TRANSCRIPTIONAL CONTROL OF ONCOGENIC PROCESSES IN BREAST CANCER CELLS BY THE ESTROGEN RECEPTORS

A Dissertation Presented to

the Faculty of the Department of Biology and Biochemistry

University of Houston

In Partial Fulfillment

of the Requirements for the Degree

Doctor of Philosophy

By

Philip Jonsson

May 2014

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Philip Jonsson

APPROVED:

____
Dr. Cecilia Williams, Chairman

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Dr. Paul Webb

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Dr. Richard Willson

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Dr. Preethi Gunaratne

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Dean, College of Natural Sciences and Mathematics

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Abstract

The estrogen receptors are fundamental factors in human biology. As transcriptional factors regulating gene programs controlling many processes in the body, they are key in both development and disease. Also, as nuclear receptors they can be activated or blocked by specific ligands, making them excellent targets for therapeutics. This dissertation focuses on the study of the estrogen receptors, both the alpha and beta isoforms (ERα and ERβ, respectively), and how they regulate gene transcription in human breast cancer.

The proliferative role of ERα in breast cancer remains poorly understood. Here we show that the ion channel KCNK5 is a direct transcriptional target of ERα in breast cancer cell lines MCF7 and T47D. Also, we show that this is reflected by changes in the ion channel's protein. Furthermore, silencing of the ion channels expression reduces cellular proliferation, as well as the estrogen-induction of proliferation. This uncovers ion channels as potential factors in the proliferation of breast cancer, as well as potential targets in novel treatment approaches.

ERα's role as a transcription factor has predominantly been studied in regards to its regulation of protein-coding genes. Herein, we show that ERα also regulates non-coding RNAs, such as long non-coding RNAs and pseudogenes. We also

potentially uncover novel protein-coding targets, by the use of novel RNA-sequencing technology, and the use of microarrays.

The other estrogen receptor, ERβ, is less characterized, but it is considered to be anti-proliferative in breast cancer and its activation suggested as a potential future therapy. However, discordant results of expression in breast tumors, correlation to prognosis, and tumor-suppressive function in cell lines have made this a debated field. We explore its role in breast cancer cells further and show that, in certain contexts, ERβ is not able to suppress breast cancer cell proliferation, nor, as often suggested, counteract ERα-mediated signaling. This warrants further studies into whether its activation in breast cancer is a desirable treatment.

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1. Introduction

1.1 Breast cancer

1.1.1 Epidemiology

Breast cancer is the second most common cancer globally and among women the cancer with highest incident. In 2012, an estimated 1.7 million new cases were diagnosed, accounting for a quarter of all cancers. Furthermore, breast cancer ranks as the fifth cancer in terms of overall lethality, with 522,000 deaths annually. For female-specific cancer deaths, it is the first and second most common cause for death, in less and more developed regions, respectively, of the world (1). In the US, according to the American Cancer Society, there were estimated 232,340 new cases of female breast cancer and 39,620 deaths, in 2013.

After lung cancer, this puts breast cancer as the most frequent cause of cancer-related death in women. A decrease in death rates has been observed since the late 1990s and is attributed to earlier detection, improved treatment, and possibly the lessened us of menopausal hormone therapy (2). Breast cancer incidence rates, however, remain essentially stable in the US since 2005, whereas they increase elsewhere (1–3).

1.1.2 Etiology and pathophysiology

The causes and development of breast cancer are still elusive. Obvious cancer-associated factors such as heredity, obesity, and exposure to exogenous agents are implied, including an increased exposure to the female sex hormones, e.g. late menopause, early menarche, delayed childbirth or nulliparity, and hormonal therapy and contraceptive usage. Additionally, socioeconomic status has been indicated (4). Although family history is the strongest predictor of breast cancer after gender and age, only 5 to 10% of cases are due to known familial mutations (4,5). These are primarily in either of the two well-established susceptibility genes *BRCA1* and *BRCA2*, although other high-penetrance breast-cancer susceptibility genes have been identified (such as *TP53*, *STK11*, *ATM*, *BRIP1*, *CHEK2*, *NBN*, *RAD51C*, and *PTEN*). This list is expected to grow as new genomic techniques are used to uncover the nature of human cancers (6,7).

Breast cancer is a highly heterogeneous disease with at least 18 different acknowledged histological types, of which the most common are mammary ductal and lobular carcinoma (8). These carcinomas develop as neoplasms from the layer of epithelial cells that line the lumen of the milk ducts or the lobules, respectively (9). The epithelial cells are enveloped by myoepithelial cells and the basement membrane. When confined by these layers, the carcinoma is said to be *in situ*. This is a precursor stage to invasive breast cancer, which is when the tumor penetrates the surrounding layers. Various genetic and epigenetic alterations, such as increased responsiveness to hormones and growth factor and abnormal expression of oncogenes and tumor-suppressor genes, can occur in the transition between these two states (10,11). Ductal and lobular breast carcinomas represent 50-80% and 5-15% of breast tumors, respectively, and are therefore the most studied types (8,12,13). The other, so called, “special types” of breast cancer are associated with particular tumor characteristics, but the utility of these in diagnostics and treatment has not been realized due to lack of standards and variability in classification (14).

Breast cancers are also classified according to grade and expression of biomarkers. The grade of a breast cancer is scored based on morphological differentiation, nuclear atypia, and mitotic rate. Pathologists also assess include the size, surgical margins, and spread axillary lymph nodes (15). Although fast and reliable in

determining cancers of different prognosis, the histopathology of breast cancer is limited in informing the choice of therapy. Hence, the trio of biomarkers consisting of estrogen receptor alpha (ERα), progesterone receptor (PR), and human epidermal growth factor receptor (HER2), is assessed by immunohistochemistry in the clinical setting to determine treatment approach. Accordingly, breast cancers are divided into ER-positive (ERα+/PR+), HER2-positive (ERα−/HER2+), and triple negative (ERα−/PR−/HER2−) tumors. Patients with ER-positive tumors have the best overall outcome, followed by those with HER2-positive. Triple-negative tumors have the poorest average outcome and lack a targeted therapy (16).

1.1.3 Molecular subtypes

Aside from histological types and grade, breast cancers can also be classified according to molecular characteristics. Additional methods to systematically classify tumors do so by use of gene-expression signatures. These so called molecular subtypes of breast cancers, categorizes tumors according to their gene-expression patterns into luminal A, luminal B, basal-like, HER2, and normal-breast-like tumors. Like the histological classes, these subtypes have prognostic value. The luminal A and B subtypes represent both ends of the continuum made up of most ERα-positive tumors, with the former subtype exhibiting better outcome and the latter worse outcome, respectively (17–20). Conversely, the

basal-like subtype, which is similar to the triple-negative type, has the worst prognosis of all subtypes (18–20). These so called gene signatures have been employed in testing platforms, which so far serve supplemental roles to traditional histopathological methods (21). It is of note that this approach to characterizing breast cancer is suboptimal, emphasizing the heterogeneous nature of the disease, for example in showing that different histological types may have similar transcriptomes, yet associate with different patient prognosis (13). Among current efforts to extensively characterize the complexity of breast cancers is The Cancer Genome Atlas Network's integrative analysis of genomic, proteomic, and transcriptomic data from thousands of samples to better understand the tumor etiology (7). Ultimately, the goal is to utilize the information gained from this type of tumor characterization to tailor therapy by predicting outcome and response to treatment (22).

1.1.4 Role of estrogen

Expression of hormone receptors in the tumor is among the most important biomarkers for treatment decisions and prognosis. The involvement of the hormone estrogen in mammary carcinogenesis was proposed in the middle of last century, based on finding in studies with mice (23), and was later supported by epidemiological findings (24). The hypothesis is that abnormal exposure to the hormone stimulates cellular proliferation, resulting in neoplasia. The increased

cell division increases the chance of somatic mutations, which lead to transformation and breast cancer (25). Aforementioned estrogen-associated risk factors also support this notion. As an alternative to the mitogenic role of estrogen in the development of breast cancer, there also exists a hypothesis that estrogen and its metabolites are genotoxic, thereby triggering neoplastic transformation (26).

1.1.5 Available treatments

Current treatments options for breast cancer are surgery, radiation therapy, chemotherapy, hormonal therapy, and targeted treatments. Adjuvant systemic treatment is particularly beneficial for patients at high risk of relapse. Depending on hormone-receptor status (see more below), these patients are candidates for chemotherapy or endocrine therapy. Endocrine treatment options include tamoxifen, aromatase inhibitors, and ovarian ablation or suppression (discussed below). The former two are also options for low-risk patients (27). Targeted therapies for tumors of certain molecular characteristic are available and also the forefront of pharmacological development. For $HER2^{+}$ tumors, the monoclonal antibody trastuzumab is demonstrated to improve survival, and other agents, such as lapatinib, targeting the same molecular pathway are available. Clinical tests of agents acting on other targets are in clinical trials (28). Importantly, the different types of breast cancer, be it histological or molecular, represent the intertumor

heterogeneity of the disease and guide decisions for treatment in the clinic. However, the intratumor heterogeneity of breast cancer, i.e. the diversity in genetic and epigenetic changes between cells within one tumor, is a novel aspect to consider in the design of new therapeutic options (29). In certain cases it might be favorable to target a certain subset of tumor cells, for instances those that exhibit a proliferative or metastatic phenotype, instead of the bulk of the tumor.

1.2 Estrogen

1.2.1 Estrogen and its receptors

Estrogens are produced by the aromatization of testosterone or androstenedione, a reaction carried out by aromatase enzymes (*CYP19A1*). In the premenopausal woman, this conversion occurs mainly in the ovaries, and estradiol (17β-estradiol, E2) is the estrogen of highest concentration and potency. After menopause, estrone (E1) is the predominant estrogen, and its sources are extragonadal sites, especially adipose tissue (30,31).

The estrogen receptors alpha and beta (ERα and ERβ) are encoded from the genes *ESR1* and *ESR2*, respectively. Both ERs are expressed and functioning in multiple tissues in both sexes, despite estrogen being recognized as the female sex hormone. Human and rodent studies have detected expression of either or both

ERs, at mRNA or protein levels, in a wide host of tissues, including the brain and spinal cord, the cardiovascular system, the urogenital tract, the skeletal system, the gastrointestinal tract, adipose tissue, pancreas, liver, kidney, mammary gland, ovary, lung, and prostate (32,33). However, the data on ER expression, in both normal and cancer tissue, are in some cases controversial and complex. Additionally, this field is hampered by technical issues and unspecific reagents related to detection of ERβ.

1.2.2 Structure and function of the estrogen receptors

ERα and ERβ are ligand-activated transcription factors. Their proteins possess similar domains but do not exhibit high sequence similarity in all (Figure 1.1). Notable, their zinc finger-containing DNA-binding domains (DBDs) are 96% similar, whereas the C-terminal ligand-binding domain (LBD) and the N-terminal transactivation function 1 (AF-1) are similar to 53 and 30%, respectively. A hinge domain connecting the LBD and DBD is 30% similar between the two ERs (34). These forms of ERα and ERβ measure 595 (66 kDa) and 530 amino acids (59 kDa), respectively. Both receptors exist in other, less common, isoforms, which will not be discussed here.

The so-called classical action of the ERs is activation through binding to estrogen. Despite the differences in the LDBs, ERα and ERβ bind E2 with similar affinities,

although this is not the case for various other natural or synthetic compounds (35–37). Subsequent to ligand binding, the ERs bind directly to DNA or tethers to other DNA-bound factors, to regulate transcription of target genes. As predicted by the similarity in their DBD, the receptors share many genomic binding sites, but also have unique target sites (38–40). The diverging AF-1 domains, the ligand-independent activation domain, are suggested as explanation to different, and sometimes opposite response, of the receptors to various ligands (41,42). Correspondingly, differences in interactions with coregulatory proteins are explained by differences in the LBD, wherein lies the ligand-dependent activation domain (AF-2) (42). Both ERs are active in homo- or heterodimeric complexes that interact with the transcriptional machinery. In addition to the classical, genomic, function of the ERs, there are also non-genomic, extranuclear, functions of the receptors, whereby they crosstalk with various kinase-signaling pathways (43). Furthermore, these pathways can also impinge on the nuclear actions of the ERs (44). A membrane-bound estrogen receptor has been described. This G protein-coupled estrogen receptor (*GPER1*) has been demonstrated to be activated by E2 and selective ligands, and confer cytosolic signaling through various pathways (45). A schematic of ER signaling pathways is shown in Figure 1.2.

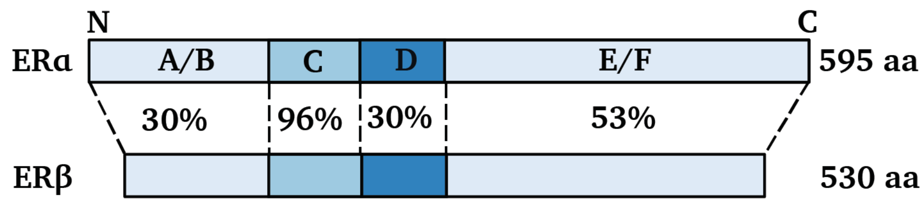


Figure 1.1. Structure of the estrogen receptors. Percentages show amino acid sequence identity. The N-terminal domain (A/B) possesses the ligand-independent AF-1 domain, whereas the AF-2 and LBD domains are C-terminal (E/F). These are connected by the DBD (C) and the hinge domain (D)

1.2.3 Role of estrogen receptors in breast development and disease

Estrogen is a major regulator of the postnatal development of the mammary gland. During this process, ducts stemming grow out to form a branched structure in the mammary fat pad. Pregnancy and lactation causes further structural changes in the mammary gland, to allow for secretion of milk. A role for ERα in the estrogen-controlled process is verified by the fact that ERα knock-out (αERKO) mice have glands akin to that of a newborn (46). The stimulation of glandular development by estrogen in ovariectomized mice and its inhibition by antiestrogens further support this notion (47,48). Unlike that of αERKO mice, the mammary gland of ERβ knock-out mice seems to develop and be capable of lactation, although there are data showing incomplete development (49–51). Disruption of both genes causes a phenotype similar to the αERKO phenotype (52). However, mammary gland development can be restored in the αERKO mice by pituitary grafting, suggesting that the mammogenic process is activated

directly at the gland, as well as by endocrine signals from the pituitary gland (53). In mouse tumor models, lack of ERα delays or disrupts the tumorigenesis, although its expression, in these models, unexpectedly declines throughout tumor progression (54,55).

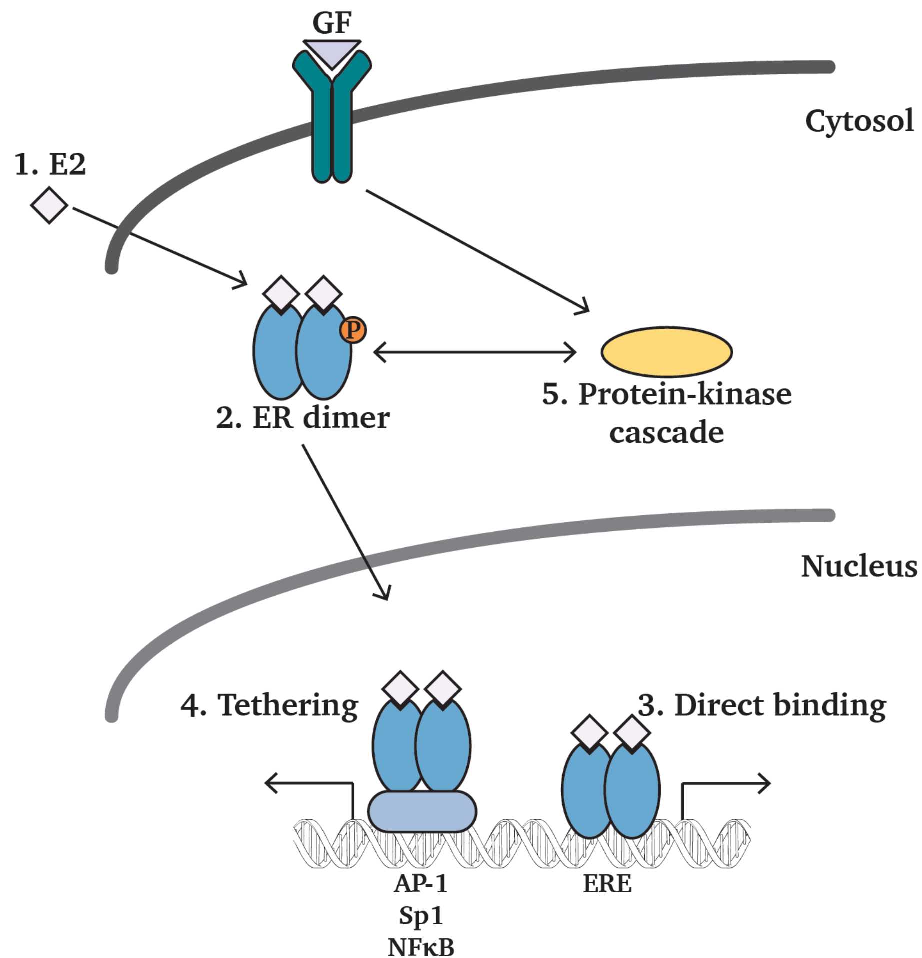


Figure 1.2. Estrogen receptor signaling. The estrogen receptors are activated by ligand binding (1), which causes homo- or heterodimerization (2), followed by transcriptional modulation of target genes through direct (3) and indirect (4) DNA binding to *cis*-regulatory elements. The ERs can also be activated by phosphorylations, and themselves trigger kinase cascades (5).

ERα-positive tumors are, despite the receptors mitogenic function, associated with more differentiated tumors and better prognosis. However, ERα-positive breast cancer remain the majority of cases, its incidence, for unclear reasons, is increasing in the US, and it is the type responsible for most deaths (56,57).

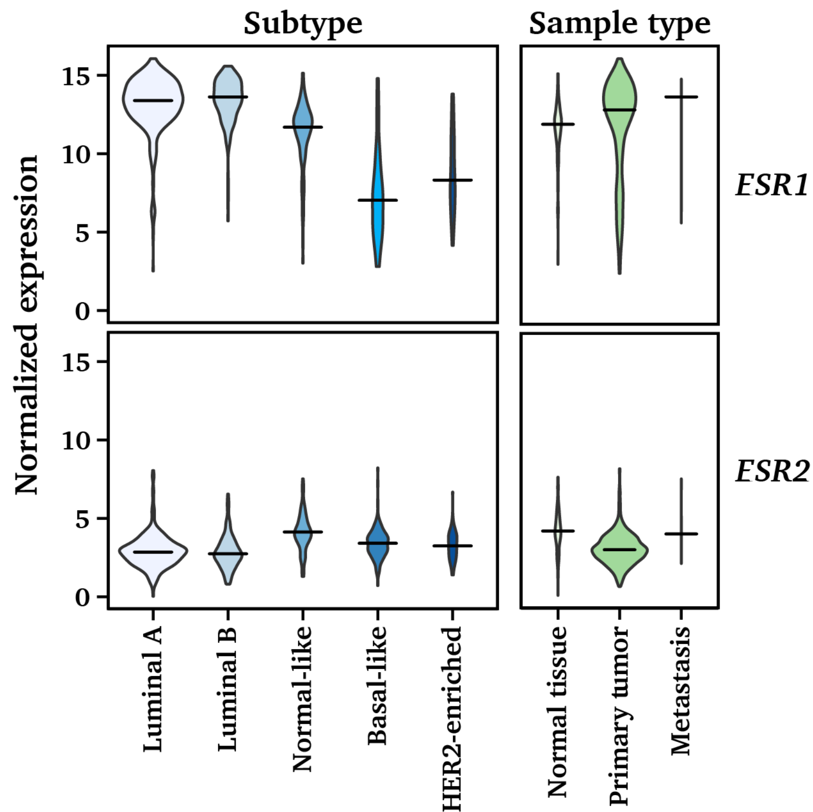


Figure 1.3. Estrogen receptor mRNA expression in clinical samples. The two left panels show normalized mRNA expression for ERα (ESR1) and ERβ (ESR2) in human female breast tumor samples, stratified by molecular subtype. The two right panels similarly show expression of the ERs, in samples from normal and breast cancer tissue. Figure areas in violin plots represent size of subset and crossbars the median value. Expression values are normalized transcript count estimates, according to the RSEM algorithm for RNA-seq analysis (58). Data consisting of 946 and 107 tumor and normal samples, respectively, acquired from TCGA (7).

The role of ERβ is more elusive, though it appears, as mainly supported by *in vitro* experiments, to be a tumors suppressor in the breast (59). Conflicting and confounding data exist regarding its expression and function, and a contradicting relationship between protein levels, as per antibody-based assays, and mRNA levels is often found. Compared to ERα, the mRNA levels of ERβ in tumor samples seem to be consistently low, even lower than levels of ERα in ERα-negative tumors (Figure 1.3). Nonetheless, the common finding in the most recent body of data from immunohistochemical studies of clinical samples is that ERβ is expressed in a majority of breast cancers, either as sole ER or together with ERα (Figure 1.4) (60–73). Mouse models also indicate this to be the case (55). However, studies also find that ERβ expression is decreased, but not lost, in the progression from normal to invasive lesions (51,59,62,74–77). The paradoxical relationship between ERβ's proposed tumor-suppressor function and prevalent tumor expression is further complicated by its association with clinical parameters; there are studies correlating ERβ expression to both proliferative and anti-proliferative markers, possibly suggesting a variable and context-dependent role for ERβ (61,63,65,66,78,79). Ultimately, both ERs function may differ in function in the presence of the other ER, as well as in the presence of various coregulatory proteins.

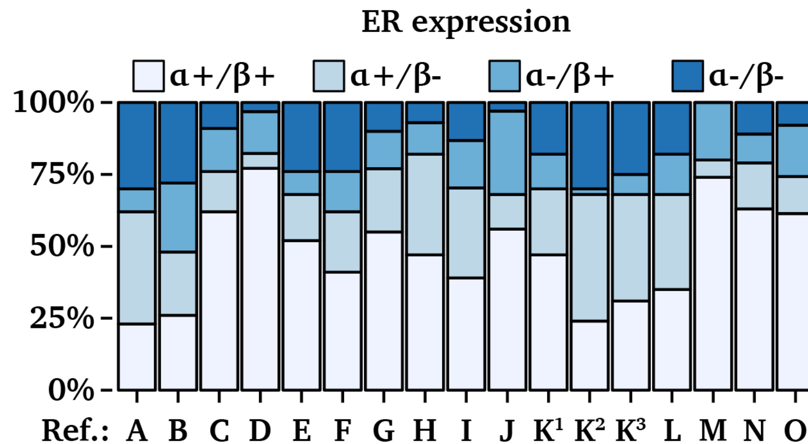


Figure 1.4. Distribution of ER-protein positive tumors in studies of clinical samples. Shown is percentage of total study population classified as according to expression of the two ERs. Sources A-O (61,63,65,66,69,78,80–88). Data from K are results of analyses with different antibodies, accordingly: 1) C-terminal antibody, 2) N-terminal antibody, and 3) total amount.

1.2.4 Targeted therapeutics

Because of the demonstrated effect of ERα on the proliferation of breast tumors, it is an obvious therapeutic target. Aforementioned use of aromatase inhibitors, which ablate the supply of estrogen, and tamoxifen and fulvestrant, which antagonize ERα, are proven to improve survival (89). Nevertheless, relapse and treatment resistance hamper their efficacy in the long term. Almost half of ERα-positive patients fail to respond to tamoxifen, and at least 40% of patients treated with adjuvant tamoxifen, and nearly all with metastatic disease, eventually relapse and die from the disease (90,91). The use of novel selective estrogen-receptor modulators (SERMs), small compounds with both tissue- and receptor-specific effects, remains a promising venue in breast cancer prevention and

treatment (92). Raloxifene, with an efficacy similar to tamoxifen's but lower risk of side effects, is an example of the development in this field. However, as for other treatment alternatives, the possibility of resistance exists for SERMs as well, as is shown by development of raloxifene and tamoxifen resistance (93).

1.2.5 Cell line models of breast cancer

In vitro culture of human breast cancer cell lines is a commonplace model for the study of breast cancer biology. The first estrogen-responsive cell line, named MCF7, was established in the 1970s and it is now a main tool in many molecular-biology laboratories (94). The MCF7 and T47D cells are characterized by a gene expression pattern indicating a luminal epithelial origin and express markers typical of this phenotype, such as E-cadherin and desmoplakin (95–98). They are also tumorigenic in mouse models and may be predictive of clinical performance of drugs (99). Caution should be taken when extrapolating data from breast cancer cell lines, however, as they, for example show higher grades of aneuploidy than tumor cells, exhibit more aggressive phenotypes than their origin, and obviously are not exposed to the tumor microenvironment (97,98,100).

2. Material and methods

2.1 Reagents

All cell culture reagents were from Life Technologies (Carlsbad, CA). 17β-estradiol (E2), and tamoxifen were from Sigma (St. Louis, MO). ICI 182,780 (ICI), PPT, and DPN were from Tocris Bioscience (Bristol, United Kingdom). KB101471 and LY3201 were gifts from KaroBio (Huddinge, Sweden) and Eli Lilly (Indianapolis, IN), respectively. Anti-ERα antibody HC-20, anti-ERβ antibody NBP1-04936, and anti-β-actin antibody AC-15 were from SantaCruz Biotechnology (Dallas, TX), Novus Biologicals (Littleton, CO), and Sigma, respectively. Secondary anti-mouse and anti-rabbit antibodies (NA931V and NA934V, respectively) were from GE Healthcare (Piscataway, NJ), and secondary anti-chicken antibody 31401 from Thermo Scientific (Rockford, IL).

2.2 Cell culture and ligand treatments

Human breast cancer cell lines T47D and MCF7 were acquired from ATCC (Manassas, VA). T47D cells were grown in DMEM/F12 (mixed 1:1) supplemented with 5% heat-inactivated fetal bovine serum (FBS, Sigma) and 100 U/ml penicillin-100 µg/ml streptomycin (PenStrep). MCF7 cells were grown in DMEM supplemented with 5% heat-inactivated FBS and PenStrep. Cells were cultured in a humidified incubator at 37°C in 5% CO2. All cell culture experiments were carried out before 30 passages and with culture free from mycoplasma, as verified by qPCR analysis with primer pair amplifying rRNA gene from multiple mycoplasma species (F: 5'-TCTGAATYTGCCGGGACCACC-3', R: 5'-CTTTCCMTCACKGTACTRGTTCACT-3').

T47D and MCF7 cells were synchronized before ligand treatment by incubation for 24 h in respective medium without phenol red supplemented with 5% dextran-coated charcoal-stripped FBS (DCC-FBS) and PenStrep, followed by 48 h incubation in medium supplemented with 0.5% DCC-FBS. After synchronization, cells were treated with ligands dissolved in ethanol or DMSO (with respective vehicle used as control), in 0.5% DCC-FBS supplemented medium.

2.3 Cell counting and viability assays

Counting of cells for quantitative purpose or seeding was carried out with automatic cell counter Countess (Life Technologies). Trypsinized cells were mixed 1:1 with tryphan blue before counting in two or three replicates.

Cell viability was determined using the MTS assay (Celltiter 96 AQueous One Solution Cell Proliferation Assay, Promega, Madison, WI) following the manufacturer's instructions. The assay was always run in at least triplicates and absorbance readings were carried out before saturation on a SpectroMax M5 Microplate Reader (Molecular Devices, Sunnyvale, CA).

2.4 RNA extraction and cDNA synthesis

RNA was extracted from cultured cells with QIAzol (Qiagen, Germantown, MD) or TRIzol (Life Technologies) and purified with RNeasy Mini Kit (Qiagen), according to the manufacturer's instructions, including on-column DNA digestion with deoxyribonuclease I (DNase I, Qiagen). For microarray and sequencing experiments, the RNA integrity was verified to be > 8, using a 2100 Bioanalyzer and application-appropriate kit (Agilent, Santa Clara, CA). For other experiments, the purity of extracted RNA was verified at measurement of concentration with NanoDrop 1000 Spectrophotometer (NanoDrop, Wilmington, DE).

Synthesis of cDNA was carried out in a total volume of 20 µl using 0.4–1 µg of RNA to which 0.1 nmol of random-hexamer primers (Integrated DNA Technologies, Coralville, IA) were added. The mixture was incubated for 10 min at 70°C and 5 min on ice after which 1× first-strand buffer, 5 mM dithiothreitol (both from Life Technologies), and 0.5 mM deoxynucleotide triphosphates (Sigma) were added. After addition of 200 U Superscript III (Life Technologies), the reaction was started at 25°C for 10 min, and continued at 46°C for 1 h. The enzyme was deactivated at 70°C for 15 min.

2.5 Quantitative polymerase chain reaction

For each qPCR, 10 ng of cDNA were used, with 1 pmol of forward and reverse primer and 1× Fast SYBR Green Master Mix (Life Technologies). Samples were run in duplicates or triplicates with no-template control on a 7500 Fast Real-Time PCR (Life Technologies). Melt-curve analysis was performed to ascertain specific amplification. Primers for cDNA were, if possible, designed to span introns to avoid amplification of genomic DNA. Analysis of the relative expression levels was made using the $\Delta\Delta C_T$ method, thereby determining differences in fold change and standard deviation in transcript levels. C_T values were obtained from the linear phase of the logarithmic amplification using the 7500 software. Gene expression was normalized to *GAPDH*, *ARHGDIA*, or *36B4* mRNA. Biological replicates

analyzed in separate qPCR runs were combined according to previously described standardization procedures (101).

2.6 Immunoblotting

Cells were washed twice in cold PBS, then scraped of the plate in cold PBS and transferred to a microcentrifuge tube. Subsequently, they were pelleted by centrifugation at 1,000 RPM for 10 min at 4°C. Pellets were resuspended in 50-200 µl RIPA buffer (Thermo Scientific, volume according to size of pellet) with 1× complete protease inhibitor (Roche Applied Science, Indianapolis, IN). Cell suspension was incubated at 4°C for 20 min on an agitating thermomixer before centrifugation at full speed for 20 min at 4°C. The supernatant was collected and protein concentration measured by the Pierce 660 nm Protein Assay (Thermo Scientific), according to manufacturer's instruction and with a BSA standard.

2.7 Statistical analyses

Unless otherwise stated, statistical significance of data was assayed using two-sample Student's t-test with two-tailed distribution, assuming homoscedasticity, error bars show one standard deviation, and asterisks are used as follows: * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

3. Chapter 1: Estrogen receptor alpha regulates pro-proliferative ion channel KCNK5

3.1 Introduction

Our group has previously reported a microarray study aimed at understanding the interactions of ERα and ERβ at the level of gene regulation in breast cancer cells (102). In this screen, one of the genes most highly induced by estrogen in T47D cells was the two-pore domain potassium channel $K_{2P}5.1$, more commonly known as KCNK5 or TASK-2. Interestingly, in T47D cells overexpressing ERβ, we found downregulation of KCNK5, suggesting that this gene is regulated in opposite ways by both types of ER (102).

KCNK5 is a pH-sensitive potassium channel expressed in many different tissues including liver, pancreas, small intestine, and kidney (103). KCNK5 currents are activated by alkaline intra- or extracellular pH and inhibited by acidic pH on either side of the membrane (104,105). KCNK5 channels are insensitive to the classical potassium channel blockers tetraethylammonium (TEA) and 4-aminopyridine but are inhibited by quinidine (103) and the antiarrhythmic agent clofilium (106). The expression and function of KCNK5 channels in cells derived from mammary epithelium have not been previously studied. Aside from important functions of this ion channel in the control of HCO_3^- excretion by the kidney (107), KCNK5 is required for the regulatory volume decreases in response to hypotonicity (106,108) and during apoptosis (109). Recently, a role for this channel in central chemoreception has been described in mice (110).

We hypothesized that this ion channel may be important in breast cancer, based on literature proposing that potassium channels are potential targets for cancer therapeutics (111). Different channels, although not KCNK5 specifically, have been shown to have effects on proliferation, sensitivity to growth signals, evasion of apoptosis, angiogenesis, and metastasis and invasion in some cancers (112), and their inhibition is associated with decreased cell proliferation (113,114). The mechanisms underlying the role of potassium channels in cell proliferation are poorly understood and could result from effects on membrane potential, calcium

homeostasis, and/or cell volume regulation, all of which can influence proteins directly involved in the cell cycle (112,115–120). Potassium channels can also control cell proliferation by regulating the activity of transporters involved in pH control (121–124). In the present study we describe the induction of KCNK5 by 17β-estradiol (E2) in ERα-positive breast cancer cell lines and provide evidence that this channel is required for estrogen-induced proliferation.

3.2 Supplemental material and methods

3.2.1 siRNA transfection

A pool of four siRNAs (ON-TARGETplus SMARTpool) was used to silence KCNK5. As a control, a non-specific siRNA was used. Transient transfections were carried out using DharmaFECT 1 Reagent (all siRNA products from Dharmacon, Waltham, MA) according to manufacturer's instructions. All transfections were done with cultures in antibiotic-free medium and transfection reagent was washed out 24 h after transfection.

3.2.2 ChIP-qPCR

ChIP was carried out using 2 µg anti-ERα antibody or normal rabbit IgG (Santa Cruz Biotechnology), bound to 10 µl Dynabeads Protein G (Life Technologies). Binding was carried out by overnight incubation at 4°C with gentle agitation, after

washing of beads with BSA/PBS (0.5% weight/volume) thrice. Synchronized cells in 15-cm plate were treated with 10 nM E2 for 45min or 10 nM ICI for 24 h. Cells were crosslinked with 1.5% formaldehyde in PBS. Crosslinking was stopped with 125 mM glycine after which cells were scraped in 100 mM Tris-HCl (pH 9.4) and 10 mM dithiothreitol. Nuclei were isolated by sequential resuspension in NCP buffer I (10 mM EDTA; 0.5 mM EGTA; 10 mM HEPES, pH 6.5; 0.25% Triton X-100), NCP buffer II (10 mM EDTA; 0.5 mM EGTA; 10 mM HEPES, pH 6.5; 200 mM NaCl), and lysis buffer [10 mM EDTA, 20 mM Tris-HCl (pH 8.0), 0.5% Empigen BB, 1% sodium dodecyl sulfate, 1× complete protease inhibitor (Roche Applied Science))]. Chromatin was sonicated to an approximate fragment size of 200-1000 bp and incubated overnight at 4°C with bead-bound antibody in 2 mM EDTA (pH 8.0), 20 mM Tris-HCl (pH 8.0), 150 mM NaCl, 1% Triton X-100, and 1× complete protease inhibitor. The beads were washed before elution with different buffers, and samples were eluted with 0.1 mM $NaHCO_3$, 1% sodium dodecyl sulfate at 65°C. Crosslinking was reversed overnight at 65°C, and DNA was purified using MinElute PCR Purification Kit (Qiagen). Input samples were diluted 1:50 before. Enrichment of binding was calculated as percentage of input (125).

3.2.3 Immunoblot

Primary antibody against KCNK5 (Alomone Laboratories, Jerusalem, Israel) was used at 1:200 dilution for incubation overnight.

3.2.4 qPCR primers

For detection of *KCNK5* mRNA the following primer pair was used: F: 5'-CGGCTTCGGTGACTTTGTG-3' and R: 5'-AGCTCCACGAAGTAGCGGTACA-3'.

Primers used for ChIP-qPCR were pS2 promoter (F: 5'-GGCCATCTCTCACTATGAATCACT-3', R: 5'-GGATTTGCTGATAGACAGAGACGA-3'), KCNK5 enhancer (F: 5'-AGGTGGAGTGGAAGCTCAAAAC-3', R: 5'-CCTCTCCCTGGTCTTTTGCA-3'), pS2 3'-UTR (F: 5'-CCCCAGCACGGTGATTAGTC-3', R: 5'-GATCTGCCTGCATCCTGAC-3'), and KCNK5 exon 4 (F: 5'-GCAGATCACGTGCACAGTCAT-3', R: 5'-TTCCACCCCTCAGTCACCAT-3').

3.3 Results

3.3.1 Estrogen induced an increase in KCNK5 at the mRNA and protein levels in T47D and MCF7 cells

We have in a previous study reported that *KCNK5* mRNA is highly regulated by E2 in T47D cells (102). To confirm this result, we measured *KCNK5* mRNA levels in

E2-treated T47D and MCF7 cells using qPCR. A time series over 72 h showed that the transcription of *KCNK5* is rapidly induced by E2 in both cell lines (Figure 3.1A). Additional experiments indicate that this is likely a direct effect of ERα-mediated gene regulation. At least three prior genome-scale studies of ERα binding in breast cancer cell lines have found the receptor to bind in a potential enhancer region upstream of the transcription start site of *KCNK5* (126–128). We verified that 15 min of E2 treatment causes ERα to bind to this region of the genome by chromatin immunoprecipitation (ChIP)-qPCR. We found that ERα binding is enriched in the region, as well as on the promoter of the prototypical E2-target gene pS2 (*TFF1*), but not in exonic or 3'-untranslated regions of these genes (Figure 3.1B). Moreover, ERα binding to the enhancer of *KCNK5* was not observed in MCF7 cells pretreated with the ERα-degrading compound ICI 182,780 (ICI) (Figure 3.1B). In accordance with this, the transcription of *KCNK5* in MCF7 cells treated with either ICI or antagonist tamoxifen, in regular non-starved medium, was reduced below the levels of vehicle treatment, further supporting the notion that ERα is regulating this gene (Figure 3.1C).

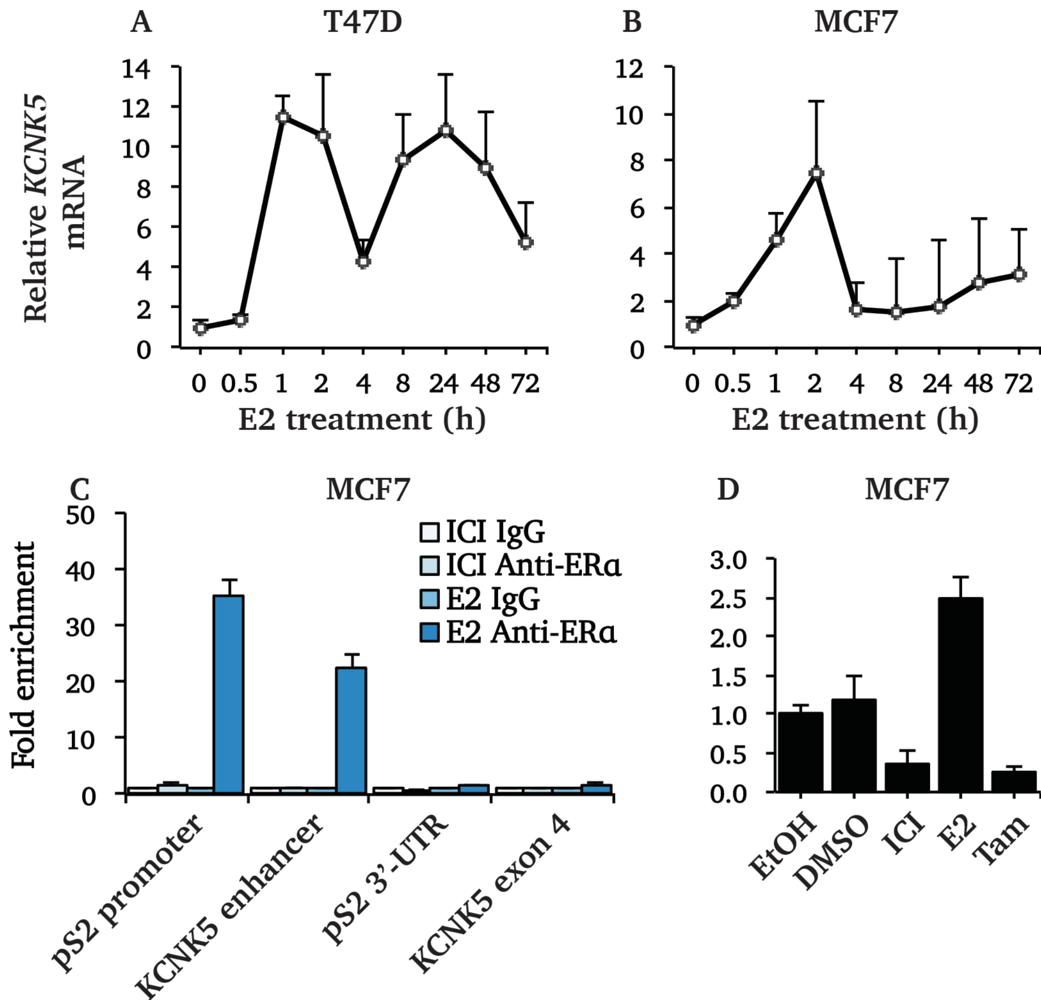


Figure 3.1 KCNK5 is induced by estrogen in ERα-positive breast cancer cells. Time course for E2 induction of KCNK5 mRNA in A) T47D and B) MCF7, after treatment with 10 nM of E2 after synchronization of cells (n = 2). C) ChIP-qPCR showing binding of ERα at the promoter of its archetypical target gene pS2, as well as in an enhancer element of the KCNK5 gene. IgG used as negative control for the immunoprecipitation, antagonist ICI for the treatment, and two negative-control sites for the specificity of the binding. (n = 3). D) qPCR showing mRNA levels of KCNK5 after 24 h of indicated treatments. Cells were grown in complete medium and without synchronization prior to treatment. Ethanol and DMSO are vehicle controls and ICI and tamoxifen (Tam) antagonists for ERα. Error bars represent SEM in this and following figures in this chapter.

To test whether E2 also increased KCNK5 protein, we carried out immunoblot analyses of extracts obtained from synchronized MCF7 and T47D cells at different time points after the start of E2 treatment. An increase in KCNK5 protein was observed in both cell lines at 12 h after the start of treatment and lasting for 48–72 h (Figure 3.2).

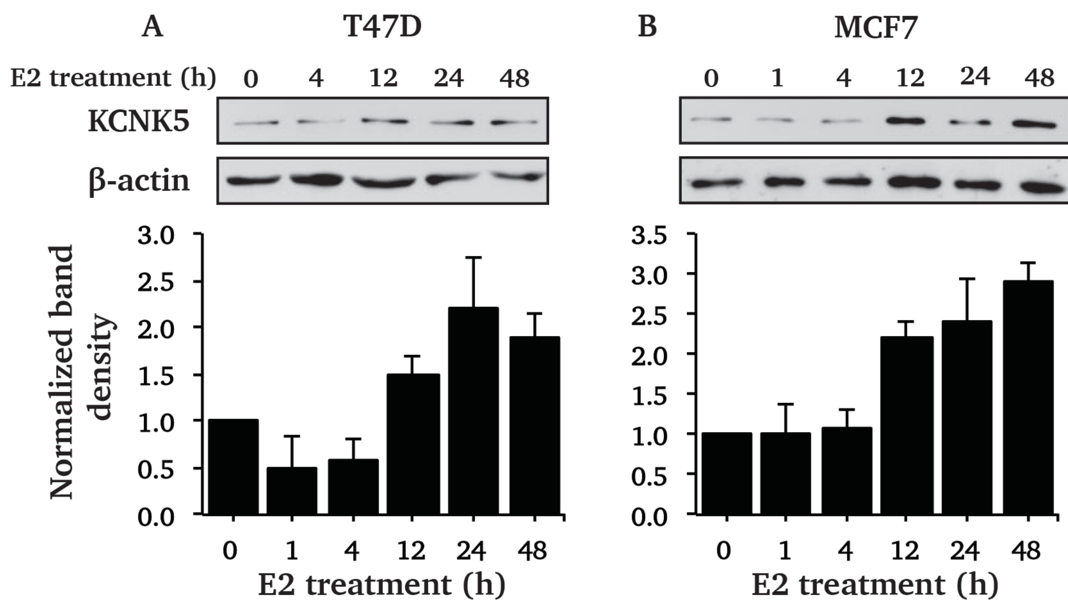


Figure 3.2. Estrogen-induced increase in KCNK5 mRNA reflected on protein levels. Time course for induction of KCNK5 at protein level, after treatment with 10 nM of E2 over indicated time, for A) T47D and B) MCF7. Cells were synchronized prior to treatment. Bar graphs show quantification of replicated immunoblots ($n \geq 3$).

3.3.2 Silencing of KCNK5 reduced E2-induced proliferation of T47D cells

To further examine the effect on the ERα-mediated regulation of KCNK5 we chose to silence its expression using siRNA. Transient transfection with siRNA targeting

KCNK5 effectively reduced the mRNA levels up till at least 72 h (Figure 3.3A). However, this change was not consistently reflected by a decrease in protein levels (Figure 3.3.B). This is likely explained by the fact that the KCNK5 protein's half-life is long. Three repeated transfections over 6 days did produce a significant decrease in protein levels (129).

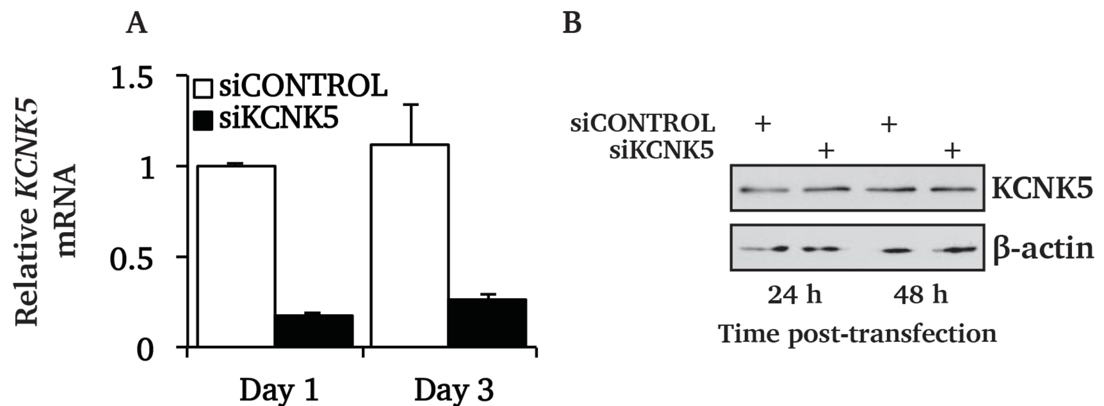


Figure 3.3. Reduction in KCNK5 expression following siRNA treatment. Treatment of T47D cells with control siRNA (scrambled sequence) or siRNA targeting KCNK5. A) Bar graph illustrating effects on KCNK5 mRNA following one and three days of siRNA treatment. B) Immunoblots showing effect on KCNK5 protein levels after 24 and 48 h of siRNA treatment.

We next assessed the effect of the reduced ion channel levels on the cell cycle of the cells. Both cell counts and MTS assay results indicated lower proliferation in cells treated with siRNA for KCNK5, and the E2-induced cellular proliferation was partially blocked (129). We then carried out flow-cytometric analysis to further dissect this change. E2 stimulated cells treated with KCNK5 siRNA were more

likely to remain in the G1 phase of the cell cycle, compared to cells treated with control siRNA (Figure 3.4). This indicates that reduction in KCNK5 levels induces G1/S cell cycle arrest.

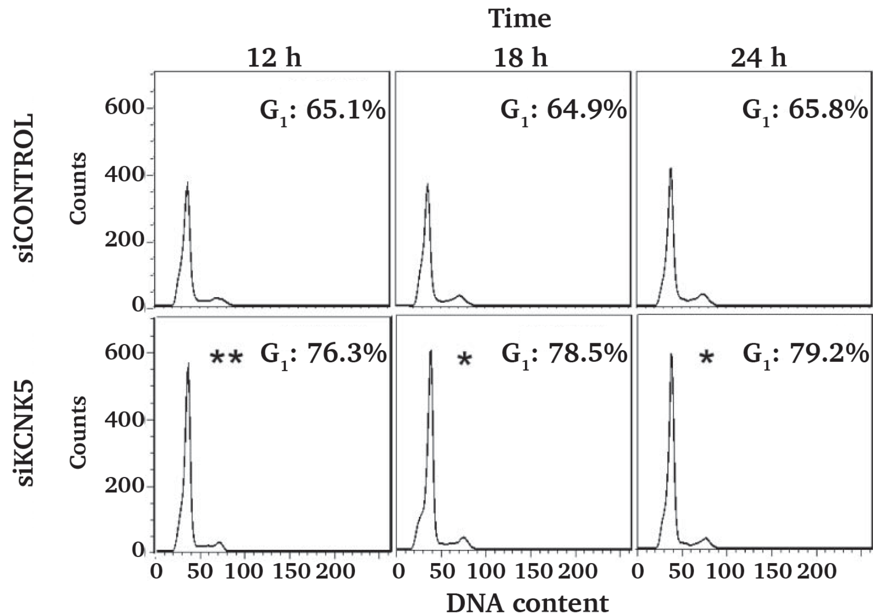


Figure 3.4. Cell-cycle analysis of siRNA-treated cells. Cells were treated with control or KCNK5-targeting siRNA over 24 h, followed by flow-cytometric analysis of DNA content. Numbers in graphs show percentage of cells in the G1 phase of the cell cycle at indicate time point and treatment. Significance indicated according to Material and methods section.

3.4 Conclusion

Other results from this study, obtained from C. Alvarez-Baron, also showed that E2 treatment of T47D and MCF7 cells increases currents indicative of KCNK5 activity at the plasma membrane. This increase was also partially blocked in T47D

cells by clofilium, an inhibitor of KCNK5. Additionally, immunofluorescent labeling of the ion channel demonstrated that it was localized at the membrane, as expected, but also largely in intracellular compartments.

KCNK5 channels may contribute to the pathogenesis of cancer. For example, a recent study of a large number of primary human cancers and cancer cell lines identified an amplification in a region located on 6p21.2 that contained eight genes including KCNK5, as well as the closely related genes KCNK16 and KCNK17 (130). Moreover, they found upregulation of KCNK5 transcripts in several of the cancer cell lines that they examined. Other channels in the two-pore domain potassium channels family have also been implicated in cancer. For example, the *KCNK9* gene, which encodes a potassium channel that is also inhibited by extracellular acidification, is significantly amplified in 10% of breast tumors, and its transcript is markedly overexpressed in 44% of breast tumors (131). Cells over-expressing KCNK9 are resistant to hypoxia. Given the similarities in function, it is possible that multiple members of this family of channels may play a role in allowing cells to adapt to the relatively anoxic microenvironment of solid tumors. In this regard, KCNK5 and KCNK9 have been reported to play a similar role in protecting cells from stress (132), and the potential mechanisms whereby these channels may regulate proliferation and apoptosis need to be further studied, but may be unrelated to channel activity *per se* (133).

Our results, therefore, offer an enhanced view of the mechanisms whereby ERα induces proliferation. In addition to the known direct upregulation of the pro-proliferative proto-oncogene *MYC*, we show that ERα induces the KCNK5 ion channel and that this is necessary for normal estrogen-induced proliferation. The effect of KCNK5 on proliferation suggests that this channel could be a useful pharmacological target for treatment of ERα-positive breast cancer. ERα-positive tumors account for the majority of breast tumor diagnoses. At present, the only targeted therapy is treatment with antiestrogens or aromatase inhibitors. The side effects of these treatments and the development of resistance over time suggest the importance of finding new strategies to target ERα-positive tumors. Targeting KCNK5 would block the proliferation-inducing ability of ERα, specifically, while preserving more beneficial activities, such as the differentiation- and apoptosis-inducing effects of ERα. Such a targeted therapy would be an alternative also for refractory ERα-positive cancers. Unfortunately, adequately specific blockers for KCNK5 are not currently available, and the available inhibitors exhibit high toxicity *in vivo* at the doses required to block KCNK5 (134).

In summary, we have shown that KCNK5, a pH-sensitive potassium channel in the two-pore superfamily, is induced by estrogen in ERα-positive breast cancer cell lines, and that this channel is required for normal E2-evoked proliferation in these

cells. KCNK5 and related channels may therefore represent potential targets for breast cancer therapeutics.

4. Chapter 2: Single-molecule sequencing of the estrogen-induced transcriptome in breast cancer cells

4.1 Introduction

The effort to characterize ERα's role in the cellular biology of BC is mainly concerned with understanding the gene-regulatory role it plays, being that it is a ligand-regulated transcription factor. The arrival of next-generation sequencing (NGS) facilitates this endeavor, by allowing for assessment of DNA binding and expression of any genomic element on a global scale and in an un-biased manner. A wide number of studies have dissected the genomic binding of ERα

(126,135,136), revealing that the receptor can bind to anywhere from 1,000 to 15,000 different loci. Not as many studies have been done to study the ensuing effect on gene regulation with said NGS technology, but it has been shown that ERα can regulate 3,386 protein-coding genes (137). Another study, assessing nascent transcripts upon stimulation with E2, found approximately 6,000 transcripts (coding and non-coding) to be regulated (138).

Although the existence of non-coding RNAs (ncRNAs) has been known for decades, it was not until recently that their importance in development and disease was revealed. Initially believed to be transcriptional "noise", the extensive transcription across the whole genome is now, by most, considered to be of biological relevance. Aside from components of the splicing and translational machinery, the class of ncRNAs comprises small non-coding RNAs (e.g. miRNAs) and long non-coding RNAs (lncRNAs), the latter being defined as RNAs larger than 200 bp. Like mRNAs, lncRNAs have exons and introns, are largely polyadenylated, and exist in splice variants, yet in most instances lack even the potential to code for protein (139,140). They are expressed at lower levels than mRNAs (141). Although still scarce, the evidence of well-characterized functional roles for lncRNAs is increasing. Their demonstrated functions are diverse, ranging from chromatin modification, regulation of transcription and translation, to modulation of protein function through direct interactions (142). Importantly, it

has been shown that lncRNAs exhibit higher tissue-specific expression than protein-coding genes, and are expressed at specific stages of development (139,142–144).

The fact that the majority of cancer-risk associated loci lie outside of protein-coding regions of the genome, together with the findings of cancer-regulatory roles of individual lncRNAs, highlight the potential of this class of molecules to impinge on tumor progression (145,146). The lncRNA HOTAIR, aside from implied roles in other cancers, is associated with metastasis in ERα-positive breast cancer and has been reported to be induced by E2 (147,148). One demonstrated function of HOTAIR is the binding to polycomb repressive complex 2, by which breast cancer cells turn on a metastatis-promoting set of genes (149).

In this study, we aimed to utilize novel sequencing technologies in order to uncover new aspects of the E2-ERα transcriptome. We chose to single-molecule sequence the polyadenlyated RNA fraction for precision, lack of amplification-induced biased, and to cover both protein-coding and non-coding RNAs (150,151). Also, in a further attempt to understand the transcriptional program controlled by ERα in breast cancer cells, we studied its interaction with the pro-proliferative transcription factor activator protein-1 (AP-1). Also, we studied how endocrine-disrupting chemicals (EDCs) can activate ERα, in comparison to

estradiol and in combinatory treatments. This in order to further understand the possible health effect of human exposure to such compounds (152).

4.2 Supplementary material and methods

4.2.1 Library preparation and sequencing

Total RNA was extracted as described above, and thereafter twice enriched for the polyA+ fraction by separation with oligo(dT) magnetic beads. Enrichment was ensured by use of the mRNA Nano kit for Agilent Bioanalyzer 2100. Sample libraries were prepared and subjected to direct RNA sequencing by Helicos Biosciences, whose technology has been described (150,153–155). In short, this included heat fragmentation, cDNA synthesis, and the addition of a poly(dA) tail to cDNA, before poly(dT) capture on the sequencing flow-cell, and sequencing by synthesis through addition of fluorescently labeled nucleotides. Biological as well as technological duplicates were analyzed.

4.2.2 Data analysis and bioinformatics

Raw reads were filtered according to length and base composition, as previously described (154). Remaining reads were aligned to the human transcriptome (assembly hg19 with Gencode 19 as annotation) using the GSNAP (156) and STAR (157). GSNAP was run with parameters allowing for 3 mismatches and

detection of novel splice sites, with all other alignment parameters at default value. STAR was run with parameters allowing for 3 mismatches, no multi-mapped reads, with all other parameters at default value.

Remaining bioinformatics analyses were carried out in R, using packages edgeR (158) and DESeq2 (159) for normalization of count data, and to test for differential expression. A cut-off value for expressed transcripts was set at 1 read per million reads (RPM) per sequenced sample. For differential expression, the union of transcripts from analysis with both programs, with absolute fold change > 1.5 at FDR 5%, were used.

4.3 Results

4.3.1 Single-molecule sequencing of the E2-ERα-transcriptome

Samples were verified to show typical E2 induction of the classical ERα target gene pS2. The polyadenylated fraction of RNA was subjected to sequencing, in technical and biological duplicates. The resulting sequencing reads were filtered for technical artifacts, the resulting number of reads per sample is listed in Table 4.1. Subsequently, the reads were aligned to the annotated human transcriptome, with alignment software allowing for splice alignment, i.e. reads spanning spliced sites. The resulting number of aligned reads is shown in Table 4.1. Aside from

reads containing sequencing errors, the remaining number of sequences could possibly be un-annotated RNA (155). To assess the biological variation, we analyzed the correlation between replicated treatments (Figure 4.1). For all but one treatment, the correlation coefficient was larger than 0.90. Apparent technical variation in one replicate for the vehicle-treated T47D led us to omit this from the remaining analyses.

Table 4.1. Summary of RNA-sequencing and sequence alignment. Shown are total number of sequencing reads after quality filtering, and reads aligned with either of the indicated aligners.

	MCF7				T47D			
	Vehicle		E2		Vehicle		E2	
Total	50M	50M	42M	45M	59M	52M	50M	39M
GSNAP	19M	19M	20M	19M	26M	19M	20M	21M
STAR	17M	18M	17M	17M	24M	17M	17M	17M

For the remainder of the analyses, we sat a cut-off for expression at 1 sequence read per total million reads (RPM) (150). At that cut-off, we found 18,402 and 17138 transcripts to be expressed in MCF7 and T47D, respectively, in either of two replicates per treatment. The majority of the transcripts were protein-coding genes, followed by non-coding RNAs and pseudogenes (Figure 4.2A). A fraction of small non-coding RNAs (including e.g. miRNAs) were detected too. Ribosomal and mitochondrial reads were discarded.

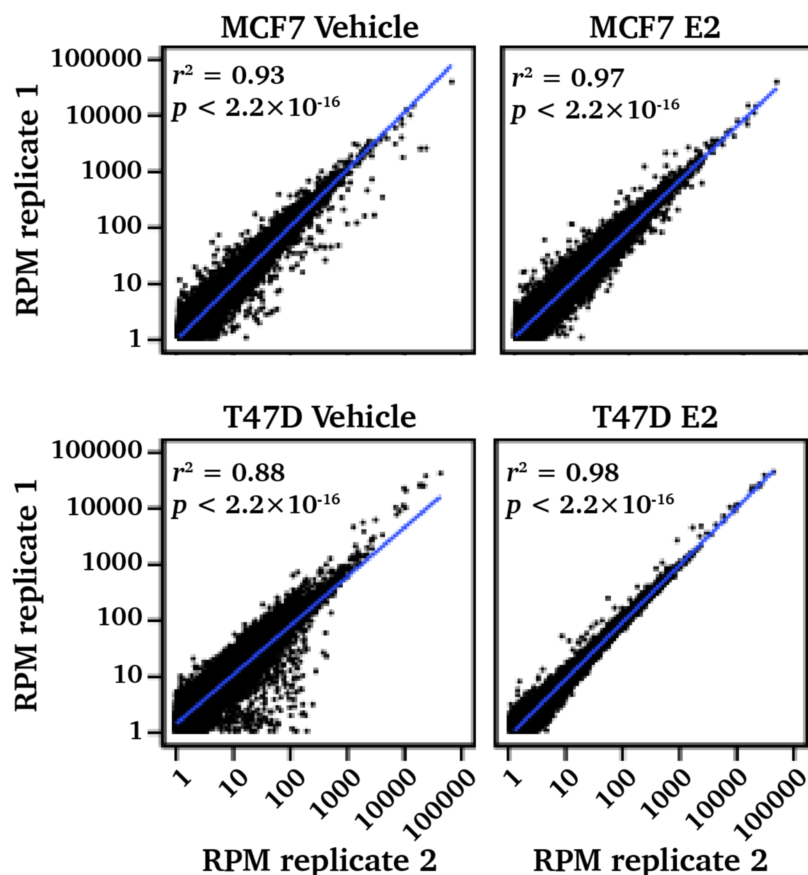


Figure 4.1. Correlation between biological replicates. Biological and technical duplicates were sequenced. Scatter plots show tag count per million reads (RPM) for features in genomic annotation. Data plotted on log scale, omitting transcripts with RPM < 1.

4.3.2 E2-ERα induced changes in the coding and non-coding transcriptome

Next, we conducted analysis of differential expression (DE) between E2- and vehicle-treated cells. For MCF7 and T47D, we observed 2,462 and 3,369 DE transcripts, respectively (Figure 4.2B-C). The higher number in T47D likely reflects the omitted vehicle replicate, i.e. a higher likelihood of false positives. The

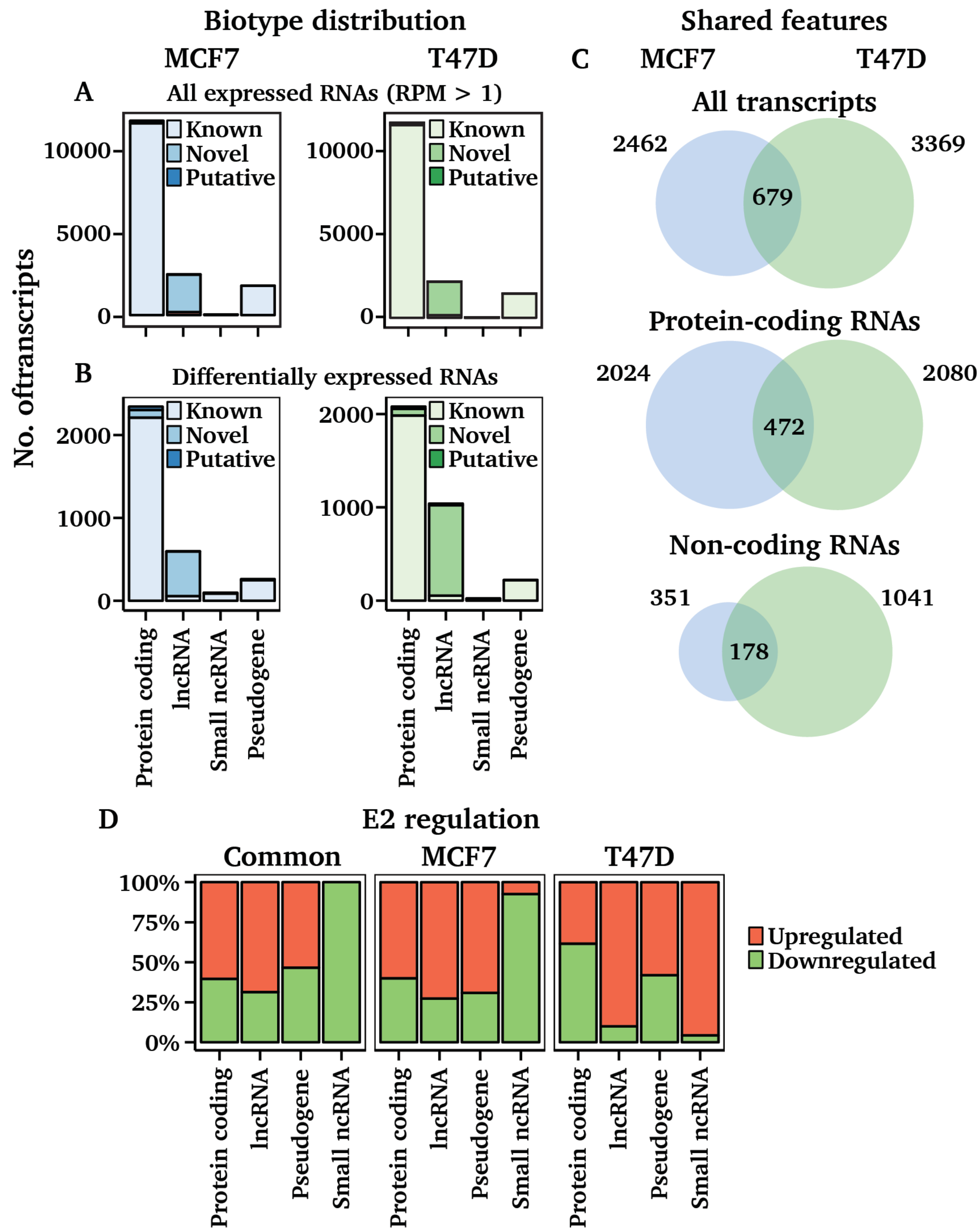


Figure 4.2. Full MCF7 and T47D transcriptome and E2-mediated changes. A) Distribution of transcript types among genes expressed in at least one of two replicates per treatment, in both cells lines. Ribosomal and mitochondrial RNA omitted. B) Similar to A), but for transcripts determined to be differentially expressed. C) Venn diagrams showing overlaps between cell lines of all differentially expressed transcripts, as well as only protein-coding and non-coding RNAs,

respectively. D) Fraction plots illustrating percentage of up- and downregulated transcripts, respectively, among the sets of common or cell-line specific transcripts.

distribution of transcript types largely reflected that of the total expressed transcriptome, i.e. consisting predominantly in protein-coding RNAs but also other RNA species. However, relative to all expressed transcripts there was possibly an overrepresentation of DE lncRNAs in T47D. We found overlaps of 472 protein-coding and 178 and non-coding DE RNAs, respectively, between the MCF7 and T47D comparisons (Figure 4.2C). We found the majority of regulated genes to be induced, rather than suppressed, by E2 (Figure 4.2D) (138). This is likely a result of the chosen time point (8 h) and the assay technology as repressed genes are commonly found in larger numbers at later time points (Hah *et al*. measure nascent transcripts, as opposed to processed ones).

The biological function of the DE transcripts was assessed by enrichment analysis of gene ontology (GO) biological processes among the common protein-coding genes, as well as among the whole sets of DE transcripts for MCF7 and T47D (Table 4.2). As expected, there was a significant overrepresentation of terms associated with cellular division and proliferation, indicative of the mitogenic role of E2 and ERα. Also, apoptosis-related GO terms were enriched for, concordant with the fact that ERα is capable of stimulating both proliferation and cell death (160,161).

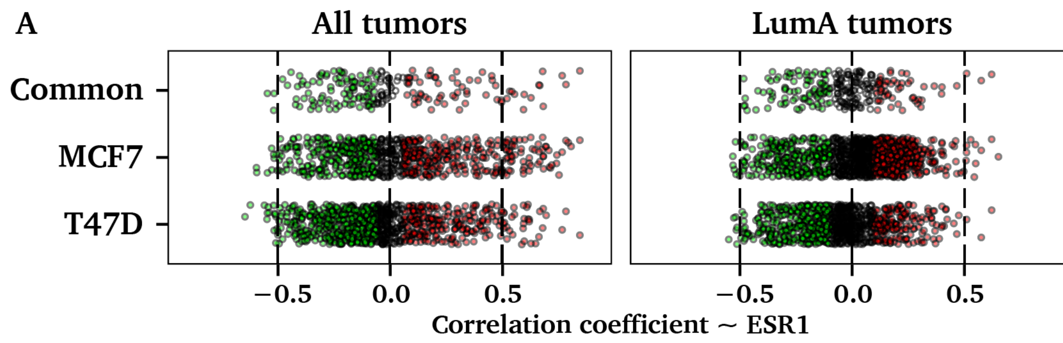
Table 4.2. Enrichment of gene ontologies among differentially expressed transcripts. Select GO terms for biological processes overrepresented in DE analysis, from common, MCF7, and T47D datasets. Note that most non-coding RNA species are not annotate to GO terms. Overlapping terms were omitted. *N* shows number of genes in category represented in DE analysis.

	GO term	Description	N	*P* value
Common	GO:0006412	Translation	50	9.1E-14
	GO:0022411	Cellular component disassembly	47	1.6E-13
	GO:0010467	Gene expression	209	1.9E-10
	GO:0019438	Aromatic compound biosynthetic process	177	2.2E-09
	GO:0019222	Regulation of metabolic process	192	4.6E-03
MCF7	GO:0007049	Cell cycle	271	1.8E-40
	GO:0034660	ncRNA metabolic process	97	1.1E-35
	GO:0050896	Response to stimulus	6819	4.2E-35
	GO:0006915	Apoptotic process	220	1.2E-18
	GO:0008283	Cell proliferation	225	3.4E-18
T47D	GO:0022904	Respiratory electron transport chain	28	3.7E-07
	GO:0045216	Cell-cell junction organization	46	5.7E-05
	GO:0050896	Response to stimulus	837	8.2E-04
	GO:0008219	Cell death	243	1.2E-03
	GO:0007049	Cell cycle	207	7.7E-03

4.3.3. RNA-seq of E2-ERα transcriptome uncovered potential novel targets

In order to explore the set of changed transcripts further, we integrated prior data into our dataset. We analyzed The Cancer Genome Atlas's (TCGA) available data of gene expression in breast tumors (7) for correlation of our DE transcripts to the expression of ERα (*ESR1*) (Figure 4.3A). We carried out this analysis for all tumors, as well as for the luminal A-only cohort, revealing 161 genes, out of the common set of 472 genes, to be correlated significantly correlated with ERα mRNA in all tumors, in a direction concordant with their regulation in the cell lines. Of the aforementioned 161 genes, 108 were not identified in two previous studies aimed to define ERα targets (136,138). This number was slightly lower,

142, when only the luminal A tumors were included. For the MCF7 and T47D gene sets, the equivalent numbers were 576 and 704, respectively, for all tumors, and 765 and 546, respectively, for the luminal A tumors. Together, these numbers indicate that many of our identified DE transcripts are *de facto* targets of ERα in human breast tumors. The higher number of significantly correlated genes for MCF7 among the luminal A tumors likely reflects the more "luminal nature" of this cell line, and possibly the effect of the omitted replicate. Thereafter we annotated our set of DE genes with known genomic binding sites for ERα, as assessed by ChIP-seq in both cell lines from previous publications (162,163). Of the differentially expressed protein-coding genes, a majority had ERα-binding sites within 50 kb of their transcription start sites, according to at least one of the two datasets used, and this was also reflected by the high proportion of protein-coding genes regulated in either cell line with binding sites (Figure 4.3B). The same was true for lncRNAs. However, as ERα often binds distal to promoters and interacts with target genes through chromatin looping, it is not always clear which, if any, genes whose promoters ERα binds that it also regulates (164).



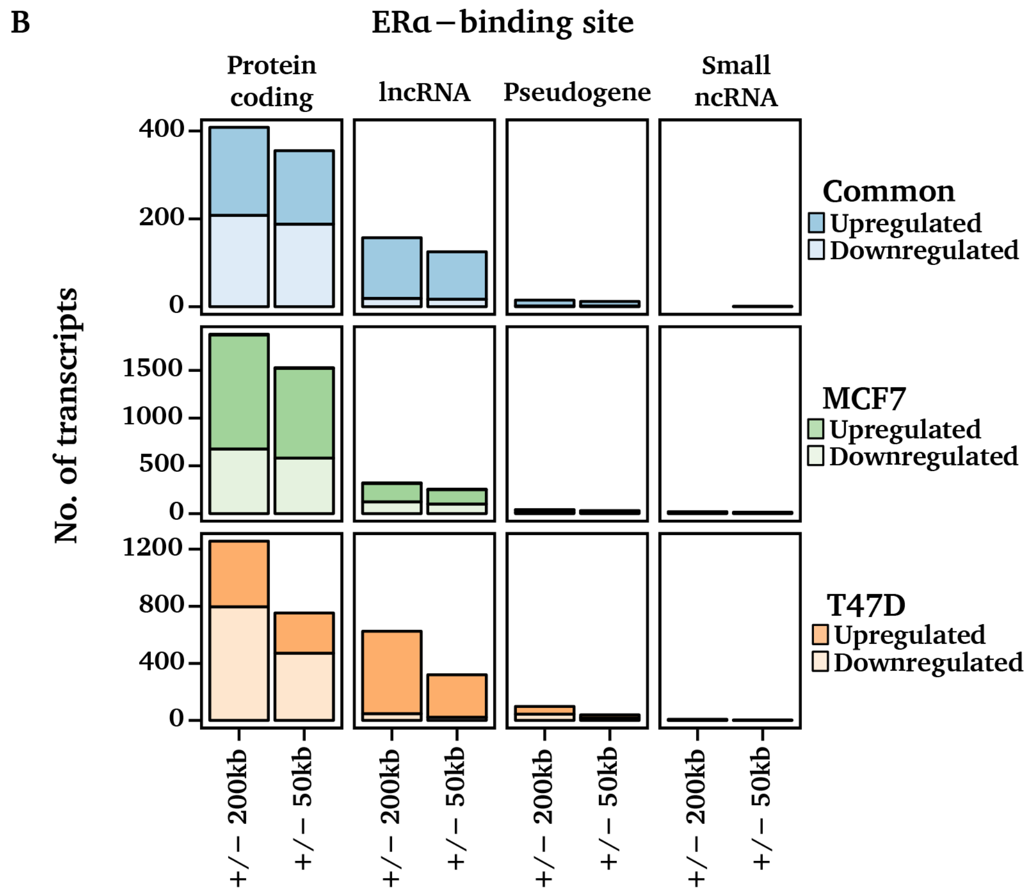


Figure 4.3. Integrative analyses of our sets of differential expression and prior data. A) Plots showing protein-coding transcripts whose expression correlates significantly with ERα mRNA (ESR1; negative correlation in green, positive in red, and non-significant correlations in black) in the TCGA dataset of human breast tumor gene expression (7). Left plot shows data for all tumors and right plot shows data for only the luminal A tumors. B) Histograms showing the number of DE transcripts from our study that also have ERα-binding sites according to published ChIP-seq

studies (162,163). For common genes, binding sites found in either cell line was included, and for the cell-line specific gene sets only binding sites from corresponding cell line was included.

We confirmed the RNA-seq data for select protein-coding genes (Figure 4.4A). The regulation of some targets, e.g. PKIB and GPR132, were exclusive for either cell line. Two long intergenic RNAs (lincRNAs) were confirmed to be upregulated by E2 in both MCF7 and T47D. Both also have ERα-binding sites, intergenic and in their promoter regions We confirmed the upregulation of both lincRNAs and noted that they are both induced significantly within an hour of E2 treatment strongly suggesting them being direct targets of ERα (Figure 4.4B). We also found ERα-binding sites close to both lncRNAs (162,163), and these binding sites overlapped with DNase-hypersensitivity regions, which suggest open chromatin at the sites. Indeed, ChIP-qPCR confirmed ERα binding at three out of four assessed sites, further supporting the notion of these lncRNAs as direct targets of ERα.

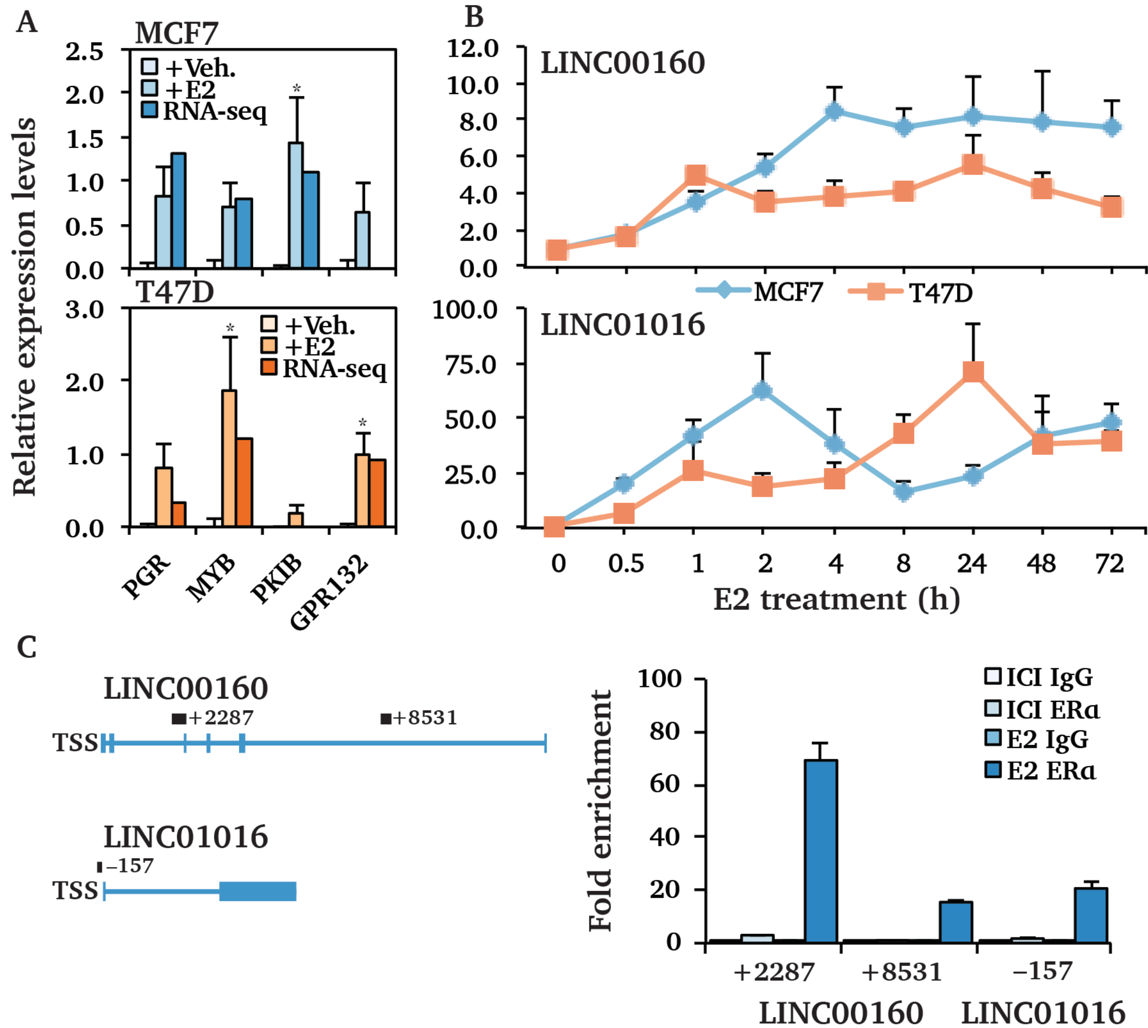


Figure 4.4. Confirmation of RNA-seq data by qPCR. A) Protein-coding genes induced by E2 according to RNA-seq in either both cell lines (*PGR*, *MYB*), MCF7 only (*PKIB*), or T47D only (*GPR132*). First two bars for each gene represent data from qPCR analysis, average for two replicates. B) Time series over 72 h of E2 induction of select lncRNAs. C) Left, gene models of indicated lncRNAs showing ER)-binding sites from the literature and their positions relative to the TSS (162,163). Right, ChIP-qPCR for indicated genomic sites for the two lncRNAs, average of two replicates.

4.3.4. Identification of the ERα/AP-1 transcriptome and regulation of endocrine-disrupting chemicals

In another effort to dissect the ERα transcriptome in breast cancer cells, we studied the receptor's interaction with the AP-1 transcription factor. By silencing one of the heterodimeric partners of AP-1, c-Fos, we showed that 37% of ERα-regulated genes were attenuated, pointing at AP-1's role in cooperatively with ERα induce or repress their expression. Moreover, we showed that AP-1 independently influences the regulation of 5% of the protein-coding transcriptome. Analysis for enrichment of gene ontology biological processes revealed that E2/ERα/AP-1-induced genes were involved in cellular proliferation and that for repressed genes biological processes associated with apoptosis and negative regulation of cell proliferation were overrepresented. Genes regulated by AP-1 independently of E2/ERα were also involved in cell proliferation. Additionally, E2F1 was identified as a direct target of ERα and AP-1 (165) and we demonstrated a network of E2F1 target genes were affected by silencing of c-Fos, both in a E2/ERα-dependent and –independent manner.

In another part of this study, we aimed to understand to what extent the transcriptional response to EDCs differ from that of E2. We studied the changes in the protein-coding transcriptome to bisphenol A (BPA), genistein, and phytoestrogen-containing soy formula by microarrays (166). This study found that

these compounds essentially regulate the same set of genes as E2, through ERα, and do not elicit extensive, if at all, non-genomic effects at lower concentrations. Moreover, it was shown that the EDCs were additive in their transcriptional effects on target genes, and that they were able to induce cellular proliferation comparable to the effect of E2.

4.4 Conclusion

We herein describe out effort to characterize the E2-ERα transcriptome in two human breast cancer cell lines by means of single-molecule RNA-sequencing. The development in sequencing technologies allows for a wide host of assays to be performed at genome-wide scale. In our study we treated MCF7 and T47D cancer cell lines for 8 h with E2, an assessed the ensuing effects on the polyadenylated transcriptome.

Our findings include a large set of E2-regulated protein-coding genes, in either cell line, as well as a set of common genes. This group of genes includes both know ERα-target genes, but also targets that have previously not been defined as direct targets. Moreover, the analysis revealed multiple lncRNAs as potential direct targets of ERα, and we confirmed the regulation of two of these, as well as ERα binding in their promoter/gene. Further ERα DNA-binding studies need to be carried out to confirm that the receptor in fact binds at these sites upon E2

stimuli. Interrogation of the whole set of lncRNAs can possibly find more targets of interest and interestingly, we have indications that E2-induced lncRNAs can exhibit high association with patient outcome (Cristian Coarfa, Baylor College of Medicine). Ultimately, this can aid in the characterization of ERα-regulation of non-coding RNAs, beyond protein-coding genes and small non-coding RNAs, such as miRNA. Due to the potent regulatory ability of lncRNAs, these are of interest to further understand ERα's role in regular biology as well as in disease.

Furthermore, the data on AP-1's role in regulating breast cancer cell proliferation, dependently and independently of ERα, suggest a critical role for this transcription factor in the proliferation of these cells, concordant with previous data (39,167,168). Also, we showed that part of this proliferative response might be mediated by direct regulation of E2F1, which in its turn regulates a proliferative program. This is in line with proposed E2F1-targeting small molecules for breast cancer treatment (169).

Finally, the examination of the ability of EDCs to regulated E2/ERα target genes showed that these compounds do not regulate subsets of genes distinct from E2, but that they can have additive effect when cells are exposed to more than one. They are also capable of inducing cellular proliferation similarly to E2, warranting further research into human exposure to EDCs out of a health perspective.

5. Chapter 3: Support of a bi-faceted role of ERβ in ERα-positive breast cancer cells

5.1 Introduction

The role of estrogen receptor alpha (ERα, *ESR1*) as a biological marker and target in breast cancer therapy is clear. ERα antagonists, such as tamoxifen, or estrogen ablation using aromatase inhibitors are efficient therapeutic approaches in the treatment of ERα-positive breast cancer. A second estrogen receptor, ERβ (*ESR2*), was discovered in 1996 (35,170) and is considered the predominant ER in normal breast (35). Clinical studies indicate that whereas ERβ expression is low in breast and decreases further during tumor progression (51,59,75–77), a large proportion, between 39% and 77%, of all breast cancer tumors co-express both

ERs (Figure 1.3) (61–73). ERβ is therefore a potential marker and target, in these tumors, that could enhance the use of endocrine therapy. Albeit both ERs are activated by estrogen, their ligand-binding domains allow for receptor-selective ligands. Their DNA-binding domains are highly conserved and they share between 46% and 73% of chromatin-binding sites (Figure 1.1) (42), although distinct regions of binding have also been defined for each receptor (38–40). The receptors' N-terminal domains are structurally different and lead to different abilities in their interaction with coregulators (171), resulting in a higher transactivation capacity for ERα (170).

The function of ERβ in breast cancer is not clearly understood (79) and ERβ is currently not used in the diagnosis or treatment of breast cancer patients. One obstacle in the field is that available breast cancer cell lines do not express sufficient levels of endogenous ERβ for consistent mechanistic and functional studies (172) and mRNA levels in tissues are also persistently low in comparison to ERα (173). To explore its role, ERβ thus has to be introduced exogenously. The majority of studies has used transient or inducible induction of ERβ and indicates that ERβ in ERα-positive breast cancer cells counteracts the proliferative and transcriptional functions of ERα (102,174–178). Whereas some clinical studies link ERβ expression to better outcomes (84,179,180), others have correlated ERβ expression to the proliferation marker Ki67 in primary breast tumors (63,78,181)

and associated its expression with a higher risk of relapse in node-positive breast cancer patients (66). In ERα-negative tumors, ERβ expression has been correlated to a higher aneuploidy, indicative of a more aggressive phenotype (61). The body of data correlating ERβ to both anti-proliferative and proliferative parameters has led to the suggestions of a bi-faceted role for ERβ (79). One clinical study indicated that tamoxifen treatment of ERβ-positive tumors may be beneficial: ERβ was associated with better survival after long-term tamoxifen-treatment in post- but not pre-menopausal women (65). A deeper understanding of the role and mechanism of ERβ may help us improve the treatment for breast cancer patients.

We set out to investigate the effect of constitutive expression of ERβ in breast-cancer cells. Our aim in this study was to complement the studies of transient or short-term effects of ERβ and gain additional insight into its mechanism and potential clinical applications.

5.2 Supplementary material and methods

5.2.1 Cell culture, ERβ expression and treatments

Duplicate T47D-ERβ and T47D-control, and triplicate MCF7-ERβ and MCF7-control mixed-cell populations were generated by lentiviral transductions (Dr. A. Ström, University of Houston) with CMV-driven, FLAG-tagged full-length ERβ

cDNA (530 aa) or empty vector, respectively, and selected as previously described (182). Two or three separate transduction replicates in the shape of mixed-cell populations, for each control and ERβ cell line, were used for all experiments. T47D and MCF7 cells were passaged and serum starved prior to ligand treatments as previously described (183,184). E2 (17β-estradiol, Sigma, Saint Louis, MO, USA) at 10 nM concentration was used as agonists for both receptors, PPT and DPN (Tocris Bioscience, Bristol, UK) at 1-10 nM as selective ligand for ERα and ERβ, respectively, KB101471 (gift from KaroBio, Sweden) at 0.5 nM and LY3201 (gift from Eli Lilly, Indianapolis, IN, USA) at 1 nM were used as selective ligands for ERβ. As antagonists, tamoxifen (Sigma) at 1 µM and ICI (Tocris Bioscience) at 10 nM were used.

5.2.2 ERβ immunoprecipitation

For immunoprecipitation experiments, cells were harvested from 15-cm plates. Cells were incubated in RIPA buffer 5 min, washed twice with cold PBS, collected in microcentrifuge tubes, passed through a syringe, and incubated at least 4 h on a nutator at 4°C. Supernatants were collected as described above, and protein concentration determined. Equal amounts of protein were used for each IP, which was performed with Anti-FLAG M2 affinity gel (Sigma), as described by manufacturer. Essentially, part of the input was saved before incubating lysates

overnight on a nutator at 4°C. Samples were boiled in Laemmli sample buffer (Bio-Rad, Hercules, CA) 5 min before performing immunoblotting.

5.2.3 Ligand-binding assay

Ligand-binding assays were carried out with tritium-labeled E2. Cells were plated in 6- or 12-well plates and grown to an approximate confluency of 50%, thereafter washed thrice with PBS before incubation for 3 h with 0.5 nM [^{3}H]-17β-estradiol (American Radiolabeled Chemicals, St. Louis, MO), with the addition of 1 µM unlabeled E2 in half of the wells. Treatment was carried out in medium supplemented with 5% DCC-FBS and PenStrep. Subsequently, the cells were carefully washed thrice with cold PBS, lysed with lysis buffer (5 M NaCl, 0.1 M Tris-phosphate, 1 M DTT, 500 mM EDTA, 50% glycerol, 10% Triton X-100), and lysates were added to liquid scintillation cocktail (MP Biomedicals, Santa Ana, CA). Radioactivity was measured using a liquid scintillation counter (LS6000, Beckman-Coulter, Pasadena, CA) and normalized to total protein content. All measurements were carried out in at least triplicates.

5.2.4 Luciferase assay

Luciferase estrogen-response element (ERE) transactivation assays were carried out with cells in 12-well plates at an approximate confluency of 80%. Each well

was transfected with 500 ng ERE-TATA-Luc plasmid and 50 ng RSV-gal plasmid using Lipofectamin LTX and Opti-MEM (Life Technologies). Cells were treated with vehicle and E2 as previously described, before washing with PBS and lysing. Lysis buffer and luciferase reagents were from BioVision (Luciferase Reporter Assay Kit, Milpitas, CA, USA). Luciferase and X-gal activity was read on a Victor X4 Multilabel Plate Reader (PerkinElmer). Luciferase activity was normalized to X-gal activity.

5.2.5 Microarray analysis and methodology

Operon's long-nucleotide spotted arrays, covering the whole human transcriptome (35 000 genes and variants), were used (Microarray Inc., Huntsville, AL). Each comparison (ERβ vs. mock) was performed in duplicate and dye-swapped to account for variance in sampling and labeling efficiency.

For each sample cDNA synthesis was carried out using 20 µg total RNA as starting material. This was mixed with 5 µg random hexamer primer (Operon, Ebersberg, Germany), incubated at 70°C for 10 min, on ice for 5 min before adding reverse-transcription mixture to a total volume of 30 µl. The mixture contained 400 units Superscript III, 1× first-strand buffer, 0.01 M DTT (all Life Technologies) and 0.5 mM dNTP mixture with a ratio of aminoallyl-modified dUTP to dTTP of 4:1 (Sigma). Subsequent to 10 min of incubation at 25°C the enzymatic reaction was

run 2 h at 46°C. The RNA strand was hydrolyzed in 16 mM EDTA pH 8.0 using 120 mM NaOH for 15 min at 70°C. The reaction was cooled to room temperature and neutralized with 120 mM HCl whereafter cDNA was purified using MinElute PCR Purification Kit (Qiagen), where provided wash and elution buffers were replaced by 80% ethanol and 100 mM $NaHCO_3$ pH 9.0. Elution was repeated, yielding 20 µl in total. To the eluate one tenth of either of the Cy3 or Cy5 monoreactive NHS-ester dye tubes, dissolved in DMSO, and dried in vacuum centrifuge, was added. Labeling mixture was incubated for at least 30 min in dark at room temperature prior to purification with MinElute PCR Purification Kit, using provided solutions.

Cy3 and Cy5 samples were pooled and vacuum-centrifuged to 13.6 µl or below. Thereafter a hybridization mixture of 5× SSC, 50% formamide (both Sigma), 0.1% SDS pH 6.6 (Life Technologies), 0.1 µg/µl tRNA (Sigma) and 0.2 µg/µl Cot-1 DNA (Life Technologies) was added to a total volume of 65 µl. Samples were then denatured for 3 min at 95°C and incubated 2 min on ice. Microarray slides were pre-hybridized for 30 min at 42°C using a 0.45 µm-filtered solution containing 10 g/l BSA (Sigma), 5× SSC and 0.1% SDS pH 6.6, this to minimize unspecific hybridization. Thereafter the slides were washed with water and isopropanol (Sigma) prior to drying in a slide centrifuge. Slides were put in hybridization chambers (Corning, Corning, NY), samples applied under LifterSlip

covers (Erie Scientific Company, Portsmouth, NH) and then hybridized for at least 40 h in water bath at 42°C. Post-hybridization washing of slides was carried out sequentially using three buffers with decreasing SDS/SSC concentration. First slides were washed for 5 min at 42°C agitated at 60 RPM with buffer containing 2× SSC and 0.1% SDS. Next wash step was for 5 min at room temperature at 60 RPM using buffer containing 0.1× SSC and 0.1% SDS. Finally slides were washed 1 min at 60 RPM using buffer containing 0.1× SSC. The ultimate washing step was repeated four times.

Slides were scanned using an Axon GenePix 4400A (Molecular Devices, Sunnyvale, CA) to obtain TIFF-images for data processing. Scanning was carried out at 10-µm resolution, focus position 10 µm and the PMT gain adjusted to obtain a balanced image in terms of both scanned channels (635 and 532 nm respectively), separately for each scan. TIFF-images were analyzed in GenePix Pro 6.1 (Molecular Devices). The images were manually inspected for irregularities and then software analysis was performed calculating foreground and background intensities for the spots. For further identification of differentially expressed (DE) genes, the data was analyzed in the R environment using the packages: limma (185), aroma (186) and custom scripts. A spot on the array was removed from the data if GenePix had flagged it as “Bad” or “Not found”, or if it did not fulfill following requirements: a) its foreground intensity was to low compared to the

background, b) its mean and median intensity values, for both channels, deviated too much, c) the ratio of median intensity for the two channels differed too much from the median of the ratio of the two channels, d) it was not within the size 70 to 171 µm, or if e) it was saturated in one or both channels. This was done using functions in the KTH-package. Remaining intensity data was normalized using the print-tip group lowess method within the aroma package (187). Thereafter a linear model using least-square method was fit for each gene and the empirical Bayes moderated t-test applied to the data, both using the limma package. This generated a list of DE genes with B scores – the probability for DE as calculated by Bayes statistics. Genes of interest, subjects for confirmation using real-time PCR, were chosen among the genes with B values over 0 and an M value (= log_2[Cy5/Cy3]) higher than 0.4 or smaller than -0.4. Genes that were not detected on at least three out of four arrays were discarded.

Overrepresentation/enrichment analyses were carried out in Pathway Studio (Elsevier, Philadelphia, PA), using the software's Gene Ontology gene sets and the ResNet motif-based database, and the transcription factor target gene set provided by Broad Institute's Molecular Signatures Database based on TRANSFAC motifs (188). P-values indicated in these data are calculated with Fisher's exact test. Microarray data is available on NCBI's GEO data repository under accession numbers GSE45047 and GSE45557.

5.2.6 Cell counting and MTS assay

Cells were seeded at indicated density in 25-cm^2 flasks and grown over the indicated number of days before counting of viable cells using trypan-blue staining. For the MTS assay, cells were seeded in a 96-well plate at density 2,500-5,000 cells per well and thereafter treated as described above with indicated ligands.

5.2.7 Wound-healing assay

Cell migration was measured using wound-healing (*in vitro* scratch) assays. The assay was carried out with cells seeded in a 12-well plate. Upon confluency, a scratch was made with a pipette tip and pictures of the scratch were taken with microscope camera at 0 and 24 h. Cells were treated with vehicle or E2 in 0.5% DCC-FBS-supplied medium. ImageJ (189) was used to analyze the scratch area, which was used to calculate migration.

5.2.8 PARP cleavage

The cleavage of PARP was examined using anti-PARP antibody (#9542, Cell Signaling, Danvers, MA), dilution 1:1,000, detecting full PARP (116 kDa) and cleaved fragment at 89 kDa. Relative cleavage was calculated, normalized to β-

actin. Western blotting was performed as previously described. Ligand treatment was done according to standard procedure with the addition of cisplatin at 10 μg/ml at 0 h.

5.2.9 qPCR primers

The following primers were used for the qPCR analysis of mRNA expression:

ERα:	F: 5'-GCTACGAAGTGGGAATGATGAAAG-3',
	R: 5'-GCTACGAAGTGGGAATGATGAAAG-3'
ERβ:	F: 5'-ACTTGCTGAACGCCGTGACC -3'
	R: 5'-CAGATGTTCCATGCCCTTGTT-3'
pS2:	5'-CATCGACGTCCCTCCAGAAGAG-3'
	R: 5'-CTCTGGGACTAATCACCGTGCTG-3'
KCNK5:	F: 5'-CGGCTTCGGTGACTTTGTG -3'
	R: 5'-AGCTCCACGAAGTAGCGGTACA-3'
MYC:	F: 5'-CCACGTCTCCACACATCAGC-3'
	R: 5'-CTTGGCAGCAGGATAGTCCTT-3')
BCL2:	F: 5'-GTACCTGAACCGGCACCTGC-3'
	R: 5'-GCAGAGTCTTCAGAGACAGC-3'
Claudin 1:	F: 5'-TCTTTGACTCCTTGCTGAATCTGA-3'
	R: 5'-TTGCTATCACTCCCAGGAGGAT-3'
APOD:	F: 5'-ATCCAGGCCAACTACTCACT-3'

R: 5’-GATTCACAGTTCCATCAGCT-3’

Cathepsin D: F: 5’-AAGCTGTCCCCAGAGGACTACA-3’

R: 5’-GGATGTCCATGCCCATGAA-3’

CCNA2: F: 5’-TGCAAACTGCAAAGTTGAAA-3’,

R: 5’-TAAATGAAAGGCAGCTCCAG-3’

5.3 Results

5.3.1 Lentiviral expression of ERβ was functional and changed ERα levels in MCF7 cells

We assessed the functional and transcriptional effects of stable ERβ expression in two models for the luminal subtype of breast tumors: the human epithelial ERα-positive breast cancer cell lines: T47D and MCF7 (95,97,98,190). The cell lines are derived from ductal carcinoma and adenocarcinoma, respectively, and both are dependent on ERα and estrogen for growth. Previous studies have characterized the ligand-activated gene regulation by ERα and corresponding promotion of cell proliferation and cell survival, along with anti-proliferative effect of ER antagonists (191,192). The effects of transient or inducible ERβ expression in these cell lines have also been characterized (174,177,178,184).

We utilized lentivirus-transduction followed by selection with blasticidin to generate T47D and MCF7 cells stably expressing full-length, FLAG-tagged ERβ in replicated mixed-cell populations, in duplicates for T47D and triplicates for MCF7 cells. ERβ transcript and protein levels were significantly increased in the ERβ-transduced cells compared to the barely detectable levels in parental or control-transduced cells (Figure 5.1A-B). Competitive ligand-binding assay with tritium-labeled E2 showed that the T47D-ERβ cells contained more than double as many

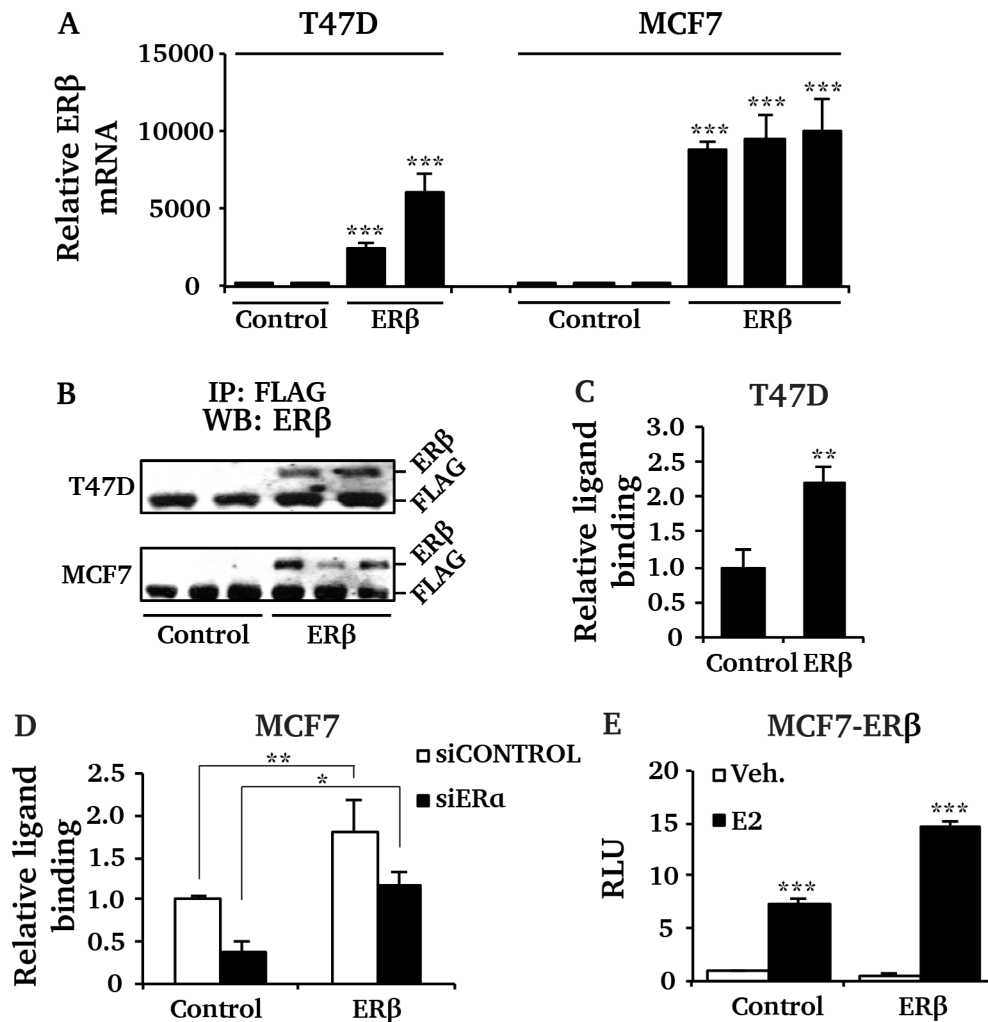


Figure 5.1 Characterization of ERβ expression in breast cancer cell lines T47D and MCF7. Using lentivirus transduction ERβ was expressed in duplicate (T47D) and triplicate (MCF7) mixed-cell populations. A) ERβ mRNA levels were increased in T47D-ERβ and MCF7-ERβ mixed-cell populations compared to in cells transduced with only control vector, as measured by qPCR. ERβ mRNA in parental and control cells of both cell lines were barely detectable (C_T values>32). B) ERβ protein was readily detectable in T47D-ERβ and MCF7-ERβ cells, measured by immunoblot of FLAG IP. Cell lysates were subjected to immunoprecipitation (IP) with a FLAG antibody prior to blotting with ERβ antibody. The expressed ERβ band is at 1 kDa higher than recombinant ERβ (at 59 kDa, not shown), due to the 8-aa FLAG tag. FLAG antibody heavy chain is co-eluted and crossreacts with secondary antibody, as indicated. IP/WB was carried out in duplicate for each mixed-cell population, representative experiment shown. C-D) Radioactive ligand-binding assay shows that ligand-binding capacity is enhanced by ERβ expression in both cell lines, and remains

high also when ERα is silenced in MCF7 cells. Efficacy of silencing is shown in Figure 5.6C. E) Luciferase ERE-transactivation assay in MCF7 cells confirms enhanced transactivation through ERβ expression upon E2 treatment.

ligand-binding receptors as the T47D control cells (Figure 5.1C), indicating an ERβ-to-ERα ratio of approximately 1:1. MCF7-ERβ also exhibited increased ligand binding after expression of ERβ, and siRNA of ERα visualized the contribution by ERβ (Figure 5.1D). In MCF7 cells, an ERE-luciferase transactivation assay showed that ERβ-expression enhanced transcriptional transactivation from an ERE (Figure 5.1E). Both receptors are known to form hetero- and homodimers with each other, and to co-localize in the cell nuclei of clinical breast tumors and cell lines (71). We showed, using co-immunoprecipitation (Co-IP) experiments after vehicle and E2 treatment, that ERβ and ERα formed heterodimers in both MCF7-ERβ and T47D-ERβ cells, with or without E2 stimuli (Figure 5.2), supporting a previous report (193).

ERα has an ERE-containing promoter and can be regulated by both ERs (194,195). We assessed whether the levels of ERα changed upon expression of ERβ. In T47D cells, the levels of ERα were not affected by neither ERα itself nor ERβ, as measured using qPCR and immunoblotting 24 h after vehicle or 10-nM E2 treatment, in cells with and without ERβ (Figure 5.3A-C).

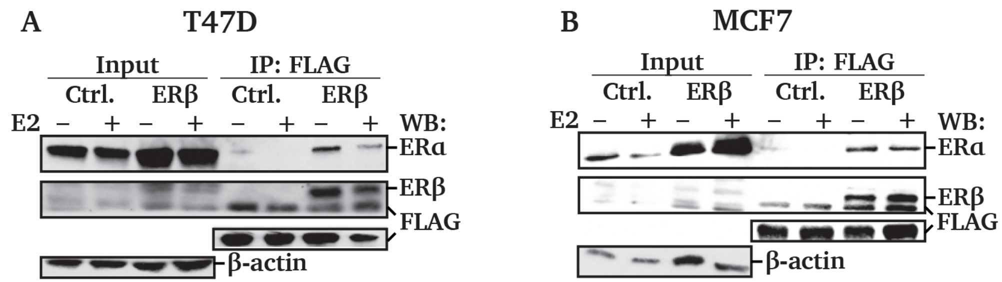


Figure 5.2. ERβ and ERα heterodimerize in T47D and MCF7 cells. Co-IPs using antibodies directed against ERα and FLAG (for ERβ) show that the ERs form heterodimers when co-expressed, both in absence or presence of ligand, in A) T47D and B) MCF7 cells. Co-IPs were performed after 24-h treatment with vehicle or 10 nM E2. ERα was detectable in Co-IPs of the FLAG-tagged ERβ from both vehicle and E2-treated T47D-ERβ and MCF-ERβ cells. In the control cells where ERβ was not expressed, no Co-IP bands were detected, supporting the specificity of the experiment. Gels were loaded with equal volumes of input lysate and immunoprecipitate. Light and heavy chain (26 and 54 kDa, respectively) of FLAG antibody, FLAG-tagged ERβ at 60 kDa, and ERα at 64 kDa are indicated.

In MCF7 cells ERβ expression doubled the basal levels (under estrogen-depleted conditions) of ERα (Figure 5.3A, D-E). Although ERα is usually repressed by E2 treatment, this regulation can vary across experimental conditions and be affected by e.g. seeding density of cells and cell synchronization protocols. Small E2-mediated upregulation of ERα mRNA, as noted in Figure 3A, has been reported in MCF7 cells previously (196). ICI treatment promoted degradation of ERα protein in all cases, whereas, under the experimental conditions used here, ERα protein degradation by E2 was minimal in all cells. We conclude that expression of ERβ significantly affected the levels of ERα in MCF7 cells, but not in T47D cells.

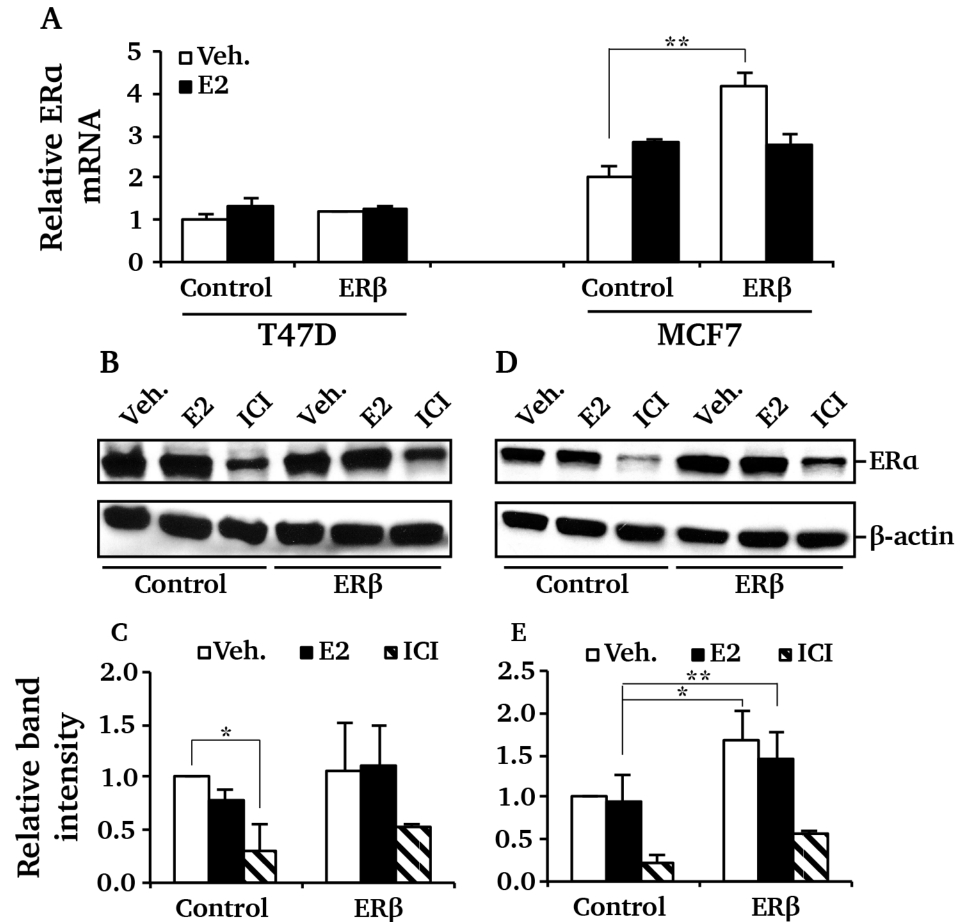
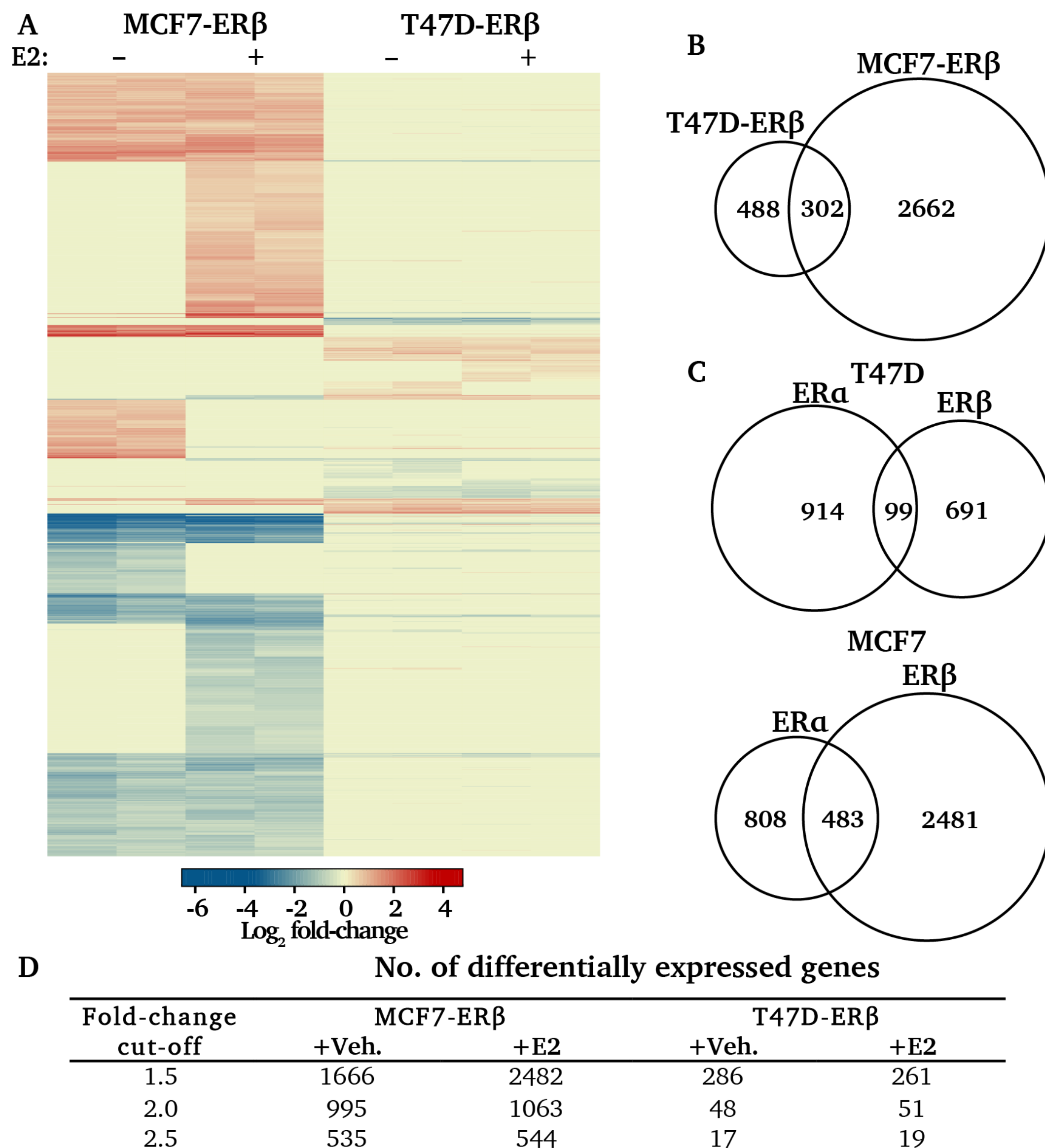


Figure 5.3 ERβ upregulates ERα in MCF7 but not in T47D cells. A) Relative ERα mRNA levels as measured by qPCR in T47D and MCF7 cells after 24 h vehicle and E2 treatment. Bar graphs illustrate the average and SD for all mixed-cell populations. B) ERα protein in T47D (left) and MCF7 (right) after 24-h treatment with vehicle, 10 nM E2 or 10 nM ICI, Two mixed-cell populations were analyzed for each condition and representative experiments are shown. C-E) Quantifications of all Western blots are shown for T47D and MCF7, respectively. Band intensities were normalized to β-actin.

5.3.2 ERβ exerted genome-wide effects on gene regulation and altered signaling pathways

We next examined the effect of ERβ over the transcriptome. Using microarray analysis, we assessed changes both at basal level (Control+Veh. vs. ERβ+Veh.) and after 24-h estrogen stimulation (Control+E2 vs. ERβ+E2) in biological and technical duplicates of both cell lines. The results, illustrated in Figure 5.4A and C, indicate that ERβ expression mediated an impact on both basal and E2-mediated gene expression in both cell lines. A total of 302 genes were regulated in both T47D and MCF7 cells (Figure 5.4B and Figure 5.5A), including repression of *AHR* (Aryl hydrocarbon receptor), the Wnt inhibitor *DKK1* (Dickkopf 1) and *TGFB2* (Transforming growth factor beta 2). The repression of both *TGFB2* and *DKK1* indicates potential effects on proliferation (197). Other genes were regulated in opposite manner in the two cell lines: The cell-cycle progression and poor-prognosis gene *CCNA2* (Cyclin A2) (198) was upregulated in MCF7-ERβ but downregulated in T47D-ERβ cells and *PIK3R1* (p85, a subunit of Phosphatidylinositol 3-kinases [PIK3]) that can contribute to non-genomic ERα signaling (199,200) was downregulated in MCF7-ERβ but upregulated in T47D-ERβ cells.



Fold-change cut-off	MCF7-ERβ +Veh.	MCF7-ERβ +E2	T47D-ERβ +Veh.	T47D-ERβ +E2
1.5	1666	2482	286	261
2.0	995	1063	48	51
2.5	535	544	17	19

Figure 5.4 Large transcriptome effects of ERβ in T47D and MCF7 cells. Microarrays were used to analyze changes in gene expression between cells with and without ERβ, in the absence and presence of 10 nM E2. Both biological (different mixed-cell populations) and technical replicates were used for each condition and cell line. A) A heatmap based on hierarchical clustering of transcripts (rows) and samples (columns), illustrates the genome-wide changes of both upregulated (red) and downregulated (blue) genes. B) Genes affected by ERβ expression (in presence of E2) in both T47D and MCF7 are illustrated using Venn diagram. C) Comparison of

genes regulated by ERα (after 24 h of 10-nM E2 treatment) in parental cells with genes affected by ERβ expression, is illustrated by a Venn diagram for T47D and MCF7. D) Table showing the number of differentially expressed genes under the different conditions, at different cut-off values for the fold-change comparison ERβ vs. control cells.

To determine the signaling pathways that were affected by ERβ, we analyzed for enrichment of predicted transcription factor (TF) target genes. We found that putative targets of E2F and AP-1 were significantly enriched among ERβ-affected genes in both cell lines, whereas more Smad3, FoxJ2, and FoxO4 target genes were overrepresented in T47D cells and MYC, NFY, and Sp1 target genes predominated in MCF7 cells (Table 5.1). ERβ expression resulted in enhanced regulation of E2F target genes (data not shown), and although E2F1 is an AP-1 target gene, many other AP-1 target genes (including *APOD*, *ANXA1*, and *SYNPO*, all previously reported as regulated by AP-1 in MCF7 cells (165)) were repressed in both cell lines along with the two AP-1 factors c-Fos and c-Jun in MCF7 cells (data not shown). In addition, MYC signaling which is imperative in tumor cell cycle progression, and regulated by ERα in complex with Nuclear factor-Y (NFY) (201), was enhanced along with ERα, and NFY activity (data not shown) in MCF7-ERβ cells.

Table 5.1. Enrichment of transcription factor target genes in ERβ gene expression profiles. Transcription factors whose predicted targets were overrepresented upon expression of ERβ, in the presence of E2. *N* indicates number of genes. The list is ranked according to ascending *p*-value, starting from the top for T47D-ERβ and from the bottom for MCF7-ERβ, and redundant instances and motifs for unknown factors are removed. Overrepresentation is considered significant if $p < 0.05$, italics indicate no significance.

TF	Motif	T47D-ERβ		MCF7-ERβ	
		N	P-value	N	P-value
	Upregulated				
PRRX2	V$S8_01	9	3.84E-03	7	***9.36E-01***
STAT5A	V$STAT5A_03	9	3.97E-03	8	***8.81E-01***
GTF2A1	V$TFIII_Q6	8	4.22E-03	5	***9.60E-01***
TFDP1	V$E2F_Q6_01	8	4.89E-03	11	***3.54E-01***
FOXO4	TTGTTT_V$FOXO4_01	37	4.98E-03	80	***8.52E-01***
EBF2	CCCNNGGGAR_V$OLF1_01	10	4.99E-03	9	***9.40E-01***
ZEB1	CAGGTA_V$AREB6_01	18	5.23E-03	21	***9.95E-01***
E2F1	V$E2F1_Q3	8	7.38E-03	14	***1.42E-01***
IRF1	V$IRF1_01	8	1.17E-02	4	***9.96E-01***
SREBF1	V$SREBP1_Q6	8	1.28E-02	11	***5.74E-01***
NRF1	RCGCANGCGY_V$NRF1_Q6	10	***4.91E-01***	43	***7.84E-02***
PAX3	CGTSACG_V$PAX3_B	1	***8.06E-01***	10	***6.68E-02***
TFDP1	V$E2F_Q4_01	5	***1.34E-01***	15	***6.32E-02***
STAT6	V$STAT_01	3	***6.13E-01***	17	***6.19E-02***
E2F1	V$E2F1_Q6_01	2	***7.73E-01***	17	***2.40E-02***
USF1	V$USF_C	5	***2.53E-01***	20	***2.10E-02***
SP1	GGGCGGR_V$SP1_Q6	40	***1.52E-01***	146	***1.15E-02***
AR	V$AR_02	2	***7.70E-02***	6	5.29E-03
MYC	CACGTG_V$MYC_Q2	12	***5.34E-01***	60	4.71E-03
NFYB	GATTGGY_V$NFY_Q6_01	18	***1.17E-01***	77	1.76E-05
	Downregulated				
RUNX1	V$AML_Q6	9	1.20E-03	18	***2.39E-01***
IKZF1	V$IK1_01	8	5.99E-03	18	***3.00E-01***
FOXA2	V$HNF3B_01	7	7.32E-03	16	***2.18E-01***
STAT1/STAT2	V$ISRE_01	7	7.32E-03	19	***5.64E-02***
SMAD3	V$SMAD3_Q6	7	8.01E-03	16	***2.38E-01***
NKX6-1	V$NKX61_01	7	8.75E-03	13	***5.80E-01***
HNF4A	V$HNF4_01_B	7	1.07E-02	27	5.95E-04
POU3F2	V$BRN2_01	7	1.16E-02	28	3.38E-04
SRF	V$SRF_Q4	7	1.19E-02	25	3.70E-03
LHX3	YTAATTAA_V$LHX3_01	6	1.23E-02	13	3.02E-01
TCF7	V$TCF1P_Q6	1	***9.11E-01***	27	4.59E-04
MTF1	V$MTF1_Q4	2	***7.15E-01***	28	4.02E-04
MAZ	GGGAGGRR_V$MAZ_Q6	21	***6.55E-01***	163	2.51E-04
SOX9	V$SOX9_B1	2	***7.04E-01***	29	1.19E-04
LEF1	CTTTGT_V$LEF1_Q2	22	***2.81E-01***	146	1.12E-04
PAX2	V$PAX2_02	5	***1.18E-01***	31	6.35E-05
FOXO4	TTGTTT_V$FOXO4_01	24	***1.86E-01***	153	3.50E-05
NFATC1	V$NFAT_Q4_01	21	***3.00E-01***	33	1.68E-05
TCF3	CAGGTG_V$E12_Q6	28	***1.94E-01***	180	1.42E-05
CEBPB	V$CEBPB_02	4	***2.83E-01***	34	8.94E-06

In conclusion, ERβ had major impact on the transcriptome in both cell lines, with significant similarities but also cell-specific characteristics. Several known ERα- and ERβ-affected transcription factor modules, including AP-1, E2F, MYC, TGFβ, NFκB, and FoxO4, were affected as a consequence of ERβ expression in both cell lines.

Others and we have previously shown that transient expression of ERβ opposes ERα-regulated transcription (174,184). Here, we explored what proportion of direct ERα targets were affected by stable ERβ expression. We found that about one third of genes that ERβ affected in both cell lines (112 of the 302 genes, including *TGFB2*, *PIK3R1* and *GATA3*) were identified as direct transcriptional targets of ERα using GRO-seq analysis (138), as illustrated in Figure 5.5A. Further, using microarrays to identify the genes that ERα regulated (24-h E2 treatment) in each cell line, we compared how ERβ affected these genes. In MCF7, 37% of ERα-E2 regulated genes (483 out of 1291, including ERα itself) were significantly affected by ERβ expression, whereas in T47D cells only 9% of ERα-regulated genes (99 out of 1103 genes) were affected (Figure 5.4C). Although the more extensive effects on ERα targets in MCF7-ERβ cells are likely attributable to the increase of ERα, ERβ expression appeared to enhance ERα signaling for the majority of the affected genes in both cell lines (84% or 406 genes out of 483 in MCF7, and 75% or 73 out of 97 genes in T47D). ERβ-

enhanced ERα-target genes included many well-known direct targets (pS2, PR, *GREB1*, *MYBL1*), and genes involved in functions such as cell proliferation (e.g. *BCL2*, c-Myc, *IGFBP4*), cell migration/cell adhesion and DNA repair in both cell lines (data not shown). ERβ opposed ERα-E2-induction for only 77 genes in MCF7-ERβ cells and 24 genes in T47D cells (data not shown). These genes include growth- and proliferation-associated *TGFB2*, *SLC3A2*, *B4GALT1* and *CCNA2* in T47D-ERβ, and genes associated to cell migration and motion, cell death, and hypoxia (e.g. *IGF1R*, *THBS1*, *KLF10*, *AEN, CAV1* and *PLOD2)* in MCF7 cells. We conclude that while ERβ did attenuate a small proportion of ERα gene-regulations, this was not its predominant action.

We confirmed the microarray data and dissected the manner in which ERβ influences ERα signaling further using qPCR on selected genes. Data for four genes where expression of ERβ enhances ERα-E2 regulation, and four genes where ERβ expression results in an opposing or divergent E2 response are shown in Figure 5.6. The ERE-controlled target gene pS2, the anti-apoptotic *BCL2* and the proliferative *MYC,* were increased at both basal and E2-regulated transcriptional levels by ERβ in both cell lines. Similarly, ERβ expression enhanced the regulation of the proliferative ion-channel *KCNK5* (Potassium channel, subfamily K, member 5) (129) and the cell-cycle gene *CCNA2* (Cyclin A2) in MCF7 cells, but attenuated these regulations in T47D cells. ERβ could also

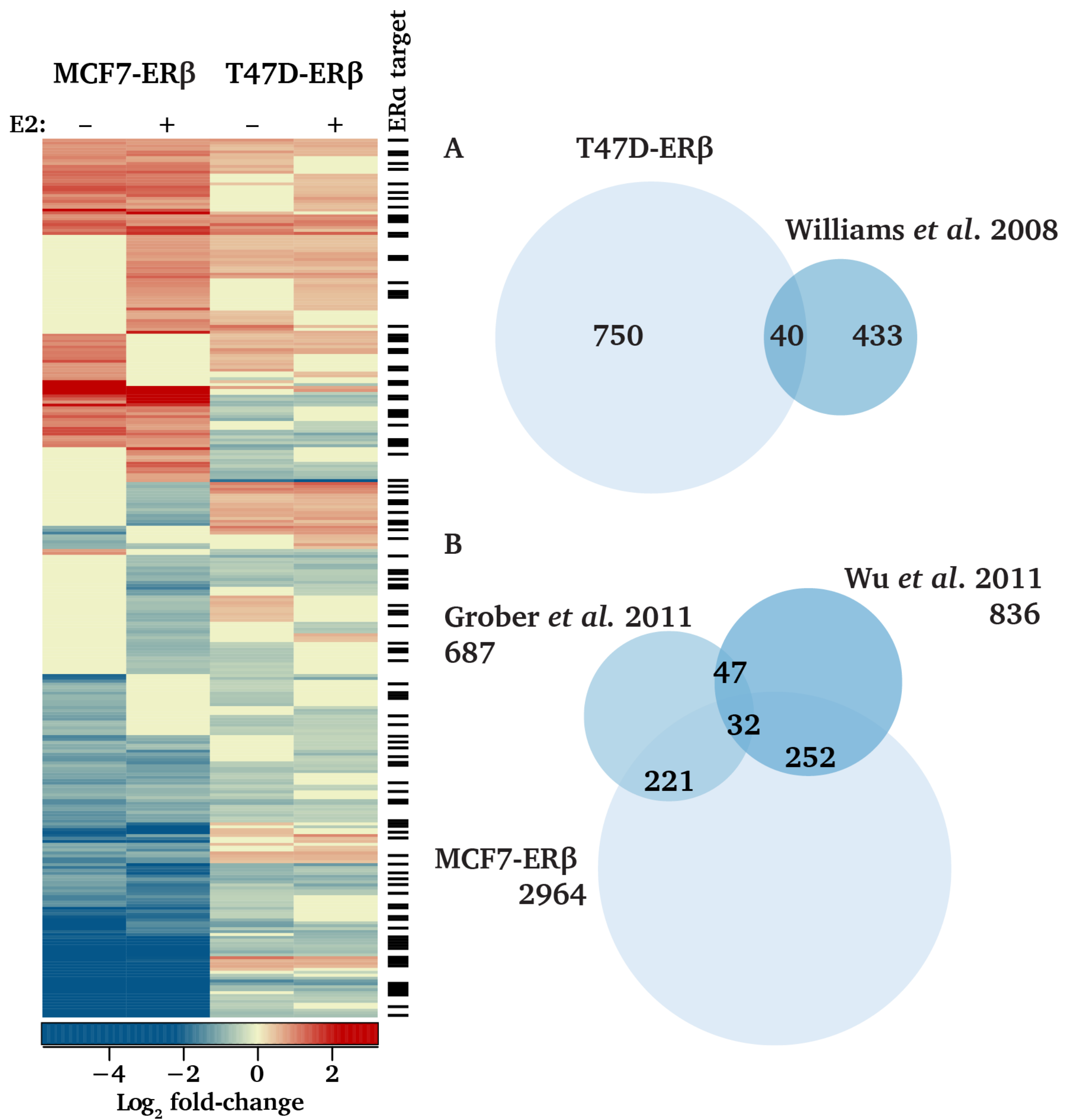


Figure 5.5. Overlap between prior genome-wide studies of ER-regulated genes and ours. A) Heatmap showing expression of the common set of differentially expressed genes in our study, with annotation for genes identified as E2-ERα transcriptional targets previously (138). In B) and C) Venn diagrams showing overlap of differentially expressed genes in present study and prior studies of gene expression in ERβ-expressing T47D and MCF7 (38,102,202).

enhance ERα repression, as illustrated by *APOD* (Apolipoprotein D) in both cell lines. On the other hand, ERβ expression reversed the effect of ERα regulation for *CLDN1* (Claudin 1), and attenuated the E2-induction of the poor-prognosis gene Cathepsin D in both cell lines (all regulations shown in Figure 5). Overall, the qPCR analysis confirmed the microarray data and illustrated that ERβ augmented ERα-signaling for many critical genes, while mediating differential regulation on others.

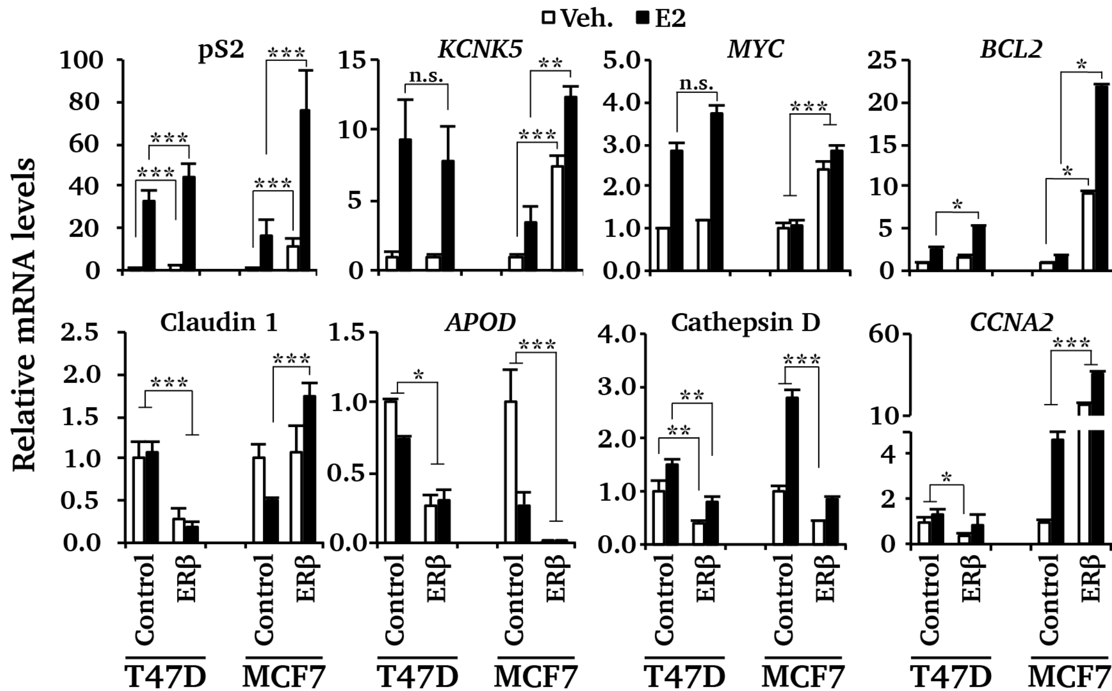


Figure 5.6. Gene-specific transcriptional effects by ERβ. Known ERα-target genes are affected by ERβ expression in absence and presence of E2. The regulation of pS2, *KCNK5*, *MYC, BCL2*, Claudin 1, *APOD*, Cathepsin D and *CCNA2* are illustrated by bar graphs. Statistically significant differences between ERβ and non-ERβ expressing cells are indicated by asterisks. The significance of changes by E2 treatment alone is not indicated for simplicity.

Discriminating the effects of ERα- and ERβ-mediated regulation was complex because ERβ upregulated ERα in in MCF7 cells, and E2-treatment downregulated ERβ expression in both cell lines (data not shown). We therefor attempted siRNA treatment to investigate receptor-specific gene regulations. Silencing of the endogenous ERα in MCF7 cells was effective, reducing its mRNA levels by 80-90% (Figure 5.7A), and ablating detectable protein expression (Figure 5.7C) along with reduction of downstream target genes pS2, *CCNA2,* and *BCL2* (exemplified in Figure 5.6D). As ERβ is stably expressed from a CMV promoter, its transcript levels could only be mildly reduced by siRNA (by 30% using double transfections, Figure 6B). This relatively slight silencing still resulted in reduction of the target genes pS2 (Figure 5.7D), *CCNA2,* and *BCL2***.** Using the ERβ-selective ligand KB101471 or E2, in combination with siERα, we demonstrated ERβ homodimer transactivation of pS2 (Figure 5.7E). The same experiment in control (no ERβ) cells ablated pS2 regulation (Figure 5.7E). Tamoxifen treatment reduced ERβ-mediated gene regulations (Figure 5.7F). In conclusion, we distinguish gene-specific effects of both ERs that can be quenched upon silencing of the receptors.

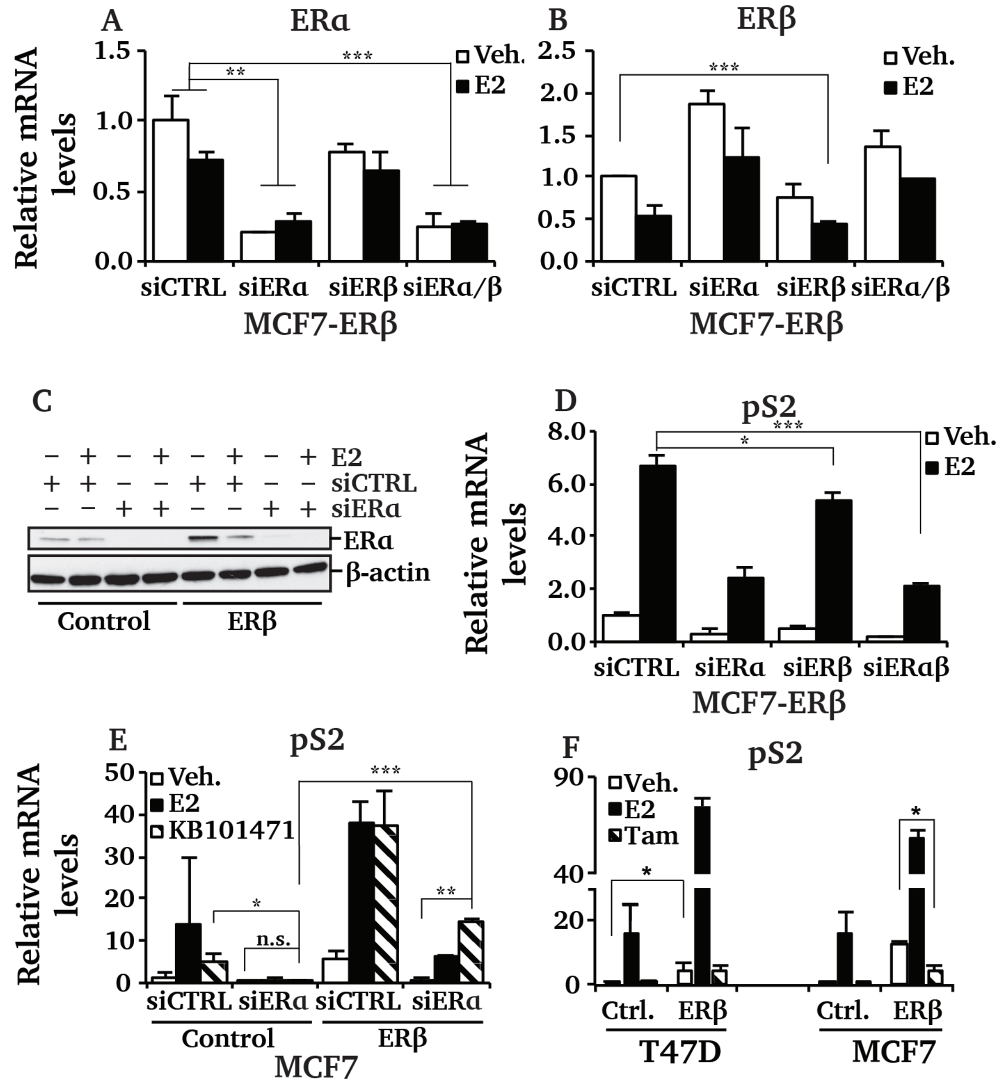


Figure 5.7. Receptor-specific effects on gene transcription identified through receptor silencing and SERM treatments in MCF7 cells. ER knockdown and ligand treatments with E2 (10 nM), ERβ-selective ligand KB101471 (0.5 nM) and ER antagonist tamoxifen (1uM) were used to identify the contribution by each receptor. qPCR measurements of relative mRNA levels of A) ERα and B) ERβ in MCF7-ERβ cells indicate effectiveness of RNA silencing C) Measurements of ERα protein levels using Western blot confirm effective ERα silencing in in MCF7-ERβ. D) Both ERα and ERβ silencing in MCF7-ERβ cells affect the regulation of direct target gene pS2. E) Silencing of ERα and treatment with vehicle, E2 (10 nM) or ERβ-specific agonist KB101471 (0.5 nM) in MCF7 control and MCF7-ERβ cells reveals ERβ regulation of pS2. F) Similar to E), with tamoxifen treatment (1 μM). Panels A, B, and D show average of triplicate experiments.

5.3.3 Effect of ERβ expression on genes associated with proliferation, apoptosis and adhesion, and respective phenotype

After establishing that, under the conditions analyzed ERβ enhanced rather than attenuated ERα signaling for most target genes, we proceeded to explore the biological outcome that stable expression of ERβ generated in these cells. Enrichment analyses of gene ontology biological processes, presented in Tables 5.2 and 5.3, indicated that ERβ affected genes involved in the response to estradiol, cell adhesion, apoptosis, proliferation, transcription, and inflammatory response in both cell lines. Functional studies were carried out to determine the effect of stably expressed ERβ on proliferation, apoptosis, and migration.

To determine the effect of ERβ on cellular proliferation, we subjected cell lines grown in full-serum medium for cell counting over a period of 6 to 8 days after seeding. T47D-ERβ cells grew at a pace similar to both the control mixes and the parental cell line, while MCF7-ERβ cells grew faster than both control and parental cells (Figure 5.8A). Thus, ERβ did not exhibit anti-proliferative properties when stably expressed in our experiment.

Table 5.2. Enriched biological processes in ERβ gene expression profiles. Gene Ontology functions that were overrepresented among genes affected by ERβ in both cell lines, in the absence of ligand. The list is ranked according to ascending p-value, starting from the top for T47D-ERβ and from the bottom for MCF7-ERβ. Redundant terms omitted, *N* indicates number of genes. Overrepresentation is considered significant at $p < 0.05$.

Term	T47D-ERβ		MCF7-ERβ	
	N	P-value	N	P-value
Positive regulation of apoptosis	19	2.72E-09	32	5.08E-09
Regulation of cell proliferation	16	1.54E-08	33	1.24E-12
Response to estradiol stimulus	14	3.61E-08	27	6.71E-11
Cell adhesion	31	6.49E-08	71	5.79E-12
Collagen fibril organization	7	4.94E-07	6	2.40E-03
Branching morphogenesis of a tube	7	1.37E-06	5	2.10E-02
Cellular response to insulin stimulus	5	8.24E-03	31	2.74E-11
Response to drug	21	1.62E-05	56	1.45E-11
Interferon-gamma-mediated signaling pathway	5	5.71E-03	22	4.68E-13
Phosphorylation	13	***5.86E-02***	63	2.48E-13
Axon guidance	17	1.11E-05	50	4.99E-15
Blood coagulation	20	4.13E-05	66	3.35E-17
Positive regulation of transcription from RNA polymerase II promoter	25	6.88E-06	81	1.01E-19
Cell cycle	21	3.58E-04	113	3.87E-41

Next, we evaluated the effect of ERβ under serum-starved conditions, and after treatment with E2, tamoxifen or ICI. Using MTS assays, we again observed that the expression of ERβ did not significantly change the proliferation of T47D (Figure 5.8B). ERβ did, however, slightly reduce the E2-dependent induction of cell growth. The growth inhibitory effect of tamoxifen or ICI was similar in the ERβ-expressing cells as in the control cells. MCF7-ERβ cells showed an increased level of proliferation also in estrogen-depleted media, and an enhanced response to E2 (Figure 5.8B). This growth was also reduced by addition of tamoxifen or ICI. Control experiments, to verify that control-transduced cells behaved similarly

to parental cell lines and to previous experiments using a synchronizing protocol (10 nM ICI for 24 h) before E2 treatment, were carried out (Figure 5.8E). The effect of ERβ expression on the proliferative phenotype of the two cell lines reflected the changes observed at the transcriptome level.

As ERα drives proliferation and the level of ERα was doubled in MCF7-ERβ cells, we speculated that this might be the primary driving event for the increased growth noted in these cells. We silenced both ERs, and investigated their respective impact on proliferation using the MTS assay (Figure 5.8C). Silencing of ERα in MCF7-ERβ cells brought down its level to one fifth, yielding 2-fold less ERα levels compared to MCF7 control cells. As expected, this silencing significantly reversed the increased basal and E2-induced proliferation of the MCF7-ERβ cells. ERβ alone could thus not sustain the elevated proliferation. The slight silencing of ERβ that we accomplished did not significantly affect proliferation. We conclude that ERβ did not mediate anti-proliferative events, and that the increased proliferation noted in MCF7-ERβ cells is, at least partly, attributed to increased ERα levels.

Both ERα and ERβ have previously been shown to induce, or sensitize different type of cells to apoptosis (160,203–208). Apoptosis-associated genes were also overrepresented among genes affected by ERβ (Table 5.2). To assess for altered

susceptibility to apoptotic events, we examined PARP cleavage in MCF7 cells in presence and absence of ERβ, E2, and the DNA-damaging agent cisplatin. We did observe increased PARP levels overall by ERβ expression, but the fraction of cleaved PARP remained unchanged (data not shown). These experiments, thus, did not support an effect on apoptosis in MCF7-ERβ cells.

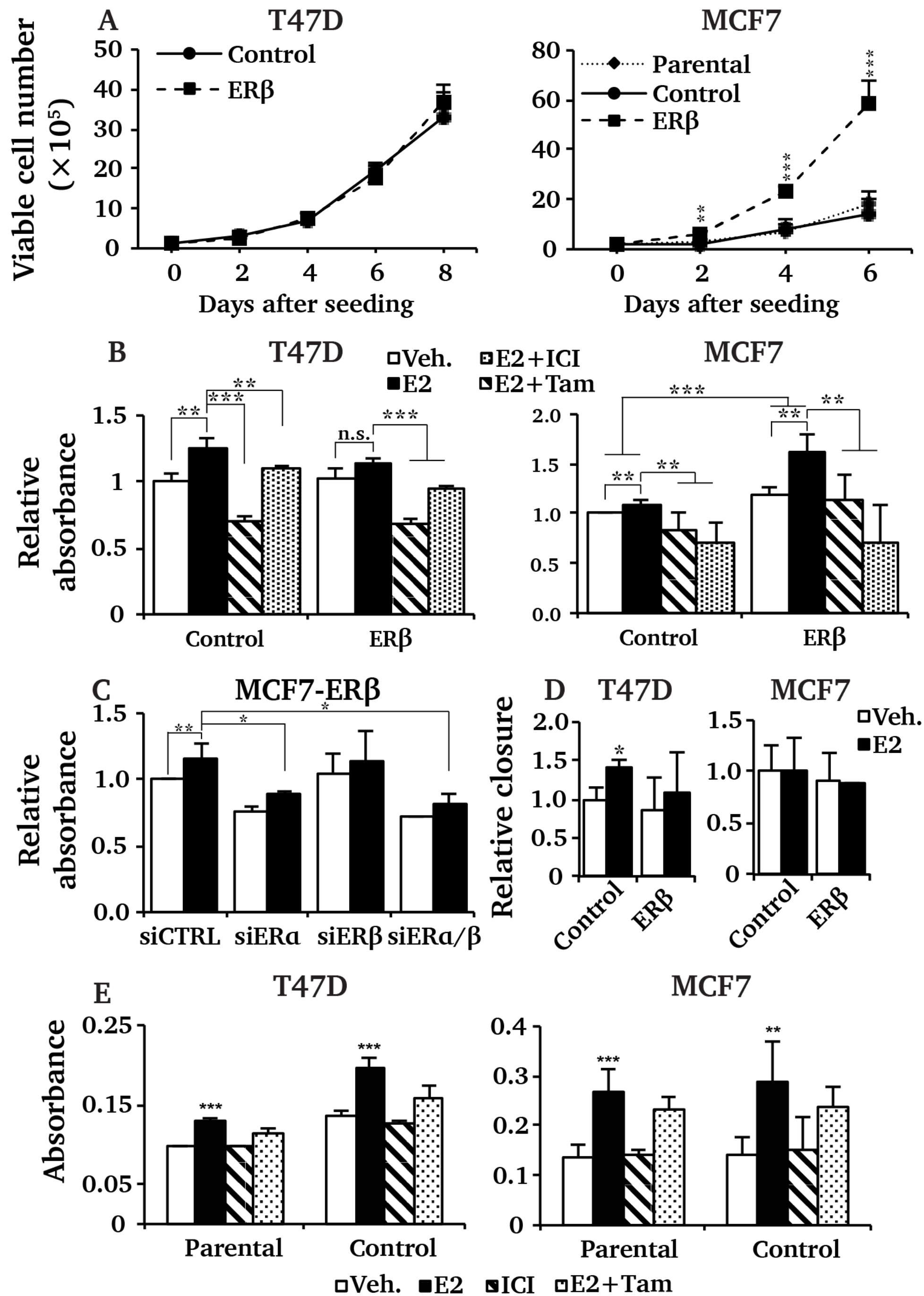


Figure 5.8. ERβ does not repress proliferation in T47D and MCF7 cells. Proliferation was measured using both cell counting and MTS assay under varying conditions. A) T47D cells and MCF7 cells, with and without ERβ expression, were counted over 6 or 8 days of growth in full-serum medium. Duplicate (T47D) and triplicate (MCF7) mixed-cell populations were analyzed,

and parental MCF7 cells were included. Each measurement was performed in duplicate or triplicates, and data for one representative mixed-cell population is shown. Proliferation of B) T47D cells and MCF7 cells, with and without ERβ expression, were measured using MTS assay. Cells were grown in estrogen-depleted medium and treated with vehicle, 10 nM E2, 10 nM E2+1 μM tamoxifen or 10 nM E2+1 μM ICI for 3 to 6 days. The average for all mixed-cell populations (two for T47D, each repeated twice, three for MCF7, each repeated 4 times) is shown in figure. C) Proliferation was measured after silencing of each receptor using MTS assay. Each bar illustrates the average for two of the mixed-cell populations, each performed in four replicates and measurement made in technical triplicates. D) The influence of ERβ on migration was measured using wound-healing assay for T47D and MCF7 cells with and without ERβ, after 24-h E2 (10 nM) or vehicle treatment. E) MTS assay to verify that the control cells proliferated similarly to the parental cell lines, with treatments as in B).

ERβ has been implicated as a repressor of migration in breast cancer cells (209–211) and of invasiveness in models of inflammatory breast cancer (212). The gene expression profiles of T47D-ERβ and MCF7-ERβ cells suggested altered ability of the cells to migrate, as indicated by enrichment of related biological processes (Tables 5.3). We performed a wound-healing assay to assess the influence of ERα+E2 on migration in control cells, and the corresponding impact of ERβ. Our experiments showed that in T47D control cells, E2 treatment significantly stimulated ERα-mediated migration (Figure 5.87D), confirming previous studies (213). When ERβ was expressed, however, E2 no longer mediated a significantly increased migration. This aligns with the observed gene regulations in T47D cells where ERβ repressed migratory Claudin 1 expression and attenuated the ERα induced TGFβ (*TGFB2*). In MCF7 cells, on the other hand,

migration was not affected by either ERα or ERβ (Figure 7D). The ability of the assay to detect effects on migration in MCF7-ERβ cells was, however, occluded by increased proliferation under serum-starved conditions upon expression of ERβ. In conclusion, ERβ appears to have the capacity to attenuate E2-stimulated migration in T47D cells.

Table 5.3. Enriched biological processes among ERβ-regulated genes in both cell lines. Overrepresentation analysis based on Gene Ontology gene sets for the common (both MCF7-ERβ and T47D-ERβ) differentially expressed genes. *N* indicates number of genes. Ranking according to *p*-value.

Term	N	P-value
Blood coagulation	20	2.99E-09
Response to estradiol stimulus	11	2.86E-08
Regulation of cell proliferation	12	3.30E-08
Cholesterol biosynthetic process	7	4.67E-08
Kidney development	9	3.66E-07
Positive regulation of apoptosis	12	5.21E-07
Axon guidance	14	6.00E-07
Epithelial cell differentiation	7	1.03E-06
Metabolic process	43	1.08E-06
Sterol biosynthetic process	5	4.19E-06
Collagen fibril organization	5	6.83E-06
Leukocyte migration	8	7.13E-06
Regulation of catalytic activity	13	1.08E-05
Blood vessel development	6	1.40E-05
Bone development	4	1.89E-05
Negative regulation of mitosis	3	2.91E-05
Response to hypoxia	10	3.17E-05
Anti-apoptosis	10	3.40E-05
In utero embryonic development	10	3.40E-05
Lens fiber cell development	3	4.34E-05

5.4 Conclusion

In this study we aimed to further our understanding of ERβ's role in ERα-positive breast cancer. We generated T47D and MCF7 cell lines co-expressing the two ERs constitutively, each in replicated mixed-cell populations. Previous studies have indicated that transiently expressed ERβ opposes ERα signaling and corresponding proliferative function (as referenced above). After assessing constitutive expression at mRNA and protein levels (Figure 5.1), we showed that ERβ formed heterodimer with ERα (Figure 5.2) and induced large transcriptomic changes in both cell lines (Figure 5.4). Our gene-expression analysis showed that general ERα signaling was not attenuated by stably expressed ERβ (Figures 4 and 5). Enrichment analysis indicated ERβ's involvement in previously reported processes, including proliferation, DNA repair, adhesion, and modulation of the inflammatory response (Tables 5.2 and 5.3). Collectively, however, our results diverge from the body of data, including our own, that suggests that ERβ in breast cancer cell lines opposes ERα gene regulations in a genome-wide manner and thereby reduces proliferation.

The enrichment analysis indicated that ERβ-affected genes were involved in proliferation, and that key cell-cycle driving genes, including ERα, *CCNA2,* and MYC, were upregulated in MCF7-ERβ cells. Also in T47D-ERβ, proliferative genes

were upregulated but a concurrent downregulation of other pro-proliferative factors was also observed (e.g. *CCNA2*). E2F targets were increased in both cell lines and the E2F family plays crucial roles in the control of cell cycle and tumor progression. The increased E2F activity (data not shown) may be attributed to the downregulation of the repressing member, E2F4, in both cell lines. In addition, the activating E2F1 was upregulated in MCF7-ERβ cells (data not shown) and the repressive E2F7 was upregulated in T47D-ERβ cells, contributing to the more extensive effects noted in MCF7 cells. E2F1 is a known target of ERα, regulated in tandem with AP-1 in MCF7 cells (165), and constitutes an essential part of ERα-mediated cellular proliferation of breast cancer cells (165,214,215). AP-1 also has a central role in in ERβ-mediated signaling (39,165), and 60% of ERβ-chromatin binding sites have been found to contain both ERE- and AP-1-like sites (39). Functional analysis demonstrated that ERβ did not affect overall proliferation in T47D-ERβ cells, whereas in MCF7-ERβ cells an increased proliferation was recorded (Figure 5.8A-B). This was consistently observed in the independent mixed-cell populations. We primarily attributed the increased proliferation in MCF7 cells to the two-fold increased levels of ERα. Overexpression of ERα in MCF7 cells has previously been shown to increase cell proliferation (216,217). When we silenced ERα in MCF7-ERβ cells, the cells reduced their proliferation to the levels of the control cells. However, since this reduced level of ERα was two-fold lower than its levels in control or parental cells without corresponding

change in proliferation, we cannot exclude that ERβ contributes to a growth advantage.

The reasons for the divergent results of ERβ in terms of proliferation can be several. We note that most studies concluding anti-proliferative abilities have been performed using transient transfection or inducible systems, and we propose two factors that may contribute to the divergent results: selection of cells with proliferative advantage under stable conditions and/or cofactor squelching during transient conditions. The selection of stably expressing ERβ cells could enrich for cells that can proliferate in the presence of ERβ, thereby obscuring anti-proliferative properties. We initially noted, within a week of transduction, that ERβ opposed ERα signaling in the T47D-ERβ cells, as reported previously (184). However, as the cells adapted to ERβ expression this was reverted to a pro-ERα activity for many target genes in both T47D and MCF7 cells. It is possible that the selection favors cells that can proliferate in the presence of ERβ in a non-physiological manner. However, the fact that we observed the same results in each of the mixed-cell populations analyzed, and did not note an anti-proliferative effect in either cell population, indicates that this is not an infrequent characteristic. Possibly, similar characteristics may evolve in clinical tumors. As we have used the same system and selection procedure and found ERβ to be anti-proliferative in colon cancer cells (182,204), we know that this approach can be

used to detect anti-proliferative functions of ERβ. We need to also consider the impact of cofactor squelching during transient expression (218). When significant levels of ERβ are introduced, ERβ will compete for interaction with many of the same cofactors that ERα also requires. This, by itself, can result in an attenuation of the ERα-mediated gene regulation and proliferation. When ERβ is stably expressed, on the other hand, the cells can adapt and reach a steady-state level of needed factors. Cells with stable expression may therefore evade the squelching effect. Our result that ERβ is not anti-proliferative when stably expressed in breast cancer cells aligns with clinical findings where ERβ *in vivo* often does not correlate with an anti-proliferative phenotype (as referenced in introduction and reviewed by Leygue and Murphy (79)). Although analysis of stable ERβ expression in MCF7 cells was recently reported (38,202) and a reduced proliferative response to E2 was noted (38), effects on basal proliferation or interplay with ERα were not explored in detail. In triple-negative breast cancer cell lines, several studies have reported a lack of anti-proliferative effects by ERβ (219–221). We suggest that our study offers a potential mechanistic model for ERβ's role in a subset of breast tumors. Our result does not preclude that ERβ can posses anti-proliferative abilities in other cells, in other circumstances, or when activated *de novo*.

Aligning with our results that stably expressed ERβ did not reduce proliferation in ERα-positive breast cancer cells, we did not observe extensive attenuation of ERα's transcriptional regulation in our experiments. On the contrary, we noted an enhancement of ERα target gene regulation when ERβ was expressed in both cell lines (Figure 5). It is established that homo- and heterodimers of both ERs bind at the promoter of e.g. pS2 (38,39,222,223), but conflicting data have been reported as to whether ERβ opposes or enhances ERα's regulation (38,184,202,222,223). Our data indicate that ERβ enhances the ERα-E2 response for most co-regulated genes, in both cell lines. Of note is that ERα levels were induced 2-fold by ERβ in MCF7 cells, which contributed to an enhanced E2 response in these cells. ERα regulation was however attenuated by ERβ for a small proportion of target genes in both cell lines (Figure 5.5). In T47D cells, further, the ERα levels were unchanged, but the regulations of known ERα-targets were still enhanced. Our data align with a previous suggestion that the ERα-ERβ heterodimer largely acts in a manner similar to the ERα homodimer (224). It is likely that the type of regulation depends on whether ERβ binds as homo- or heterodimer, and whether it binds to *cis*-regulatory sequences at ERE sites or tethers with AP-1 or Sp1 transcription modules.

Three groups have compared the DNA-binding sites of both receptors in MCF7 cells at a genomic scale (38–40). They found that 33-73% of ERβ-binding sites

were sites where ERα homodimer can also bind, and that the majority of binding sites contained full or half EREs. Zhao et al. found significant enrichment of AP-1 and Forkhead motifs, and that 60% of ERβ-binding sites contained both ERE- and AP-1-like sites (39). Thus, the two ERs can bind many common regions that possibly are dominated by full EREs, whereas sites unique to each ER might be enriched for other type of motifs. ERα is known to activate AP-1 sites (165), whereas ERβ has been reported both to repress (225) and activate (226) such transcription. Our data support that AP-1 targets, with E2F1 as one exception, were repressed by ERβ. Further, we note that genes with Sp1 motifs in their promoters were upregulated in MCF7-ERβ cells, correlating with findings of ERβ activity in osteosarcoma cell line U2OS (227). That one of the few genes where ERβ opposed ERα in both cell lines, Cathepsin D, is regulated in complex with Sp1 (228–230) , may indicate that ERβ's influence on ERα-mediated regulation via Sp1 tethering is different from that of direct regulation. Global ChIP-reChIP would need to be carried out to fully characterize genomic binding of the ER heterodimer, and to differentiate it from common binding by ERα and ERβ homodimers.

We compared our gene expression dataset to the recently published data from MCF7 cells also engineered to express ERβ stably (38,202) as well as our previous study of inducible ERβ expression in T47D cells (184). Whereas the overlap of

transcriptional changes between transient and stable ERβ expression in T47D cells was low (10% of the transient-ERβ regulated genes were observed), comparison between studies of stable expression in MCF7 cells rendered a relatively large overlap. Here, 37% and 34%, respectively, of genes previously identified as regulated by ERβ expression were changed in our study (data not shown), supporting the generality of these data

We further observed that E2-induced migration was opposed in T47D cells, that the MCF7-ERβ-expressing cells adhered to each other more strongly than the control cells, and that migration and proliferation assays was more dependent on cellular confluency when the cells expressed ERβ. ERβ also affected several cell-adhesion genes in both cell lines, including repression of *TGFB2* and, in MCF7-ERβ cells, repression of the TGFβ-induced KLF10, which has been demonstrated as an ERβ-specific target in U2OS cells (231). These properties are in line with evidence that ERβ has a role in cellular adhesion and TGFβ signaling (174,210,211,232). Due to the dual roles of TGFβ in cellular proliferation, cellular migration and cancer metastasis (233–236), this aspect of ERβ regulation merits further investigation.

Our study suggests that ERα signaling and estrogen-induced proliferation can be unaffected or enhanced by ERβ, and that tamoxifen or ICI treatment is still able to

block these functions (Figure 5.). In addition, the previously described upregulation of SPINK4 by tamoxifen-liganded ERα (237) was maintained or enhanced in the presence of ERβ in both cell lines (data not shown). Several studies have concluded that tamoxifen is an antagonist to ERβ (238), but other has suggested that ERα-positive tumor cells become resistant to tamoxifen when ERβ is expressed (239). Our data support that patients with ERα/ERβ-positive tumors may benefit from tamoxifen or fulvestrant treatment, in line with the clinical evidence presented by Honma *et al.* (65).

In conclusion, we present evidence that breast cancer cells are able to continue proliferating and thrive while stably expressing significant levels of ERβ. Our analysis shows that ERβ can enhance rather than oppose ERα-signaling, and this knowledge aids in the understanding of the role ERβ mediates in some ERα/ERβ-positive breast cancer cells. Our study supports that the better survival noted in tamoxifen-treated patients with ERβ-positive tumors is because tamoxifen antagonizes both ERα and ERβ signaling. However, as also beneficial functions appeared mediated by ERβ, e.g. repression of Cathepsin D and migration, the optimal treatment approach will need to be carefully evaluated. *In vivo* studies are needed to investigate how stable ERβ expression impacts tumor metastasis in ERα-positive breast cancer cells. As ERβ is a highly druggable target, a better understanding of its function is critical. Currently, only ERα is utilized in the clinic

and our study supports the accumulating data that ERβ is a promising target for breast cancer therapy.

6. Concluding remarks and future directions

The estrogen receptors are of high interest for their pivotal role in many biological processes, as well as their involvement in multiple human diseases. As transcription factors the control large transcriptional programs, the effect upon which is modulated by a plethora of factors. Such factors include the presence of receptor ligands of various specificity and potency, coregulatory proteins that interact with the ERs at the DNA, as well as proteins from other signal transduction pathway that crosstalk with the ERs. Collectively, this makes the ER signaling a complex system to dissect.

In order to study the signaling of the ERs in breast cancer, and the pathways by which they regulate oncogenic processes, we utilize immortalized cell lines, which allows for manipulations of various kinds, in order to understand this biology.

In chapter one, we describe how ERα directly regulates the expression of the ion channel KCNK5, and how this ion channel is involved in cell proliferation. Silencing the KCNK5's expression reduced basal and E2-induced proliferation. This discovery suggests that ion channels, as ER targets, are potential proliferative factors and thus also potential therapeutic targets. Further studies are required to elucidate whether KCNK5, or other ion channels, are under ER control in human breast tumors, and, if so, whether the blocking of the channel has any beneficial effect for the patient.

The second chapter describes our effort to extend out knowledge of the transcriptional program that ERα controls in breast cancer cells. Until recently, most studies of this kind have been biased, in the sense that they only have interrogated the known, protein-coding transcriptome. RNA-seq, which we applied here, allows for an unbiased view of all or a select subset of RNAs. In our study we observed the commonplace ERα–mediated regulation of protein-coding genes, but more interestingly, the regulation of lncRNAs (and pseudogenes). How lncRNAs function and what roles they have in human biology, is still a field in its

infancy, and only approximately one percent of *bona fide* or computationally predicted lncRNAs have experimentally been ascribed a function. To extend upon our research, the regulation of individual lncRNAs should be verified and their possibly role in tumorigenic processes in breast cancer assessed. Additionally, studies should also, albeit technically challenging, focus on the whole transcriptome to uncover other transcriptional targets of ERα, as well as other aspects of transcriptional regulation such as alternative splicing and allele-specific expression.

Finally, the third chapter revolves around ERβ. Studies of ERβ are hampered by its elusive expression pattern, the paradoxical findings of its near-absence of mRNA yet presence of protein, and the lack of specific and commercially available antibodies. The lion's share of studies of ERβ's function in breast cancer point to it being an anti-proliferative factor that possible counteracts the adverse effects of ERα. However, clinical data remain inconclusive and contradictory on this topic, and no ERβ-targeted therapy is available for breast cancer. If ERβ is expressed at the levels it has been demonstrated to be, in breast tumors, it would make it a unique tumor suppressor to be so ubiquitously expressed in tumors. In our study, we find that long-term established stable cell lines expressing ERβ do, in fact, suggest that the receptor not always counteracts the effects of ERα, not on regulation of individual genes, nor on the cell proliferation. Much remains to be

done in order to develop our understanding of ERβs function, in normal as well as disease biology.

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