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Exploring the Genome-Wide Impact of Estrogen Receptor Alpha and Estrogen Receptor Beta in Breast and Colon Cancer Cells

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In memoriam,
Mormor Ruth

ABSTRACT

Estrogen signaling is involved in the development and progression of breast cancer and is implicated to be protective in colon cancer. Estrogenic actions are conveyed through transcriptional regulation by ligand stimulated estrogen receptors (ERα and ERβ). ERα is upregulated in most breast cancers and is responsible for the proliferative effect of estrogen. ERβ on the other hand is usually downregulated, and studies indicate an antiproliferative function. Therapies targeting ERα are available and commonly used in the treatment of breast cancer. In the normal colonic epithelia, however, ERβ is the most abundant estrogen receptor and the suggested mediator of the protective effects of estrogen in colon cancer. The role of ERβ in breast cancer and colon cancer is not well understood. Thus, exploring the genome-wide impact and contribution of both receptors in estrogen responsive cancers would substantially help to identify novel therapeutic and preventive strategies for these cancers.

In **Paper 1**, we examined differences in transcriptional regulation between ERα and ERβ in the breast cancer cell line T47D. We could show that ERβ often exhibited an opposing effect on ERα-regulated genes within proliferation and regulation of cell cycle. We also demonstrated a set of genes only regulated by ERβ, indicating that, despite the high homology between the two receptors, there are differences in their transcriptional targets. The fact that ERβ opposed ERα indicates that ERβ activation may be of value in the treatment of breast cancer. To further explore the transcriptional role of ERα in breast cancer, we performed large-scale analyses of microRNA in 24 hours estrogen treated ERα-expressing T47D cells, **Paper II**. However, we found no evidence of direct and rapid regulation of mature miRNAs by ERα.

In **Paper III**, we studied ERβ gene regulation in colon cancer cells. We could show that ERβ-expressing xenografts grew significantly slower than those lacking ERβ. Further we demonstrated that ERβ induced a transcriptional response independently of ERα and induced inhibition of the proto-oncogene MYC and other G_1-phase cell cycle genes. In **Paper IV**, we dissected the regulatory networks of ERβ-induced transcriptional changes in human colon cancer cells. The set of genes changed by ERβ varied in different colon cancer cell lines, however, corresponded to the same biological processes such as cell cycle regulation and kinase activity. In addition, we identified the ERβ-driven downregulation of the transcription factor PROX1 as a key mechanism behind a large proportion of the transcriptional changes. In **Paper V**, we studied the effect of long term expression of ERβ on the miRNA pool in SW480 colon cancer cells. While we could not show a direct and rapid effect of ERα on the miRNome, we showed that long term expression of ERβ did induce large changes in the miRNA pool in colon cancer cells. In particular, we found the oncogenic miR-17-92 cluster to be downregulated and proposed this to be a consequence of the ERβ-induced downregulation of MYC.

In conclusion, we have shown that ERβ is antiproliferative in breast and colon cancer cells, both when co-expressed with ERα and alone, as well as identified key signaling pathways. We suggest that activation of ERβ will have a beneficial effect for treatment or prevention of estrogen dependent cancers.

LIST OF PUBLICATION

I. Williams C, **Edvardsson K**, Lewandowski S, Ström A, Gustafsson JÅ. *A genome-wide study of the repressive effects of estrogen receptor beta on estrogen receptor alpha signaling in breast cancer cells.* Oncogene (2008), 27, 1019-1032.

II. Katchy A, **Edvardsson K**, Aydogdu E, Williams C. *Estradiol-activated estrogen receptor α does not regulate mature microRNAs in T47D breast cancer cells.* The Journal of Steroid Biochemistry and Molecular Biology (2011), doi:10.1016/j.jsbmb.2011.10.008.

III. Hartman J, **Edvardsson K**, Lindberg K, Zhao C, Williams C, Ström A, Gustafsson JÅ. *Tumor Repressive Functions of Estrogen Receptor β in SW480 Colon Cancer Cells.* Cancer research (2009), 69, 6100-5106.

IV. **Edvardsson K**, Ström A, Jonsson P, Gustafsson JÅ, Williams C. *Estrogen Receptor β Induces Antiinflammatory and Antitumorigenic Networks in Colon Cancer Cells.* Molecular endocrinology (2011), 25, 969-979.

V. **Edvardsson K**, Vu HT, Kalasekar SM, Ponten F, Gustafsson JÅ, Williams C. *Estrogen receptor beta expression induces changes in the microRNA pool in human colon cancer cells.* Manuscript.

Related publications not included in thesis:

Williams C, Helguero L, **Edvardsson K**, Haldosén LA, Gustafsson JA. *Gene expression in murine mammary epithelial stem cell-like cells shows similarities to human breast cancer gene expression.* Breast Cancer Res. 2009;11(3):R26.

Ström S, **Edvardsson K**, Inzunza J and Williams C. *Estrogen receptor alpha and beta expressed but transcriptional inactive in human pluripotent embryonic stem cells.* Manuscript.

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LIST OF ABBREVIATIONS

AF-1/2	Activation function-1/2
AI	Aromatase inhibitor
AP1	Activator protein 1/ JUN
APC	Adenomatous polyposis coli
BRAF	v-raf murine sarcoma viral oncogene homolog B1
CD	Crohn's disease
CDH1	E-cadherin
CDK	Cyclin-dependent kinase
ChIP	Chromatin immunoprecipitation
CLU	Clusterin
CRC	Colorectal cancer
DBD	DNA-binding domain
E2	Estradiol
ERE	Estrogen response element
ERK1/2	Extracellular signal-regulated kinase
ERα	Estrogen receptor alpha, NR3A1
ERβ	Estrogen receptor beta, NR3A2
FACS	Fluorescence activated cell sorter
FAP	Familial adenomatous polyposis
HAT	Histone acetyltransferases
HDAC	Histone deacetyltransferases
HER2	ERBB2, v-erb-b2 erythroblastic leukemia viral oncogene homolog 2
HRT	Hormone replacement therapy
IBD	Inflammatory bowel disease
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog
LBD	Ligand-binding domain
MAPK	Mitogen activated protein kinase
MCRC	Metastatic colorectal cancer
miRNA	microRNA
mRISC	miRNA-induced silencing complex
MYC	v-myc myelocytomatosis viral oncogene homolog
NCOA3	Nuclear receptor coactivator 3, SRC-3
NCoR	Nuclear receptor corepressor
NR	Nuclear receptor
PR	Progesterone receptor
pre-miRNA	Precursor microRNA
pri-miRNA	Primary microRNA
PROX1	Prospero homeobox 1
RE	Response element
SERM	Selective estrogen receptor modulator
SP1	Specificity protein 1
SRC	Steroid receptor coactivator
TSS	Transcription start site
UC	Ulcerative colitis
ZEB1	Zinc finger E-box binding homeobox 1

1 INTRODUCTION

1.1 NUCLEAR RECEPTORS

Nuclear receptors (NR) belong to a class of evolutionary conserved transcription factors. Generally, regulation of gene transcription occurs when a ligand, such as thyroid and steroid hormones or free fatty acids, binds to the receptor thus driving it to undergo conformational change leading to its activation. NRs regulate gene transcription involved in a wide array of biological processes, such as metabolism, development and reproduction. Therefore, deregulation in NR signaling has immense consequences in diseases such as diabetes, obesity, inflammation and cancer.

1.1.1 Structural and functional organization

The NRs share a similar structure containing a N-terminal region, a DNA-binding domain (DBD), a hinge region, a ligand-binding domain (LBD) and a short C-terminal domain (Figure 1) (126).

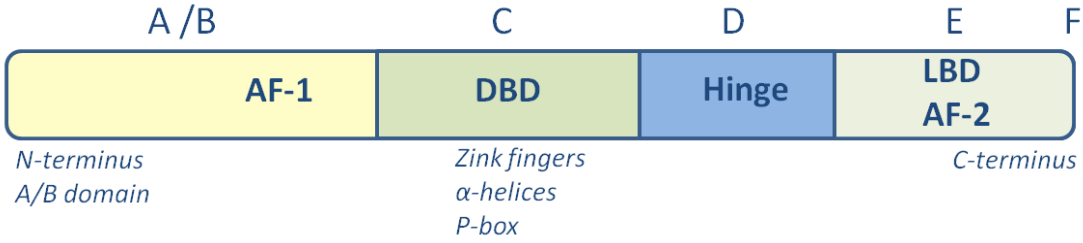

Fig 1. General structural organization of NR consists of five domains named A-F. The A/B domain contains a section important for cofactor interaction, the C domain contains the DNA-binding domain, the D domain contains the hinge connecting DBD with LBD, and is also the target for different post-translational modifications and the E domain contains the ligand binding domain and is also important for cofactor interactions. The function of the F domain is not fully understood.

The N-terminal A/B region varies both in length and sequence between different NRs and contains, for the majority of NRs, a transcriptional activation domain known as AF-1. AF-1 is important for interaction with coregulatory proteins as well as for ligand-independent transcriptional activity. The DBD is responsible for the binding to specific DNA sequences. Several residues, amongst others two zink-finger domains, build up the core of the DBD; two α-helices. The first helix contains the P-box, the residues critical for interaction with the two DNA half sites, while the second α-helix stabilizes the DBD structure (D-box) (4).

Initially, it was believed that the primary purpose of the hinge was to serve as a linker between the DBD and the LBD. However, several studies have now shown that the hinge may change the receptor function through providing interaction surfaces for cofactors and/or post-translational modifications such as SUMOylation (205). The ligand-binding domain contains the ligand-binding pocket and a ligand-dependent

activation function domain (AF-2). AF-2 is necessary for recruitment of a variety of coactivating proteins.

Both the size and shape of the binding-pocket as well as the hydrophobic/hydrophilic nature of the pocket surface correlates with ligand specificity of the receptor (33, 240, 260). The steroid receptors GR, AR, PR, and ER have smaller volumes within their binding pockets compared to other NRs, as well as specific polar side chains of the pocket surface, which provides them with a high affinity towards a small number of ligands. Some of the adopted orphan receptors exhibit a larger volume binding-pocket compared to the steroid receptors, giving them the possibility to interact with a large number of ligands with different structures and of different sizes (159).

1.1.2 A superfamily subdivided into three classes

The NRs can be classified in different ways. Sequence alignment and phylogenetic tree construction reveals six evolutionary groups based solely on sequence homology (92). The NRs can also be divided into three classes based on the type of ligand they bind and/or where they are found in the unliganded state (20). Class I is known as the steroid receptor family, binds to steroids (estrogens, glucocorticoids, progestins, androgens, mineralocorticoids) and includes the estrogen receptor (ER), glucocorticoid receptor (GR), progesterone receptor (PR), androgen receptor (AR) and mineralocorticoid receptor (MR) (169). Class II is known as the thyroid/retinoid family, which binds to non-steroids (thyroid hormone, retinoids, prostaglandines) (20) and includes the thyroid receptor (TR), retinoic acid receptor (RAR), vitamin D receptor (VDR) and peroxisome proliferation-activated receptor (PPAR). The third class is a group of receptors to which no known ligands have been found (true orphans) or just recently been found (adopted orphans).

Generally, unliganded class I receptors can be found in complex with heat shock proteins in the cytoplasm. Upon ligand binding, the receptor undergoes a conformational change leading to dissociation from the heat shock proteins and translocation to the nucleus where the activated receptor can bind to its response element (RE) and activate transcription of a gene. Unliganded receptors from group II are most commonly found associated with corepressors at their RE (in the nucleus) consequently leading to a repression of activation. Ligand binding results in conformational changes leading to dissociation of corepressors, recruitment of coactivators and thereby transcription of a gene (4). Recent studies have shown that unliganded class I receptors shuttles between the cytoplasm and the nucleus (173), however it has not been fully established if they have the same ability as class II receptors by binding to RE in the unliganded form.

Class I receptors bind as homodimers to two hexanucleotide half sites, organized as inverted repeats often upstream of the promoter site, separated by a 3bp spacer. Class II receptors and some of the orphan receptors form heterodimers with the retinoid X receptor (RXR) and bind to two direct repeats of the consensus half site sequence separated by 1-5 bp spacer (169).

1.2 ESTROGEN RECEPTORS

Almost 50 years ago it was discovered that estrogen signaling was mediated through a specific high-affinity receptor (132). This estrogen receptor (ER) was one of the first NRs to be cloned in 1986 (97, 98). A second estrogen receptor was identified ten years later, in 1996, (151) and the two receptors received their names estrogen receptor alpha (ERα; NR3A1; ESR1) and estrogen receptor beta (ERβ; NR3A2; ESR2), respectively. The two ERs are highly homologous in their DNA binding domain, with approximately 97% identical amino acid sequence, and in their ligand-binding domain where they have 56% similarity. However, they only have 24% identity in the N-terminus where the AF-1 domain is found (61).

The two ERs are found expressed in various tissues and cells throughout the body. However, ERα and ERβ exhibit different tissue- and cell-type specific expression, sometimes both may be present in the same tissue but in different cell types. ERα can, for instance, be found in the uterus, kidney, prostate, testes, bone, mammary gland, placenta, ovary, liver, certain regions of the brain and white adipose tissue (53, 61, 245). ERβ can be found in the ovary (granulosa cells), mammary gland, testis, colon, prostate, lung, bladder, bone marrow and certain regions of the brain (53, 61, 75, 245).

The ERs are functionally unique amongst class I receptors since they can function both as homodimers as well as heterodimers (200). Both receptors can bind to the DNA through the classical estrogen response element (ERE) GGTCAnnnTGACC, and they are both activated by the ligand 17β-estradiol. Still, activation of the two receptors triggers different responses. When they are co-expressed, ERβ often appears to exhibit an inhibitory action and oppose the effect of ERα (175, 193). In addition, ER homodimers and ER heterodimers give rise to different sets of regulated genes (196, 252). Differences in the LBD and AF-1domain, and thereby differences in cofactor interactions, can be the reasons to the various responses from the receptors.

1.2.1 Ligands

Estrogen is a steroid hormone and is, as all steroid hormones, derived from cholesterol. Figure 2 illustrates the biosynthesis of estrogens (148, 188). Even though estrogen is considered to be a female sex hormone, it also plays an important role in development of male sex characteristics. Both male ER knock-out mice, and mice lacking the enzyme used in estrogen production have impaired fertility, indicating a role for estrogen in sperm maturation (69, 213).

17β-estradiol (E2) is a natural non-selective ligand for ERα and ERβ and is the most potent ER ligand, followed by the two metabolites estrone (E1) and estriol (E3). The last step in the steroid biosynthesis of estrogen involves aromatization of androgen and testosterone to estrone and estradiol with the enzyme aromatase cytochrome P450 (222). The main source of production of estrogens in the premenopausal woman is the granula cells in the ovaries. In men and in postmenopausal women, estrogen is also produced at extragonadal sites in mesenchymal cells, in adipose tissue, osteoblasts and in the brain. In men, estrogens are also produced in the testis (221, 222).

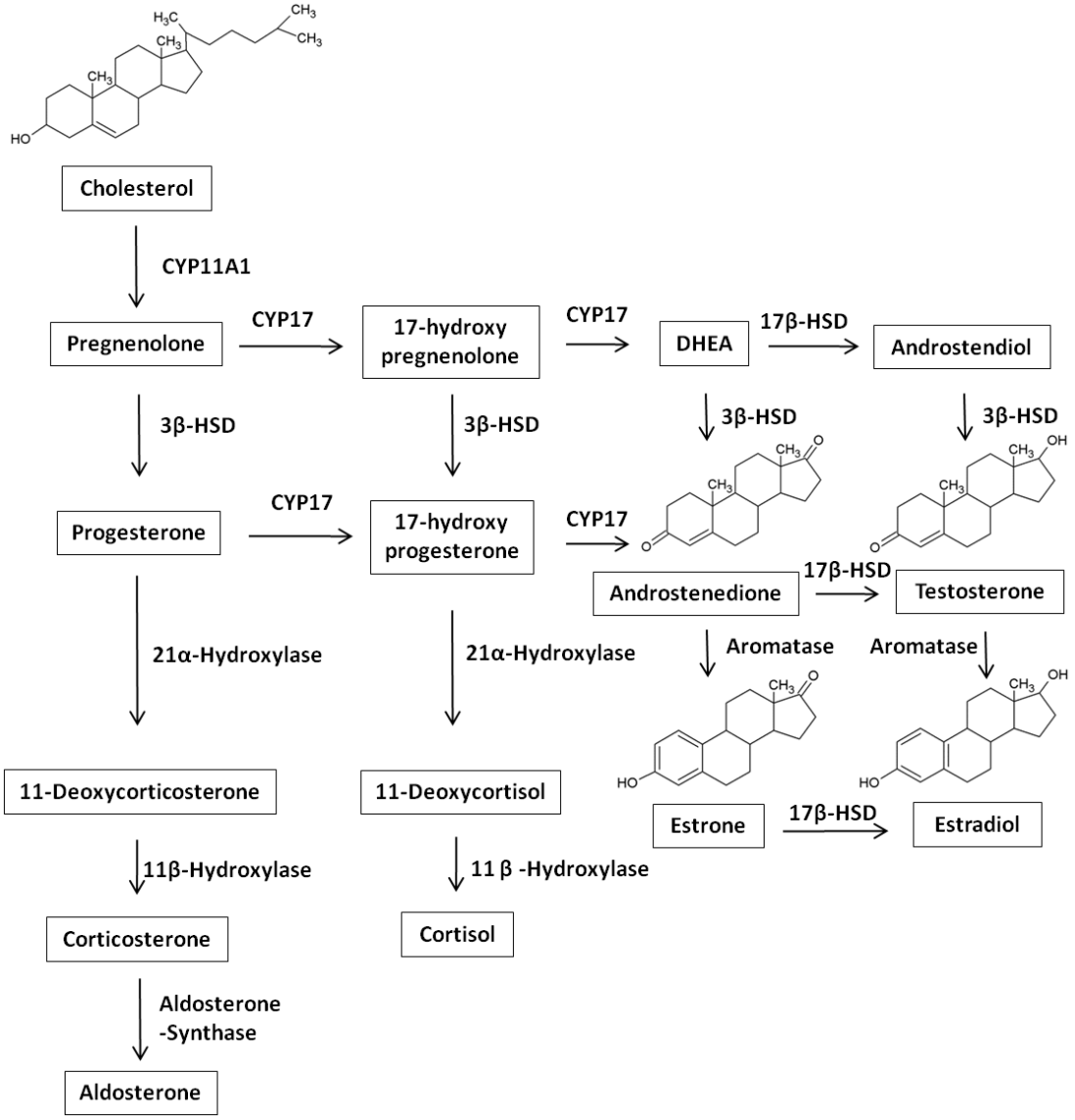

Fig 2. Cholesterol is the first step in steroid biosynthesis. Several enzymes catalyze the different products into different steroids such as glucocorticoids, mineralocorticoids, androgens and estrogens.

In addition to the natural ligands, there are several synthetic compounds able to function as agonist and/or antagonist on the two ERs. For instance, the synthetic compound ICI 182 780 (fulvestrant) is a pure antiestrogen for ERα in the sense that it reduces ERα protein levels in a dose dependent manner (62). Tamoxifen is a selective estrogen receptor modulator (SERM) that acts on both of the ERs but gives different response in various tissues. Its active metabolite, 4-hydroxytamoxifen, is a pure antagonist for ERβ and for ERα in breast tissue but an agonist for ERα in bone and uterus (167, 201).

After ligand binding, an important α-helix in the C-terminus, α-helix 12, undergoes a reposition, thereby affecting the ability of AF-2 to bind to coregulators. For example, antagonist bound to ERα will reposition helix 12 so that it overlaps with the coactivator binding sites thus preventing binding of coactivators and consequently prevents transcriptional activity (218). A ligand (agonist or antagonist) may inhibit one or both AF domains. Since the N-terminus AF-1 differs between ERα and ERβ, an antagonist-

driven reposition of helix 12 may reduce transcriptional activity differently in the two ERs depending on if helix 12 occludes the coactivator recognition groove or not.

1.2.2 Transcriptional regulation by estrogen receptors

Transcriptional regulation by the two estrogen receptors may occur through several different pathways, see Figure 3 (32, 118). The classical estrogen signaling pathway occurs when liganded ER dimerizes and either binds directly to an ERE or to other response elements through tethering to other transcription factors such as AP-1 or SP1. In addition to the genomic regulation, there is also a rapid non-genomic regulation where a signal cascade is activated, following activation of secondary messengers (183). The non-genomic ER activation is too rapid to involve activation by mRNA or synthesis of protein. Instead, this often involves activation of various protein-kinase cascades. An example is ER activation of endothelial nitric oxide synthase through protein kinase-mediated phosphorylation (44). The fourth way of estrogen receptor activation includes ligand-independent posttranslational modifications such as phosphorylation, SUMOylation, methylation and acetylation of the estrogen receptor (19, 92, 138, 233). The different ways of transcriptional regulation by the ERs result in varying outcomes; e.g. different sets of genes can be regulated by liganded ERβ and unliganded ERβ (252).

The ERE is a *cis*-regulatory element. Whilst it can be found less than 10 kb upstream of estrogen regulated genes, it is estimated that half of all conserved non-coding elements in vertebrates are >250 kb away from their associated genes (242). Theories on how distal binding sites physically can participate in transcriptional regulation suggest that it may be through coregulator recruitment to the target promoters via DNA looping (39, 42). Many estrogen responsive genes have both proximal and distal binding sites (43, 124, 160). Actually, only a small faction (5%) of ERα binding sites are located < 5 kb upstream of transcription start site (TSS) of the closest gene while 38% maps to intronic regions and 23% are within 100 kb from 5' start site and 19% are within 100 kb of the 3' polyadenylation site (160). This indicates that ERα can regulate transcription through DNA interactions both proximal and distal of the TSS, not limited to orientation (5' or 3'). Most ChIP studies have been performed in ERα-expressing MCF7 cells and since the ERs have high homology in their DNA binding domain it has been assumed that these binding sites also are true for ERβ. There are no known ERβ-expressing breast cancer cell lines, therefore ERβ binding studies have been performed by overexpression of ERβ in MCF7 cells. These studies have confirmed that the ERα pattern with distal and proximal binding sites also is true for ERβ binding sites, in which only 3% of all ERβ binding sites are within 1kb from either end of a gene (259). Further, a substantial overlap of DNA binding sites between the two liganded receptors has been identified (47, 100).

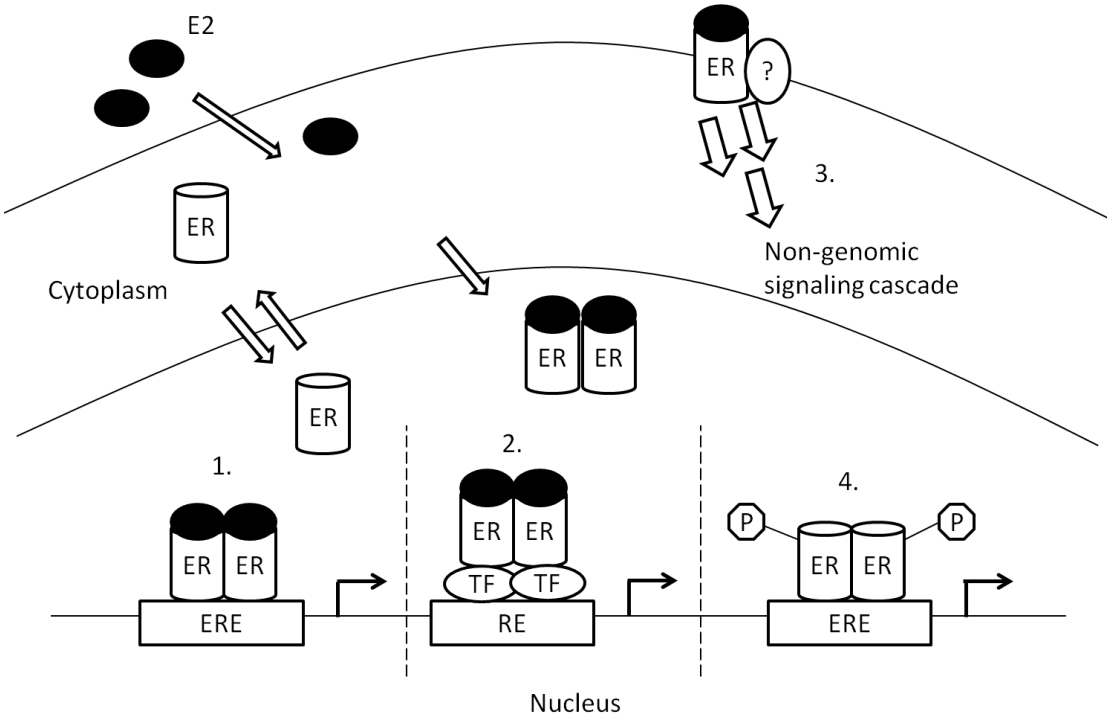

Fig 3. Different mechanisms of ER action. 1, 2 and 4 illustrate the genomic action while 3 illustrates the non-genomic action. 1) Estrogen binds to ER. The receptor dimerizes and binds to estrogen response elements (ERE). 2) Ligand/ER tethers to other transcription factors such as c-Fos/c-Jun which in turn binds to respective response element (AP-1/SP1). 3) Ligand activated membrane bound ER in complex with other factors activates non-genomic signaling cascades. 4) Several different activated cascades lead to phosphorylation of ER which binds to ERE and results in ligand independent transcription.

Transcriptional regulation by the ERs also involves recruitment of coregulators, histone deacetyltransferases (HDACs) and histone acetyltransferases (HATs) (108). Coregulators function as either coactivators or corepressors. Coactivators (e.g. SRC-1, SRC-2, SRC-3) activate transcriptional regulation (141) and the first ones believed to be recruited to activated ERs are the p160 family of proteins and p300 (92). Corepressors (e.g. NCoR, SMRT) on the other hand decrease transcription (153). The coactivators have HAT activity while corepressors mediate HDAC activity (104, 246, 253, 261). NCoR and SMRT does not themselves have enzymatic activities but rather resides in/or recruits transcriptional complexes that contain specific HDACs (92, 111). In addition, the retinoic acid receptor α (RARα) can associate with the ERs to maintain ER cofactor interactions, although it is not needed for ER recruitment to DNA (214).

ERα transcriptional profiles in breast cancer cells, predominantly MCF7 cells, have been established, with several accepted estrogen stimulated ERα regulated genes such as pS2, SIAH2, GREB1, BCL2 and MYC (45, 89). Even though the ERs have high homology in the DNA binding domain, they still give rise to different transcriptional profiles. This has been illustrated in transcriptional studies in MCF7 cells and U2OS osteosarcoma cells with ERα and ERβ expression in which only 25% and 23% of all ERβ regulated genes were shared with ERα, respectively (100, 232). One problem when studying ERβ transcriptional regulations in cancer cells is that there are no cancer cell lines endogenously expressing significant levels of ERβ. The few studies published

on ERβ transcriptional regulation have poor correlation, possibly caused by different techniques for introduction of ERβ as well as varying expression levels of the protein. Therefore, there is no general accepted transcriptional profile for ERβ in neither breast nor colon cancer cells. One study with ERβ overexpression in U2OS osteosarcoma cells have suggested that ERβ gene regulation can be divided into three classes: class I which is primarily regulated by unliganded ERβ, class II which is regulated only with E2 and class III which is regulated both by unliganded and liganded ERβ. Interestingly, AP-1 binding sites are more enriches in class I genes whereas ERE, SP1 and NFκB1 are enriched in class II genes, suggesting that ERβ regulates different sets of genes through interaction with different transcription factors and coregulators (252). However, this has not been confirmed in other cell lines and further studies are needed to define and confirm ERβ's transcriptional profile in different cell types.

1.3 microRNA

1.3.1 miRNAs regulate gene transcription

MicroRNAs, miRNAs, were discovered about 20 years ago (155, 249), but their gene regulatory properties were not understood until about seven years ago (208). miRNAs are short (19-25 nt) single stranded non-coding RNA molecules. They suppress gene regulation through complementary binding between the miRNA 5' sequence and the 3' UTR of the target mRNA thereby mediating mRNA degradation and/or gene translation suppression. The target section of the mRNA can occasionally be located in the coding region. The miRNA 5' sequence is known as the 'seed' sequence. The seed consists of nucleotides 2-7 and is the most important part needed in miRNA target recognition (26).

The miRNAs are highly conserved amongst species, e.g. the miRNA Let-7 is identical between human, fly and *C. elegans* (198). Some miRNAs, however, are specific to primates only. Many of the primate specific miRNA are expressed in placenta, brain, testis and epididymis, and some are enriched in human embryonic cells. However, most of the primate specific miRNAs are expressed at low levels in adult tissues compared to embryonic cells, indicating a specific role in reproduction and embryonic development of primates (162). Human, mouse, zebrafish and fruit fly all have approximately the same sized genome. This tells us that other factors are important when it comes to the complexity of the animal. Animals with a more simple body plan tend to have a smaller miRNome., e.g. the human genome has 1424 miRNA genes, mouse 720, zebrafish 358 and fruit fly 238 (mirbase.org, October 2011). Taken together, this point towards the fact that miRNAs may work as "fine-tuners" in regulation of gene expression.

There are 1424 miRNA genes in the human genome (mirbase.org), and together they may target up to 60% of all protein-coding genes (91). A miRNA usually has a relatively mild effect on expression of the target gene. However, each miRNA can target several different genes and many of these targets are often in the same pathway (133), thus the total impact of miRNAs on the transcriptome and proteome is significant.

miRNAs can, through their regulation of target mRNAs, be involved in the regulation of many biological processes, e.g. cell differentiation, fat metabolism, apoptosis, stress and cell proliferation (10, 25, 36). Mice with a non-functional miRNA machinery die early with severe developmental defects (30), while knock-out of a specific miRNA only has mild effects. Thus, a functional miRNA machinery is critical in normal biological processes. Consequently, deregulated miRNA expression may lead to several diseases. Altered miRNA levels have been detected in several different cancers, infection, cardiovascular disorders, diabetes, inflammation and autoimmunity (58, 67, 103, 107, 250).

1.3.2 miRNA biogenesis

miRNA genes are transcribed from the DNA by RNA polymerase II into primary miRNAs (pri-miRNAs). A pri-miRNA can be several hundreds of nucleotides in length and may contain several imperfect hairpin loops corresponding to several precursor miRNAs (pre-miRNA). The nuclear protein DGCR8 and the enzyme Drosha introduce a cleavage to liberate the hairpin loop from the pri-miRNA. The resulting hairpin, pre-miRNA, is about 70 nucleotides in length and is exported from the nucleus to the cytoplasm. The protein Dicer then cleaves the loop, leaving a double stranded miRNA/miRNA* duplex. One strand of the duplex (passenger or miRNA*) is usually released and degraded while the other strand, the mature miRNA, is incorporated into the Argonaute containing miRNA-induced silencing complex (miRISC), which facilitates the interaction between the mature miRNA and its mRNA target (115). The biogenesis of miRNAs is illustrated in Figure 4.

1.3.3 The role of ER in miRNA regulation

ER-induced gene regulation involves transcription by RNA polymerase II. Breast cancer patients expressing ERα have been reported to express a distinct miRNA pattern compared to ERα negative patients (129, 154, 176, 263). Deregulated miRNA has also been found in colon cancer (178, 184, 257). Both breast and colon cancers have been associated with deregulation of ERα/ERβ. Taken together, this suggests that the ERs may exert some of their transcriptional effects via regulation of miRNAs.

Most studies have focused on the effect of miRNAs on ERα protein or mRNA levels, such as miR-221/222, miR-206, miR-27a, miR-22 and miR145 (5, 158, 195, 228, 263) and the repressive effect of miR-92 on ERβ (7). However, the regulatory effect of ERα and ERβ on miRNAs has not thoroughly been examined. So far, there are no studies looking into ERβ-driven regulation of miRNAs, and the published studies on ERα regulated miRNAs are somewhat contradictory (31, 248). miRNA-induced downregulation of ERα makes MCF-7 and T47D breast cancer cells more resistant to tamoxifen induced apoptosis (263). This suggests that these miRNAs might be potential targets for improved antiestrogen therapy in breast cancer. It is essential to further explore ER regulation of miRNAs since identification of ER regulated miRNAs might reveal new biomarkers for diagnosis, success of treatment as well as potential targets for novel therapeutics in several different estrogen responsive cancers.

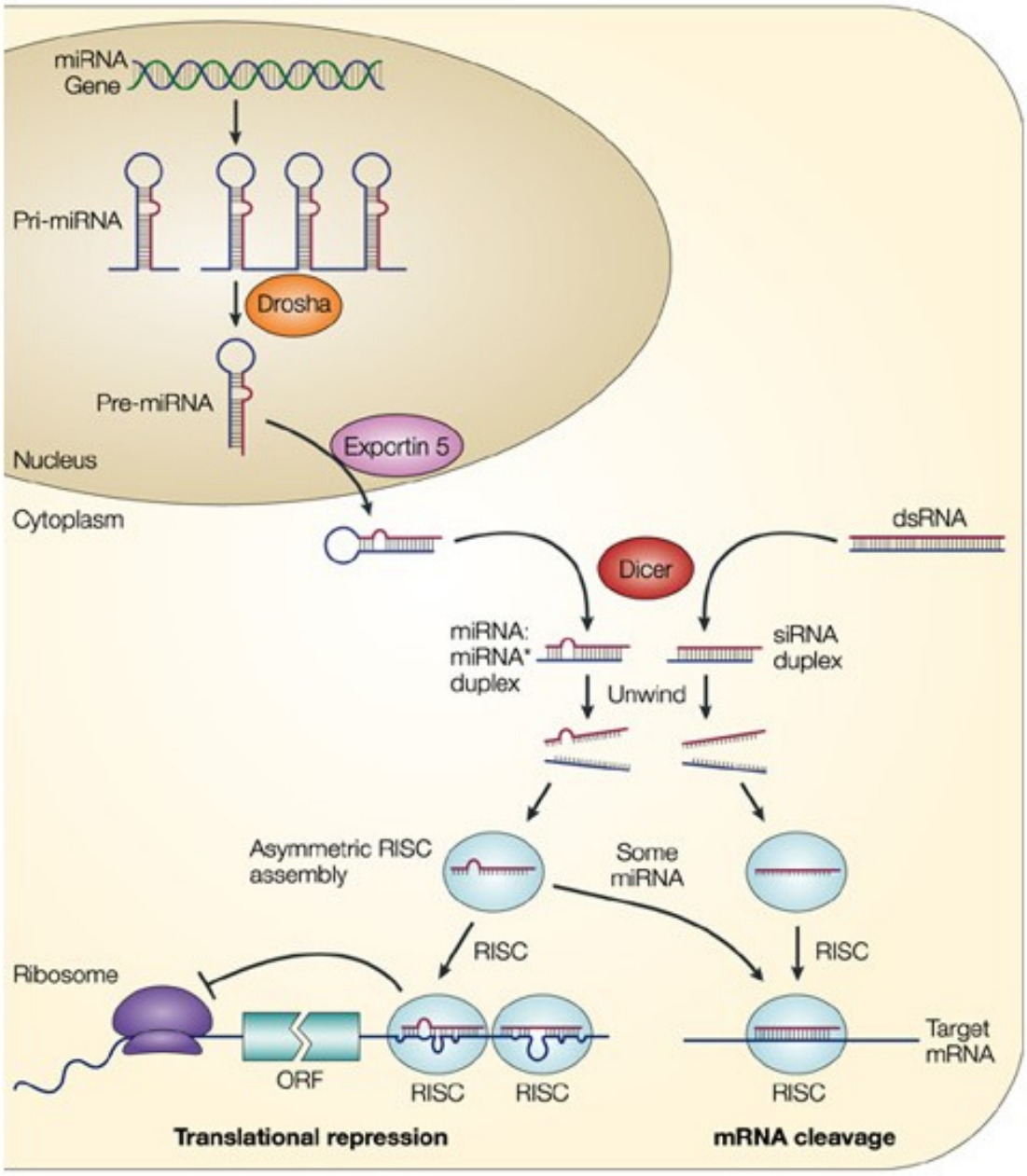

Figure 4. The biogenesis of miRNAs. miRNAs are first transcribed into pri-miRNAs in the nucleus by RNA polymerase II. Drosha cleaves off an imperfect hairpin loop, the pre-miRNA, which then is exported to the cytoplasm where Dicer cleaves off the loop leaving a miRNA/miRNA* duplex. The duplex is separated into single strands and the miRNA associates with the RISC complex and is transported to its target gene resulting in translational repression or degradation. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Genetics (He L, Hannon GJ. MicroRNAs: small RNAs with a big role in gene regulation), copyright (2004) (115).

1.4 BREAST CANCER

Cancer is a major health problem in the western world, and trends show that the rest of the world is following in this direction. Breast cancer is the most commonly diagnosed form of cancer in women, and is accountable for 29% of all diagnosed cancer cases (1, 220). Between 15 and 20 women in Sweden are diagnosed with breast cancer every day. This form of cancer is much less common amongst men, and approximately 40 men are diagnosed yearly in Sweden (3).

Most forms of breast cancer are believed to emerge from mutations accumulating during the lifetime. The female sex hormones are involved; use of contraceptives increases the risk for development of breast cancer while pregnancy at young age decreases the risk. 5-10% of all breast cancers are hereditary. Genes that most often are mutated are BRCA1 and BRCA2. Women with one or both of these mutations not only have higher risk of developing both breast and ovarian cancer, but also tend to be affected at an earlier age (13, 177).

In Sweden the incidence of breast cancer has increased with a yearly average of 1.2% during the last two decades (1). The reasons behind this are unclear, but two major contributing factors are the aging population and introduction of mammography screening programs. Screening programs lead to an earlier detection of cancer, and might detect tumors in elderly women that previously would die of other causes before the cancer was progressed enough to be detected. In addition, a change of dietary intake, environmental factors, delay of child birth and the use of hormone replacement therapy (HRT) may also contribute to this increase.

The incidence of breast cancer varies widely between more developed regions such as western/northern/southern Europe, Australia and northern America and less developed regions such as central America, middle/eastern Africa and Asia (81). The incidence per 100,000 women is 19.3 in Eastern Africa and 89.7 in Western Europe. Even though incidence of breast cancer among Japanese women has increased over the past decades, Japan is still considered to be a low-risk country. It has been suggested that the high dietary intake of soy foods significantly has contributed to the low incidence of breast cancer in Asian countries. Soy foods are rich in isoflavones; plant-derived non-steroidal compounds (phytoestrogens) that have estrogen-like properties (6).

1.4.1 Breast cancer and estrogen

There are two major epithelial cell lineages in the mammary gland; luminal and myoepithelial. The latter is adjacent to the basal membrane. The major parts of mammary gland development take place during three distinct phases; puberty, pregnancy and lactation, and is under control of growth factors and the steroid hormones estrogen and progesterone (120, 210). A mammary stem cell can self-renew and differentiate into all cell types constituting the mammary gland. Estrogen and progesterone have been shown to regulate mammary stem cells both in number and in ability to propagate into all cell types constituting the mammary gland, despite the lack of expressed ER and PR (18, 135). This indicates that steroid over-stimulation of mammary stem cells increases the risk for breast cancer development. ERα is critical

for regulation of the breast epithelial cell differentiation and proliferation. Females with aromatase deficiency will not develop breasts at puberty unless given estrogen replacement therapy (134). Studies on ERα knock-out mice have shown that ERα is important for normal mammary gland development and is required for development of a normal ductal structure (34, 53, 80). ERβ knockout mice showed that ERβ is involved in terminal differentiation of the mammary gland but is not involved in ductal growth (86). During menstrual cycle, the epithelial ducts and branches will increase, and during pregnancy and lactation they will differentiate into milk secreting alveoli cells (120, 121). The main ER contributor to the first part is ERα, while ERβ has a prominent role in the second one.

ERβ is the predominant ER in the human breast. ERα is only expressed in 10-20% of normal resting mammary gland cells (202). In addition to differences in protein expression levels, the distribution of the two receptors differs; in rodent, ERβ is found in both epithelial and stromal cells while ERα only is expressed in epithelial cells (48). The ratio between ERα and ERβ is changed in breast cancer, 70% of all new breast cancers express ERα, whereas ERβ is decreased in advanced stages of breast cancer (76, 114, 194, 197).

Since estrogen plays such an important role in the mammary gland development, it is not surprising that estrogen signaling has a prominent role in breast cancer. Upregulation of ERα results in increased proliferation and subsequent progression of breast cancer. Estrogen treatment of ERα positive breast cancer cells stimulates proliferation while ERβ has been shown to suppress ERα transcriptional regulation (105, 113, 183, 230). ERβ levels are decreased or lost in breast cancer progression, which may result in a limited inhibitory effect of ERβ on ERα-driven proliferation, thus contributing to the pathogenesis of breast cancer.

Most breast cancers, especially in an early stage, are estrogen dependent. This has lead to the development of many new therapies targeting the estrogen signaling pathway. It has been stated that breast cancer tumors expressing both ERα and the estrogen regulated progesterone receptor (PR) will benefit the most from endocrine therapy (16, 22, 54). In general, tumors expressing ERα have a better prognosis than ERα negative tumors and are associated with lower-grade tumors, longer disease-free survival and a better overall survival. ERα negative tumors on the other hand have a higher risk for metastases and recurrence (22, 51, 143, 202, 247). It is not entirely clear what role ERβ plays in breast cancer. However, ERβ KO mice and *in vitro* studies show a connection between decreased or lost levels of ERβ and a more invasive phenotype, tamoxifen resistance and overall poor survival (87, 146). However, ERβ expression seems to have multiple effects depending on ERα status and invasiveness of the tumor. ERβ reduces proliferation in ERα positive cells, yet reintroduction of ERβ in more invasive ERα negative breast cancers has the opposite effect and increases cell proliferation (123, 234).

1.4.2 Treatment of breast cancer

Even though the incidence for breast cancer has increased during the last decades, the chance to be cured from this form of cancer has conversely increased. Detection at an earlier stage is one major contributing factor, and also a better understanding of the molecular mechanisms behind breast cancer has lead to improved treatments. Breast cancer tumors can be divided into different molecular classes depending on the expression status of ERα, PR and HER2. Tumors status is determined by molecular profiling and therapeutic strategy is then determined based on the molecular class of the tumor. The HER2 protein is amplified in 25-30% of all human primary breast cancers (225), and promotes growth and invasion. VEGF is a protein important to tumors by stimulating formation of new blood vessels. New treatments for breast cancer comprises monoclonal antibodies targeting HER2 and VEGF (e.g. trastuzumab and bevacizumab), and can, at least short term, prevent tumor growth and blood supply to the tumor. In addition, since most breast cancers express ERα, therapies targeting estrogen signaling is widely used in the treatment of breast cancers (tamoxifen, raloxifene and aromatase inhibitors) (114). About 15-20% of all breast cancers have a triple negative profile (ERα, PR and HER2 negative) (59) and these patients have poorer survival compared to hormone responsive subtypes.

1.4.3 Endocrine therapies

Almost 70% of all early breast cancer tumors express ERα, and breast cancer therapies targeting this protein have been widely used for decades (202). Early treatment of estrogen responsive breast cancer included surgery, but in early 1970's a new therapeutic approach was started through to the introduction of the SERM tamoxifen (187). Five years of adjuvant tamoxifen treatment significantly reduces mortality and recurrence for the first 15 and 10 years, respectively (63). Even though tamoxifen gives ERα positive breast cancer patients a better overall survival, there still are some drawbacks. Endocrine treatment with tamoxifen has been shown to be associated with an increased risk of endometrial cancer, blood clots and stroke (83, 84).

ICI 182 780 promotes degradation of the ERα protein and is used as an endocrine treatment to ERα positive breast cancer patients who fail to respond to tamoxifen. Aromatase is the enzyme responsible for conversion of androgens to estrogen. Aromatase inhibitors (AI's) such as anastrozole, exemestane and letrozole are widely used therapies against estrogen responsive breast cancers. They reduce the primary source of estrogen in postmenopausal women, thereby limiting estrogen levels in both plasma and tumors. Studies have shown that advanced breast cancer patients favors from AI's compared to tamoxifen as a first line agent when it comes to overall response rate (88, 187, 259). Tamoxifen is today the most widely used initial therapeutic in early breast cancer, but combination with AI's might improve the overall outcome.

1.4.4 Endocrine resistance

Only approximately 50 to 70 % of all patients with ERα-expressing tumors respond well to hormonal therapy (164, 189). The majority of all patients that initially respond

to tamoxifen develop resistance during treatment (acquired resistance) (128, 211). Also, in some tumors tamoxifen might instead stimulate growth of the tumor. ERα-expressing tumors that are not responding to tamoxifen may be caused by lack of and/or modifications in the allele carrying the gene for the enzyme CYP2D6 (239). This enzyme is responsible for conversion of inactive tamoxifen to its active metabolite; consequently lack of this enzyme will result in lower levels of or no active tamoxifen metabolites. Another explanation to endocrine resistance is the presence of a tamoxifen resistant clone that during time will take over the tumor cell population (52).

Loss of ERα expression, or mutations of the ERα protein, is one known factor contributing to endocrine resistance, although this is only a small fraction of all breast cancers (20%) (190). Instead it has been suggested that post-translational modification of ERα is responsible for most of the resistance. Phosphorylation of ERα results in activation of the receptor independently of estrogen or tamoxifen (41, 181, 223, 244). Activation of alternative pathways that can stimulate proliferation and survival are involved in endocrine resistance. Resistance can be achieved through crosstalk and modulation between these pathways, such as growth factors and kinase pathways that phosphorylate ER (17, 174). In addition, these pathways can during endocrine treatment develop into drivers of tumor growth independent of ER. Some pathways involve HER tyrosine kinase receptor family (e.g. HER2 and EGFR), fibroblast growth factor and stress-related kinases (190). HER2 regulates ERα expression and activity which in turn stimulates phosphatidylinositol 3-kinase (PI3-K)/Akt signaling (74, 229). This ligand-independent activation of ERα results in ERα genomic functions regardless of hormone, thus leading to ERα-induced proliferation independent of tamoxifen treatment (15, 50, 219). ER+/HER2+ breast cancer tumors might therefore benefit from a combined treatment of tamoxifen and inhibitor of EGFR, HER2 and VEGFR (74, 77). Further, reduced levels of ERβ might be involved in resistance to tamoxifen treatment (122, 163). However, other studies suggest that tamoxifen has a negative effect in ERβ-expressing breast cancers, by antagonizing ERβ activated growth-inhibitory genes (157). Therefore, the mechanism behind the potential involvement of ERβ in endocrine resistance is not fully understood, and more research is needed.

1.5 COLON CANCER

Colorectal cancer (CRC) is the third most common form of cancer amongst both men and women. Colon cancer corresponds to almost 7% of all Swedish cancer cases reported to the cancer registry in 2009 (1). Equivalent number for men and women in the world is 10% and 9.4%, respectively (81). CRC is a disease more common in developed regions, covering almost 60% of all CRC cases in the world (81). Each year about 608,000 persons die from colon cancer worldwide, accounting for 8% of all cancer deaths. This numbers makes it the fourth most common cause of death from cancer. Just as breast cancer, colon cancer incidences in Sweden have been steadily increasing during the last decade with an average yearly increase of 1.7 % for women and 1.2% for men (1). Each year, about 2,000 men and 2,000 women are diagnosed with colon cancer in Sweden. Also, if rectum and anus cancer were included the number would increase to 3,200 and 2,900, respectively (1). Risk factors

for developing colorectal cancer include smoking, diet, age, alcohol intake, sex, genetic background and intake of hormonal replacement treatment.

1.5.1 Cause of colorectal cancer

Colorectal cancer is caused by uncontrolled growth of the epithelial cells lining the colon. It starts as transformation from normal colonic epithelia into benign adenomatous polyps which may develop into an advanced adenoma and eventually into invasive cancer (170). Development of sporadic CRC is a long multistep process involving several different mutations and requires years before it fully develops into cancer. Mutations include different tumor suppressors and oncogenes, and one of the earliest events in colorectal carcinoma is the inactivating mutation of both alleles of the adenomatous polyposis coli (APC) gene. The APC protein is found mutated in 50% and 80% of all colorectal adenomas and carcinomas (142). This mutation can either be acquired, or inherited. APC was first described 20 years ago through its association with an inherited form of colorectal cancer known as familial adenomatous polyposis (FAP) (35, 101, 136). FAP is an inherited syndrome characterized by an early onset of multiple adenomatous polyps of the colon, and a high risk of developing colorectal carcinoma (96, 136).

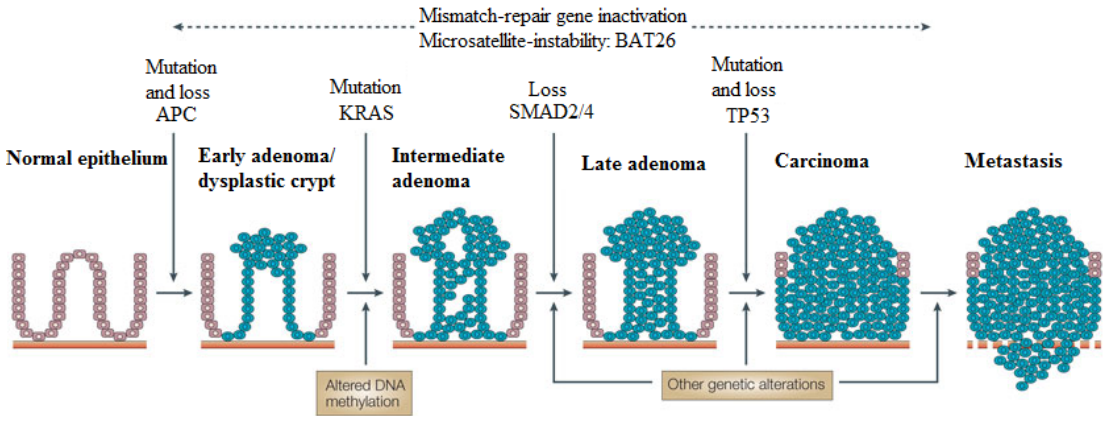

Figure 5. Development of spontaneous CRC from normal epithelium to metastatic carcinoma is a process that takes many years, and requires several steps with gene mutations and/or gene loss. Removal of early polyps may prevent the progression to carcinoma. Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Cancer (Davies RJ, Miller R, Coleman N, Colorectal cancer screening: prospects for molecular stool analysis), copyright (2005) (64).

Most mutations of APC are nonsense mutations resulting in a truncated, non-functional APC protein (101). APC is a major binding partner and regulator of β-catenin in the β-catenin-dependent Wnt signaling pathway (14, 203). Loss of APC will lead to an accumulation of the β-catenin protein which in turn will activate genes important in stem cell renewal and differentiation. When overexpressed, these genes will contribute to cancer development. The impact of accumulating β-catenin, by the non-functional APC gene, was further demonstrated *in vivo* where β-catenin knock-in mice showed formation of multiple polyps morphologically similar to those found in Apc-knock-out mice (109, 191). Kinzler and Vogelstein suggest that APC acts as a "gatekeeper" of colonic epithelial cell proliferation (142). The function of a gatekeeper gene is to keep a constant cell number in renewing cell populations. A mutation in such gene will result

in imbalance between cell division and cell growth. Some colorectal cancers have instead a mutated β-catenin pathway leading to its overexpression due to blocked degradation or mutations in genes responsible for β-catenin regulation such as AXIN1, AXIN2, TCF7L2 or NKD1 (57, 217).

Activating mutations of KRAS and BRAF are other steps involved in transformation and progression of CRC. Both these mutations will lead to constitutive activation of the RAS/MAPK pathway (28). KRAS is a signal-transduction molecule that stimulates cell proliferation, hence an active mutation of this gene will lead to uncontrolled cell proliferation (11). Patients with KRAS mutations are more likely to overexpress p53 and 95% of these adenomas are classified as advanced (71). Mutations of KRAS are not seen in smaller adenomas, and is only seen in 37.7% of colorectal carcinomas and advanced adenomas (11).

In CRCs, inactivation mutations of SMAD2 and SMAD4 are found in 5% and 10-15%, respectively. In addition, it has recently been suggested that inactivation mutations can be found in the SMAD3 gene in 5% of CRCs (78). SMAD2 and SMAD3 are important mediators of the TGFβ signalling pathway and form, after activation, a complex with SMAD4. The complex translocates to the nucleus where it together with coregulators regulates transcription (117).

More mutations besides deregulation of the APC-β-catenin-Wnt signaling pathway, KRAS and SMAD2/4 are needed for the cell to transform from an adenoma into an invasive carcinoma. One late mutation is in the TP53 gene coding for the tumor suppressor p53, which is responsible for monitoring cell division and cell cycle. The mutation of p53 results in loss of the wild-type protein, and gain of a missense protein. The missense p53 has been suggested to contribute to decreased apoptosis, increased tumor angiogenesis and affect tumor cell proliferation (78). p53 mutations are not seen in adenomas, but occur in later stages of colorectal carcinogenesis and can be detected in 50-70% of all CRC (79). Mutations of other parts of the p53 regulatory pathway, such as PUMA, p21 and BAX, may also be involved in the transformation to colorectal carcinoma (256). Figure 5 illustrates the progression of normal colonic epithelium to metastatic colonic cancer, and the mutations involved.

Most CRC are either inherited (10-30%) or sporadic (65-85%), but some are also caused by chronic inflammation (180). Patients with any of the two major forms of inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC), have an increased risk of developing colorectal cancer (72, 152). The mechanism behind this is not fully understood, but release of several cytokines (IL-6, TNF-α) during chronic colitis and low expression of immunosuppressive cytokines (TGF-β and IL-10) have been shown to promote tumor growth (27, 29, 149, 204, 212, 231).

1.5.2 The role of estrogen in colon cancer

Low intake in fruit and vegetables and a high intake in red and processed meat have been associated with elevated risks of developing CRC (55, 241). However, the impact of diet on initiation and development of colon cancer is being debated. In the last few

years several prospective epidemiological studies have demonstrated that diet might not be as important as previously thought (8, 9, 147, 171).

Since diet might not be as strongly associated with CRC, other etiological factors might be more important in the initiation and development of CRC. The gender distribution in CRC is quite even, although slightly more men than women are diagnosed (81). Interestingly, women have a later onset than men; median age for men to develop CRC is 63 while it for women is 73 years (37). After menopause a woman's body produces less of the sex hormones estrogen and progesterone. For most women, this happens between the age of 45 to 50 (95). A screening of 52,882 US patients with metastatic colorectal cancer (MCRC) from the Surveillance, Epidemiology and End Results registry revealed that younger women (18-44 years old) with MCRC had a better overall survival compared to men of the same age. This was however reversed at older age (>55 years old) where females diagnosed with MCRC lived shorter than men of the same age (119). These results supported previously published data from a study with 2,050 CRC patients that demonstrated an opposing effect of gender on the overall survival at either side of the age of 50 years (145).

Several studies have shown an association between postmenopausal hormone replacement therapy (HRT) and a reduced incidence of CRC (102, 130, 165, 182). The Women´s Health Initiative trial was a large randomized trial designed to study the effect of hormone replacement therapy with estrogen and progestin in postmenopausal women (2). Although the trial was terminated due to an increased risk of developing breast cancer for the group receiving the HTR compared to the placebo group, a reduced risk for colon cancer amongst the group receiving the HTR was shown (49). There has also been several reports on an inverse association between oral contraceptives and CRC (161). This indicates that hormonal status seems to play an important role in the development of colorectal cancer. This has been supported in several *in vivo* and *in vitro* studies with estrogen or phytoestrogens: Distribution of estrogen to ovariectomized rats reduced the number of chemically induced tumors in the colon (227). Phytoestrogens are weak ligands to the ER with chemopreventive properties against estrogen related cancers such as breast cancer and colon cancer (150, 179, 235). Treatment with a dietary fiber that by colonic bacterial enzymes is converted to a phytoestrogen, counteracted the intestinal tumorigenesis in ApcMin/+ mice (24) and treatment with phytoestrogen from soy resulted in suppressed colon tumor growth in male rats (207). Further, one small pilot study with five FAP patients receiving a combination of the phytoestrogens curcumin and quercentin showed reduced size and number of adenomas after treatment (56). All this support that estrogen has a protective role in the development of CRC.

In the normal colonic epithelia, ERβ is the most common form of the two estrogen receptors (40, 82, 139, 226, 255). ERβ expression decreases in the progression of normal colonic tissue to colon cancer. This decrease of ERβ expression directly correlates to apoptosis, correlates to the differentiation grade of the tumor and inversely correlates with cell proliferation (23, 85, 131, 144, 185). This suggests that ERβ is the mediator of the protective effect of estrogen in CRC. This has further been supported in *in vitro* and *in vivo* studies: ERβ overexpression in HCT8 colon cancer cells lead to decreased cell proliferation (172). Treatment with an ERβ specific agonist,

diarylpropionitrile (DPN), in mice that spontaneously develop intestinal adenomas (ApcMin/+) resulted in reduced intestinal tumorigenesis (93), and deletion of ERβ in these mice had the opposite effect increasing both number and size of adenomas (94). It has also been demonstrated that estrogen stimulated ERβ reduced cell growth in nontransformed colonocytes, implying a role for ERβ in the protection of the colonocytes from malignant transformation (243). All together, this supports the role of ERβ in colon cancer; both as a protector against CRC initiation as well as inducer of decreased proliferation in already transformed colon cancer cells. However, the mechanisms behind this are still rather unexplored. To further establish ERβ's role as a target for CRC prevention or therapy, more studies are needed, both *in vitro*, *in vivo* and in the clinic using ERβ specific ligands. In addition, a better understanding of gene pathways activated by ERβ will help to further dissect the impact of ERβ in CRC and might serve as a tool to improve diagnostics, adapt and select proper therapeutic treatments and help to predict clinical outcome of CRC patients.

1.6 GENOME-WIDE TECHNIQUES TO STUDY GENE EXPRESSION

The focus in this thesis has been to explore genome-wide effects of the estrogen receptors. There are several different techniques that can be applied and below are short explanations of a few of them.

1.6.1 Microarray

The microarray technology has been widely used in genome-wide gene expression studies since the late 1990s (215, 216). This technique offers an easy and rather cheap method to study differences in gene expression between samples. The basics behind microarrays are deposition of DNA corresponding to the full transcriptome, where each gene is represented by one or several probes, on an array. A sample cDNA, labeled with fluorescent dyes, is hybridized onto the probes. The fluorescent intensity of each probe corresponds to the abundance of that gene transcript in the sample. Independent of platform used, microarray data needs to be confirmed with other techniques such as real-time PCR. This is especially true for low abundant genes.

Several different companies offer varying techniques and manufacturing of microarrays. Affymetrix is one of the most commonly used microarray platforms (73). Probes on the GeneChip from Affymetrix contain 25-mer oligonucleotides, synthesized directly on the microarray surface, where the probe sequence is built one base per cycle. Each gene is represented by several different oligonucleotides known as a probe set, both perfectly matched to the target transcript as well as mismatched sequences (73). Specific binding is determined through the signal difference between matched and mismatched transcripts in a probe set. One sample is hybridized per array, meaning that a comparison between two samples requires two arrays. While Affymetrix uses photochemical synthesis to print arrays with short oligonucleotides, Agilent offers printed arrays with 60-mere oligonucleotides using InkJet technology. In addition to offering single-color arrays, Agilent also provides dual-color arrays where the two samples to be compared are hybridized on the same array slide. Another technique is the spotted array where (25-70 mere) pre-synthesized oligonucleotide probes are

attached onto the glass array. These probes can be produced “in-house” of academic laboratories or companies, or can be bought as ready-made collections of oligonucleotides (e.g. Operon’s 35k 70mer library covering all known human genes). Spotted arrays are usually two-channeled which means that each of the two samples are labeled with different fluorescent dyes and hybridized onto the same array. A different technique is offered by Illumina, where the oligonucleotides are attached to small beads instead of a glass slide. One benefit of Illumina is the possibility to process several samples in parallel.

The major benefit of printed arrays is the possibility to add more probes onto an array that what mechanically is possible for spotted arrays. Spotted arrays have the capacity to cover all know genes. However, the benefit with more probes, as offered by printed arrays, is the possibility to add replicates as well as splice variants. Both Affymetrix and Illumina offer multiple replicates for each gene, thereby increasing the possibility to detect differentiated genes despite of one bad probe. The drawback of Illumina compared to Affymetrix is that each transcript is detected with multiple probes with the same sequence instead of multiple probes with different sequences (73). Benefits of spotted arrays are the cheaper price and the possibility to customize the array for each experiment. However, results from custom made spotted arrays may not be so easily compared to results from commercially printed arrays due to reduced sensitivity, reduced printing efficiencies and reduced quality with missing spots (21, 73). Commercially available printed arrays have been quite expensive, but during the last years, both Affymetrix and Agilent arrays have drastically dropped in price. Still, purchasing spotted arrays are considerably cheaper.

microRNA microarrays are commonly used in large-scale miRNA studies and several companies offer commercial arrays for miRNA expression analysis. miRNA arrays are rather new in the field of microarrays, and it has not been thoroughly established how to analyze the data. Because of the small size of the miRNome compared to the genome, one challenge is the normalization of miRNA microarray data (125, 206). Therefore, it is essential to validate miRNA microarray data with other techniques available.

1.6.2 Real-time PCR

Real-time PCR (quantitative real-time PCR/qPCR) is the most sensitive method to study the relative level of a specific RNA between two samples. Total RNA extracted from cells or tissues of interest is reversely transcribed to cDNA and then amplified using forward and reverse primers and analyzed in real-time. The data is measured and presented while the run is ongoing, thus the name ‘real-time’.

There are several different chemistries available for real-time PCR, the two most common being TaqMan and SYBR green. Both these chemistries generate fluorescence which will be relative to the amount of PCR product produced. TaqMan utilizes a sequence specific probe as well as primers specific for the gene of interest. The probe is an oligonucleotide containing a 5' fluorescent dye and a 3' quenching dye, designed to hybridize to an internal region of a PCR product. As long as the dyes are in close

proximity there will be no fluorescence. As the PCR product extends to the probe, it will be cleaved off by Taq polymerase and the dyes will separate resulting in fluorescence (127). SYBR green, on the other hand, binds to all double stranded DNA and will give a stronger signal as more DNA is amplified for each cycle of the PCR run. SYBR green primers therefore need to be carefully designed for optimal selectivity, and checked with melt curve analysis to ensure a pure PCR product, not containing unspecific fragments or primer dimerization. The advantages and disadvantages of the two techniques are that TaqMan is more specific but more expensive, while SYBR green is more cost effective but requires additional controls to ensure gene specificity.

Advantages of real-time PCR compared to microarrays are the sensitivity, specificity, and simplicity. However, some important considerations for real-time PCR are the need for a good reference gene and well designed, gene specific primers. This, together with the higher cost for primers and reagents for a large number of genes, limits the use of this method to studies exploring changes in a small set of genes such as confirmation of microarray data, rather than for a screening study of genome-wide changes. However, this limitation has somewhat been bypassed through the development of large scale real-time PCR plates. Real-time PCR plates already containing optimized gene specific primers covering either parts of the genome, a specific pathway or genes involved in a specific disease, are commercially available. The TaqMan low density arrays (TLDA) microRNA cards from Applied biosystems, covering most known miRNAs, are an example of large-scale analysis of the miRNome. One limitation with large-scale real-time PCR analysis is the decreased accuracy and specificity for low copy number genes, resulting in higher ΔCt variances between intra-plate assays compared to high copy number genes (166).

1.6.3 RNA sequencing

Both microarrays and real-time PCR are widely used in analyses of gene expression due to their simplicity and low price. However, a growing field for genome-wide analyses is RNA sequencing (RNAseq). RNAseq has both advantages and disadvantages where one of the biggest advantages is that while microarrays only can detect transcripts present on the array, RNAseq can detect known and unknown genes as well as different splice variants and mutations (168). However, the cost for RNAseq is much higher than a microarray experiment, and might lead to a reduction of biological replicates being analyzed. In addition, since microarrays have been around for almost two decades, strategies for analyzing the data with minimal biases have been optimized, while it for RNAseq still is under development. Another major drawback is the magnitude of data generated from an RNAseq run. RNAseq data analysis requires knowledge in bioinformatics and data programming. It will take some time to develop tools for easy analysis of the data, but the more research that is done with this technique, the simpler and cheaper it will be (168). Despite the present drawbacks for RNAseq, this is a growing field that will become more common in the future.

2 AIMS OF THESIS

The general aim of this thesis was to increase the overall knowledge about the functions of ERα and ERβ by studying their effects on gene transcription, with focus on their implications in breast- and colon cancer cells. Specifically, our objectives were:

I. To investigate the differences and similarities in transcriptional regulation by the two estrogen receptors in T47D breast cancer cells (Paper I).

II. To dissect the ERα-driven regulatory networks, through analyzing miRNA expression following estrogen stimulation of ERα in T47D breast cancer cells (Paper II).

III. To study the role of re-introduction of ERβ in the human colon cancer cell line SW480, with focus on cell cycle regulation and impact in xenograft tumors (Paper III).

IV. To gain a better understanding of ERβ transcriptional regulation in three colon cancer cell lines through transcriptome analyses and bioinformatics (Paper IV).

V. To explore ERβ-driven changes of the miRNA pool and correlating mRNA regulations in SW80 colon cancer cells (Paper V).

3 METHODOLOGICAL CONSIDERATIONS

In any experimental system, significant considerations need to be taken into account when deciding on the methodological layout as well as for data interpretation. Considerations and limitations of some of the major methods and systems used in the studies in this thesis are described below. Other considerations are discussed throughout each study.

3.1 CELL LINES

The cell lines used in this thesis were immortalized from different breast and colon cancer tumors, and purchased from American Type Culture Collection (ATCC). Cell lines are a useful tool for *in vitro* models in cancer research since they are easy to grow and can be cultured infinitely. However, due to their continuous culturing they are prone to undergo genotypic changes thereby creating different subpopulations. Two different labs working with the same cell line might get different results depending on culture conditions and passage number of the cells.

In addition, cancer is a complex disease with diverse genetic backgrounds and many separate, activated signaling pathways leads to the progression of the disease. Therefore, an immortalized cell line cannot be seen as the perfect model system for that particular type of cancer. An alternative approach is to generate primary cultures directly from tumors. However, since primary cultures grow slowly and have a definite lifespan of just a few passages, the use of primary cultures is limiting when large amounts of cells are needed.

Since ERβ expression decreases during breast and colon cancer progression, no cancer cell line exist that express significant amounts of endogenous ERβ. The lack of immortalized cell lines expressing ERβ is one of the challenges when studying its transcriptional regulation. We therefore used systems where we introduced ERβ into the cells, thereby restoring the lost endogenous expression of ERβ. These methods will most likely not fully restore the physiological situation of the lost ERβ, but serves as an excellent tool for *in vitro* mechanistic studies and as guidance for future *in vivo* studies.

The two breast cancer cell lines used, MCF-7 and T47D, both express significant levels of endogenous ERα, and in our study express ERβ under the control of an inducible tet-off system. The three colon cancer cell lines used, SW480, HT29 and HCT-116 have low or no expression of endogenous ERα or ERβ, and were made to express ERβ through lentiviral transduction.

3.2 BIOINFORMATICS

Bioinformatics is a powerful *in silico* tool to analyze vast amounts of data. In this thesis, bioinformatics have been employed for microarray analyses, for gene ontology overrepresentation analyses, for genome-wide comparisons of ChIP with our

microarray data, for identifying predicted miRNA mRNA targets, for incorporation of literature searches and for building regulatory networks and pathways. However, as with any other method there are some limitations to take into consideration. There are many databases freely available with large amounts of data from different *in vivo* and *in vitro* experiments. However, tissue and cell specific effects are often not accounted for, and data that is true in one cell line might not be true in another cell line, even if they both come from the same type of cancer. In addition, chromatin binding site studies for a particular transcription factor might be similar in different cell lines, but *will* differ depending on presence of e.g. cofactors and packing of the chromatin. miRNA target predictions are algorithms calculating the most statistical probable target. All the different miRNA target predicting sites have different algorithms, thereby resulting in different responses despite the same in-data.

Bioinformatics can be used to find new angels and insights on obtained *in vitro*/*in vivo* results, and build new experiments based on that.

4 RESULTS AND DISCUSSION

4.1 PAPER I: A GENOME-WIDE STUDY OF THE REPRESSIVE EFFECTS OF ESTROGEN RECEPTOR BETA ON ESTROGEN RECEPTOR ALPHA SIGNALING IN BREAST CANCER CELLS

ERα mediates a proliferative effect in estrogen responsive breast cancers, and therapies targeting ERα are used in the clinic. It has been suggested and demonstrated *in vitro* that that ERβ opposes this effect of ERα. Most patients that initially respond to ERα targeting therapies develop resistance over time. New therapies are needed, and ERβ is a potential target. However, the clinical significance of ERβ in diagnostics and treatment has not been established. Thus, there is a need for a better understanding on the underlying mechanism of the supposedly antiproliferative effect of ERβ. In Paper I, we performed a genome-wide analysis of ERα and ERα/ERβ transcriptional activity, with the aim to elucidate the different regulatory networks of the two receptors (251).

We used T47D cells and expressed ERβ through a tet-off system. ERβ was expressed for 24 hours prior 24 hours treatment of ICI or E2 in control cells and ERβ-expressing cells. Changes in gene expression were studied using microarray and real-time PCR. Our analysis identified 1434 ERα regulated transcripts, out of which 998 genes were inhibited by ERβ expression. In addition, we found a large proportion of genes regulated by ERα alone or by ERβ. 80% of the 50 strongest ERα regulated genes were induced by E2, while the corresponding number for ERβ was 26%. This illustrates that ERα predominantly had an upregulatory effect while ERβ negatively modulated a majority of its targets. This pattern was also seen in the genes commonly regulated by the two ERs, where 76% were upregulated by ERα and attenuated by ERβ. In addition to genes regulated both by ERα and ERβ, we also detected altered expression of genes in ERβ-expressing cells that were not affected by ERα, revealing an ERβ specific regulation. After removing boarder-line ERα regulated transcript, we had a list with 49 transcripts regulated by ERβ alone, out of which 71% were upregulated.

Gene ontology analysis of all ERα regulated genes showed that the most overrepresented category for downregulated genes was related to the 'cell cycle', and for upregulated genes to 'cell adhesion', 'oncogenesis' and 'apoptosis'. A corresponding analysis for all ERβ regulated genes showed an enrichment of downregulated genes within 'cell proliferation' and an enrichment of upregulated genes within 'negative regulation of cell growth' and 'apoptosis'. Further, a parallel analysis of genes regulated both by ERα and ERβ showed an enrichment of genes within the 'cell cycle'. The fact that gene ontology analyses for both ERs showed an enrichment of genes within categories such as cell cycle and cell growth, further supported ERα's proliferative effect and ERβ's antiproliferative effect. In addition, we could confirm this antiproliferative effect with a proliferation assay in which the estrogen stimulated ERα-induced proliferation in T47D cells was completely abolished when ERβ was expressed.

Many of the ERα-regulated genes opposed by ERβ, such as several cyclins (CCNA2, CCNB1, CCNB2, CCND1 and CNF), pS2, BCL2, GREB1, MYC and TP53INP1, are involved in proliferation and cell-cycle regulation. In addition, ERβ alone regulated several antiproliferative genes, such as QSCN6, NDRG3, SEPT9, KCTD11 and STK3. IL-20 was one of the most strongly induced ERα-regulated genes that was opposed by ERβ. IL-20 stimulates epithelial cell proliferation, antiapoptotic response, can enhance wound-healing activities and is involved in skin inflammations (192, 209). Deregulated IL-20 expression may lead to uncontrolled tissue repair processes resulting in diseases (192). Thus, the opposing effect by ERβ on the proinflammatory cytokine IL-20 as well as the regulation of several genes involved in proliferation and cell-cycle regulation may therefore contribute to the antiproliferative properties of ERβ.

In an effort to elucidate the mechanism behind ERβ's opposing effect on ERα signaling, we transfected T47D cells with an ERβ protein with a mutated DNA-binding domain. We saw that the opposing action of ERβ on some of the ERα regulated genes was in fact accentuated when ERβ had a mutated DNA binding domain and was unable to bind to the DNA. One possibility is that ERβ heterodimerizes with ERα resulting in reduced binding efficiency to the ERE compared to an ERα homodimer, thus explaining ERβ's opposing effect and lack of full inhibition of the ERα-driven transcription. However, when the mutated ERβ heterodimerizes with ERα, the ability to bind to ERE is fully abolished.

Comparison of our T47D microarray data with previously published studies on ERα transcriptional regulation in MCF7 cells showed many common regulations (89). These were found despite the use of different cell lines, different treatments (ICI or vehicle) and different technology platforms. In addition to already published data, this study also contributed with a large number of novel ERα regulated genes, possibly cell specific. Few studies of ERβ transcriptional regulation had been published at the time. Comparison with the one published study in, MCF7 breast cancer cells, found many discrepancies (45). This may be contributed to different cell lines, different mode of ERβ delivery (tet-off construct versus transient adenoviral gene delivery) and the use of different microarray platforms. Nonetheless, both studies could show the same gene groups, such as cell-proliferation and ion homeostasis, being affected by introduction of ERβ in ERα-expressing cells.

In conclusion, we could show that ERβ opposed the effect of ERα-driven upregulation of genes involved in cell proliferation and cell cycle in T47D breast cancer cells, which we also functionally confirmed with a proliferation assay. Further, we demonstrated that ERβ not only opposes ERα transcription, but also induced transcriptional changes independently of ERα.

4.2 PAPER II: ESTRADIOL-ACTIVATED ESTROGEN RECEPTOR α DOES NOT REGULATE MATURE MICRORNAS IN T47D BREAST CANCER CELLS

microRNAs (miRNAs) have been shown to be involved in several different cellular processes and is often deregulated in cancers, including breast cancer. Both mRNA and

miRNA are transcribed from the DNA by RNA polymerase II. Since estrogen can give rise to transcriptional regulation of protein coding genes in breast cancer (by RNA polymerase II), it is possible that miRNAs are transcribed by the same mechanism. Identification of miRNAs associated with disrupted estrogen signaling could therefore contribute to an enhanced understanding of the molecular processes underlying development of estrogen related breast cancer. Some studies have investigated ERα regulation of miRNAs in MCF7 breast cancer cells, but have presented conflicting results. In Paper II, we performed a thorough analysis of miRNAs regulated following 24 hours estrogen stimulation of T47D cells endogenously expressing ERα (137). We utilized several different techniques to identify changes in the miRNA pool. For the full miRNome analysis, we used both dual-color microarrays and TaqMan Low Density Arrays. Real-time PCR confirmations were then extensively performed with both SYBR Green and TaqMan chemistry.

First, we confirmed that 24 hours estrogen stimulation of T47D cells induced expected transcription of protein coding genes. pS2, KCNK5 and SPINK4 have all previously been shown to be regulated by ERα (251) and were therefore used as positive controls of gene transcription. We could confirm that 24 hours of estrogen treatment of T47D gave rise to a strong and significant transcriptional response on these genes. Further, treatment with ICI showed a decreased response on these genes. Thus, we could show that ERα induced transcription of mRNA in these cells. Our miRNA microarray revealed no significantly changed miRNAs after estrogen treatment whereas TLDA analysis detected 57 regulated miRNAs. However, these TLDA detected regulations could not be confirmed with real-time PCR (neither SYBR Green nor TaqMan).

Since the combined analysis with microarray, TLDA and real-time PCR did not show any significantly changed miRNAs, a literature study was performed to identify miRNAs previously indicated to be changed by ERα. Real-time PCR could not confirm an ERα-driven regulation on these miRNAs in T47D cells. Given that most published studies have been performed in MCF-7 cells, we also analyzed these miRNAs with real-time PCR in these cells. Despite the use of identical cell line, we could not show a direct regulation by ERα on these miRNAs. To further explore if estrogen exposure time would affect miRNA regulations, we performed times series with estrogen treatment in T47D cells. These studies did not show any change on miRNA regulation. This conflicts with previously published articles, highlighting the technical challenges when it comes to miRNA expression profiling.

In conclusion, even though there was a strongly induced estrogen-activated ERα-mediated gene transcription at 24 hours in T47D cells, no mature miRNAs were found to be significantly regulated.

4.3 PAPER III: TUMOR REPRESSIVE FUNCTIONS OF ESTROGEN RECEPTOR β IN SW480 COLON CANCER CELLS

The two estrogen receptors are sometimes co-expressed, often in varying ratios in different cell types. The development of estrogen dependent cancer can be facilitated

by changes in ratio of the two receptors. Both ERα and ERβ are expressed in the normal breast epithelia but as breast cancer progresses, the ratio between them changes, often by a decrease of ERβ and an increase of ERα. It is therefore of interest to study transcriptional effects by both receptors expressed in breast cancer cells. However, in the normal colonic epithelium where levels of ERα are significantly lower or not expressed at all, ERβ is the major estrogen receptor, and is often decreased or lost as colon cancer progresses. Hormone replacement therapy has been shown to reduce the risk for colon cancer, implying a protective role for estrogen in development of colon cancer. This point towards a potential tumor suppressor function of ERβ in colon cancer. For that reason, it is of interest to study the effect of ERβ alone in colon cancer cells. The aim of Paper III was to study the molecular effect of reintroduction of ERβ in SW480 colon cancer cells and especially its implication on cell cycle regulations (112).

Following lentiviral transduction with an ERβ-expressing construct in SW480 colon cancer cells, proliferation was significantly decreased compared to control cells. FACS analysis confirmed this, showing that 75% of the ERβ-expressing cells were halted in G_1 phase while the equivalent number for the control cells was 52%. Cells transfected with an ERβ DBD-mutant did not show this antiproliferative effect, and resulted in a nearly complete removal of the ERβ-driven G_1 arrest (58%). This demonstrates that the ERβ-driven antiproliferative effect in SW480 colon cancer cells was ERE-dependent.

To corroborate the *in vitro* proliferation results *in vivo*, we performed a xenograft study with SW480 ERβ and control cells in immunodeficient mice. This study showed a reduction in tumor weight by 65% in the ERβ-expressing cells. Apoptosis was not affected by ERβ, as demonstrated with a TUNEL assay, which made us conclude that a changed apoptosis potential was not the cause of the smaller size of the ERβ-expressing xenografts. However, ERβ-expressing tumors exhibited a significant reduction of Ki67 positive cells compared to control xenografts, confirming that ERβ mediated a reduced proliferation, resulting in the smaller sized xenograft tumors.

Western blots reviled ligand-independent effects by ERβ. This may be caused by the overexpression of ERβ, but alternative causes will be further discussed in a section of Paper IV.

Stimulation of cells with growth factors will result in upregulation of cell-cycle and cell proliferation genes. One early response gene is MYC, and this gene was strongly downregulated by ERβ. MYC has a central role in colon cancer growth (238) where it is an activator of many G_1-S phase genes. The ERβ-driven downregulation of MYC is thus an important event contributing to the antiproliferative function of ERβ in SW480 cells. Other key regulators of cell cycle are cyclin-dependent kinases (CDKs), where CDK2, 4, 5 and 6 are involved in G1 phase progression. Western blot showed that the MYC target gene CDC25A was repressed in ERβ-expressing cells. CDC25A activates CDK2 and CDK4; thus, low expression of this protein results in high levels of the inactive form of CDK2/CDK4 thereby halting the cells in G_1 phase. Further, ERβ-induced downregulation of the p45 protein, a regulator of p27 degradation, resulted in a strong induction of the p27 protein. ERβ also upregulated $p14^{ARF}$, a tumor suppressor involved in cell-cycle arrest. $p14^{ARF}$ in turn downregulates MDM2, resulting in a stabilization of the p53 protein that, consequently, was strongly induced in ERβ-

expressing cells. p53 promotes p21 expression, which also was upregulated. The two upregulated proteins p27 and p21 function as CDK2 inhibitors. ERβ expression did not induce a change on p15 and p16. Cyklin D1 was upregulated by ERβ, while cyclin E and A, both important in late phase of G_1, were not. Figure 6 illustrates CDKs and regulators of cell cycle progression, as well as the effect ERβ expression had on respective protein level, indicating that the antiproliferative effect mediated by ERβ is primarily through halting the cells in G_1 through the changed expresison of several CDK regulators.

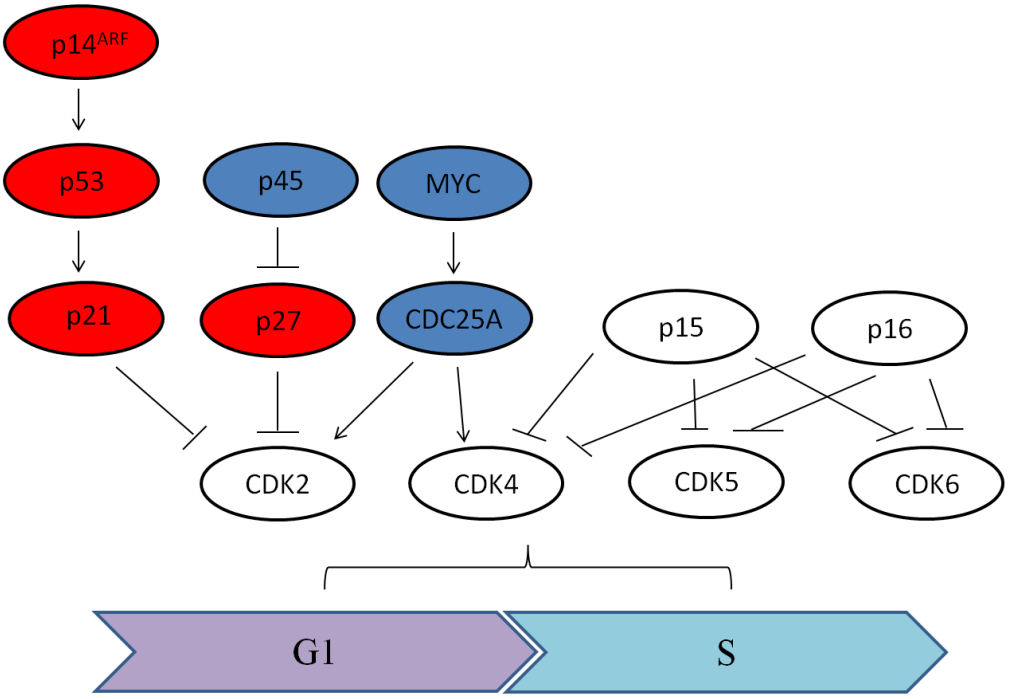

Figure 6. The cyclin-dependent kinases CDK2/4/5/6 are all important in regulation of cell cycle and the G1/S progression. Several CDK regulators were changed following ERβ expression (genes in red were upregulated, and genes in blue were downregulated), suggesting an explanation for the G1 phase arrest and following decrease in proliferation in SW480 colon cancer cells.

Taken together, ERβ demonstrated an antiproliferative role in SW480 colon cancer cells *in vitro* as well as in xenografts and affected proliferation partly through transcriptional regulation of genes involved in cell cycle regulation such as MYC, $p14^{ARF}$, p53, p21, p45, p27 and CDC25A. Further, we saw that ERβ had significant ligand-independent effects.

4.4 PAPER IV: ESTROGEN RECEPTOR β INDUCES ANTI-INFLAMMATORY AND ANTITUMORIGENIC NETWORKS IN COLON CANCER CELLS

Introduction of ERβ in both breast and colon cancer cells results in reduced proliferation and changes in key cell cycle proteins (112, 230, 251). The genome-wide study performed in T47D cells (Paper I) dissected ERβ transcriptional effects in combination with endogenously expressed ERα. ERβ genome-wide regulation on its

own is, however, not known. To further elucidate the mechanism behind ERβ's antiproliferative effect in colon cancer cells, and regulation by ERβ homodimers alone, we performed a genome-wide analysis on three colon cancer cell lines with induced expression of ERβ (Paper IV) (70).

Stable ERβ expression resulted in large genome-wide transcriptional changes in all three cell lines. Although the impact of ERβ was significant, few regulated transcripts were found in common between the different cell lines. Just like there were differences in ERβ regulated genes in our T47D study compared to published ERβ regulation in MCF-7 cells, we here demonstrated that ERβ gene regulation is cell specific also in different colon cancer cells. In an effort to further understand ERβ regulations, we analyzed enriched gene ontology groups in the three cell lines. Even though different genes were regulated, the GO analysis showed an enrichment of genes within protein binding, apoptosis, regulation of cell cycle, cell differentiation and kinase activity in all three cell lines. ERβ therefore seems to regulate similar biological events, but uses different genes in the process. Cell specific activity could be caused by already activated pathways, mutations, presence of coregulators and cell specific genes in that particular cell. Some known differences in these three cell lines are mutations in p53 and varying levels of RAS, PROX1, MYC and DNA repair genes.

Once established that there were large variations between the different cell lines, we focused our studies on SW480 colon cancer cells. Enriched sub-network analysis revealed regulation of many downstream targets of IL-6. Real-time PCR could confirm a strong ERβ-driven downregulation on IL-6. In addition, we also found reduction of mRNA levels of downstream targets (SP-1, VEGF, JUN1) in the MAPK/ERK pathway in ERβ-expressing cells in the two other colon cancer cell lines HT29 and HCT-116. Previously we have revealed that ERβ opposed the ERα upregulatory effect on IL-20 in breast cancer cells, and here we have shown that ERβ downregulates IL-6 and many target genes in the MAKP/ERK pathway. This suggests a role for ERβ in inflammation. A randomized clinical trial with hormone therapy as treatment for rheumatoid arthritis in post-menopausal women showed suppressed signs of inflammation (60), supporting the role for estrogen in treatment of inflammation. A key component in inflammatory signaling is NFκB. The ERs are known to inhibit NFκB inflammation response through several different pathways such as competing for the transcription coactivator CREB binding protein (CBP) (110), prevention of NFκB DNA binding (106) or as a positive transcriptional cross-talk between NFκB and ER (90). Most of these studies have however been performed in ERα positive breast cancer cells, and the mechanism behind the potential inhibitory role of ERβ on NFκB inflammation response in colon cells is not well studied. There is a connection between inflammation and tumorigenesis, where chronic intestinal inflammation proceeds tumor development. An ERβ-mediated regulation of the inflammatory pathways could, in normal colonic epithelia *in vivo,* significantly contribute to the suggested colon cancer protective effect of estrogen and should be examined more thorough.

PROX1 is a transcription factor and nuclear receptor coregulator. It is often overexpressed in colon adenomas, in which high PROX1 expression is associated with poor tumor differentiation (199, 224). We found that re-expression of ERβ in SW480 colon cancer cells led to a downregulation of PROX1 both at mRNA and protein level.

To our knowledge, this is the first time it has been shown that ERβ expression can affect PROX1 levels. It is plausible that the ERβ-driven downregulation of PROX1 is responsible for some of ERβ's antitumorigenic properties. Comparison of ERβ-regulated genes to a PROX1 silencing microarray study (199) revealed many commonly changed genes, suggesting that regulation of these genes was a direct consequence of the ERβ-driven downregulation of PROX1. Further, PROX1 chromatin binding sites (as published (46)) were enriched in the proximity of ERβ regulated genes. Interestingly, several of these transcripts had both a PROX1 and an ERβ binding site. With PROX1-ChIP we could confirm CITED2 as a gene regulated by both PROX1 and ERβ, with a PROX1 binding site, in SW480 cells, less than 50 kb from TSS. It is therefore possible that PROX1 and ERβ compete and/or co-regulate each other to influence the regulation of common target genes.

An ideal model to study the role of ERβ in colon cancer would be a colon cancer cell line endogenously expressing ERβ. However, to our knowledge no such cell line exists. We therefore need to rely on a model system where we express ERβ. The model we used, with stable expression of ERβ in mixed cell populations, made it impossible to distinguish between directly regulated transcript and secondary events. As a part of the analysis we utilized data from an ERβ binding study in MCF-7 cells with the assumption that ERβ binding sites in MCF-7 also exist in SW480, and compared it to our genome-wide data. Based on the MCF-7 dataset, 11% of the genome had an ERβ binding site. We found an enrichment of genes with an ERβ binding site in our ERβ regulated genes (17%). The regulated genes that possessed an ERβ binding site are potentially directly regulated by ERβ.

Further, no ligand-dependent ERβ regulatory effects were detected. Several studies have published ligand-independent gene regulations by both of the ERs. Mechanism behind this is not fully understood, but includes phosphorylation of the AF-1 domain via the MAPK pathway and ligand-independent association with the steroid receptor coactivators SRA, SRC and CBP to the AF-1 domain (65, 236, 237). We suggest that the observed ERβ ligand-independent regulation is caused by high MAPK phosphorylation activity. A recent study showed that estrogen stimulated ERβ had a protective effect in noncancerous colonocytes (243). We hypothesize that ERβ has a ligand-dependent protective role in normal colon epithelial cells, but that this ligand-dependence for some unknown reason is lost upon re-expression in colon cancer cell lines.

In conclusion we have shown that reintroduction of ERβ in colon cancer cells resulted in large and cell specific transcriptional changes. These regulations reduced proliferation and tumorigenic potential and included, for instance, genes in the MAPK/ERK signaling pathway. We could also conclude that the colon carcinogenic activity of PROX1 in SW480 cells was affected by ERβ in dual ways; through down-regulation of PROX1 itself, and through co-regulation of the target genes.

4.5 PAPER V: ESTROGEN RECEPTOR BETA EXPRESSION INDUCES CHANGES IN THE MIRNA POOL IN HUMAN COLON CANCER CELLS

No studies so far have looked at a possible ERβ regulation of miRNAs. We speculate that some of ERβ's antiproliferative effect and some of the transcriptional mRNA changes we detected after ERβ re-expression may be attributed to miRNA regulations. In Paper V, we performed a large-scale miRNA analysis in SW480 colon cancer cells that stably expressed ERβ, and correlated the changes to the transcriptome analysis previously performed (70).

We could show that ERβ expression induced significant changes in the miRNA pool in SW480 cells, and defined 25 downregulated miRNAs and 3 upregulated miRNAs using miRNA real-time PCR confirmations. To investigate the impact of the changed miRNA pool, we used bioinformatics to identify predicted target genes and related these to the regulated mRNAs (70). We found an extensive overlap of predicted miRNAs targets and ERβ regulated mRNAs. We believe that this finding can serve as a guide for future studies exploring the complexity of ERβ transcriptional regulation.

As a first step to examine direct ERβ regulatory effects on the changed miRNAs, we investigated whether or not there were ERβ binding sites nearby the chromosomal location of the miRNAs. We found several miRNAs with proximal and distal binding sites, and 66% of the pre-miRNAs had an ERβ binding site within 100kb. This indicated a possible direct ERβ regulation for many of the regulated miRNAs. Other miRNAs may not be primary targets of ERβ, but rather secondary events of ERβ-regulated transcription factors. MYC is downregulated by ERβ via a chromatin binding site less than 70kb from its promoter (100, 262). MYC is a key regulator of miRNAs (38, 140, 186) and miR-200a, miR-200b, miR-9-3, miR-106a, miR-221, miR-135a-1 and the miR-17-92 cluster have all been shown to be regulated by MYC in other cell lines. Thus, these twelve miRNAs may be changed as a consequence of the ERβ-driven downregulation of MYC in SW480 cells.

miR-200a and miR-200b were strongly downregulated in ERβ-expressing cells. The expression of these miRNAs is decreased in some cancers (68). They have both, in other cell lines, been shown to target ZEB1 and thereby lead to the upregulation of CDH1 (E-cadherin). Thus, the miR-200 family is believed to be involved in tumor suppression through the inhibition of the epithelial-mesenchymal transition (EMT) therefore inhibit formation of metastases. Using miR-200a and miR-200b mimics, we confirmed ZEB1 to be a target gene, resulting in the subsequent upregulation of E-cadherin. This regulation does not explain the protective role of ERβ, and the impact of miR-200 downregulation in colon cancer needs to be further explored. However, it has been demonstrated that miR-200 expression also can promote metastatic properties of some cancer cells, suggesting that changed expression of the miR-200 family will have different impact on disease outcome depending on cellular context (68).

Another interesting finding was the strong downregulation of the miR-17-92 cluster, also known as Oncomir-1, consisting of miR-17, miR-18a, miR-19a, miR-19b, miR-20a and miR-92. This cluster is known to act as an oncogene (116) and has been shown to be involved in enhanced cell proliferation and suppression of apoptosis in human B-

cell lymphomas and in colon cancer cells (66, 116). The strong downregulation of this cluster may contribute to the complex pathway in which ERβ generates the antiproliferative effect, as demonstrated in the xenograft study (112). We identified and confirmed that the ERβ regulated genes CLU and NCOA3 (SRC-3) both were induced when miR-17 was silenced. NCOA3 is reported to affect the expression of other transcription factors and regulators such as NCOA1 (SRC-1), AHR, PPARG, CEBPD and MYB. These genes were all changed in ERβ-expressing cells, pointing towards an ERβ-driven regulation of these genes partially through the downregulation of miR-17 and the subsequent upregulation of NCOA3. NCOA3 is a nuclear receptor coactivator, affecting the transcriptional activity of both ERα and ERβ (156, 254). NCOA3 has been associated with a more locally advanced disease in CRC, and elevated levels of the protein have been reported in many different cancers (258) (99). However, elevated levels of NCOA3 have also been found to be associated with a better overall survival in CRC (99). Therefore, the role of NCOA3 as an ERβ coregulator and the upregulatory effect of ERβ regulated miRNAs on NCOA3 in colon cancer needs to be explored further.

We also found that treatment with cisplatin, a DNA damaging agent, led to a higher cell death in miR-17 silenced cells compared to wild type cells. This finding indicates an interesting role for co-treatment with miR-17 inhibitor and chemotherapy in colon cancer patients resistant to chemotherapy.

In conclusion, we have shown that stable re-expression of ERβ in SW480 colon cancer cells resulted in a large change of the miRNA pool. To our knowledge, this is the first report of ERβ affecting miRNAs. We further detected changed mRNA levels of many miRNA predicted target genes, illustrating the impact of the ERβ regulated miRNA pool on mRNA levels.

5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

ERα was discovered more than 50 years ago and major efforts have been put into describing the molecular network by which it works. The regulatory networks of ERα are therefore relatively well studied, and ERα is used as a therapeutic target in breast cancer. ERβ was discovered 15 years ago, and the molecular mechanisms behind ERβ's effect are still far from clear. Normal breast epithelia expresses both ERα and ERβ, but the ratio between them changes during cancer progression. A standard procedure in breast cancer diagnostics consists of identifying those patients expressing ERα, thereby more likely to respond to tamoxifen treatment. At this time, ERβ is not used in diagnostics, and the clinical significance of ERβ in breast cancer has not been established. Clinical trials with progestin-estrogen HRT indicate a protective role against colorectal carcinoma. As ERβ is the predominant ER in the colonic epithelium and is lost during cancer progression, it is reasonable to assume that these protective effects are conveyed by ERβ. This has further been supported *in vivo* studies where deletion of ERβ in mice that spontaneously develops intestinal adenomas leads to an increase both in size and number of adenomas (94). Despite these facts, the role of ERβ in colon cancer is not well explored.

Since ERα has been shown to be involved in the development and progression of breast cancer, and ERβ has been indicated to mediate a protective effect in colon cancer, it is of importance to elucidate the molecular mechanism behind their tumorigenic and anti-tumorigenic properties, respectively. The aim of this thesis was to dissect the regulatory networks of ERα and ERβ and thereby gain a better knowledge of the molecular and cellular mechanisms of estrogen signaling in breast and colon cancer. A better understanding of the regulatory networks by which ERα and ERβ work, would not only aid in development of novel therapeutics based on downstream targets of the ER pathways but also help identify those patients that might benefit from treatment aiming at ERα and/or ERβ.

When this study was initiated, it had been established that ERα was proliferative. It had been suggested that ERβ was antiproliferative, but this notion had not yet been widely accepted. We could show that ERβ often opposed the transcriptional effect of ERα at the transcription levels in T47D cells, especially for genes within proliferation and regulation of cell cycle. We also found a set of genes regulated by ERβ alone, indicating that in spite of the high homology in their DBDs, there are differences in their transcriptional targets. The significance of ERβ in breast cancer has been debated, but our data implies that ERβ may play an important role in the treatment and diagnostics of breast cancer. Stimulation of ERβ through ERβ specific agonists is one future direction for the treatment of estrogen responsive breast cancers. However, it is not established to what extent ERβ is expressed in breast cancer tumors due to the varying specificity and efficiency of commercially available ERβ antibodies. Therefore, there is a need for development of new ERβ antibodies with a better specificity. This would facilitate further exploration of ERβ occurrence in breast cancer tumors and lead to a better knowledge if ERβ is expressed at those levels that ERβ targeting therapies would be beneficial as an alternative treatment method.

Most studies on ERβ so far had been performed in cell lines expressing endogenously ERα in addition to the transduced ERβ. Therefore, they could only detect how ERβ behaved in combination with ERα. We demonstrated that ERβ not only is antiproliferative when co-expressed with ERα in breast cancer, but also by itself in colon cancer cells. We further showed that this also was true *in vivo* with xenograft ERβ-expressing tumors. Our studies substantially contribute to a better understanding of the mechanism behind ERβ's antiproliferative effect in colon cancer cells by the discovery that ERβ halts the cells in G_1-phase through the strong regulatory inhibition on MYC and several other cell cycle genes.

ERα has been widely studied, and there are several accepted estrogen target genes. However, no transcriptional profile had been established and accepted for ERβ. To further explore the impact of ERβ regulation in colon cancer, we performed genome-wide analyses. This showed that ERβ is capable of regulating a large set of genes independently of ERα, and that the set of ERβ regulated genes is cell type and cell line specific. We therefore could not establish one specific ERβ transcriptional profile in colon cancer cells. However, the different sets of ERβ regulated genes in three different colon cancer cell lines were all involved in similar biological processes, such as regulation of cell cycle, kinase activity and apoptosis. We therefore suggest that ERβ regulates similar biological processes in different cells, but uses different pathways and genes to reach them. The exact mechanism behind ERβ's regulatory network in colon cancer cells is still not clear, but some key factors such as regulation of cell cycle, MAKP signaling and PROX1 play important roles. The biological role of these target genes in the development and progression of colon cancer as well as correlation between them and ERβ expression should be further explored in normal colon cells. In addition, for a full understanding of the ERβ regulatory pathways, future studies should include a thorough analysis of ERβ binding sites in colon cells, such as with RNAseq and ChIP-Seq, as well as proteomic studies and analysis of post-translational modifications, especially involving ERβ regulation on kinases and subsequent effect on protein phosphorylations.

The finding of gene inhibition by miRNAs is rather new in the field of gene regulation. As the number of known miRNAs increases for each year, the understanding of their role in different biological pathways and processes is improved. miRNAs are involved in most biological processes and are often deregulated in both breast and colon cancer. One recent study showed that expression of the mir302/367 cluster efficiently could reprogram human and mouse fibroblasts to an induced pluripotent stem cell state (12). This demonstrates that changes in the miRNA pool of just a few miRNAs might be sufficient for a cell to switch into a different type of cell, or possibly turn cancerous. Identification of miRNAs that are associated with normal or disrupted estrogen signaling would be of great importance for prognosis and diagnosis. Previous studies on ERα regulation of miRNAs in breast cancer cells revealed conflicting results. We could show a strong estrogen activated ERα transcriptional response in T47D cells, but we did not detect any rapid changes of mature miRNA expression. However, we found that stable re-expression of ERβ in colon cancer cells led to a significant change of the miRNA pool. Many of the changed miRNAs were indirectly regulated via MYC, but others had an ERβ binding site within 100kb indicating a possible direct ERβ regulation. This finding offers insight into the long term effect of ERβ activation in

colon. In addition, we could show that genes regulated by ERβ could be enhanced through ERβ regulated miRNAs, illustrating how ERβ can fine tune gene expression through the regulation of miRNAs. To our knowledge, this is the first study presenting miRNAs regulated by ERβ. This opens up for an entirely new field of potential therapeutic targets of ERβ signaling in colon cancer. Future mimic and inhibitor studies, *in vitro* and *in vivo*, with detected changed miRNAs are interesting approaches to further explore miRNAs as potential therapeutic targets in estrogen dependent colon cancers.

The findings in this thesis significantly contribute to elucidating the regulatory pathways of the two estrogen receptors. We now know, e.g., that ERβ not only opposes the effect of ERα but also induces transcriptional response as a homodimer, that ERβ is antiproliferative in both breast and colon cancer cells, partly through ERβ-induced inhibition of PROX1, MYC and other G1-phase cell cycle genes, that ERβ may fine-tune mRNA expression through the regulation of miRNAs and that ERβ has anti-inflammatory properties. All this supports the notion that ERβ is a potential target in breast and colon cancer prevention and/or inflammation proceeding CRC development. ERβ expression might be lost in many cancers especially at later stages, thus not a possible target for novel therapeutics. One approach would be reintroduction of ERβ in these tumors. However, this is currently not a practical or realistic solution. Instead, investigations should be done to explore if specific ERβ agonists have treatment potential or if novel therapeutics could be aimed at targets downstream of ERβ, such as those discovered in this thesis.

Only a few studies have explored the use of ERβ specific ligands or phytoestrogens in animal studies, and just one small pilot study has explored the combinatory effect of two phytoestrogens on CRC in humans. Given that we have shown that ERβ exhibits an antiproliferative effect we suggest that a specific ERβ agonist might prove to be an interesting complement in future therapies against early stages of ERβ-expressing breast and colon cancer tumors as well as in treatment of inflammation. In addition, ERβ specific agonists may potentially be used as a preventive approach for persons with an increased risk of developing CRC, such as IBD patients. Undoubtedly, there is a need for development and characterization of ERβ specific ligands to be used *in vitro* and in subsequent *in vivo* randomized clinical trials to fully understand and utilize the clinical significance of ERβ in estrogen responsive cancer.

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