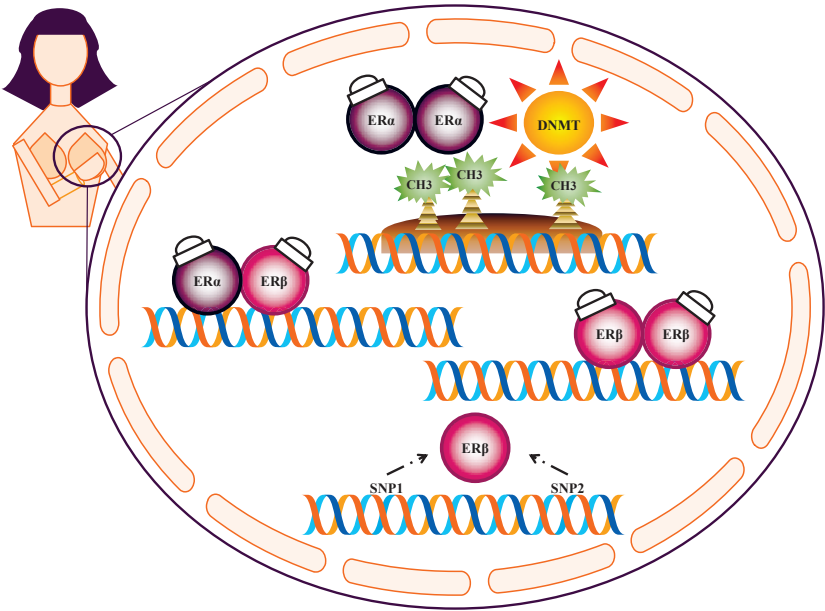


MOLECULAR CHARACTERIZATION OF ESTROGEN RECEPTORS WITH FOCUS ON BREAST CANCER



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CANCER**

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To my mother,
and other heroes,
who fight the Breast Beast

ABSTRACT

Estrogen signaling is mediated by estrogen receptors (ERs), ER α and ER β . Aberrant estrogen signaling is involved in breast cancer development. ER α is one of the key biomarkers for diagnosis and treatment of breast cancer. Unlike ER α , ER β is still not introduced as a marker for diagnosis and established as a target of therapy. Numerous studies suggest antiproliferative effects of ER β , however its role remains to be fully explored. Albeit important, ER α is not a perfect marker, and some aspects of ER α function are still unclear. This thesis aims to characterize distinct molecular facets of ER action relevant for breast cancer and provide valuable information for ER-based diagnosis and treatment design.

In **PAPER I**, we analyzed the functionality of two common single nucleotide polymorphisms in the 3' untranslated regions of ER β , rs4986938 and rs928554, which have been extensively investigated for association with various diseases. A significant difference in allelic expression was observed for rs4986938 in breast tumor samples from heterozygous individuals. However, no difference in mRNA stability or translatability between the alleles was observed.

In **PAPER II**, we provided a more comprehensive understanding of ER β function independent of ER α . A global gene expression analysis in a HEK293/ER β cell model identified a set of ER β -regulated genes. Gene Ontology (GO) analysis showed that they are involved in cell-cell signaling, morphogenesis and cell proliferation. Moreover, ER β expression resulted in a significant decrease in cell proliferation.

In **PAPER III**, using the human breast cancer MCF-7/ER β cell model, we demonstrated, for the first time, the binding of ER α / β heterodimers to various DNA-binding regions in intact chromatin.

In **PAPER IV**, we investigated a potential cross-talk between estrogen signaling and DNA methylation by identifying their common target genes in MCF-7 cells. Gene expression profiling identified around 150 genes regulated by both 17 β -estradiol (E2) and a hypomethylating agent 5-aza-2'-deoxycytidine. Based on GO analysis, CpG island prediction analysis and previously reported ER binding regions, we selected six genes for further analysis. We identified BTG3 and FHL2 as direct target genes of both pathways. However, our data did not support a direct molecular interplay of mediators of estrogen and epigenetic signaling at promoters of regulated genes.

In **PAPER V**, we further explored the interactions between estrogen signaling and DNA methylation, with focus on DNA methyltransferases (DNMT1, DNMT3a and DNMT3b). E2, via ER α , up-regulated DNMT1 and down-regulated DNMT3a and DNMT3b mRNA expression. Furthermore, DNMT3b interacted with ER α . siRNA-mediated DNMT3b depletion increased the expression of two genes, CDKN1A and FHL2. We proposed that the molecular mechanism underlying regulation of FHL2 and CDKN1A gene expression involves interplay of DNMT3b and ER α .

In conclusion, the studies presented in this thesis contribute to the knowledge of ER β function, and give additional insight into the cross-talk mechanisms underlying ER α signaling with ER β and with DNA methylation pathways.

LIST OF PUBLICATIONS

- I. **Putnik M**, Zhao C, Gustafsson J-Å, Dahlman-Wright K
Effects of two common polymorphisms in the 3' untranslated regions of estrogen receptor beta on mRNA stability and translatability.
BMC Genetics, 2009, 10:55
- II. Zhao C, **Putnik M**, Gustafsson J-Å, Dahlman-Wright K
Microarray analysis of altered gene expression in ERbeta-overexpressing HEK293 cells.
Endocrine, 2009, 36(2), 224-32
- III. Papoutsis Z, Zhao C, **Putnik M**, Gustafsson J-Å, Dahlman-Wright K
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- IV. **Putnik M**, Zhao C, Gustafsson J-Å, Dahlman-Wright K
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Effects of estrogen on gene expression profiles in mouse hypothalamus and white adipose tissue: target genes include glutathione peroxidase 3 and cell death-inducing DNA fragmentation factor, alpha-subunit-like effector A.
J Endocrinol. 2008, 196(3), 547-57

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LIST OF ABBREVIATIONS

AF	Activation function
AI _s	Aromatase inhibitors
AP-1	Activator protein 1
BRCA	Breast cancer susceptibility protein
ChIP	Chromatin immunoprecipitation
Co-IP	Co-immunoprecipitation
DAC	5-aza-2'-deoxycytidine
DBD	DNA-binding domain
DNMT	DNA methyltransferase
E2	17 β -estradiol
ERE	Estrogen response element
ERK	Extracellular signal-regulated kinases
ER α	Estrogen receptor α
ER β	Estrogen receptor β
ER β 2	Estrogen receptor β 2 (ER β cx)
FHL2	Four and a half LIM domains 2
FOXA1	Forkhead box protein A1
GO	Gene ontology
GWAS	Genome-wide association studies
HDACs	Histone deacetyltransferases
HER2	Human epidermal growth factor receptor 2
LBD	Ligand-binding domain
LD	Linkage disequilibrium
MAP kinase	Mitogen-activated protein kinase
MBD	Methyl-CpG-binding domain protein
MeCP2	Methyl CpG binding protein 2
miRNA	microRNA
NCoR1	Nuclear receptor corepressor 1
NR	Nuclear receptor
PR	Progesterone receptor
pS2	TFF1, trefoil factor 1
siRNA	Small interfering RNA
SMRT	Silencing Mediator for Retinoid and Thyroid-hormone receptors
SNP	Single nucleotide polymorphism
Sp1	Specificity protein 1
SRC	Steroid receptor coactivator
TF	Transcription factor
Tet	Tetracycline
UTR	Untranslated region

1 INTRODUCTION

1.1 BREAST CANCER

Breast cancer is the form of cancer with the highest global incidence [1]. Of all cancer types diagnosed in women, it has the highest incidence (22%) and mortality rate (13%), and presents one of the biggest medical challenges of the modern world. According to estimates of lifetime risk, about 12% of women in the general population will develop breast cancer sometime during their lives. Established risk factors are age, high mammographic density, older age at first child, low number of children, hormone replacement therapy, ethnicity, high body mass index, exposure to ionizing radiation, increased alcohol consumption, low physical activity and genetic factors [2]. Only 5-10% of breast cancer cases are hereditary. Mutations in BRCA1 and BRCA2 tumor-suppressor genes are the prime examples of hereditary causes of breast cancer. These mutations are uncommon, but associated with high risk of early development of breast and ovarian cancer [3, 4].

Breast cancer is a complex disease, with heterogeneous molecular background. This, in combination with individual variations in origin, grade and stage at diagnosis are reasons for the lack of a universal cure for breast cancer. Breast cancer classifications based on histopathology include tumor stage (size, invasiveness, metastatic status), grade and origin. Breast cancer can originate in the lobular or ductal epithelium of the mammary gland, which represents the basis of the histopathological classification to lobular and ductal carcinoma, respectively.

Mammary ductal carcinoma is the most common type of breast cancer in women [5]. The mammary gland duct epithelium is comprised of two distinct cell types, **basal-like (myoepithelial)** and **luminal cells**. Breast cancer can originate from either cell type and it can be classified by cellular features and molecular features (Table 1) [6, 7]. Myoepithelial cells form an outer layer of the mature mammary duct, surrounding an inner layer of milk-secreting luminal cells. It is believed that mammary stem cells reside in a basal position between these two cell types and give rise to progenitor cells and both lineages of fully differentiated cells (*Figure 1*) [8-10]. Female mammary gland growth, development and function are regulated by hormones and growth factors. Elevated levels of estrogen, progesterone and growth factors, such as epidermal-, fibroblast- and insulin-like growth factors, induce proliferation and differentiation of mammary stem cells, suggesting possible mechanisms of cancer development [11-13].

The majority of hormones and growth factors exert their biological functions by binding to specific proteins - receptors, which became crucial biomarkers for diagnosis and treatment of breast cancer. Tumor biomarkers are usually proteins measured either in serum, plasma or tumor tissue and they are used to identify individuals with increased predisposition to develop a cancer, screen for early malignancies and/or assist

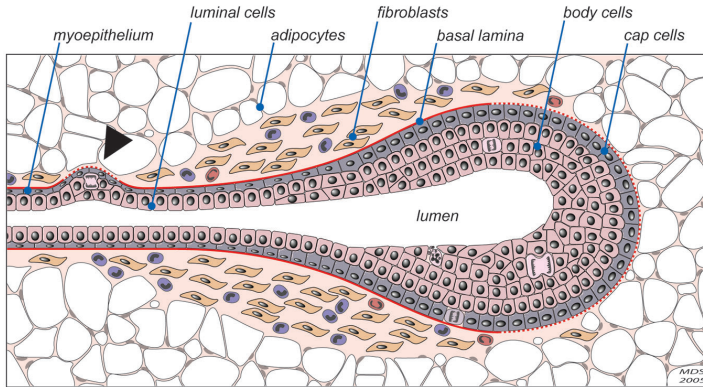


Figure 1. Simplified cellular organization of a mammalian gland terminal duct-lobular unit.
Copyright by Mark D. Sternlicht [10].

in cancer diagnosis, therapeutic strategy and prognosis. **Estrogen receptor (ER), progesterone receptor (PR) and human epidermal growth factor receptor 2 (HER2)** are the most significant biomarkers in breast cancer diagnostics and treatment. Hence, breast cancers are classified by ER, PR and HER2 status (Table 1) [6, 7, 14]. About 70% of breast cancer types belong to the ER+ luminal subtype. The luminal A subtype shows good prognosis and it is less invasive than the luminal B and ER-subtypes.

Table 1. Classification of breast cancer.

Classification of breast cancer					
	ER+		ER–		
	<i>Luminal A</i>	<i>Luminal B</i>	<i>Basal-like</i>	<i>HER2+</i>	<i>Normal-like/ unclassified</i>
Markers [6, 7, 14]	ER+PR+HER2–	ER+PR±HER2±	ER–PR–HER2– (triple negative)	ER–PR–HER2+	?
Incidence rate [6]	56-61%	9-16%	8-20%	8-16%	6-10%
5-year survival rate [14]	95%	50%	10%	30%	50%
Therapy [7, 14]	Hormonal	Hormonal, Chemotherapy, HER2 blockers	Chemotherapy	Chemotherapy, HER2 blockers	?

In general, early detection and diagnosis, and surgery remain the first-line and most successful strategies in breast cancer management. However, adjuvant treatments are required in most cases, in order to increase the survival rate, eliminate (micro)metastases, or where surgery cannot be performed. These include radiotherapy and systemic therapies - chemotherapy and targeted treatments.

Chemotherapy of breast cancer includes treatment with cytotoxic drugs, which target inhibition of cell mitosis or promoting apoptosis. Chemotherapy decreases mortality rate by 20-30% in 10 years [15]. The advantage of chemotherapy is low incidence of resistance, due to rather general biological effects, which can however lead to a wide range of severe side-effects [16].

ER and HER2, the most significant biomarkers, were essential for development of **targeted treatments** of breast cancer - *hormonal therapy* and *HER2 blockers*, respectively. Hence, ER and HER2 status is crucial for choice of therapy. PR status is usually positively associated with that of ER, and is considered as a marker of functional ER.

Amplification and/or overexpression of the HER2 gene are associated with the pathogenesis and progression of the aggressive HER2+ types of breast cancer [17]. Thus, the HER2 protein, a transmembrane tyrosine kinase exposed on the cell surface, became an important biomarker and target of therapy. HER2 blockers, such as trastuzumab (trade name Herceptin) and pertuzumab (trade name Omnitarg), are monoclonal antibodies, binding selectively to HER2 and blocking the dimerization, required for HER2 function [18, 19]. It has been shown that one year of treatment with trastuzumab after adjuvant chemotherapy has a significant overall survival benefit after a median follow-up of 2 years [20]. However, the majority of patients with metastatic breast cancer who initially respond to trastuzumab develop resistance within one year of treatment [21, 22]. Furthermore, HER2-blockers are considered expensive and can cause cardiac dysfunction [23].

ER status is a most crucial marker for breast cancer classification and treatment. Most breast cancers are ER+ and those patients typically receive hormonal (endocrine) therapy after completion of chemotherapy. Hormonal therapy is used to block the estrogen production or estrogen receptor signaling.

1.2 ESTROGEN RECEPTORS

The ERs belong to the nuclear receptor (NR) superfamily of ligand-regulated transcription factors. **ER α** was the first ER to be characterized, and it is used as a marker for diagnosis and treatment of breast cancer [24-26]. **ER β** , discovered in the mid 1990's [27], remains less characterized including its relation to breast cancer development.

Both ERs are co-expressed in a number of tissues including the mammary gland, epididymis, thyroid, adrenal, bone, and certain regions of the brain [28]. However, within some of these tissues, such as the endometrium and the prostate, they are expressed in different cell types [29, 30].

ER α is a dominant ER in the uterus, liver, kidney, and heart, whereas ER β is a dominant ER in the ovaries, prostate, lung, gastrointestinal tract, bladder, and

hematopoietic and central nervous system [31]. Estrogen signaling plays a critical role in many physiological processes, including regulation of development, growth and function of many organ systems in the body. Therefore, aberrations in estrogen signaling are associated not only with different types of cancer, including breast, endometrial and ovarian cancers, but also with diseases such as osteoporosis, depression and eating disorders [32].

1.2.1 Structural and functional organization

ER α and ER β encoding genes are located on different chromosomes (6 and 14, respectively) [27, 33], having a typical NR domain organization and sharing relatively high protein domain homology. ER proteins include six functional domains referred to by letters A-F [34-36]. The A and B protein domains contain the ligand-independent transcription activation function-1 (AF-1), and sites for phosphorylation. The C domain contains the DNA-binding domain (DBD). The D domain contains nuclear localization sequences. The E domain is the ligand-binding domain (LBD) and it contains the ligand-dependent transcription activation function-2 (AF-2). The F domain is involved in coregulator recruitment. ER α and ER β share 96% amino acid identity in the DBD, approximately 53% amino acid identity in the LBD and 30% or less in other domains, involved in transactivation and localization (*Figure 2*) [35]. The latter may explain some of the observed differences in transcriptional activation potential of the two receptors.

Activated estrogen receptors form dimers. It is assumed that in cell types where the two receptor subtypes are co-expressed, the formation of α/β heterodimers plays an important role in estrogen signaling, affecting patterns of gene regulation distinct from those regulated by the ER homodimers [28].

Both ER encoding genes have complex promoter structures. The ER α gene is transcribed from at least nine promoters (A, B, C, D, T2, T, E1, F and E), into multiple transcripts that can vary in their 5' untranslated regions (5'UTRs) [37]. The significance of the multiple promoters in the ER α gene is still unclear. However, there are many examples of a tissue-specific usage of particular promoters and with associated production of different ER α mRNA variants. For instance, ER α transcripts derived from promoter B showed the highest expression in human breast cancer MCF-7 cells [38, 39], and promoters E1 and E2 are used predominantly in the liver [40, 41]. The ER β gene is transcribed from at least two promoters, named 0K and 0N [42], giving rise to two different messenger RNAs, which display distinct tissue distribution [43].

Both ER full-length mRNAs are encoded by eight exons. Additionally, both ERs are expressed in a variety of isoforms, due to alternative splicing [34]. The most relevant ER α splice variants, with regard to functionality and occurrence, are ER α 46 and ER α Δ3. ER α 46 was named after its predicted molecular weight of 46 kDa and corresponds to a deletion of the first coding exon [44]. It inhibits the function of the wild-type ER α and forms dimers with ER α and β [45]. ER α Δ3 is a result of a deletion

of exon 3, hence it lacks part of the DNA-binding domain. It inhibits the function of ER α [46].

ER β 2, also known as ER β cx, is the best characterized ER β isoform [47]. It utilizes an alternative last exon, and consequently encodes a variant receptor with an altered C-terminus. The amino acids corresponding to exon 8 are replaced with 26 unique amino acids, giving rise to an altered F domain and truncated protein [48]. ER β 2 has undetectable affinity for ligands and cannot activate transcription of an estrogen response element-driven reporter [47, 49]. ER β 2 has been found to bind ER α and inhibit ligand-induced ER α transcriptional activity, most likely by mediating ER α protein degradation [50, 51]. This suggests that ER β 2 has an important role in neutralizing the function of ER α , hence ER β 2 may be significant for diagnosis and treatment of breast cancer [52].

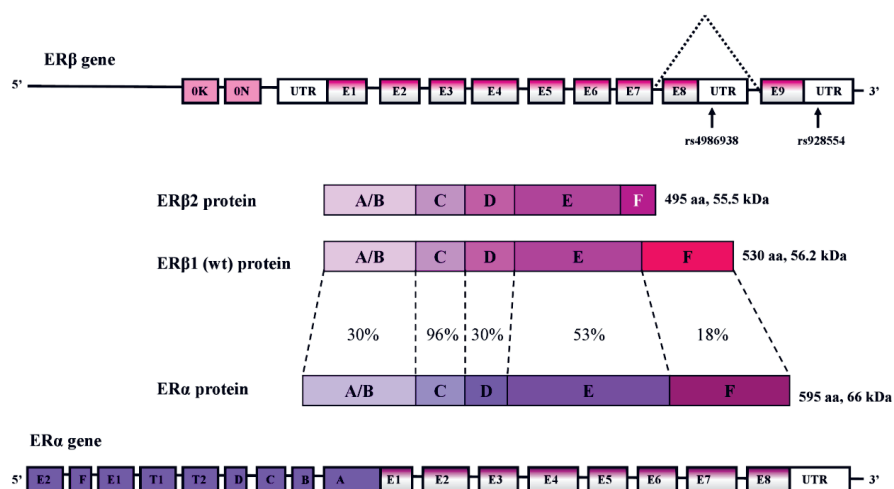


Figure 2. Structural organization of ER α and ER β genes and proteins. For the genes, exons, including UTRs, are indicated with boxes, and introns as lines in between. For the protein, the numbers on the right indicate the total size of the protein in amino acids (aa) and kilodaltons (kDa). Functional protein domains are marked by letters A-F. Homology between ER α and ER β domains is marked in %. The position of two SNPs studied in this thesis (**PAPER I**), rs4986938 and rs928554, is indicated.

1.2.2 Ligands

The main natural endogenous ER ligands (agonists) are estrogen hormones. The predominant estrogen in the body is 17 β -estradiol (E2), secreted by the ovaries during the female reproductive period. E2 metabolites, estrone (E1) and estriol (E3) are also ER ligands. E1 is the main estrogen before puberty and after menopause and is synthesized by the ovaries and the adipose tissue. E3 is produced mainly by the placenta during pregnancy. Of all three estrogens, E2 has the highest affinity for ER α , and equal binding affinity for ER α and ER β . E1 has preferential binding affinity for ER α over ER β , whereas E3 has preferential binding affinity for ER β over ER α [53].

Antiestrogens (ER antagonists) bind ERs in a manner similar to estrogens, but induce a different conformation of the ligand-binding domain [54, 55]. This results in a lack of recruitment of coactivators by the AF-2 domain. Most ER antagonists act by targeting the ER competitively, by binding and blocking access of other possible ligands. Some ER antagonists possess partial agonist activity, dependent on the cell type and tissue, and they are referred to as selective ER modulators (SERMs). The most common SERMs are tamoxifen and raloxifene. Fulvestrant/ICI 182,780, is a complete ER antagonist, and a SERD - selective estrogen receptor down-regulator. It binds to ER and inhibits its activity by nuclear export and degradation [56].

Relatively low ligand-binding domain homology between the two ERs allowed the development of ER subtype-selective ligands. PPT (propylpyrazole triol) is a commonly used synthetic ER α selective agonist, with a 410-fold relative binding affinity for ER α versus ER β [57]. It shows E2-like properties in many different tissues [58]. DPN (diarylpropionitrile) is the most commonly used synthetic ER β selective agonist, with 70-fold binding higher binding affinity for ER β compared to ER α [59]. Some phytoestrogens, plant-derived compounds with steroid structure and estrogen-like properties, such as genistein and coumestrol, have higher affinities for ER β than for ER α [60]. Selected ER ligands are presented in *Figure 3*.

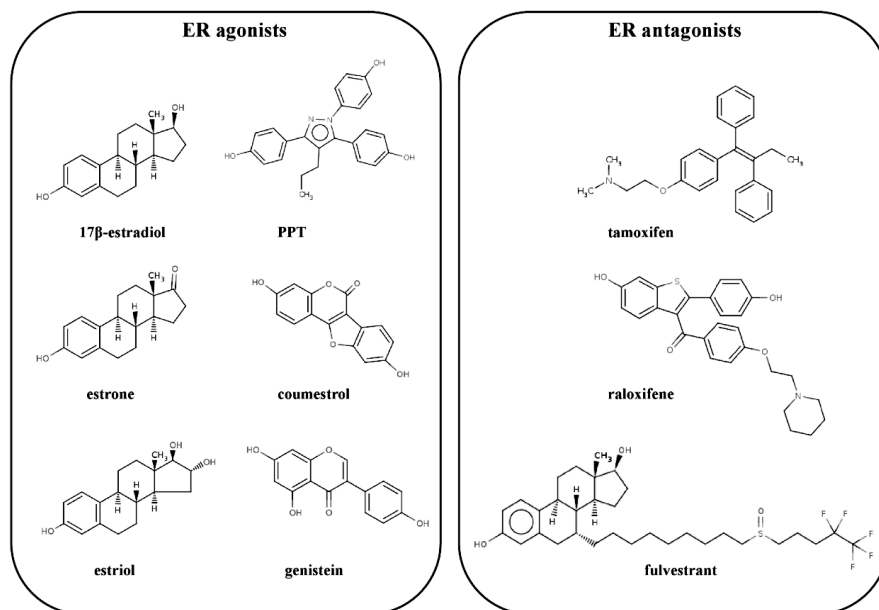


Figure 3. Chemical structures of representative ER agonists and antagonists. Chemical formulas are obtained from METLIN, Metabolite and Tandem MS Database [61].

1.2.3 Signaling pathways

ER activation can either be ligand-dependent or independent. The **classical model** of ER action involves activation of the ER by a ligand, upon which the receptors form dimers and to bind specific DNA sequences, such as estrogen response elements (EREs). By **transcription factor cross-talk**, ligand-activated ERs can regulate transcription indirectly, through interaction with other transcription factors (TFs), such as members of the activating protein-1 (AP-1) and specificity protein-1 (Sp1) families [62-64]. By **non-genomic mechanisms**, ligand binds to ERs localized in the cell membrane, which leads to activation of signal transduction pathways in the cytoplasm [65, 66]. **Ligand-independent pathways** include receptor phosphorylation by growth factor signaling via activation of kinases, such as HER2-regulated mitogen-activated protein (MAP) kinases ERK1 and ERK2 [67] (Figure 4).

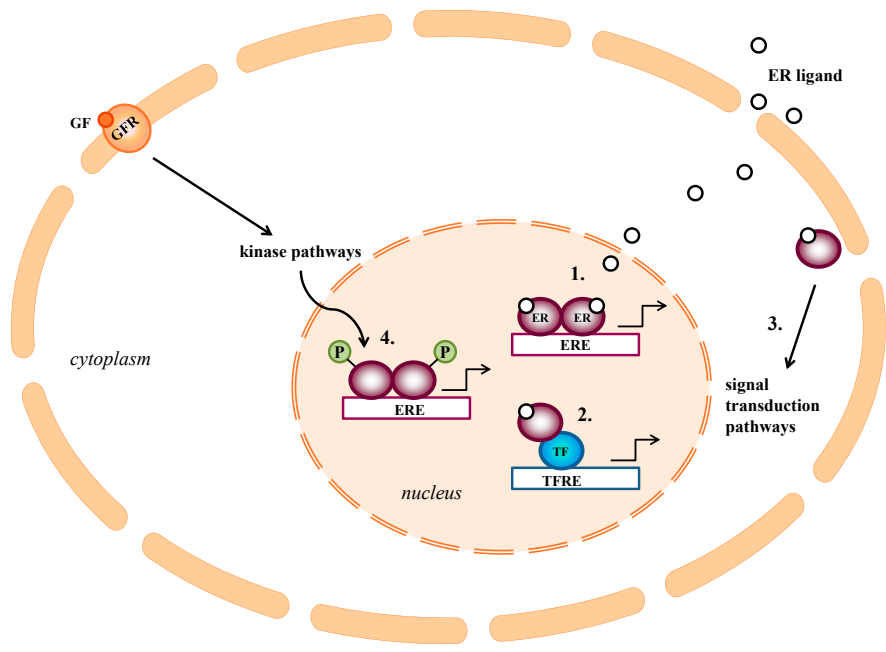


Figure 4. Simplified models of ER signaling pathways. 1. *Classical model*. Liganded nuclear ERs bind directly to EREs in target gene promoters. 2. *Transcription factor cross-talk*. Liganded nuclear ER complexes are tethered through protein-protein interactions to a TF-complex that contacts the target gene promoter containing TF-response element (TFRE). 3. *Non-genomic mechanisms*. Liganded extranuclear ER complexes activate protein-kinase cascades. 4. *Ligand-independent pathways*. Growth factors (GF) activate protein-kinase cascades via growth factor receptors in the membrane, leading to phosphorylation (P) and activation of nuclear ERs at EREs.

ER-mediated gene transcription also requires the recruitment of coregulatory proteins, which form complexes with ER through protein-protein interactions. Coregulators can be either coactivators, required for transcriptional activation, or corepressors, which are involved in decrease of the transcriptional activity.

The best characterized coactivators belong to the steroid receptor coactivator family (SRC), which binds to the AF-2 domain of the ER protein. SRC coactivators were found to recruit enzymes that mediate histone acetylation and methylation, associated with an open chromatin structure and increased transcriptional output [68, 69]. Antagonist-bound receptors interact with corepressors, such as nuclear receptor corepressor 1 (NCoR1) and silencing mediator of retinoid and thyroid hormone receptors (SMRT). NCoR1 and SMRT in turn recruit large repressor complexes including histone deacetylases (HDACs) that repress gene activity by maintaining or reinforcing a repressive chromatin state [70, 71].

1.3 ESTROGEN RECEPTOR SIGNALING AND BREAST CANCER

Normally, ER α is found to be expressed in only 7-10% of luminal cells in the mammary gland, although its levels vary during the menstrual cycle [72-74]. ER α levels are often found to be increased in breast cancer and it is regarded as a positive regulator of cell proliferation [75]. However, high ER α expression is considered a marker of good prognosis, since such patients (ER+) have higher chance to benefit from antiestrogen therapies and have an increased survival rate. DNA methylation of the ER α promoter has been proposed as a mechanism for the development of ER- cell lines as well as primary tumors [76-78]. Patients diagnosed with ER- breast cancer have generally poor survival rate, increased metastasis and relapse occurrence [79].

ER β is found to be expressed in 80-85% of the cells in the normal breast tissue, and hence it is considered to be a predominant ER in breast [34]. Yet the role of ER β in the breast remains largely unclear, and it is still not introduced as a marker and a target in diagnostics and treatment. Reported antiproliferative effects, interplay with ER α and other mechanistic features of ER β have a potential to contribute to our understanding of breast cancer and improvement of current treatments. Protein assays generally suggest that the ER β protein expression is a favorable prognostic factor, correlating with known biomarkers such as low histological grade, PR expression, longer disease-free survival, and response to antiestrogen therapy [80, 81]. Decreased expression of ER β in preinvasive *in situ* carcinoma, and its antiproliferative and anti-invasive properties *in vitro* suggest that ER β has a role in maintaining the benign phenotype, perhaps as a tumor suppressor [82]. It has also been shown that the ER β promoter is methylated MCF-7 cells, as well as in some breast cancer tumors [43, 83], leading to loss of ER β expression.

1.3.1 Cistrome

Cistrome is a term used to define a set of DNA-binding sites (*cis*-regulatory elements) recruiting a certain transcription factor. The ER cistrome includes the ER binding sites associated with the classical model of ER action, as well as those associated with transcription factor cross-talk.

The genome-wide maps for ER binding sites have recently been described using chromatin-immunoprecipitation assays (ChIP) combined with high-throughput genomic technologies. The number of ER α binding regions, primarily in MCF-7 breast cancer cell lines, ranges from approximately 10,000 to 15,000 [84, 85]. Additionally, these studies show that the ERE is the most predominant motif in ER α -binding regions, but also identify other motifs, such as AP-1, Sp-1 and Forkhead motifs as enriched in ER α -binding regions. Recently, ER α binding events were assayed in different subtypes of ER+ breast tumors for the first time [86]. Interestingly, more than 70% of ER α binding sites enriched in the samples with poor outcome (ER+PR-HER2-, ER+PR+HER2-) and metastasis overlapped with ER-binding events in MCF-7 cells. In addition, it was shown that breast tumors with different clinical outcome have distinct ER α -binding profiles.

Several studies showed that 50-60% of the FOXA1 (Forkhead box protein A1) binding regions overlap with ER α -binding regions [86-88]. It is suggested that FOXA1 acts as a pioneering factor of ER signaling, facilitating ER α binding [89].

Using the ChIP-chip approach, Krum et al. compared the ER α -binding site profile in MCF-7 cells with that in the U2OS osteosarcoma cell line. Strikingly, less than 15% of the ER α -binding regions were common between the two cell lines. Importantly, FOXA1 is not expressed in U2OS cells and its DNA motif was not enriched within ER α binding regions in these cells [90].

ER β binding regions in breast cancer cells have so far been studied only in cell lines with overexpressed ER β , due to the lack of an appropriate breast cell line that expresses ER β endogenously. Several studies examined ER β -binding sites in MCF-7 cells engineered to express ER β . Charn et al. [91] examined the location of ER α and ER β DNA-binding regions in MCF-7 cells engineered to express one or both ERs upon E2 treatment. They identified a higher number of sites bound by ER α than by ER β . More than 70% of ER β binding regions overlapped with ER α binding regions when either ER subtype was expressed alone. However, only about 30% of the binding regions were shared when both ERs were expressed together, suggesting a competition between the ER subtypes with regard to selection of DNA-binding regions.

Our group identified ER β -binding regions on a genome-wide scale in MCF-7 cells using the ChIP-chip approach [92]. Interestingly, 60% of the genomic regions bound by ER β contained AP-1-like binding sites together with ERE-like sites. Co-occupancy of ER β and AP-1 on chromatin was demonstrated and siRNA-mediated knockdown of expression of AP-1 family members (c-Fos and c-Jun) decreased ER β recruitment to chromatin. These results suggest that the transcription factor AP-1 collaborates with ER β in mediating estrogen responses in breast cancer cells.

Most of the studies investigated ER cistromes in MCF-7 breast cancer cells under similar conditions of E2 treatment. However, the number of regions detected differs between the studies and the binding profiles show a limited overlap. These differences could be due to biological variation between the MCF-7 (sub)lines, different cell handling protocols used in different laboratories, choice of ER α antibody or different platforms for detection of ChIP DNA sequences [93]. Additionally, MCF-7 cells with

exogenous ER β vary in the employed ER β -inducible systems and the ratio of ER α versus ER β levels.

Despite the differences, several observations are consistent between the studies: (1) both ERs bind across the genome; (2) many regulated genes are associated with multiple ER binding regions; (3) ER α binding regions are more significantly associated with estrogen up-regulated, than down-regulated genes and 4) ER α and ER β share many common binding regions.

These findings suggest that in estrogen-responsive breast cancer the final cellular response to estrogen is likely to depend upon the relative concentration of the two ERs in the cell, their activation status, DNA binding kinetics and the presence of other factors, such as FOXA1 and AP-1, influencing their respective functions.

1.3.2 Transcriptome

The transcriptome defines the set of RNA molecules, including mRNA, rRNA, tRNA, and additional non-coding RNAs, present in one or a population of cells at a given time.

Several reports have described global gene expression profiles in ER α -expressing breast cancer cell lines in response to E2 treatment [94-96]. These studies have reported different numbers of E2-regulated genes via ER α in MCF-7 breast cancer cells, ranging from 200 to 1500. Gene expression profiling have confirmed the regulation of several well known ER target genes in breast cancer cells such as pS2 (trefoil factor 1; TFF1), ADORA1 (adenosine A1 receptor), GREB1 (growth regulation by estrogen in breast cancer 1), MYC (v-myc myelocytomatosis viral oncogene homolog), CCND1 (cyclin D1) and IGFBP4 (insulin-like growth factor binding protein 4). ER α -regulated genes can be categorized into those that modulate the cell cycle, transcriptional regulation, morphogenesis, and apoptosis, compatible with a role of estrogen, via ER α , in inducing breast cancer cell proliferation and survival [97].

ER β signaling is studied mainly in cell lines stably expressing ER β either with or without ER α , due to the lack of cell lines expressing endogenous ER β . Studies examining ER β gene expression profiles in ER α -positive breast cancer cell lines stably expressing ER β have provided insights into the interplay between ER α and ER β in gene regulation. It is established that ER α and ER β share some target genes, although each receptor also appears to have distinct sets of downstream target [98-101]. In these studies, co-expression of ER β with ER α was found to significantly impact the E2-induced transcriptional response by ER α .

Monroe et al. attempted to identify targets unique for each ER in the ER α -osteosarcoma U2OS cell line that was stably transfected with either ER α or ER β [102]. Only 17 common genes were identified, suggesting that the transcriptional effects of E2 via ER α and ER β , are largely distinct in these cells.

In other ER α cells, such as HEK293 (human embryonal kidney, **PAPER II**) and Hs578T (human ductal breast carcinoma, [101]), ER β alone has been overexpressed in order to identify its specific targets. Only three genes (PTGER4, ENPP2, and DKK1) were found to be commonly regulated in these cell lines, suggesting that ER β evokes distinct gene responses in different types of target cells. Despite the differences, both studies reported inhibition of cell proliferation by ER β expression independently of ER α , suggesting a similar function of ER β in different cell types. Further studies are needed to clarify the molecular mechanisms by which ER β elicits inhibitory effects on cell proliferation.

1.4 DNA METHYLATION

DNA methylation, an epigenetic modification, is a chemical change of the DNA sequence catalyzed by the enzymes DNA methyltransferases (DNMTs), which most commonly occurs at CpG dinucleotides in mammals [103].

Three DNMTs, encoded by distinct genes, have been identified in mammals, DNMT1, DNMT3a and DNMT3b. DNMT1 maintains the CpG methylation pattern during DNA replication and repair, whereas DNMT3a and DNMT3b establish the initial CpG methylation pattern *de novo* [103]. Furthermore, DNMT3b has been shown to be expressed as several splice variants. Most of them have altered catalytic activity, adding to the functional complexity of DNMTs. Methyl-CpG-binding domain protein family (MBD) members (MeCP2, MBD1, MBD2) which share a methyl-CpG-binding domain, have a specific affinity for methylated CpG sites, are shown to regulate gene expression by interacting with other epigenetic modulators, HDACs in particular [104].

DNA methylation is a post-replication modification, almost exclusively found on cytosines within CpG dinucleotides [105]. Genomic regions rich in CpG dinucleotides are called CpG islands. A CpG island is a region at least 200 bp long, with a GC percentage that is greater than 50% and with an observed/expected CpG ratio that is greater than 60%. CpG islands are located in the 5'-regions of 70% of all genes [106].

In cancer cells, CpG islands that are normally unmethylated can become methylated (hypermethylation) [107], which may result in repression of tumor-suppressor genes and genomic instability, through silencing of DNA repair genes, and chromatin condensation [108]. Additionally, CpG dinucleotides in other regions can become unmethylated (hypomethylation), leading to gene reactivation and ultimately to the up-regulation or overexpression of proto-oncogenes, increased recombination and mutation rates [109].

Gene silencing by hypermethylation of promoter genes is an important mechanism of carcinogenesis that offers opportunities for novel diagnostic and therapeutic strategies. More than 100 genes have been reported to be hypermethylated in breast tumors or breast cancer cell lines. Many of them play important roles in cell-cycle regulation, apoptosis, tissue invasion and metastasis, angiogenesis and hormone signaling [110,

111]. Two established inhibitors of DNA methylation, 5-azacytidine (trade name Vidaza) and 5-aza-2'-deoxycytidine (trade name Decitabine), used in treatment of myelodysplastic syndromes, are in phase I/II of clinical trials for breast cancer treatment [76, 112]. Vidaza and Decitabine are cytosine analogues and act by their incorporation into DNA in the place of the natural base, cytosine, during DNA replication leading to covalent trapping of DNMTs. This causes the depletion of active DNMTs and demethylation of genomic DNA through cell division [113]. One disadvantage of these for treatment regimens is that the compounds are highly unstable in neutral aqueous solutions, and more stable derivatives and compounds are under development, such as zebularine and procainamide [112, 114].

1.4.1 Cross-talk between DNA methylation and estrogen signaling in breast cancer

Many studies have investigated the cross-talk between epigenetic modifications and estrogen-mediated gene regulation, mostly in breast cancer cell lines. However, the majority of studies focus on mediators of histone modification. Histone H3K9 acetylation and H3K4 methylation, associated with an open chromatin structure and increased transcriptional output, were observed at the promoters and enhancers of the active ER α target genes pS2 and GREB1 [115, 116]. Enzymes that mediate these modifications, such as arginine methyltransferase CARM1 and H3K4 methyltransferase SMYD3, were found recruited to the promoters of these genes upon treatment with E2 [68, 69]. Conversely, marks of repression (HDACs, H3K9 methylation) were observed in the promoters of E2-repressed genes [70, 71].

It was suggested that histone H3 and H4 acetylation and methylation, as well as DNA methylation, of the pS2 promoter are added and removed in a cyclical fashion [117]. The recruitment of cofactors, ER α , and RNA Polymerase II also occurred in a cyclical manner, producing transcriptional 'waves'. The authors observed DNA methylation at the end of each productive transcription cycle. DNA methylation correlated with the occurrence of the MBD proteins, DNMT1, DNMT3a/b and the chromatin remodeler SWI/SNF. Furthermore, the authors suggest that DNMT3a/b is involved in both methylation and demethylation of the pS2 promoter. These data suggest that both histone modifications and DNA methylation may be intricate parts of the regular ER α transcriptional cycle.

Some studies show indirectly a relationship between DNA methylation and estrogen signaling, e.g. PR promoter methylation after ER α loss [118], or global methylation pattern changes in antiestrogen-resistant breast cancer cells [119]. The promoter of the CXCR4 gene, involved in the induction of proliferation, was found to be demethylated by tamoxifen in MCF-7 cells [120]. A genome-wide study, which combined DNA methylation and ER α binding assays in mammosphere-derived epithelial cells and MCF-7 cells, identified 11 large chromosomal zones, including a total of 108 genes that might undergo estrogen-mediated epigenetic repression [121].

Interestingly, ER α gene expression can be reactivated by inhibition of methylation in ER $-$ cells, using demethylating agents or reducing DNMT expression [122, 123]. However, clinical data remains contradictory. For example, Lapidus et al. found hypermethylation of the ER α promoter region in ER $-$ tumors [124], whilst other reports show no correlation between the gene methylation pattern and ER α gene expression in breast tumors [125].

Modified DNA methylation patterns in the ER β promoters have been described for several forms of cancer [43]. Our group has previously shown that ER β expression in tumors was inversely correlated with promoter methylation and that treatment with 5-aza-2'-deoxycytidine led to increased ER β expression [126], which was confirmed by others [83, 127].

1.5 SINGLE NUCLEOTIDE POLYMORPHISMS

Single nucleotide polymorphisms (SNPs) are defined as a single base change in the DNA sequence that occurs more frequently than in 1% of individuals in a population. The corresponding nucleotides that differ within individuals are referred to as alleles, and chromosomal position of an allele is called locus. Alleles of loci in close vicinity tend to be inherited together. A set of SNPs that are statistically associated and therefore transmitted together is defined as a haplotype. Linkage disequilibrium (LD) is used to describe the non-random association of alleles at two or more loci. Unlike SNPs, genetic mutations that are recognized to increase e.g. breast cancer risk within families, such as those in BRCA genes, are much less frequent in the population. However, mutations confer higher risk [3].

Candidate gene association studies assay the effects of genetic variants in a gene potentially contributing to disease in a case-control material. These studies can be performed relatively quickly and inexpensively. However, the candidate gene approach is limited by the still incomplete knowledge on the genetic background of the investigated diseases. An example of a gene variant identified by this approach and confirmed in a large Breast Cancer Consortium study is a SNP in the caspase 8 gene (CASP8) coding region, which results in a substitution of aspartic acid for histidine [128]. The functional implications of this SNP on CASP8 protein function have not yet been identified.

Genome-wide association studies (GWAS) use high-throughput technologies to assay alleles of a large number of SNPs, typically 1 million in a case-control material. In one such study, the strongest association with breast cancer susceptibility was found for a SNP in the fibroblast growth factor receptor 2, FGFR2, positioned in intron 2, suggesting no direct effect on FGFR2 protein [129]. However, SNPs within introns may affect alternative splicing. The FGF signaling pathway has been shown to be important in mammary tumorigenesis and FGFR2 encodes a transmembrane tyrosine kinase involved in mammary gland development and breast carcinogenesis. Interestingly, FGFR2 expression, as well as the identified FGFR2 SNP, is associated

with ER+ tumors, suggesting interplay between these pathways. The possible function of the identified SNP in relation to FGFR and ER signaling remains to be determined.

1.5.1 SNPs in estrogen receptor genes

Approximately 2800 SNPs have been identified in the ER α gene [130]. Polymorphisms in ER α are associated with breast cancer, endometrial cancer, lupus nephritis, menstrual disorder, Alzheimer's disease, osteoporosis and coronary artery disease [131, 132].

Several potentially functional SNPs in ER α have been evaluated for their association with breast cancer as well as other estrogen-related diseases. The most commonly studied are rs2234693 (T \leftrightarrow C) and rs9340799 (A \leftrightarrow G), both located in the first intron [133-135]. A potentially functional ER α SNP, rs2747648, was analyzed for association with breast cancer risk using a large familial study population [136]. It is located in the last exon and in silico studies show that it affects the binding of microRNA miR-453, which is stronger when the C allele is present, which can be associated with relative increased miRNA-mediated ER α repression, and decreased breast cancer risk. Another ER α breast cancer associated SNP (rs851987) is located in the promoter CpG island [137], in a predicted binding site for MeCP2. The T allele was associated with a protective effect regarding breast cancer risk. However, it remains to be shown that the different alleles of the SNP actually display different binding of MeCP2 with associated effects on DNA methylation and potentially ER α gene expression. Of 14 ER α SNPs associated with disease in GWAS, 4 are associated with breast cancer [138].

Around 720 SNPs have been identified in the ER β gene [130]. Polymorphisms in the ER β gene have been correlated to pathological states such as ovulatory dysfunctions, hypertension, bone mineral density, androgen levels and breast cancer. rs4986938, rs928554 and rs1256049 are frequent ER β polymorphisms that have been associated with diseases, including breast cancer [132, 139-141]. None of these polymorphisms change the amino acid sequence of the ER β protein. rs4986938 is a G \leftrightarrow A exchange in the 3'-UTR of exon 8. rs928554 is a G \leftrightarrow A exchange in the 3'UTR of exon 9. These SNPs display strong LD. rs1256049 is a G \leftrightarrow A exchange in the coding region of exon 6. A recent meta-analysis suggested that rs4986938 is related to breast cancer risk, acting as a modifier of the relationship between breast cancer risk and environmental factors, while the rs1256049 association is only due to high LD with rs4986938 [139]. Furthermore, rs4986938 has been found to be associated with psychiatric disorders, such as bulimia, Parkinson disease and Alzheimer disease [142].

Several groups have studied SNPs located in the ER β promoter region, including rs2987983 and rs35036378, for association with breast cancer, proposing their effect on transcriptional regulation of gene expression [143, 144]. To date, no GWAS have revealed association between ER β SNPs and disease [145].

Overall, the functional significance of many disease-associated SNPs has not been clarified. There is evidence that intronic SNPs may contribute to alternative splicing and 5'UTR SNPs to gene promoter activity. SNPs in coding regions and regulatory

regions are non-synonymous and likely to affect gene function [146]. Finally, SNPs in the 3'UTR may contribute to transcript stability and translatability [147].

1.6 HORMONAL THERAPY OF BREAST CANCER

Drugs used in hormonal therapy target estrogen signaling, and they include antiestrogens and aromatase inhibitors (AIs).

Antiestrogens disrupt estrogen signaling, including its down-stream proliferative effects. According to the producer's (AstraZeneca) market analysis, the antiestrogen ***tamoxifen*** (trade name Nolvadex) is today's best-selling hormonal anti-cancer drug. It is used in treatment of ER+ positive cancers, both in pre- and postmenopausal women. Tamoxifen itself has relatively low affinity for the ER, and whilst its metabolites, 4-hydroxytamoxifen and endoxifen, bind the ER with 30-100 times higher affinity [148].

The antagonistic activity of tamoxifen is crucial for breast cancer treatment, whereas in the bone, the endometrium and the cardiovascular system tamoxifen displays agonistic properties [149]. In the bone, tamoxifen was found to prevent osteoporosis [150, 151]. However, tamoxifen has a proliferative effect on the endometrium, increasing the risk for uterine cancer [152]. It is believed that the reason for tamoxifen being only a partial antagonist lies in the differential response of the two ERs. It has been demonstrated that tamoxifen can act as an agonist through ER α , and as an antagonist through ER β [98], which can be due to their structural differences in the N-terminal domain that regulates interactions with coregulators. This suggests that the co-expression of the two ERs may affect the response to tamoxifen therapy.

Raloxifene (trade name Evista) is a SERM used in treatment of ER+ breast cancer. Side-effects of raloxifene are reportedly less profound than tamoxifen, particularly decreased risk of endometrial cancer and thrombosis. However their overall therapeutic effects are similar [153].

Fulvestrant (trade name Faslodex) is used as second-line therapy in postmenopausal women who had relapsed or progressed after previous hormonal therapy [154]. In ER+ breast cancer postmenopausal patients, fulvestrant was shown to have similar efficacy to tamoxifen [155, 156].

Aromatase inhibitors target estrogen production, by blocking aromatases, enzymes that catalyze estrogen synthesis. AIs are used in treatment of almost exclusively postmenopausal women, where the estrogen production is "outsourced" from ovaries to other tissues, such as liver, adrenal glands and fat. AIs are ineffective in premenopausal women, since the ovarian estrogen production is still active and under control of the hypothalamus and pituitary axis, via positive feedback. The AI-induced decrease in estrogen would activate the axis to stimulate estrogen production in the ovary, counteracting the AI effect. Acquired resistance to AIs eventually occurs, as well as some of the side effects: hot flushes, sweating, joint and muscle pain, and osteoporosis

[61]. However, it has been suggested that AIs are highly effective in those ER+ tumors where tamoxifen, the most commonly used ER antagonistic drug, “switched sides” - displaying agonistic activities and inducing tumor growth [157]. It is proposed that optimizing the combinational AI+tamoxifen therapy may lead to improved treatments [158, 159].

1.6.1 Resistance to antiestrogen therapy

About 40% of ER+ tumors fail to initially respond to tamoxifen therapy and breast tumors initially responding to tamoxifen will in many cases develop resistance to this treatment. *De novo* and acquired resistance to antiestrogen therapy has been extensively studied, particularly for tamoxifen, however the underlying molecular mechanisms remain elusive.

About 10% of the patients who possess variant forms of the gene CYP2D6, whose cytochrome product is involved in the tamoxifen metabolism, may not receive full benefit from tamoxifen due to impaired CYP2D6 activity [160]. That is described as ***de novo* (intrinsic) resistance**. Additionally, it has been suggested that overexpression of HER2 can be another mechanism of intrinsic tamoxifen resistance [161], which is consistent with the lower efficacy of tamoxifen in ER+HER2+ patients.

Many different mechanisms can contribute to **acquired resistance**. There is evidence that high levels of coactivators, such as SRC-1 and SRC-3, may enhance the agonistic activity of tamoxifen and contribute to resistance [162, 163]. On the other hand, progressive reductions in corepressor activity during tamoxifen therapy may enhance the agonist effects of tamoxifen on the ER contributing to resistance [164]. For example, NCoR1 only weakly associated with ER in the absence of ligand, but did so avidly in the presence of hydroxytamoxifen [165]. When NCoR activity was blocked using a purified specific antibody, hydroxytamoxifen was converted into an agonist in MCF-7 cells.

Cross-talk between ER signaling and the growth factor receptor pathways, such as HER2, epidermal growth factor receptor (EGFR) and insulin-like growth factor receptor (IGFR) pathways, can affect cell growth and patterns of acquired resistance [166, 167]. For example, ER can be phosphorylated by the downstream components of the HER2 signaling pathway, the MAP kinases ERK1 and ERK2, leading to ligand-independent ER activation [67]. Therefore, increased ERK activity could potentially contribute to resistance to endocrine therapy, shown in several breast cancer cell-line models of endocrine resistance and suggested by clinical observations [168-170]. In addition to activating ER directly, kinase-mediated growth factor signaling may also modulate ER activity indirectly by phosphorylation of coregulators, enhancing the activity of coactivators and attenuating corepressor activity [171]. Experimental findings show that HER2 and EGFR levels rise after administration of tamoxifen and fulvestrant to breast cancer cells, suggesting a possible mechanism of acquired resistance [172]. Additionally, ER can activate HER2 and EGFR, and their downstream kinases, which was also implicated in resistance to tamoxifen [65, 66].

Acquired tamoxifen resistance can occur due to loss of ER α expression, but this is observed in only about 15% of breast cancer patients [173]. In fact, up to 20% of patients who have relapsed on tamoxifen, respond to AIs or fulvestrant, suggesting that ER continues to regulate growth even after the acquired resistance [174, 175]. DNA methylation of the ER α promoter has been proposed as a mechanism for the development of ER- cell lines as well as primary tumors [76-78]. MCF-7 cell lines resistant to either tamoxifen or fulvestrant were shown to have distinct epigenetic profiles compared to sensitive MCF-7 cell lines, suggesting that epigenetic mechanisms might be involved in antiestrogen resistance [119].

Loss of ER β expression, commonly observed in breast cancer, has also been implicated in tamoxifen resistance. In line with this, it has been shown that high levels of ER β may improve disease-free and overall survival in patients treated with tamoxifen [176]. Interestingly, another hypomethylating agent, procainamide, was found to decrease tamoxifen resistance by inducing ER β overexpression in breast cancer patients [177].

2 AIMS OF THE THESIS

Clarifying the molecular pathways of estrogen signaling is important for our understanding of breast cancer development, diagnosis and treatment. These molecular pathways display significant complexity, including the existence of two distinct ERs, ER α and ER β , their numerous splice variants and polymorphisms, as well as their interplay, both mutual and with other signaling pathways. ER status is not a perfect marker for responsiveness to antiestrogen therapy, and additional markers are needed to more accurately define patients who will benefit from hormone therapy. Additionally, intrinsic and acquired resistance to this treatment represents a significant clinical challenge. This work attempts to address some molecular details and mechanisms relating to the role of ERs in breast cancer with the ultimate hope that this knowledge will in the future contribute to stratify diagnosis and improve therapy for breast cancer patients.

The general aim of this thesis was to characterize molecular mechanisms of ER action, focusing on breast cancer. It addresses the interplay of ER α with ER β and DNA methylation pathways, the role of ER β in global gene expression, and the functionality of two common ER β SNPs. Specifically, the aims were:

PAPER I Functional characterization of two common SNPs positioned in the ER β 3'UTRs that have been shown to be associated with several diseases.

PAPER II Description of ER β regulatory functions independent of ER α .

PAPER III Demonstration of the recruitment of ER α / β heterodimers to various DNA-binding regions in intact chromatin.

PAPER IV Investigation of global gene regulation by estrogen signaling and DNA methylation.

PAPER V Investigation of the interplay between ER α and main regulators of DNA methylation, DNMTs.

3 METHODOLOGICAL CONSIDERATIONS

3.1 CELL LINES

The majority of the constituent studies in this thesis are based on experiments in immortalized human cell lines. Cell lines offer attractive systems for molecular mechanistic studies due to their ease of manipulation and propagation.

The **MCF-7** (**M**ichigan **C**ancer **F**oundation **7**) cell line is the most commonly used cell line in ER signaling studies related to breast cancer. The receptor status of MCF-7 is ER+ and the cell line is of luminal origin. There are different MCF-7 (sub)lines used in different laboratories, varying in E2-response, proliferation rate and chromosomal structure [178]. In **PAPER I**, **PAPER IV** and **PAPER V**, we used the MCF-7S strain, originally generated by Dr. S. Shafie at the NIH in 1981 [179]. MCF-7 cells with an inducible Tet-system used in **PAPER III** were obtained from Clontech.

The **HEK293** (**H**uman **E**mbryonic **K**idney **293**) cell line is of epithelial origin. It was selected for the studies in **PAPER II** due to the lack of endogenous ERs. In this paper we used HEK293 cells with an inducible Tet-system obtained from Clontech. Due to high transfection efficiency [180], the HEK293 cell line from American Type Culture Collection (ATCC) was selected for the majority of the experiments in **PAPER I** due to its convenience in transfection assays.

HeLa (**H**enrietta **L**acks) is the first generated human cell line, derived from cervical cancer of the patient that the line was named after [181]. Similar to HEK293, it lacks endogenous ERs and can be efficiently transfected. It was used in **PAPER I**, to confirm data obtained in HEK293 cell line.

COS-7 (**CV-1** **O**rigin **SV40** **7**) is a monkey kidney cell line, obtained by immortalizing a CV-1 cell line derived from kidney cells of the African green monkey [182]. Similar to HEK293 and HeLa cell lines, this cell line is often used for in vitro studies, especially transient transfection-based assays including co-immunoprecipitation, which we employed in **PAPER V**.

3.1.1 Tet gene expression systems

Due to lack of a breast cancer cell line that expresses significant amounts of ER β , we have generated stable cell lines expressing ER β (**PAPER II** and **PAPER III**). The expression of ER β in these cell lines were controlled by Tet gene expression systems.

Tet gene expression systems are commercially available as Tet-Off and Tet-On cell lines, providing regulated, high-level gene expression [183]. In the Tet-Off system,

gene expression is turned on when the antibiotics tetracycline (Tet) or doxycycline (Dox; a Tet derivative) are removed from the culture medium. In contrast, expression is turned on in the Tet-On system by the addition of Dox. The first critical component of the Tet Systems is the tetracycline-controlled transactivator (tTA), a modified E.coli TetR protein. In the Tet-Off system, tTA is encoded by the pTet-Off regulator plasmid. In the Tet-On system, tTA is modified into rtTA (“reversed” tTA) and it is encoded by the pTet-On regulator plasmid. rtTA protein is capable of binding the operator only when bound by doxycycline, hence the Tet-On system is not responsive to Tet (*Figure 5*). In both Tet-On and Tet-Off systems, transcription is turned on or off in response to the antibiotic in a precise and dose-dependent manner [184].

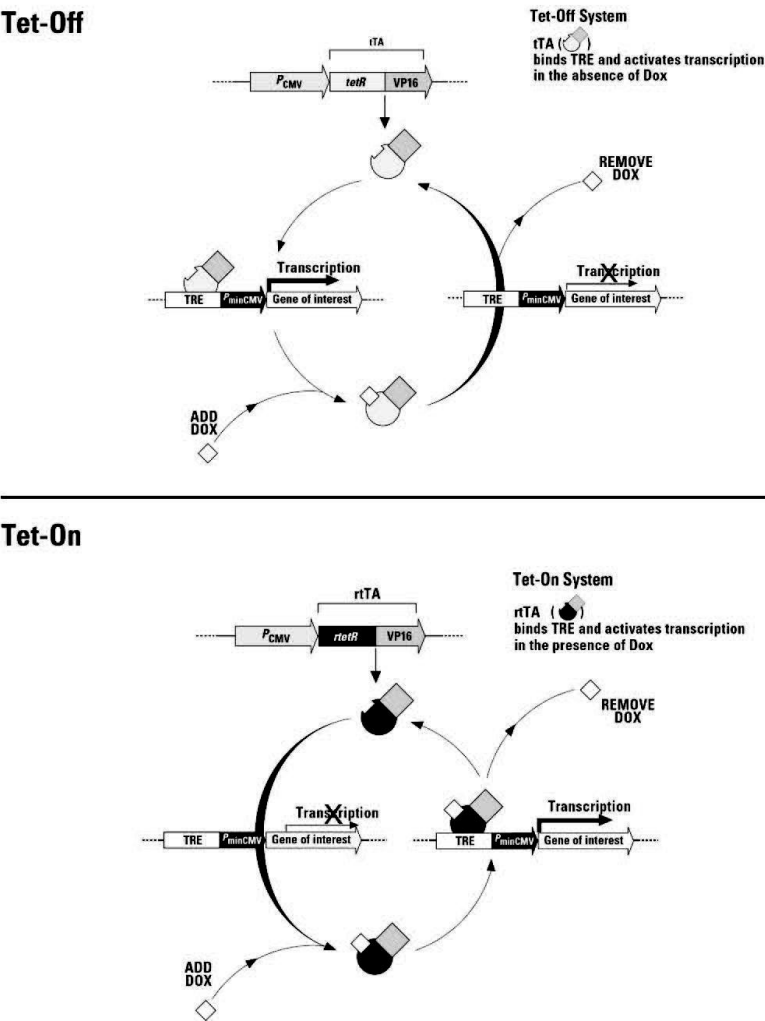


Figure 5. Schematic outline of gene regulation in the Tet-Off and Tet-On Systems. *Tet-Off:* tTA binds the TRE and activates transcription in the absence of Tet or Dox. The TRE is located upstream of the minimal immediate early promoter of cytomegalovirus (PminCMV), which is silent in the absence of activation. tTA binds the TRE - and thereby activates transcription of Gene X - in the absence of Tet or Dox. ***Tet-On:*** rtTA binds the TRE and activates transcription in the presence of Dox. From Clontech Laboratories, Inc.® Tet-Off and Tet-On Gene Expression Systems User Manual.

The second critical component is the response plasmid which expresses the gene of interest (in our case ER β) under control of a tetracycline-response element (TRE). A response plasmid without the gene of interest is used as a negative control (Mock). The response plasmid is usually pBI-EGFP, where TRE is bidirectional (*Figure 6*), i.e. designed to co-express both the gene of interest and enhanced green fluorescent protein (EGFP). Hence, when TRE is activated, both the gene of interest and EGFP are expressed, and the cells are visible as green, which facilitates clone selection.

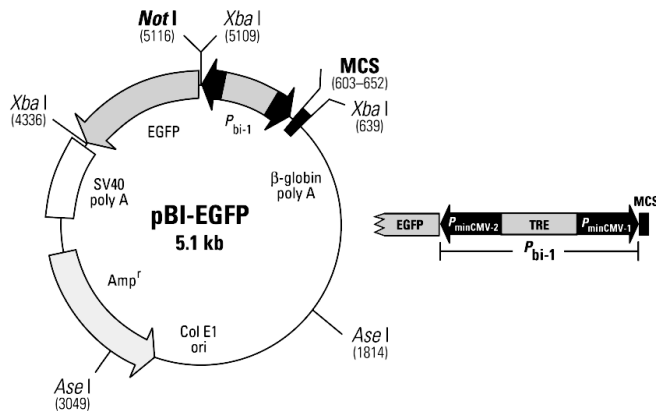


Figure 6. Restriction map of pBI-EGFP (left) and detailed organization of the Pbi-1 bidirectional promoter (right). MCS is acronym for Multiple Cloning Site into which the ER β cDNA is cloned. Pbi-1 bidirectional promoter contains the TRE. From Clontech Laboratories, Inc.® pBI-EGFP Vector Information.

3.2 GENE EXPRESSION ASSAYS

Gene expression assays can target either a single gene or have a genome-wide approach. Both approaches were extensively used in the studies described in this thesis.

3.2.1 Quantitative polymerase chain reaction

Quantitative polymerase chain reaction (qPCR, real-time PCR) is a widely used approach for expression analysis at low throughput. It is based on detection of produced PCR products in real time by fluorescence produced by reporter molecules, the most common being the SYBR Green dye and TaqMan probes. The SYBR Green dye functions by non-specifically binding to double-stranded DNA. TaqMan is a fluorophore- and quencher-containing probe that specifically hybridizes to the gene of interest.

To quantify the PCR product, we used the standard $2^{-\Delta\Delta C_t}$ method, which assumes 100% efficiency for the PCR reaction. The C_t (Cycle threshold) value represents the number of cycles required for the fluorescent signal to reach a set threshold in the linear phase of the qPCR production. ΔC_t is the difference between the C_t values of two

samples. The exponential value, $2^{-\Delta Ct}$, represents the relative fold change between two samples. To normalize for individual sample variations, Ct values from a housekeeping gene are used, which is then subtracted from the first one, giving the $\Delta\Delta Ct$ value. The final exponential value, $2^{-\Delta\Delta Ct}$, represents the relative fold change between two samples normalized by the Ct values from a housekeeping gene. Three of the most commonly used human housekeeping genes, Gus, 18S and 36B4 (RPLP0), were used in our studies, due to high and stable expression levels in the used cell lines.

3.2.2 Expression microarrays

Microarray technology is used to measure gene expression on a genome-wide scale. It is based on high-density arrays that contain thousands to millions of oligonucleotide probes to detect cDNA targets. Differences in expression levels determined by microarrays are usually confirmed by qPCR. In most cases, qPCR confirms the general trend of a change obtained from a microarray, but the absolute changes can vary, due to different sensitivities of the two assays and different designs of probes and primers. While qPCR employs two primers and optionally a probe amplifying an approximately 100bp long sequence, microarrays used in this study employ multiple probes that cover the longer parts, and sometimes the entire mRNA transcripts.

Two Affymetrix® expression array types were used in our studies. In **PAPER II**, we employed the Gene 1.0 ST Array, which contains approximately 26 probes spread across the transcripts of 28,869 annotated genes. GeneChip HT HG-U133+ PM 96-Array Plate was used in **PAPER IV**. This array contains probe sets with 9 or 10 probe pairs per transcript of more than 47,000 transcripts and variants of more than 33,000 well-characterized genes and UniGene clusters. It is a 3'-based array, which means that all probes are positioned at the 3' end of mRNAs. Sample processing and data analysis was performed at the Bioinformatics and Expression Analysis core facility at the Karolinska Institutet (www.bea.ki.se).

3.3 IMMUNOPRECIPITATION ASSAYS

Immunoprecipitation assays employ antibodies in order to isolate proteins and screen for their interactions, including with other proteins (Co-IP) or chromatin (ChIP).

3.3.1 Protein complex immunoprecipitation (Co-IP)

We used Co-IP in **PAPER V**, to identify possible protein complexes between ER α and DNMTs. The method is based on precipitation of a protein complex, using an antibody specific for one of the complex members, in our case ER α . Precipitated proteins are then separated by SDS PAGE and proteins present in the complex are detected by a Western blot assay, using an antibody specific for other possible member of the complex, in our case a DNMT. When an interaction is detected, it can be confirmed by

switching the antibodies at the respective steps. However this is not always successful as an antibody may work for protein precipitation but not in Western blot assays, and vice versa.

3.3.2 Chromatin immunoprecipitation (ChIP)

ChIP was critical central assay in three of our studies: **PAPER II**, **PAPER III** and **PAPER IV**. It was used to determine the location of ER α and/or ER β DNA binding sites.

This assay is designed to isolate DNA sequences that DNA-associated proteins bind to. An antibody, or in some cases a pool of antibodies, specific for a putative DNA-binding protein is used to immunoprecipitate the protein-DNA complex from cellular lysates after cross-linking, usually by formaldehyde. The cells are then lysed and the protein-bound DNA is broken into pieces of 0.2-1.0 kb in length using sonication. Protein-DNA complexes are then precipitated with a specific antibody, followed by reversing cross-links using high temperature (65°C), which allows the DNA to be separated from the protein. The identity and quantity of the DNA fragments isolated are then determined by classical PCR, qPCR or using high-throughput assays. The latter include ChIP-chip and ChIP-DSL (DNA selection and ligation), based on high-density oligonucleotide arrays, and ChIP-PET and ChIP-Seq, based on high-throughput sequencing [84]. Re-ChIP is used to investigate the simultaneous binding of two proteins to the same DNA region(s), employing two, or more, sequential precipitation steps as exemplified in **PAPER III**.

4 RESULTS AND DISCUSSION

4.1 PAPER I

EFFECTS OF TWO COMMON POLYMORPHISMS IN THE 3' UNTRANSLATED REGIONS OF ESTROGEN RECEPTOR BETA ON mRNA STABILITY AND TRANSLABILITY

This study was the first report to functionally characterize two common SNPs in the 3'UTRs of ER β , focusing on the differences between alleles with regard to mRNA stability and translatability. The goal was to define the molecular mechanisms by which SNPs in ER β are associated with disease.

ER β 1 and ER β 2 use different last exons, exons 8 and 9, respectively, and thus have different 3'UTRs. The SNPs in focus of this study are referred to as rs4986938 and rs928554, positioned in the 3'UTR of exon 8 and 9, respectively. Both are G \leftrightarrow A transitions. A large number of studies report associations of these two SNPs with diseases, including bulimia, cardiovascular diseases, osteoporosis and breast cancer [140, 141, 185-187]. The SNPs are found to be associated either individually or within a haplotype block.

We examined allelic expression for the two SNPs in breast tumor samples from heterozygous individuals. For each sample and SNP, the relative levels of the cDNA (as a measure of the mRNA levels) versus the genomic DNA were determined for both alleles. A significant difference in allelic expression level was observed for rs4986938, but not for rs928554.

To further investigate potential molecular effects of the two SNPs, a cell model system was employed. We used a modified PGL3 basic vector, where the ER β promoter and the ER β 3'UTRs are flanking the luciferase gene including the different alleles of the investigated SNPs. mRNA stability was assayed by determining mRNA levels after inhibition of transcription by actinomycin D. A luciferase assay was used to determine protein levels as a measure of mRNA translability. We did not observe any differences in mRNA stability or translability between the two alleles for the investigated SNPs. Although the allelic expression assay suggested that rs4986938 alleles had an impact on mRNA levels the sample number was small (n=5) and the observed result could not be accounted for by molecular mechanisms investigated in cell models.

These results indicate that the observed associations between ER β 3'UTR SNPs and disease susceptibility are most likely due to linkage disequilibrium with another gene variant, rather than the variant itself being the susceptibility factor.

4.2 PAPER II

MICROARRAY ANALYSIS OF ALTERED GENE EXPRESSION IN ER β -OVEREXPRESSING HEK293 CELLS

The aim of this study was to gain a global understanding of ER β -dependent gene regulation independent of ER α . The experimental approach was a global gene expression profiling analysis for HEK293 Tet-On cell lines with inducible version of ER β 1 and ER β 2. The analysis showed that ER β 2 had no effect on mRNA expression under the applied experimental conditions and with the filters employed to identify differentially expressed mRNAs (unpublished data).

A total of 332 genes and 210 genes were found to be up-regulated and down-regulated by ER β , respectively. The Gene Ontology analysis revealed that ER β -induced and ER β -repressed genes were involved in cell-cell signaling, morphogenesis, and cell proliferation. The ER β repressive effect on genes related to proliferation was further studied by functional proliferation assays, where ER β expression resulted in a significant decrease in cell proliferation.

To identify primary ER β target genes, we examined 20 ER β -regulated genes selected from the global gene expression profiling experiment, using ChIP assays for regions bound by ER β . Our results showed that ER β recruitment was significant to regions associated with 8 ER β up-regulated genes and 5 ER β down-regulated genes. Six binding regions were located within genes, four downstream and two upstream of genes.

Our findings on ER β regulatory functions independent of ER α were consistent with previous reports in two ER- cell lines, the human breast cancer Hs578T cell line and the osteoblastic U2OS cell line, in which stably expressed ERs were investigated for their global gene expression profiles [101, 102]. However, of 95 genes found to be regulated 2-fold or more, by ER β in Hs578T/ER β cells, only 3 (PTGER4, ENPP2, and DKK1) were identified in our study. One of them (ENPP2) was identified as a primary ER β target gene in our study. For another gene (PTGS2), we show down-regulation by ER β , whereas the study in Hs578T cells reports up-regulation by ER β . The observed discrepancies may be the result of different origin of the cell systems (HEK293 versus Hs578T breast cancer cells), achieved levels of stably expressed ER β , number of genes on the array (28,869 genes in our study versus 8,700 genes in the Hs578T study), and applied filters for identifying regulated genes (1.5-fold vs. 2-fold). However, both studies report inhibition of cell proliferation by ER β expression independently of ER α , suggesting a similar function of ER β in different cell types.

This study provides novel information on the gene regulatory function of ER β independent of ER α and identified a number of primary ER β target genes. The results of GO analysis and proliferation assays are consistent with an antiproliferative role of ER β independent of ER α , which supports ER β as a potential marker of good prognosis in breast cancer treatment.

4.3 PAPER III

BINDING OF ESTROGEN RECEPTOR α/β HETERODIMERS TO CHROMATIN IN MCF-7 CELLS

This study was the first demonstration of ER α/β heterodimers recruitment to various DNA-binding regions in intact chromatin. Although the formation of ER α/β heterodimers has been previously demonstrated using GST-pull down and gel-shift assays [35, 188], their exact role in estrogen signaling remains unclear.

We applied the Re-ChIP assay to study the simultaneous presence of ER α and ER β on various DNA-binding regions in intact chromatin. Following E2-treatment for 45 min, ER α/β heterodimers were isolated by precipitation with an anti-ER β antibody followed by an anti-ER α antibody in an MCF-7 Tet-Off cell line that stably expresses an inducible version of ER β (MCF-7/ER β) together with endogenous ER α . MCF-7/Mock was used as a negative control.

We initially applied the Re-ChIP method to assay ER α/β heterodimers to a promoter region of the pS2 gene, known to bind both ER α and ER β . Recruitment to the pS2 promoter was only detected in ER β -ER α immunoprecipitated fractions from the MCF-7/ER β cells, but not from the MCF-7/Mock cells. The fold-enrichment for heterodimers was significantly lower than when the interaction was assayed for each ER alone, which could be explained by loss of material during the Re-ChIP assay, low fraction of heterodimers compared to homodimers or lower DNA binding affinity of heterodimers compared to homodimers, which has been previously suggested [176].

We further examined the binding of ER α/β heterodimers to 12 binding regions, identified as regions recruiting both ER α and ER β by a ChIP-chip assay (from [92] and unpublished data). Among the 12, 8 binding sites were located within genes, and 4 were located in the promoter regions. The Re-ChIP assay demonstrated recruitment of ER α/β heterodimers to all selected regions. Additionally, all tested binding regions exhibited significant recruitment of ER α and ER β individually. However, similarly to what was observed for the pS2 binding region, significantly lower recruitment of heterodimers was observed in comparison to when the interaction was assayed for each ER alone. Furthermore, it is notable that different binding regions exhibited differences in fold enrichment of ER α/β heterodimers. This could be attributed to variations in affinity of heterodimers to various ER-binding regions depending upon the sequence of the binding regions.

Using the CONSITE program, we found that all of the selected binding sites contained half-ERE motifs, 70% contained AP1-motifs, and only 23% contained full ERE motifs. Furthermore, our data showed that the ratio of enrichment in the ER α/β Re-ChIP assay versus the enrichment in the ER α and ER β ChIP assays varied among the selected sites. For example, for genes such as NBPf4, NOTCH2, NBPf15, and PRUNE, this ratio is high, suggesting that heterodimer recruitment may play a more significant role in ER-mediated transcriptional regulation of these genes.

To verify the effect of ER β on transcriptional activity of ER α , we evaluated the mRNA levels of some selected genes after E2 treatment. Our data showed that mRNA levels of genes that are implicated in cell proliferation, like pS2, and ADORA1 were down-regulated in the MCF-7/ER β cell line compared with the MCF-7/Mock cell line. The mRNA levels of NBPF1 and NBPF4, whose function is not fully explored, were also decreased in the presence of ER β . mRNA levels of BCL9, which is participating in cell proliferation and development, were up-regulated when ER β is expressed. Finally, the mRNA levels of NOTCH2 and PRUNE were unaffected by the presence of ER β . Interestingly, both these genes have been implicated in the aggressiveness of breast neoplasm.

Considering that all examined binding regions exhibited significant ER α / β heterodimer recruitment, our data suggested that heterodimerization is a frequent mechanism by which ER α and ER β interact in estrogen signaling.

4.4 PAPER IV

GENE REGULATION BY ESTROGEN SIGNALING AND DNA METHYLATION IN MCF-7 BREAST CANCER CELLS

In this study we investigated a potential regulatory cross-talk between estrogen signaling and DNA methylation by identifying their common target genes and exploring potential underlying molecular mechanisms in human MCF-7 cells.

We compared effects on global gene expression profiles in response to E2 and the hypomethylating agent 5-aza-2'-deoxycytidine (DAC). We focused on the up-regulated genes in the DAC-regulated group, since DAC-induced hypomethylation leads to up-regulation of genes that are normally directly silenced by DNA methylation. Changes in gene expression profiles in response to E2 and DAC co-treatment were not further explored, as DAC down-regulated both mRNA and protein levels of ER α in our experiments (data not shown), in accordance with previous findings for MCF-7 cells [189].

A total of 88 genes were up-regulated by both E2 and DAC (E \uparrow D \uparrow group) and 58 genes were down-regulated by E2 and up-regulated by DAC (E \downarrow D \uparrow group). Candidate common target genes were selected for further analysis using GO Analysis, previously reported association with breast cancer, estrogen signaling and/or DNA methylation, reported ER recruitment, and predicted CpG islands. Based on these selection criteria, six genes were selected for a detailed investigation in relation to regulation by E2 and DAC: Three from the E \uparrow D \uparrow group (BTG3, FHL2 and PMAIP1) and three from the E \downarrow D \uparrow group (BTG2, CDKN1A and TGFB2). Real-time PCR analysis confirmed changes in gene expression derived from microarray data for selected genes. We further confirmed that the selected genes were regulated through ER by E2 and tamoxifen co-

treatment. Finally, a dose-response effect of DAC on the induction of the expression of these genes was observed.

As the regions of the selected genes previously shown to recruit ER α do not contain predicted CpG islands, we assessed ER α recruitment to the CpG island containing promoter regions of these genes. ER α showed significant recruitment to all investigated promoters, however the observed recruitment was much lower to the CpG island containing promoter regions than to the previously reported ER binding regions of these genes. The lack of an effect of E2 in this assay suggests ligand-independent ER α recruitment. Additionally, DAC treatment did not affect ER α recruitment to the promoter regions, suggesting that the promoter methylation status has no effect on ER α recruitment.

Although DAC treatment activated the expression of all selected genes, the bisulfate sequencing assay showed that only the promoters of the BTG3 and FHL2 genes were methylated. However, E2 treatment had no effect on the methylation status of these promoters. PMAIP1, BTG2, CDKN1A and TGFB2 are also found to be up-regulated by DAC. However, as their promoters were not methylated even in the absence of DAC, this suggests that DAC regulated the expression of these genes either via hypomethylation of other methylated DNA regions, such as CpG shores, shelves and open seas, or indirectly, through hypomethylation of other genes.

Our results support the previous report that BTG3 (B-cell translocation gene 3) is directly regulated by DNA methylation in MCF-7 cells [211]. Furthermore, we show that E2 affects the expression of this gene. However, our data does not support that this effect involves changes in DNA methylation status. Genistein, an ER ligand, has been shown to have the same hypomethylating effect as DAC on the BTG3 promoter in renal and prostate cancer cells, hence estrogen effects on DNA methylation might display cell type selective mechanisms [190, 191]. Interestingly, the BTG gene family encodes proteins that appear to have antiproliferative properties [192]. Apart from BTG3, another member of the family, BTG2, was identified in our study.

We confirm the previous data that FHL2 (four and a half LIM domains 2) gene is regulated by E2 in MCF-7 cells [119]. Fan et al. observed that long term disruption of estrogen signaling using fulvestrant can lead to hypermethylation of the FHL2 promoter with the associated loss of E2 responsiveness. However, they showed no changes in FHL2 methylation upon long term tamoxifen treatment, suggesting that diverse antiestrogens can exert different effects on the DNA methylation status of the FHL2 promoter. Our study, focusing on regulation of promoter methylation upon short term E2 treatment, does not support a connection between short term E2 treatment and FHL2 promoter methylation.

In summary, we identified a set of genes regulated by both estrogen signaling and DNA methylation. However, our data does not support a direct molecular interplay of mediators of estrogen and epigenetic signaling at promoters of regulated genes.

4.5 PAPER V

INTERPLAY OF ESTROGEN RECEPTOR α AND DNA METHYLTRANSFERASES WITH FOCUS ON DNMT3B

In this study, we explored the interplay of estrogen signaling and DNMTs.

Reports on the effect of estrogen signaling on DNMT expression are limited, and most of them study the endometrium, since its lining undergoes cyclic regeneration and it is hypothesized that this process is regulated epigenetically [193]. Additionally, prolonged exposure to estrogen is a risk factor for endometrial cancer [194]. We observed that in MCF-7 cells estrogen signaling affects the mRNA expression of the two DNMT classes, the maintenance DNMT, DNMT1 and the *de novo* DNMTs, DNMT3a and DNMT3b, differently. E2 induces the expression of DNMT1, and decreases the expression of DNMT3a and DNMT3b. E2 increased DNMT1 mRNA expression only at 24h, but not at 48 and 72h, which could be due to additional regulatory mechanisms contributing at later time points. It is well established that estrogen signaling controls the cell cycle in MCF-7 cells by mediating the G1-S phase transition [195], which can be related to the increase in DNMT1 expression observed after 24h E2 treatment, and due to a requirement for maintenance of the DNA methylation pattern during replication. ER cistrome assays and gene expression profiling assays of potential E2-regulated genes did not detect DNMTs as primary ER targets in breast cancer cells.

Using a Co-IP assay, we identified protein-protein interactions between ER α and DNMT3b1. We could not detect protein-protein interactions between endogenous ER α and transfected c-myc tagged DNMT3b in MCF-7 cells, possibly be due to low transfection efficiency in these cells. The interaction between ER α and endogenous DNMT3b could not be studied due to the lack of an antibody against endogenous DNMT3b suitable for the Co-IP assay.

Interestingly, in comparison to DNMT1 and DNMT3a, DNMT3b has been more clearly implicated in breast cancer. It was shown that about 30% of breast cancer patients had increased DNMT3b expression in tumor tissue compared to normal breast tissue, while DNMT1 and DNMT3a were overexpressed in only 5% and 3% of breast carcinomas, respectively [196]. Elevated expression of DNMT3b was shown to be significantly associated with hypermethylation and subsequent reduced ER α expression and higher histological grade, pointing to a potential involvement of DNMT3b in breast tumor progression and aggressiveness [197]. A strong correlation between total DNMT activity and overexpression of DNMT3b was reported in a subset of breast cancer cells correlating with hypermethylation of methylation-sensitive genes, including ER α . No correlation between total DNMT activity with the expression of DNMT3a or DNMT1 was observed. It was recently demonstrated by the same group that inhibition of DNMT3b by siRNA-mediated knockdown can increase the chemotherapeutic efficacy in breast cancer cells [198]. The authors suggested that DNMT3b has a predominant role over DNMT3a and DNMT1 in breast tumorigenesis.

Since DNA methylation is involved in the process of gene silencing, we investigated whether depletion of DNMT3b can lead to up-regulation of expression of a set of genes we identified as regulated by both estrogen signaling and DNA methylation in **PAPER IV**. We found that the expression of FHL2 and CDKN1A was increased after siRNA-mediated DNMT3b knockdown.

In line with our results, the mRNA levels of CDKN1A were previously found to be up-regulated in MCF-7 cells after DAC-treatment or siRNA-mediated depletion of DNMT3b [199]. In **PAPER IV**, the CDKN1A promoter was found to be unmethylated, suggesting that DNMT3b regulate CDKN1A expression indirectly, which has also been proposed by others [200]. Alternatively, regulation of DNA methylation might be mediated via DNA-methylation of non-CpG island motifs such as CpG shores, shelves and open seas [201, 202].

Our results suggest that E2 treatment and DNMT3b depletion did not display synergistic effects on FHL2 and CDKN1A expression. This is line with the results of **PAPER IV**, where we demonstrated that the E2-mediated effect of ER α recruitment is selective to the enhancers of these genes, which contain no predicted CpG islands.

The FHL2 promoter was found to be methylated in MCF-7 cells in **PAPER IV**, as suggested by others [119]. However, to our knowledge, the molecular mechanisms that regulate FHL2 promoter methylation have not been described. Interestingly, FHL2 was found to be overexpressed in patients with ICF (Immunodeficiency, Centromere Instability, Facial abnormalities) syndrome, caused by mutations in the DNMT3b gene that lead to impaired catalytic activity of the enzyme [203]. This supports our findings and suggests that FHL2 promoter methylation, and subsequent gene expression, is regulated by DNMT3b. Considering that DNMT3b and ER α may be in the same complex, we can hypothesize that ER α can regulate FHL2 expression together with DNMT3b on a same regulatory element, possibly a CpG island in the promoter region. Whether promoter methylation pattern and the protein expression of FHL2 are directly regulated by DNMT3b, remains to be confirmed. In line with our hypothesis, additional approach could be to assay FHL2 promoter methylation status after ER α depletion.

5 GENERAL CONCLUSIONS AND FUTURE PERSPECTIVES

5.1 ESTROGEN RECEPTOR B SNPs AND DISEASE SUSCEPTIBILITY

Several studies have associated SNPs in ER β 3'UTRs with various diseases including breast cancer. However the function of these SNPs in disease etiology remains unknown. We have addressed the function of two SNPs in the ER β 3'UTRs that have been frequently assayed and associated with disease in candidate gene association studies. Our studies do not support a function of these SNPs in disease etiology. Future studies should address additional potential functions of these SNPs as well as the potential function of SNPs in LD with these SNPs. In general, development in our understanding of SNP function has been rather slow in comparison with the generation of data about associations between SNPs and disease. Novel approaches might be needed to address the function of SNPs, ideally in a high throughput format.

5.2 ESTROGEN RECEPTOR B FUNCTIONALITY

Antiproliferative properties of ER β have been shown in a number of *in vitro* studies and ER β has been correlated with markers of good prognosis in many clinical studies. However, the general clinical significance of ER β is not yet established. Unlike ER α , ER β is not generally introduced as a biomarker in breast cancer diagnostics. Reasons include the lack of well validated ER β antibodies, and still rather limited knowledge about the molecular mechanism of action of ER β and its function in breast cancer. In general, ER β levels are decreased in breast cancer. It is believed that ER β is the predominant ER in the mammary epithelium, but that its expression is reduced and possibly ultimately lost during cancer progression, which would restrict its exploration as a drug target.

In our studies we have addressed the function of ER β in the absence of ER α as well as the cross-talk between ER α and ER β at the level of heterodimer binding to DNA in the context of the intact chromatin. We used HEK293/ER β cells to confirm the antiproliferative function of ER β as well as to identify a palette of ER β -target genes in the absence of ER α . We used MCF-7/ER β cells to provide further evidence for the existence of ER α /ER β heterodimers.

These findings, as well as many others that study the function of ER β in the context of cell model systems that stably overexpress ER β , remain to be further investigated, in appropriate cell line models that express endogenous ER β and more importantly *in vivo*. There is a constant influx of data on ER α genome-wide expression profiles and DNA binding events in various cell lines and breast tumors, but such information is still

lacking for ER β . Future effort should focus on the identification of appropriate model systems to study the endogenous ER β , where re-activation of ER β by hypomethylating drugs could constitute one approach. The identification of antibodies compatible with the ChIP assay on breast cancer samples should also be prioritized.

5.3 ESTROGEN SIGNALING AND DNA METHYLATION

Interplay between the pathways of estrogen signaling and DNA methylation has been explored, but it is not well understood. Aiming to identify common targets of estrogen signaling and DNA methylation on a genome-wide scale in breast cancer cells, we used gene expression profiling and identified about 150 genes that were regulated by both pathways. After selecting and analyzing a subset of six genes, we concluded that there was no direct molecular interplay of mediators of estrogen and epigenetic signaling at these promoters. We cannot exclude the possibility that some of the remaining ~140 genes could be targets for a direct molecular interplay of these two pathways. Additionally, our studies were limited to a single time point of E2 exposure and ER ligands, including E2, could have direct effects on methylation patterns at other time points.

Most of the methylation screening assays used so far assessed the methylation changes in the promoter regions, which was also the focus of our study. It is possible that estrogen signaling affects methylation of other regions in the genome reported for differential methylation patterns, such as shores, shelves and open sea. Platforms covering wider DNA regions, such as Infinium Human Methylation 450 BeadChip, or the MeDIP assay, which assays methylation across the complete genome, could be used to test this hypothesis.

Instead of genomically aberrant breast cancer cells, another relevant approach would be assessing the estrogen signaling-related changes in global DNA methylation in mammary epithelial cells.

Nevertheless, we have identified one significant gene, FHL2, regulated by both pathways, but via distinct regulatory elements. E2-activated ER α is recruited to its distant enhancer and we hypothesize that this is the mechanism behind E2-induction of FHL2 expression. Unliganded ER α is recruited to the CpG island within the FHL2 promoter. Furthermore, we found that DNMT3b depletion increases FHL2 mRNA expression, suggesting that this DNA methyltransferase regulates FHL2 promoter methylation, which remains to be confirmed. Interestingly, we show that ER α and DNMT3b can be in the same complex, implying that the unliganded ER α could also be involved in methylation of FHL2 promoter. FHL2 is a transcription factor found to interact with many other factors, including estrogen receptor [204]. It has been suggested to repress ER α and ER β transcriptional activity in breast cancer cells, together with the corepressor Smad4 [205]. ERs and FHL2 seem to regulate each other through a complex feedback mechanism that involves DNA methylation, and their intriguing relationship is worth exploring further.

We also show that ER α signaling affects the mRNA expression of DNMTs, suggesting global interplay between estrogen signaling and regulation of methylation. Future studies should focus on assaying DNMT protein expression and enzymatic activity in response to ER ligands in breast cancer cells.

In summary, this thesis addresses various mechanistic aspects of estrogen signaling in breast cancer cells. Hopefully, knowledge about estrogen and ER signaling, including the cross-talk between the two ERs and their respective cross-talk with other signaling pathway will suggest hypothesis for improved diagnostic criteria as well as therapeutic strategies that can be tested in an appropriate clinical setting.

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7 REFERENCES

1. Jemal A, Bray F, Center MM, Ferlay J, Ward E, *et al.*: Global cancer statistics. *CA Cancer J Clin* **2011** 61:69-90.
2. Tirona MT, Sehgal R, Ballester O: Prevention of breast cancer (part I): epidemiology, risk factors, and risk assessment tools. *Cancer Invest* **2010** 28:743-50.
3. Ripperger T, Gadzicki D, Meindl A, Schlegelberger B: Breast cancer susceptibility: current knowledge and implications for genetic counselling. *Eur J Hum Genet* **2009** 17:722-31.
4. Mavaddat N, Antoniou AC, Easton DF, Garcia-Closas M: Genetic susceptibility to breast cancer. *Mol Oncol* **2010** 4:174-91.
5. Ehemann CR, Shaw KM, Ryerson AB, Miller JW, Ajani UA, *et al.*: The changing incidence of in situ and invasive ductal and lobular breast carcinomas: United States, 1999-2004. *Cancer Epidemiol Biomarkers Prev* **2009** 18:1763-9.
6. Raica M, Jung I, Cimpean AM, Suci C, Muresan AM: From conventional pathologic diagnosis to the molecular classification of breast carcinoma: are we ready for the change? *Rom J Morphol Embryol* **2009** 50:5-13.
7. Schnitt SJ: Classification and prognosis of invasive breast cancer: from morphology to molecular taxonomy. *Mod Pathol* **2010** 23 Suppl 2:S60-4.
8. Dontu G, Al-Hajj M, Abdallah WM, Clarke MF, Wicha MS: Stem cells in normal breast development and breast cancer. *Cell Prolif* **2003** 36 Suppl 1:59-72.
9. Tiede B, Kang Y: From milk to malignancy: the role of mammary stem cells in development, pregnancy and breast cancer. *Cell Res* **2011** 21:245-57.
10. Sternlicht MD: Key stages in mammary gland development: the cues that regulate ductal branching morphogenesis. *Breast Cancer Res* **2006** 8:201.
11. Stingl J: Estrogen and progesterone in normal mammary gland development and in cancer. *Horm Cancer* **2011** 2:85-90.
12. LaMarca HL, Rosen JM: Minireview: hormones and mammary cell fate--what will I become when I grow up? *Endocrinology* **2008** 149:4317-21.
13. Hynes NE, Watson CJ: Mammary gland growth factors: roles in normal development and in cancer. *Cold Spring Harb Perspect Biol* **2010** 2:a003186.
14. Nakshatri H, Srour EF, Badve S: Breast cancer stem cells and intrinsic subtypes: controversies rage on. *Curr Stem Cell Res Ther* **2009** 4:50-60.
15. Peto R, Davies C, Godwin J, Gray R, Pan HC, *et al.*: Comparisons between different polychemotherapy regimens for early breast cancer: meta-analyses of long-term outcome among 100,000 women in 123 randomised trials. *Lancet* **2011** 379:432-44.
16. Hassan MS, Ansari J, Spooner D, Hussain SA: Chemotherapy for breast cancer (Review). *Oncol Rep* **2010** 24:1121-31.
17. Harari D, Yarden Y: Molecular mechanisms underlying ErbB2/HER2 action in breast cancer. *Oncogene* **2000** 19:6102-14.
18. Le XF, Pruefer F, Bast RC, Jr.: HER2-targeting antibodies modulate the cyclin-dependent kinase inhibitor p27Kip1 via multiple signaling pathways. *Cell Cycle* **2005** 4:87-95.
19. Keating GM: Pertuzumab: in the first-line treatment of HER2-positive metastatic breast cancer. *Drugs* **2012** 72:353-60.
20. Ross JS, Slodkowska EA, Symmans WF, Pusztai L, Ravdin PM, *et al.*: The HER-2 receptor and breast cancer: ten years of targeted anti-HER-2 therapy and personalized medicine. *Oncologist* **2009** 14:320-68.

21. Tagliabue E, Campiglio M, Pupa SM, Menard S, Balsari A: Activity and resistance of trastuzumab according to different clinical settings. *Cancer Treat Rev* **2012** 38:212-7.
22. Nahta R, Esteva FJ: HER2 therapy: molecular mechanisms of trastuzumab resistance. *Breast Cancer Res* **2006** 8:215.
23. Telli ML, Witteles RM: Trastuzumab-related cardiac dysfunction. *J Natl Compr Canc Netw* **2011** 9:243-9.
24. Green S, Walter P, Kumar V, Krust A, Bornert JM, *et al.*: Human oestrogen receptor cDNA: sequence, expression and homology to v-erb-A. *Nature* **1986** 320:134-9.
25. Greene GL, Gilna P, Waterfield M, Baker A, Hort Y, *et al.*: Sequence and expression of human estrogen receptor complementary DNA. *Science* **1986** 231:1150-4.
26. Osborne CK: Steroid hormone receptors in breast cancer management. *Breast Cancer Res Treat* **1998** 51:227-38.
27. Mosselman S, Polman J, Dijkema R: ER beta: identification and characterization of a novel human estrogen receptor. *FEBS Lett* **1996** 392:49-53.
28. Matthews J, Gustafsson JA: Estrogen signaling: a subtle balance between ER alpha and ER beta. *Mol Interv* **2003** 3:281-92.
29. Taylor AH, Al-Azzawi F: Immunolocalisation of oestrogen receptor beta in human tissues. *J Mol Endocrinol* **2000** 24:145-55.
30. Latil A, Bieche I, Vidaud D, Lidereau R, Berthon P, *et al.*: Evaluation of androgen, estrogen (ER alpha and ER beta), and progesterone receptor expression in human prostate cancer by real-time quantitative reverse transcription-polymerase chain reaction assays. *Cancer Res* **2001** 61:1919-26.
31. Dahlman-Wright K, Cavailles V, Fuqua SA, Jordan VC, Katzenellenbogen JA, *et al.*: International Union of Pharmacology. LXIV. Estrogen receptors. *Pharmacol Rev* **2006** 58:773-81.
32. Nilsson S, Makela S, Treuter E, Tujague M, Thomsen J, *et al.*: Mechanisms of estrogen action. *Physiol Rev* **2001** 81:1535-65.
33. Menasce LP, White GR, Harrison CJ, Boyle JM: Localization of the estrogen receptor locus (ESR) to chromosome 6q25.1 by FISH and a simple post-FISH banding technique. *Genomics* **1993** 17:263-5.
34. Herynk MH, Fuqua SA: Estrogen receptor mutations in human disease. *Endocr Rev* **2004** 25:869-98.
35. Ogawa S, Inoue S, Watanabe T, Hiroi H, Orimo A, *et al.*: The complete primary structure of human estrogen receptor beta (hER beta) and its heterodimerization with ER alpha in vivo and in vitro. *Biochem Biophys Res Commun* **1998** 243:122-6.
36. Hewitt SC, Korach KS: Estrogen receptors: structure, mechanisms and function. *Rev Endocr Metab Disord* **2002** 3:193-200.
37. Kos M, Reid G, Denger S, Gannon F: Minireview: genomic organization of the human ERalpha gene promoter region. *Mol Endocrinol* **2001** 15:2057-63.
38. Tanimoto K, Eguchi H, Yoshida T, Hajiro-Nakanishi K, Hayashi S: Regulation of estrogen receptor alpha gene mediated by promoter B responsible for its enhanced expression in human breast cancer. *Nucleic Acids Res* **1999** 27:903-9.
39. Gustafsson N, Zhao C, Gustafsson JA, Dahlman-Wright K: RBCK1 drives breast cancer cell proliferation by promoting transcription of estrogen receptor alpha and cyclin B1. *Cancer Res* **2010** 70:1265-74.
40. Grandien K: Determination of transcription start sites in the human estrogen receptor gene and identification of a novel, tissue-specific, estrogen receptor-mRNA isoform. *Mol Cell Endocrinol* **1996** 116:207-12.

41. Flourirot G, Griffin C, Kenealy M, Sonntag-Buck V, Gannon F: Differentially expressed messenger RNA isoforms of the human estrogen receptor-alpha gene are generated by alternative splicing and promoter usage. *Mol Endocrinol* **1998** 12:1939-54.
42. Hirata S, Shoda T, Kato J, Hoshi K: The multiple untranslated first exons system of the human estrogen receptor beta (ER beta) gene. *J Steroid Biochem Mol Biol* **2001** 78:33-40.
43. Swedenborg E, Power KA, Cai W, Pongratz I, Ruegg J: Regulation of estrogen receptor beta activity and implications in health and disease. *Cell Mol Life Sci* **2009** 66:3873-94.
44. Flourirot G, Brand H, Denger S, Metivier R, Kos M, *et al.*: Identification of a new isoform of the human estrogen receptor-alpha (hER-alpha) that is encoded by distinct transcripts and that is able to repress hER-alpha activation function 1. *Embo J* **2000** 19:4688-700.
45. Taylor SE, Martin-Hirsch PL, Martin FL: Oestrogen receptor splice variants in the pathogenesis of disease. *Cancer Lett* **2010** 288:133-48.
46. Erenburg I, Schachter B, Mira y Lopez R, Ossowski L: Loss of an estrogen receptor isoform (ER alpha delta 3) in breast cancer and the consequences of its reexpression: interference with estrogen-stimulated properties of malignant transformation. *Mol Endocrinol* **1997** 11:2004-15.
47. Moore JT, McKee DD, Slentz-Kesler K, Moore LB, Jones SA, *et al.*: Cloning and characterization of human estrogen receptor beta isoforms. *Biochem Biophys Res Commun* **1998** 247:75-8.
48. Ogawa S, Inoue S, Watanabe T, Orimo A, Hosoi T, *et al.*: Molecular cloning and characterization of human estrogen receptor betacx: a potential inhibitor of estrogen action in human. *Nucleic Acids Res* **1998** 26:3505-12.
49. Peng B, Lu B, Leygue E, Murphy LC: Putative functional characteristics of human estrogen receptor-beta isoforms. *J Mol Endocrinol* **2003** 30:13-29.
50. Omoto Y, Eguchi H, Yamamoto-Yamaguchi Y, Hayashi S: Estrogen receptor (ER) beta1 and ERbetacx/beta2 inhibit ERalpha function differently in breast cancer cell line MCF7. *Oncogene* **2003** 22:5011-20.
51. Zhao C, Matthews J, Tujague M, Wan J, Strom A, *et al.*: Estrogen receptor beta2 negatively regulates the transactivation of estrogen receptor alpha in human breast cancer cells. *Cancer Res* **2007** 67:3955-62.
52. Zhao C, Dahlman-Wright K, Gustafsson JA: Estrogen signaling via estrogen receptor {beta}. *J Biol Chem* **2010** 285:39575-9.
53. Zhu BT, Han GZ, Shim JY, Wen Y, Jiang XR: Quantitative structure-activity relationship of various endogenous estrogen metabolites for human estrogen receptor alpha and beta subtypes: Insights into the structural determinants favoring a differential subtype binding. *Endocrinology* **2006** 147:4132-50.
54. Leclercq G, Lacroix M, Laios I, Laurent G: Estrogen receptor alpha: impact of ligands on intracellular shuttling and turnover rate in breast cancer cells. *Curr Cancer Drug Targets* **2006** 6:39-64.
55. Wiseman H, Duffy R: New advances in the understanding of the role of steroids and steroid receptors in disease. *Biochem Soc Trans* **2001** 29:205-9.
56. Osborne CK, Wakeling A, Nicholson RI: Fulvestrant: an oestrogen receptor antagonist with a novel mechanism of action. *Br J Cancer* **2004** 90 Suppl 1:S2-6.
57. Stauffer SR, Coletta CJ, Tedesco R, Nishiguchi G, Carlson K, *et al.*: Pyrazole ligands: structure-affinity/activity relationships and estrogen receptor-alpha-selective agonists. *J Med Chem* **2000** 43:4934-47.
58. Harris HA, Katzenellenbogen JA, Katzenellenbogen BS: Characterization of the biological roles of the estrogen receptors, ERalpha and ERbeta, in estrogen target

- tissues in vivo through the use of an ERalpha-selective ligand. *Endocrinology* **2002** 143:4172-7.
- 59. Meyers MJ, Sun J, Carlson KE, Marriner GA, Katzenellenbogen BS, et al.:** Estrogen receptor-beta potency-selective ligands: structure-activity relationship studies of diarylpropionitriles and their acetylene and polar analogues. *J Med Chem* **2001** 44:4230-51.
- 60. Kuiper GG, Carlsson B, Grandien K, Enmark E, Haggblad J, et al.:** Comparison of the ligand binding specificity and transcript tissue distribution of estrogen receptors alpha and beta. *Endocrinology* **1997** 138:863-70.
- 61. Smith CA, O'Maille G, Want EJ, Qin C, Trauger SA, et al.:** METLIN: a metabolite mass spectral database. *Ther Drug Monit* **2005** 27:747-51.
- 62. Schultz JR, Petz LN, Nardulli AM:** Cell- and ligand-specific regulation of promoters containing activator protein-1 and Sp1 sites by estrogen receptors alpha and beta. *J Biol Chem* **2005** 280:347-54.
- 63. Bjornstrom L, Sjoberg M:** Mechanisms of estrogen receptor signaling: convergence of genomic and nongenomic actions on target genes. *Mol Endocrinol* **2005** 19:833-42.
- 64. Safe S, Abdelrahim M:** Sp transcription factor family and its role in cancer. *Eur J Cancer* **2005** 41:2438-48.
- 65. Chung YL, Sheu ML, Yang SC, Lin CH, Yen SH:** Resistance to tamoxifen-induced apoptosis is associated with direct interaction between Her2/neu and cell membrane estrogen receptor in breast cancer. *Int J Cancer* **2002** 97:306-12.
- 66. Kahlert S, Nuedling S, van Eickels M, Vetter H, Meyer R, et al.:** Estrogen receptor alpha rapidly activates the IGF-1 receptor pathway. *J Biol Chem* **2000** 275:18447-53.
- 67. Bunone G, Briand PA, Miksicek RJ, Picard D:** Activation of the unliganded estrogen receptor by EGF involves the MAP kinase pathway and direct phosphorylation. *Embo J* **1996** 15:2174-83.
- 68. Kim H, Heo K, Kim JH, Kim K, Choi J, et al.:** Requirement of histone methyltransferase SMYD3 for estrogen receptor-mediated transcription. *J Biol Chem* **2009** 284:19867-77.
- 69. Ma H, Baumann CT, Li H, Strahl BD, Rice R, et al.:** Hormone-dependent, CARM1-directed, arginine-specific methylation of histone H3 on a steroid-regulated promoter. *Curr Biol* **2001** 11:1981-5.
- 70. Stossi F, Madak-Erdogan Z, Katzenellenbogen BS:** Estrogen receptor alpha represses transcription of early target genes via p300 and CtBP1. *Mol Cell Biol* **2009** 29:1749-59.
- 71. Sun JM, Chen HY, Davie JR:** Effect of estradiol on histone acetylation dynamics in human breast cancer cells. *J Biol Chem* **2001** 276:49435-42.
- 72. Markopoulos C, Berger U, Wilson P, Gazet JC, Coombes RC:** Oestrogen receptor content of normal breast cells and breast carcinomas throughout the menstrual cycle. *Br Med J (Clin Res Ed)* **1988** 296:1349-51.
- 73. Petersen OW, Hoyer PE, van Deurs B:** Frequency and distribution of estrogen receptor-positive cells in normal, nonlactating human breast tissue. *Cancer Res* **1987** 47:5748-51.
- 74. Ricketts D, Turnbull L, Ryall G, Bakhshi R, Rawson NS, et al.:** Estrogen and progesterone receptors in the normal female breast. *Cancer Res* **1991** 51:1817-22.
- 75. Miyoshi Y, Murase K, Saito M, Imamura M, Oh K:** Mechanisms of estrogen receptor-alpha upregulation in breast cancers. *Med Mol Morphol* **2010** 43:193-6.
- 76. Pathiraja TN, Stearns V, Oesterreich S:** Epigenetic regulation in estrogen receptor positive breast cancer--role in treatment response. *J Mammary Gland Biol Neoplasia* **2010** 15:35-47.

77. Giacinti L, Claudio PP, Lopez M, Giordano A: Epigenetic information and estrogen receptor alpha expression in breast cancer. *Oncologist* **2006** 11:1-8.
78. Zhou Q, Shaw PG, Davidson NE: Epigenetics meets estrogen receptor: regulation of estrogen receptor by direct lysine methylation. *Endocr Relat Cancer* **2009** 16:319-23.
79. Putti TC, El-Rehim DM, Rakha EA, Paish CE, Lee AH, *et al.*: Estrogen receptor-negative breast carcinomas: a review of morphology and immunophenotypical analysis. *Mod Pathol* **2005** 18:26-35.
80. Fuqua SA, Schiff R, Parra I, Moore JT, Mohsin SK, *et al.*: Estrogen receptor beta protein in human breast cancer: correlation with clinical tumor parameters. *Cancer Res* **2003** 63:2434-9.
81. Omoto Y, Kobayashi S, Inoue S, Ogawa S, Toyama T, *et al.*: Evaluation of oestrogen receptor beta wild-type and variant protein expression, and relationship with clinicopathological factors in breast cancers. *Eur J Cancer* **2002** 38:380-6.
82. Speirs V, Carder PJ, Lane S, Dodwell D, Lansdown MR, *et al.*: Oestrogen receptor beta: what it means for patients with breast cancer. *Lancet Oncol* **2004** 5:174-81.
83. Skliris GP, Munot K, Bell SM, Carder PJ, Lane S, *et al.*: Reduced expression of oestrogen receptor beta in invasive breast cancer and its re-expression using DNA methyl transferase inhibitors in a cell line model. *J Pathol* **2003** 201:213-20.
84. Welboren WJ, Sweep FC, Span PN, Stunnenberg HG: Genomic actions of estrogen receptor alpha: what are the targets and how are they regulated? *Endocr Relat Cancer* **2009** 16:1073-89.
85. Zwart W, Theodorou V, Carroll JS: Estrogen receptor-positive breast cancer: a multidisciplinary challenge. *Wiley Interdiscip Rev Syst Biol Med* **2011** 3:216-30.
86. Ross-Innes CS, Stark R, Teschendorff AE, Holmes KA, Ali HR, *et al.*: Differential oestrogen receptor binding is associated with clinical outcome in breast cancer. *Nature* **2012** 481:389-93.
87. Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, *et al.*: Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell* **2005** 122:33-43.
88. Carroll JS, Meyer CA, Song J, Li W, Geistlinger TR, *et al.*: Genome-wide analysis of estrogen receptor binding sites. *Nat Genet* **2006** 38:1289-97.
89. Lupien M, Eeckhoute J, Meyer CA, Wang Q, Zhang Y, *et al.*: FoxA1 translates epigenetic signatures into enhancer-driven lineage-specific transcription. *Cell* **2008** 132:958-70.
90. Krum SA, Miranda-Carboni GA, Lupien M, Eeckhoute J, Carroll JS, *et al.*: Unique ERalpha cistromes control cell type-specific gene regulation. *Mol Endocrinol* **2008** 22:2393-406.
91. Charn TH, Liu ET, Chang EC, Lee YK, Katzenellenbogen JA, *et al.*: Genome-wide dynamics of chromatin binding of estrogen receptors alpha and beta: mutual restriction and competitive site selection. *Mol Endocrinol* **2010** 24:47-59.
92. Zhao C, Gao H, Liu Y, Papoutsis Z, Jaffrey S, *et al.*: Genome-wide mapping of estrogen receptor-beta-binding regions reveals extensive cross-talk with transcription factor activator protein-1. *Cancer Res* **2010** 70:5174-83.
93. Cheung E, Kraus WL: Genomic analyses of hormone signaling and gene regulation. *Annu Rev Physiol* **2010** 72:191-218.
94. Frasor J, Danes JM, Komm B, Chang KC, Lyttle CR, *et al.*: Profiling of estrogen up- and down-regulated gene expression in human breast cancer cells: insights into gene networks and pathways underlying estrogenic control of proliferation and cell phenotype. *Endocrinology* **2003** 144:4562-74.

- 95. Stender JD, Frasor J, Komm B, Chang KC, Kraus WL, et al.:** Estrogen-regulated gene networks in human breast cancer cells: involvement of E2F1 in the regulation of cell proliferation. *Mol Endocrinol* **2007** 21:2112-23.
- 96. Kininis M, Kraus WL:** A global view of transcriptional regulation by nuclear receptors: gene expression, factor localization, and DNA sequence analysis. *Nucl Recept Signal* **2008** 6:e005.
- 97. Carroll JS, Brown M:** Estrogen receptor target gene: an evolving concept. *Mol Endocrinol* **2006** 20:1707-14.
- 98. Barkhem T, Carlsson B, Nilsson Y, Enmark E, Gustafsson J, et al.:** Differential response of estrogen receptor alpha and estrogen receptor beta to partial estrogen agonists/antagonists. *Mol Pharmacol* **1998** 54:105-12.
- 99. Chang EC, Frasor J, Komm B, Katzenellenbogen BS:** Impact of estrogen receptor beta on gene networks regulated by estrogen receptor alpha in breast cancer cells. *Endocrinology* **2006** 147:4831-42.
- 100. Lin CY, Strom A, Li Kong S, Kietz S, Thomsen JS, et al.:** Inhibitory effects of estrogen receptor beta on specific hormone-responsive gene expression and association with disease outcome in primary breast cancer. *Breast Cancer Res* **2007** 9:R25.
- 101. Secreto FJ, Monroe DG, Dutta S, Ingle JN, Spelsberg TC:** Estrogen receptor alpha/beta isoforms, but not betacx, modulate unique patterns of gene expression and cell proliferation in Hs578T cells. *J Cell Biochem* **2007** 101:1125-47.
- 102. Monroe DG, Getz BJ, Johnsen SA, Riggs BL, Khosla S, et al.:** Estrogen receptor isoform-specific regulation of endogenous gene expression in human osteoblastic cell lines expressing either ERalpha or ERbeta. *J Cell Biochem* **2003** 90:315-26.
- 103. Jurkowska RZ, Jurkowski TP, Jeltsch A:** Structure and function of mammalian DNA methyltransferases. *Chembiochem* **2011** 12:206-22.
- 104. Dhasarathy A, Wade PA:** The MBD protein family-reading an epigenetic mark? *Mutat Res* **2008** 647:39-43.
- 105. Bird A:** DNA methylation patterns and epigenetic memory. *Genes Dev* **2002** 16:6-21.
- 106. Saxonov S, Berg P, Brutlag DL:** A genome-wide analysis of CpG dinucleotides in the human genome distinguishes two distinct classes of promoters. *Proc Natl Acad Sci U S A* **2006** 103:1412-7.
- 107. Hinshelwood RA, Clark SJ:** Breast cancer epigenetics: normal human mammary epithelial cells as a model system. *J Mol Med (Berl)* **2008** 86:1315-28.
- 108. Esteller M, Corn PG, Baylin SB, Herman JG:** A gene hypermethylation profile of human cancer. *Cancer Res* **2001** 61:3225-9.
- 109. Portela A, Esteller M:** Epigenetic modifications and human disease. *Nat Biotechnol* **2010** 28:1057-68.
- 110. Evron E, Umbricht CB, Korz D, Raman V, Loeb DM, et al.:** Loss of cyclin D2 expression in the majority of breast cancers is associated with promoter hypermethylation. *Cancer Res* **2001** 61:2782-7.
- 111. Fackler MJ, McVeigh M, Evron E, Garrett E, Mehrotra J, et al.:** DNA methylation of RASSF1A, HIN-1, RAR-beta, Cyclin D2 and Twist in in situ and invasive lobular breast carcinoma. *Int J Cancer* **2003** 107:970-5.
- 112. Brueckner B, Lyko F:** DNA methyltransferase inhibitors: old and new drugs for an epigenetic cancer therapy. *Trends Pharmacol Sci* **2004** 25:551-4.
- 113. Christman JK:** 5-Azacytidine and 5-aza-2'-deoxycytidine as inhibitors of DNA methylation: mechanistic studies and their implications for cancer therapy. *Oncogene* **2002** 21:5483-95.

114. Billam M, Sobolewski MD, Davidson NE: Effects of a novel DNA methyltransferase inhibitor zebularine on human breast cancer cells. *Breast Cancer Res Treat* **2010** 120:581-92.
115. Kwon YS, Garcia-Bassets I, Hutt KR, Cheng CS, Jin M, *et al.*: Sensitive ChIP-DSL technology reveals an extensive estrogen receptor alpha-binding program on human gene promoters. *Proc Natl Acad Sci U S A* **2007** 104:4852-7.
116. Li Y, Sun L, Zhang Y, Wang D, Wang F, *et al.*: The histone modifications governing TFF1 transcription mediated by estrogen receptor. *J Biol Chem* **2011** 286:13925-36.
117. Metivier R, Gallais R, Tiffocche C, Le Peron C, Jurkowska RZ, *et al.*: Cyclical DNA methylation of a transcriptionally active promoter. *Nature* **2008** 452:45-50.
118. Leu YW, Yan PS, Fan M, Jin VX, Liu JC, *et al.*: Loss of estrogen receptor signaling triggers epigenetic silencing of downstream targets in breast cancer. *Cancer Res* **2004** 64:8184-92.
119. Fan M, Yan PS, Hartman-Frey C, Chen L, Paik H, *et al.*: Diverse gene expression and DNA methylation profiles correlate with differential adaptation of breast cancer cells to the antiestrogens tamoxifen and fulvestrant. *Cancer Res* **2006** 66:11954-66.
120. Kubarek L, Jagodzinski PP: Epigenetic up-regulation of CXCR4 and CXCL12 expression by 17 beta-estradiol and tamoxifen is associated with formation of DNA methyltransferase 3B4 splice variant in Ishikawa endometrial adenocarcinoma cells. *FEBS Lett* **2007** 581:1441-8.
121. Hsu PY, Hsu HK, Singer GA, Yan PS, Rodriguez BA, *et al.*: Estrogen-mediated epigenetic repression of large chromosomal regions through DNA looping. *Genome Res* **2010** 20:733-44.
122. Yang X, Phillips DL, Ferguson AT, Nelson WG, Herman JG, *et al.*: Synergistic activation of functional estrogen receptor (ER)-alpha by DNA methyltransferase and histone deacetylase inhibition in human ER-alpha-negative breast cancer cells. *Cancer Res* **2001** 61:7025-9.
123. Yan L, Nass SJ, Smith D, Nelson WG, Herman JG, *et al.*: Specific inhibition of DNMT1 by antisense oligonucleotides induces re-expression of estrogen receptor-alpha (ER) in ER-negative human breast cancer cell lines. *Cancer Biol Ther* **2003** 2:552-6.
124. Lapidus RG, Ferguson AT, Ottaviano YL, Parl FF, Smith HS, *et al.*: Methylation of estrogen and progesterone receptor gene 5' CpG islands correlates with lack of estrogen and progesterone receptor gene expression in breast tumors. *Clin Cancer Res* **1996** 2:805-10.
125. Gaudet MM, Campan M, Figueroa JD, Yang XR, Lissowska J, *et al.*: DNA hypermethylation of ESR1 and PGR in breast cancer: pathologic and epidemiologic associations. *Cancer Epidemiol Biomarkers Prev* **2009** 18:3036-43.
126. Zhao C, Lam EW, Sunters A, Enmark E, De Bella MT, *et al.*: Expression of estrogen receptor beta isoforms in normal breast epithelial cells and breast cancer: regulation by methylation. *Oncogene* **2003** 22:7600-6.
127. Rody A, Holtrich U, Solbach C, Kourtis K, von Minckwitz G, *et al.*: Methylation of estrogen receptor beta promoter correlates with loss of ER-beta expression in mammary carcinoma and is an early indication marker in premalignant lesions. *Endocr Relat Cancer* **2005** 12:903-16.
128. Cox A, Dunning AM, Garcia-Closas M, Balasubramanian S, Reed MW, *et al.*: A common coding variant in CASP8 is associated with breast cancer risk. *Nat Genet* **2007** 39:352-8.
129. Easton DF, Pooley KA, Dunning AM, Pharoah PD, Thompson D, *et al.*: Genome-wide association study identifies novel breast cancer susceptibility loci. *Nature* **2007** 447:1087-93.

130. Riva A, Kohane IS: A SNP-centric database for the investigation of the human genome. *BMC Bioinformatics* **2004** 5:33.
131. Deroo BJ, Korach KS: Estrogen receptors and human disease. *J Clin Invest* **2006** 116:561-70.
132. Alcazar LP, Arakaki PA, Godoy-Santos A, Santos M: Estrogen receptor polymorphism and its relationship to pathological process. *Am J Med Sci* **2010** 340:128-32.
133. Casazza K, Page GP, Fernandez JR: The association between the rs2234693 and rs9340799 estrogen receptor alpha gene polymorphisms and risk factors for cardiovascular disease: a review. *Biol Res Nurs* **2010** 12:84-97.
134. Li N, Dong J, Hu Z, Shen H, Dai M: Potentially functional polymorphisms in ESR1 and breast cancer risk: a meta-analysis. *Breast Cancer Res Treat* **2010** 121:177-84.
135. Wedren S, Lovmar L, Humphreys K, Magnusson C, Melhus H, *et al.*: Estrogen receptor alpha gene polymorphism and endometrial cancer risk--a case-control study. *BMC Cancer* **2008** 8:322.
136. Tchatchou S, Jung A, Hemminki K, Sutter C, Wappenschmidt B, *et al.*: A variant affecting a putative miRNA target site in estrogen receptor (ESR) 1 is associated with breast cancer risk in premenopausal women. *Carcinogenesis* **2009** 30:59-64.
137. Harlid S, Ivarsson MI, Butt S, Hussain S, Grzybowska E, *et al.*: A candidate CpG SNP approach identifies a breast cancer associated ESR1-SNP. *Int J Cancer* **2011** 129:1689-98.
138. Hindorff LA, Sethupathy P, Junkins HA, Ramos EM, Mehta JP, *et al.*: Potential etiologic and functional implications of genome-wide association loci for human diseases and traits. *Proc Natl Acad Sci U S A* **2009** 106:9362-7.
139. Yu KD, Rao NY, Chen AX, Fan L, Yang C, *et al.*: A systematic review of the relationship between polymorphic sites in the estrogen receptor-beta (ESR2) gene and breast cancer risk. *Breast Cancer Res Treat* **2011** 126:37-45.
140. Maguire P, Margolin S, Skoglund J, Sun XF, Gustafsson JA, *et al.*: Estrogen receptor beta (ESR2) polymorphisms in familial and sporadic breast cancer. *Breast Cancer Res Treat* **2005** 94:145-52.
141. Nilsson M, Naessen S, Dahlman I, Linden Hirschberg A, Gustafsson JA, *et al.*: Association of estrogen receptor beta gene polymorphisms with bulimic disease in women. *Mol Psychiatry* **2004** 9:28-34.
142. Westberg L, Eriksson E: Sex steroid-related candidate genes in psychiatric disorders. *J Psychiatry Neurosci* **2008** 33:319-30.
143. Treack O, Elemenler E, Kriener C, Horn F, Springwald A, *et al.*: Polymorphisms in the promoter region of ESR2 gene and breast cancer susceptibility. *J Steroid Biochem Mol Biol* **2009** 114:207-11.
144. Philips S, Richter A, Oesterreich S, Rae JM, Flockhart DA, *et al.*: Functional characterization of a genetic polymorphism in the promoter of the ESR2 gene. *Horm Cancer* **2012** 3:37-43.
145. Hindorff LA MJEBI, Wise A, Junkins HA, Hall PN, Klemm AK, and Manolio TA.: A Catalog of Published Genome-Wide Association Studies. Available at: www.genome.gov/gwastudies. . Accessed April 2012.
146. Wang Z, Moulton J: SNPs, protein structure, and disease. *Hum Mutat* **2001** 17:263-70.
147. Chen JM, Ferec C, Cooper DN: A systematic analysis of disease-associated variants in the 3' regulatory regions of human protein-coding genes I: general principles and overview. *Hum Genet* **2006** 120:1-21.

- 148. Desta Z, Ward BA, Soukhova NV, Flockhart DA:** Comprehensive evaluation of tamoxifen sequential biotransformation by the human cytochrome P450 system in vitro: prominent roles for CYP3A and CYP2D6. *J Pharmacol Exp Ther* **2004** 310:1062-75.
- 149. Fabian CJ, Kimler BF:** Selective estrogen-receptor modulators for primary prevention of breast cancer. *J Clin Oncol* **2005** 23:1644-55.
- 150. Michael H, Harkonen PL, Kangas L, Vaananen HK, Hentunen TA:** Differential effects of selective oestrogen receptor modulators (SERMs) tamoxifen, ospemifene and raloxifene on human osteoclasts in vitro. *Br J Pharmacol* **2007** 151:384-95.
- 151. Zidan J, Keidar Z, Basher W, Israel O:** Effects of tamoxifen on bone mineral density and metabolism in postmenopausal women with early-stage breast cancer. *Med Oncol* **2004** 21:117-21.
- 152. Lasset C, Bonadona V, Mignotte H, Bremond A:** Tamoxifen and risk of endometrial cancer. *Lancet* **2001** 357:66-7.
- 153. Vogel VG, Costantino JP, Wickerham DL, Cronin WM, Cecchini RS, et al.:** Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP Study of Tamoxifen and Raloxifene (STAR) P-2 trial. *Jama* **2006** 295:2727-41.
- 154. Croxtall JD, McKeage K:** Fulvestrant: a review of its use in the management of hormone receptor-positive metastatic breast cancer in postmenopausal women. *Drugs* **2011** 71:363-80.
- 155. Vergote I, Robertson JF:** Fulvestrant is an effective and well-tolerated endocrine therapy for postmenopausal women with advanced breast cancer: results from clinical trials. *Br J Cancer* **2004** 90 Suppl 1:S11-4.
- 156. Howell A, Robertson JF, Abram P, Lichinitser MR, Elledge R, et al.:** Comparison of fulvestrant versus tamoxifen for the treatment of advanced breast cancer in postmenopausal women previously untreated with endocrine therapy: a multinational, double-blind, randomized trial. *J Clin Oncol* **2004** 22:1605-13.
- 157. Carpenter R, Miller WR:** Role of aromatase inhibitors in breast cancer. *Br J Cancer* **2005** 93 Suppl 1:S1-5.
- 158. Baum M:** The ATAC (Arimidex, Tamoxifen, Alone or in Combination) adjuvant breast cancer trial in postmenopausal patients: factors influencing the success of patient recruitment. *Eur J Cancer* **2002** 38:1984-6.
- 159. Baum M, Buzdar A, Cuzick J, Forbes J, Houghton J, et al.:** Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early-stage breast cancer: results of the ATAC (Arimidex, Tamoxifen Alone or in Combination) trial efficacy and safety update analyses. *Cancer* **2003** 98:1802-10.
- 160. Hoskins JM, Carey LA, McLeod HL:** CYP2D6 and tamoxifen: DNA matters in breast cancer. *Nat Rev Cancer* **2009** 9:576-86.
- 161. Dowsett M:** Overexpression of HER-2 as a resistance mechanism to hormonal therapy for breast cancer. *Endocr Relat Cancer* **2001** 8:191-5.
- 162. Font de Mora J, Brown M:** AIB1 is a conduit for kinase-mediated growth factor signaling to the estrogen receptor. *Mol Cell Biol* **2000** 20:5041-7.
- 163. Kojetin DJ, Burris TP, Jensen EV, Khan SA:** Implications of the binding of tamoxifen to the coactivator recognition site of the estrogen receptor. *Endocr Relat Cancer* **2008** 15:851-70.
- 164. Dobrzycka KM, Townson SM, Jiang S, Oesterreich S:** Estrogen receptor corepressors -- a role in human breast cancer? *Endocr Relat Cancer* **2003** 10:517-36.

- 165. Lavinsky RM, Jepsen K, Heinzel T, Torchia J, Mullen TM, et al.:** Diverse signaling pathways modulate nuclear receptor recruitment of N-CoR and SMRT complexes. *Proc Natl Acad Sci U S A* **1998** 95:2920-5.
- 166. Arpino G, Wiechmann L, Osborne CK, Schiff R:** Crosstalk between the estrogen receptor and the HER tyrosine kinase receptor family: molecular mechanism and clinical implications for endocrine therapy resistance. *Endocr Rev* **2008** 29:217-33.
- 167. Osborne CK, Shou J, Massarweh S, Schiff R:** Crosstalk between estrogen receptor and growth factor receptor pathways as a cause for endocrine therapy resistance in breast cancer. *Clin Cancer Res* **2005** 11:865s-70s.
- 168. Gee JM, Robertson JF, Ellis IO, Nicholson RI:** Phosphorylation of ERK1/2 mitogen-activated protein kinase is associated with poor response to anti-hormonal therapy and decreased patient survival in clinical breast cancer. *Int J Cancer* **2001** 95:247-54.
- 169. Coutts AS, Murphy LC:** Elevated mitogen-activated protein kinase activity in estrogen-nonresponsive human breast cancer cells. *Cancer Res* **1998** 58:4071-4.
- 170. Shim WS, Conaway M, Masamura S, Yue W, Wang JP, et al.:** Estradiol hypersensitivity and mitogen-activated protein kinase expression in long-term estrogen deprived human breast cancer cells in vivo. *Endocrinology* **2000** 141:396-405.
- 171. Girault I, Bieche I, Lidereau R:** Role of estrogen receptor alpha transcriptional coregulators in tamoxifen resistance in breast cancer. *Maturitas* **2006** 54:342-51.
- 172. Knowlden JM, Hutcheson IR, Jones HE, Madden T, Gee JM, et al.:** Elevated levels of epidermal growth factor receptor/c-erbB2 heterodimers mediate an autocrine growth regulatory pathway in tamoxifen-resistant MCF-7 cells. *Endocrinology* **2003** 144:1032-44.
- 173. Kuukasjarvi T, Kononen J, Helin H, Holli K, Isola J:** Loss of estrogen receptor in recurrent breast cancer is associated with poor response to endocrine therapy. *J Clin Oncol* **1996** 14:2584-9.
- 174. Howell A, Robertson JF, Quaresma Albano J, Aschermannova A, Mauriac L, et al.:** Fulvestrant, formerly ICI 182,780, is as effective as anastrozole in postmenopausal women with advanced breast cancer progressing after prior endocrine treatment. *J Clin Oncol* **2002** 20:3396-403.
- 175. Osborne CK, Pippin J, Jones SE, Parker LM, Ellis M, et al.:** Double-blind, randomized trial comparing the efficacy and tolerability of fulvestrant versus anastrozole in postmenopausal women with advanced breast cancer progressing on prior endocrine therapy: results of a North American trial. *J Clin Oncol* **2002** 20:3386-95.
- 176. Jisa E, Jungbauer A:** Kinetic analysis of estrogen receptor homo- and heterodimerization in vitro. *J Steroid Biochem Mol Biol* **2003** 84:141-8.
- 177. Hopp TA, Weiss HL, Parra IS, Cui Y, Osborne CK, et al.:** Low levels of estrogen receptor beta protein predict resistance to tamoxifen therapy in breast cancer. *Clin Cancer Res* **2004** 10:7490-9.
- 178. Osborne CK, Hobbs K, Trent JM:** Biological differences among MCF-7 human breast cancer cell lines from different laboratories. *Breast Cancer Res Treat* **1987** 9:111-21.
- 179. Shafie SM, Grantham FH:** Role of hormones in the growth and regression of human breast cancer cells (MCF-7) transplanted into athymic nude mice. *J Natl Cancer Inst* **1981** 67:51-6.
- 180. Thomas P, Smart TG:** HEK293 cell line: a vehicle for the expression of recombinant proteins. *J Pharmacol Toxicol Methods* **2005** 51:187-200.
- 181. Masters JR:** HeLa cells 50 years on: the good, the bad and the ugly. *Nat Rev Cancer* **2002** 2:315-9.

- 182. Gluzman Y:** SV40-transformed simian cells support the replication of early SV40 mutants. *Cell* **1981** 23:175-82.
- 183. Gossen M, Bujard H:** Tight control of gene expression in mammalian cells by tetracycline-responsive promoters. *Proc Natl Acad Sci U S A* **1992** 89:5547-51.
- 184. Gossen M, Freundlieb S, Bender G, Muller G, Hillen W, et al.:** Transcriptional activation by tetracyclines in mammalian cells. *Science* **1995** 268:1766-9.
- 185. Reimann M, Vafeiadou K, Hall WL, Dierkes J, Nilsson M, et al.:** Evidence for associations between common polymorphisms of estrogen receptor beta gene with homocysteine and nitric oxide. *Climacteric* **2006** 9:215-23.
- 186. Silvestri S, Thomsen AB, Gozzini A, Bagger Y, Christiansen C, et al.:** Estrogen receptor alpha and beta polymorphisms: is there an association with bone mineral density, plasma lipids, and response to postmenopausal hormone therapy? *Menopause* **2006** 13:451-61.
- 187. Curro M, Marini H, Alibrandi A, Ferlazzo N, Condello S, et al.:** The ESR2 AluI gene polymorphism is associated with bone mineral density in postmenopausal women. *J Steroid Biochem Mol Biol* **2011** 127:413-7.
- 188. Tremblay GB, Tremblay A, Labrie F, Giguere V:** Dominant activity of activation function 1 (AF-1) and differential stoichiometric requirements for AF-1 and -2 in the estrogen receptor alpha-beta heterodimeric complex. *Mol Cell Biol* **1999** 19:1919-27.
- 189. Pryzbylowski P, Obajimi O, Keen JC:** Trichostatin A and 5 Aza-2' deoxycytidine decrease estrogen receptor mRNA stability in ER positive MCF7 cells through modulation of HuR. *Breast Cancer Res Treat* **2008** 111:15-25.
- 190. Majid S, Dar AA, Shahryari V, Hirata H, Ahmad A, et al.:** Genistein reverses hypermethylation and induces active histone modifications in tumor suppressor gene B-Cell translocation gene 3 in prostate cancer. *Cancer* **2010** 116:66-76.
- 191. Majid S, Dar AA, Ahmad AE, Hirata H, Kawakami K, et al.:** BTG3 tumor suppressor gene promoter demethylation, histone modification and cell cycle arrest by genistein in renal cancer. *Carcinogenesis* **2009** 30:662-70.
- 192. Winkler GS:** The mammalian anti-proliferative BTG/Tob protein family. *J Cell Physiol* **2010** 222:66-72.
- 193. Munro SK, Farquhar CM, Mitchell MD, Ponnampalam AP:** Epigenetic regulation of endometrium during the menstrual cycle. *Mol Hum Reprod* **2010** 16:297-310.
- 194. Ito K, Utsunomiya H, Yaegashi N, Sasano H:** Biological roles of estrogen and progesterone in human endometrial carcinoma--new developments in potential endocrine therapy for endometrial cancer. *Endocr J* **2007** 54:667-79.
- 195. Doisneau-Sixou SF, Sergio CM, Carroll JS, Hui R, Musgrove EA, et al.:** Estrogen and antiestrogen regulation of cell cycle progression in breast cancer cells. *Endocr Relat Cancer* **2003** 10:179-86.
- 196. Girault I, Tozlu S, Lidereau R, Bieche I:** Expression analysis of DNA methyltransferases 1, 3A, and 3B in sporadic breast carcinomas. *Clin Cancer Res* **2003** 9:4415-22.
- 197. Roll JD, Rivenbark AG, Jones WD, Coleman WB:** DNMT3b overexpression contributes to a hypermethylator phenotype in human breast cancer cell lines. *Mol Cancer* **2008** 7:15.
- 198. Sandhu R, Rivenbark AG, Coleman WB:** Enhancement of chemotherapeutic efficacy in hypermethylator breast cancer cells through targeted and pharmacologic inhibition of DNMT3b. *Breast Cancer Res Treat* **2012** 131:385-99.
- 199. Chik F, Szyf M:** Effects of specific DNMT gene depletion on cancer cell transformation and breast cancer cell invasion; toward selective DNMT inhibitors. *Carcinogenesis* **2011** 32:224-32.

- 200. Jiemjit A, Fandy TE, Carraway H, Bailey KA, Baylin S, et al.**: p21(WAF1/CIP1) induction by 5-azacytosine nucleosides requires DNA damage. *Oncogene* **2008** 27:3615-23.
- 201. Sandoval J, Heyn H, Moran S, Serra-Musach J, Pujana MA, et al.**: Validation of a DNA methylation microarray for 450,000 CpG sites in the human genome. *Epigenetics* **2011** 6:692-702.
- 202. Irizarry RA, Ladd-Acosta C, Wen B, Wu Z, Montano C, et al.**: The human colon cancer methylome shows similar hypo- and hypermethylation at conserved tissue-specific CpG island shores. *Nat Genet* **2009** 41:178-86.
- 203. Ehrlich M, Buchanan KL, Tsien F, Jiang G, Sun B, et al.**: DNA methyltransferase 3B mutations linked to the ICF syndrome cause dysregulation of lymphogenesis genes. *Hum Mol Genet* **2001** 10:2917-31.
- 204. Kobayashi S, Shibata H, Yokota K, Suda N, Murai A, et al.**: FHL2, UBC9, and PIAS1 are novel estrogen receptor alpha-interacting proteins. *Endocr Res* **2004** 30:617-21.
- 205. Xiong Z, Ding L, Sun J, Cao J, Lin J, et al.**: Synergistic repression of estrogen receptor transcriptional activity by FHL2 and Smad4 in breast cancer cells. *IUBMB Life* **2010** 62:669-76.