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ANTIPROLIFERATIVE ACTION OF ESTROGEN RECEPTOR β AND HES-1 IN BREAST CANCER

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ABSTRACT

Breast cancer is the most common malignancy among Swedish women. Although the mechanism behind the tumorigenesis remains unclear, estrogen receptor α (ERα) plays an important role in the progression of breast cancer and is regarded as a target for endocrine therapy. In this thesis, focus is on the second estrogen receptor, ERβ and its function in breast cancer. In addition, the significance of the transcription factors Hes-1 and Hes-6 in breast cancer and their relation to ERα has been studied.

By using T47D breast cancer cells with inducible ERβ expression, the role of ERβ has been characterised with respect to proliferation and cell-cycle regulation. In contrast to ERα, expression of ERβ inhibited the proliferation of 17β-estradiol (E2) treated breast cancer cells and caused significantly changed levels of cell-cycle regulators. In response to ERβ expression, the levels of the Cdk2-activating phosphatase Cdc25A as well as cyclin E and E2F1 were reduced with a subsequent decrease of the Cdk2-activity.

Moreover, expression of ERβ reduced the number of tumor associated blood vessels as well as tumor volume in a mouse xenograft model. In addition to the anti-tumorigenic effects, expression of ERβ reduced the levels of secreted growth factors *in vitro* as well as *in vivo*.

The transcriptional repressor Hes-1 has been described as an essential factor during embryonic development. However, Hes-1 is also important in breast cancer cells where it inhibits proliferation. Downregulation of Hes-1 by ERα is a crucial step in E2 stimulated proliferation of breast cancer cells. We expressed Hes-1 in breast cancer cells to study the mechanism behind its antiproliferative properties upon E2 treatment. Hes-1 expression induced a G_1 cell-cycle phase arrest and a concomitant reduction of the E2F1-level. By real-time quantitative PCR and electrophilic mobility shift assay, we can conclude that Hes-1 inhibits E2F1 at the promoter level.

Hes-6 is an inhibitor of Hes-1 and has been associated with tumorigenesis and metastasis. When Hes-6 was expressed in T47D breast cancer cells, proliferation was increased as well as tumor growth in immunodeficient mice. Furthermore, E2F1 was identified as an important target gene, induced by Hes-6 in breast cancer cells.

In conclusion, these studies have significantly contributed to the knowledge of estrogen receptor function in breast cancer as well as to elucidate important roles of Hes-1 and Hes-6 in estrogen signalling.

LIST OF PUBLICATIONS

I. Anders Ström, **Johan Hartman**, James S Foster, Jay Wimalasena and Jan-Åke Gustafsson. Estrogen receptor β inhibits 17β-estradiol-stimulated proliferation of the breast cancer cell line T47D. Proc Natl Acad Sci U S A. 2004 Feb 10;101(6):1566-71

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LIST OF ABBREVIATIONS

AF	Activator function
AP-1	Activator protein 1
atRA	All-*trans*-retinoic acid
bHLH	Basic helix-loop-helix
CBP	Creb-binding protein
Cdk	Cyclin dependent kinase
Ch-IP	Chromatin immunoprecipitation
DBD	DNA binding domain
DCIS	Ductal carcinoma in situ
E2	17β-estradiol
EGF	Epidermal growth factor
ERE	Estrogen response element
G-phase	Gap phase
HAT	Histone acetylase
HDAC	Histone deacetylase
Hes	*Hairy* and *Enhancer of Split* homolog
HRG	Heregulin-β1
HRT	Hormone replacement therapy
IDC	Invasive ductal carcinoma
INK	Inhibitors of kinase
LBD	Ligand binding domain
M	Mitosis
NED	Neuroendocrine differentiation
N-CoR	Nuclear receptor co-repressor
PKC	Protein kinase C
S-phase	DNA synthesis phase
SHBG	Sex-hormone binding globulin
SMRT	Silencing mediator for retinoid and thyroid hormone receptors
SRC	Steroid receptor co-activator
TLE	Transducin like enhancer of split
VEGF	Vascular endothelial growth factor
WHI	Women´s Health Initiative trial

1 INTRODUCTION

1.1 ESTROGENS AND THEIR RECEPTORS

1.1.1 Introduction to estrogens

Estrogens are physiologically important hormones in both females and males. Although several different types of estrogens are synthesised throughout life, 17β-estradiol (E2) is generally considered as the most important and potent estrogen (1).

The main source of E2 is the ovarian follicles of the fertile woman, in which a P-450 enzyme catalyses the synthesis of pregnenolone from cholesterol (2), which is the rate-limiting step in estrogen production. In the subsequent reactions, androstenedione and testosterone are formed, which in turn are aromatized into estrogens.

After production and secretion into the circulation, the vast majority of E2 is transported in the blood bound to serum albumin and sex-hormone binding globulin (SHBG). E2 reaches its target tissues via the blood circulation, and enters the cells by dissociation across the cell membranes. Inside the cells, estrogens bind the estrogen receptors (ERs) and regulate gene expression. Since ERs participate in numerous biological systems in the body, defects in estrogen signalling may result in many diseases. In addition, humans are exposed to environmental compounds or chemicals with ability to bind the ERs, thereby causing estrogenic effects, which might also play a role in the etiology of several diseases.

1.1.2 Estrogen receptors

ERs are members of the nuclear receptor superfamily. Two different ERs exist, namely ERα and ERβ. The first ER was identified in 1958 by Elwood Jensen and colleagues (3), later named ERα. Since then, substantial efforts have been made to explore its functions. Today, a considerable knowledge exists about its physiological roles. With antibody-based techniques such as immunohistochemistry, the tissue distribution of ERα has been extensively studied (4). Hence, ERα has been found in several organ systems, from brain to ovaries (5, 6).

Estrogen signalling through ERα plays a central role in many diseases such as breast and endometrial cancer, osteoporosis and cardiovascular disease. Also, inhibition of ERα activity has proven an effective treatment option in hormone dependent malignancies such as breast and endometrial cancer (7).

In 1996, a second estrogen receptor was discovered, subsequently named ERβ (8). Surprisingly, ERβ was shown to sometimes mediate opposite effects to ERα (9, 10). In addition, the two receptors are frequently distributed in different cells and organs (11, 12).

The two receptors are encoded by different genes and are located on different chromosomes. ERα is encoded at 6q25.1 and ERβ at 14 q23.2 (13, 14). However, structurally the two receptors have much in common. They consist of several individual domains, each with important functions. Close to the COOH-terminus (called the F-domain) is the ligand binding domain (LBD-domain) or E-domain with 59% homology between the two receptor subtypes. This region allows the receptors to dimerize and form functional homo- or heterodimers (15, 16). Furthermore, it contains one of the two transcriptional activating domains of the receptors, the activator function 2 (AF-2). The AF-2 domain induces ligand-dependent activation of promoter elements. Differences in the LBD-domain and hence, the ligand binding pocket, generate ER subtype specificity for both natural and synthetic ligands.

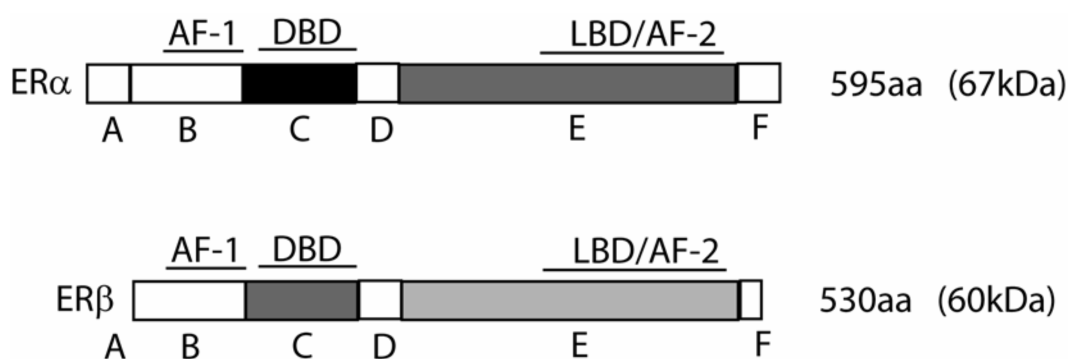

Figure 1. *Schematic representation of the ERα and ERβ structures. The domains of the receptors include the DBD, LBD and the two transcriptional activator domains AF-1 and AF-2. Full-length ERα is 595 amino acids long whereas ERβ is 530 amino acids long.*

The D-domain, or hinge-region, joins the E-domain to the C-domain, which comprises the DNA-binding domain (DBD-domain). This region is the most well-conserved region between the receptor subtypes with 97% homology. The NH_2-terminal A/B-domain contains the AF-1 domain, which is constitutively active and mediates transcriptional activation/inactivation independent of ligand binding. However, this area has only 16 % sequence homology between the receptor subtypes and the AF-1 domain of ERβ has a weaker function than that of ERα (17). Consequently, ERα and ERβ bind to estrogen response elements with similar affinity.

As identified with chIP-on-chip analysis, there is significant overlap between ERα and ERβ bound DNA-regions. However, there are regions which preferentially bind ERα or ERβ. Most often, the ERα-binding regions are rich in TA-rich motifs while the ERβ-binding regions are rich in GC-rich motifs (18).

Several human splice variants of ERα and ERβ have been found and characterized. Most of the ERα splice variants differ in the 5'-untranslated region (5'-UTR). These isoforms have not yet been found as functional proteins in human tissue, therefore their importance *in vivo* has been questioned. On the other hand, several ERβ-isoforms have been characterized as proteins in tissue (19, 20). One of these variants, called ERβcx (ERβ2), has a unique C-terminus with exon 8 replaced by an alternative exon of 26 amino acids (21). ERβcx forms non-functional dimers preferentially with ERα *in vitro* and therefore functions as a dominant negative inhibitor of ERα. The ERα/ERβcx heterodimer is unble to activate estrogen response elements and is subsequently degraded through the proteasomal pathway (22).

Although ERβcx does not bind E2, it cannot be excluded that it might bind to a yet unidentified ligand.

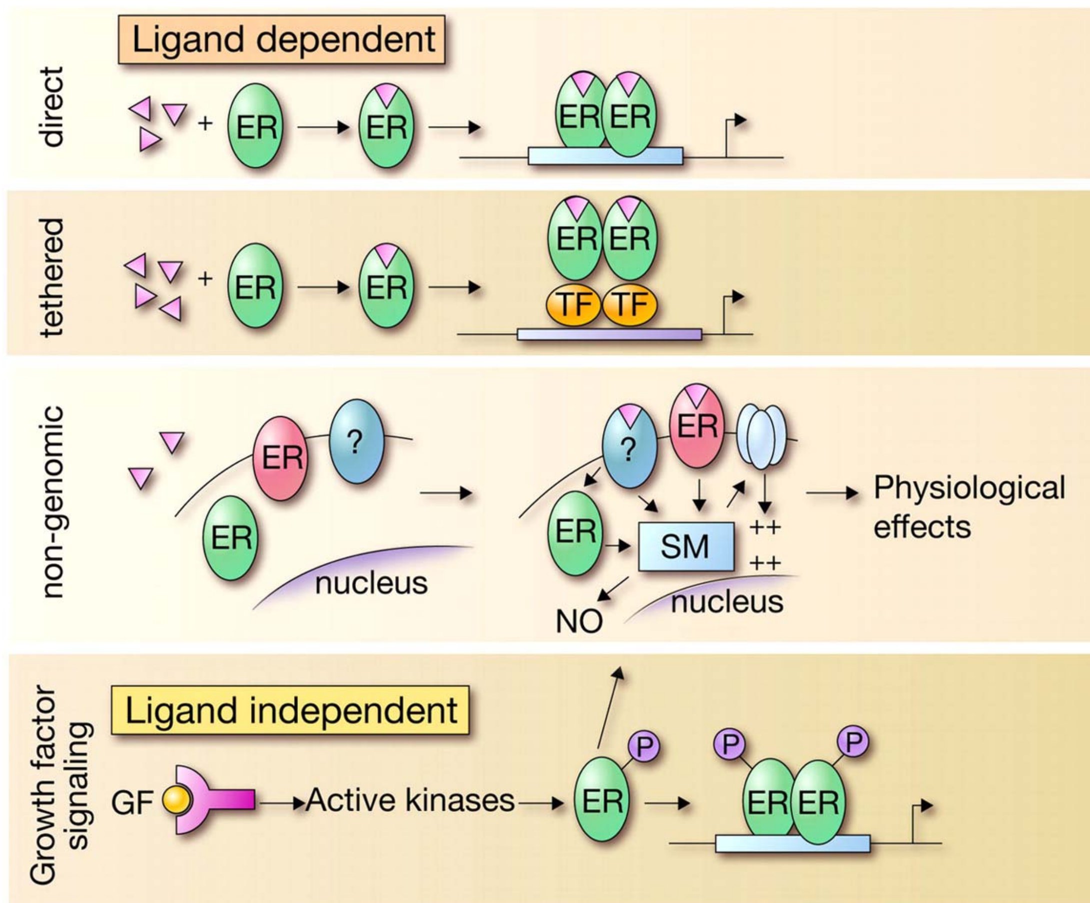

Figure 2. *Four different pathways of ER action: The classical (direct) pathway includes ligand activation and a direct DNA binding to estrogen response elements (ERE). The tethered pathway involves protein-protein interaction of ERs with other transcription factors and indirect DNA binding. The non-genomic pathway involves rapid estrogenic effects, which have been observed in some cells. ER-activity can also be regulated through a ligand-independent pathway by growth factor signalling. ER; estrogen receptor, GF; growth factor, P; phosphate, SM; second messager and TF; transcription factor. Used with permission from Heldring N, et al. Physiol. Rev. 2007; 87: 905-931.*

1.1.3 Estrogen receptors and promoter elements

In their unliganded state, ERs are associated with inhibitory protein complexes containing heat shock proteins (hsps) in the cytosolic or nuclear compartments. Upon ligand activation, the receptors dissociate, change conformation and form functional dimers at certain DNA-elements (23). Depending on the presence of ERα and ERβ or both in a specific cell, the receptors form functional homo- or heterodimers on the promoter elements. The classical pathway involves binding of ER-dimers to an estrogen response element (ERE), a palindrome with the sequence GGTCAnnnTGACC, where n can be any nucleotide.

In addition, ER can bind to DNA directly or indirectly through alternative elements. ER-binding at Activator Protein 1 (AP-1) responsive elements involves indirect binding through Jun/Fos-proteins (24). In addition, ER can activate transcription through Specificity Protein 1 (SP-1) (9).

When the receptors bind to DNA-response elements, transcription is affected through recruitment of co-regulatory proteins. Depending on the promoter context, type of ligand and receptor subtype, these co-regulatory proteins can be co-activators and co-repressors (25, 26). Co-activators modify the chromatin to facilitate recruitment of RNA-polymerase II, with subsequent transcription. Two ER-associated co-activators, the SRC-family and p300/CBP-associated factor have intrinsic histone acetylase (HAT) activity.

In contrast, co-repressors decrease acetylations in the chromatin, resulting in inhibition of the transcription machinery. ER-associated co-repressors such as SMRT and N-CoR recruit histone deacetylases (HDACs) which block the recruitment of the RNA-polymerase machinery to the promoter.

1.2 CELLULAR PROLIFERATION IN HEALTH AND DISEASE

1.2.1 The cell-cycle

A fundamental step in eukaryotic organisms is cellular proliferation or the division of one cell into two identical daughter cells, through a process known as mitosis. Uncontrolled proliferation is, however, the hallmark of cancer (27) and proliferation is therefore a tightly regulated process. Cellular proliferation is governed by the cell-cycle, which is divided into four different phases; Gap 1 (G_1), DNA-synthesis (S), Gap 2 (G_2) and the Mitosis (M-phase) (28). The G_1-phase is the only cell-cycle phase dependent on growth factors and external stimuli. In the G_1-phase, there is an active synthesis of proteins necessary for DNA-replication. In normal cells, incorrect G_1-progression or uncontrolled signalling will halt the cell-cycle in this phase. During the S-phase, DNA is replicated and in the G_2-phase there is a substantial protein production to prepare for mitosis. Finally, in the M-phase, the cell divides into two daughter cells.

In the adult organism, the majority of cells are non-proliferative. They are positioned in a quiescent state termed the G_0-phase, with the notable exception of stem cells. Upon the appropriate stimulation, some cells have the potential to re-enter the cell-cycle and proliferate. In humans, hepatocytes are an example of cells which are normally resting, but enter the cell-cycle during the right conditions, for example after partial hepatectomy (29, 30). Other fully differentiated cells, such as neurons, seldom or never enter the cell-cycle. Yet, it is now clear that even neuronal tissue has stem cells. Epithelial stem cells are potentially able to proliferate continuously in response to stimuli and both breast and colon cancer cells are of epithelial origin.

1.2.2 Cell-cycle regulation

Several check-points exist at which the cell-cycle can be arrested (31, 32). The cyclins and their associated cyclin dependent kinases (Cdks) are a class of key-regulatory molecules within the cell-cycle. These proteins were first described in yeast, and later in eukaryotes, a discovery that resulted in the Nobel Prize in medicine 2001 (33).

The cyclin molecule is the regulatory subunit of the cyclin/Cdk complexes and harbours an intrinsic preference for certain target factors, which are phosphorylated by the associated Cdks. Both Cdk 1 and 2 are normally kept inactive by inhibitory phosphorylations (34), which are reversed by the Cdc25-family of Cdk-phosphatases,

resulting in an active cyclin-Cdk1/2-complex (35). Therefore, in the late G_1-phase, activation of Cdk2 by Cdc25A is a crucial step in the G_1/S-phase transition (36). The two other members of the Cdc25-family, Cdc25B and C, are active in the late G_2-phase. In response to external mitogens such as E2, c-Myc is one of the first genes to be expressed within the G_1-phase (37). Both c-Myc and cyclin D have promoters that contain binding sites for several signal molecules responding to hormones and growth factors. Therefore, both these proteins are important initiators of the G_1-phase. When c-Myc is expressed, it in turn activates transcription of genes such as cyclin D1 and D2, cyclin E, $p21^{CIP1}$ and Cdc25A (38-42) as a Myc/Max-dimer. As cyclin D1 is induced it associates with Cdk4/6 and catalyzes the partial phosphorylation of the retinoblastoma protein (pRb) (43).

Subsequently, newly synthesized cyclin E associates with Cdk2 and catalyzes the phosphorylation of pRB (44).

In the normal non-proliferating state, pRb is hypophosphorylated and thereby keeps the transcription factors E2F in an inactive state (45). The phosphorylated pRB on the other hand, is unable to bind E2F-factors, which dissociate and activate transcription of numerous S-phase genes.

1.2.3 E2F1 and pRb

The members of the E2F-family have an important role in the G_1/S-phase transition and were first identified as factors with the ability to bind the adenovirus E2 promoter (46). The E2F-family is composed of 8 different members, encoded by separate genes. E2F1-3 are classically regarded as transcriptional activators whereas E2F4-6 are transcriptional repressors. But today we know that there is substantial overlap in the function of the different E2F-factors (47-50) and the two most recently found E2F-factors, E2F7 and -8, have important functions during developmental processes (51).

The free E2F-factors form heterodimers with the DRTF-polypeptide family of proteins (DP-family) (52, 53), and activate transcription at E2F-binding sites. E2F1 is the best studied E2F-factor and overexpression of E2F1 drives cells into S-phase (54). Overexpression of E2F1 can also rescue cells arrested by the factors $p16^{INK4B}$, $p21^{Cip1}$, $p27^{Kip1}$and TGFβ (47, 55, 56). Several genes important for cell-cycle progression are transcriptionally regulated by E2F1 including cyclin E, cyclin A and Cdc25A (57-59).

Since unrestrained E2F-activity could potentially lead to uncontrolled proliferation, the cells have developed control mechanisms. If E2F1 is overexpressed in a normal cell (60), E2F1 activates the transcription of $p14^{ARF}$, which is the product of the alternative

reading frame of $p16^{INK4A}$. $p14^{ARF}$ in turn, directly interacts with and inhibits MDM2, which normally targets p53 for degradation (61). The increase in p53-levels will initiate apoptosis or cell cycle arrest through induction of $p21^{CIP1}$. On the other hand, most tumors have a mutated p53-pathway and consequently, overexpression of E2F1 will then induce proliferation, and apoptosis to less extent.

The pRb-family of pocket proteins consists of pRB, p130 and p107. In quiescent cells, E2F1 forms complex with pRB, making E2F1 unable to work as a transcriptional activator (62). Instead, the E2F/pRb-complex might even actively repress transcription at E2F-binding sites.

In proliferating cells however, pRb is phosphorylated by cyclin D1-Cdk4/6 and subsequently by cyclin E-Cdk2, causing dissociation of free E2F (43, 44). The E2F-promoter is regulated by c-Myc as well as E2F1 itself. Therefore, dissociation of E2F1 from pRb leads to a positive feedback with transcriptional upregulation of E2F1 (63-65).

One of the most important steps in the regulation of E2F is its inactivation following DNA-synthesis. In the S-phase, cyclin A-Cdk2 phosphorylates both E2F1 and DP-1, thereby inhibiting binding of the heterodimer to DNA (66).

1.2.4 Cdk-inhibitors

The cyclins and their associated kinases are essential regulators of the cell-cycle. To control their activity, two families of cyclin/Cdk-inhibitors exist within the G_1-phase. The Inhibitors of kinase 4 (INK4) family of Cdk inhibitors are specific inhibitors of Cdk4 and 6 (67, 68) and consists of $p16^{INK4a}$, $p15^{INK4b}$, $p18^{INK4c}$ and $p19^{INK4d}$. The INK4-members form dimers with Cdk4 and 6, thereby preventing formation of active complexes with cyclin D.

The second group of Cdk inhibitors are the CIP/KIP-family, containing three members; $p21^{CIP1,}$ $p27^{KIP1}$ and $p57^{KIP2}$. They form inactive trimeric complexes with Cdk2 and cyclin E or cyclin A (69). $p21^{CIP1}$ is primarily regulated at transcriptional level and is induced in response to c-Myc. $p27^{KIP1}$ on the other hand, is regulated both at transcriptional and translational levels as well as through degradation. One important pathway is via $p45^{Skp2}$, which targets $p27^{KIP1}$ for ubiquitination and degradation (70, 71). However, the CIP/KIP-family members are not solely negative regulators of Cdks. Although overexpression of $p21^{CIP1}$ or $p27^{KIP1}$ inhibits proliferation, low levels of $p21^{CIP1}$ and $p27^{KIP1}$ are sequestered to the cyclin D/Cdk-complex (69) and thereby increase the Cdk-activity.

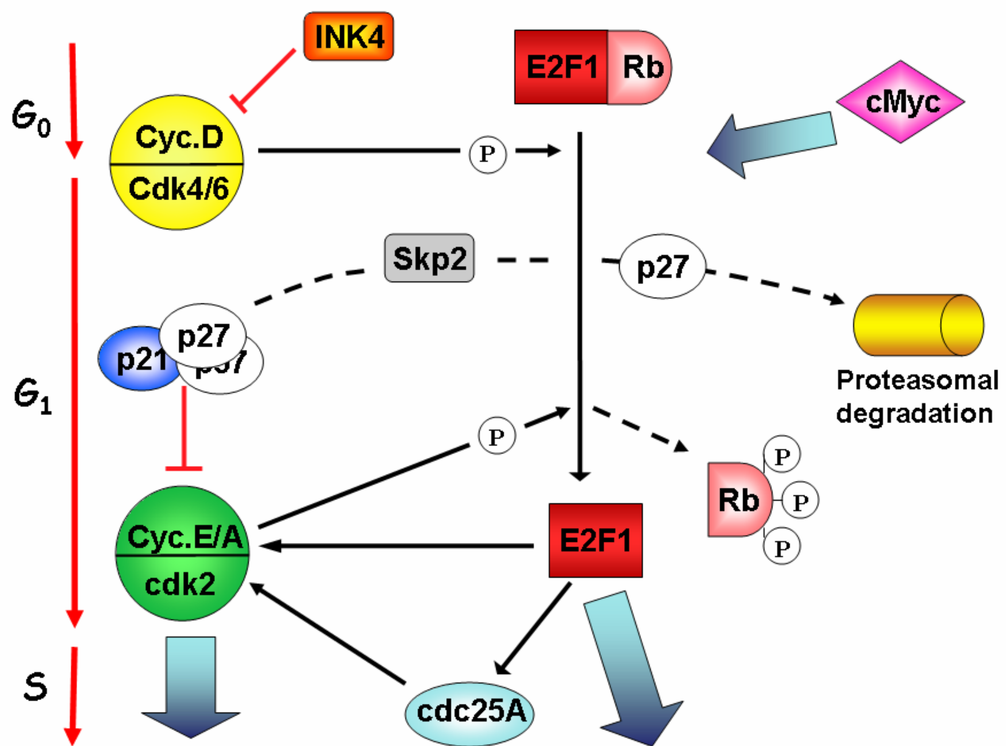

Figure 3. *Overview of the G_1-phase of the cell-cycle. Mitogen induced cell-cycle stimulation results in elevated expression of early cell-cycle genes such as cyclin D1 and c-Myc, which in turn regulates downstream factors. Phosphorylation of pRb by the cyclins with subsequent activation of E2F-factors is considered as the central mechanism in the activation of cell-cycle progression.*

1.2.5 The cell-cycle in cancer

Through proliferation, tumors grow and every new cell-cycle means a possibility for new, spontaneous mutations to occur. Without proliferation, mutant clones cannot accumulate and metastasis cannot occur. Additional mutations and genetic abnormalities mean a more malignant phenotype with increased risk for an aggressive disease. Therefore, dysregulation of the cell cycle and reduction in apoptosis are important components in tumorigenesis.

Uncontrolled cell-cycle progression can occur through different mechanisms. In tumors, several oncogenes are mutated, causing accumulation of active proteins, such as members of the Ras-family or its downstream effectors (72, 73). The tumor suppressor pRb is often mutated, causing a dysfunctional protein (74). Other genes involved in repression of tumorigenesis have methylated promoters, and hence, altered gene expression. These events may lead to overexpression of oncogenes or inhibition of tumor suppressors.

Since the G_1-phase is sensitive to external stimuli, increased growth factor signalling through overexpressed receptors can also induce proliferation. In breast cancer, the transmembrane EGF-receptor and Her2/neu are often overexpressed, leading to increased intracellular tyrosine kinase activity (75, 76).

Her2/neu is a ligand-independent transmembrane dimerisation partner for other members of the EGF-receptor family and overexpression of Her2/neu occurs through gene-amplification, resulting in permanently increased tyrosine kinase activity.

In cancer, the p53 signalling pathway is most often inactivated due to mutations or altered expression (77, 78). For that reason, the cancer cell will have a reduced capacity to enter the apoptotic pathway. An example of the central role of p53 and pRb as tumor suppressors is cervical cancer, which is caused by the human papilloma viruses (HPV) 16 and 18. HPV-encoded proteins E6 and E7 inhibit p53 and pRb, causing uncontrolled proliferation with low rate of apoptosis (79).

But pRb can get inactivated through other pathways as well. Overexpression of cyclin D1 and cyclin E are common in cancer (80-84). Since the cyclins are important regulators of Cdk activity, high cyclin D1 or cyclin E levels result in overactive cyclin-Cdk complexes and sequential neutralisation of pRb through phosphorylations.

The main function of pRb is to regulate the free E2F-factor concentration. Overexpression of free E2F1 occurs in cancer (85, 86). In a paper by Zhang *et al*, E2F1-expression was gradually increased in breast ductal carcinoma in situ (DCIS)

progressing to invasive ductal carcinomas (IDC). E2F1 also showed a positive correlation with Mib-1, which is an established proliferation marker in clinical pathology (87).

Within the INK4-family of Cdk-inhibitors, $p16^{INK4A}$ is often mutated in human cancer and has been identified as a tumor suppressor (88, 89). In the CIP/Kip family, $p27^{KIP1}$ is frequently reduced in breast cancer (90), most often as a result of increased $p45^{Skp2}$-dependent degradation (91).

1.2.6 Estrogen regulation of the cell-cycle in breast cancer

Many of the breast cancer cell lines cultured *in vitro* are dependent on estrogens for their proliferation (92). Hence, treatment of breast cancer cells with anti-estrogens leads to cell-cycle arrest or apoptosis. Estrogen dependent breast cancer cell-lines such as MCF7 and T47D are ERα positive but express low ERβ levels (93). To our knowledge, in all breast cancer cells studied *in vitro*, the proliferative effect of E2 is mediated by ERα. However, ERα induces proliferation by regulating multiple cell-cycle factors, which makes the mechanism complex.

The first G_1-phase factor acutely regulated by E2 is c-Myc, which is regulated at the transcriptional level (37). In synchronized MCF7 cells, expression of c-Myc mRNA starts upon 30 minutes after treatment with E2. As described above, c-Myc in turn, is a transcriptional activator of multiple cell-cycle factors.

Within three hours of E2 treatment, cyclin D1 expression is activated. Since cyclin D1 is a known target gene of c-Myc, c-Myc is proposed to play an important role in ERα-mediated induction of cyclin D1. However, E2-stimulated cyclin D1 transcription is also dependent on ERα binding to a cAMP-responsive element in the cyclin D1 promoter (94, 95).

E2F1-expression is activated by ERα both secondarily and through direct ER interaction with promoter elements. Direct activation of E2F1 is dependent on the promoter region between basepairs (bp) -146 and -54, which is activated by ERα in different ways depending on the cell type (96).

Cdc25A is another important down-stream target gene of c-Myc. However Cdc25A is also induced by ERα/SP-1 binding in concert with activation of an E2F1 binding site in the promoter region between bp -151 and -12 (97).

The central importance of cell cycle regulation by ERα could be a reason for the efficacy of anti-estrogens in breast cancer, since these drugs hit several pathways concurrently.

1.3 ESTROGEN RECEPTOR FUNCTION IN HEALTH AND DISEASE

1.3.1 Breast cancer

The incidence of breast cancer has steadily increased in Sweden during the recent 50 years. Although the reason is unclear, this alarming trend has raised the suspicion that dietary factors and hormone replacement therapy are in part responsible for the present situation.

Today, in Sweden, approximately 7000 women will be diagnosed with breast cancer and 1500 women die every year in this disease (98). Presumably, improved diagnostic methods such as continuous breast checks with mammography are one reason for the increased registered incidence of breast cancer, and also contribute to earlier detection. The increased incidence could also be related to environmental factors, diet and the increase in obesity among the population (99).

Most breast cancers are believed to develop spontaneously through accumulation of mutations and perhaps epigenetic changes throughout life. Early menarche and late menopause increase the risk of breast cancer whereas pregnancy especially at young age decreases the risk.

It is interesting to note that women in eastern Asia have lower risk of acquiring breast cancer compared to women of the western, industrialised world (100). There is now considerable evidence that the difference in incidence cannot be explained solely by genetic factors. Instead, the western diet could be an important risk factor. The typical Asian diet contains high levels of phytoestrogens, which are low in the western diet. It has been suggested that high levels of phytoestrogens, which have a binding preference for ERβ, could work as cancer preventing agents.

It is estimated that approximately 10% of all breast cancers are inherited. The most frequently mutated genes are BRCA1, BRCA2 and p53. Carriers of BRCA1 have a more than 80% risk of breast cancer at age 70 (101).

In the last ten years, several new treatment options for breast cancer have become available. Two of these are the monoclonal antibodies bevacizumab and trastuzumab. Bevacizumab (Avastin®) is an inhibitor of VEGF, which stimulates new blood vessel

formation. As a result, inhibition of VEGF by bevacizumab decreases blood supply to the tumor. Trastuzumab (Herceptin®) inhibits the transmembrane protein HER2/neu, which is amplified in 20-30 % of early stage breast cancers. Amplified and overactive HER2/neu promotes growth and invasion through the PI3K/Akt-pathway and other signalling molecules.

Still, the best treatment option for localised breast cancer is surgery. The surgical method of choice depends on tumor stage. Classically, the tumor has been removed with complete removal of the diseased breast (mastectomy). Today, tumors localised to the breast without lymph node metastasis are removed with partial mastectomy (lumpectomy) followed by radiation therapy. In addition, these patients are often treated with adjuvant anti-estrogens for five years to minimise the risk of recurrence (98) in ERα+ tumors.

Pathologically, breast cancers are divided into different subtypes depending on expression of ERα, PR and Her2/neu. This separation makes sense from a medical perspective since the treatment options are dependent on receptor expression.

Another classification is based on molecular profiling, separating cancers into luminal, basal and Her2+ subtypes, which is a more biologically based classification (102).

The majority of breast cancers are diagnosed within the perimenopausal or postmenopausal period. 80% of these tumors are ERα-positive and dependent on estrogen for growth. Therefore, the first line of pharmacological treatment of these patients is anti-estrogens. This is an effective treatment with few side-effects compared to cytotoxic drugs.

Finally, breast cancer prognosis is dependent on many factors; the most important is the tumor stage; in recurrent disease, therapy is focused on prolonged life and palliative care.

1.3.2 Estrogens, health and breast cancer

In the menopausal period, the decreasing estrogen production of the ovaries leads to negative effects on mood and well-being for many women. But the declining estrogen production has other physiological effects as well. In elderly people, there is a gradual loss of cognitive functions, and this process is more pronounced in women after menopause. The hippocampus is a part of the brain involved in memory processing. In a study performed with PET/MRI-imaging, there was a more pronounced decrease of hippocampus volume in postmenopausal women compared to men of the same age (103). The authors also described a changed metabolism in the hippocampus area of

postmenopausal women. In a meta-analysis performed on several RCT-studies, Yaffe *et al* showed than cognitive performance was better in estrogen-treated than in non-estrogen treated postmenopausal women (104).

Low estrogen level which is often seen in postmenopausal women is also a strong risk factor for osteoporosis. Normally, bone turnover is a permanently ongoing process. Osteoblasts synthesize and osteoclasts degrade bone continuously (105). However, in conditions with low circulating estrogen levels such as after menopause, bone resorption outweighs bone synthesis and results in osteoporosis. Osteoporosis is a serious condition and is the major cause of fractures in old people. Since these patients have to be treated for long time, treatment of osteoporosis related fractures is extremely expensive for the health care system. Hence, hormonal replacement therapy (HRT) with estrogen has been shown to be an effective therapy to prevent osteoporosis in these women. HRT is also the only efficient treatment for women with menopausal symptoms on mood and well-being. To protect the endometrium, estrogen treatment is combined with progesterone in non-hysterectomised women (106).

Since millions of women are treated with HRT every year, it was necessary to investigate the potential health effects in a randomised, placebo controlled and blinded study.

The Women´s Health Initiative (WHI) trial was initiated by the National Institute of Health (107). One of the objectives was to investigate possible health effects of estrogens and progesterone in postmenopausal women. This clinical trial was performed on more than 10,000 women in the US.

The trial showed that combined estrogen-progesterone treatment of healthy postmenopausal women increased the risk of breast cancer. This report caused a huge drop in the prescription of HRT worldwide (108). However, the WHI study also showed that treatment with estrogen alone does not increase the risk of breast cancer (109). Today, in Sweden, HRT is only recommended for women with severe menopausal symptoms, not as a first line therapy for osteoporosis (110).

Even if estrogens do not cause breast cancer, and might even be protective, the role of estrogens in breast cancer progression is well-characterised. Blocking endogenous estrogens by anti-estrogens or castration is an effective treatment for the majority of postmenopausal breast cancer patients. But this treatment is sometimes associated with adverse effects such as depression, sleeping disorders and atherosclerosis-related vascular disease (111).

1.3.3 Estrogen receptors in the normal and cancerous breast

In the human breast, ERβ is the predominant ER. In rodents, ERβ is expressed in luminal epithelial cells of the ducts as well as in the surrounding stromal compartment. In contrast to ERβ, ERα is expressed in low levels and only within the luminal cells (112-114). In breast cancer, on the other hand, ERα is the predominant ER and approximately 80% of newly diagnosed breast cancers contain ERα within the cancer epithelial cells. ERβ is expressed at low levels in advanced breast cancer. However, the reason for this discrepancy between normal and cancerous breast is unclear (115). Interestingly, Zhao *et al* showed that the ERβ promoter region was hypermethylated in breast cancer cells compared to normal breast epithelial cells and the methylation pattern was inversely correlated to the ERβ mRNA-level. Furthermore, treatment of breast cancer cells with a demethylating agent reactivated the ERβ-expression (116). We therefore suggest that promoter methylation must be a partial explanation to the transcriptional downregulation of ERβ found in breast cancer.

1.3.4 SERMs in breast cancer treatment

The Selective Estrogen Receptor Modulators (SERM) are compounds with mixed ER mediated agonist and antagonist activity, depending on tissue, ER subtype and the presence of co-activators or co-repressors. The most well known SERM is tamoxifen, a so-called anti-estrogen used in adjuvant and palliative treatment of breast cancer. Tamoxifen has proven to be effective against micrometastatic disease and five years treatment reduces the recurrence by 47 % compared to placebo (117). Tamoxifen is a pro-drug metabolised to its active forms 4-hydroxytamoxifen and endoxifen in the liver (118) and is secreted from the body as hydroxylated metabolites.

Tamoxifen is well-studied and widely used as an anti-estrogen in the clinic today. Tamoxifen displays antagonist-activity in the breast but agonist activity in other tissues including the bone, uterus, liver and the coagulation system (7). This means that tamoxifen is an efficient therapy for ERα+ breast cancer and at the same time, diminishes osteoporosis and causes a positive serum lipid profile with reduced levels of low-density lipoprotein (LDL) cholesterol and total cholesterol (119, 120). On the other hand, tamoxifen increases the risk of endometrial cancer as well as thromboembolic events (121).

Tamoxifen was introduced already during the 1970s and is therefore by far the best studied SERM. The mechanism behind the anti-tumorigenic function of tamoxifen is

through inhibition of the AF-2 activity of ER, causing a specific conformational change of the receptor and recruitment of corepressors (122). The AF-1 domain of ER still remains active in the presence of tamoxifen.

In Sweden, tamoxifen is the first line adjuvant therapy for ERα+ breast cancer with a standard treatment regime of five years. However, it is estimated that 30% of the treated patients do not respond to tamoxifen treatment, which is called primary resistance.

Raloxifen is a newly developed SERM with the same agonist profile in bone and liver but with antagonist activity in breast and uterus (123). Because of its non-tumorigenic effects in uterus, this drug has been approved for prevention of osteoporosis in high-risk women. However, raloxifen is not approved for treatment of ERα+ breast cancer since sufficient information of potential side-effects are lacking (124).

The aromatase inhibitors are a new treatment option for postmenopausal women with ERα+ breast cancer. Several clinical trials have shown that these drugs are superior to tamoxifen in preventing recurrence (125-127), but are associated with higher incidence of fractures. Fulvestrant (ICI 182,780) is another endocrine treatment option, functioning as a complete ER-antagonist with no agonist effects (121).

Findings from adjuvant studies have revealed that tamoxifen also reduces the incidence of breast cancer in the contralateral breast. Therefore, several randomised clinical trials have been performed with tamoxifen as a preventive agent for breast cancer in high risk women. The largest of these, the National Surgical Breast & Bowel Project P-1 trial, was performed with 13 888 women with high risk of developing breast cancer (128). The trial revealed a 49 % relative risk reduction of developing invasive breast cancer in women treated with tamoxifen compared to placebo. One additional trial showed a relative risk reduction of 32 % (129), whereas two other trials have failed to show any significant risk reduction (130, 131).

In conclusion, tamoxifen is still the most important endocrine treatment option for breast cancer patients, but the role of ERβ as a mediator of tamoxifen response is unclear.

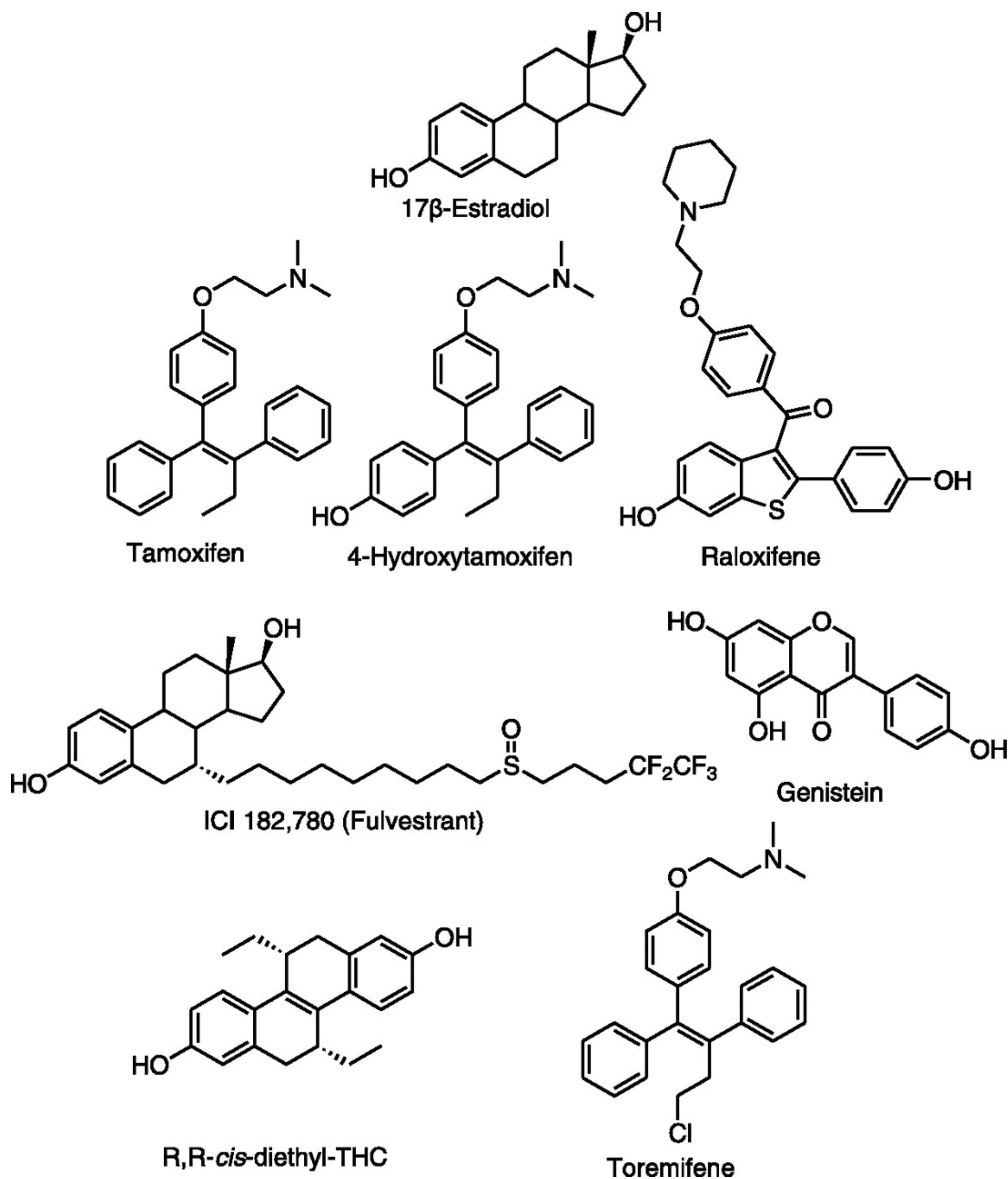

Figure 4. *Structure of the ER-ligands 17β-estradiol, tamoxifen, 4-hydroxy-tamoxifen, ramoxifene, ICI 182.780 (fulvestrant), toremifene, R,R-cis-diethyl-THC and genistein. Used with permission from Heldring N, et al. Physiol. Rev. 2007; 87: 905-931.*

1.3.5 Endocrine resistance

Endocrine resistance in breast cancer means tumor cells which grow in the presence of anti-estrogens. Resistance can be divided into primary resistance or acquired resistance. The luminal breast cancer subtype is mostly estrogen dependent, tamoxifen responsive, whereas the basal and Her2+-subgroups seldom respond to adjuvant tamoxifen.

If treatment with adjuvant tamoxifen does not fully eradicate all tumor cells, it is likely that the patient will acquire tamoxifen resistant breast cancer cells. These cells will finally grow even in the presence of tamoxifen and cause recurrent disease. At this point, complete remission is not possible.

Different pathways have been suggested for this mechanism:

1. Stimulation of the agonist activity of ERα: Tamoxifen inhibits the AF-2 activity of ERα, thereby causing an overall antagonistic profile in the breast. But tamoxifen works as an agonist for AF-1 activity. Therefore it has been speculated that the AF-1 domain has a central role in the agonist profile of tamoxifen in tissues such as the uterus, but could also be a mechanism for acquired resistance. Phosphorylation of the AF-1-domain can further stimulate its activity (132), causing a constitutively active protein.

2. Changed cellular co-factor dynamics: Tamoxifen-bound ERα represses transcription by interacting with co-repressors. Inhibition of expression of co-repressors and stimulation of expression of co-activators are potential pathways to tamoxifen resistance (133, 134).

The co-activators of the SRC-family seem to be particularly important. AIB1 (SRC-3) is overexpressed in more than 50% of breast tumors, pointing at central role in tumor development (135).

3. ER-crosstalk with growth factor receptors: Overexpression of Her2/neu in MCF7 breast cancer cells *in vitro* causes tamoxifen resistant cells and treatment of Her2+ MCF7 cells with an anti-Her2 antibody reverses tamoxifen resistance (136, 137).

In addition, expression of the EGF receptor and Her2/neu correlates inversely to ERα expression in breast cancers (138).

The Her2-receptor affects ER-signalling in several ways (139). Increased signalling through Her2 or the EGF receptor causes increased intracellular protein kinase activity, which targets residues within the AF-1 domain of ER for phosphorylation (140, 141). This activity has been shown to make both ERα and ERβ unresponsive to estrogens, although through different signalling pathways (142). Furthermore, signal transduction

through protein kinases leads to phosphorylation of ER co-regulators, which can change their activity or localisation (143, 144).

Interestingly, tamoxifen can also increase the transcription of Her2 through the co-activator SRC1 (145), which is in line with the hypothesis that crosstalk between growth factor receptors and ER is a central mechanism of endocrine resistance. Moreover, some evidence from clinical trials have shown that Her2 expression is associated with tamoxifen resistance and that tamoxifen treatment might even harm patients with Her2+ breast cancer (146).

The aromatase inhibitors have proven to be superior to tamoxifen in prevention of endocrine resistance. This is especially important in patients co-expressing ERα and Her2. However, even patients treated with aromatase inhibitors will finally acquire endocrine resistance (147).

Treatment of endocrine resistance, i.e. cancer cells which are not dependent on estrogens to grow, and especially tamoxifen resistance remains as a great challenge for cancer researchers and clinicians. So far, focus has been on the coupling between ERα and tamoxifen resistance, since model systems for ERβ+ breast cancer have been absent. Furthermore, as ERβ does not harbour significant AF-1 activity, it has been considered less important in the endocrine resistance process.

In the coming years, it will be important to get insight into ERβ-function in endocrine resistance. If not, we will not get a complete picture of the endocrine resistance mechanism, a key component in the development of new drugs.

1.3.6 Phytoestrogens

The phytoestrogens are naturally occurring estrogens (148). Several studies indicate that in countries with high intake of phytoestrogens in food there is a lower incidence of breast cancer (149, 150). Therefore, it has been suggested that phytoestrogens may have a potential role in preventing breast cancer.

There are several families of phytoestrogens, of which the isoflavones, found predominantly in soy beans and red clover, and the lignans found in fruit and vegetables are the best studied and most frequently found in food. Other families of phytoestrogens include the coumestans, found in bean shoots and resveratrol, the phytoestrogen of red wine (151, 152). The phytoestrogens are metabolised by the intestinal microflora before they are absorbed by the enterocytes. The main isoflavones, genistein and daidzein, are subsequently metabolised in the liver before they are

converted to their active metabolites (151). There is a great variation in the metabolism of phytoestrogens in humans due to genetic factors, use of antibiotics and composition of the intestinal microflora (153, 154).

Phytoestrogens bind to ERα and ERβ with low affinity compared to that of E2. On the other hand, most of them have greater affinity for ERβ than for ERα, whereas E2 binds to these receptors with similar affinity. The two isoflavones genistein and daidzein, bind to ERβ with ten times higher affinity than for ERα (148).

1.3.7 Phytoestrogens and breast cancer

In several studies the effects of phytoestrogens on proliferation *in vitro* have been examined. In doses less than 1 μM, the isoflavones stimulate proliferation of MCF7 breast cancer cells whereas higher doses inhibit proliferation (155, 156). In *in vivo* xenograft models, performed with MCF7 cells, several researchers have observed tumor growth stimulatory effects after genistein treatment (157, 158). However, in other studies performed with chemically induced endogenous tumors in rats, inhibitory effects of phytoestrogens have been reported (159). One explanation for these inconsistent findings could be that the anti-carcinogenic and tumor suppressive effects of some phytoestrogens are mediated by ERβ, whereas ERα stimulates proliferation. Since MCF7 cells are ERα+ ERβ-, the tumor suppressive effects cannot be studied with this system. On the other hand, chemically induced tumors in rats contain endogenous ERβ and could therefore be growth-inhibited by an ERβ agonist.

It should be pointed out that phytoestrogens could have other, ER independent effects mediated by protein-tyrosine kinases, and these effects are believed to occur with higher concentrations of phytoestrogens (160).

Several case control studies with soy have been performed. Generally, those studies show a protective effect of phytoestrogens on breast cancer incidence, especially in premenopausal women (161, 162), but the results are inconsistent and there are reports in which no relation between soy consumption and breast cancer have been found (163).

In a meta-analysis of published cohort and case-control studies, high versus low soy intake was associated with lower breast cancer incidence. In addition, the breast cancer preventive effects were more pronounced in premenopausal women (164).

Only a few epidemiological studies of lignan consumption and breast cancer have been published, but these studies consistently report a protective effect associated with lignan consumption (165, 166).

It has been speculated that the breast cancer protective effect of phytoestrogens is dependent on the age at exposure. In prepubertal rats treated with genistein, the breast cancer incidence was lower in the adult animal (167). Furthermore, in a study performed in Canadian women, high consumption of phytoestrogens in adolescence had a protective effect against development of breast cancer later in life (168).

In summary, results from experiments performed in cell lines and animal models are inconsistent. In many cases, this is the result of poorly planned experiments. Even if many cohort and case-control studies show a protective effect of phytoestrogens, the data are not strong enough for general recommendations. Moreover, many of the dietary supplements containing phytoestrogens include various mixtures of compounds, which makes it difficult to draw conclusions about specific phytoestrogens. Hopefully, ongoing randomised clinical trials will answer many of these questions.

1.3.8 Estrogens and angiogenesis

Angiogensis is the process by which blood vessels are formed and renewed in adult organisms. The primary components of blood vessels are the endothelial cells, lining the blood vessel wall. In the angiogenic process, endothelial cells proliferate but can also be recruited from bone marrow stem cells (169). The driving force for angiogenesis is hypoxia, causing transcriptional upregulation of HIF1α and secretion of growth factors such as vascular endothelial growth factor (VEGF). In endothelial cells, membrane bound VEGF-receptor 2 mediates these signals, which results in stimulation of the cell-cycle.

In women, the continuous growth of blood vessels within the endometrium during the proliferative phase is an example of normal angiogenesis. Another example is the growth of vessels which takes place during wound healing. But angiogenesis has also a central role in many diseases such as rheumatoid arthritis, diabetes retinopathy and cancer. In cancer, tumors cannot grow larger than a size of 1 mm in diameter without the formation of new blood vessels (170). Therefore, targeting angiogenesis is a promising new therapy in oncology.

In Sweden, the VEGF monoclonal antibody bevacixumab (Avastin®) is approved for treatment of metastatic colon and breast cancer in combination with chemotherapy (171).

Estrogens are important in angiogenesis and depending on the cellular localisation, endothelial cells contain either ERα, ERβ or both receptors. Estrogens stimulate angiogenesis through different processes. In xenograft experiments, estrogens stimulate angiogenesis of implanted breast cancer tumors and antiestrogens inhibit angiogenesis (172). In studies performed with clinical breast cancer specimens, it was found that tamoxifen reduced the vascular density within the tumor (173), thereby causing tumor regression.

In a study performed with ERα-knockout mice, loss of ERα prevented E2-stimulated angiogenesis (174). On the other hand, E2 treatment of endothelial cells only expressing ERβ did not cause increased proliferation (175). Therefore, we speculate that ERα mediates the pro-angiogenic effects on endothelial cells. One mechanism could be through ERα stimulated upregulation of VEGF receptor 2 which makes the cells more sensitive to external stimuli (176, 177). However, tumor angiogenesis is also dependent on growth factor secretion from cancer cells and the precise function of ERα and ERβ in this context is not known.

1.3.9 The role of ERβ as a diagnostic marker for breast cancer

ERα is a well-established predictive factor and a marker for hormone-dependence of breast tumors. The importance of ERβ as a marker in the pathological examination is not as well established. Today, ERβ-status is not routinely checked in breast cancers and the precise role of ERβ as a diagnostic maker is not clear.

In breast carcinogenesis, the cellular levels of ERα increase at the expense of ERβ levels which decrease (178, 179). The mechanism by which ERβ is downregulated is not fully understood, but epigenetic changes could play a role. In a paper by Zhao *et al*, a correlation was found between ERβ promoter methylation and mRNA expression in both cell lines and breast cancer tissues (116). Since there has been a lack of well-characterised ERβ antibodies, many researchers have tried to correlate ERβ mRNA with treatment and survival parameters. Studies have described ERβ mRNA as a marker for poor prognosis and endocrine resistance, whereas other studies have found a correlation of ERβ mRNA with good prognosis (115, 180, 181). The problems linked to mRNA measurements of whole tumors are apparent since the mRNA level does not fully predict the protein level. Also, a tumor consists of several cell types: cancer cells, endothelial cells, fibroblasts, lymphocytes and normal mammary epithelial cells.

In recent years, several ERβ specific antibodies have become available, opening up for immunostaining of breast cancer samples. With this method, several investigations have supported a role for ERβ protein as a marker for good prognosis (182-186). But in other studies, no significant correlations have been found (187, 188). Interestingly, increased levels of ERβcx have been found in breast cancer compared to normal breast epithelium (189).

A major obstacle in medical oncology of today is to decide whether a patient will respond to tamoxifen or not, since expression of ERα does not tell the whole truth. Therefore, the potential function of ERβ within this context has been investigated. In a study performed on 50 breast tumors from patients treated with adjuvant tamoxifen, significantly lower ERβ but not ERβcx expression was found in tamoxifen resistant tumors (190). In another study, the expression of ERβ was associated with tamoxifen resistance (191).

One problem with the detection of ERβ is the lack of standardized scoring systems for immunohistochemistry. An even larger problem is the need for standardized antibodies. Cross-reaction of ERβ antibodies against different isoforms is a major problem since at least ERβ1 and ERβ2 (ERβcx) have different functions as described above.

1.4 ESTROGEN SIGNALLING AND HES-1

1.4.1 The Hes-family

First described as inhibitor of neuronal differentiation in drosophila, *Hairy* and *Enhancer of Split 1* (Hes-1), is a member of the helix-loop-helix (bHLH) family of transcriptional regulators (192). This evolutionarily conserved family is composed of 125 members in humans. The Hes-family, Hes-1-7 are transcriptional repressors and have been studied thoroughly in neurogenesis and during embryonic development (193). The Hes-members bind as dimers directly to specific DNA-sequences, CACGAG or CACAAG, called N-boxes (194).

Structurally, Hes-1 has rather low homology with the other Hes-family members. In the COOH-terminus, Hes-1 has a repression domain with the tetrapeptide sequence WRPW. Close to the NH_2-terminus, Hes-1 has a basic domain with a proline residue, important for DNA-binding. This proline residue is a well conserved domain throughout all Hes-family members. The bHLH domain is responsible for dimerisation of Hes-1 with other bHLH-factors. The central part of Hes-1 has an Orange-motif, a well-conserved sequence involved in repression (195-198).

In humans, Hes-1 works as a transcriptional repressor by binding as homodimer to N-boxes within or outside of promoters. DNA-bound Hes-1 recruits the corepressor transducin-like enhancer of split homologue (TLE), through its WRPW-motif (199). TLE in turn, is phosphorylated by the casein kinase 2 (CK2) and engages histone deacetylase-1 (HDAC-1) which modifies chromatin and makes the DNA-helix inaccessible to the transcriptional complex (200).

In human cells, Hes-1 expression is not continuously on or off. Instead, Hes-1 expression seems to oscillate with a 2-hour cycle (201). Since both Hes-1 mRNA and protein are rapidly degraded with a half-life of less than 25 minutes, this oscillation can be regulated at the promoter level. Within the Hes-1 promoter, four independent N-boxes are located. By binding to these N-boxes, Hes-1 represses its own transcription. Furthermore, Hes-1 is expressed in response to serum treatment, which induces an oscillatory expression. The exact mechanism behind growth factor activated transcription of Hes-1 is unclear.

1.4.2 Hes-factors in Notch signalling

Notch signalling is an important mechanism during developmental cell-fate decisions. During embryonic neurogenesis, Notch acts as an anti-differentiating factor (202). By a

process known as lateral inhibition, a population of pro-neuronal cells can suppress the surrounding cells from developing (203). This is done through inhibitory signals, which are transduced through the Notch receptors. The process of lateral inhibition is important to limit the number of neuronal cells. The Notch genes encode the transmembrane proteins which act as receptors for the human ligand families Delta and Jagged. Upon activation of a Notch receptor, the intracellular part of the protein is cleaved off and released into the cytoplasm (204). The Notch intracellular domain (NICD) enters the nucleus where it binds to the transcription factors CBF1, Suppressor of Hairless and Lag-2 (CSL). The CSL-transcription factors are turned into transcriptional activators and stimulate expression of specific genes. One of its targets is the gene encoding Hes-1, which is expressed in response to Notch signalling (205).
Notch signalling has been extensively studied *in vitro* and today, it is believed that Notch signalling goes through other pathways as well. Since Notch signalling is an important, anti-differentiating process during embryonic development, its potential role in cancer has been investigated. Abnormal Notch signalling has been observed in some cancers such as acute T-cell lymphoblastic leukaemia and cervical cancer. In human breast cancer however, the potential involvement of Notch signalling is not fully understood (206-209).

1.4.3 Hes-6, an inhibitor of Hes-1

Hes-6 is a non-DNA-binding member of the Hes-family, exhibiting structural homology to other Hes-factors. It has the conserved proline residue within the basic region and the COOH-terminal WRPW domain. However, the loop region of Hes-6 is five amino acids shorter than that of Hes-1. Hes-6 was first described in the mouse embryo, where it is expressed in multiple tissues (210, 211). According to the expression pattern of Hes-6, it was assumed to play a role in neurogenesis and myogenesis. Later on, Hes-6 was established as an inhibitor of Hes-1. As we know today, Hes-6 acts as a suppressor of Hes-1 activity through two different mechanisms: First, Hes-6 binds to Hes-1 and prevents the association with the co-repressor TLE; second, Hes-6 increases degradation of Hes-1 (212, 213). As a consequence, Hes-6 promotes neuronal differentiation during embryonic development.

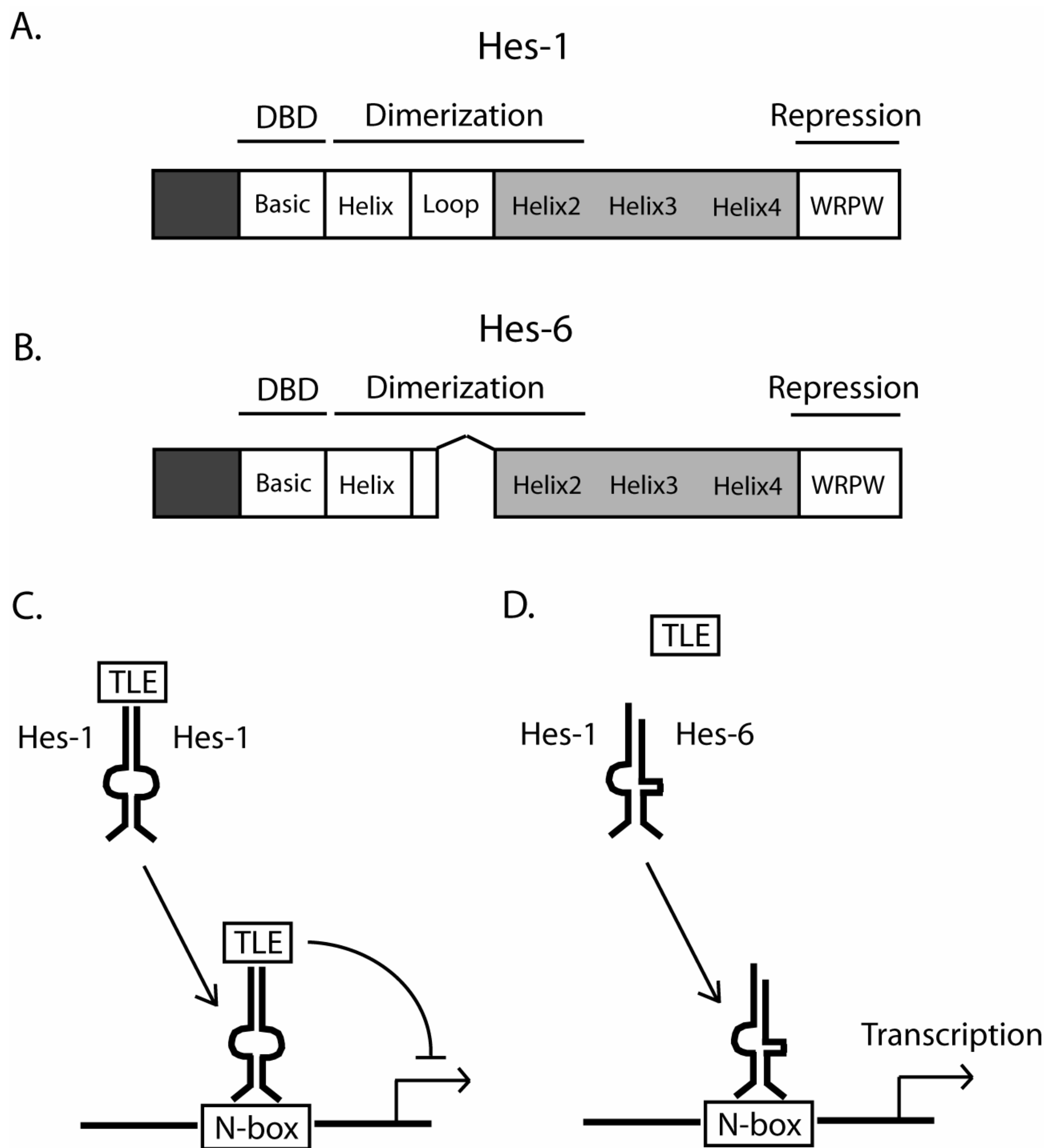

Figure 5. A. *Schematic representation of the Hes-1 structure.* ***B.*** *Schematic representation of the Hes-6 structure.* ***C.*** *Human Hes-1 dimers bind to N-boxes within gene promoters, thereby repressing transcription through recruitment of the co-repressor TLE.* ***D.*** *The Hes-1-Hes-6 heterodimer binds to N-boxes but cannot recruit the co-repressor TLE. Thereby, Hes-6 inhibits the activity of Hes-1.*

1.4.4 Hes-1 as a mediator of estrogen signalling

As discussed above, Hes-1 is a transcriptional repressor. In the estrogen-dependent, human breast cancer cell lines T47D and MCF7, Hes-1 expression causes inhibition of proliferation (214). E2 treatment causes repression of Hes-1 protein in these cells, and the repression can be reversed with anti-estrogens. As T47D and MCF7 cells contain ERα but no ERβ, the effect is most likely mediated by ERα. All-*trans*-retinoic acid (atRA) opposes the proliferative effects of E2 and prevents estrogen-mediated downregulation of Hes-1 in MCF7 cells (215). These results indicate that downregulation of Hes-1 is a necessary step in the proliferative response to E2. The validity of this conclusion was confirmed by the use of a dominant negative version of Hes-1 (dnHes-1) (mutated basic region). In cells expressing dnHes-1, E2 still caused proliferation but co-treatment with atRA could no longer prevent estrogen-induced proliferation. Thus estrogen-induced proliferation requires the downregulation of Hes-1 and atRA opposes estrogen's proliferative action by preventing the downregulation of Hes-1.

In primary endothelial cells (HUVEC), Hes-1 was upregulated in confluent, non-proliferating cells via a mechanism independent of Notch signalling (216). This points at multiple regulatory pathways of Hes-1 and it is likely that Hes-1 regulation is cell and tissue specific.

1.4.5 Expression of bHLH-factors in cancer

Given that Hes-1 functions as an inhibitor of proliferation in the experimental setting, it could be assumed that Hes-1 should be downregulated in human cancers. But the situation could be more complicated since inhibition of Hes-1 has not been found in breast cancer. In our laboratory, we have examined Hes-1 mRNA levels in a large set of breast cancers and normal breast tissue, without finding any correlation to clinical parameters (unpublished data). However, in pancreatic endocrine tumors, Hes-1 is downregulated at the protein level compared to normal pancreatic tissue. This downregulation of Hes-1 also correlates with the cytoplasmic localisation of Hes-1, which is normally found in both cytoplasm and nucleus (217).

By using a method called suppression subtractive hybridisation, Weinstein and colleagues searched for differentially expressed genes in colon cancer metastases compared to their primary tumors (218). A mouse xenograft model with HT29 colon cancer cells was used as model. They observed several genes that were upregulated in

different metastatic samples, but the only gene which was upregulated in all metastases compared to their primary tumor was Hes-6.

Another type of cancer in which Hes-6 has been identified is the CD4+CD56+ hematodermic neoplasm; an aggressive haematological malignancy initially found in the skin (219). This malignancy has poor prognosis and is resistant to chemotherapy. It has been difficult to differentiate this tumor from coetaneous myelomonocytic leukaemia, and therefore, a gene expression study was performed to compare these two malignancies. Different gene expression profiles were found and most interestingly, Hes-6 was identified as one of the two most overexpressed genes in the hematodermic neoplasm (220).

Recently, Hes-6 was also identified as a marker for an aggressive phenotype of prostate cancer (221). In this study, the authors used a hormone independent metastatic LNCap cell line in a xenograft model. They also found overexpressed Hes-6 in clinical specimens of metastatic prostate cancer compared non-metastatic tumors. Altogether, these data point at a central role for Hes-6 in human cancer and especially during metastasis. With our current knowledge, Hes-6 seems to be a potential oncogene, the expression of which results in an aggressive disease.

In conclusion, the bHLH factors are involved in several types of cancer. Based on current knowledge, bHLH factors seem to be especially important in tumors with a neuroendocrine phenotype.

2 AIMS OF THE STUDY

General aims

Estrogen signalling through ERα in breast cancer has been investigated thoroughly during the last decades, whereas estrogen signalling through ERβ and its effects on proliferation has been considerably less studied. Therefore, the overall objective of this thesis has been to investigate the function of ERβ in breast cancer proliferation. Since Hes-1 has been shown to play an essential role in ERα-mediated proliferation in breast cancer cells, the second major objective has been to explore the mechanism by which Hes-1 inhibits estrogen stimulated proliferation and how Hes-1 expression is regulated.

Specific aims

- In **paper I**, the aim has been to study how ERβ affects proliferation in ERα+ breast cancer cells, as well as to identify how central G_1-phase cell-cycle regulators are affected by ERβ-expression.

- The purpose of **paper II** has been to explore the function of ERβ in the context of *in vivo* tumor growth, focusing on angiogenesis and growth factors.

- In **paper III,** we investigated the mechanism by which Hes-1 inhibits E2 and heregulin-β1 mediated proliferation in breast cancer cells.

- In **Paper IV,** our aim was to investigate whether Hes-6 was regulated by E2 and to study the function of Hes-6 in breast cancer cells.

3 MATERIALS AND METHODS

Design and generation of tetracycline-inducible expression

To generate ERβ-inducible cells, normal T47D breast cancer cells were initially transfected with the transcriptional *trans*-activator (pTet-tTAk-vector) (GIBCO). By integrating puromycin resistance into the vector, cells containing the pTet-tTAk-vector could be selected with 0.5 μg/ml puromycin. Subsequent transfection of the selected cells with the luciferase vector pUHC13-3 revealed the presence of clones with high tTAk-expression. The 485 amino acid version of ERβ (**paper I** and **II**) or full-length Hes-1 (**paper III**) was fused to the FLAG-tag and cloned into the PBI-EGFP vector (Clontech). These constructs were co-transfected together with a neomycin resistance plasmid into the previously selected clones, and finally grown in presence of 500 μg/ml geneticin (G418) for selection.

Generation of breast cancer cells with continuous Hes-6 expression

T47D and MCF7 breast cancer cells were lentivirally transduced to obtain cells with continuous Hes-6 expression, as used in **paper IV**. This was performed by cloning a PCR amplified fragment of Hes-6 into the EcoRI site of pcDNA3-FLAG and then amplifying the FLAG-tagged Hes-6 with primers flanked by SpeI sites for cloning into the SpeI site of pLenti6/V5-D vector.

Lentivirus was produced with the ViraPower Lentivirus Expression system (Invitrogen) and virus titer was estimated according to instructions. The T47D or MCF7 cells were incubated with lentivirus in growth medium supplemented with polybrene, according to the manufacturer. Following the viral transduction, the cells were grown in presence of 10 μg/ml blasticidine to select for Hes-6 expressing cells.

Proliferation assays

Measuring the proliferation rate of breast cancer cells has been essential in this thesis. First, cells were syncronised by incubation with charcoal-stripped medium in presence of 10 nM ICI 182,780 for 48 h. Next, the cells were grown in normal medium +/- tetracycline for 6 days, after which the proliferation rate was measured. In **papers I** and **III**, cellular ATP was extracted using 5% trichloroacetic acid followed by measurement of the amount of ATP, which is proportional to the cell number. The measurement of

ATP was performed using Biothema's ATP kit and a microplate luminometer (Berthold). In **paper IV**, proliferation was measured by incubating the cells with the tetrazolium compound MTS, and subsequently analyzed by measuring the absorbance of the cell culture medium at 490 nm (MTS-assay, Promega).

Flow cytometric analysis

To investigate the cell-cycle phase distribution of the Hes-1 and ERβ expressing cell lines, flow cytometric analysis was performed. Growth arrested or stimulated cells were fixed in 70% cold ethanol for 30 min on ice. The fixed cells were washed in PBS and stained with 50 µg/ml propidium iodide (Sigma, St. Louis, MO) supplemented with RNase A (50 µg/ml, Sigma) for 30 min at 37°C. Fluorescence-activated cell sorting (FACS) was performed using a FACS Calibur flow cytometer (Becton–Dickinson). Cell-cycle analysis was performed using CELLQuest software (Becton–Dickinson).

Real-time quantitative PCR

mRNA expression of individual genes was measured with real-time quantitative PCR. First, total cellular RNA was extracted using TRIzol-reagent (Invitrogen, Carlsbad, USA). The obtained RNA was purified and subsequently reverse transcribed with Superscript III according to manufacturer’s instructions (Invitrogen). Expression was quantitatively analysed with real-time PCR run on an ABI 7500 Sequence detection system (Applied Biosystems, USA). 18s rRNA or GAPDH was used as reference genes. Further information of specific primer sequences and conditions can be obtained in the Materials and Methods section of respective paper.

Western immunoblot

Western blot was performed to detect specific proteins in tissue and cellular extracts. Nuclear or whole cell extracts of cells were separated on 12% SDS/PAGE gel and subsequently transferred onto a nitrocellulose filter (Amersham Biosciences Inc., Piscataway, NJ). The membrane was blocked with 5% milk powder in phosphate-buffered saline-Tween 20 (0.1%) to reduce unspecific binding of antibodies. The membrane was incubated with primary and secondary antibodies according to the Materials and Methods section of respective paper.

Immunohistochemisty

To detect specific proteins in xenograft tissues, immunohistochemistry was performed. Antigen retrieval of tissue sections was done by microwave boiling in 0.01 M citric acid (pH 6.0). Endogenous peroxidase activity was blocked by incubating sections shaking in 3% hydrogen peroxide in methanol and then blocked in 2% Bovine Serum Albumin (BSA) and 0.1% NP40 for 1 h at room temperature. The tissue sections were incubated with primary antibody overnight at 4°C. Sections were then washed consecutively in PBS, 0.1% NP40, followed by incubation with a biotinylated secondary antibody in 0.1% NP40 for 1 h. After this, sections were incubated in the streptavidin-horseradish peroxidase ABC complex (Vectastain Elite, Vector Laboratories) for 1 h, stained in DAB and counterstained with Mayer hematoxylin (Sigma, St. Louis, MO). Finally, the sections were dehydrated in ethanol and mounted in Pertex (Histolab, Gothenburg, Sweden). The mounted tissue sections were subsequently viewed in microscope. Concentrations and conditions of antibodies can be obtained in the Materials and Methods section of **paper II**.

Experimental xenograft model

The xenograft model system was used to study effects on tumor growth, angiogenesis and gene regulation *in vivo*. 5-10x10^6 cells were diluted in 200 µl normal medium + 200 µl Matrigel (BD Biosciences, Bedford, MA) and injected into the abdominal fat close to the mammary tissue of 9-12-week-old pathogen free SCID/beige mice (Taconic, Denmark) on day 0. E2 pellets, 0.72 mg/pellet (IRA, Sarasota, Fl), were inserted subcutaneously into the neck with a pellet trochar (IRA, Sarasota, Fl). After 14 days, the mice were sacrificed and the tumor weight was measured. All tumors were fixed in 4% paraformaldehyde and stored in 75% ethanol. After this, tissues were paraffin-embedded and subsequently sliced into 4.5 µm sections according to standard protocol. The sections were analyzed with immunohistochemistry.

4 RESULTS AND DISCUSSION

4.1 *ERβ INHIBITS THE PROLIFERATION OF BREAST CANCER CELLS (PAPER I)*

E2 is a potent cell-cycle stimulator for the majority of breast cancer cell-lines. Considering the fact that these cells express ERα but not ERβ, it has been concluded that ERα mediates the proliferative effect of E2.

To investigate how ERβ affects proliferation, we developed ERβ-expressing cells by stable transfection of a tetracycline-inducible ERβ expression vector into T47D breast cancer cells.

In the examination of this newly developed cell-line, our first step was to perform a proliferation assay. Most interestingly, expression of ERβ resulted in an inhibition of proliferation and reduced the proliferative fraction of cells as measured with flow cytometric analysis (FACS) with propidium iodide staining.

Normally, treatment of ERα+ breast cancer cells with tamoxifen inhibits proliferation. However, when ERβ was expressed in our T47D cells, tamoxifen reduced the proliferation even more, pointing at an anti-proliferative role of ERβ in the presence of anti-estrogens. Treatment with the anti-estrogen ICI 182,780 on the other hand, which is known to degrade estrogen receptors, had similar anti-proliferative effects independently of ERβ expression.

Remarkably, cyclin D1 which is regularly induced in the early phase of the cell-cycle was increased in the presence of ERβ. On the other hand, both the level of cyclin E as well as the activity of the cyclin E associated kinase Cdk2 was decreased by ERβ expression. Importantly, the reduced Cdk2-activity correlated to increased protein level of $p27^{Kip1}$, an inhibitor of Cdk2.

$p45^{Skp2}$ is a central factor in the degradation process of $p27^{Kip1}$ and the level of $p45^{Skp2}$ was reduced as a consequence of ERβ-expression. We therefore conclude that ERβ-mediated regulation of the $p45^{Skp2}$/$p27^{Kip1}$-pathway is important in reduction of Cdk2-activity.

In addition, we found that both E2F1 and Cdc25A were repressed by ERβ. However, we cannot conclude whether these genes are repressed by ERβ directly or through indirect signal pathways.

In summary, we have characterized the function of ERβ in breast cancer cell proliferation and conclude that ERβ is a strong anti-proliferative factor, in many ways antagonising the function of ERα.

4.2 REDUCTION OF TUMOR GROWTH BY ERβ IN A XENOGRAFT MODEL (PAPER II)

In **paper II**, we used the T47D cell-line with tetracycline-inducible ERβ expression as previously described. These cells and normal T47D cells as a control were implanted into the mammary fat pad of immunodeficent SCID/beige mice and grown in the presence of E2. After 30 days incubation, continuous expression of ERβ reduced the tumor xenograft volume by 80%.

When the tumors grown for 4, 8, 16 and 30 days respectively were analysed, we observed that the number of ERβ+ cells decreased gradually from day 8 to day 30. This was in contrast to the number of Ki67+ cells, which was lower in the tumors grown for 8 and 16 days compared to the control and the cells with 30 days ERβ expression.

The most likely reason for the continuous reduction of ERβ+ cells *in vivo* is a gradual overgrowth by normal T47D cells in expense of the ERβ+ cells. Moreover, the numbers of intratumoral blood vessels were reduced significantly in ERβ-expressing xenografts compared to the parental T47D xenografts. To investigate the pathway by which ERβ might affect angiogenesis we analysed several pro-angiogenic factors by western blot of the xenograft tissues. We found that the pro-angiogenic factor PDGFβ was reduced at both the protein and mRNA levels upon ERβ expression whereas VEGF was not reduced significantly by ERβ.

To exclude the possibility that PDGFβ was reduced only as a consequence of reduced tumor growth, we performed *in vitro* studies with non-synchronised T47D-ERβ cells. PDGFβ levels were reduced during both normal and hypoxic conditions in presence of ERβ. Also, in transient transfections with a -981/+25 PDGFβ-promoter construct, ERβ opposed ERα stimulated PDGFβ expression.

These results indicate an anti-proliferative role of ERβ *in vivo*, possibly as a consequence of reduced growth factor production and secretion. As a consequence, suppression of growth factors by ERβ would also reduce angiogenesis in breast cancer xenografts, which could have important medical implications.

4.3 HES-1 INHIBITS PROLIFERATION THROUGH REGULATION OF G_1-PHASE CELL-CYCLE FACTORS (PAPER III)

Hes-1 has been shown to be an essential factor in ERα-mediated proliferation of breast cancer cells. In MCF7 cells, treatment with E2 downregulates Hes-1 and thereby initiates proliferation. However, upregulation of Hes-1 on the other hand, inhibits proliferation of breast cancer cells.

As shown by Müller *et al,* (215) the vitamin A derivate all-*trans* retinoic acid (atRA) induces the expression of Hes-1 and as a consequence, atRA inhibits proliferation as well as expression of cell-cycle factors in breast cancer cells. However, the target genes for Hes-1, responsible for the anti-proliferative effect have not yet been identified.

We used tetracycline-regulated, Hes-1 inducible T47D breast cancer cells (T47D-Hes-1) to investigate potential cell-cycle regulation by Hes-1. In the presence of tetracycline, the expression of Hes-1 is shut off and in absence of tetracycline; Hes-1 is fully expressed.

Flow cytometric analysis of the T47D-Hes-1 cells revealed a complete cell-cycle arrest in the G_1-phase in response to Hes-1 expression.

Next, we analysed the potential Hes-1 regulation of individual cell-cycle factors. As described within the literature, E2 treatment of T47D cells causes E2F1-dissociation from pRB as well as transcriptional activation of E2F1. We found that expression of Hes-1 reduced this induction and restored E2F1 protein to its basal level. Further analysis revealed that E2F1 mRNA was reduced as well, pointing at transcriptional regulation by Hes-1.

Treatment of T47D cells with atRA resulted in similar inhibition of E2F1, indicating a potential regulation through Hes-1. Interestingly, by expression of a tetracycline-regulated dominant negative version of Hes-1 (dnHes-1), the repressive effect of atRA was abolished.

Heregulin-β1 (HRG) is a ligand for the ErbB3 receptor, a member of the EGF-family of tyrosine kinase coupled transmembrane receptors. Upon ligand binding, ErbB3 dimerises with ErbB2 (Her2/neu), which results in the initiation of a phosphorylation cascade and subsequent hormone independent proliferation of breast cancer cells.

As discussed within the introduction of this thesis, the ErbB-family plays an important role in the progression into endocrine resistant breast cancer. We found that HRG

treatment of T47D cells increased their proliferation, which could be prevented by Hes-1 expression. However, in contrast to E2 treatment, HRG treatment did not result in downregulation of Hes-1. It has been shown by Ström *et al* (222) that Hes-1 was phosphorylated by protein kinase C (PKC) in response to nerve growth factor (NGF) treatment, which inhibited the DNA binding capacity of Hes-1.

Therefore, we suggest that HRG treatment of T47D cells causes phosphorylation of Hes-1 and thereby prevents its DNA binding activity. As with E2 treatment, treatment with atRA was able to oppose the effects of HRG on T47D cells, but was inefficient in the presence of dnHes-1. In transient transfections of T47D cells with an E2F1 promoter construct, Hes-1 was able to inhibit both E2 and HRG stimulated upregulation of E2F1.

Finally, we mutated the Hes-1 binding site in the E2F1 promoter region which prevented Hes-1 from inhibiting E2F1 expression.

Our data indicate that E2F1 is a central factor in Hes-1 mediated inhibition of proliferation.

As E2F1 is not only a central factor in G_1/S-phase transition but also of importance during differentiation, tumorigenesis and apoptosis (223, 224), we believe that this pathway is of significance for the anti-tumorigenic properties of Hes-1.

4.4 HES-6; A NOVEL LINK BETWEEN ERα AND HES-1 (PAPER IV)

Based on our *in vitro* experiments, we conclude that Hes-1 has strong anti-proliferative properties in breast cancer cells and functions as a potential tumor suppressor (214). Nevertheless, according to our mRNA-expression analyses, Hes-1 does not seem to be suppressed in breast cancer compared to normal breast tissue. Consequently, it is possible that another factor or signal-pathway regulates Hes-1 action in tumors. Since Hes-6 has been described as a posttranscriptional inhibitor of Hes-1 during embryogenesis, we investigated the function of Hes-6 and its regulation in breast cancer.

First, Hes-6 levels in clinical breast cancer samples were analysed. However, since functional antibodies against Hes-6 are not available, real-time quantitative PCR was used to determine the mRNA levels of Hes-6 in breast tumors. Interestingly, Hes-6 expression was significantly higher in malignant breast tumors compared to normal breast cells, but did not correlate to any other clinical parameters investigated. In

addition, we found that Hes-6 was more strongly expressed in estrogen independent, metastatic breast cancer cell lines compared to estrogen dependent, non-metastatic cancer cell lines.

Using lentivirus, a Hes-6 expression cassette was transduced into T47D breast cancer cells and the cellular proliferation was increased. Moreover, the Hes-1 target genes E2F1 and hASH-1, which are normally repressed by Hes-1, were induced as a consequence of Hes-6 expression.

In xenograft experiments where breast cancer cells were implanted into the breast tissue of SCID/beige immunodeficient mice, expression of Hes-6 resulted in increased tumor size.

Because of estrogen dependence and high expression of ERα, MCF7 cells are often used as model system for mechanistic studies of estrogen receptor signalling. We found that E2 treatment of MCF7 cells increased the expression of Hes-6, which suggests that Hes-6 is a novel breast cancer supportive factor regulated by ERα.

To further prove the tumorigenic function of Hes-6 in breast cancer, MCF7 breast cancer cells were transfected with siRNA towards Hes-6, resulting in great reduction of Hes-6 mRNA level as well as suppression of the E2F1 mRNA level. We conclude that Hes-6 is a strong activator of proliferation and is overexpressed in breast cancer. Therefore, we propose that Hes-6 is important in tumorigenesis as an inhibitor of Hes-1.

5 GENERAL DISCUSSION

5.1 *ERβ AND ENDOCRINE THERAPY*

Our study **(paper I)** indicates that tamoxifen treatment of ERα+ breast cancer cells has an even stronger effect in the presence of ERβ. As ERα and ERβ have profound dissimilarities in structure and affinity for promoter elements, we suggest this might explain their difference in tamoxifen response.

ERE activity is repressed by both ERα and ERβ in presence of tamoxifen, while AP-1 elements are activated by both receptors in the presence of tamoxifen (10). When ERβ is expressed in parallel with ERα, which is often the case in breast cancer cells, the activation of AP-1 elements is inhibited by ERβ (225). Since AP-1 elements are important in the transcriptional activation of many genes, the discrepancy in ER-function could play an important role in the proliferative behaviour of breast cancer cells in response to tamoxifen.

Perhaps ERβ can be used as marker for tamoxifen responsiveness in clinical diagnostics? In the published literature, there is substantial evidence for ERβ as a marker for endocrine sensitivity (184, 190). In a newly performed study based on 442 breast cancers from women treated with adjuvant tamoxifen, ERβ-positivity was associated to significantly better survival compared to ERβ-negativity (226). Based on our findings, a tissue specific, selective ERβ agonist and ERα antagonist would be the optimal endocrine treatment option for ERα+, ERβ+ breast cancer. Perhaps, this would not just be a more effective therapy than tamoxifen, but would also be associated with fewer side-effects. Tamoxifen has significant side-effects such as increased risk of thrombosis and alternative adjuvant breast cancer treatments are needed.

As mentioned within the introduction of this thesis, the level of ERβ is reduced in the majority of breast cancers compared to normal breast tissue. Nevertheless, the receptor is still present within the tumor although at low levels (115). Consequently, ERβ could be a promising target for a novel endocrine treatment regime.

Our papers as well as many other *in vitro* studies support an anti-proliferative role of ERβ when re-introduced into breast cancer cell lines. However, as there are no completed clinical trials with ERβ-selective agonists/antagonist as treatment of breast

cancer, we cannot be certain about the medical implications of ERβ in human breast cancer. On the other hand, several ERβ-agonists have been evaluated in animal models. One of these agents prevented several inflammatory diseases as well as uterus hyperplasia and did not stimulate growth of mammary glands in a rat mammotrophic assay (227).
In the clinic, there is a great need for new hormone replacement therapies (HRT), with the health-promoting benefits of estrogens but without the risks of long-term estrogen treatment. In the future, that drug could be a selective ERβ-agonist, which would hopefully even prevent the initiation of breast cancer in treated women.

Before the introduction of tamoxifen, high-dose estrogen treatment was the therapy of choice for postmenopausal breast cancer patients. Today, estrogen treatment is occasionally used in late stage, tamoxifen resistant breast cancer patients (228).
In a clinical study performed on patients insensitive to estrogen deprivation therapy (aromatase inhibitors, tamoxifen etc), patients were treated with diethylstilbestrol (DES), a synthetic estrogen. Of a total number of 32 patients included, 31% of the patients responded to estrogen therapy with complete or partial response (229).
Moreover, in at least one randomised clinical study, E2 treatment have been compared to tamoxifen treatment for breast cancer, and shown a similar efficacy (230). In these cases, it would be of great importance to examine the ERβ-status of the tumors. Based on our studies, we propose that the anti-proliferative, anti-tumorigenic effects of estrogens are mediated by ERβ. If this is the case, ERβ-specific agonists should be an even more effective therapy for this group of patients. Based on our current knowledge that ERβ-expression is repressed in the majority of breast tumors through promoter methylation (116), we believe that a combination therapy of ERβ-agonist and demethylating agent would be a promising treatment option.

5.2 ERβ, ANGIOGENESIS AND TUMOR GROWTH

In **paper II**, we have described how ERβ inhibits tumor growth as well as angiogenesis in a xenograft model. The process of *in vivo* tumor growth, studied with a xenograft system is much more complex than *in vitro* growth in a cell-culture. Tumor growth is dependent on several biological processes such as angiogenesis, paracrine and

endocrine growth factor signalling, proliferation and immune cells. Therefore, inhibition of proliferation *in vitro* is not synonymous with inhibition of tumor growth.

Based on our findings, we conclude that the tumor suppressive effects of ERβ *in vivo,* are most likely a consequence of the combined action of reduced angiogenesis and proliferation. The reduction in the number of blood vessels is in turn a result of inhibition of growth factor expression and secretion by ERβ.

PDGF-related factors, which are reduced in ERβ-expressing cells *in vitro* and *in vivo*, have other important functions than angiogenesis in tumor growth as PDGF-factors are potent stimulators of proliferation and invasive properties of cancer cells. Therefore, the paracrine effects mediated by ERβ expression such as changed growth factor secretion may be essential in the reduction of tumor growth.

Our xenograft model summarises several aspects of tumor growth, but does not include all factors important for tumor growth in humans. Since the mice used in the xenograft experiments are immunodeficient, they lack or have dysfunctional lymphocytes, normally present within tumors. Therefore, this system does not give a complete picture of the possible anti-tumorigenic effects of ERβ.

The immune system has been shown to play important roles in the prevention and repression of cancer. Nevertheless, immune cells such as macrophages can also promote growth and metastasis of cancer cells. The presence of ERβ in mature lymphocytes (231) raises the question whether ERβ might play a role in the tumor associated immune cells. Although preliminary experiments performed in our laboratory strengthen this notion, the exact function of ERβ within tumor associated immune cells is still unclear. Further experimental studies are needed to clarify the role of ERβ in the interaction between immune cells, cancer cells and tumor growth.

In **paper II**, we have focused on the anti-angiogenic properties of ERβ in tumors, but ERβ could affect tumor blood vessels in other ways as well. Endothelial cells, the main targets for angiogenic growth factors, express both ERα and ERβ (232). Therefore, estrogens can also affect endothelial cells directly.

In endothelial cells, ERα stimulates the expression of VEGF-receptor-2 (Flk-1) as well as the angiopoetin receptor-2 (Tie-2) (175-177), resulting in increased endothelial sensitivity for VEGF and angiopoetin, respectively. However, the function of ERβ in endothelial cells is currently not known.

Today, one of the most intensively studied areas within cancer research is angiogenesis and several drugs with anti-angiogenic profile have been developed (171). We propose

that activation of ERβ could have a similar effect, reducing angiogenesis. However, further experimental studies are needed to explore the function of ERβ within the pathways involved in tumor angiogenesis.

5.3 WHAT ARE THE TARGET GENES FOR ERβ IN INHIBITION OF PROLIFERATION?

Our data clearly demonstrate that ERβ has an anti-proliferative function when re-introduced into breast cancer cells. In many ways, ERβ appears to oppose the action of ERα. Also, in clinical specimens, there is a significant downregulation of ERβ in breast cancer epithelium compared to normal breast epithelium, supporting the notion that ERβ plays a central role in tumorigenesis. However, there is no unequivocal correlation of ERβ with proliferation associated parameters such as Ki67-staining, indicating that the situation is complex.

In the presence of E2, ERα stimulates proliferation by regulating numerous cell-cycle genes. ERβ on the other hand, binds to the same promoter elements but sometimes modulates gene expression differently. Therefore it is likely that ERβ inhibits proliferation in cancer cells by affecting several cell-cycle factors in parallel. Also, it is likely that the target genes for ERβ differ between tissues and cancer types.

Nevertheless, we have identified one pathway that is regulated by ERβ in the same way in breast and colon cancer (unpublished data, our lab). As shown in **paper I,** the Cdk2 inhibitor $p27^{Kip1}$ is induced in response to ERβ expression. However, $p27^{Kip1}$ is not regulated at the transcriptional level since its mRNA level does not change significantly in response to ERβ. Instead, we observed that $p45^{Skp2}$, which is a critical regulator of $p27^{Kip1}$-degradation, is inhibited by ERβ both in breast and colon cancer cells. We therefore consider this pathway to be of particular importance in ERβ-mediated inhibition of proliferation. Although we have not identified the regulatory mechanism behind the ERβ-mediated repression of $p45^{Skp2}$, this gene is a transcriptionally activated target for E2F1 (233). Since ERβ decreases the level of E2F1, $p45^{Skp2}$ is a likely secondary target for ERβ, regulated through E2F1. Further studies are needed to clarify whether ERβ interacts with the $p45^{Skp2}$-promoter directly or through E2F-factors.

c-Myc is the other cell-cycle regulator which is strongly reduced by ERβ in breast cancer and also in colon cancer cells (234). c-Myc is expressed in the early cell-cycle and in turn regulates the transcription of other cell-cycle genes. Hence, inhibition of c-

Myc by ERβ is probably responsible for the multiple cell-cycle gene regulatory events which occur in response to ERβ expression.

To receive a more complete view of the cell cycle signalling pathways regulated by ERβ in cancer, global gene expression analysis or proteomics on a large-scale using clinical samples with different ERβ-status would be useful.

5.4 THE ROLE OF HES-1 AND HES-6 IN BREAST CANCER

As presented in **paper IV**, we observed higher levels of Hes-6 mRNA in breast cancer compared to normal breast tissues. According to earlier reports, ERα-levels are higher in breast cancer compared to normal mammary gland (235), which made us consider a connection between Hes-6 and ERα. We identified Hes-6 as an ERα induced target gene; ERα mediated upregulation of Hes-6 is a possible reason for the increased Hes-6 levels in breast cancer.

However, we were not able to find any correlation between Hes-6 mRNA and ERα-status in breast cancer (data not shown), indicating an alternative regulation of Hes-6 *in vivo*. A second possibility is that ERα-mediated regulation of Hes-6 also occurs at posttranscriptional level. Therefore, the relevance of Hes-6 and its potential association to ERα must also be studied at the protein level. However, with the current knowledge we can conclude that ERα is important in the regulation of Hes-6 *in vitro*.

In **paper IV,** we also describe the consequence of forced Hes-6 expression, which in many ways counteracts the antiproliferative effects of Hes-1. In our cell system, Hes-6 is a strong inducer of proliferation *in vitro* as well as *in vivo*. One of the most strongly affected cell-cycle regulators *in vitro* and *in vivo* is E2F1, which is also the target gene for Hes-1.

Although the mechanism remains unclear, Hes-6 has been shown to be important during metastasis and growth towards a high-grade cancer in prostate and colon (218, 221).

Based on our studies and the literature, we suggest that neuroendocrine differentiation (NED) may play a role in this process. Neuroendocrine differentiation occurs in many types of cancers and is often correlated with poor prognosis (236), such as in prostate cancer, where NED is associated with androgen resistant tumors (237).

It is possible to identify NED by the immunohistochemical markers chromogranin A and synaptophysine. NED occurs in breast cancer as well, but less frequently. It is

estimated that approximately 12% of all diagnosed breast cancers contain NED cells, but in contrast to other cancer types, NED of breast cancer does not seem to be associated with any prognostic parameters (238). Expression of Hes-6 and another bHLH-factor, hASH1 (Achaete-scute complex homolog 1) is associated with NED and is important for the differentiation towards the NED-phenotype (239). In contrast to Hes-1, hASH-1 functions as a transcriptional activator, inducing transcription through E-boxes (240) and is negatively regulated by Hes-1 at the promoter level. Therefore, in cancer, Hes-1 is important both as an inhibitor of estrogen stimulated proliferation and as an inhibitory factor for NED of cancer cells.

A targeted therapy towards the Hes-1 pathway could be a promising alternative to the classical treatment regimes for breast cancer. How do we find an agent which induces the transcription factor Hes-1? A retinoid-based compound could be that agent. Retinoids are vitamin A derivates, mediating their activity through nuclear receptors of the retinoic acid or retinoid x receptor family. A wide range of retinoids display anti-proliferative, apoptosis-inducing effects in cancer cells *in vitro*. Furthermore, retinoids are used as standard therapy for acute promyelocytic leukaemia (241) and have been used as experimental therapy for other cancer types such as neuroblastoma, prostate and lung cancer with varying degrees of success.

In breast cancer cells, treatment with the retinoid all-*trans*-retinoic acid (atRA) prevents ERα-mediated suppression of Hes-1 (215) *in vitro*. Yet, randomised clinical trials with retinoids alone or in combination with chemotherapy for breast cancer have not been successful (242). Perhaps atRA is efficient only in a sub-group of breast cancers, namely tumors with neuroendocrine differentiation. These tumors have low Hes-1 expression and/or high expression of Hes-6 together with hASH-1. Therefore, atRA-treatment might be useful in clinical trials with NED+ breast cancers.

5.5 HOW ARE HES-1 AND ERβ CONNECTED?

In an increasing number of studies, Hes-family members have been identified as factors involved in metastasis and malignant behaviour of cancer. To our knowledge, no correlation of Hes-1 expression to prognostic parameters has been reported.

However, as discussed in **paper IV**, Hes-6 has been identified as a Hes-1 inhibitor, and is frequently overexpressed in human cancers. As reported in **paper IV**, we observed that in the presence of E2, ERα upregulated Hes-6 in MCF7 breast cancer cells. This

might be a novel pathway by which ERα induces proliferation and perhaps, endocrine resistance.

As described within the introduction, increased growth factor signalling or overexpression of the EGF-family receptors Her2/neu and EGFR are common events in endocrine resistance and subsequent metastasis. Therefore, it should be pointed out that Hes-1 DNA-binding activity is regulated through phosphorylations by PKC, which in turn is activated by growth factor signalling (222). Hence, inactivation of Hes-1 might have a function in endocrine resistance and cancer in general. Phosphorylations of Hes-1 are not identified through measurements of mRNA or protein levels, which could explain why Hes-1 levels are not suppressed in breast cancer.

In conclusion, the relation between Hes-1 and ERβ expression is still somewhat unclear. However, cell-cycle regulation by Hes-1 and ERβ, as described within **papers I** and **III**, have a much in common. E2F1 has been identified as a target gene for Hes-1, shown to be directly inhibited at the promoter level. Furthermore, E2F1 is downregulated in response to ERβ overexpression in T47D cells. Cyclin E shows a similar pattern of transcriptional repression in response to ERβ and Hes-1 expression. Furthermore, cyclin E is a transcriptional target gene for E2F1.

Hes-1, Hes-6, ERs and growth factor receptors are connected through diverse pathways which are only partially identified. Knowledge about the signalling pathways between these factors will have implications in the identification of new markers for breast cancers responding to endocrine treatment. Moreover, the current development of subtype and tissue specific ER-ligands by the pharmaceutical industry will provide clinicians with new therapeutics for several diseases. The data presented in this thesis highlights the importance of ERβ as a strong, anti-proliferative factor in breast cancer cells. Therefore, re-expression and activation of ERβ in breast cancer should be an accurate and tumor suppressive pathway.

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