C</i>	2	1	
16	BCCIP, HIST1H2BN	2	1	Cell Signaling, Cancer, Cellular Growth and Proliferation
17	Cl--transporting ATPase, PCYOX1	2	1	Amino Acid Metabolism, Small Molecule Biochemistry, Cell-To-Cell Signaling and Interaction
18	CLIC3 , MAPK15	2	1	Molecular Transport, Post-Translational Modification, Cancer
19	ARPM1, KIF21A , MIB1	2	1	Genetic Disorder, Ophthalmic Disease, Skeletal and Muscular Disorders
20	<i>KIAA1324</i> , MIR128-1 (includes EG:406915), MIR128-2 (includes EG:406916)	2	1	Cardiac Arteriopathy, Cardiovascular Disease, Genetic Disorder

Expected networks overlap between datasets was found. In depth analysis of these overlapping networks based on protein expression values (Tables 2, 3 and 4), showed that proteins belonging to overlapping networks were regulated in opposite directions (i.e. up-respectively downregulated). Proteins listed in functions like cell cycle, cellular assembly and organization, apoptosis or cellular growth and proliferation showed opposite expression profiles with high scores when comparing all datasets. Networks involved in cell proliferation and growth were shown to be regulated in both genistein exposed cells expressing ER β and in genistein exposed cells not expressing ER β albeit in opposite directions. These biological effects elucidated by the present protein-network based approach were in line with our previous results obtained on the basis of analysis of modification of individual proteins also demonstrating that in genistein exposed T47D-ER β cells not expressing ER β processes of cell proliferation and cell growth were stimulated, whereas in genistein exposed T47D-ER β cells expressing high levels of ER β proteins involved in cell proliferation and cell death were

downregulated and proteins involved in apoptosis and cell death were upregulated (chapter 5). The results are also in line with our previous data on genistein mediated effects on cell proliferation as a biological endpoint, showing genistein induced cell proliferation in cells in which ER β expression was absent whereas genistein induced cell proliferation was inhibited upon increased ER β expression in the T47D-ER β breast cancer cells (8).

Table 4: IPA network analysis of second approach for ER β target proteins. Bold protein names and underlined italic indicate up- and down-regulated expression, respectively. Regular black colour indicates intermediate molecules incorporated through relationships with other molecules.

ID	Molecules in Network	Score	Focus Molecules	Top Functions
1	ACY1 , ANXA6 , Caspase, CAT , Cytochrome c, DCD , EIF2AK2 , EIF2B1 , ERK, GANAB , GDI1 , GIPC1 , GOT2 , Hdac, Hsp70, Hsp90, HSP90B1 , HSPA4 , HSPA9 , HSPA1A , IFN Beta, ILF3 , ILF2 (includes EG:3608), Lamin b, LMNA, LMNB1 , LMNB2 , MVD , MYO6 , PA2G4 , PPIB , PTGES3 (includes EG:10728), TOR1AIP1 , TTC1 , VDAC1	48	27	Infection Mechanism, Cell Morphology, Cellular Assembly and Organization
2	ACP2 , Akt, Alpha actin, Alpha catenin, ANP32A , ANXA2 , ATPIF1 , BLVRB , CIRBP , CLU , COX7A2L , ERK1/2, ERO1L , ESR2 , ETFDH , FDFT1 , FSH, hCG, HYOU1 , LASP1 , LDL, MAP4 , MUC1 , MVK , MX1 , NPC2 , Oxidoreductase, PP2A, PPP1R2 , PTGR2 , RBMX , RPL22 , Tgf beta, VCL , Vegf	40	24	Lipid Metabolism, Small Molecule Biochemistry, Vitamin and Mineral Metabolism
3	ANXA1 , CA12 , CBX5 , CORO1B , COTL1 , DIAPH1 , Dynamin, F Actin, FLNA , GAPDH (includes EG:2597), GFPT1 , GSTM2 , HK1 , lfn, Insulin, Jnk, LNPEP , Mapk, MED20 , NAE1 , OGT , PDGF BB, PI3K, Pkc(s), PREX1 , Rab5, Rac, Ras, RBBP4 , RNA polymerase II, S100A6 , S100A8 , TAF9 , UGDH , VPS45	36	22	Carbohydrate Metabolism, Molecular Transport, Nucleic Acid Metabolism
4	Ap1, APOE , Arf, ASS1 , BID , CD59 , Ck2, CRABP2 , DHX9 , EIF4B , FKBP2 , GBF1 , HADH , HNRNPC , IFIT1 , IFN TYPE 1, IgG, IL1, IL12 (complex), Interferon alpha, ISG15 , MIR124, NPM1 (includes EG:4869), NUMA1 , P38 MAPK, Pdgf, PRSS8 , SCARB2 , SMC1A , STOM , SWAP70 , TACSTD2 , TWF2 , WARS , ZMPSTE24	36	24	Genetic Disorder, Ophthalmic Disease, Cellular Assembly and Organization
5	ALDH7A1 , Alpha Actinin, Calpain, CD3, CD9 , Collagen(s), DBNL , DMD , DSP , FAK, FGR , FHL2 , Fibrinogen, Integrin, Integrin alpha 3 beta 1, Integrin alpha 6 beta 1, Integrinα, Integrinβ:, ITGA2 , ITGAV , ITGB1 , ITGB5 , KPNA4 , KRT8 , KRT18 , KRT19 , Laminin, NFkB (complex), NME3 , PEBP1 , Raf, Ras homolog, RRAS , TCR, TPMT	29	19	Tissue Development, Cell-To-Cell Signaling and Interaction, Endocrine System Development and Function
6	ACSS1 , ALAD , ANKHD1-EIF4EBP3 , butyric acid, CNPY2 , COTL1 , DAK , DCXR , DEK , EHD1 , FAM49B , GBP1 (includes EG:14468), GMPS , HNRNPAB , IFIH1, IFIT1 , IFNA5 (includes EG:3442), IFNB1, IKBKE , ISG15 , ITGAD, KLF15, NFKBIE, PHGDH , PURA , RIG1, RPL38 (includes EG:6169), SLK , SP1, TALDO1 , TBCB , TNF, TP53I3 , TPR , YARS	25	17	Infection Mechanism, Infectious Disease, Antigen Presentation
7	2-hydroxyestradiol, AARS , ABAT , ACOX1 , AHSA1 , AIMP2 , ANXA3 , AP1B1 , beta-estradiol, C8ORF55 , CFTR, CLCN3, CRK, CSTB , CTPS , CYB5A , dehydroepiandrosterone sulfate, FADS2, Glutathione peroxidase, GMFB , GPX2, GPX3, GPX7, GPX8 , GSTZ1 (includes EG:2954), KARS , LGALS4, MB , MGP , MGST3 , PTP4A2 , RAB15 , RARS , THOC2 , WFS1	20	15	Gene Expression, Lipid Metabolism, Small Molecule Biochemistry
8	ACLY , AGR3 , APOA1, APOA4, APOC1, APOC2, CLYBL , CRIP1 , CSK , DAG1, DHX38 , EPPK1 , FGA, GALM , GPT, GSTK1 , HNF4A, HSDL2 , ISOC1 , LBP, LIPC, PCMT1 , PHB2 , PNPO , PON1, PSAT1 , QDPR , RAB18 , SCARB1, SERTAD3, SRPR , SSR2, TBC1D15 , TOMM20 , TTR	19	14	Lipid Metabolism, Small Molecule Biochemistry, Molecular Transport

9	<u>ALDH18A1</u> , <u>CCT2</u> , <u>CCT3</u> , <u>CCT4</u> , <u>CCT5</u> , <u>CCT8</u> , <u>CCT6A</u> , <u>CSDA</u> , <u>FAM118B</u> , <u>FAM129A</u> , <u>GAMT</u> , <u>GCN1L1</u> , <u>HDHC3</u> , <u>HIGD1A</u> , <u>IFI16</u> , <u>IFI202B</u> , <u>IMPACT</u> , <u>MAPKAP1</u> , <u>MYC</u> , <u>NDUFA5</u> , <u>NDUFA8</u> , <u>NDUFAF2</u> , <u>NDUFS1</u> , <u>NDUFS2</u> , <u>NDUFS3</u> , <u>NMI</u> , <u>PCBP2</u> , <u>PHB (includes EG:5245)</u> , <u>PTBP1</u> , <u>RPS27</u> , <u>SHMT1</u> , <u>SHMT2</u> , <u>TCP1</u> , <u>TKT</u> , <u>TP53I3</u>	19	14	Amino Acid Metabolism, Small Molecule Biochemistry, Cancer
10	<u>APP</u> , <u>ARL2</u> , <u>BLMH</u> , <u>CLDN1</u> , <u>CRAT</u> , <u>CTNNB1</u> , <u>DCI</u> , <u>DNM1L</u> , <u>EHHADH</u> , <u>FRUCTOSE 1,6 BIPHOSPHATASE</u> , <u>HYOU1</u> , <u>IDE</u> , <u>INS1</u> , <u>ISYNA1</u> , <u>KCNJ11</u> , <u>KYNU</u> , <u>Ldh</u> , <u>LDHB</u> , <u>LGALS9</u> , <u>NCSTN</u> , <u>PECI</u> , <u>Pka</u> , <u>PPME1</u> , <u>PPP1R2</u> , <u>PPP1R1A</u> , <u>PPP2CA</u> , <u>PTGFRN</u> , <u>RAF1</u> , <u>SLC1A5</u> , <u>TBCD</u> , <u>THOP1</u> , <u>UBE2D2</u> , <u>UBE2E2</u> , <u>WBP2</u> , <u>ZNRF1</u>	17	14	Lipid Metabolism, Small Molecule Biochemistry, Cell Cycle
11	<u>AHNAK</u> , <u>AK3</u> , <u>AP3B1</u> , <u>ARMCX3</u> , <u>ATP6V1G1</u> , <u>CLIC4</u> , <u>CLTC</u> , <u>CLTCL1</u> , <u>CMPK1</u> , <u>CSR1</u> , <u>DDX1</u> , <u>DNASE2</u> , <u>EHD1</u> , <u>EIF3A</u> , <u>ETF1</u> , <u>IL9R</u> , <u>KPNA1</u> , <u>LARP1</u> , <u>MIR16-1</u> , <u>MIR206 (includes EG:406989)</u> , <u>MYOF</u> , <u>NUFIP1</u> , <u>PFN1</u> , <u>RP11-529110.4</u> , <u>RUVBL1</u> , <u>RUVBL2</u> , <u>SEC24A</u> , <u>SH3BP4</u> , <u>SNX5</u> , <u>TNFSF11</u> , <u>TRAP1</u> , <u>UTRN</u> , <u>WIBG</u> , <u>WWC2</u> , <u>YWHAZ</u>	17	13	Antigen Presentation, Cell Death, Cell Morphology
12	<u>ANXA11</u> , <u>ARF4</u> , <u>ASPM</u> , <u>CCAR1</u> , <u>DDX19A</u> , <u>EGFR</u> , <u>FDXR</u> , <u>H2AFZ</u> , <u>HARS</u> , <u>HINT2</u> , <u>KCNJ4</u> , <u>LETMD1</u> , <u>LMCD1</u> , <u>LRRFIP1</u> , <u>MBNL2</u> , <u>NCAPG (includes EG:64151)</u> , <u>NEDD8</u> , <u>P4HA1</u> , <u>PMM1</u> , <u>REEP5</u> , <u>RNH1</u> , <u>RPSZ</u> , <u>RPS15A</u> , <u>RYR1 (includes EG:6261)</u> , <u>S100A11</u> , <u>SCAMP1</u> , <u>SHISA5</u> , <u>SMURF2</u> , <u>SNX2</u> , <u>SPG20</u> , <u>SRM</u> , <u>TGFB1</u> , <u>TP53</u> , <u>UBE4B</u> , <u>VPS24</u>	17	13	Cancer, Cell Cycle, Tumor Morphology
13	<u>AK3L1</u> , <u>ALDOC</u> , <u>B2m-Mhc1a</u> , <u>BCAT2</u> , <u>C11ORF79</u> , <u>CANX</u> , <u>CBR3</u> , <u>COX6B1</u> , <u>CPSF7</u> , <u>DECR1</u> , <u>EFHD1</u> , <u>ELP2</u> , <u>ELP5</u> , <u>ERBB2</u> , <u>Histone h3</u> , <u>Histone h4</u> , <u>HLA-A</u> , <u>HSD17B12</u> , <u>HYOU1</u> , <u>IKBKAP</u> , <u>KIAA1107</u> , <u>LSR</u> , <u>ME2</u> , <u>NOC2L</u> , <u>PHYH</u> , <u>PIP</u> , <u>RPL17</u> , <u>SDHB</u> , <u>SDHD</u> , <u>SGK1</u> , <u>SNUPN</u> , <u>TUFM</u> , <u>VEGFA</u> , <u>WBP5</u> , <u>WWOX</u>	16	12	Cancer, Hematological Disease, Reproductive System Disease
14	<u>ACTBL2</u> , <u>Actin</u> , <u>AKT1</u> , <u>AP3B1</u> , <u>AP3D1</u> , <u>ARRB1</u> , <u>ARRB2</u> , <u>BOLA2</u> , <u>CMBL</u> , <u>DGKE</u> , <u>DGKZ</u> , <u>DMD</u> , <u>EEF1A2</u> , <u>GMPPB</u> , <u>GRHPR</u> , <u>H1FX</u> , <u>HIST1H1C</u> , <u>HIST1H2BO</u> , <u>HNRNPA0</u> , <u>IKBKB</u> , <u>KTN1</u> , <u>MATR3</u> , <u>NOP10</u> , <u>RBM10</u> , <u>RPL7A</u> , <u>RPS8</u> , <u>RPS17 (includes EG:6218)</u> , <u>S100A16</u> , <u>SCYL2</u> , <u>SLC9A5</u> , <u>TCOF1</u> , <u>TPM3</u> , <u>TUBA1C</u> , <u>UBA52</u> , <u>XPOT</u>	15	12	Cellular Development, Cell Signaling, DNA Replication, Recombination, and Repair
15	4-phenylbutyric acid, <u>AIF1</u> , <u>APRT</u> , <u>BCL2L1</u> , <u>BNIP3 (includes EG:664)</u> , <u>CA9</u> , <u>CAPG</u> , <u>CAPN1</u> , <u>CCND1</u> , <u>CDV3</u> , <u>FAF2</u> , <u>GBE1</u> , <u>GPI</u> , <u>GTPBP4</u> , <u>HIF1A</u> , <u>HIF1AN</u> , <u>HIF3A</u> , <u>HINT1</u> , <u>HISTONE</u> , <u>HUWE1</u> , <u>LYPLA1</u> , <u>PEBP1</u> , <u>PGAM5</u> , <u>PITPNB</u> , <u>PLEC1</u> , <u>PRDX5</u> , <u>PRKCA</u> , <u>QPRT</u> , <u>S100A11</u> , <u>TPT1 (includes EG:7178)</u> , <u>Ube3</u> , <u>UBR2</u> , <u>VHL</u> , <u>VHL-Cul2-Elongin-RBX1</u> , <u>ZNF197</u>	12	11	Cellular Growth and Proliferation, Cancer, Cell Death
16	<u>ACAD8</u> , <u>ACAD9</u> , <u>ACAD10</u> , <u>ACADL</u> , <u>ACADS</u> , <u>ACADSB</u> , <u>ACADVL</u> , <u>ACOT8</u> , <u>ACOT9</u> , <u>ACP2</u> , <u>Acyl-CoA dehydrogenase</u> , <u>CRIP1</u> , <u>HNF4A</u> , <u>IL4</u> , <u>IVD</u> , <u>KPTN</u> , <u>LTA4H</u> , <u>MED23</u> , <u>MIR1</u> , <u>NDFIP1</u> , <u>NP</u> , <u>OTC</u> , <u>PAH</u> , <u>PPARA</u> , <u>PPM1G</u> , <u>RAB25</u> , <u>RAB11A</u> , <u>RAB11B</u> , <u>RAB11FIP1</u> , <u>RAB11FIP2</u> , <u>SRM</u> , <u>TAGLN2</u> , <u>TRAPPC3</u> , <u>UCHL5</u> , <u>XPNPEP3</u>	12	10	Lipid Metabolism, Small Molecule Biochemistry, Amino Acid Metabolism
17	<u>METTL7A</u> , <u>MME</u>	2	1	Cancer, Cell Death, Neurological Disease
18	Cob(I)alamin adenosyltransferase, <u>MMAB</u>	2	1	Genetic Disorder, Metabolic Disease
19	Cl--transporting ATPase, <u>PCYOX1</u>	2	1	Amino Acid Metabolism, Small Molecule Biochemistry, Cell-To-Cell Signaling and Interaction
20	<u>AMDHD2</u> , <u>N-acetylglucosamine-6-phosphate deacetylase</u>	2	1	Dermatological Diseases and Conditions, Infectious Disease, Embryonic Development
21	<u>GLTP</u> , <u>MIR373</u>	2	1	Lipid Metabolism, Molecular Transport, Small Molecule Biochemistry
22	<u>ACTL6B</u> , <u>ANO8</u> , <u>COQ9</u>	2	1	

Subnetwork markers corresponding to the hallmarks of immune/inflammatory system deregulation.

When comparing the IPA network-based classification from all the datasets (Table 2, 3 and 4), other protein networks than those involved in cell proliferation and apoptosis also appeared to be affected upon genistein treatment. Especially evident was a marked enrichment of molecular mechanisms correlated with immune system regulation or inflammatory response apparently affected upon genistein treatment of the cells.

Infection mechanism was the most significant function (scores 58, 53 and 48) shared between the three datasets (sample CA, DB and ED). This immune function was affected, reflected by downregulation of ILF2, ILF3, DLAT, DHX9, HNRNPC and KIAA1967, upon genistein exposure of T47D-ER β cells without ER β (sample CA, table 2), and by upregulation upon genistein exposure of T47D- ER β cells that do express ER β (sample DB, table 3 and sample ED table 4), the latter reflected by downregulation of ILF2, ILF3, LMNA and LMNB1 proteins in the inhibited-ER β dataset.

In table 3, presenting the results for genistein exposed T47D-ER β cells expressing high levels of ER β (sample DB), network IDs 1, 2, 4 and 5 indicated also additional effects of genistein on immune response mechanisms. Functions related with immune responses such as antigen presentation (CNN2, ANXA1 and DCTN2)(score 44), cell-mediated immune response (STAT1, STAT3, NF- κ B, IL18, B2M, MHC class I and HSP90B1) (score 32), and immunological disease (MGMT and TOP2B) (score 30) were found to be downregulated in genistein exposed ER β expressing T47D-ER β cells.

This type of immune regulation processes were not found as functional terms in the genistein exposed cells not expressing ER β (sample CA, table 2), only inflammatory response (score 20) was found regulated (IL18, TXN, S100A8 and S100A9). Enrichment of additional immune-related terms were also found in the second sample reflecting genistein induced ER β -mediated effects (sample ED, table 4) and included molecular functions such as infection mechanism, infectious disease and antigen presentation.

Immune-response differences between genistein exposed ER β and non-ER β expressing T47D-ER β cells

In addition of the protein-network based analysis presented above, the SILAC datasets were also analyzed using a protein interaction network based approach. Using this approach, it becomes possible to select all the associated proteins from a specific term and construct a unique merged protein interaction network. Initially the four main immune system related networks regulated by the expression of ER β (Network IDs 1, 2, 4 and 5 from sample DB)

were merged using the IPA tool into one. Expression values and protein relationships could be visualized in the connectivity map (Figure 1).

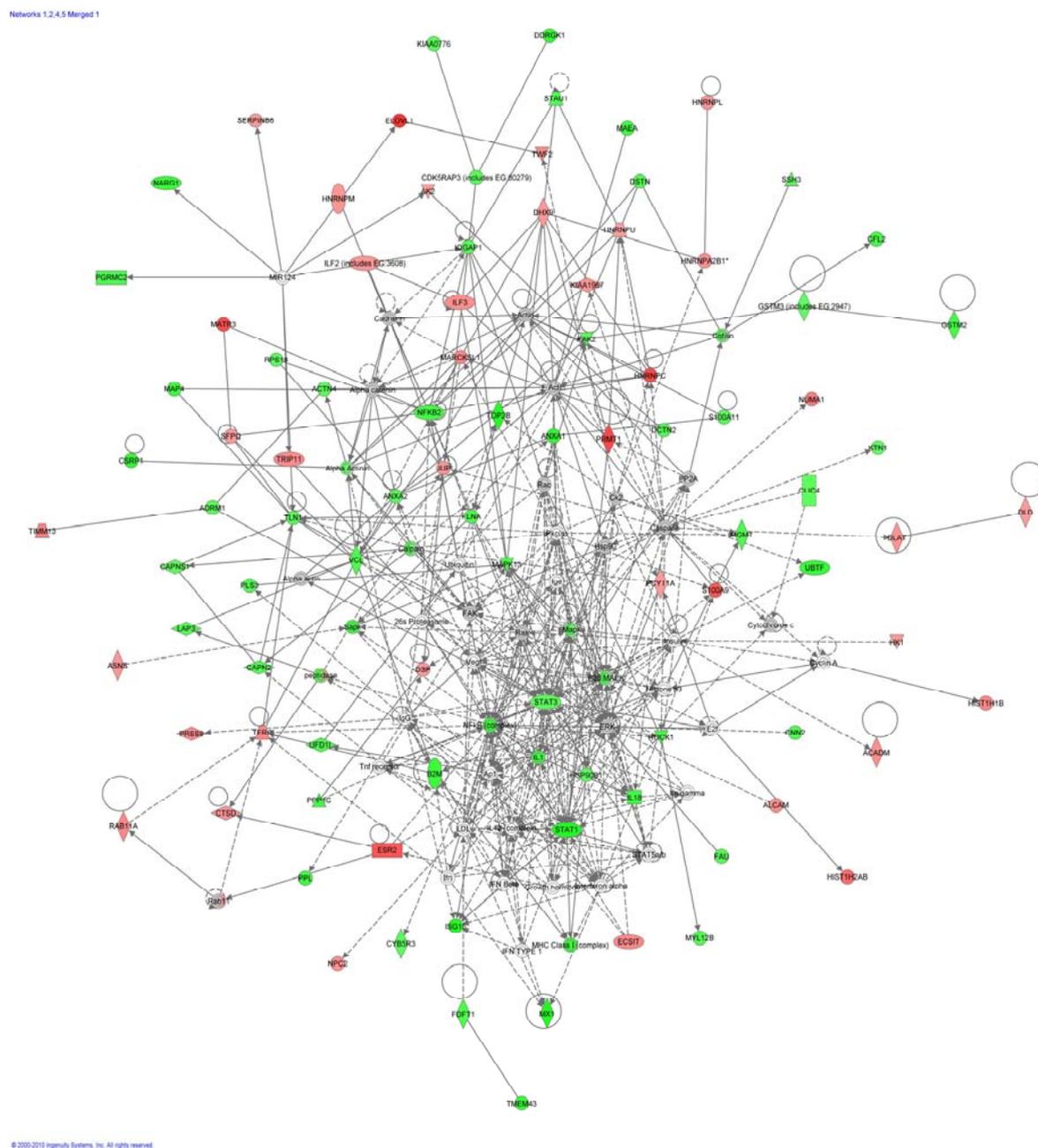


Figure 1: Connectivity map of the responses by Ingenuity Pathway assistant analysis. Merged networks 1, 2, 4 and 5 from genistein-ER α /ER β regulated proteins experiment. Red colored proteins indicates upregulated, green indicates downregulated proteins, white represents not detected proteins whereas grey indicates detected proteins that do not pass our statistical cut off. A more detailed legend to the connectivity map in figure 1 can be found in Supplementary Fig. S1, explaining the symbols.

Consistent with other systems biology methodologies, and based on the results above (Figure 1), we conducted a thorough functional study of the highly scored nodes from our merged immune-related protein expression using functional analysis in Ingenuity. For this, IPA analyses of the original dataset was done without extra molecules added by ingenuity that might decrease the p-value of the networks and become more meaningful. The molecular function and disease analysis inquired into the functions of all significantly affected proteins, regardless of their mutual interaction, confirmed the implication of the selected proteins in immune-related processes (Table 5), terms such as infection mechanism, inflammatory response or immune trafficking were found confirmed.

Table 5: IPA Top molecular functions and diseases from ER β -merged network.

Relative function & disease	p-value	Molecules
Cellular assembly and organization	6.69E-05	25
Cardiovascular disease	1.52E-04	12
Genetic disorder	1.52E-04	20
Gene expression	5.54E-04	9
Infection mechanism	5.54E-04	7
Cell-to-cell signaling and interaction	7.79E-04	16
Hematological system development and function	7.79E-04	15
Immune trafficking	7.79E-04	13
Inflammatory response	7.79E-04	12
Cancer	2.26E-03	16
Reproductive system disease	2.26E-03	12
Antigen presentation	2.70E-03	9

In a next step, the highly complex network obtained, was reduced to give the data-model presented in Figure 2 by selecting significant ER-mediated immune protein relationships.

The molecular pathways in normal and tumor cells that control immune responses include transcription factors, such as nuclear factor kappa B (NF- κ B), and signal transducer and activator of transcription 3 (STAT3), which, in turn, control the production of other chemokines and inflammatory mediators (cytokines). These two significant transcription factors appear to be significantly regulated in both datasets (CA and DB), being both downregulated in genistein exposed T47D-ER β cells that express ER β (Figure 2B), whereas in non-ER β -expressing cells, only NF- κ B was downregulated (Figure 2A).

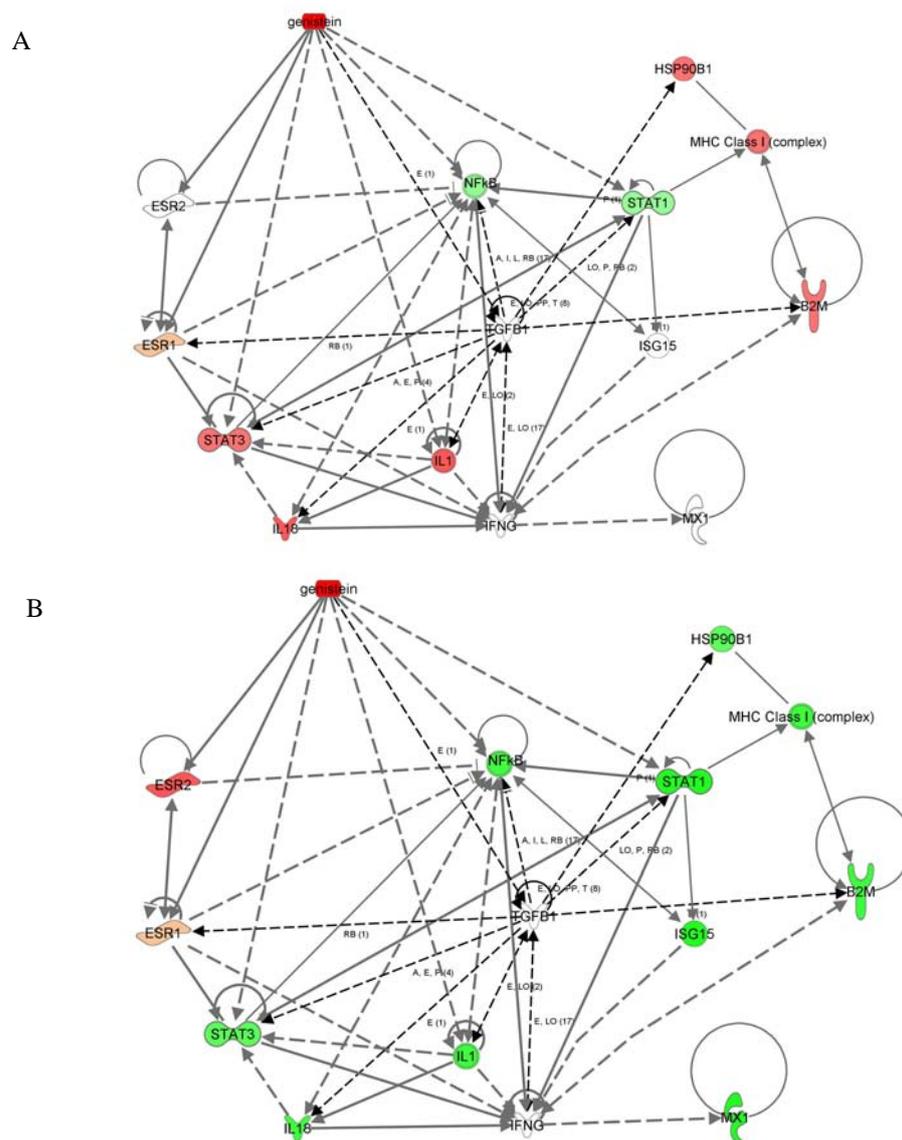


Figure 2: Connectivity map of the responses by Ingenuity Pathway assistant analysis. A) Cells expressing only low levels of ER α . Green indicates downregulated, red indicates upregulated, pink indicates low expression and white not detected. B) Cell coexpressing ER α and ER β . A more detailed legend to the connectivity map in figure 2 can be found in Supplementary Fig. S1, explaining the symbols.

Figure 2B (sample DB) also shows that in genistein exposed T47D-ER β cells that express ER β , complete downregulation of the immune system network is observed. Since the expression of ER β in this experiment was found to be much higher than ER α (chapter 5), it is likely that genistein mediated ER β activation downregulated the production of cytokines (IL1 and IL18) by inhibition of NF- κ B or STAT3. The protein interaction network analysis also revealed that genistein exposure of T47D-ER β cells expressing ER β also resulted in direct inhibition of STAT1 and NF- κ B activation (Table 3), and therefore downregulated the production of an array of inflammatory mediators, finally resulting in downregulation of interferon-stimulated proteins like MX1, ISG15, B2M.

Upregulation of cytokines IL1 and IL18 and further increased expression of interferon related proteins was found. Thus, in the absence of ER β expression, genistein induced immunosuppression was no longer observed (Figure 2A).

Cytokine production

To validate the predicted genistein-induced ER β mediated immune suppression, exposure medium from treated cells (samples A, B, C, D and E) was used to measure cytokine production. Specifically the cytokines that might be influenced by the genistein induced downregulation of the transcription factors NF- κ B and/or STAT-1 (Figure 2) were analysed, being interleukines 1 β and 8 (IL-1 β , IL-8), interferon γ (IFN- γ), and tumor necrosis factor α (TNF- α). In addition tumor growth factor β (TGF- β) known to be upregulated when IFN- γ levels go down was quantified as breast cancer patients have increased levels of regulatory T-cells that are profoundly able to inhibit T-cell dependent production of IFN- γ (17) In line with the fact that genistein exposure of ER β expressing T47D-ER β cells resulted in down regulation of NF- κ B and/or STAT-1, expression of the related cytokines IL-1 β , IL-8, IFN- γ , and TNF- α could not be detected in the medium. Only the production of TGF- β was detected (Figure 3) showing small differences between samples. Genistein was able to induce the production of TGF- β , and this production was higher in ER β expressing samples than in non-ER β expressing cells. Upregulation of TGF- β was more pronounced in genistein exposed T47D-ER β cells expressing ER β than cells not expressing ER β .

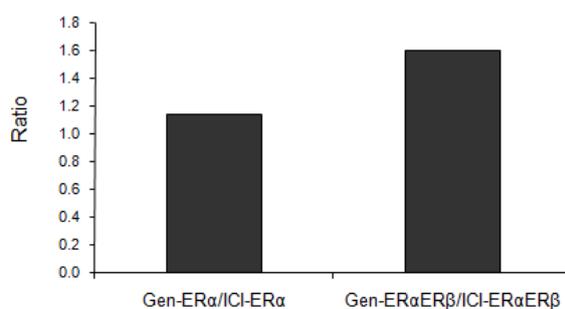


Figure 3: Cytokine TGF- β production. Ratios of cytokine production were calculated.

Discussion:

The present study presents a protein-network based analysis of the quantitative SILAC-based proteome of T47D-ER β breast cancer cells with tetracycline-dependent varying intracellular ER α /ER β ratio exposed to genistein. Genistein is a phytoestrogen found in high levels in soy products the intake of which has been frequently associated with various beneficial health effects including reduced risk on breast cancer and osteoporosis in menopausal women (18-20).

Using the T47D-ER β cell line, three different protein datasets were obtained using the SILAC approach, and signaling effects of estrogen receptor subtypes exposed to the phytoestrogen genistein were addressed based on single protein expression level analysis in a previous paper (Chapter 5). Functional analysis of the genistein induced proteome data in genistein exposed T47D-ER β cells not expressing ER β , revealed that genistein was able to induce growth of the breast epithelial cells as indicated by the activation of cellular reorganization and maintenance, whereas genistein exposure of ER β expressing T47D-ER β cells decreased the cell motility and cell proliferation. Analysis of the data by a protein network approach as presented in the present paper corroborates that pathways of cell proliferation, cell death, cell cycle and lipid metabolism are significantly affected in opposite ways in cells that do not express ER β on one hand and cells that do express higher levels of ER β (Tables 2, 3 and 4). Networks found to be upregulated in the genistein exposed T47D-ER β cells not expressing ER β belonged to processes such as cellular assembly and organization, cellular function and maintenance, and cell cycle among others, whereas networks upregulated in the genistein exposed T47D-ER β cells expressing high levels of ER β showed a clear implication in cell cycle, gene expression and cell death.

Furthermore, comparing network analysis between samples expressing or not ER β , we detected clear differences in regulatory networks among differentially expressed proteins related to immune responses. Genistein, in the presence of ER β , downregulated cellular processes and proteins implicated in antigen presentation, cell-mediated immune response, and immunological disease. Whereas, in absence of ER β , these processes were not regulated, instead, parameters such as inflammatory response or infection mechanism were found to be affected. It is well established that estrogens and phytoestrogens can affect the development and regulation of the immune system *in vivo* and *in vitro*, but how the biological function of this estrogenic compounds are mediated is still not fully understood (3, 21, 22).

During the past few years implications of ERs on inflammation and immune diseases have been proposed but not further elucidated (23, 24). It is well established that sex hormones can have a substantial impact on the immune system (25). Immune tissues and cells expressing ERs respond to estrogens (22). Several studies have reported increased activity of several immune function parameters, such as interferons and interleukins production, or T and NK cell activity after exposure to the isoflavones, daidzein and genistein (22, 26). Moreover, genistein attenuates cytokine-stimulated proliferation of both normal and cancer cells supporting its contribution to the inflammatory/immune responses as its possible mechanism of action (27, 28). Recently it has been suggested that genistein may exhibit anti-tumour activity via a cellular immune mechanism (4, 21). Therefore, estrogens and estrogen-like compounds may modulate immunological responses and the mechanisms underlying these effects remain unclear (29).

It has been shown, that ER α and ER β have an important role in immune organ development. Knock out ER α , ER β and double knock out (ER α and ER β) mice show thymus and spleen atrophy (3, 30). Moreover, recent data suggested the immunosuppressive and thymic atrophy effects as a result of genistein treatment in mice (21).

Our results are in line with the hypothesis that the immune system is a major estrogen target (29). In addition to the protein-network based analysis, in a next step a regulatory protein network model was used in order to elucidate if the immune modulating effects of genistein were ER- or non-ER-mediated. Figure 2 shows how ERs are able to directly downregulate NF- κ B after genistein binding. Moreover, genistein is able to downregulate STAT1 and NF- κ B directly and indirectly (Figure 2). These two transcription factors are the main regulators for iNOS, through an array of inflammatory mediators. Other flavonoids such as kaempferol, quercetin and daidzein have shown this anti-inflammatory property in previous studies (31). Since the downregulation of STAT1 and NF- κ B was common in cells expressing or not ER β , the possible downregulation of the immune system protein network in ER β -expressing cells could not be explain by the regulation of this two transcription factors.

Pathways different from those regulated by STAT1 and NF- κ B could be identified in the presented model (Figure 2) as the main mechanism for the regulation of the immune suppression in breast cancer cells. Among all STAT family members, STAT3 is most often correlated to tumorigenesis, and is considered as an oncogene (32). Many indications suggest a role for STAT3 in regulating cell movement, mainly by contributing to cytoskeleton reorganization and controlling cell adhesion properties (33). Expression of STAT3 was found to be upregulated in genistein treated cells in which ER β was inhibited, supporting the implication of genistein-activated ER α in cell proliferation by cytoskeleton reorganization and cell movement. Moreover, STAT3 also regulates in tumor cells the molecular pathways that control cancer-related immunity (34) which, in turn, controls the production of other cytokines. Cytokines are also expressed in breast cancer cells (35). IL-18 produced by cancer cells seems to have pro-cancer activity, promoting cell proliferation and migration, more importantly is reported to be an important marker for breast cancer progression (36, 37). IL-1 and IL-18 are pro-inflammatory cytokines and activate immune responses (38). Both cytokines were upregulated upon gensitein treatment of T47D-ER β cells that did not expressed ER β . As a consequence of this upregulation of pro-inflammatory cytokines, upregulation of interferon gamma related proteins was also found (B2M, MHC Class I and HSP90B1).

Downregulation of STAT3, IL-18 and IL-1 in genistein treated cells expressing ER β resulted in further downregulation of the overall immune protein-network. In addition, other interferon gamma stimulated proteins were detected as a result of the treatment (ISG15 and

MX1). These proteins were also found to be secreted from both non-immune and immune cell (39) in addition, MX1 present a putative steroid receptor binding site for ER and PR (40).

Furthermore, the merged immune-related network (Figure 1), also pointed at the downregulation of p38 MAP Kinase in genistein treated cells expressing ER β (an effect not observed in genistein treated cells that did not express ER β (sample CA). This is in agreement with the previously reported inhibition of p38 MAP Kinase by genistein (41).

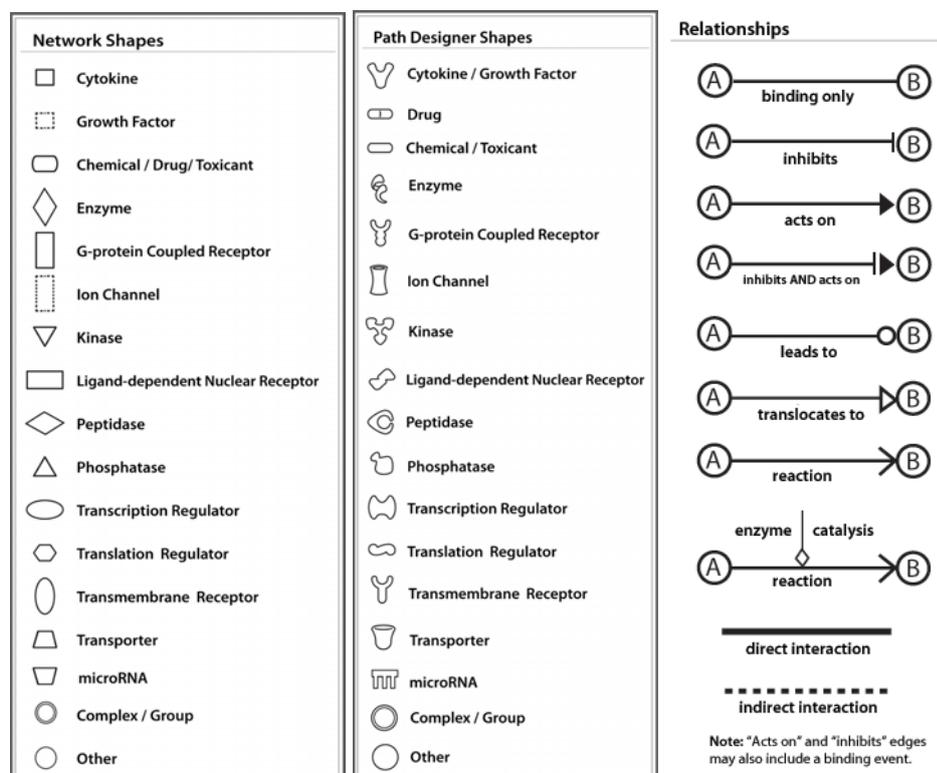
Finally in an attempt to validate the predicted genistein-induced ER β mediated immune suppression, cytokines involved in immune/inflammatory responses were analyzed. Exposure medium (supernatant) from SILAC samples were used to measure an array of different cytokines not detected by LC-MS/MS analysis. No formation of IL-1 β , IL-6, IL-8, IL-10, IL-12, IFN- γ and TNF- α could be detected. These cytokines were not detected in any of the treatment conditions. These results were in agreement with the negative regulation of interferon gamma by NF- κ B.

On the other hand, production of transforming growth factor β (TGF- β) was detected in all the samples (A, B, C, D and E). In breast cancer TGF- β presents an important immunosuppressive role (42, 43). From the independent measurements of treated samples with genistein and ICI 182780 (A, B, C, D and E) slightly different levels of TGF- β were detected (data not shown). This is in accordance with the direct antagonist effects of antiestrogens in TGF- β production. Previous studies shown that ICI 182780 (or fulvestrant) is able to induce this cytokine secretion and activation in breast cancer (44-46). TGF- β is an prognostic indicator associated with relapse in breast cancer patients supporting its important role in modulating disease activity(47). TGF- β induces MAPK and MAPK/ERK kinase dependent signal transduction in addition to the NF- κ B pathway. TGF- β signaling is highly regulated via interaction with inhibitory SMAD's or binding of the E3-ubiquitin ligases and coreceptors. TGF- β is able to cause inhibition of NF- κ B transactivation and therefore proinflammatory cytokine production, including IFN- γ , mainly through p38 MAPK (48). In addition, TGF- β , an inhibitor of epithelial cell growth, has both increased expression and production in the presence of genistein in normal and transformed breast epithelial cells (27, 28). Therefore, when calculating resulting ratios, as in SILAC comparison, genistein treated cells expressing ER β showed higher induction of cytokine production than those not expressing ER β .

Altogether the results of the present study reveal that in addition to cell proliferation and apoptosis, immune signaling appears to be another major estrogen target influenced by phytoestrogen exposure resulting in genistein induced ER β -mediated downregulation of immune signaling networks.

Supplemental Material

Figure S1: Legends from Ingenuity pathway analysis.



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Summary, Future Perspectives
and Concluding Remarks

Summary

The multiple actions of estradiol and other estrogenic compounds in mammalian physiology are brought about, on a molecular level, as a result of complex signalling pathways, and mediated by at least two receptors namely estrogen receptor (ER) α and ER β .

The aim of the work presented in this thesis was to obtain insight in the role of ER α , ER β and the ratio of ER α /ER β present within a cell, in the cellular response to estrogen-like compounds. To this end, this thesis addressed the transcriptional activity at both the gene and protein level and effects on cell proliferation under the influence of specifically-acting estrogen-like molecules when varying the ratio of ER α /ER β present in the cells under study. The ultimate aim was to link the data on cell proliferation as the biological end-point to the transcriptomics and proteomics data.

Chapter 1 of this thesis gives an introduction describing the molecular basis of ERs, their tissue distribution and their dualistic role in cancer cell proliferation.

Several studies demonstrated that estrogens stimulate the growth of a large proportion of ER α positive breast cancers (1-4) or reported a decreased ER β expression in cancer tissues as compared to benign tumors or normal tissues, whereas ER α expression persists (5, 6). This led to the hypothesis that ER β may modulate the proliferative effect of ER α . To further investigate this possible ER β mediated modulation of the proliferative effect of ER α we investigated in **Chapter 2** how variable cellular expression ratios of the ER α and ER β modulate the effects on cell proliferation induced by ER α or ER β agonists, respectively. Using human osteosarcoma (U2OS) ER α or ER β reporter cells, propyl-pyrazole-triol (PPT) was shown to be a selective ER α and diarylpropionitrile (DPN) a preferential ER β modulator. The effects of these selective estrogen receptor modulators (SERMs) and of the model compound estradiol (E2) on the proliferation of T47D human breast cancer cells with tetracycline-dependent expression of ER β (T47D-ER β) were characterized. E2-induced proliferation of cells in which ER β expression was inhibited was similar to that of the T47D wild-type cells, whereas this E2-induced cell proliferation was no longer observed when ER β expression in the T47D-ER β cells was increased. In the T47D-ER β cell line, DPN also appeared to be able to suppress cell proliferation when levels of ER β expression were high. In the T47D-ER β cell line, PPT was unable to suppress cell proliferation at all ratios of ER α /ER β expression, reflecting its ability to activate only ER α and not ER β . It was concluded that effects of estrogen-like compounds on cell proliferation are dependent on the actual ER α /ER β expression levels in these cells or tissues and the potential of the estrogen agonists to activate ER α and/or ER β .

In further studies described in the present thesis attention was focussed on food-born estrogens, genistein and quercetin. The ubiquitous and unavoidable presence of estrogenic substances in our western diet may pose a significant health concern, although others have claimed beneficial effects related to for example intake of phytoestrogens from soy (7-9). The human diet contains several plant-derived, nonsteroidal weakly estrogenic compounds (10). Moreover, phytoestrogens have been considered a natural alternative to hormone replacement therapy (HRT) since these chemicals are found in the regular diet (11). A hypothesis put forward to explain the seemingly contradictory health effects of estrogens and phytoestrogens relates to the potentially different ultimate cellular effect of activation of ER α , promoting cell proliferation and possible adverse health effects on the one hand, and ER β , promoting apoptosis and beneficial health effects on the other hand, with phytoestrogens activating especially ER β but estradiol activating preferentially ER α . This hypothesis was investigated to a further extent in **Chapter 3** of the thesis. In Chapter 3, the importance of the intracellular ratio of the two estrogen receptors ER α and ER β for the ultimate potential of the phytoestrogens genistein and quercetin to stimulate or inhibit cancer cell proliferation was investigated. This is of importance because i) ER β was shown to play a role in modulating ER α -mediated cell proliferation (Chapter 2), ii) genistein and quercetin may be agonists for both receptor types and iii) the ratio of ER α to ER β is known to vary between tissues. Using human osteosarcoma (U2OS) ER α or ER β reporter cells it was shown that, compared to estradiol (E2), genistein and quercetin have not only a relatively greater preference for ER β but also a higher maximal potential for activating ER β -mediated gene expression. Using the human T47D breast cancer cell line with tetracycline-dependent ER β expression (T47D-ER β), the effect of a varying intracellular ER α /ER β ratio on E2- or phytoestrogen-induced cell proliferation was characterised. Similar to the results obtained in Chapter 2 the data in Chapter 3 reveal that E2-induced cell proliferation of cells in which ER β expression was inhibited was similar to that of the T47D wild type cells, whereas this E2-induced cell proliferation was no longer observed when ER β expression was increased. With increased expression of ER β the phytoestrogen-induced cell proliferation was also reduced. These results pointed at the importance of the cellular ER α /ER β ratio for the ultimate effect of phytoestrogens on cell proliferation.

A remarkable observation reported in Chapter 3 was that in the U2OS ER α and ER β reporter cells genistein and quercetin resulted in a higher maximal induction of ER α or ER β -mediated luciferase activity than the maximal luciferase activity induced by E2. This phenomenon has been observed also in other studies (12, 13) and has been referred to as superinduction. This effect was consistently measured in our lab, but so far, the mechanism underlying this effect and thus also its biological relevance remained to be elucidated. Therefore, in **Chapter 4**, several hypotheses for the possible mechanisms underlying this

superinduction were investigated using genistein as the model compound known to induce the effect. These hypotheses included i) a non ER-mediated mechanism, ii) a role for an ER α and/or ER β activating genistein metabolite with higher ER-inducing activity than genistein itself, and iii) an artefact that is not biologically relevant but specific for the luciferase based reporter gene assays. The data presented in Chapter 4 indicate that induction and also superinduction of the reporter gene is ER-mediated, and that superinduction by genistein could be ascribed to stabilization (decreased degradation) of the firefly luciferase reporter enzyme increasing the bioluminescent signal during the cell-based assay at genistein concentrations above 1 μ M. This indicated that the phenomenon of superinduction may not be biologically relevant but may rather represent a post-transcriptional effect on protein stability.

In order to elucidate the mechanisms underlying the genistein induced effects on cell proliferation in T47D-ER β cells with variable cellular ER α /ER β ratio as reported in Chapter 3, **Chapter 5** addressed by transcriptomics and quantitative SILAC-based proteomics, the ER α and ER β -mediated effects on gene and protein expression in T47D-ER β breast cancer cells exposed to the phytoestrogen genistein. Using the T47D-ER β human breast cancer cell line with tetracycline-dependent ER β expression, the effect of a varying intracellular ER α /ER β ratio on genistein-induced gene and protein expression was characterised. Results obtained revealed that in ER α -expressing T47D-ER β cells genistein induced transcriptomics and proteomics signatures pointing at rapid cell growth and migration by dynamic activation of cytoskeleton remodeling. The data revealed an interplay between integrins, focal adhesion kinase (FAK), CDC42 and actin cytoskeleton signalling cascades, occurring upon genistein treatment, in the T47D-ER β breast cancer cells with low levels of ER α and no expression of ER β . In addition, data presented in Chapter 5 indicated that ER β -mediated gene and protein expression counteract ER α -mediated effects, because in T47D-ER β cells expressing ER β and exposed to genistein a clear downregulation of genes and proteins involved in cell growth and induction of genes and proteins involved in cell cycle arrest and apoptosis was demonstrated. Thus, genistein induced ER β mediated effects resulted in decreased expression of genes and proteins involved in cell motility and metastatic potential as well as of genes and proteins involved in cell survival of the breast cancer cell line. It was concluded that the effects of genistein on proteomics and transcriptomics endpoints in the T47D-ER β cell model were comparable to those reported previously for estradiol, and that the ultimate estrogenic effect will be dependent on the receptor phenotype (ER α /ER β ratio) in the cells or tissue of interest.

In a subsequent study of the proteomics results, data analysis was performed based on a protein-network approach. Results from this protein-network analysis revealed additional protein networks to those involved in cell proliferation and apoptosis to be affected upon

genistein exposure of the T47D-ER β breast cancer cells. These results are described in Chapter 6.

Chapter 6, presents a protein-network based analysis of the quantitative SILAC-based proteome of T47D-ER β breast cancer cells with tetracycline-dependent varying intracellular ER α /ER β ratio exposed to the isoflavone genistein. Genistein is a phytoestrogen found in high levels in soy products, the intake of which has been frequently associated with various beneficial health effects including reduced risk on breast cancer and osteoporosis in menopausal women. In our study protein networks involved in cell proliferation and apoptosis appear to be affected upon genistein exposure of the T47D-ER β breast cancer cells. In addition the data point at genistein-induced ER β mediated immune suppression. Genistein induced ER β -mediated downregulation of the expression of the transcription factors NF- κ B and STAT3 as well as down regulation of a variety of cytokines including IL-1 β , IL-18, IFN- γ , and TNF- α . On the other hand, a considerable increase of TGF- β induction was found in genistein treated T47D-ER β cells expressing ER β compared with cells with no expression of ER β , further corroborating the genistein induced ER β mediated downregulation of immune signaling. Altogether, the results of the present study reveal that in addition to cell proliferation and apoptosis, immune signaling appears to be another major estrogen target influenced by phytoestrogen exposure.

Concluding remarks and future perspectives

After more than ten years since the discovery of the second estrogen receptor, named ER β , the importance of the complex balance in estrogen signalling through the two receptors ER α and ER β for the regulation of human development and reproduction becomes more evident. It has been demonstrated that ER β is an anti-proliferative transcription factor in ER α positive breast cancer cells. Hence, estrogen signalling is dependent on the balance between two opposing receptors (ER α and ER β). With our studies, we provided some insight into the mechanism of differential biological responses induced by food-borne, xeno- and phyto-estrogenic compounds using the latest state-of-the-art techniques. In the present thesis emphasis was on especially the nature of the estrogen, activating preferably ER α (PPT), ER β (DPN, quercetin and genistein) and/or both receptors (E2), as well as on the importance of the ratio between the two receptors. It is however, important to note that the overall ultimate biological effects of estrogenic compounds in cancer cells will be the result of an even more complex interplay between various mechanisms, which may depend on the cellular context, balance between ER subtypes, but also on coactivators and corepressors present in the cell, and splice variants of the two receptors present in the cells. All these factors taken together

enable accurate and targeted responses to hormones or antiestrogens. Given these considerations it seems logic that future research should be directed at the following topics:

- i) the role of coactivators and corepressors that may vary with the cells or tissues of interest,
- ii) the role of splice variants of the two receptors ER α and ER β , which may vary within the cells or tissue of interest,
- iii) a comparison of the actual ER α /ER β ratio in the T47D-ER β model system compared to ER α /ER β ratios actually observed in *in vivo* tissues,
- iv) integration of the knowledge obtained into the risk assessment of phytoestrogen preparations already sold on the market.

ERs can associate with distinct subsets of coactivators and corepressors depending on binding affinities and relative abundance of these factors (14, 15). Several ER coactivators and corepressors have been described (16). Differences between ER α and ER β in coactivators and corepressors recruitment have also been reported (17, 18), and therefore this preferential binding of certain coactivators and corepressors to one of the ERs will have consequences for specific ligand signalling and the ultimate biological effect elicited by ligand binding.

NCoR and SMRT corepressors and the p160 family coactivators are widely expressed (19-21). Low levels of SRC-3 have been demonstrated for human proliferating endometrium with increased expression in the late secretory phase (22) while overexpression of SRC-3 is frequently observed in breast, ovarian, and prostate cancers (23-25). Similar expression levels of CBP, p300, AIB1, GRIP1, p300, NCoR, and SMRT have been measured for Ishikawa uterine and MCF-7 breast cancer cells (26). High levels of SRC-1 expression is found in the Ishikawa cells, and this might correlate with the agonist activity of tamoxifen in this cell line (26). We have seen in our studies (Chapter 4), that the T47D breast cancer cells express the ER coactivator PRMT1. Recruitment of this coactivator is accompanied by histone methylation (27, 28). Recently, PRMT1 gene expression has been used as a marker of unfavourable prognosis for colon cancer patients (29).

Thus, other signalling events within the cell may affect nuclear receptor transcriptional responses via alteration in the expression of certain coregulators, and therefore it is predicted that significant differences in coactivator and corepressor expression found in various cell and tissue types would be important determinants of specific receptor modulator activity.

In addition, distribution of particular splicing variants of both ERs should be taken into account when considering tissue response to estrogens and cofactor recruitment as they have

differential and sometimes antagonistic properties and their relative abundance might significantly influence biological responses to hormones. The main physiological role of ER splice variants in breast cancer development is however far from clear and might be a crucial determinant for clinical parameters.

Full length ER α and ER β proteins are approximately 66 and 59 kDa respectively (30, 31), although as a result of alternative splicing both receptors can form different isoforms. ER α has been shown to form over 20 alternative splice variants in breast cancer and other tumors (32), three of them with relevant functionality, while at least five ER β variants have been reported in human (33). The two most referenced ER α isoforms that seem to be of particular significance are ER α 46 and ER α 36 as they were reported to oppose genomic actions of full length ER α 66 (Figure 1).

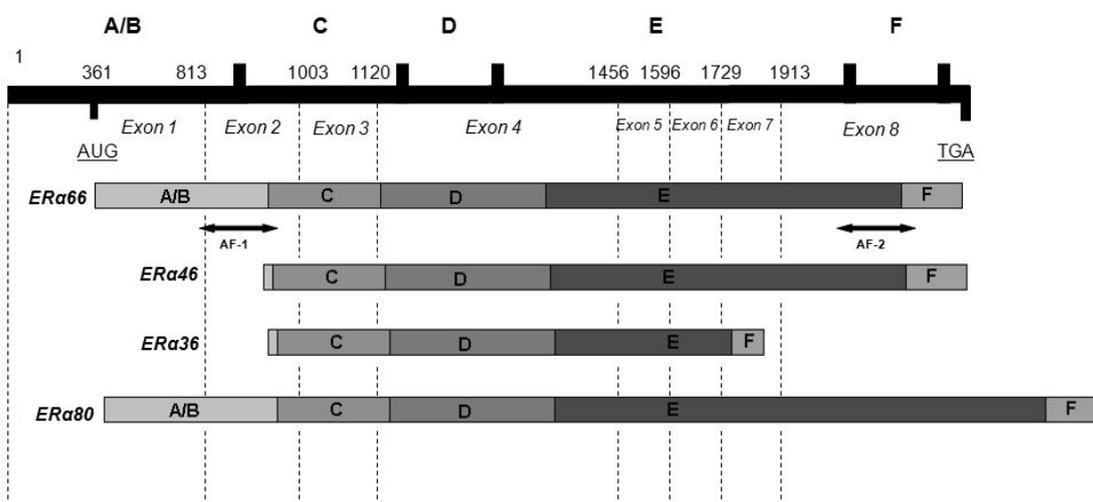


Figure 1: Schematic comparison between full length ER α and its most referenced truncated isoforms.

The **ER α 46** isoform has been identified in the MCF7 breast cancer cell line (34) in which it is coexpressed with full length ER α 66. The presence of ER α 46 has also been confirmed in osteoblasts (35) and endothelial cells (36). This isoform is devoid of AF-1 (ligand independent transactivation domain) activity. In contrast with full length ER α 66, truncated isoform ER α 46 does not mediate E2 dependent cell proliferation and high levels of this isoform have been shown to be associated with cell cycle arrest in the G0/G1 phase. Similarly to ER β , ER α 46 is a potent ligand-dependent transcription factor in promoter and cell context sensitive to AF-2 (ligand dependent transactivation domain) and a powerful inhibitor of AF-1 dependent transcription (36). Coexpression of ER α 46 with ER α 66 in a SaOs osteoblast cell line results in receptor-concentration dependent inhibition of E2 stimulated cell proliferation (37).

The second truncated ER α isoform, **ER α 36**, was described recently (35), and it has been shown to lack both the AF-1 and AF-2 transactivation functions of full length ER α . However it has functional DNA binding and ligand binding domains (DBD and LBD). ER α 36 contains an exon coding for myristoylation sites, hence suggesting an interaction with the plasma membrane. With no functional AF-1 and AF-2, ER α 36 does not have any direct transcriptional activity. However, it is a powerful inhibitor of full length ER α and ER β dependent transactivation (38). Even though it lacks transcriptional activity it can activate non genomic ER pathways such as MAPK/ERK signaling in response to E2 which is of particular significance in response to antiestrogens such as tamoxifen, 4OH-tamoxifen and ICI-182.780 (38).

The **ER α 80** isoform was detected in the MCF7:2A cell line, which is a subclone of the MCF7 cell line derived from long term growth in the absence of E2. This ER α 80 isoform was produced by duplication of exons 6 and 7 (39). No evident functionality has been described so far. A list of selected ER α splice variants and their expression in various breast tissues (normal and tumor) and breast cancer cell lines is given in Table 1.

Table 1: List of selected ER α splice variants and their expression in various breast tissues (normal and tumour) and breast cancer cell lines.

Splice variant								References	
	Breast	MCF7	T47D	MDA-MB-231	MDA-MB-435	BT-474	BT20		ZR-75
ERα36	+			+					(40, 38, 41)
ERα46 (or ER$\alpha$$\Delta$1)		+							(34)
ER$\alpha$$\Delta$2	+	+	+		+				(42, 43, 44, 45, 46, 32)
ER$\alpha$$\Delta$3	+	+	+	+					(42, 45, 44, 43, 47, 48, 46, 49)
ER$\alpha$$\Delta$4		+			+			+	(50, 43, 44, 32, 45)
ER$\alpha$$\Delta$5	+	+	+	+	+	+	+	+	(51, 43, 44, 45, 43, 52, 53)
ER$\alpha$$\Delta$6	+								(45, 44)
ER$\alpha$$\Delta$7	+	+	+	+					(42, 54, 45, 44, 54, 46)
ER$\alpha$$\Delta$5,7	+								(43)

The presence of ER β isoforms has been confirmed in various human cell lines as well as in a broad range of tissues at different levels (55, 56). At present their functional significance is poorly understood. The only fully functional ER β isoform is **ER β 1**, which is a full length protein with LBD and active AF-2 domain (Figure 2). **ER β 2, 4 and 5** have a shortened Helix 11 and a full length Helix 12 is present only in ER- β 1 and β 2. Although in ER β 2 it has a different orientation than in ER β 1 due to the shorter Helix 11. It has been reported that displaced Helix 12 in ER β 2 limits ligand access to the binding pocket. As a consequence of this altered structure, ER β 2, 4 and 5 cannot form homodimers and have no transcriptional activity on their own, although they have been shown to heterodimerize with ER β 1 upon E2 binding and enhance its AF-2 mediated transcriptional activity (56). Studies of interactions between different ER β isoforms with ER α are very limited. However **ER β 2** (also named **ER β cx**) was shown to limit DNA binding of ER α 66 and inhibit its transcriptional activity in a manner similar to that described for ER β 1 (57).

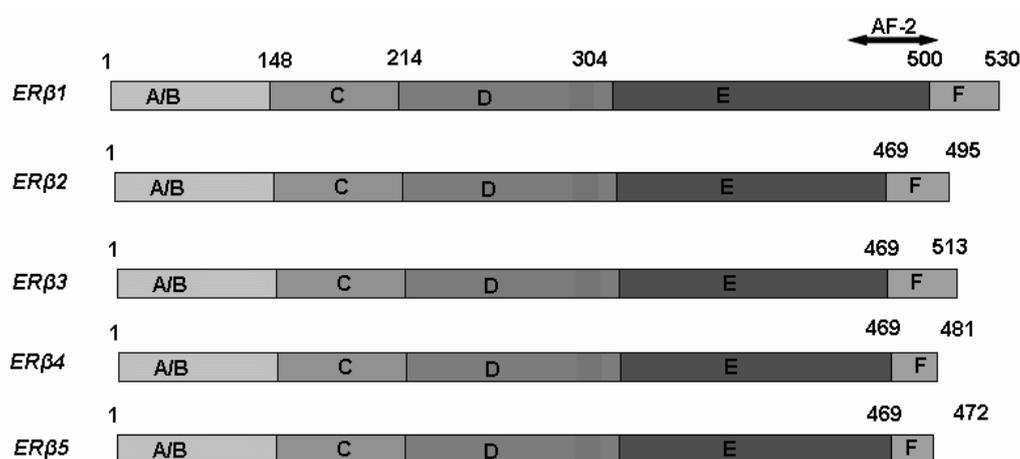


Figure 2: Comparison between full length ER β and its most referenced truncated isoforms.

Two new exon-deleted variants were detected in the cancer cell line MDA-MB-231, **ER β Δ 1,2,5** and **ER β Δ 1,2,5,6** of approximately 35 and 28 kDa (58). Both proteins are predicted not to contain AF-1, and be the result of deletions in the DBD and LBD. Therefore, these two variants are expected to be devoid of or have significantly reduced ligand-dependent and ligand independent activities, and their expression did not affect growth of cancer cell lines tested (Table 2). A list of selected ER β splice variants and their expression in various breast tissues (normal and tumour) and breast cancer cell lines is given in Table 2.

All together it can be concluded that more in depth analysis of the mechanisms of estrogen receptor splicing variants and corepressors/coactivators is needed because they provide possible additional mechanisms underlying the tissue-dependent modulation of the ER response.

In future research, it might be possible that knowing the nature of the ER subtype by specific receptor isoform transfection, and by using technologies such as chromatin immunoprecipitation studies (Chip), the expression and the association of coactivators and repressors in a specific given cell type might provide further insight about the mechanism whereby estrogens and estrogen like compounds produce tissue-specific effects.

Moreover, literature data reveal that physiological levels of ER α to ER β may vary in such a way that either one of the two receptors is dominant. It remains to be established in what way the range of ER α to ER β ratios in the T47D-ER β line with increasing concentrations of tetracycline reflect physiologically relevant variations in the receptor ratio compared to *in vivo* tissues.

Table 2: List of selected ER splice variants and their expression in various breast tissues (normal and tumour) and breast cancer cell lines.

Splice variant							References
	Breast	MCF7	T47D	MDA-MB-231	MDA-MB-435	BT20	
ER β 2	+	+	+	+		+	(59, 60, 61, 62, 7, 56)
ER β 3	+						(61)
ER β 4	+		+		+		(55, 61, 63)
ER β 5	+	+	+	+	+	+	(59, 61, 55, 30, 56, 7)
ER $\beta\Delta$ 2	+			+			(64)
ER $\beta\Delta$ 3	+						(64, 65)
ER $\beta\Delta$ 4	+						(64, 65)
ER $\beta\Delta$ 5	+	+		+			(64, 66, 67)
ER $\beta\Delta$ 6	+						(64, 67)
ER $\beta\Delta$ 1,2,5		+		+			(58)
ER $\beta\Delta$ 1,2,5,6		+		+			(58)

Various studies reveal that physiologically levels of ER α and ER β may vary depending on the cell or tissue type, with for example ER α being dominant in the liver or within the ovary in the thecal and interstitial cells and ER β being dominant in isolated granulosa cells

(68), in cells from human umbilical vein endothelium, prostate tumours (69) as well as in colon, epithelium of prostate cells or granulose cells within the ovary (68) in normal secretory luminal prostate cells (70) or human testis in the developing spermatids (71).

Since the T47D-ER β cell line is derived from a human breast tumor (T47D) the levels of ER α and ER β in the cell line when grown in the presence of 1000 ng/ml tetracycline (no additional ER β expressed) are expected to reflect the physiological levels in this tumour tissue. Expression of ER β in the T47D wild type cells appeared to be below the detection limit (72). These low levels of ER β expression in breast tumour tissue is in line with the loss of ER β expression in malignant cancer tissues as compared to benign tumours or normal tissues, where the ratio of ER α /ER β expression is higher than in normal tissues (6, 67, 73, 74). Information on the levels of ER α and ER β in the T47D cells when grown in the absence of tetracycline (full ER β expression) can be derived from data reported in a previous paper (72), where it was demonstrated that, based on mRNA levels, the T47D-ER β cell line at 0 ng/ml of tetracycline (full expression of ER β) expresses only 4 times more ER β than ER α . Such a 4-fold difference in the level of the two receptors does appear physiologically relevant when compared to literature data on other tissues. Quantification of the levels of both receptor types in the T47D-ER β cells grown in the presence of varying levels of tetracycline and comparing them to the levels quantified by the same technique in the same study in relevant tissues would validate and optimise the model system used in the present study to a further extent.

Finally, one may question whether the results obtained in the present thesis provide some clues to the presently ongoing discussion on the risk-benefit of phytoestrogen preparations already sold on the market. At present the consumer tends to believe that just because a product can be bought from the health food store, it is healthy and that "natural" equals "safe". Nothing could be further from the truth. Although several health claims are connected to phytoestrogen preparations, such as lowering the risks of developing breast cancer or decreasing menopause symptoms, there is also evidence that suggests that phytoestrogens may stimulate cancer progression and other undesired side effects concerning the central nerve system and immune system among others. The results of the present thesis clearly indicate that the differences between phytoestrogens on the one hand and estradiol on the other hand may be smaller than generally suggested, and that the ultimate biological effect of these estrogens strongly depends on the tissue of interest and could even be similar. The results obtained argue against the general idea that phytoestrogens are beneficial because they activate ER β , while estradiol may be adverse because it activates ER α . The ultimate biological effect is influenced by a complex interplay between the agonist characteristics but also characteristics of the cells and tissues of interest, including their ratio of the two receptors, receptor splice variants and coactivators and corepressors being present. Thus it

seems likely that the effects of phytoestrogens will vary with the tissue of interest and can be beneficial for one tissue and biological endpoint but at the same dose adverse for another tissue and biological endpoint. Furthermore, there is a need for a better understanding of the health implications of the dose, bioavailability and long-term effects of these phytoestrogen based food supplements, and as a consequence, a need for a better and more detailed evaluation of their risks and benefits.

Results of the present thesis have elucidated possible similarities between E2 and the phytoestrogen genistein pointing at not only beneficial health effects of phytoestrogens and this should not be ignored in the safety assessment of phytoestrogen supplements.

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Samenvatting, concluderende opmerkingen en
toekomstige perspectieven

Samenvatting

De verscheidene acties van estradiol en andere oestrogene stoffen in de zoogdierfysiologie worden bewerkstelligd, op een moleculair niveau, via complexe routes, en de werking van ten minste twee oestrogeenreceptoren ER α en ER β .

Het doel van het onderzoek beschreven in dit proefschrift was om meer inzicht te krijgen in de invloed van ER α en ER β en de intracellulaire ER α /ER β -ratio op de cellulaire reactie op oestrogeenachtige stoffen. Dit proefschrift beschrijft de invloed van oestrogenen op de transcriptionele activiteit op zowel het gen- als het eiwitniveau en op de celproliferatie wanneer de intracellulaire verhouding van ER α /ER β wordt gevarieerd. Het uiteindelijke doel was de gegevens over celproliferatie als biologisch eindpunt te correleren met de transcriptomics- en proteomicsgegevens.

Hoofdstuk 1 van dit proefschrift beschrijft de moleculaire basis van ERs, hun weefselverdeling en hun dualistische rol in kankercelproliferatie.

Verscheidene studies tonen aan dat oestrogenen de groei van een groot aantal ER α -positieve borstkankers bevorderen (1-4), of zij rapporteren een verminderde expressie van ER β in kankerweefsels in vergelijking met goedaardige tumoren of normale weefsels, terwijl de expressie van ER α in die weefsels hetzelfde blijft (5, 6). Dit leidde tot de hypothese dat ER β het stimulerende effect van ER α op de celproliferatie zou kunnen moduleren. Om deze mogelijke ER β -gemedieerde modulatie van het proliferatieve effect van ER α verder te onderzoeken, wordt in **Hoofdstuk 2** bestudeerd hoe een variabele intracellulaire ER α /ER β -ratio de door respectievelijk ER α - en ER β -agonisten geïnduceerde celproliferatie beïnvloedt. Met behulp van humane osteosarcoom ER α - of ER β -reportercellijnen werd allereerst aangetoond dat propyl-pyrazole-triol (PPT) een ER α -selectieve agonist en diarylpropionitrile (DPN) een preferentiële ER β -agonist is. Vervolgens werden de effecten gekarakteriseerd van deze selectieve oestrogeenreceptormodulatoren (SERMs) en van de modelstof estradiol (E2) op de proliferatie van cellen van de T47D menselijke borstkankercellijn met tetracycline-afhankelijke expressie van ER β (T47D-ER β). De E2-geïnduceerde proliferatie van T47D-ER β -cellen waarin ER β -expressie was geblokkeerd, was vergelijkbaar met die van de T47D wild type cellen, terwijl deze E2-geïnduceerde celproliferatie niet langer werd waargenomen als ER β in de T47D-ER β -cellen tot expressie kwam. In de T47D-ER β -cellijn was ook DPN in staat om celproliferatie te onderdrukken wanneer de ER β -expressieniveaus hoog waren. In de T47D-ER β -cellijn was PPT, ongeacht de intracellulaire ER α /ER β -ratio, niet in staat om celproliferatie te onderdrukken, en dit is in lijn met de waarneming dat PPT alleen in staat is ER α te activeren en niet ER β . Geconcludeerd kon worden dat de effecten van oestrogeenachtige stoffen op celproliferatie afhankelijk zijn van de intracellulaire ER α /ER β -

expressieniveaus in de betreffende cellen of weefsels en de potentiële activatie van ER α en/of ER β door de oestrogene agonist.

In verdere studies die in dit proefschrift staan beschreven, wordt de aandacht gevestigd op de voedingsgeassocieerde oestrogenen, genisteïne en quercetine. De alomtegenwoordige en onvermijdelijke aanwezigheid van oestrogene stoffen in ons westerse dieet, brengt een significante zorg met zich mee rond de mogelijke gezondheidseffecten die dat teweeg kan brengen, hoewel sommige studies juist een gunstig effect claimen voor bijvoorbeeld de in de voeding aanwezige phyto-oestrogenen van soja (7-9). Het humane dieet bevat verscheidene plantgerelateerde zwak oestrogene stoffen (10). Deze phyto-oestrogenen worden wel gezien als een mogelijk natuurlijk alternatief voor hormoonvervangings therapie aangezien deze stoffen ook van nature in ons dieet worden aangetroffen (11). Een hypothese om de schijnbaar tegenstrijdige gezondheidseffecten van oestrogenen en phyto-oestrogenen te verklaren stelt, dat er verschillende uiteindelijke cellulaire effecten zijn van enerzijds ER α -activatie, wat zou leiden tot promotie van celproliferatie en mogelijke nadelige gezondheidseffecten, en van anderzijds ER β -activatie, wat apoptose en gunstige gezondheidseffecten zou bevorderen. Phyto-oestrogenen die specifiek ER β activeren hebben dan een positief effect en stoffen die preferentieel ER α activeren hebben een nadelig effect. Deze hypothese is verder onderzocht in hoofdstuk 3 van dit proefschrift. In **hoofdstuk 3** werd het belang van de intracellulaire verhoudingen onderzocht van de twee oestrogene receptoren ER α en ER β voor het uiteindelijke vermogen van de phyto-oestrogenen genisteïne en quercetine om celproliferatie te stimuleren of te remmen. Dit is van belang omdat i) ER β een rol blijkt te spelen in de modulatie van middels ER α -activatie gestimuleerde celproliferatie (hoofdstuk 2), ii) genisteïne en quercetine agonisten kunnen zijn voor beide receptortypes en iii) de verhouding van ER α en ER β tussen weefsels kan variëren. Door humane osteosarcoom (U2OS) ER α - of ER β -reportercellen te gebruiken, werd aangetoond dat, vergeleken met estradiol (E2), genisteïne en quercetine niet alleen een relatief grotere voorkeur hebben voor ER β , maar ook een hogere maximale inductie van de reporterrespons tot stand brengen dan E2. Door gebruik te maken van de menselijke T47D borstkankercellen met tetracycline-afhankelijke expressie van ER β (T47D-ER β), werd het effect van een variërende intracellulaire ER α /ER β -ratio op door E2- of phyto-oestrogen veroorzaakte celproliferatie gekarakteriseerd. Voor de modelstof E2 komen de data van hoofdstuk 3 overeen met de resultaten van hoofdstuk 2, en laten zien dat E2-geïnduceerde ER α -afhankelijke celproliferatie in cellen zonder ER β -expressie gelijk is aan de E2-geïnduceerde celproliferatie van T47D wildtype-cellen, terwijl de E2-geïnduceerde celproliferatie geremd wordt in cellen waarin behalve ER α ook ER β tot expressie wordt gebracht. Met verhoogde ER β -expressie werd de phyto-oestrogen geïnduceerde ER α -afhankelijke celproliferatie ook verminderd. Deze resultaten wijzen op het belang van de

cellulaire verhouding ER α /ER β voor het uiteindelijke effect van phyto-oestrogens op de celproliferatie.

Een opmerkelijke observatie, beschreven in Hoofdstuk 3, was dat in de U2OS ER α - en ER β -reportercellen door genisteïne en quercetine een hogere maximale ER α - of ER β -gemedieerde inductie van luciferaseactiviteit wordt gemeten, dan de maximale luciferaseactiviteit die door E2 wordt geïnduceerd. Dit fenomeen is ook in andere studies waargenomen (12, 13) en wordt superinductie genoemd. Dit effect werd in ons laboratorium al meerdere malen waargenomen in reportergerbioassays, maar het mechanisme dat aan dit effect ten grondslag ligt, evenals de biologische relevantie van het effect, was nog onduidelijk. Daarom werden in **Hoofdstuk 4** de verscheidene hypothesen voor de mogelijke mechanismen die aan deze superinductie ten grondslag liggen onderzocht met gebruik van genisteïne als modelstof. Deze hypothesen omvatten i) een niet ER-gemedieerd mechanisme, ii) een rol voor een ER α - en/of ER β -activerende genisteïne metaboliet met hogere activiteit dan genisteïne zelf, en iii) een artefact dat niet biologisch relevant is, en specifiek is voor de op luciferasereportergeractiviteit gebaseerde assays. De gegevens die in Hoofdstuk 4 worden gepresenteerd, wijzen erop dat ER betrokken is bij de inductie en ook de superinductie van de luciferase reportergeractiviteit, en dat de superinductie door genisteïne kan worden toegeschreven aan stabilisatie (verminderde degradatie) van het luciferase reporterenzym, waarbij deze stabilisatie en dus ook de extra verhoging in de luciferaseactiviteit in de bioassays optreedt bij genisteïneconcentraties boven 1 μ M. De resultaten wijzen erop dat het fenomeen van superinductie niet biologisch relevant is, maar eerder het gevolg van een posttranscriptioneel effect op de eiwitstabiliteit.

Om de mechanismen die ten grondslag liggen aan de door genisteïne geïnduceerde effecten op de celproliferatie van T47D-ER β -cellen met variabele cellulaire ER α /ER β -ratio, zoals beschreven in Hoofdstuk 3, nader op te helderen wordt in **Hoofdstuk 5** gebruik gemaakt van transcriptomics en van kwantitatieve op SILAC-gebaseerde proteomics. Daarmee werden de via ER α en ER β veroorzaakte gevolgen op gen- en eiwitexpressie in T47D-ER β -borstkankercellen, die aan het phyto-oestrogen genisteïne werden blootgesteld, geanalyseerd. Gebruik makend van de humane kankercellijn T47D-ER β met tetracycline-afhankelijke expressie van ER β , werd het effect van een variërende intracellulaire ER α /ER β -ratio op genisteïne-geïnduceerde gen- en eiwitexpressie gekarakteriseerd. De verkregen data laten zien dat in T47D-ER β -cellen die alleen ER α tot expressie brengen, transcriptomics- en proteomics- data aangeven dat genisteïne vooral genen en eiwitten beïnvloedt die betrokken zijn bij snelle celgroei en bij celmigratie door dynamische activering van cytoskeletremodellering. De gegevens openbaarden een interactie tussen integrins, focal adhesiekinase (FAK), CDC42 en actin cytoskeleton signalling cascades, dit als gevolg van

blootstelling aan genisteïne van de T47D-ER β -cellen met alleen ER α - en geen ER β -expressie. Bovendien wezen de gegevens die in Hoofdstuk 5 worden beschreven erop dat via ER β -beïnvloede gen- en eiwitexpressie de via ER α -geïnduceerde effecten tegengaan. Dit omdat in T47D-ER β -cellen die ER β tot expressie brengen en die aan genisteïne werden blootgesteld, een duidelijke downregulering werd aangetoond van genen en eiwitten die betrokken zijn bij de celgroei en een inductie van genen en eiwitten die betrokken zijn bij het afbreken van de celdeling en apoptosis. Op deze wijze leidt de door genisteïne gemedieerde activering van ER β tot een verminderde expressie van genen en eiwitten betrokken bij celmotiliteit en het vermogen tot metastase evenals van genen en eiwitten betrokken bij celoverleving. Uiteindelijk kan worden geconcludeerd dat de gevolgen van genisteïne voor proteomics en transcriptomics eindpunten in het T47D-ER β celmodel vergelijkbaar waren met eerder vermelde data voor estradiol, en dat het uiteindelijke estrogene effect afhankelijk zal zijn van het receptorfenotype (de verhouding ER α /ER β) in de bestudeerde cellen of weefsels.

In een verdere studie van de proteomicsresultaten, werd de gegevensanalyse uitgevoerd op basis van een eiwitnetwerkbenadering. De resultaten van deze eiwitnetwerkanalyse openbaarden extra eiwitnetwerken, behalve diegenen die betrokken zijn bij celproliferatie en apoptosis, welke werden beïnvloed door blootstelling van de T47D-ER β -borstkankercellen aan genisteïne. Deze resultaten worden beschreven in Hoofdstuk 6.

Hoofdstuk 6, beschrijft een op eiwitnetwerk gebaseerde analyse van het kwantitatieve SILAC-gebaseerde proteome van T47D-ER β -cellen met tetracycline-afhankelijke variërende intracellulaire ER α /ER β -ratio blootgesteld aan genisteïne. Genisteïne is een phyto-oestrogen dat in hoge concentraties wordt gevonden in sojaproducten. Blootstelling aan genisteïne wordt vaak geassocieerd met diverse gunstige gevolgen voor de gezondheid, met inbegrip van verminderd risico voor borstkanker en osteoporose bij vrouwen tijdens de menopauze. Ook in de netwerkanalyse van de proteomicsdata, beschreven in Hoofdstuk 6, bleken de eiwitnetwerken, betrokken bij celproliferatie en apoptosis, beïnvloed te worden door blootstelling van de T47D-ER β -cellen aan genisteïne. Daarnaast wijzen de data op een genisteïne-geïnduceerde ER β -gemedieerde immuunsuppressie. Genisteïne geïnduceerde ER β activering resulteerde in downregulering van de expressie van de transcriptiefactoren NF- κ B en STAT3, evenals in downregulatie van een verscheidenheid aan cytokines met inbegrip van IL-1 β , IL-18, IFN- γ , en TNF- α . Ook werd een aanzienlijke verhoging van TGF- β -inductie gevonden in genisteïne- behandelde T47D-ER β -cellen die ER β tot expressie brachten in vergelijking met cellen zonder ER β -expressie, en dit was een verdere aanwijzing dat genisteïne geïnduceerde ER β -gemedieerde eiwitexpressie leidt tot downregulering van de immuunsignalering. Alles bij elkaar laten de resultaten van deze studie zien dat naast

celproliferatie en apoptosis, de immuunsignalering een ander belangrijk oestrogeen eindpunt lijkt te zijn dat door blootstelling aan phytoestrogenen wordt beïnvloed.

Concluderende opmerkingen en toekomstige perspectieven

Meer dan tien jaar, sinds de ontdekking van de tweede oestrogeenreceptor, ER β , wordt het belang van de complexe balans in oestrogeensignalering door de twee receptoren ER α en ER β voor de regulering van de menselijke ontwikkeling en reproductie steeds duidelijker. Het is aangetoond dat ER β een antiproliferatie transcriptiefactor in ER α -positieve borstkankercellen is. Vandaar dat oestrogeensignalering afhankelijk is van het evenwicht tussen twee verschillende receptoren (ER α en ER β). Met de studies, beschreven in het huidige proefschrift, is extra inzicht verkregen in het mechanisme van differentiële biologische reacties die door voedingsgeassocieerde, xeno- en fyto-oestrogene stoffen geïnduceerd worden. Hiervoor gebruikten wij de meest recente state-of-the-art technieken. In de huidige thesis werd de nadruk vooral gelegd op de aard van het oestrogeen, dat bij voorkeur ER α (PPT), ER β (DPN, quercetine en genisteïne) en/of beide receptoren (E2) activeert, evenals op het belang van de verhouding tussen de twee receptoren. Het is nochtans belangrijk om op te merken dat de algemene uiteindelijke biologische gevolgen van oestrogene stoffen in (kanker)cellen het resultaat zullen zijn van een complexe interactie tussen diverse mechanismen, die afhangen van de cellulaire context, de ratio tussen de ER-subtypes, maar ook van coactivatoren en corepressoren in de cel, evenals van splicing varianten van de twee receptoren in de cellen. Al deze factoren samen maken nauwkeurige en gerichte reacties op hormonen of anti-oestrogenen mogelijk. Gezien deze overwegingen is het belangrijk dat toekomstig onderzoek ook aandacht besteedt aan de volgende aspecten:

- I) de rol van coactivatoren en corepressoren die kunnen variëren in de relevante cellen of de weefsels,
- II) de rol van splicing varianten van de twee receptoren ER α en ER β die kunnen variëren binnen de relevante cellen of het weefsel,
- III) een vergelijking van de daadwerkelijke ER α /ER β -verhouding in het T47D-ER β -modelsysteem in vergelijking met de ER α /ER β -verhoudingen die in *in vivo* weefsels worden waargenomen,
- IV) integratie van de reeds verkregen kennis in de risicobeoordeling van supplementen met phyto-oestrogen die reeds op de markt worden verkocht.

ERs associëren met verschillende soorten coactivatoren en corepressoren, afhankelijk van bindingsaffiniteiten en relatieve overvloed van deze factoren (14, 15). Er zijn verscheidene coactivatoren en corepressoren van ERs (16). Verschillen tussen ER α en ER β in de rekrutering van coactivatoren en corepressoren zijn ook beschreven (17, 18) en daarom zal

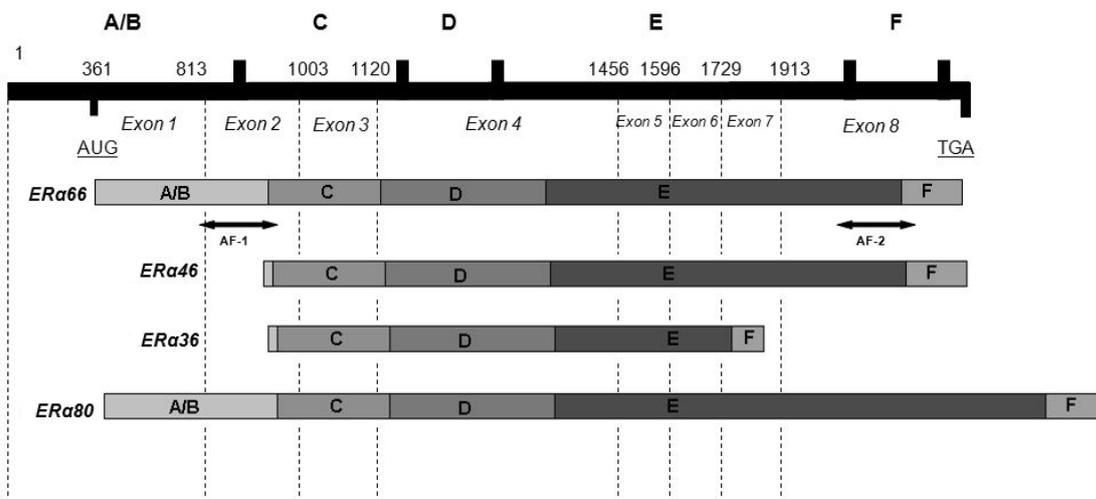
preferentiële binding van bepaalde coactivatoren en corepressoren aan één van de ERs, gevolgen hebben voor de specifieke ligandsignalering en het uiteindelijke biologische effect dat door de ligandbinding wordt veroorzaakt.

NCoR en corepressors SMRT en de p160 familie van coactivatoren worden in veel biologische systemen tot expressie gebracht (19-21). Lage niveaus van SRC-3 zijn aangetoond in menselijk prolifererend endometrium met verhoogde expressie in de late secretorische fase (22) terwijl overexpressie van SRC-3 vaak in borst-, ovaria-, en prostaat kanker (23-25) wordt waargenomen. De gelijke expressieniveaus van CBP, p300, AIB1, GRIP1, p300, NCoR, en SMRT zijn gemeten voor de Ishikawa- baarmoeder- en MCF-7 borstkankercellen (26). Hoge niveaus van SRC-1-expressie zijn gevonden in Ishikawa-cellen en dit zou met de agonistactiviteit van tamoxifen in deze cellenlijn kunnen correleren (26). Wij hebben in onze studies (Hoofdstuk 4) expressie van de ER coactivator, PRMT1 door de T47D-borstkankercellen gezien. De rekrutering van deze coactivator gaat vergezeld van histonmethylering (27, 28). Onlangs is de genexpressie van PRMT1 gebruikt als marker voor een ongunstige prognose voor darmkankerpatiënten (29).

Aldus kunnen andere gebeurtenissen in de cel de transcriptionele respons van de nucleaire receptor beïnvloeden via wijziging in de expressie van bepaalde coregulatoren en daarom voorspelt men dat significante verschillen in coactivator- en corepressorexpressie in specifieke cel- en weefseltypes belangrijke determinanten zouden zijn van de specifieke receptormodulatoractiviteit.

Bovendien zou de distributie van bepaalde splicing varianten van beide ERs in acht moeten worden genomen in het kader van de weefselreactie op oestrogenen en cofactorrekrutering, aangezien zij differentiële en soms antagonistische eigenschappen hebben en hun relatieve concentraties de biologische reacties op hormonen beduidend zou kunnen beïnvloeden. De belangrijke fysiologische rol van de splicing varianten van ERs in de ontwikkeling van borstkanker is echter momenteel verre van duidelijk en zou een essentiële determinant voor klinische parameters kunnen zijn.

De volledige ER α - en ER β -eiwitten zijn ongeveer 66 en 59 kDa, respectievelijk (30, 31), maar als gevolg van alternatieve splicing kunnen van beide receptoren verschillende isovormen ontstaan. Voor ER α zijn er meer dan 20 alternatieve splicing varianten in borstkanker en ander tumorweefsel aangetoond (32), drie van hen met relevante functionaliteit, terwijl minstens vijf varianten van ER β bekend zijn in de mens (33). De twee meest gerefereerde ER α - isovormen, die een bijzondere rol lijken te hebben, zijn ER α 46 en ER α 36 aangezien van deze varianten wordt gemeld dat ze de genomische acties van de volledige de ER α 66 kunnen tegenwerken (Figuur 1).



Figuur 1: Schematische vergelijking tussen de volledige ER α en zijn meest gerefereerde kortere isovormen.

De **ER α 46** isoform is geïdentificeerd in de MCF7-borstkankercellijn (34), waarin de isoform tegelijk met de volledige ER α 66 tot expressie komt. De aanwezigheid van ER α 46 is ook bevestigd in osteoblasten (35) en endotheelcellen (36). De ER α 46 isoform heeft geen AF-1-(ligand-onafhankelijk transactivatiedomein)activiteit. In tegenstelling tot de volledige ER α 66, medieert isoform ER α 46 geen E2-afhankelijke celproliferatie en hoge niveaus van deze isoform zijn aangetoond in de G0/G1-fase van de celcyclus. Net als ER β , is ER α 46 een potente ligandafhankelijke transcriptiefactor die gevoelig is voor AF-2 (ligand-afhankelijk transactivatiedomein) en een krachtige remmer van AF-1-afhankelijke transcriptie (36). Coexpressie van ER α 46 met ER α 66 in een SaOs-osteoblastcellijn resulteerde in receptorconcentratie-afhankelijke remming van E2-gestimuleerde celproliferatie (37).

De tweede verkorte ER α -isoform, **ER α 36**, werd onlangs beschreven (35), en het is aangetoond dat deze vorm noch de AF-1- noch de AF-2- transactivatiefuncties van de volledige ER α heeft. Nochtans heeft het de functionerende DNA-bindende en ligandbindende domeinen (DBD en LBD). ER α 36 bevat een exon coderend voor myristoyleringsplaatsen, waarmee een interactie met het plasmamembraan wordt gesuggereerd. Zonder functionele AF-1 en AF-2, heeft ER α 36 geen directe transcriptionele activiteit. Desalniettemin is het een krachtige remmer van volledige afhankelijke ER α - en ER β -transactivatie (38). Hoewel het de transcriptionele activiteit niet heeft, kan het de wel de niet-genome ER-routes activeren zoals MAPK/ERK- signalering als respons op E2, wat van bijzondere betekenis is als respons op antioestrogenen zoals tamoxifen, 4OH-tamoxifen en ICI-182.780 (38).

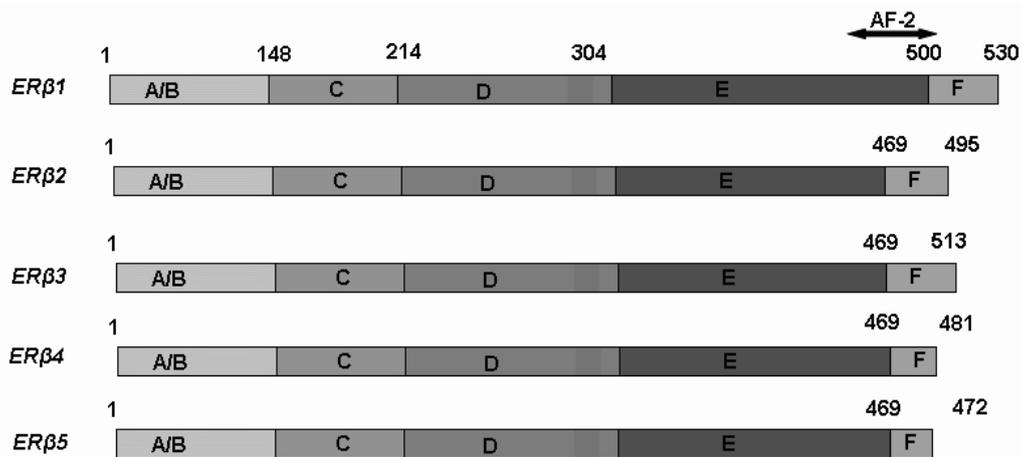
De **ER α 80** isoform werd ontdekt in de MCF7: 2A-cellijn, een subkloon van de MCF7-cellijn, geïsoleerd na lange tijd groei zonder E2. Deze ER α 80 isoform werd geproduceerd

door verdubbeling van exons 6 en 7 (39). Tot dusver is er geen duidelijke functionaliteit beschreven. Een lijst van geselecteerde ER α -splicing varianten en hun expressie in diverse borstweefsels (normaal en tumor) en in borstkankercellijnen is weergegeven in Tabel 1.

Tabel 1: Lijst van geselecteerde ER α -splicing varianten en hun expressie in diverse borstweefsels (normale en tumor) en in borstkankercellijnen.

Splicing varianten								Referenties	
	Borst	MCF7	T47D	MDA-MB-231	MDA-MB-435	BT-474	BT20		ZR-75
ERα36	+			+					(40, 38, 41)
ERα46 (or ER$\alpha$$\Delta$1)		+							(34)
ER$\alpha$$\Delta$2	+	+	+		+				(42, 43, 44, 45, 46, 32)
ER$\alpha$$\Delta$3	+	+	+	+					(42, 45, 44, 43, 47, 48, 46, 49)
ER$\alpha$$\Delta$4		+			+			+	(50, 43, 44, 32, 45)
ER$\alpha$$\Delta$5	+	+	+	+	+	+	+	+	(51, 43, 44, 45, 43, 52, 53)
ER$\alpha$$\Delta$6	+								(45, 44)
ER$\alpha$$\Delta$7	+	+	+	+					(42, 54, 45, 44, 54, 46)
ER$\alpha$$\Delta$5,7	+								(43)

De aanwezigheid van ER β isovormen is bevestigd in diverse menselijke cellijnen evenals in een breed scala van weefsels (55, 56). Momenteel is hun functionele betekenis niet duidelijk. De enige volledig functionele ER β -isovorm is ER β 1, een volledig eiwit met LBD en actief AF-2-domein (Figuur 2). **ER β 2, 4 en 5** hebben een verkorte Helix 11 en een volledige Helix 12 is slechts aanwezig in ER- β 1 en β 2. Hoewel de Helix 12 in ER β 2 een andere oriëntatie heeft dan in ER β 1, doordat die een kortere Helix 11 heeft. Men heeft gerapporteerd dat een verplaatste Helix 12 in ER β 2 de toegang van de ligand tot de bindingspocket beperkt. Als gevolg van deze veranderde structuur, kunnen ER β 2, 4 en 5 geen homodimeren vormen en hebben ze geen transcriptionele activiteit van zichzelf, hoewel wel is getoond dat zij kunnen heterodimeriseren met ER β 1 na E2-binding en de AF-2 bemiddelde transcriptionele activiteit kunnen verbeteren (56). De studies over de interactie tussen de verschillende ER β isovormen met ER α zijn zeer beperkt. Echter van **ER β 2** (ook wel **ER β cx** genoemd) is aangetoond dat het de DNA-binding van ER α 66 beperkt en zijn transcriptionele activiteit remt op dezelfde manier als beschreven voor ER β 1 (57).



Figuur 2: Vergelijking tussen volledige lengte ERβ en zijn gerefereerde isovormen.

Twee nieuwe exon-deleted varianten werden ontdekt in de kankercellijn MDA-MB-231, **ERβΔ1,2,5** en **ERβΔ1,2,5,6** van ongeveer 35 en 28 kDa (58). Beide eiwitten bevatten mogelijk geen AF-1, en zijn het resultaat van deleties van DBD en LBD. Daarom wordt van deze twee varianten verwacht dat ze geen, of beduidend verminderde ligandafhankelijke en ligandonafhankelijke activiteiten hebben, en hun expressie beïnvloedde de groei van geteste kankercellijnen dan ook niet (Tabel 2). Een lijst van geselecteerde ERβ-splicingvarianten en hun expressie in diverse borstweefsels (normale en tumor) en borstkankercellijnen wordt gegeven in tabel 2.

Alles bij elkaar kan geconcludeerd worden dat meer diepgaande analyse van de mechanismen en consequenties van oestrogenreceptor splicingvarianten en corepressoren/coactivatoren nodig is, omdat zij mogelijk extra mechanismen aan het licht brengen die aan de weefselafhankelijke modulatie van de ER response ten grondslag liggen.

In de toekomst zou het mogelijk kunnen zijn de aard van het ER-subtype door specifieke receptor-isovormtransfectie te bepalen, door technologieën zoals chromatin-immunoprecipitatie studies (ChIP), en dan ook de effecten van gelijktijdige expressie van coactivatoren en repressoren in een specifiek celtype te gebruiken om verder inzicht te verkrijgen in het mechanisme op basis waarvan oestrogenen en oestrogenachtige stoffen weefsel-specifieke gevolgen veroorzaken.

Literatuurgegevens laten verder zien dat de fysiologische niveaus van ERα en ERβ zodanig kunnen variëren, dat één van beide dominant is. Het moet nog worden onderzocht op welke manier de mogelijk range van ERα/ERβ-ratio's in de T47D-ERβ-cel lijn met

toenemende concentraties tetracycline representatief zijn voor fysiologisch relevante variaties in de receptorverhouding in cellen en weefsels *in vivo*.

Tabel 2: Lijst van geselecteerde ER splice-varianten en hun expressie in diverse borstweefsels (normale en tumor) en borstkankercellijnen.

Splicing varianten	Referenties						
	Borst	MCF7	T47D	MDA-MB-231	MDA-MB-435	BT20	
ERβ2	+	+	+	+		+	(59, 60, 61, 62, 7, 56)
ERβ3	+						(61)
ERβ4	+		+		+		(55, 61, 63)
ERβ5	+	+	+	+	+	+	(59, 61, 55, 30, 56, 7)
ER$\beta$$\Delta$2	+			+			(64)
ER$\beta$$\Delta$3	+						(64, 65)
ER$\beta$$\Delta$4	+						(64, 65)
ER$\beta$$\Delta$5	+	+		+			(64, 66, 67)
ER$\beta$$\Delta$6	+						(64, 67)
ER$\beta$$\Delta$1,2,5		+		+			(58)
ER$\beta$$\Delta$1,2,5,6		+		+			(58)

Diverse studies laten zien dat fysiologische niveaus van ER α en ER β afhankelijk kunnen zijn van het cel- of weefseltype, met bijvoorbeeld ER α die dominant is in de lever of in thecale en interstitiële cellen in de eierstok en ER β die dominant is in geïsoleerde granulosecellen (68), in cellen van menselijk umbilical ader-endotheel, prostaattumoren (69) en in het epitheel van de dikke darm, prostaatcellen of granulosecellen binnen de eierstok (68) in normale secretorische lumenale prostaatcellen (70) of in de menselijke testis in zich ontwikkelende spermatiden (71).

Aangezien de T47D-ER β -cellijn uit een menselijke borsttumor (T47D) komt, reflecteren naar verwachting de niveaus van ER α en ER β in de cellijn, wanneer deze gekweekt wordt in aanwezigheid van 1000 ng/ml tetracycline (geen extra ER β - expressie) de fysiologische niveaus in dit tumorweefsel. De expressie van ER β in de T47D wildtype cellen bleek onder

de detectielimiet (72) te liggen. Deze lage niveaus van ER β -expressie in borsttumorsewefsel komen overeen met het verlies van ER β -expressie in kwaadaardige kankerweefsels in vergelijking met goedaardige tumoren of normale weefsels, en het feit dat de verhouding van ER α /ER β -expressie in kwaadaardig weefsel hoger is dan in normale weefsels (6, 67, 73, 74). De informatie over de niveaus van ER α en ER β in de T47D-cellen wanneer ze gekweekt worden zonder tetracycline (volledige expressie van ER β) kan worden afgeleid uit de literatuur (72) waar men aantoonde dat, gebaseerd op mRNA-niveaus, de T47D-ER β -cellijn bij 0 ng/ml tetracycline (volledige expressie van ER β) slechts 4 keer meer ER β dan ER α bevat. Een viervoudig verschil in het niveau van de twee receptoren lijkt fysiologisch relevant wanneer dit vergeleken wordt met literatuurgegevens over andere weefsels. Kwantificering van de niveaus van beide receptortypes in T47D-ER β -cellen die worden gekweekt in aanwezigheid van variërende niveaus van tetracycline en het vergelijken van de niveaus die met dezelfde techniek in dezelfde studie in relevante weefsels worden gekwantificeerd, zal het modelsysteem dat in de huidige studies werd gebruikt verder optimaliseren en valideren.

Tot slot kan men zich afvragen of de resultaten die in het huidige proefschrift worden verkregen, aanwijzingen verstrekken voor de huidige discussie over de zogenoemde riks-benefit van phyto-oestrogensupplementen die reeds op de markt worden verkocht. Momenteel neigt de consument te geloven dat alleen omdat een product in de natuurlijke voedingswinkel verkocht wordt, het gezond is en dat "natuurlijk" equivalent is voor „veilig”. Niets is minder waar. Hoewel er verscheidene gezondheidsclaims worden gekoppeld aan phyto-oestrogene supplementen, bijvoorbeeld dat ze het risico om borstkanker te ontwikkelen verminderen of overgangssymptomen verminderen, is er ook bewijsmateriaal dat aangeeft dat phyto-oestrogenen kankerontwikkeling kunnen bevorderen en andere ongewenste bijwerkingen op bijvoorbeeld het centrale zenuwstelsel en het immuunsysteem kunnen hebben. De resultaten van het huidige proefschrift wijzen er duidelijk op dat de verschillen tussen phyto-oestrogenen enerzijds en estradiol anderzijds kleiner kunnen zijn dan over het algemeen wordt gedacht, en dat het uiteindelijke biologische effect van deze oestrogenen sterk zal afhangen van het weefsel waarnaar wordt gekeken en dat de effecten van phytoestrogenen en estradiol mogelijk zelfs vergelijkbaar zouden kunnen zijn. De verkregen resultaten spreken daarmee het algemene idee tegen dat phyto-oestrogenen gezond zijn omdat zij ER β activeren, terwijl estradiol ongunstig kan zijn omdat het ER α activeert. Het uiteindelijke biologische effect wordt beïnvloed door een complexe interactie tussen de agonist kenmerken maar ook de kenmerken van de cellen en de weefsels zijn van belang, met inbegrip van de intracellulaire verhouding van de twee receptoren, de aanwezige receptor-splicing varianten en de coactivatoren en corepressoren die aanwezig zijn. Daarom lijkt het waarschijnlijk dat de gevolgen van phytoestrogenen per weefsel zullen variëren en voor het ene weefsel en biologische eindpunt gunstig kunnen zijn, terwijl ze bij eenzelfde dosis juist ongunstig kunnen zijn voor een ander weefsel en biologisch eindpunt. Voorts is er behoefte

aan een beter inzicht in de gezondheidsimplicaties van de dosis, de biologische beschikbaarheid en de gevolgen op lange termijn van deze op phyto-oestrogen gebaseerde voedingssupplementen, en als gevolg daarvan, een behoefte aan een betere en meer gedetailleerde evaluatie van hun risico's en voordelen.

De resultaten van het huidige proefschrift hebben mogelijke gelijkenissen tussen E2 en het phyto-oestrogen genisteïne laten zien, die zich niet alleen beperken tot gunstige effecten en dit zou moeten worden meegenomen in de veiligheidsbeoordeling van phyto-oestrogene voedingssupplementen.

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Ana María Sotoca Covalada was born on the 25th of July 1976, in the city of Palencia, Spain. In 1994, she completed her secondary education. The same year she entered the University of Valladolid to study “Agricultural Technical Engineer”, and finished her Bachelor of Sciences (BSc) with the specialization of agricultural enterprises in 2000. During several years she worked as an engineer at the companies of Ibérica de Tecnología Avícola S.A. (Valladolid, Spain) and later at Alvarez de la Lama S.L. (Palencia, Spain).

In 2003 she moved to the Netherlands to start her MSc studies in “Bioinformatics” at Wageningen University. During this period, she completed her master thesis at the department of Molecular Design and Informatics in N.V. Organon, and in 2005 she received her MSc degree in Bioinformatics.

She continued working at the department of Molecular Design and Informatics as a scientific researcher until she joined the section of Toxicology at Wageningen University to start her PhD studies. She carried out her research on “Unravelling the mechanism of differential biological responses induced by food-borne xeno- and phyto-estrogenic compounds” in collaboration with the Laboratory of Biochemistry. The results from this project are summarized in this thesis.

In 2010, Ana was appointed as a post-doctoral researcher at the Department of Applied Biology at Radboud University in Nijmegen.



Overview of completed training activities

Discipline specific activities

General toxicology (Wageningen, 2006)
Pathobiology (PET-Utrecht, 2007)
Medical & Forensic toxicology (PET-Utrecht, 2007)
Organ toxicology (PET-Utrecht, 2006)
Mutagenesis and carcinogenesis (PET-Leiden, 2007)

Meetings

User meeting Wageningen NMR center (Wageningen, 2007)
Program proteomics tour, Thermo Fisher Scientific (Utrecht, 2008)
NPC meeting (Utrecht, 2009)
LC-MS/MS user meeting, Thermo Fisher Scientific (Breda, 2009)
Nuclear Receptor Meeting Benelux (Leiden, 2009)
Prote MMX: Strictly Quantitative (Chester-UK, 2010)

General courses

VLAG AIO-week (VLAG-Ermelo, 2006)
Proteomics (VLAG-Wageningen, 2008)
Techniques for writing and presenting a scientific paper (VLAG,-Wageningen, 2007)
Advanced visualisation, integration and biological interpretation of –omics data (VLAG-Wageningen, 2009)

Training in mass spectrometry techniques (Biqualy-Wageningen, 2009)

Optionals

Two scientific presentations at Schering Plough
Attending research discussions at Toxicology
Attending research discussion at Biochemistry
Preparation of research proposals



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