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Role of cyclin D1 as an estrogen receptor cofactor and the influence of hypoxia on estrogen receptor regulation, with focus on prognositic and treatment predictive features in breast cancer

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Role of cyclin D1 as an estrogen receptor cofactor and the influence of hypoxia on estrogen receptor regulation, with focus on prognostic and treatment predictive features in breast cancer

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Abstract

Estrogen receptor (ER) status can define breast cancer patients who would benefit from adjuvant tamoxifen therapy. However, resistance to tamoxifen is often observed and possible mechanisms may be loss or reduction of ER, dysfunctional ER-signaling and ligand independent activation of the receptor. Hypoxia and hypoxia inducible factor-1α (HIF-1) expression has been correlated to loss of ER in breast tumors. Cyclin D1, initially described as a cell cycle regulator, might also function as a cofactor to ER inducing ligand independent activation of the receptor. We therefore determined the relation between ER, cyclin D1 and HIF-1-expression in primary breast tumors and cell lines. Further, the prognostic and treatment predictive value of cyclin D1 and HIF-1 was analyzed in breast cancer patients receiving two years of tamoxifen versus no adjuvant treatment. The results indicated that ER-heterogeneity in primary breast tumors was associated with cyclin D1 and HIF-1 expression. Further, breast cancer patients with cyclin D1 high tumors did not benefit from tamoxifen treatment. The survival for untreated patients with cyclin D1 high tumors was nevertheless slightly better than for patients with cyclin D1 low tumors. Hypoxia was also strongly linked to ER downregulation in DCIS and invasive breast cancer and caused ER downregulation in breast cancer cell lines. Interestingly, hypoxic cells were less differentiated, showing changes in morphology, proliferation and cytokeratin 19 expression. The hypoxia induced ER reduction was due to both proteasomal degradation and decreased transcription and active extracellular regulated kinase (ERK1/2) was involved in the transcriptional regulation of ER. Consequently, tamoxifen treatment did not affect proliferation as efficiently in hypoxia as in normoxia, but ERK1/2 inhibitors efficiently increased the tamoxifen effect in hypoxia. Unexpectedly, tumor specific HIF-1 expression was not a predictive marker for tamoxifen response in premenopausal breast cancer patients but associated with a worse recurrence free survival. These results suggest that cyclin D1 is a predictive marker for tamoxifen resistance and HIF-1 a marker of poor prognosis in breast cancer. Targeting cyclin D1 and/or ERK1/2 in conjunction with tamoxifen represent new treatment strategies for improving the tamoxifen response.

Key words:
breast cancer, ER, Tamoxifen, HIF-1, Cyclin D1, predictive marker, prognosis

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TABLE OF CONTENTS

LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text by their respective roman numerals.

I. **Kronblad Å, Helczynska K, Nielsen NH, Emdin S, Påhlman S and Landberg G:** Regional cyclin D1 overexpression or hypoxia correlate inversely with heterogeneous oestrogen receptor-alpha expression in human breast cancer. In Vivo. 2003 Jul-Aug;17(4):311-8.

II. **Stendahl M*, Kronblad Å*, Rydén L, Emdin S, Bengtsson NO and Landberg G:** Cyclin D1 overexpression is a negative predictive factor for tamoxifen response in postmenopausal breast cancer patients. Br J Cancer. 2004 May 17;90(10):1942-8.

III. **Helczynska K, Kronblad Å, Jögi A, Nilsson E, Beckman S, Landberg G and Påhlman S:** Hypoxia promotes a dedifferentiated phenotype in ductal breast carcinoma in situ. Cancer Res. 2003 Apr 1;63(7):1441-4.

IV. **Kronblad Å, Hedenfalk I, Nilsson E, Påhlman S and Landberg G:** ERK1/2 inhibition increases anti-estrogen treatment efficacy by interfering with hypoxia-induced down-regulation of ERα. A novel combination therapy for breast cancer. Oncogene. 2005 Oct 13;24(48):6835-41.

V. **Kronblad Å, Jirström K, Rydén L, Nordenskjöld B and Landberg G:** Hypoxia inducible factor-1alpha is a prognostic marker in premenopausal patients with intermediate to highly differentiated breast cancer but not a predictive marker for tamoxifen response. Int J of Cancer. 2006 May 15;118(10): 2609-16.

*Authors contributed equally

ABBREVIATIONS

AF1	Activating function site 1
	AF2Activiating function site 2
AIB-1	Amplified in breast cancer-1
AKT/PKB	Protein kinase B
AP-1	Activator protein-1 (jun/fos)
ARNT	Aryl hydrocarbon receptor nuclear translocator
BCSS	Breast cancer specific survival
BRCA	Breast cancer gene
CALLA	Common acute lymphoblastic leukemia antigen
c-AMP	Cyclic AMP
CBP/p300	CREB binding protein
CCND1	Cyclin D1 gene
Cdc2	Cell division cycle protein 2
CDK	Cyclin dependent kinase
CK	Cytokeratin
CMF	Cyclophosphamide-methotrexate-flourourcil
DCIS	Ductal carcinoma in situ
DNA	Deoxyribonucleic acid
DNMT1	DNA methyltransferase 1
E2F	E2 promoting binding factor
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ER	Estrogen receptor
ERE	Estrogen responsive element
ERK	Extracellular signal regulated protein kinase
ESA	Epithelial specific antigen
FIH	Factor inhibiting hif
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HER2/neu	Heregulin receptor 2 (protein encoded from Cerb-B2)
HIF	Hypoxia inducible factor
HRE	Hypoxia responsive element
Hsp	Heat shock protein
IGF	Insulin growth factor
IGFR	Insulin growth factor receptor
ILR	Interleukin receptor
JAK	Janus kinase
JNK	c-Jun terminal kinase
LBD	Ligand binding domain
LCIS	Lobular carcinoma in situ
LHRH	Luteinizing-hormone releasing hormone
MAPK	Mitogen activated protein kinase
MEK-1/2	Mitogen-activated protein kinase kinase
MUC1	Mucin 1
NCOA	Nuclear-receptor co-activator
NCOR	Nuclear-receptor corepressor
NGF	Nerve growth factor
NHG	Nottingham histological grade
OS	Overall survival
P/CAF	p300/CBP associated factor
p65-PAK	p65-p21-activated kinase
p90-rsk	90-kDa ribosomal S6 protein kinase
PDGF	Platelet derived growth factor
PI3K	Phosphatidylinositol-3 kinase
PKA	Protein kinase A
PPAR	Peroxisome proliferative activated receptor
PR	Progesterone receptor
Rb	Retinoblastoma protein
RFS	Recurrence free survival
SAPK	Stress-activated protein kinase
SF-1	Steroidogenic factor 1
SFRE	SF-1 response elements
SMRT	Silencing mediator for retinoid and thyroid hormone receptors
Sp1	Specificity protein-1
SRC-1	Steroid receptor coactivator
STAT	Signal transducer and activator of transcription
SV40t	Sesmian virus 40 small t
SWI/SNF	Swithching/sucrose non-fermenting
TDLU	Terminal duct lobulo-alveolar units
TGF	Transforming growth factor
TRAP	Thyroid-hormone-receptor-associated protein
TyrK	Tyrosine kinase
VEGF	Vascular endothelial growth factor
VHL	Von Hippel Lindau-protein
α-SMA	α-smooth muscle actin

INTRODUCTION

Inappropriate cellular proliferation is a simple way of defining what cancer is, but there is nothing simple about cancer development. Tumor progression is a multi step process, which enables cells to evolve into malignant tumors. This progression has been suggested to depend on six essential characteristics identified as the ”hallmarks of cancer,” which include: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis [1]. It has long been known that cancer cells gain these traits by mutations in either tumor suppressor genes [2] or activation of proto-oncogenes [3]. In certain tumors this happens in an orderly fashion [4] whereas in breast cancer no such orderly progressive accumulation of events has been defined. Breast cancer is characterized by a very heterogeneous clinical course influenced by both exogenous and endogenous risk factors [5]. Exogenous diagnostic factors include race, diet, age, environmental factors, cumulative exposure to sex hormones (early menarche, pregnancy, late menopause, oral contraceptives, estrogen replacement) and endogenous risk factors are for example inherited mutations in BRCA1/2, estrogen receptor (ER) or progesterone receptor (PR) expression, cerbB-2 amplification. The ideal would be to use these biological factors to individualize the treatment and to classify the patients into subgroups based on their risk of recurrence. Until now there are unfortunately very few such biomarkers that dispose prognostic features (time to recurrence RFS, over-all survival OS and breast cancer specific survival BCSS) or predictive response (predicts the response to therapies, e.g. as tamoxifen). The standard prognostic factors used for primary breast cancer today are; lymph node status, tumor size, histological grade [6] (where mitotic index, tubular formation and nuclear atypia are taken into account), ER and PR status. ER and PR are also used as predictive markers together with HER2-expression and c-erbB2 gene amplification [7]. The reality is that very few markers in the current years have made it into clinical practice. This thesis will shed light on a promising predictive marker for tamoxifen response, cyclin D1 and a second potentially prognostic marker, HIF-1, that also regulates the expression of ER. The rationale for studying cyclin D1 lies in its ability to directly interact with ER causing the receptor to be active without estrogen stimulation and not possible to block with tamoxifen. HIF-1 is expressed in tumors with oxygen deficiency, hypoxia, and this has been correlated to a worse prognosis in several different tumor types. Further, in breast cancer it was observed that ER and HIF-1 were not coexpressed.

Breast cancer

Breast cancer is the most common female malignancy approximately affecting 1 in 8 women in the Western world. The worldwide incidence is about a million cases a year [8]. The total Swedish breast cancer incidence in the female population was 6,869 cases in 2003, attributing to 30 % of all female cancers (Socialstyrelsen, Statistics, Health and Diseases 2004:10). The annual increase in incidence is 1.5% but the mortality is decreasing. The increase in incidence is partly due to better screening

techniques, such as mammography and partly due to increased exposure to risk factors (e.g. estrogen replacement and oral contraceptives). The lowered mortality rate is due to better screening leading to earlier detection, prevention and better treatment strategies, most importantly the introduction of tamoxifen. The hereditary cancers comprise about 10% of all cases whereas the rest are sporadic cases [9]. The mortality of breast cancer is highly dependent of the tumors' ability to metastasize to distant body sites [10]. In breast cancer the most common metastases occur in lymph nodes, the bones, the chest, the liver and the brain [11]. Detecting metastases in the axillary lymph nodes is the best marker now available for distant disease; still this marker is not a reliable indicator, since about 25% of the node negative patients do have metastases and will relapse, and 25% of the node positive cases will not relapse even without adjuvant treatment.

The breast

The structure and development of the breast is important for understanding breast cancer. The development of the breasts begins in week 7-8 of pregnancy. Small clusters of cells start to branch in week 12-16 and these will be the foundation for future ducts, milk producing glands, muscle cells will form the nipple and the areola. Later in pregnancy hormones will cross the placenta causing the fetal breast to form milk ducts, and in late pregnancy, lobules (terminal duct lobulo-alveolar units (TDLU) or milk producing glands) mature. In the beginning of female puberty circulating estrogens and progesterone, from the ovaries, will cause the breast to become fully mature, consisting of lobules, milk ducts, connective tissue and fat (see figure 1). The lobules group into lobes and each breast consists of about 20 lobes. This is the site for half of all breast cancers. In pregnant women there is a secondary branching of the ducts and increasing numbers of glands, and at lactation the glands are full of milk. After pregnancy the epithelium is eliminated by apoptosis and the glands are remodeled until they once again resemble the mature virgin tissue. In younger women most of the breasts consists of glands but in elderly, postmenopausal women the glands involute and are replaced by fat. The breast consists of different cell types, where the inner layer of the ducts consists of luminal epithelial cells, with milk secreting capacity and the outer layer of myoepithelial cells, with contractile capacity when stimulated with oxytosin. Luminal epithelial cells are suspected to be the origin of most breast cancers. Apart from the morphological difference the breast cells can be separated by different marker proteins, luminal epithelial cells are positive for MUC1, epithelial specific antigen (ESA), and cytokeratins (CK) 7, 8, 18, 19, ER and PR. MUC-1 is a mucin glycoprotein with a transmembrane domain and an extracellular part that can be shed from the cell, and therefore is exclusively expressed on the apical surface of luminal epithelial cells [12]. Myoepithelial cells express α-smooth muscle actin (α-SMA), that exerts contractile capacity in response to oxytocin and move milk into the ducts [13]. Further, they also express vimentin, common acute lymphoblastic leukemia antigen (CALLA), Thy-1 and CK5 and CK14 (reviewed by [14]). Cytokeratins and vimentin are part of the intermediate filaments in the cytoskeleton.

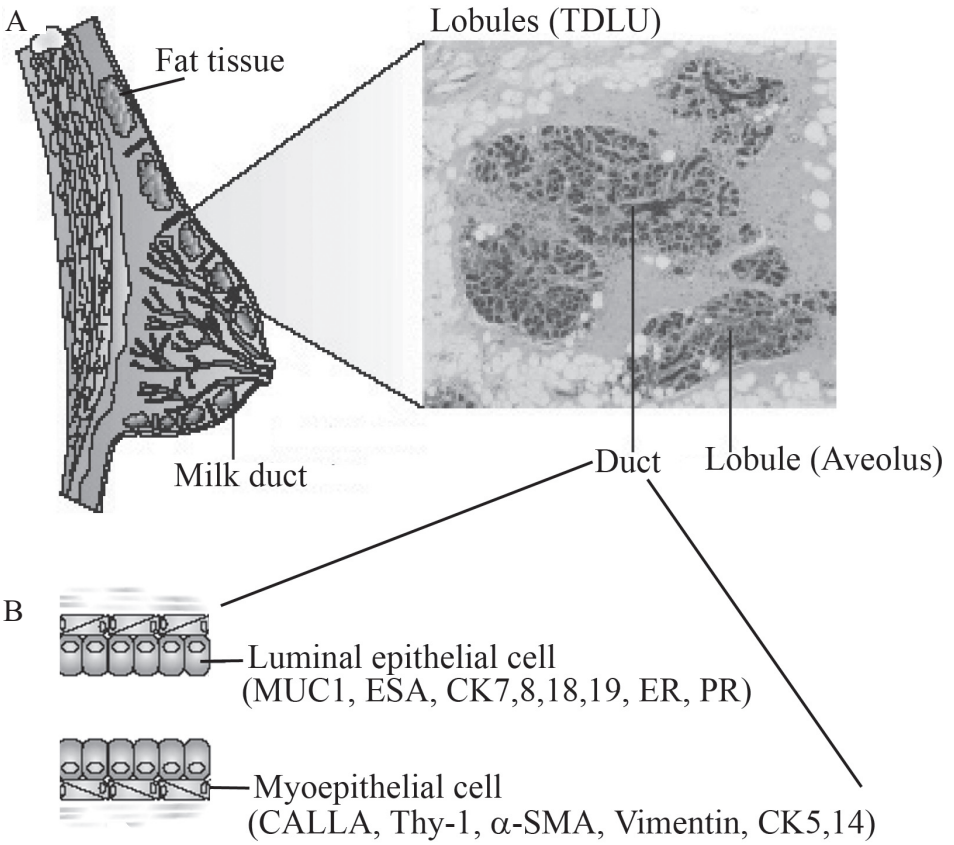

Figure 1. The human breast. A) Schematic illustration of the epithelial structure of normal human breast, with photomicrograph of a cross section through ducts and lobules, B) with their inner luminal epithelial and outer myoepithelial cell layers. Modified from ref [14].

Steroid receptors in the normal breast

Estrogens and progesterone bind to their nuclear receptors in the luminal epithelial cells. Upon binding the receptor changes conformation and acts as a transcription factor binding to specific steroid response elements in the DNA sequences. There are two different ERs, the classical ERα [15] and the newer ERβ [16]. ERα and PR are known to colocalize in a subset of the luminal epithelial cells. PR is expressed in two different isoforms transcribed from the same gene, PRA and PRB [17]. PRB is the longer isoform that is thought to be the active receptor whereas the role of PRA is not completely defined. ERα and PR positive cells account for 10-20% of the epithelial cells (whereof 96% coexpress the proteins) and are distributed throughout the breast lobule. About 2% of the epithelial cells are proliferating, and these cells do not express the receptors. Interestingly, proliferating cells are often found close

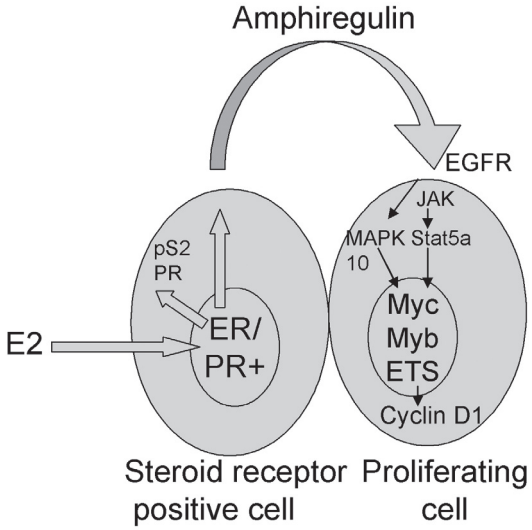

Figure 2. A model of steroid hormone stimulation of normal breast cell growth by paracrine signaling. Estrogen diffuses into the hormon receptor positive cell and the ligand activated ER transactiv ates specific genes, such as PR, pS2 and growth factors like amphiregulin, which is secreted and acts in a paracrine fashion on the adjacent cell. Proliferation may be induced via intracellular signaling pathways (MAPK, STAT) that lead to activation of nuclear transcription factors (Myc, ETS, Myb) and consequently cell cycle genes (cyclin D1). Modified from ref [18].

to cells expressing ERα and PR, suggesting secretion of paracrine signals from the steroid receptor positive cells. Studies have shown that a possible candidate for mediating this paracrine signal is the growth factor amphiregulin, which can bind to epidermal growth factor receptors on adjacent cells, thus inducing a proliferative signal cascade via MAPK, JAK and STAT, to induce myc, myb, ETS and the cell cycle regulator cyclin D1 (see figure 2). The separation between steroid receptor expression and cell proliferation observed in the normal mammary epithelium is altered at an early stage in human breast tumorigenesis such that estrogen directly drives cell proliferation in ERα-positive cancers [18].

Breast stem cells and progenitor lineages

There is current research ongoing into the origin and differentiation process of the breast epithelial cells, where breast stem cells and breast progenitor lineages are beginning to be identified, reviewed by [19,20]. The adult breast stem cell is thought to be a small, round cell without much organelles, localized in the luminal suprabasal compartment, never reaching the lumen of the ducts. Studies of these suprabasal cells using cell-surface markers supported an ESA+/MUC1– staining pattern and also indicated that they were positive for cytokeratin 19. These cells also retained the ability to form complete lobule-like structures, forming both luminal epithelial and myoepithelial cells when put into 3-dimensional culture. This plasticity indica-

tes the potential of stem-cell like capacities, perhaps progenitor derived. Cytokeratin 19 shows a heterogeneous expression pattern in the human breast, and some are located suprabasaly. Interestingly, in the majority of breast carcinomas the neoplastic epithelial cells stain positive for CK19. If the progenitor cells contain ER or not remains highly controversial. Steroid receptor expression is a characteristic of terminally differentiated cells but recently a study showed that the suprabasal cells coexpressed ER and CK19 [21], implying that they could be the origin of the common CK19/ER positive breast cancer. The theory that breast cancers arise from stem cells or progenitor cells is a rather inviting thought based on the slow renewal, capacity to accumulate mutations over time that terminally differentiated cells do not have. The answers to these questions will not be found until the true hierarchy of normal breast differentiation has been outlined.

Breast cancer types and prognostic markers

Breast cancer microarray analysis has revealed two major subclasses of tumors, luminal like cancers (ER-positive, with rather good prognosis) and basal like cancers (ER-negative, with luminal, myoepithelial markers and poor prognosis) [22-24]. These characteristics are also preserved in the molecular profile of the tumors. Traditional cancer diagnostic techniques include assessment of histological appearance, identification of specific tumor subtypes, tumor grading [6], tumour size [25], lymph node status [26], and presence of metastases (TNM-staging).

Identification of subtypes is preformed according to the WHO classification of invasive breast cancer into ductal carcinomas, the most common subtype (80-85%), lobular carcinomas, tubular and mucinous carcinomas, and medullary carcinomas (atypical medullary carcinoma). There are also two types of non-invasive disease, ductal carcinoma in situ (DCIS) and lobular carcinoma in situ (LCIS). DCIS and LCIS are known to be able to develop into invasive disease if left untreated. The progression from non invasive disease to invasive disease and the correlation to prognosis is very complex and can be reviewed in [27].

Tumor grading is based on a grading system first described by Richardsson and Bloom in the 1950s [28] and then modified by Elston and Ellis [6]. The parameters considered are tubule formation, nuclear atypia, and mitotic count. Each of these parameters receives a score from 1-3 and the sum of the scores will divide the tumors into grade 1 (score 3-5) well differentiated, grade 2 (score 6-7) intermediate differentiation or grade 3 (score 8-9) poor differentiation; and these grades are collectively called the Nottingham histological grade (NHG). NHG is closely related to prognosis and is used as a prognostic marker.

TNM-staging is a combination of tumor size, presence of lymph node metastases and distant metastases, and is used to measure the extent of the disease [29]. Tumor size is measured in mm and is correlated to the prognosis of the disease. Lymph node status is based on presence of micro- or macrometastases in the axillary lymph nodes. There is a correlation between the number of positive lymph nodes and the

progression of the disease. Last, the involvement of bone marrow metastases is examined, and if it is positive there is a correlation to relapse and death. Taken together the TNM assessment classifies a tumor as a stage I if the tumor is 0-2 cm and node negative, stage II if the tumor is 2-5 cm, or involves positive lymph nodes or stage III disease with advanced tumors attached to chest or skin, and positive lymph nodes. Stage IV tumors display distant metastases and positive lymph nodes.

The age of the patient is also important at diagnosis, such that the younger the patient (below 35) the worse the prognosis [30] and above 70 the prognosis tends to be worse as well [31].

ER and PR are both prognostic and predictive markers in invasive breast cancers [32]. It has been shown that women with ER-positive tumors have a better disease free survival and OS than women with ER-negative tumors; however this difference might not persist in long term follow-ups. ER and PR are strong predictive factors to the benefit of tamoxifen [33]; this will be discussed in detail later.

The cerbB-2 (HER2/neu) proto-oncogene is a tyrosine kinase protein similar to EGFR. It is amplified and/or overexpressed in 30% [34] of all breast cancers and is associated with increased tumor aggressiveness, recurrences, and mortality in node-positive patients [35]. Different studies have shown a resistance to chemotherapy when the tumor is HER2 positive [36] but this was not shown in a large long time follow-up study [37]. The predictive value of HER2 regarding tamoxifen treatment is a bit narrow since HER2 and ER are inversely related, thus limiting the number of patients that were treated with tamoxifen. One trial with postmenopausal breast cancer patients showed resistance to tamoxifen therapy in HER2 positive patients [38]. Trastuzumab (Herceptin), is a recombinant humanized monoclonal antibody directed against the extracellular part of HER2 and it reduces signaling from PI3K and MAPK cascades, promoting cell cycle arrest and apoptosis (reviewed by [39]). In the metastatic setting HER2 directed treatment with trastuzumab increases the response rate of patients [40]. Clinical trials were recently reporting that trastuzumab in the adjuvant therapy of early-stage breast cancer improves disease free survival as well as a reduces the risk of breast cancer death [41,42].

Breast cancer treatment

Primary surgery of the breast

Over the last 30 years breast cancer patients have been introduced to mammography as a diagnostic tool. Because of mammography most women now present with small tumors, inducing a shift from radical breast surgery (mastectomy) to breast conserving therapy where a good cosmetic result can be achieved, preserving the woman's body self image and allowing for rapid recovery. Breast conservative surgery is often complemented with postoperative radiotherapy. Unfortunately, postoperative radiotherapy is also a risk factor for development of contralateral breast cancer [43].

While mastectomy is still the method of choice when the tumor is large and locally advanced, both methods are equally effective in terms of survival [44,45].

Axillary surgery
All patients with invasive breast cancer receive some surgery to the axillary lymph nodes and removal of up to ten lymph nodes has been recommended. But sentinel lymph node biopsy is rapidly gaining popularity as a diagnostic procedure since it enables selective targeting of the first tumor-draining lymph node, where the initial metastases will form. A negative sentinel node predicts the absence of tumor metastases in the other regional lymph nodes with a high degree of accuracy (reviewed by [46,47]). This means that in the case of a negative sentinel node, regional lymph node dissection can be avoided. This will prevent the many side effects of a complete lymph node dissection, with decreased morbidity. In stage I/II breast cancer, the sentinel node biopsy is already used as a definite staging procedure [48]. Only in case of a tumor positive sentinel node on histo-pathological investigation is axillary lymph node dissection performed.

Postoperative radiotherapy
Radiotherapy after breast conservating surgery is given to the breast to destroy any residual breast cancer cells and is known to decrease the risk of recurrences [43]. After mastectomy radiotherapy can be applied to the chest wall for the same purpose. Occasionally radiotherapy is also given to the axillary lymph nodes in patients with four or more positive nodes. Rare side effects with radiotherapy, including lung scarring and heart damage, and newer methods with partial breast irradiation have shown fewer of these unwanted side-effects and might have the potential to replace the longer, whole breast treatments [49].

Adjuvant chemotherapy
In endocrine non-responsive tumors, chemotherapy is the systemic adjuvant treatment of choice, independent of patient age or lymph node status [50]. In endocrine-responsive disease, chemotherapy plays an important role next to endocrine treatment. The question of which patients need combined chemo-endocrine therapy, and for whom endocrine therapy alone is sufficient, remains unsolved. Anthracyclines (doxorubicin and epirubicin) act to prevent cell division by disrupting the structure of the DNA and terminate its function in two ways; by intercalation into the base pairs in the DNA minor grooves, and also cause free radical damage of the ribose DNA. Cyclophosphamide is an alkylating agent that also targets the DNA of cancer cells. Methotrexate and fluorouracil are antimetabolites. Taxanes (taxotere and taxol) function by their ability to immobilize the microtubules in dividing cells. Anthracyclines are standard adjuvant chemotherapy, superior to other combined drugs as CMF (cyclophosphamide, methotrexate, fluorouracil) [51]. It is generally accepted that taxanes are among the most active chemotherapy agents in the management of metastatic breast cancer, as they appear to improve overall survival, time to progression and overall response in women with metastatic disease. Recent published evidence suggests that adding taxanes to anthracyclin regimens

may benefit patient survival and that taxanes are a valid therapeutic option in node-positive, hormone receptor negative breast cancer, reviewed by [52].

Endocrine therapy

Adjuvant endocrine therapy is recommended for ER positive tumors (more than 10% positive tumor cells), regardless of age, menopausal status, node involvement and tumour size. The therapies are directed to either block or downregulate the ER, or reduce the circulating estrogens within and around the tumor. In premenopausal patients the major supply of steroid hormones is attributed to the ovaries and ovarian ablation or medical suppression using LHRH agonists are effective endocrine treatments [51]. Tamoxifen (a selective estrogen receptor modulator) is also an effective adjuvant endocrine therapy in both pre and post-menopausal patients and 5 years of treatment is currently favored. Adjuvant tamoxifen for 5 years appears to reduce relapse and death, with benefits for several years after its cessation [51]. In postmenopausal patients ovarian secretion of estrogens is ended and therefore ovarian ablation is not necessary, instead the supply of hormones is produced in peripheral tissues (fat cells) and in the tumor itself. The enzyme converting androstenedione and testosterone to estrone and estradiol is aromatase, and new aromatase inhibitors might be a more effective treatment than tamoxifen in postmenopausal patients [53]. Chemo and tamoxifen treatment is still the first-line recommended treatment regime in postmenopausal patients. Anastrozole (an aromatase inhibitor) is used as second line treatment for 5 years. The pure estrogen receptor blocker fulvestrant (ICI 182,780) is currently used in metastatic disease, and was recently shown to be as effective as anastrozole as second-line treatment for time to progression, and was well tolerated [54]. A critical observation that distinguishes the pure estrogen antagonist fulvestrant from tamoxifen is that fulvestrant leads to degradation of ER through the proteasome and also fails to elicit a compensatory stimulation of ER mRNA, thus causing a sustained depletion of ER. Tamoxifen does not downregulate ER but rather stabilizes the nuclear form of the receptor.

Estrogen receptor expression and cofactors

About 70% of all breast cancers are positive for ER and/or PR and estrogen is the main stimulus for development and growth of these tumors. Not much is known about the novel ERβ and here we will only briefly discuss the possible role of this receptor (ERα will be referred to as ER). The first estrogen receptor (ER) was identified in 1967 and the gene is located on chromosome 6q25 [15,55]. Mammary glands of ER KO mice have normal embryonic and fetal development, but these glands never develop beyond the newborn stage [56,57]. The majority of efforts to understand ER action have focused on its functional domains and protein-protein interactions because mutations in the receptor were considered a very rare event, present in less than 1% of the primary tumors. Due to newer and more sophisticated techniques it has been shown that deletions of different exons and naturally occurring mutations can cause constitutive receptor activity and proliferation even in the absence or low levels of estrogen, as for example the K303R mutation [58]. ER negative tumors

Table 1. Effects of hormones and hormon receptor modulators on ER expression.

Agent	Pathway	Tissue/Cell line	ER mRNA	ER protein
Estradiol	Estrogen	MCF-7	Increase	Decrease
		Endothelium	Increase	Decrease
		Hypthalamus	Increase	Increase
		Osteoblast	Increase	
ICI 182 780	Estrogen antagonist	MCF-7	No effect	Decrease
Tamoxifen	SERM	MCF-7	No effect/	Stablization
		Uterus	Increase	Decrease
Raloxifen	SERM	MCF-7	Increase	

and tamoxifen resistant tumors may be caused by additional mutations leading to a dominant negative receptor (review by [59]).
Over the past years, many investigators have separately identified and named as many as eight upstream untranslated ER exons [60]. To date, at least seven different promoters have been described; the most common promoter found expressed in human tissues and cell lines is encoded in exon 1 and is termed promoter A [61]. Considering this complexity it is not surprising that numerous factors may affect ER expression. Loss of estrogen receptor expression in breast cancer and in breast cancer cell lines is often attributed to methylation of the estrogen receptor gene and DNMT1 (DNA methyltransferase 1) is upregulated 2-10 fold in ER negative breast cancer cell lines [62,63]. Gene silencing by methylation also involves the assistance of HDACs, resulting in a closed DNA-histone structure. Two transcription factors, ERF-1, which transactivates promoter A, and ERBF-1 which control the transcription of promoter B, are also involved in ER-expression [64,65]. Hyperactivation of MAPK signaling has been shown to downregulate the expression of ER in breast caner cell lines [66]. Hormones also influence the levels of ER expression and estrogen induces a rapid ER turn-over mediated by the ubiquitin ligase 26S proteasome system [67]. The degradation is linked to an ER-mediated transcription response, this predicts a balance between novel ER synthesis via transcription and degradation in mammary epithelium [68]. How different hormones, anti-estrogens and growth factors regulate ER expression is summarized by [69], (see table 1). Overexpression of cerbB2 (HER2) in advanced breast cancer is associated with hormone unresponsiveness, implicating the epidermal growth factor (EGF) pathway in ER regulation, and cell line studies have shown that EGF [70] and heregulin [71] cause a decrease in ER gene transcription. Hypoxic conditions are also linked to downregulated ER expression [72-76], this will be discussed in detail further on.

After entering cells, estrogen binds to and activates the ER, a member of the steroid/thyroid/retinoid nuclear receptor family of transcription factors. Estrogen binding

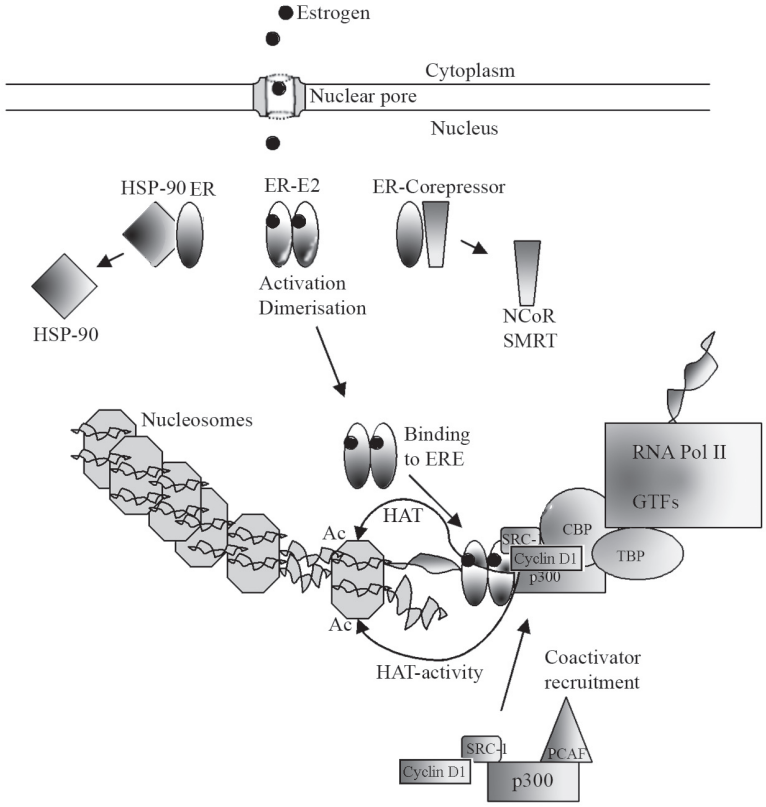

Figure 3. Schematic diagram of the mechanism of ER action in a target cell nucleus. E2 is generally believed to diffuse freely through the capillary wall and the cell membrane and the cytoplasm and enters the nucleus by two processes: 1) passive diffusion through the central channel of the nuclear pore or 2) active transport. Free ER resides in the nucleus in complex with the hsp90 chaperon complex or bound to co-repressors such as SMRT or NCoR. Binding of E2 to the ER activates the receptor by inducing alterations in the conformation of the receptor protein, triggering dimerization. ER dimer binds to EREs in the regulatory region of the target gene. Once bound to the ERE, ER recruits co-activator proteins, such as SRC-1, P/CAF, cyclin D1. SRC-1 is a component of a co-activator complex that includes p300 and PCAF. The histone acetyltransferase (HAT) activity of co-activators acetylates lysine residues in histones within the nucleosome, relieving chromatin repression and facilitating the binding of general transcription factors and interaction with components of the RNA polymerase II initiation complex.

results in disassociation from chaperones (Hsp90, Hsp70) [77], dimerization and a conformational change in the receptor, allowing it to bind to specific sequences, small palindromic DNA motifs, called estrogen responsive elements (EREs) reviewed by [78], in the promoters of estrogen regulated target genes (see figure 3). In addition to

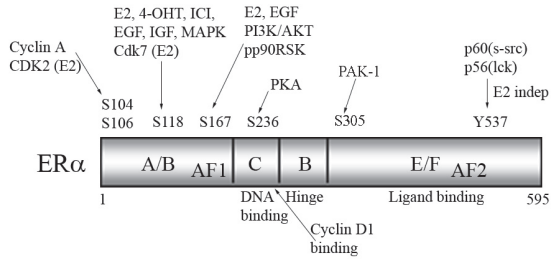

Figure 4. Kinases induce ER phosphorylation at specific sites. Phosphorylation of the ER occurs within AF1 at S104/S106, S118 and S167 by a variety of kinases as indicated. S236, in the DNA binding domain, is phosphorylated by PKA and Y537, in the AF2 domian, is phosphorylated by src family kinases. PAK-1 phosphorylates ER at S305 in the AF2 domian. The co-activator, Cyclin D1, binds to the receptor in the DNA-binding domain.

classical ER-dependent gene transcription via ERE upstream of regulated genes, ER can interact with Jun/Fos proteins at the AP-1 promoter site of certain genes, such as the collagenase gene. A number of estrogen-responsive genes that lack EREs contain ERE half-sites, or binding sites for the orphan nuclear hormone receptor SF-1, SF-1 response elements (SFREs) that serve as direct ER binding sites. Finally, estrogen responsiveness can be driven by a half-ERE site in strict combination with a nearby Sp1 site, both of which must be occupied for maximal activation, reviewed by [79]. Two different activation domains, AF1 and AF2 of ER, mediate the transcriptional activation. AF2 contains the ligand-binding domain and requires estrogen to be activated. The AF1 domain is regulated by phosphorylation to be activated (see figure 4). Both these domains can start the transcription of target genes, independently or synergistically, and this is regulated by the promoter type and the cell type where binding occurs, reviewed by [59]. The AF1 domain can be activated/phosphorylated at key positions, such as serine 118, serine 167 and threonine 311, by different growth factor signaling cascades. These include the MAPK/extracellular-regulated kinase (ERK) pathway [80], which can be activated by growth factors such as hergulin (via HER2), epidermal growth factor (via EGFR) [81], the AKT/PI3K pathway [82], which can be activated via the insulin like growth factor pathway, and the p38 MAPK pathway, which is activated by stress and various cytokines. New findings show that PAK-1 also has the ability to phosphorylate ER at serine 305 [83]. Recruitment of the general transcription machinery starts when ER binds to EREs and this is facilitated by co-activator complexes. Several co-activator complexes also mediate remodeling of chromatin, such as the SWI/SNF complex [84], the TRAP/DRIP/SMCC complex [85] which associates with RNA polymerase II and CBP [86,87] and P/CAF [88] which are histone acetyltransferase complexes (HATs). P160 co-activators interact with ER in the liganded AF2 domain [89,90], these include NCOA1 (SRC-1), NCOA2 (TIF2, GRIP1) and NCOA3 (P/CIP, ACTR, AIB-1, RAC3, TRAM-1), and associate with CBP. Cyclin D1 can bind directly to SRCs and ER, promoting ligand-independent binding of ER to EREs [91,92]. Cyclin D1 can also associate with P/CAF and form a complex with ER [93]. NCOA1 and NCOA3 have intrinsic histone acetylase activity. Co-activator recruitment to the LBD is mediated by a short motif in co-activators,

characterized by the amino-acid sequence leucine–X–X–leucine–leucine (where X is any amino acid). Unliganded nuclear receptors recruit co-repressors (NCOR1, NCOR2 or SMRTs) to the AF2 domain [94] that interacts with histone deacetylase complexes (HDACs) [95].

ERβ, the second estrogen receptor was identified in 1996, and the gene is not located on the same chromosome as ER but on 14q22-24 [16,96]. The DNA binding domain of ERβ differs from ER by one amino acid only, and there is 53% homology in the AF2 domain whereas the AF1 domain is not well conserved [97]. Both ligand bound receptors can mediate transcription via EREs but have opposite effects through the AP-1 sites, with ER promoting transcription and ERβ blocking it. Conversely, antiestrogens such as tamoxifen, having bound to ERβ, actually promote gene transcription through the AP-1 pathway—exactly the opposite to the intended effect [98]. In receptor positive breast tissue the ERα:ERβ ratio differs between benign and malignant tissues, with a dominance of ERα in malignant and ERβ in benign tissue [99]. There are 5 different isoforms of ERβ [100] and ERβ has been found to inhibit cell migration in the normal cell, inhibit proliferation in a ligand independent fashion and promote an epithelioid-like shape [101]. ERβ and ER have been demonstrated to form heterodimers, as well as homodimers, further complicating their individual and/or combined function within a cell. The prognostic significance of ERβ remains the most fundamental and challenging question in the clinical setting and present studies have shown diverging results.

Tamoxifen and tamoxifen resistance mechanisms

Tamoxifen and raloxifene are clinically important selective estrogen receptor modulators (SERMs) that inhibit the activation of the ER AF2 domain and thereby the ligand bound receptor activity, but they do not prevent the activation of the AF1 domain. In the breast epithelium ER activity is mainly due to the AF2 domain so tamoxifen acts as an antagonist to the receptor, whereas in other tissues such as the uterus, AF1 activity is the important activation pathway rendering tamoxifen into a stimulatory agonist instead of an antagonist [102]. Tamoxifen binds to the LBD and covers the co-activator binding pocket, preventing co-activator assembly and transcriptional activation by AF2. Tamoxifen also interacts with co-repressors but the mechanism is not fully understood. Given the complexity of ER activation, there are several mechanisms by which resistance to endocrine therapies might evolve. Either the ER itself or any number of ER-interacting proteins might be deregulated to tip the balance towards ER activation, even in the absence of estrogen or in the presence of an ER antagonist, reviewed by [103,104]. Two forms of antiestrogen resistance occur; about 50% of breast cancers do not respond initially to antiestrogen therapy (de novo resistance), whereas acquired resistance occurs when tumors initially responding eventually relapse. Absence of estrogen receptors/progesterone receptors is the most common mechanism of de novo resistance. In the case of acquired resistance, however, the problem is not well understood and is likely to involve several alterations in the structure and function of the estrogen receptor, genetic and epigenetic

alterations in the tumor cells, altered pharmacokinetics as well as receptor crosstalk. An important early finding was that ER expression was lost at relapse in about 15% of breast cancer patients who developed acquired tamoxifen resistance, providing an explanation for relapse in this group. However, functional ER was retained in the other patients, and the clinical evidence that response might still occur to endocrine therapy indicated that tumor regrowth might still involve functional ER signaling, reviewed by [105]. Mutations correlated to tamoxifen resistance are rare but Y537K in the LBD results in a receptor that is active without estrogen and in the presence of tamoxifen, reviewed by [59]. K303R leads to increased estrogen sensitivity and this mutation has been detected in 34% of premalignant hyperplastic lesions, in one recent study [58]. The clinical relevance regarding resistance is still under determination.

There is growing evidence that crosstalk between ER and growth factor signaling pathways, in particular the EGFR-family, is a mechanism by which tamoxifen resistance can develop [104]. Growth factors such as EGF, TGFα and Amphiregulin bind to the external part of EGFR, which dimerizes either as a homodimer or a heterodimer with HER2 or HER3 and Heregulin binds to HER3 in a similar manner. This will result in activation of the AKT and the MAPK pathways, leading to phosphorylation of the ER, as described earlier, and increases tumor growth and survival. ERK1 and ERK2 phosphorylate ER on serine 118 contributing to ligand independent activity [80,81] and ERKs also activate RSK, which phosphorylates ER on serine 670 [106]. The MAPK pathway also leads to the activation of coregulators such as AIB1 [107] or SRC-1, further contributing to tamoxifen resistance since the agonistic properties of tamoxifen are enhanced in cells with increased levels of these coactivators [102,108]. ERK1/2 expression and/or activity is increased in several breast cancer cell-line-derived models of endocrine resistance [109,110], as well as in human breast cancers compared with non-malignant breast tissue [111]. Increased ERK1/2 activity also correlates with poorer quality and shorter duration of response to endocrine therapies [112], as well as decreased survival [113]. Other factors involved in tamoxifen resistance are PKA-signaling [114], PAK-1 expression [83], high levels of c-AMP [115] and CDK7 [116].

Recent studies have identified a small pool of ER that resides in the plasma membrane or the cytoplasm, that contributes to very short term effects of estrogen (within minutes) [117,118]. The membrane bound ER can interact with IGFR, the p85 of PI3K, Src, EGFR and HER2, and this can lead to secondary EGF release which initiates growth factor signaling, reviewed by [104]. The membrane bound ER reacts to tamoxifen as if it was an estrogen; this might not be important in cells with low levels of EGFR or HER2 but in tumors with enhanced crosstalk this might possibly contribute to tamoxifen resistant tumor growth [119]. MCF-7 cells overexpressing HER2 become resistant to tamoxifen treatment, and both estrogen and tamoxifen recruited co-activators to the receptor explaining the agonist effect [119]. Interestingly, gefinitib, an EGFR inhibitor reversed this effect indicating that patients with enhanced HER2 or EGFR signalling would benefit from this treatment. Further, perhaps aromatase inhibitor treatment would be a therapeutic advantage compared to tamoxifen in such patients.

Cyclin D1 and tamoxifen in breast cancer

Cyclin D1 is overexpressed in about 50% of all breast cancers and the gene CCND1 at 11q13 is amplified in about 15% of primary tumors [120-122]. Transgenic mice overexpressing cyclin D1 in the mammary glands develop adenocarcinoma [123] and cyclin D1 deficient mice do not develop the mammary epithelium properly during pregnancy and have abnormal retinas [124,125]. Further, cyclin D1 deficient mice are totally resistant to breast tumors induced by Neu and RAS, but not to tumors induced by Wnt-1 and Myc [126]. Mice deficient in cyclin D2 or cyclin D3, the other subtypes of cyclin Ds, were still susceptible to all four oncogenes, indicating that lack of cyclin D1 was unique in this context. In conclusion to this, Neu and RAS tumourogenisis is dependent on cyclin D1 to promote tumour growth in breast epithelium. Cyclin D2 expression is fairly rare in breast cancer due to methylation [127], whereas cyclin D3 is frequently overexpressed in breast cancer and is associated with high grade carcinomas [128]. There appears to be a correlation between CCND1 gene amplification and poor disease outcome [129,130]. Cyclin D1 protein expression has been reported to be associated with both better prognosis and worse prognosis. Overexpression of cyclin D1 is known to correlate with the early onset of cancer [131] and to risk of tumor progression and metastasis [132-134] in some studies. However, a number of studies have shown a surprising lack of correlation between increased cyclin D1 expression and increased tumor DNA synthesis [135] and even correlated cyclin D1 to a better prognosis [136,137]. These discrepancies could be attributed to the strong relationship between cyclin D1 and ER-status [138,139], since ER itself is a prognostic marker. Activation of ER by estrogen leads to transcriptional induction of cyclin D1. Hence it is possible that cyclin D1 expression merely reflects the presence of a functional ER [140], alternatively that cyclin D1 gains its oncogenic function through interaction with ER. Cyclin D1 is a target gene for ER transcription via Jun/Fos proteins at the AP-1 promoter site [141] and also via half-ERE sites in combination with a nearby Sp1 site [142]. It was also shown that in tamoxifen resistant HER2 overexpressing MCF-7 cells, tamoxifen treatment contributed to cyclin D1 transcription [119]. Further, cyclin D1 has the ability to increase ER activity independent of cdk-function and hormone [143,144], by recruiting SRC-1 [91] and P/CAF [93] to the ER. P/CAF has HAT activity to unwind the DNA, increasing the cyclin D1 induced transcriptional activity.

Cyclin D1 is a major player in bridging of mitogenic signals to the early G1-phase in the Rb-regulated cell cycle. Apart from being activated by estrogens via ER [140], cyclin D1 transcription can be activated by other growth factors such as IGF I [145] and II, and by oncogeneic signals such as PPAR [146], HER2/Neu [147], SRC [148], Ras [149], β-catenin [150], STATs [151] and SV40t [152]. It has been shown that overexpression of cyclin D1 will increase proliferation [153,154] and inhibition of cyclin D1 will arrest cells in G1-phase [155]. Cyclin D1 associates with cyclin dependent kinase (CDK) 4 and 6 and phosphorylate the Rb-protein (and other members of the Rb-family as p107, p130), thus starting the inactivation of pRb-E2F-binding and promotes transcription of genes like cyclin E. Cyclin D1´s initial phophorylation of Rb will not lead to transcription of cyclin A, because it will not remove BRG1 from the Rb complex,

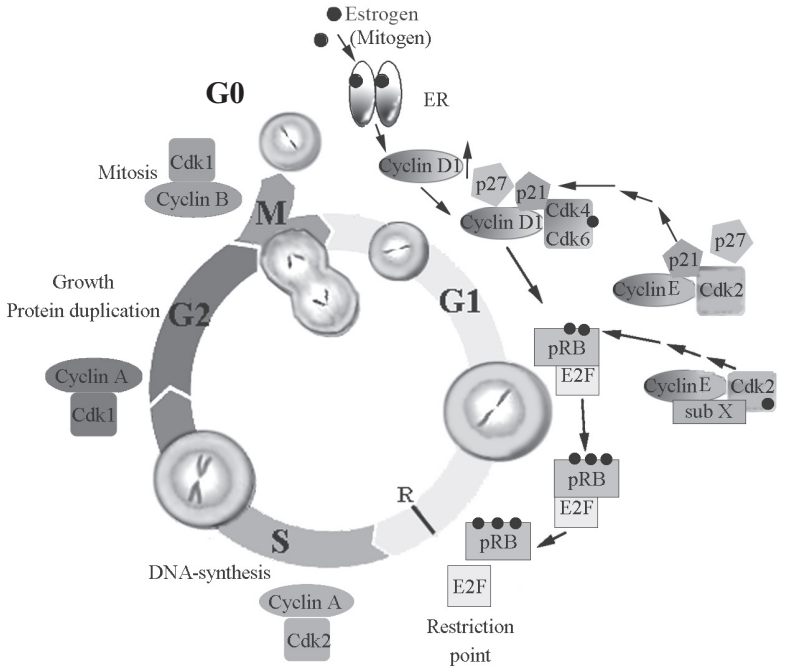

Figure 5. Illustration of how estrogen signaling through activation of cyclin D1 leads to cell cycle progression. Cdk4 or Cdk6 in complex with cyclin D1 can be viewed as mitogen sensors, that act together with p21, p27, during G1 phase to initiate phosphorylation of pRB releasing E2F from the inhibitory complex. Activation of cyclin E by E2F enables formation of the cyclin E-cdk2 complex. This is accelerated by the continued sequestration of p21, p27 into complexes with assembling cyclin D-cdk complexes. Cyclin E-cdk2 completes the phosphorylation of Rb, further activating E2F-responsive genes needed for progression into S-phase, including cyclin A. The major cyclin-CDK complexes active in human cells are illustrated. The different stages of the cell cycle are mitosis (M), quiescence (G0), G1, the DNA synthetic phase (S) and G2.

as this will only occur when Rb is heavily phosphorylated. Cyclin D1 also titrates p27 and p21 away from cyclin E/CDK2 complexes, leading to their activation and in the cyclin D1/CDK complex p27 and p21 functions as assembly factors stabilizing the complex. Cyclin E in association with CDK2 sequentially phosphorylates Rb further, thereby releasing the gate keeping function of Rb in G1, and passage through the restriction point leads to DNA synthesis and cell cycle progression, reviewed by [156,157], figure 5.

In breast cancer there appears to be different subtypes of tumors with variable characteristics, those with a more favorable outcome display an intact Rb-pathway, cyclin D1 in close correlation to proliferation and ER-expression, p16 silencing via methylation and decreased expression of p27 CDK inhibitor. In the other arm are tumors with an inactivated Rb-pathway, high cyclin E expression and ER-negative phenotype with a more aggressive prognosis [158]. Since these proteins appear

to have redundant functions in the mammary gland, as evidenced by the ability of cyclin E to substitute for cyclin D1 [159], it is tempting to speculate that they might be exerting similar roles in tumorigenesis in these two different breast cancer phenotypes. Regarding tamoxifen response in cyclin D1 overexpressing tumors the clinical results point in diverging directions [134,160], this could be due to analysis within relatively small cohorts, with including patients with different stages of breast cancer, and exposed to different treatment regimes. Further, there have been implications that cyclin D1 overexpression may lead to tamoxifen resistance [161]; therefore we set out to investigate this relation in a prospective randomized clinical trial.

Hypoxia and HIF-1

Like most solid cancers, breast cancers require new blood vessel growth if they grow beyond a few millimeters in diameter [162]. Neovascularization of tumors is usually not very sufficient, because the new vessels are leaky, disorganized and have blunt endings; still they supply the tumor with nutrients and routes for metastases. High grade DCIS has a rim of microvessels around the ducts that are filled with proliferating epithelial cells, with a characteristic necrosis in the center, indicating that there is oxygen deficiency in this region, defined as hypoxia (pO_2 <7 mmHg, 1% O_2) [163,164]. In normal breast epithelium the pO_2 is approximately 65 mmHg (7% O_2), but in locally advanced breast tumors the mean fluctuates around 10 mmHg (1.5% O_2) with several tumors between 0-2.5 mmHg (0-0.2% O_2) [165]. To mimic hypoxia in our cell line cultures we exposed the cells to 1% O_2. 1% O_2 induces a rather mild hypoxia, minimizing the acute hypoxic effects of lower oxygen tension but still very well in line with the in vivo situation. Unfortunately, our control cells are maintained in traditional cell line culture systems in normal air oxygen, 21% O_2, when the most preferable control conditions would be about 7% O_2.

As the tumor develops into an invasive form a correlation between poor prognosis and amount of microvessels or other factors that stimulate vessel growth arises. In understanding the molecular and biochemical signaling involved during hypoxia one of the major players, hypoxia inducible factor-1α (HIF-1α, here abbreviated as HIF-1) was found [166,167]. HIF-1 is a heterodimeric transcription factor that dimerizes with HIF-1β (or ARNT) under hypoxic conditions [168]. HIF-1β is a nuclear protein that is constitutively expressed and is independent of O_2 tension. Under oxygenated conditions, or normoxia, HIF-1 is bound to von Hippel-Lindau (VHL)-protein, which leads to ubiquitination and rapid degradation of HIF-1 [169]. In contrast, during hypoxia, HIF-1 is stabilized because VHL cannot interact with HIF-1 due to inactivation of prolyl hydroxylases that during normoxia hydroxylate HIF-1 (at Pro-402 and Pro-564) promoting the binding of VHL [170]. Prolyl hydroxylases serve as oxygen sensors to the cell [171]. Acetylation of HIF-1 at lysine-532 enhances the interaction of VHL with HIF-1, promoting its ubiquitination and degradation. HIF-1 transactivation-domain function is also O_2-regulated via the action of FIH-1 (factor inhibiting HIF-1) [172]. FIH-1 mediates this effect by hydroxylation of asparaginase-803, which prevents the interaction of HIF-1 with co-activators p300 and CBP [173]. In hypoxia, HIF-1 localizes to the nucleus, and active HIF-1 dimers bind DNA pro-

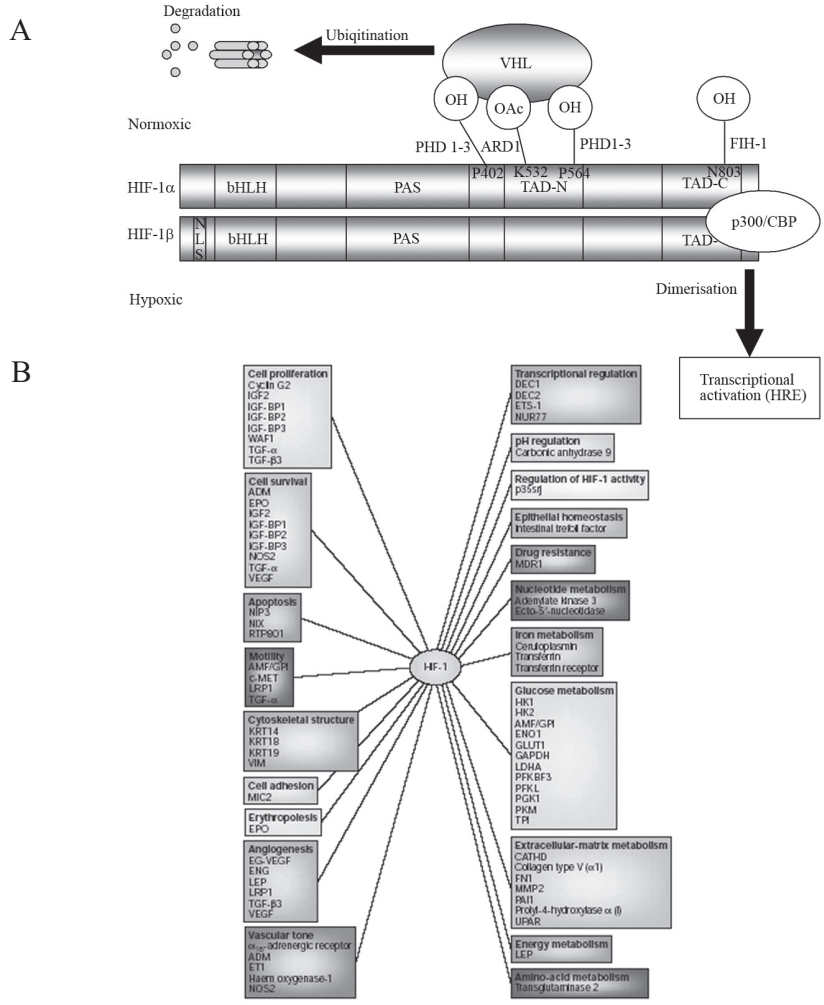

Figure 6. O_2-dependent regulation of HIF-1 activity. A) O_2 regulates the rate at which HIF-1 protein is degraded. In normoxic conditions, O_2-dependent hydroxylation of proline (P) residues 402 and 564 in HIF-1 by the enzymes PHD 1–3 is required for the binding of the VHL tumour-suppressor protein, which is the recognition component of an E3 ubiquitin-protein ligase. VHL binding is also promoted by acetylation of lysine (K) residue 532 by the ARD1 acetyltransferase. Ubiquitination of HIF-1 targets the protein for degradation by the 26S proteasome. O_2-dependent hydroxylation of asparagine (N) residue 803 in HIF-1 by the enzyme FIH-1 (factor inhibiting HIF-1) blocks the binding of p300 and CBP to HIF-1α and therefore inhibits HIF-1-mediated gene transcription. Under hypoxic conditions, the rate of asparagine and proline hydroxylation decreases. VHL cannot bind to HIF-1 that is not prolyl-hydroxylated, resulting in a decreased rate of HIF-1 degradation. By contrast, p300 and CBP can bind to HIF-1 that is not asparaginyl-hydroxylated, and HIF-1 dimerizes with HIF-1β allowing transcriptional activation of HIF-1 target genes, illustrated in different processes in B, modified from ref [175]

moter regions containing hypoxia response elements (HRE's) containing the core recognition sequence 5 ′-RCGTC-3 ′ and then recruit coactivator molecules (p300, CBP), resulting in increased transcription, mRNA synthesis, leading ultimately to the biosynthesis of proteins that mediate responses to hypoxia [174], including over 60 putative genes involved in metabolic adaptation (glycolysis), apoptotic resistance, angiogenesis, invasion and metastasis, reviewed by [175], see figure 6. Additionally, HIF-1 activity can be increased through phophorylation, for example HIF-1 can be directly phophorylated by p42/p44 MAPK, increasing the transcriptional activity at HRE's [176,177], partly through interaction with p300 [178]. In light of these features it is not surprising that HIF-1 is overexpressed in many human cancers. A significant association between HIF-1 expression and patients' mortality has been shown for example in breast [179,180], cervix [181] and endometrial [182] cancers. HIF-1 KO mouse embryos suffer from severe cardiovascular malformations and deficiencies in neuronal development; they die at E 10.5, indicating the major role of HIF-1 during normal development [183]. The embryos display failure in key functions of downstream target genes, such as erythropoietin in oxygen delivery and maturation of erythrocytes and VEGF in angiogenetic activiation and motility of endothelial cells, remodeling of extracellular matrix through activation of matrix metalloproteinase 2, cathepsin D, urokinase plasminogen activator receptor, and CK 14/18/19.

Non-hypoxic activation of HIF-1.

Even if hypoxia is the main activation of HIF-1 there is increasing evidence indicating that growth factor stimulation, cytokines, vascular hormones and viral proteins can turn on HIF-1 expression and activity, reviewed by [184]. In normoxia HIF-1 accumulation is due to an increase in protein translation, rather than decreased degradation, shifting the balance toward accumulation of normoxic HIF-1. The PI3K pathway has been implicated in the increased translation of HIF-1, via activation of the ribosomal S6 protein through phosphorylation of p70S6K that regulates translation of mRNA's with an extreme 5' terminus with a stretch of 4-14 pyrimidines, which is found in the mRNA of HIF-1. Recently also the MAPK pathway was shown to activate HIF-1 transcription [185,186]. This appears to be the main mechanism responsible for HIF-1 induction by vasoactive hormones such as angiotensin II, thrombin (involved in wound healing) and endothelin and by lipopolysaccarides in macrophages (involved in inflammation). Growth factors like heregulin [187], insulin-like growth factor 2 [188] and TGF-α, are all activators and gene targets of HIF-1, promoting proliferation and survival. Interleukin-1 has also been shown to promote activation of HIF-1 in normoxia [189].

HIF-1 and MAPK

MAPKs were identified through their activation in response to growth factor stimulation, e.g. EGF, PDGF, NGF, insulin, ILR, phorbol esters, and thrombin. Maximal MAPK activity requires both phosphorylation on tyrosine and threonine residues. The best characterized MAPKs fall into three subgroups. First, the extracellular

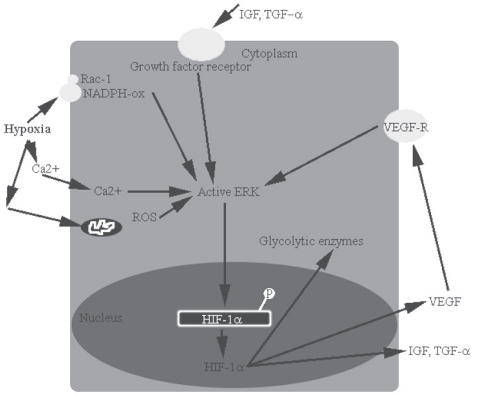

Figure 7. Schematic representation of upstream activators of the ERK pathway that could be involved in the regulation of HIF-1 phosphorylation and HIF-1 transcriptional activity. Under hypoxia, HIF-1 is stabilized and translocated into the nucleus. In hypoxic, or in growth factor activated cells, the ERK pathway is activated and ERK phosphorylates HIF-1. This phosphorylation enhances HIF-1 transcriptional activity. The hypoxic activation of the ERK pathway may be due to calcium influx triggered by a glucose/sodium symport or by membrane depolarization. Mitochondrial ROS or NADPH-oxidase associated to Rac1 are activators of the ERK pathway. Finally, the hypoxia-induced VEGF production as well as other growth factors and oncogenes are ERK activators that can participate in enhancing HIF-1 activity, modified from ref [191].

signal-regulated kinase-1 (ERK1 or p42), and ERK2 (p44) and their relatives; this subgroup is referred to as ERKs. The second subgroup consists of Jun N-terminal kinases (JNKs), because they can activate the jun transcription factor. The third subgroup is the p38 MAPKs. In 1997 it was discovered that hypoxia and hypoxia followed by reoxygenation rapidly activated upstream Src-family TyrKs and p21ras [190], which was followed by a sequential activation of Raf-1, MEK-1/2, ERK1/2 and S6 kinase (p90-rsk). Further, hypoxia also caused a rapid activation of stress activated kinases such as p65-PAK, p38 and SAPK. ERK1/2 can control the expression of VEGF at two levels, in normoxia ERK1/2 can activate the VEGF promoter and in hypoxia ERK1/2 is known to phosphorylate HIF-1, increasing its transcriptional activity and inducing VEGF expression (see figure 7). HIF-1 and its relation to MAPK signaling were recently reviewed by [191]. Regarding the impaired tamoxifen response seen when ERK1/2 is activated, as described earlier, we investigated the role of hypoxia induced ERK1/2 in response to tamoxifen treatment in breast cancer cell lines and the effect on ER expression.

Breast cancer and HIF-1

Increased levels of HIF-1 have been reported in breast carcinogenesis, especially in poorly differentiated lesions but not in the normal breast [192]. In poorly differentiated ductal carcinoma in situ we report that HIF-1 expression has been correlated to loss of ER expression as well as a more undifferentiated phenotype [74]. From studies of breast cancer cell lines it appears that hypoxia also correlates to loss of ER expression [73,75,76,193] and that this may induce tamoxifen resistant growth [72]. Decreased ER expression and its downstream target PR in tumor cells surrounding necrotic zones

are nevertheless obvious [194] and despite some inconsistencies between studies [192], there seems to be an inverse link between HIF-1 expression and ER in in vivo tumors [195]. The mechanism of ER reduction is attributed to proteasomal breakdown [73,75,76] but we show that over time also a transcriptional reduction is present [196]. HIF-1 is correlated to proliferation, tumor size, cyclin E and cyclin A, but not cyclin D1 [197]. The prognostic role of HIF-1 is being established and several reports show a correlation between HIF-1 expression and shorter overall survival [179,180,198,199]. Some studies show that the lymph node positive group was influenced by HIF-1 expression [179,200] and the other study showed that the lymph node negative group, but not the lymph node positive group, was influenced by HIF-1 [180]. The differing results might be due to either short follow-up or small patient cohorts. One recent study including 745 patients with long term follow-up showed that HIF-1 was indeed correlated with a worse overall survival as reported before, but no significance was shown in the lymph node negative population [198]. Further, HIF-1 was correlated to early relapse, and was predictive of risk for metastasis.

We set out to study the relation between HIF-1 and prognostic, as well as predictive importance, with regards to tamoxifen treatment response in a randomized trial.

THE PRESENT INVESTIGATION

Aims

The general aim of this thesis was to investigate the role of cyclin D1 as an estrogen receptor cofactor and how hypoxia influences estrogen receptor regulation, with focus on cyclin D1 and HIF-1's tumor specific expression, and prognostic and treatment predictive features.

The specific aims were:

- To evaluate how ER is coexpressed with cyclin D1 and HIF-1 in breast tumors with heterogenous ER expression (paper I).
- To investigate the role of the ER coactivator cyclin D1, in predicting tamoxifen response in a randomized trial including 248 postmenopausal women whose tumors were arranged into a tissue microarray (paper II).
- To investigate whether differentiation of breast cancer cells is affected by hypoxia, using DCIS with central necroses as well as hypoxia exposed cell lines as model systems (paper III).
- To examine the mechanism behind hypoxia induced ER downregulation, with regards to protein degradation and transcriptional regulation, with focus on MAPK-signaling (paper IV).
- To investigate the tamoxifen treatment effect on proliferation during hypoxia and MAPK-inhibition using breast cancer cell lines (paper IV).
- To examine the impact of HIF-1 as a prognostic and predictive marker for tamoxifen response using a randomized trial including 564 premenopausal breast cancer patients (paper V).

Cyclin D1 in breast cancer (Paper I, II)

Paper I

Breast cancer is often defined as ER positive or ER negative and ER is used as a marker for prognosis and is indicative of hormone responsiveness in adjuvant tamoxifen treatment of breast cancer. Nevertheless, resistance to SERMS, as tamoxifen, remains an unsolved challenge in disease management. Hormonal therapy resistant tumor growth is caused by several factors and one such factor is the ER content, which often is highly variable in different cells of the same tumor. The occurrence of ER negative tumor cells might be a consequence of clonal selection among tumor cells. This would lead to mosaics in the pattern/distribution of ER expression, resulting in tumor cell populations with different characteristics than the majority of the tumor cells (reviewed by [201]). These clones might not respond in a predictive way to treatment and may even have the capacity to expand during treatment giving the tumor a more aggressive phenotype. Cyclin D1 expression in breast cancer cells has consistently been associated with ER positivity (reviewed by [92]). However, it has been shown that in ER negative breast cancers, cyclin D1 was an independent prognostic marker for poor prognosis both in terms of RFS and OS [202].

In paper I, ER heterogeneity was analyzed in 134 ER-positive tumors stained immunohistocmemically for ER, cyclin D1 and HIF-1 expression (discussed in Paper III). To examine the correlation between ER and cyclin D1 protein expression in detail we further used a primary breast cancer material consisting of 114 tumors where ER was previously analyzed using an ELISA based assay and cyclin D1 was determined by Western blot analysis [139].
We found 24 tumors with heterogeneous expression of ER and 6 of these cases displayed an inverse relation between cyclin D1 and ER protein expression. Cyclin D1 high, ER-low areas within the tumors displayed higher proliferation and lower differentiation according to Elston-Ellis criteria compared with areas with low cyclin D1 and high ER expression. In 2 of the cases, cyclin D1 protein overexpression was explained by clonal expansion of CCND1 amplified tumor cells. CCND1 gene amplification as well as other amplicons have been correlated to poorer differentiation in breast cancer [203].

In the primary breast cancer material (114) ER and cyclin D1 expression were indeed associated ($p=0.001$). However, within the 81 ER positive tumors an inverse correlation was present where tumors with extreme ER expression had low cyclin D1 expression and vice versa. Tumors with high cyclin D1 expression and low ER were also correlated to higher proliferation and lower differentiation, supporting the observation from the heterogeneous tumors. Further, these tumors were more commonly c-myc amplified and c-erbB2 high. This is in line with a recent report where it was shown that HER2 and Ras transformation were dependent on cyclin D1 expres-

sion in breast cancer [126]. Heterogeneously expressed ER is a rather common event (18%), whereas patterns with inverse correlations to cyclin D1 would account for about 5% of the tumors in the investigated material (134 tumors). In this subgroup of tumors cyclin D1 has the potential to influence anti-hormonal treatment response [134,204]. Cyclin D1 has indeed been reported to be a cofactor to ER [91,205]. The role of cyclin D1 as a predictor of tamoxifen response will be investigated in paper II.

Paper II

Cyclin D1 was originally described as a cell cycle regulator as it binds cdk4/6, promotes the phosphorylation of RB, and triggers the entry of cells into G1/S-phase. More recently several cdk-independent functions of cyclin D1 have been found, as it regulates the activity of transcription factors, coactivators, (e.g.ER with and without ligand), and corepressors that are involved in chromatin remodeling and histone acetylation. Moreover, cyclin D1 is known to influence cellular metabolism, migration and fat cell differentiation, reviewed in [206]. As part of the estrogen receptor complex, cyclin D1 might have the potential to influence the response to tamoxifen, reviewed by [103].

In order to better clarify the role of cyclin D1 in breast cancer and in anti-estrogen response we analyzed a patient cohort of 248 postmenopausal breast cancer patients randomized to 2 years of tamoxifen or no treatment. Representative parts of the tumors were assembled into a tissue microarray and then stained immunohistochemically for ER and cyclin D1. The randomized study included both ER-positive and ER-negative breast cancers and we first delineated the fraction of ER-positive tumors (>10% staining) and a subgroup of patients therein with more than 90% ER-positive tumor cells, representing a homogenous group of potentially highly tamoxifen responsive tumors. Cyclin D1 was scored according to fraction and intensity. Relevant tissue array biopsies for ER/cyclin D1 analysis were obtained from 167 tumors.

In the cohort of patients with 10% ER-positive tumor cells tamoxifen only slightly improved survival, whereas the subgroup containing tumors with more than 90% ER-positive showed a positive response to tamoxifen ($p=0.1446$). Cyclin D1 fraction and cyclin D1 intensity were positively correlated ($p=<0.0001$). The fraction of cyclin D1 positive cells did not seem to influence the effect of tamoxifen treatment. When analyzing ER positive tumors and separating cyclin D1 intensity into moderate/low and cyclin D1 high cases, a clear distinction in tamoxifen response was seen. In the cyclin D1/low group there was a difference in survival between tamoxifen treated patients and controls in the entire ER-positive cohort ($p=0.0509$), and the difference was even more distinct in the highly ER-positive cohort ($p=0.0077$). Surprisingly, the difference between tamoxifen and no treatment was completely eliminated for tumors with high cyclin D1 levels, suggesting that overexpression of cyclin D1 was linked to tamoxifen resistance, despite high and homogenous ER content.

An impaired tamoxifen response has later been shown by our group [207], also in premenopausal patients with cyclin D1 high tumors, using the tumor material described in paper V. Further, another report recently showed the same results for postmenopausal patients [208].
Untreated controls can be used to investigate true prognostic information without the interference of adjuvant treatment and for the subgroup with high cyclin D1 contra low/moderate levels the mortality rates were 56% versus 71% (p=0.613). This suggests that high levels of cyclin D1 are associated with an overall better prognosis than moderate or low cyclin D1 levels in untreated patients. Interestingly, the opposite was observed in the tamoxifen treated cohort. This illustrates the importance of including an untreated cohort in prognostic studies, and might explain some of the divergent results concerning cyclin D1 in different studies.

The different results regarding cyclin D1 fraction versus intensities is not clear but the fraction of positive cells might reflect the number of cells in different phases of the cell cycle, whereas intensities reflect the maximum level of protein expression independent of proliferation. Cyclin D1 can be induced through the MAPK signaling cascade and data from our group has correlated ERK1/2 phosphorylation expression to tamoxifen resistance in this particular randomized trial [112]. Intensities might also be linked to the amplification status of the cyclin D1 gene, CCND1 [207]. In this study CCND1 amplification was by far a more powerful predictor of tamoxifen response than cyclin D1 expression, even in the absence of protein overexpression. The CCND1 amplified tumors actually identified tumors where tamoxifen seemed to have agonistic effects. These findings indicate the potential involvement of other amplified products, besides cyclin D1, at chromosome 11q13 in tamoxifen resistance. The mechanism behind the effect of cyclin D1 protein expression on tamoxifen response might be through direct interaction between cyclin D1 and the ER/SCR-1 complex [91], potentially causing an agonistic activity of tamoxifen. We recently identified another potential protein involved with tamoxifen response and cyclin D1 in breast cancer, PAK1. The PAK1 gene is located at the same locus as the cyclin D1 gene. Expression evaluation in randomized breast cancer trials and cell line studies showed that high PAK1 expression correlated to loss of tamoxifen treatment response (Holm et al, manuscript submitted). PAK1 has the ability to

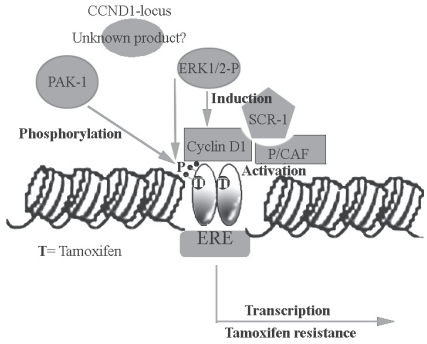

Figure 8. Schematic illustration of our current understanding of how the cross-talk between the ER pathway and cyclin D1, PAK-1 and ERK kinase pathways can contribute to active ER-transcription and tamoxifen failure in breast cancer. Further, an unknown protein residing at the CCND1 locus might also be involved in tamoxifen resistance.

phosphorylate ER at serine 305 and when tamoxifen is bound to the receptor this causes activation of the receptor [83]. Whether cyclin D1 and PAK1 are coamplified in breast cancer will be examined in the near future. Nevertheless, it seems like a proportion of patients receiving tamoxifen does not benefit from it and this is partially mediated through cyclin D1 (figure 8). Theoretically, by inhibiting cyclin D1 in ER-positive tumors with overexpressed cyclin D1 the treatment response might be improved.

Hypoxia in breast cancer (Paper I, III, IV, V)

Paper III and Paper I

Hypoxia frequently developes in solid tumor tissues and is correlated to a worse prognosis regarding recurrences, locoregional spread and metastasis reviewed by [209] and the clinical relevance of HIF-1 in breast cancer [198] is under investigation. HIF-1 is a transcription factor, with over 60 known target genes [210] that is accumulated during hypoxia and adapt cells to survival in low oxygen supply. In breast cancer HIF-1 expression has been associated with poorly differentiated tumors [192] and loss of ER expression [72]. Hypoxia has been linked to a more immature tumor phenotype in neuroblastomas [211], and potentially similar changes could be induced in breast cancer.

The purpose of these studies was to explore if hypoxia influences the differentiation grade and the ER expression in breast cancer. In paper III we chose DCIS lesions as our model system, as these consist of well-defined tumor lesions, with frequent central necrosis, lack of vascularization and stabilization of HIF-1 expression towards the necrotic regions. Tubule formation, consisting of tumor cells with the ability to polarize with radial orientation of the apex towards an intracellular lumen, is a measurement of differentiation in breast cancer. ER and cytokeratine 19 (CK19), an early marker for mammary gland development and a potential breast stem cell marker [212], were used as differentiation markers.

In paper I we stained the 24 ER heterogeneous invasive breast tumors for HIF-1 protein expression, to study the link between ER heterogeneity and hypoxia.

Further, to verify that the changes observed in the tumor models in paper III and I were indeed due to the hypoxic environment, three ER positive breast cancer cell lines were exposed to 1% O_2 in a hypoxic chamber for 72 hours and analyzed for ER-expression.

As reported in paper III, tubule formation was lost in cell layers in close proximity to the necrotic zones in DCIS. Further, the nuclear to cytoplasmic index increased significantly from the basal cell layers towards the hypoxic cell layers. Together these traditional histological differentiation criteria indicate a more malignant DCIS phenotype. 8/19 DCIS expressed ER, but the expression was decreased or lost completely in the inner most cell layers towards the necrosis. CK19 expression was present throughout the tumor lesions but greatly enhanced towards the necrotic zones. To test whether these changes were secondary to hypoxia-induced apoptosis

we stained for apoptotic cells using the TUNEL assay, detecting fragmented DNA, but no apparent gradient in apoptotic cells was present towards the necrosis. Ki-67 staining, used to monitor proliferation in the lesions, showed that cells ceased to divide towards the hypoxic regions, potentially due to poor nutrient supply and lack of growth factors.
9 out of 24 invasive tumors, showed regions with HIF-1 expression where the ER expression was downregulated, indicating that this pattern is not only present in DCIS but also in invasively growing tumors.
ER was downregulated in all three cell lines, perfectly mimicking the results seen in the DCIS (paper III) and in invasive tumors (paper I). CK19 expression was upregulated in line with data postulated in paper III. Ki-67 staining and flow cytometry showed that two of the three cell lines decreased proliferative capasity but maintained viable after hypoxic exposure. The third cell line, T47D did not show any major changes in normoxic versus hypoxic conditions.

Taken together these findings suggests that hypoxic conditions in the tumor cell microenvironment drive cells to acquire a less differentiated phenotype. Recently, a single mouse breast stem cell was isolated that could reconstitute the entire mammary organ [213]. Cytokeratin 19 expression is found in the putative progenitor cells of the luminal epithelial compartment [214] and if hypoxia indeed drives cells into a more immature phenotype it might be linked to such a progenitor. Furthermore, ER downregulation in tumor cells within a hypoxic microenvironment could have implications for tamoxifen treatment efficacy. Downregulation of ER during hypoxia has later been confirmed both in DCIS, invasive tumors and cell lines by others, and the underlying mechanism behind this has been attributed to an increased proteasomal degradation of the receptor [73,76,75].

Paper IV
ER is present in 70 % of all breast cancer and is the most important predictor of tamoxifen response [33]. However, either de novo or acquired resistance is often observed, and the possible mechanisms of actions can be summarized into three categories; reduction or loss of ER expression, dysfunction of ER-signaling and ligand independent activation of ER. Acquired tamoxifen resistance is correlated to hyperactivated growth factor signaling, induced through overexpression of HER1 and HER2 signaling [104]. Hypoxia is known to trigger the activation of the MAPK signaling cascade, resulting in active (phosphorylated) ERK1/2. ERK1/2 is known to phosphorylate the ER at serine 118, promoting ligand independent activation [80,81]. In MCF-7 cells hyperactivated ERK1/2 is indeed correlated to reduced ER expression [66]. Further, hypoxia caused downregulation of ER expression in breast cancer, through mechanisms not yet fully elucidated.

From conclusions drawn in paper III, and I, with emphasis on ER regulation in hypoxic conditions, we wanted to investigate the mechanism behind the reduced receptor expression. Using DCIS as a model system of hypoxic regions, we observed an increase in active ERK1/2 protein towards the hypoxic regions in 7 out of

21 investigated DCIS. ER positive cell lines exposed to hypoxia showed ERK1/2 phophorylation in parallel to ER decrease. The upstream activator of ERK1/2, MEK-1 was also phophorylated in all three cell lines. Our findings indicate a direct or indirect role of active ERK1/2 in the downregulation of ER in hypoxia.
From a time-based microarray analysis performed on cell lines exposed to hypoxia we got the impression that ER was not only downregulated at the protein level, as shown by [73 76 75] but also at the transcriptional level [215]. Using quantitative RT-PCR we observed a marked decrease in ER transcripts after 72 hours of hypoxic treatment in all three cell lines, indicating that both transcriptional downregulation and proteasomal-dependent degradation are involved in the regulation of ER in hypoxia.
ERK1/2 might be involved in the downregulation of ER in hypoxia and we therefore used MEK1/2 inhibitors and ERK 1/2 siRNA to try to block this pathway. U0126 inhibited phophorylated ERK1/2 in a dose dependent manner in MCF-7 cells and ER was indeed reexpressed in parallel with decreased phophorylation status. Q-RT-PCR showed that the reexpression was due to increased transcription of the ER-gene. PD184352 blocked ERK1/2 phosphorylation and increased ER-protein expression in CAMA cells, proving that the mechanism is not unique to MCF-7 cells or to U0126. siRNA directed to ERK1/2 also produced an increase in ER-expression in CAMA-1 cells during hypoxia.
Due to the effects on ER-levels by blockage of ERK1/2 we wanted to test the proliferative response to anti-estrogens, during hypoxia and after ERK1/2-blockage. We observed that in hypoxia, tamoxifen caused a 9% accumulation of G1/G0-cells and in normoxia the equivalent fraction was 18% G1/G0-cells. Adding U0126 to hypoxic cultures also caused a slight decrease in proliferation, but when using the combined treatment (tamoxifen and U0126) a massive G1/G0-block was induced. Using the relative S-phase decrease to illustrate the additional effect of U0126 in hypoxia, we delineated the effect of U0126 alone and then compared this with the effect of U0126 after compensating for the tamoxifen effect. The compensated curve showed a significant decreased relative S-phase in cultures that were U0126 treated in combination with tamoxifen compared to cultures that were only U0126 treated.
We also immunoblotted ER-expression, and unexpectedly, in the combined treatment the receptor levels did not increase but rather a decreased expression was observed. This could potentially be due to the massive G0/G1 block or attributed to a change in ER-degradation pattern. PR-levels were not upregulated by U0126 treatment indicating that the reexpressed ER might not be functioning as a transcription factor but rather prone to degradation.

We conclude that in hypoxia, ER is downregulated transcriptionally and through proteasomal degradation. Others have suggested that ER is only downregulated through the proteasomal complex and not at the transcriptional level [73,75,76]. Interestingly, downregulation of genes involved in the proteasomal complex was found specifically in the ER-positive cell lines (MCF-7, T47D) during hypoxia in the microarray experiment [215]. Hypoxia induced activation of the MAPK-signaling cascade and ERK1/2 phosphorylation seems to be involved in the transcriptional downregulation of ER (figure 9). It was shown in cells with hyperactive MAPK-signaling, that

NF-kβ might be the protein that is governing the ER repression [216]. In MCF-7 cells exposed to hypoxia the NF-kβ promoter activity was indeed increased [217]. Whether NF-kβ has a role in the repression of ER remains to be investigated.
An ER positive breast cell line exposed to hypoxia showed no difference in cell number between controls and cells treated with ICI 182 780, a specific estrogen receptor inhibitor, compared with normoxic conditions where ICI-treatment drastically decreased the cell growth [72], which is in line with our results using tamoxifen treatment. An ERK1/2 inhibitor might induce an upregulation of the main target for tamoxifen treatment, ER, and in combination these two treatments would theoretically increase the treatment efficacy, thereby better targeting hypoxic tumor cells, which would benefit the subgroup of patients with hypoxia driven downregulation of ER. Administration of hypoxic cytotoxins, that specifically target and kill cells with HIF-1 expression, has been shown to restore the ER expression in human breast cancer xenografts transplanted into athymic nude mice [218]. Unfortunately this drug was shown to have severe toxicity when used in a phase III clinical trial [219]. 103D5R, a small HIF-1 inhibitor molecule, tested on breast cancer, strongly reduced HIF-1alpha protein synthesis, whereas HIF-1alpha mRNA levels and HIF-1alpha degradation were not affected [220]. 103D5R inhibited the phosphorylation of Akt, Erk1/2, and stress-activated protein kinase/c-jun-NH(2)-kinase, without changing the total levels of these proteins, suggesting it might be functional in inhibiting the downregulation of ER that we have explored. The clinical relevance of these in vitro findings needs to be investigated in patients treated with tamoxifen. The role of HIF-

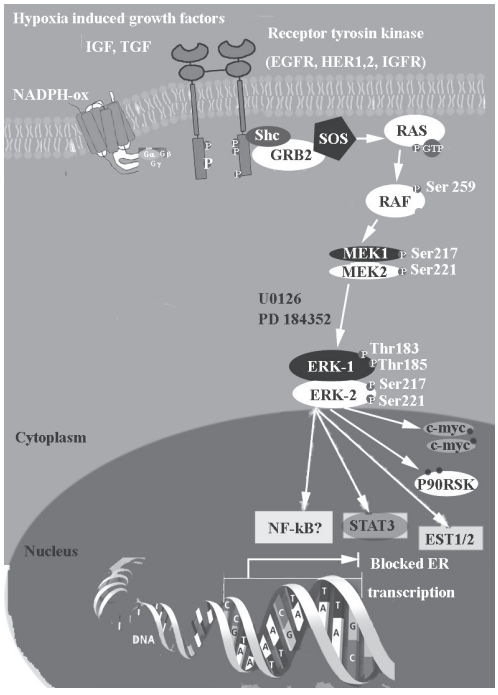

Figure 9. Schematic illustration of the ERK signaling cascade. Hypoxia induced growth factor activation processes are indicated. The growth factor ligand binds to its trans-membrane receptor and activates tyrosine kinases in the receptor molecule.White arrows stand for the main pathway upon growth factor activation. A series of protein complexes form involving Shc, GRB-2, and SOS. Subsequent to this, GDP-Ras is converted to GTP-Ras which activates Raf, MEK and then ERK. ERK activation can be inhibited by U0126 and PD184352. ERK phosphorylates a number of transcription factors such as c-myc, ETS, ELK, STAT3, p90RSK. In hypoxia ERK influences ER repression and we speculate that the protein involved might be NF-kβ.

1 as a predictor of tamoxifen response and as prognostic marker will be investigated in a randomized trial in paper V.

Paper V

HIF-1 is a transcription factor that is involved in tumor growth and metastases, and its main function is to regulate genes induced in response to hypoxia. HIF-1 has been shown to be overexpressed in several different tumor types and correlates to poor prognosis. Only a few reports have investigated the prognostic relevance of HIF-1 in breast cancer. HIF-1 has been shown to be involved in downregulation of ER and potentially induces endocrine treatment resistant tumor growth, but the predictive role of HIF-1 regarding treatment outcome has not been investigated before.

The purpose with this study was to investigate the prognostic and predictive role of HIF-1 in a randomized trial of two years adjuvant tamoxifen versus no treatment, including only premenopausal patients with stage II invasive breast cancer. The tumors were organized into a tissue microarray and immunohistochemical tumor specific HIF-1 expression could be evaluated in 377 cases (67%). HIF-1 was scored as positive in 24% of the breast carcinomas. HIF-1 correlated positively to tumor size, Nottingham histological grade (NHG), Ki-67, Her2 and cyclin E expression and negatively to lymph node metastases, cyclin D1, and ER-PR-expression. There was a trend towards a positive association between Her2-amplification ($p=0.069$) and HIF-1-expression.

HIF-1 and tamoxifen treatment response

Only patients with ER-positive tumors (<10%) responded to tamoxifen treatment and this subgroup was selected for studies of a potential link between HIF-1 expression and tamoxifen response. There was no obvious difference in tamoxifen response between patients with high HIF-1 and low HIF-1 expressing tumors in terms of RFS (recurrence free survival). HIF-1 low tumors showed a near significant tamoxifen effect ($p=0.096$) whereas in the smaller HIF-1 high group there was a significant tamoxifen effect ($p=0.011$).

HIF-1 and Survival

In the entire cohort of patients there was a significantly worse RFS for patients with high HIF-1 expression ($p=0.048$). To investigate pure prognostic information in relation to HIF-1 expression we restricted the analysis to the 288 patients that received no adjuvant tamoxifen treatment. Interestingly, there was only a trend towards worse prognosis and high HIF-1 expression in this subgroup. HIF-1 has earlier been reported as an independent prognostic marker in both lymph node negative as well as lymph node positive disease, and we therefore analyzed the subgroups of lymph node negative and lymph node positive cases separately [180, 179, 200]. A significant association between worse RFS and HIF-1 expression was found in the lymph node positive subgroup, but there was no association to RFS in lymph node negative patients. To further explore prognostic information in HIF-1 expression we analyzed the subgroups of NHG 1/2 versus NHG 3 tumors. There was no difference between NHG 1/2 in RFS, moreover when analyzing HIF-1 expression in this cohort high HIF-1 expression was significantly correlated to a worse prognosis. NHG 3 tumors had a significantly worse RFS compared to NHG 1/2 tumors, but in this cohort high

HIF-1 expression was not correlated to a worse RFS. The result indicates that the prognostic information of HIF-1 expression is restricted to grade 1/2 tumors. When performing a multivariate analysis including all untreated patients, only NHG and node status showed independent prognostic information; HIF-1 was not significant in regard to RFS. In contrast, when restricting the multivariate analysis to NHG 1/2 tumors only, HIF-1 was the only remaining independent prognostic marker for RFS.

Using tissue microarray technique to cover the expression of a marker with heterogenous expression such as HIF-1 might influence the validity of immunohistochemical results. Our observations are nevertheless in line with other studies done on whole tumor section stainings [198] and the tissue microarray technique has been used by others regarding HIF-1 expression in breast cancer [221]. CA IX is a membrane bound glycoprotein whose expression has been shown to be upregulated by HIF-1. Unpublished data shows that in this randomized material there was a positive correlation between HIF-1 and CA-IX ($p=<0.0001$), verifying that the HIF-1 expression indeed is due to functional signaling. Whether the expression is due to hyperactive growth factor signaling or true hypoxia in the tumor is, however, difficult to determine from this study. This randomized clinical trial was organized into a tissue microarray with the intention to make this rare material last longer and to provide a possibility for numerous markers to be tested.
The negative correlation between HIF-1, ER and PR expression strengthens our results from DCIS, whole tumor section stainings, and cell line studies. The fact that HIF-1 expression showed a negative correlation to ER contributes to the low number of patients in the HIF-1 high, ER positive subgroup. From this, we conclude that HIF-1 positive and ER positive tumors seem to be able to respond to tamoxifen, despite obvious links between hypoxia and ER-modulation.
Regarding HIF-1 and prognostic information a recent report showed that hypoxia in the lymph node metastases is predicted by hypoxia in the primary tumor [222]. Further, HIF-1 expression in tumors from primarily lymph node negative patients was predictive of risk of metastasis and relapse [198]. In the whole cohort we found a negative correlation between HIF-1 expression and lymph node metastases, indicating that most HIF-1 positive tumors do not metastasize. However, HIF-1 positive tumors that do metastasize seem to be predictive of a worse prognosis for the breast cancer patient. Potentially, grade 1/2 tumors that express HIF-1 will express HIF-1 also in the metastases, giving rise to a worse prognosis than grade 1/2 tumors without HIF-1 expression. In conclusion, immunodetection of HIF-1 in the primary breast tumor may be an indicator of prognosis and future systemic therapies to control breast cancer might include small inhibitor molecules targeting the HIF-1 signaling pathway [220, 223].

Conclusions

- Regional cyclin D1 expression and hypoxia can be linked to downregulated ER in breast tumors where ER is heterogeneously expressed. An inverse relation between cyclin D1 and ER protein levels was found in a larger material of ER-positive breast tumors.

- Postmenopausal breast cancer patients with ER-positive, cyclin D1 high tumors, did not benefit from tamoxifen treatment. Therefore, cyclin D1 might be a promising predictive marker for tamoxifen response. In the untreated cohort high cyclin D1 tumor content, on the other hand, was associated to a better survival.

- Hypoxia might drive tumor cells to acquire a less differentiated phenotype, including attributes such as loss of tubule formation, increased cytoplasmic to nuclear ratio, lower proliferation, loss of ER expression and increased CK19 expression in DCIS.

- Hypoxia was also strongly linked to ER downregulation in DCIS and invasive breast cancer and caused ER downregulation in breast cancer cell lines. The hypoxia induced ER reduction was due to both proteasomal degradation and decreased transcription.

- Active ERK1/2 signaling appears to be directly or indirectly involved in the transcriptional regulation of ER during hypoxia and by blocking ERK1/2 activity the receptor is partially restored.

- Tamoxifen treatment does not affect proliferation as efficiently in hypoxia as in normoxia, indicating a potential mechanism of acquired resistance. Using MEK1/2 inhibitors to block ERK1/2 signaling we could efficiently augment the tamoxifen response in hypoxia.

- Tumor specific HIF-1 expression was positively associated to tumor size, NHG, Ki-67, Her2 and cyclin E expression and negatively to lymph node metastases, cyclin D1, ER-PR-expression in premenopausal breast cancer patients .

- Tumor specific HIF-1 expression was not a predictive marker for tamoxifen response in premenopausal breast cancer patients. Few tumors coexpressed HIF-1 and ER due to an inverse correlation between the two proteins.

- HIF-1 expression in the primary tumor was associated with worse RFS for premenopausal patients with breast cancer. In patients receiving no adjuvant treatment, HIF-1 had prognostic importance in lymph node positive patients and in patients with NHG1/2 tumors.

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Bröstcancer blir allt vanligare och i Sverige diagnostiseras varje år ca 7000 kvinnor med bröstcancer. Trots detta sjunker dödligheten och de främsta orsakerna till detta är bättre diagnostik och behandling. Operation är den första behandlingen av en bröstcancer. Det opererade området kan behöva strålbehandlas för att döda de cancerceller som eventuellt finns kvar. Därefter används stödjande behandling med cytostatika och antihormonella preparat (Tamoxifen) för att minska risken för återfall. Målet med Tamoxifenbehandling är att blockera östrogen receptorerna (ÖR) så att tumörens tillväxt hämmas. Det finns dock en betydande andel patienter med hormonkänsliga tumörer som trots tamoxifenbehandling får återfall. Hur resistensen uppstår är oklart men ett par mekanismer har identifierats; tumören kan förlora sitt ÖR-innehåll, ÖR-signaleringen kan bli felaktig eller ÖR kan aktiveras oberoende av östrogen. ÖR regleras av ett antal aktiverande och inaktiverande protein. En felaktig balans av dessa kan innebära att receptorn aktiveras trots att den inte är bunden till östrogen eller till och med aktiveras när tamoxifen binder till ÖR.

Cyklin D1 överuttrycks i 50 % av all bröstcancer och har visat sig orsaka tumörer hos möss. Cyklin D1 är en viktig cellcykelreglerare och verkar genom att förmedla tillväxtsignaler till cellens delningsmaskineri. Defekter i cellcykelns tidiga fas (G1/S-regleringen) kan leda till okontrollerad tillväxt och därmed cancer. Cyklin D1 fungerar dessutom som ett aktiverande protein i ÖR. I försök utförda på cellinjer kan för mycket cyklin D1 orsaka resistens mot tamoxifen, men i patienter har förhållandet mellan cyklin D1 och tamoxifenbehandling varit svårare att påvisa.

Syrebrist, hypoxi, i tumören är ett vanligt fenomen som uppkommer när tumören växt sig större än ett par millimeter. För att kunna fortsätta växa måste nya blodkärl bildas som förser tumören med näring och syre. Tumörcellerna har egenskapen att kunna producera flera proteiner som gör att de kan överleva trots låg näring och syretillgång, samt stimulera bildningen av nya blodkärl. Hypoxia Inducible Factor-1α (HIF-1) är det protein som till stor del styr denna process. I bröstcancer verkar det råda ett inverst förhållande mellan HIF-1 och ÖR-positiva tumörceller.

Målsättningen med detta avhandlingsarbete har varit att undersöka cyklin D1 och HIF-1 i förhållande till ÖR i brösttumörer. Ytterligare en målsättning var att undesöka om cyklin D1 och HIF-1 kan påverka utfallet av tamoxifenbehandling i kliniska bröstcancerstudier.

I delarbete 1 fann vi att i ÖR-positiva tumörer med områden där tumörcellerna faktiskt var ÖR-negativa, fanns det en koppling till antingen högt cyklin D1-uttryck eller vilket var vanligare, högt HIF-1-uttryck. I hypoxiska områden i invasiva tumörer (delarbete 1) och i förstadium till bröstcancer (ductal carcinoma in situ, DCIS, delarbete 3) gick ÖR-uttrycket ner. I neuroblastom (en tumör som drabbar barn) har hypoxi visat sig orsaka förändringar i tumörcellerna som indikerar att tumörcellerna blir mer omogna. De liknar en cell i en tidigare utvecklingsfas. I delarbete 3 visar vi

att i bröstcancer kan eventuellt samma process ske, då cellerna får en annat utseende och tappar sin orientering, samt uttrycker mer protein som kopplas till en tidigare differentieringsfas. I både delarbete 1 och 3 visar vi att tre olika ÖR-receptorpositiva cellinjer odlade i hypoxi minskar sitt ÖR-uttryck.

I delarbete 2 undersökte vi förhållandet mellan tumörspecifikt cyklin D1-uttryck och behandlingseffekten av tamoxifen. Detta gjordes i en studie där 251 bröstcancer patienter fick antingen tamoxifenbehandling eller ingen stödjande behandling. Patienter, vars tumörer hade högt ÖR-innehåll och högt cyklin D1-innehåll, svarade inte på tamoxifenbehandlingen. I den obehandlade patientgruppen visade det sig att patienter vars tumörer hade högt cyklin D1 innehåll paradoxalt nog hade en god prognos. Utifrån våra resultat verkar det som att cyklin D1 direkt påverkar ÖR och kan bidra till att tamoxifen inte blockerar receptorn som var dess syfte.

I delarbete 4 har vi försökt att utreda hur HIF-1-aktivering leder till minskat ÖR-uttryck. Ett flertal arbeten tyder på att resistens mot tamoxifen kan vara relaterad till överaktivering av cellsignalering via extracellulärt reglerat kinas (ERK). Överaktivering av ERK har också kopplats till att ÖR minskar i cellinjer. I DCIS med hypoxiska regioner och i bröstcancercellinjer utsatta för hypoxi ökar ERKs aktivitet (delarbete 4). Vi visar att ÖR-uttrycket reduceras via två olika mekanismer, 1) genom att proteinet förstörs via cellens egna nerbrytningssystem 2) genom transcriptionell nedreglering på gennivå. Genom att blockera ERK-aktivering minskade inte ÖR i samma utsträckning under hypoxi. Tamoxifenbehandling på cellinjer visade att de celler som växte i ’normal’ syrenivå hade färre aktivt delande celler efter behandling än de som växte i hypoxi. Blockerades ERK-aktiveringen i de tamoxifenbehandlade, hypoxiska cellerna blev effekten en näst intill en total eliminering av celler med aktiv delning. Dessa resultat indikerar att en patient som inte svarar på tamoxifenbehandling och samtidigt har områden med hypoxi i tumören eventuellt skulle kunna gynnas av en tilläggsbehandling med ERK-blockerare.

I delarbete 5 undersöker vi om HIF-1-positiva tumörceller kan påverka patientens prognos, samt om HIF-1 kan leda till ett sämre tamoxifensvar i en bröstcancerstudie. HIF-1 visade sig dock inte medföra ett minskat svar på tamoxifen, utan patienterna med högt HIF-1 i tumören svarade lika bra på tamoxifenbehandlingen som de som inte uttryckte HIF-1 i tumören. Tumörspecifikt HIF-1-uttryck medförde dock en sämre prognos för patienten. Nyligen publicerades en stor klinisk studie (745 patienter) där tumörspecifikt HIF-1 uttryck undersöktes i bröstcancer. Denna studie visade också att HIF-1-uttryck i tumörcellerna medförde en sämre prognos för patienterna, vilket styrker våra resultat.

För närvarande utvecklas en typ av små molekyler som kan blockera HIF-1-signaleringen och förhoppningarna är att dessa skall fungera som tilläggsbehandling för patienter med cancer. Framtida kliniska studier får avgöra om HIF-1 blockad kan bli en etablerad behandling. Idag har klinikerna endast ett fåtal markörer till hjälp vid valet av behandling av en brösttumör; ÖR, progesteronreceptorn, tumörgrad,

tumörstorlek, lymfmetastaser och HER2. I framtiden kommer dessa sannorlikt att utökas med en mängd andra markörer, exempelvis cyklin D1 och HIF-1. Bröstcancer är alltså inte en specifik sjukdom, utan den består av flera subgrupper med olika prognos och behandlingsutfall. Med hjälp av nya markörer kommer det bli enklare att skräddarsy en behandling för varje patient.

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