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Abstract

Anti-oestogens are commonly used in adjuvant breast cancer treatment and for a long time tamoxifen has been the main endocrine treatment. Patients who are eligibly for endocrine treatment are selected by the tumour-specific expression of oestrogen receptor (ER) and approximately 70 % of all breast cancer patients are classified as ER positive. However, resistance to tamoxifen is common and several patients will experience tumour relapse and might also die from their disease. This stresses the need for identifying treatment predictive factors that can guide clinicians during treatment decisions.

We have identified biomarkers that were associated with tamoxifen response in a material of premenopausal breast tumours. One of the markers is Pak1, a serine/threonine protein kinase, of which high expression and particular nuclear localisation was associated with poor tamoxifen response.

Two other biomarkers are different ER phosphorylations at serine 305 and serine 118; ERS305-P and ERS118-P, respectively. Tumours that were positive for ERS305 were associated with poor tamoxifen response while tumours with high expression of ERS-118 were associated with a good response. Furthermore, we have studied associations between the activated kinases responsible for these ER phosphorylations and tamoxifen response. Our observations led us to conclude that ERS118-P and ERS305-P are better tamoxifen predictive factors than their respective phosphorylating kinases, and possibly, using a combination of the phosphorylations might be even more valuable in predicting the response.

In addition, we have identified Pak1 as a regulator of the hypoxic response in breast cancer cells. More specifically, we have observed Pak1-mediated phosphorylation of the hypoxia inducible transcription factor 1α (HIF-1α). Silencing of Pak1 led to decreased HIF-1α levels and less transcriptional activity, suggesting that Pak1 phosphorylation stabilises HIF-1α and thereby increases HIF-1α's ability to induce gene expression during tumour hypoxia.

Key words: Breast cancer, ER, ER phosphorylation, tamoxifen, predictive marker, Pak1, HIF-1α

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Journal of the National Cancer Institute: 98 (10), 671-80 (2006)

II. Holm C*, Kok M*, Michalides R, Fles R, Koornstra RH, Wesseling J, Hauptmann M, Neefjes J, Peterse JL, Stål O, Landberg G, Linn SC.
Phosphorylation of the oestrogen receptor alpha at serine 305 and prediction of tamoxifen resistance in breast cancer.
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III. Kok M*, **Holm C***, Hauptmann M, Michalides R, Stål O, Linn SC, Landberg G.
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Manuscript

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Borgquist S, **Holm C**, Stendahl M, Anagnostaki L, Landberg G, Jirström K.
Oestrogen receptors alpha and beta show different associations to clinicopathological parameters and their co-expression might predict a better response to endocrine treatment in breast cancer.
Journal of Clinical Pathology: 61 (2), 197-203 (2008)

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Elevated levels of cyclin A1 expression in childhood acute lymphoblastic leukemia.
Leukemia Research: 30 (3), 254-61 (2005)

Ekberg J, **Holm C**, Jalili S, Richter J, Anagnostaki L, Landberg G, Liao Persson J.
Expression of cyclin A1 and cell cycle regulatory proteins in hematopoietic progenitors and acute myeloid leukemia and links to disease relapse and survival.
European Journal of Hematology: 75 (2), 106-15 (2005)

Ekberg J, Jalili S, **Holm C**, Richter J, Anagnostaki L, Landberg G, Liao Persson J,
Expression of cyclin A1 and cell cycle regulatory proteins in hematopoietic progenitors and acute myeloid leukemia and links to disease relapse and survival.
Oncogene: 23 (56), 9082-9 (2004)

CONTENTS

AC	Adenylate cyclase
AF-1 or 2	Activation function 1 or 2
AI	Aromatase inhibitor
AIB1	Amplified in breast cancer
AKT/PKB	Protein kinase B
AP-1	Activating protein 1
ARD1	Arrest-defective 1
ARNT	Aryl hydrocarbon receptor nuclear translocator
BM	Basement membrane
BNIP3	Bcl-2 nineteen interacting protein
BRCA	*Breast cancer* gene
CAIX	Carbonic anhydrase IX
CALLA	Common acute lymphoblastic leukemia antigen
cAMP	Cyclic adenosine monophosphate
CBP/p300	CREB binding protein
CCND1	Cyclin D1 gene
CDK	Cyclin dependent kinase
CIS	Carcinoma *in situ*
CK	Cytokeratin
CoF	Co-factor
CREB	cAMP responsive element binding protein
CSC	Cancer stem cell
C-TAD	C-terminal activation domain
DBD	DNA-binding domain
DCIS	Ductal carcinoma *in situ*
DNA	Deoxyribonucleic acid
E2	Oestradiol (oestrogen)
ECM	Extracellular matrix
EFG	Epidermal growth factor
EMT	Epithelial-mesenchymal transition
ER	Oestrogen receptor
ERE	Oestrogen responsive element
ERK	Extracellular signal regulated kinase
ERS118-P	ER phosphorylated at serine 118
ERS305-P	ER phosphorylated at serine 305
ESA	Epithelial specific antigen
FGFR2	Fibroblast growth factor receptor 2
FIH-1	Factor inhibiting HIF-1
FSH	Follicle-stimulating hormone
HAT	Histone acetyltransferase
HDAC	Histone deacetylase
HER2	Human epidermal growth factor receptor 2
HIF	Hypoxia inducible factor
HRE	Hypoxia response element
IAP2	Inhibitor of apoptosis protein 2
IDC	Invasive ductal carcinoma
ILC	Invasive lobular carcinoma
JNK	c-Jun terminal kinase
LBD	Ligand-binding domain
LCIS	Lobular carcinoma *in situ*
LH	Luteinising hormone
LHRH	Luteinising hormone releasing hormone
LOH	Loss of heterozygosity
MAPK	Mitogen activated protein kinase
MISS	Membrane-initiated steroid signalling
MUC1	Mucin 1
NCoA	Nuclear receptor co-activator
NCoR	Nuclear receptor co-repressor
NF-κB	Nuclear factor kappa B
NHG	Nottingham histological grade
NISS	Nuclear-initiated steroid signalling
NPI	Nottingham prognostic index
N-TAD	N-terminal activation domain
ODDD	Oxygen dependent degradation domain
Pak1	p21 activated kinase 1
PDGF	Platelet derived growth factor
PgR	Progesterone receptor
PHD	Prolyl-4-hydroxylase
PI3K	Phosphatidylinositol 3-kinase
PKA	Protein kinase A
PKC	Protein kinase C
pRb	Retinoblastoma protein
pVHL	Von Hippel Lindau protein
RB	Retinoblastoma gene
ROS	Reactive oxygen species
RSK	Ribosomal s6 kinase
SAPK	Stress-activated protein kinase
SERM	Selective oestrogen receptor modulator
SMA	Smooth muscle actin
SP-1	Specificity protein 1
SRC-1	Steroid receptor co-activator
TAM	Tamoxifen
TDLU	Terminal ductal lobular unit
TGF	Transforming growth factor
TMA	Tissue micro array
TRAP	Thyroid hormone receptor associated protein

INTRODUCTION

Breast cancer accounts for almost 30 % of all diagnosed cancers in Sweden, putting a great demand on research and resources for treating this disease. The fact that breast cancer is such a heterogenous disease, individualised and tailored treatments are necessary. For this reason, factors that might predict disease prognosis and treatment response are very important. The majority of breast cancers thrive on the hormone oestrogen and several drugs have been developed to block the oestrogenic effect in breast cancer. The most widely used anti-oestrogen is tamoxifen. Although it serves as a very effective treatment in many patients, a major drawback with tamoxifen is resistance, which can be present either from the start or acquired after some time of treatment. This thesis will mainly deal with factors that predict tamoxifen response in premenopausal breast cancer patients. In our research, we have used clinical material from a randomised breast cancer trial, where patients after surgery were assigned to either control (no treatment) or two years of adjuvant tamoxifen. We have then identified markers that are significantly associated with either good tamoxifen response or no response. These types of studies are important for the overall understanding tamoxifen resistance mechanisms and also for the identification of patients that are less likely to benefit from tamoxifen who might benefit more from other endocrine treatments.

Another subject matter discussed in this thesis is regulation of hypoxia (low oxygen levels) in breast cancer. Hypoxia is very common in solid tumors, involving countless gene expression changes that promote, among other things, survival and vascularisation of the tumour among other things. Hypoxia is associated with more aggressive tumours and might also impair treatment efficacy. To study factors involved in the regulation of hypoxia and the transcription factors that govern the hypoxic response is important for the overall understanding of tumour hypoxia.

BACKGROUND

Cancer and tumour progression

Normal cells continuously undergo mutations at a slow rate during DNA replication and cell division. Such mistakes will be corrected by different DNA repair mechanisms but sometimes the repair machinery bypass mutations unintentionally. Not all mutations will have a functional effect on the cell, however if the mutation results in growth-promoting advantages, a clonal expansion of that cell will take place and these cells are then targets for further genetic and epigenetic events. This stepwise model with rounds of accumulating mutations followed by selective advantage and cellular expansion is called "the clonal evolution theory of cancer" and it is based on the assumption that cancer arises from a single mutated cell [1, 2].

Another theory of cancer propagation is the hierarchical cancer stem cell (CSC) model. This model proposes that only CSCs are capable of creating and maintaining tumour growth based on their self-renewal and proliferative capacity [3-5]. The CSC does not have to be the cell of origin (the cell that obtains the first mutation) and it is not necessarily derived from a normal stem cell. Instead, it can originate from a progenitor cell that have acquired stem-cell like character. The clonal evolution model and the CSC model are not mutually exclusive and they are both likely to exist in cancer and give rise to tumour heterogeneity. Nonetheless, there has been a substantial amount of data suggesting CSCs in a variety of solid tumours and from a clinical perspective CSCs are of importance since they are thought to be more resistant to different cancer treatments [3].

Accumulation of growth-promoting mutations is the basis of multistep carcinogenesis and while most of the evidence supports the monoclonal origin of human cancer, the possibility that some cancers are derived from several different clones (polyclonal origin) can not be disregarded[2]. The fact that normal cells have very few mutations has lead to the suggestion that spontaneous mutations are not enough to create the vast number of mutations observed in most cancer. Instead, a mutator phenotype in the very early tumour progression could be the explanation [6]. A mutator phenotype is the result of mutations in genes important for maintaining genomic stability, i.e. DNA synthesis and repair genes and chromosomal segregation genes. This would lead to a genetically instable cell type with an inherited increased mutational rate.

The number of mutations required for tumour formation is not clear, but a fare suggestion is five to six [7]. The mutations frequently occur in genes involved in cellular growth, apoptosis, replicative potential, invasion and angiogenesis [8]. The genes are either classified as proto-oncogenes or tumour suppressor genes, reflective of their normal functions in the cell [9]. Proto-oncogenes are normal genes that promote cell growth and characteristically they code for growth factors or the respective receptor or signalling proteins that convey mitogenic stimuli. Activated proto-oncogenes are called oncogenes and they drive tumour formation through dominant gain-of-function. Tumour suppressor genes act opposite to proto-oncogenes, i.e. they repress tumour formation. Genes that induce apoptosis or inhibit cell cycle are classic tumour suppressors and generally they exhibit recessive loss-of-function, i.e. both alleles have to be lost before an outcome is apparent. This was described as the "two-hit" hypothesis when it was observed that both alleles of the *RB* gene were lost in retinoblastoma [10]. However, for some tumour suppressor genes, a phenotype might arise even if only one allele is mutated or lost, a phenomenon called haploinsufficiency [11]. Another mechanism for silencing a tumour suppressor gene is by epigenetic regulation, such as hypermethylation [9]. Furthermore, proteins encoded by tumour suppressor genes can be subjected to increased degradation or inactivation by viral oncoproteins [12].

The common understanding is that cancer cells are defective in pathways that govern normal proliferation and homeostasis [8, 13]. Normal cellular proliferation is driven by extracellular

stimuli that signals through networks of proteins that drive the cells into DNA replication and cell division in a controlled manner. Cancer cells often acquire the ability to synthesise their own stimuli, e.g. growth factors like PDGF and TGFα, creating a loop of autocrine stimulation. The receptors that communicate the stimulatory signals from the outside to the inside of the cell are also often overexpressed or deregulated in cancer cells. The result of this might be hypersensitivity of the receptor to growth factor levels that normally would not elicit a response or it may generate ligand-independent signalling. Downstream of the receptors are pathways that transmit the signal into the cell nucleus. Aberrations of molecules in these pathways are also common features in cancer cells, e.g. mutated Ras proteins termed "oncogenic Ras" are expressed in ~30 % of all human cancers [14].

In addition to proliferating signals there are anti-proliferative signals, e.g. TGFβ and interferons, which normal cells respond to by going from an active cell cycle to a quiescent state. This arrest in cell cycle is mediated by induction of cell cycle inhibitors that hinder the interaction between cyclins and cyclin-dependent kinases (CDKs) that in turn are responsible for driving the cell cycle forward by phosphorylating the pRb protein. A majority of cancers have disrupted cell cycle control, either due to loss of *RB*, overexpression of cyclins or loss of cell cycle inhibitors [15]. Anti-proliferative signals not only tell the cell to stop dividing but also to enter a state of terminal differentiation, something that cancer cells never do. Conversely, cancer cells frequently display an undifferentiated phenotype [8].

An important mechanism that possibly has to be evaded in all cancers is programmed cell death – apoptosis. Apoptosis can be triggered by an extrinsic pathway from external death signals or by an intrinsic pathway, involving the mitochondrial release of cytochrome *c*. The ultimate effectors in both pathways are the caspases, which are responsible for breaking down DNA and other cellular components [16]. Factors regulating apoptosis are either pro-apoptotic or anti-apoptotic. The most commonly inactivated pro-apoptotic protein in cancer is the tumour suppressor p53, which is inactivated in more than 50 % of all types of cancer. In response to DNA damage or other cellular stress, p53 is activated and induces cellular apoptosis to avoid dissemination of cells with damaged DNA [17, 18].

Apart from having deregulated proliferation and suppressed apoptosis, cancer cells must have limitless ability to replicate. As normal cells divide, telomeres continuously shorten with each cell division, as normal DNA polymerase is unable to replicate the ends of the chromosomes. Therefore, normal cells only proliferate for a certain number of divisions before they enter a permanent state of growth arrest called cellular senescence [19]. Cancer cells can acquire unlimited proliferative capability by up-regulating telomerase. Telomerase is a reverse transcription enzyme that adds nucleotides and successively replicates the telomere ends of the chromosome and as many as 85-90 % of all cancerous cells have up-regulated telomerase.

As the tumour grows, the need for oxygen and nutrients increase and thus new blood vessels are formed by the secretion of angiogenic factors from cancer cells, such as vascular endothelial growth factor (VEGF). This process, where cancer cells induce blood vessel formation, is called the "angiogenic switch", and it is caused by a tipped balance in favour of pro-angiogenic factors [20]. The increase in blood supply will enhance the tumours' chances of metastasising to distant organs. This is an important factor in cancer management since it is well known that it is metastases, and not the primary tumour, that cause death. During tissue invasion, cancer cells are dependent on their migratory ability, degradation of the extracellular matrix and their surrounding environment. A process known as epithelial-mesenchymal transition (EMT) has received a lot of attention in cancer biology on the basis that epithelial cells are unable to migrate from neighbouring cells, while mesenchymal cells tend to be highly motile [21]. The major protein involved in EMT is E-cadherin and its loss of expression has been linked to invasive growth and cancer metastasis [22].

The sequence in which cancer cells acquire all these tumour traits is not universal and even within a tumour, and certainly among different tumours, there is a great divergence, demonstrating the complexity of cancer disease.

The breast

The major growth and development of the breast, or mammary gland, occurs at the beginning of puberty and it is not until pregnancy and lactation that the breast is fully developed [23]. The functional unit of the breast is a branched ductal system surrounded by stroma and fat (figure1). It originates from the nipple and terminates in lobules, referred to as the terminal duct lobular units (TDLUs), where each TDLU is composed of several grapelike alveoli. The ducts and lobules are composed of two cell types: the luminal epithelial cells, which are polarized cells forming a single layer of inner epithelia, and the myoepithelial cells, forming an outer layer of cells attaching to the basement membrane (BM). Myoepithelial cells lining the ducts are spindle-shaped and are aligned parallel along the ducts, whereas in the TDLU they are disconnected, enabling some luminal cells to directly contact the BM. It has recently been recognised that myoepithelial cells act as guardians of tissue polarity and may very well function as innate tumour suppressors [24, 25].

Fully differentiated, the two cell types in the breast have different functions: alveolar luminal epithelial cells are capable of producing and secreting milk after pregnancy, while myoepithelial cells help to drive the milk through the ducts by contracting in response to oxytocin. Also, myoepithelial cells contribute to the making of BM by producing and secreting laminins, collagen IV and fibronectin [24]. Both cell types are believed to arise from a common precursor cell with stem cell like character and it is generally believed that breast stem cells are responsible for the massive expansion of cells that is required during each cycle of pregnancy.

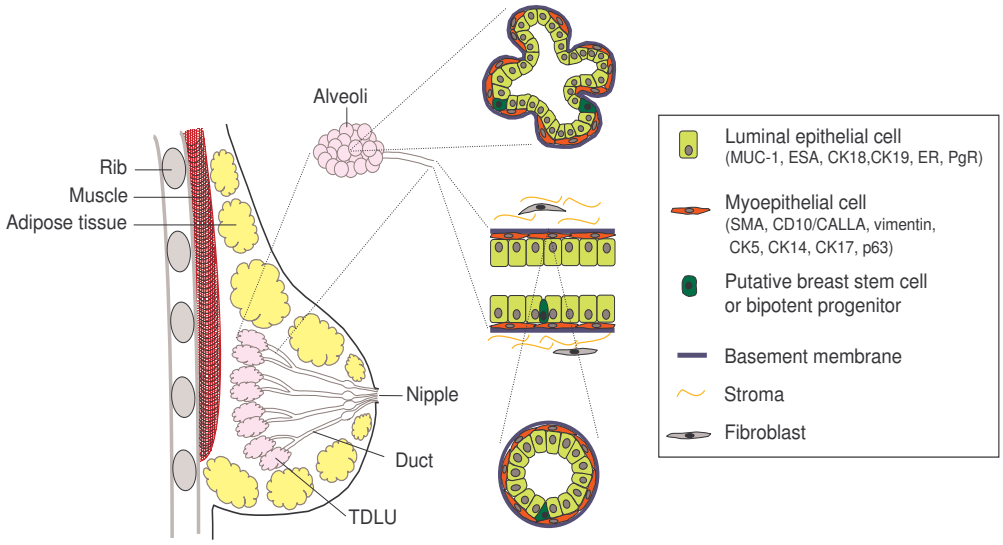

Figure 1. A schematic illustration of the normal breast. The functional units are the ducts and terminal ductal lobular units (TDLUs). Cross sections of a duct and a TDLU are showing the two cell types that are lining the ducts and lobules; the luminal epithelial and the myoepithelial cells. Specific markers that are expressed by each cell type are delineated in brackets.

In contrast to the embryonic and prepubertal stages, further development of the mammary gland during puberty relies on hormonal signalling, mainly from oestrogen and progesterone, which signal through specific hormone receptors: the oestrogen receptor (ER) and progesterone receptor (PgR), respectively. In the normal breast, about 10-20 % of the luminal epithelial cells express ER and/or PgR, and most often the receptors are co-expressed in the same cell [26]. The ER/PgR expressing cells do not proliferate although they are often in close proximity to proliferating cells. Therefore, it is believed that ER/PgR- positive cells stimulate proliferation of ER/PgR-negative cells through secretion of paracrine factors, such as amphiregulin [27, 28].

Breast cancer

Etiology

Breast cancer is the most common cancer in women and it is also one of the leading causes of female cancer related death [29]. It is estimated that more than one million are diagnosed with breast cancer each year in the world, and in Sweden approximately 7000 women are affected annually. The life-time risk of developing breast cancer up to the age of 75 is nearly 10 % and although the incidence has increased over the past 20 years the mortality rates have decreased [Socialstyrelsen, 2007]. This is probably due to a combination of earlier detection and better treatment efficacy. There are several factors, besides age, that are coupled to an increased risk of getting breast cancer, such as heredity, hormonal exposure and lifestyle. 5-10 % of all breast cancers are considered to be hereditary, mainly through mutations in the breast cancer pre-disposing genes *BRCA1* or *BRCA2* [30].

The initiation of breast cancer is thought to be dependent on oestrogen in most cases, and factors that lead to increased oestrogen exposure, e.g. early menarche, late age at first pregnancy, nulliparity, late menopause, oral contraceptives and hormonal replacement therapy (HRT), have been associated with increased breast cancer risk [31, 32]. Another suggestion as to why increased oestrogen exposure would enhance the breast cancer risk is that oestrogen metabolites, especially catechol quinones, are able to react with DNA and cause damage [33]. In addition, studies on lifestyle factors have revealed that there is a positive association between breast cancer and physical inactivity, obesity and alcohol consumption, and a plausible explanation for these associations could be increasing levels of circulation oestrogens [34]. Also, genetic variation or single nucleotide polymorphism in different genes has been associated with breast cancer risk, for instance the *CYP450* genes involved in oestrogen synthesis, the cell cycle regulatory gene *CCND1*, the apoptosis-regulating gene *CASP8* and the growth factor receptor gene *FGFR2,* to name a few [35-38].

Breast cancer progression

The current theory of human cancer development and progression is the accumulation of selective genetic events over time, resulting in increased malignancy. In breast cancer, this is manifested as a sequential progression through different clinical and pathological stages, where each step brings the cancer closer to a full blown invasive cancer (figure 2). Most breast cancers arise from the luminal epithelial cells in the TDLUs. Initiation of abnormal proliferation of cells will lead to atypical hyperplasia that will progress into carcinoma *in situ* (CIS) [39]. In CIS, the cancer cells are still separated from the stroma by a continuous layer of organised myoepithelial cells and a basement membrane (BM). This is considered a precursor-stage of invasive cancer, which is distinguished from CIS by the disappearance of myoepithelial cells and dismembered BM [40]. Although not clearly understood what the key event in this transition is, it has recently become evident that myoepithelial cells and the surrounding stromal cells are very important in the progression from non-invasive to invasive cancer.

As mentioned previously, myoepithelial cells are natural tumour suppressors in the normal breast, however, during the transition from *in situ* to invasive cancer they lose some of their differentiation markers and up-regulate genes that promote cancer progression. Finally, the differentiated myoepithelial cells are outnumbered by the cancer cells and steadily disappear [24]. The importance of myoepithelial and stromal cells is becoming clearer since changes in the tumour microenvironment have a substantial impact on the conditions that allow the cancer cells to become more malignant. Molecular changes during breast cancer progression have been examined using loss of heterozygosity (LOH) and other newly developed techniques such as microarrays, comparative genome hybridization (CGH) and laser capture microdissection, in order to address the question whether the transition from *in situ* to invasive cancer is associated with extensive molecular changes in the cancer cells [39, 41]. Although some data show that there are genetic and transcriptional changes at different stages of cancer cell progression, other results indicate that there is no major change [42-44]. The finding that gene expression signatures are similar throughout distinct stages of breast cancer supports the idea that genetic events resulting in an invasive phenotype are already present in early stages of pre-malign breast cancer, and LOH has even been identified in morphologically normal breast epithelial cells [43, 45, 46]. On the contrary, major alterations in gene expression are detected in different histological grades of breast cancer [39, 43].

The hypothesis that cancer is derived from multipotent stem cells has been supported in breast cancer by the isolation of a subset of tumour cells ($CD44^{+}/CD24^{-/low}/lin^{-}$) designated putative breast cancer stem cells. These cells are able to initiate new tumour formation when injected at low numbers into mammary fat pad of immunodeficient mice [47]. Interestingly, these mammary stem cells appear to be ER negative [48].

Genetic alterations in breast cancer

In most tumour types there is no uniform activation of a certain oncogene or deletion of a tumour suppressor gene. However, certain genetic alterations are more or less common in a specific cancer type, often depending on the organ where the cancer arises. Mutations represent one mechanism for oncogene activation, and genetic predisposition to breast cancer is mediated by mutations in high-penetrance genes, e.g. *BRCA1, BRCA2* and *TP53*, intermediate-penetrance genes, low-penetrance genes and genes of uncertain penetrance [49].

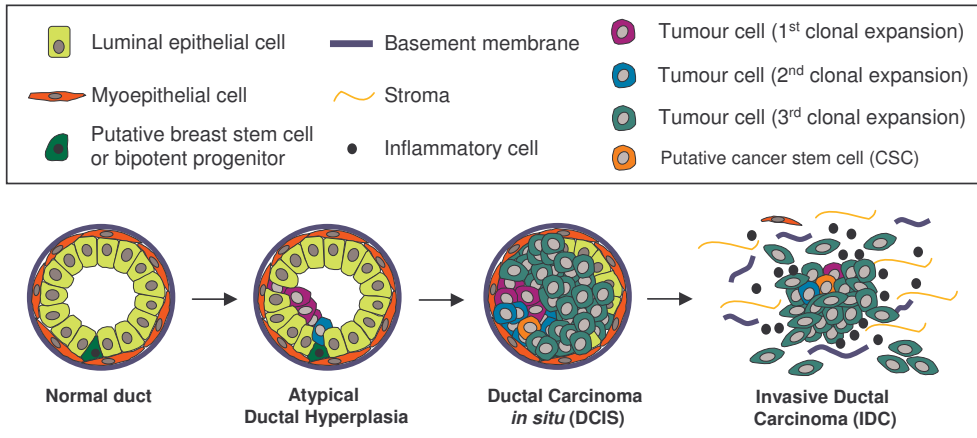

Figure 2. The development of IDC. Abnormal proliferative potential of a normal cell leads to atypical ductal hyperplasia and subsequently to ductal carcinoma *in situ* (DCIS). Breakdown of BM results in invasive ductal carcinoma (IDC). Rounds of clonal expansion of tumour cells are outlined and a putative cancer stem cell (CSC) is also defined.

Another common and important genetic alteration for oncogenic overexpression is amplification. In breast cancer, frequently amplified chromosomal regions are 8p12, 8q24, 11q13, 17q12 and 20q13, among others [50, 51]. A few of the proto-oncogenes in these amplified regions have been identified, such as *HER2* (17q12), *MYC* (8q24), *CCND* and *PAK1* (11q13) [52-55], although it is possible that more than one proto-oncogene in each region is responsible for driving tumour formation. Another gene often amplified in breast cancer is *AIB1*, which codes for the transcriptional co-activator protein SRC3/AIB1/NCoA3 [56]. Inactivation of tumor suppressor genes often involves deletions of chromosomal regions, and commonly deleted regions are 1p, 3p, 6q, 7p, 11q, 16q and 17p, to mention a few [51, 57, 58]. As previously mentioned, a prerequisite in tumor formation is uncontrolled proliferation. In breast cancer, cell-cycle regulators that are overexpressed, lost, mutated or altered by an unknown mechanism include cyclin D1, cyclin E1, CDK4, $p27^{Kip1}$ and pRB [59].

The number of genetic alterations and deregulated proteins involved in breast cancer development and/or progression are too substantial for a detailed description and the above mentioned are just few examples.

Tumour type

Breast cancer is recognised as a very heterogenous disease and by definition of morphological and histological appearance it can be divided into several subtypes where a major distinction is made between non-invasive CIS and invasive breast cancer. A histological difference is also made between ductal cancer and lobular cancer, while it is debated whether these designations are appropriate for signifying their origin, as most cancers arise in TDLUs [39, 60]. Nonetheless, a difference in classification is made between ductal CIS (DCIS) and lobular CIS (LCIS) and invasive ductal cancer (IDC) and invasive lobular cancer (ILC). As mentioned before, CIS is considered a precursor-stage of invasive cancer and today most breast cancers are discovered at an early stage, which has resulted in a tremendous increase of diagnosed DCIS [61]. Other histologically special types of breast cancer are ductal/lobular, mucinous, comedo, inflammatory, tubular, medullary, metaplastic, neuroendocrine, apocrine, adenoid cystic and papillary carcinoma [62, 63].

A molecular based categorisation of breast cancer subtypes has exploited the diversity in gene expression profiles of different breast cancers. This type of molecular classification, using different DNA microarray techniques, separates breast cancers into five classes: luminal A and B (ER-positive), HER2+ (mainly ER-negative), basal-like (mainly ER-, PgR- and HER2-negative) and normal breast-like [64, 65]. Genetic profiling of individual tumors are emerging both for prognostic profiling and for predicting therapeutic response, examples being the Rotterdam 76 gene signature, Invasive Gene Signature, Onco*type* DX™, MammaPrint® and gene signatures that predict sensitivity to anthracyclines and taxanes [66, 67].

Tumour stage, TNM classification

The purpose of using a staging system for breast cancer is to determine how far the cancer has progressed and to help physicians in treatment decisions for the patient. The tumour stage is based on the size of the primary tumour (T), whether it has spread to the lymph nodes (N) and if the cancer has spread to distant parts of the body (M). These three characteristics are each subdivided into different stages and then combined into an overall stage [Swedish Breast Cancer Group, 2008].

Histological grade

Histological grading is a widely accepted classification system for breast cancer and is routinely used at diagnosis. The Nottingham Histological Grade (NHG), first described by Bloom and Richardson and later modified by Elston and Ellis, is based on the assessment of three morphological parameters: tubule formation, nuclear atypia and mitotic count [68, 69]. Each parameter is scored

from 1 to 3, and the total sum of the scores defines the malignancy of the tumour, where grade I, II, and III corresponds to well, moderately and poorly differentiated breast tumours, respectively. Tumour grade provides useful prognostic information in breast cancer, as poorly differentiated high grade tumours are associated with significantly poorer clinical outcome [69].

Prognostic factors in breast cancer

A prognostic factor is used for envisioning the natural course of a disease. For breast cancer, valuable prognostic factors are age, histological grade, tumor size, nodal status, metastases, HER2 status and S-phase [Swedish Breast Cancer Group, 2008]. The Nottingham Prognostic Index (NPI) combines three prognostic factors: tumour size, nodal status, and histological grade [70]. Prognostic factors are valuable for the identification of those patients that only need surgical treatment, and those that need additional therapy, e.g. chemotherapy. However, even a patient with a good prognosis might relapse after surgery, stressing the need for identifying better prognostic factors in breast cancer.

Treatment of primary breast cancer

Surgery

Treatment of breast cancer involves surgical removal of the tumour, either by breast conserving surgery or mastectomy. The type of surgery is largely depending on tumour size and involvement of lymph nodes. Also, women who are at greater risk of developing breast cancer can undergo prophylactic mastectomy. With invasive cancer comes the risk that cancer cells have spread outside the breast, most likely through the axillary lymph system that filters lymph fluid from the breast. By removing the lymph nodes, cancer cells that might reside in them are also removed. However, taking out all of the axillary lymph nodes is combined with side effects such as lymphedema. Instead, removal and examination of only the first node that filters the tumour area is preferred, a procedure known as sentinel lymph node dissection [71]. If there are no cancer cells in the sentinel node, the chances of having cancer cells in the rest of the nodes are small. Even though most breast cancer patients have a localised disease at time of diagnosis, some of them will later on present a metastasised disease. Therefore, surgery is often followed by adjuvant treatment in order to target any residual cancer cells that might be left.

Radiotherapy

Radiotherapy is an effective adjuvant treatment and it is known that women who receive postoperative radiotherapy have a reduced risk of recurrence [72]. It is standard treatment after breast conserving surgery but sometimes also after mastectomy. Radiation is given locally to the tumour area and induces DNA-strand breaks in tumour cells either directly or indirectly through reactive oxygen species (ROS) [73]. Normal cells are also affected by the radiation, however, they are more capable of repairing the damage than tumour cells. Therefore, side-effects after or during radiation therapy are generally small. Tumour hypoxia (low oxygen levels) limits however the effect of radiotherapy, which will be discussed further on.

Chemotherapy

Chemotherapy is used both in the neoadjuvant and adjuvant setting. Patients with hormone receptor-negative tumours are treated with adjuvant polychemotherapy, independent of nodal status and age, and this will increase both overall and relapse-free survival, especially in premenopausal women [74]. In patients with hormone receptor-positive tumours, polychemotherapy may be delivered in combination with endocrine therapy, however, it still remains a question which patients are actually in need of the combination and which patients would benefit from endocrine therapy alone. A combination of several drugs – polychemotherapy, has considerably better effect than a single agent – monochemotherapy. Common combinations of chemotherapy for treating breast

cancer are CMF (cyclophosphamide, metotrexate, 5-fluorouracil), FEC (5-fluorouracil, epirubicin, cycloposphamide) and FAC (5-flourouracil, doxorubicin, cyclophosphamide). The different drugs target cancer cells by various mechanisms and therefore, a combination will hopefully have a synergistic effect. Anthracycline containing combinations, e.g. FAC and FEC, seem superior to non-anthracycline combinations, e.g CMF [74]. Taxanes, like docetaxel and paclitaxel, are other types of chemotherapy commonly used in locally advanced and metastatic breast cancer [Swedish Breast Cancer Group, 2008].

Endocrine therapy

Endocrine therapy was developed over a century ago, when it was discovered that ovariectomy in premenopausal women improved breast cancer prognosis. This finding could be explained by the importance of ovarian hormones, mainly oestrogen, in breast cancer growth, as nearly 70 % of all breast cancers express the receptor for oestrogen. Since then, several different therapies have been generated that target either oestrogen synthesis or the ER (figure 3) [75].

The choice of endocrine treatment depends on the patient's menopausal status. In premenopausal women, ovarian ablation by LHRH analogues, e.g. goserelin (brand name; Zoladex), is possible since the major site of oestrogen synthesis is the ovaries. This type of treatment lowers the level of oestrogen by inhibiting the release of hormones from the pituitary gland, which normally stimulates the release of oestrogen from the ovaries. In postmenopausal women, the levels of oestrogen are already low but they are still sufficient to stimulate growth of breast cancer cells [76]. The low levels of oestrogen are synthesised by adipose tissue and other sites of the body (discussed further on). Also, oestrogen is produced by a proportion of breast tumours.

Aromatase inhibitors (AIs), divided into type 1 or type 2 inhibitors, interfere with the synthesis of oestrogen by blocking the enzyme aromatase. Type 1 inhibitors, e.g. exemestane (Aromasin), are steroidal compounds that bind irreversibly to the substrate site of aromatase and are therefore primarily acting as aromatase inactivators. Type 2 inhibitors, e.g. anastrozole (Arimidex) and letrozole (Femara), are non-steroidal compounds that bind to the heme part of the enzyme and this binding is reversible [77]. The use of AIs have been more confined to postmenopausal women since it is believed that AIs are unable to fully inhibit ovarian aromatase, and might even lead to increased enzyme activity, resulting in higher oestrogen levels [78]. However, third generation AIs are extremely specific, have fewer side effects and are able to suppress oestrogen levels even in premenopausal women [79]. They are currently being investigated in combination with LHRH agonist in premenopausal women [80].

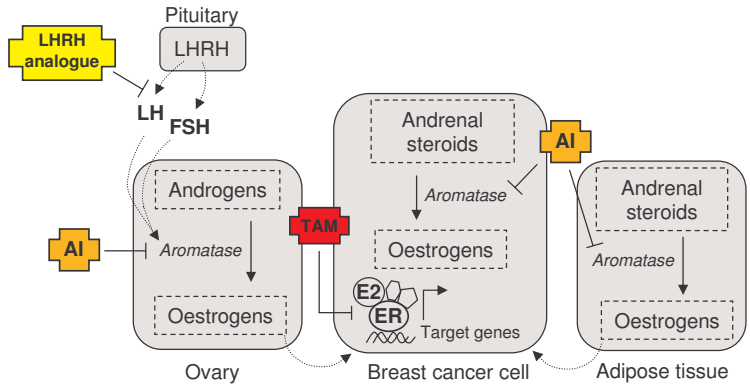

Figure 3. Mechanism of action of different anti-oestrogen treatments. Chemical ovarian ablation is achievable with LHRH analougues, which stop ovarian oestrogen production. Oestrogen synthesis is prevented with aromatase inhibitors (AIs) and the oestrogen receptor (ER) is blocked with tamoxifen (TAM). Modified from [75].

Pure anti-oestrogens, such as fulvestrant (Faslodex), act by down-regulating ER and are only used for treating postmenopausal women with recurrence or a progressed disease following other endocrine treatment [76]. The leading anti-oestrogen agent in both pre-and postmenopausal women is tamoxifen (Nolvadex), which primarily acts through competitive binding to ER, preventing oestrogen to mediate its growth stimulatory effect.

Tamoxifen belongs to a group called selective oestrogen receptor modulators (SERMs) and it has both anti-oestrogenic and oestrogenic effects depending on the tissue [81]. Tamoxifen will be discussed below in further detail. Endocrine therapy is also used in the prevention of breast cancer, initiated by the observation that tamoxifen treatment in the adjuvant setting was associated with a decreased risk of developing new contralateral breast cancer [82]. Currently, tamoxifen is the preventative treatment of choice for premenopausal women and raloxifen (Evista), another SERM, for postmenopausal women [83].

Trastuzumab

In 15-20 % of all breast cancers, the human epidermal growth factor receptor 2 (HER2) is overexpressed, most often due to amplification of the 17q locus where the coding gene is located. HER2 belongs to a family of growth factor receptors that convey growth promoting stimuli into the cell, which can be blocked by a monoclonal antibody, trastuzumab (Herceptin). HER2 may also predict response to certain chemotherapies [84], and while some reports also suggest that ER-positive tumours that are also HER2-positive respond poor to tamoxifen, others have failed to confirm this [85-88].

Oestrogen and oestrogen receptors

Oestrogens are a group of steroid hormones synthesised from the precursor cholesterol by several enzymatic pathways. In premenopausal women, the primary oestrogen is 17β-oestradiol (E2) which is converted to estrone and estriol from testosterone by the enzyme aromatase [37, 89]. The synthesis and secretion of E2 is regulated by the pituitary hormones follicle-stimulating hormone (FSH) and luteinising hormone (LH) which stimulate the conversion of androgens to oestrogens in the ovaries [75]. At menopause, the ovarian oestrogen production cease and the levels of circulating oestrogen are reduced, although, oestrogen synthesis by non-reproductive organs still remains and might even be increased in postmenopausal women [90]. Main sites of peripheral oestrogen synthesis are adipose tissue, adrenal glands, liver, muscle, bone, vascular endothelium and brain [75]. The increased local oestrogen synthesis in elder women have beneficial effects since oestrogen also maintain bone density and have a protective role in the vasculature and central nervous system. However, the increased production could also lead to development of breast tumours in some women. The main oestrogen in postmenopausal women is estrone and the synthesis is stimulated by different cytokines and prostaglandin E_2 [90].

Oestrogens are involved in the regulation of several important cellular functions such as proliferation, differentiation and apoptosis. Oestrogen stimulation leads to up-regulation of factors that promote proliferation and survival, however, the majority of genes are down-regulated in response to oestrogen and these are mostly genes coding for transcriptional repressors and anti-proliferative factors [91]. Oestrogen mediates its actions through binding to ERs. These are ligand-inducible transcription factors belonging to the superfamily of nuclear hormone receptors, where the members share many structural and functional components. The structure of nuclear receptors contains an A/B domain at the N-terminal end, a C/D domain with DNA-binding structure, and an E/F domain with a ligand-binding pocket (figure 4) [92-94].

There are two receptors for oestrogen; ERα and ERβ. They are encoded by different genes and several splice variants have been described, however, the biological function of all variants has

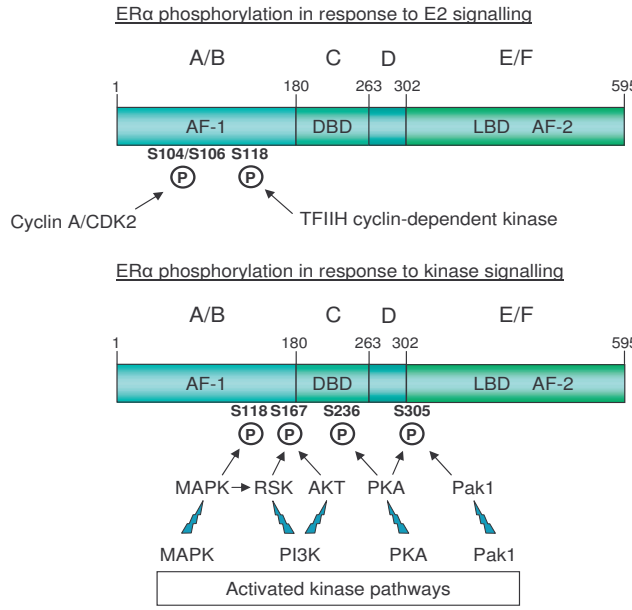

Figure 4. Illustration of the functional domains within ER and the phosphorylated residues in response to oestrogen (E2) signalling (top) and kinase activation (bottom). The two domains capable of inducing DNA transcription, AF-1 and AF-2, are situated in the N-terminal and C-terminal, respectively. AF-1 is activated in a ligand-independent manner through phopsphorylation, while activation of AF-2 is dependent on oestrogen binding to the ligand binding domain (LBD). The DNA binding domain mediates specific binding to ER target genes. The signalling pathways and kinases responsible for ER phosphorylation are also shown. Modified from [102].

not been clarified yet [95]. The two ERs share a high degree of sequence identity within the DNA binding domain, whereas the ligand-binding domains diverge, suggesting that ERα and ERβ have different affinity for certain ligands, even if they do have similar affinity for E2 [93]. The expression pattern for the two receptors in the human body is overlapping, but some tissues are more specific in their ER expression. ERα is primarily expressed in the uterus, liver, kidney and heart, whereas ERβ is expressed in ovary, prostate, lung, gastrointestinal tract, bladder, hematopoietic and central nervous system. ERα and ERβ are co-expressed in mammary gland, epididymis, thyroid, adrenal gland, bone and brain [95, 96]. The two receptor isoforms can form heterodimers, and when co-expressed, ERβ causes reduction in ERα-mediated transcription, suggesting that ERβ act as a negative regulator of E2-signalling [95]. A major understanding about ER mechanisms in different tissues has come from studies of the knock-out mouse models, i.e. the αERKO and βERKO mice [94], as well as the double knockout. This thesis will be focused on ERα (referred to as ER), but will discuss some of the differences between the two receptors.

The inactive ER is sequestered in a complex together with inhibitory heat-shock proteins. Upon ligand binding, the receptor undergoes a conformational change that enables dissociation of the heat-shock proteins and facilitates dimerisation and nuclear localisation of the receptor and interaction with other co-factors. The receptor is now able to bind to its target gene promoters, either by binding to certain DNA sequences called oestrogen response elements (EREs) or by interacting with other DNA bound transcription factors such as AP-1, SP-1 or NF-κB. This is referred to as the classical and the non-classical pathway of nuclear-initiated steroid signalling (NISS), respectively (figure 5) [97].

Opposite effects between ERα and ERβ has been observed at AP-1 regulated gene promoters [92, 93]. The ER induces or silences target genes depending on whether the bound ligand is an agonist, e.g. oestrogen, or antagonist, e.g. tamoxifen. Agonist binding to the ER facilitates the recruitment of co-activators and histone acetyltransferases (HATs), which modifies the chromatin of the target gene promoters into a transcriptionally active state. In contrast, antagonist binding leads to interaction with co-repressors and histone deacetylases (HDACs), turning the chromatin into a condensed transcriptionally inactive state [97, 98]. The ability to activate gene transcription is dependent on two functional domains within ER; the activation function 1 (AF-1) and activation function 2 (AF-2). Between the two ERs, there is less similarity in AF-1 than in AF-2, and the receptors also show a difference in their interaction with co-factors and their transcriptional activities. Furthermore, the AF-1 has been shown to have minimal activity in ERβ under conditions where the ERα AF-1 is very active, highlighting another difference between these receptors [95]. The AF domains function synergistically to mediate maximum transcriptional activity, but in some cells only one is required for gene activation. The AF-2 domain is located in the ligand-binding domain (LBD) of the ER, and thus its activation is dependent on ligand-binding. When oestrogen binds to ER, helix 12 of the LBD is placed over the ligand binding pocket and acts as a surface for co-activators to interact with [92]. Several co-activators that bind to the AF-2 domain are known, including the steroid receptor co-activator (SRC) family (SRC1/NCoA1, SCR2/TIF2/GRIP1/NCoA2 and SRC3/AIB1/RAC3/TRAM1/NCoA3), TRAPs/DRIPs, CBP and p300 [99]. The interaction of co-activators with the AF-2 domain is mediated by leucine-rich motifs (i.e. LXXLL) called “NR boxes” present in most co-activators. Some of the co-activators also possess intrinsic HAT activity, and together with the general transcription machinery, the ER complex is able to induce gene transcription. When antagonists bind to the LBD, helix 12 changes position and instead co-repressors are recruited to ER, which will silence the basal transcriptional activity of target genes [92]. Examples of such co-repressors are SMRT and NCoR and they function by recruiting HDACs, such as SIN3 [99]. The AF-1 is not regulated by ligand-binding like AF-2, but instead its activity is regulated by phosphorylation. Phosphorylation of ER on multiple sites is enhanced in response to E2-signalling and also in response to activation of signalling pathways (figure 4) [100-102]. Upon E2-binding, ER is phosphorylated on S104/S106 and S118 by cyclin A/CDK2 and TFIIH cyclin-dependent kinase, respectively [103, 104]. S118 is also phosphorylated by MAPK [105], which also phosphorylates S167 by cross-talking with p90 ribosomal S6 kinase (RSK). Another kinase that phosphorylates S167 is AKT (also known as PKB) [106]. Two serine residues, S236 and S305, are phosphorylated by PKA, the former phosphorylation being important for receptor dimerisation and the latter induces agonistic effects of tamoxifen [107-109]. Phosphorylation of ER on S305 has been shown to be associated with poorer tamoxifen response in breast cancer patients (paper II) [110]. This site has also been suggested to be phosphorylated by Pak1 [111]. Tyrosine phosphorylation of ER has been reported but this remains controversial [102].

In addition to nuclear-initiated steroid signalling (NISS), rapid effects of E2 are mediated through non-nuclear ERs situated at the plasma membrane (figure 5). It is believed that the membrane ER is identical to the nuclear ER, only transported to the membrane where they are either bound to the inner lipid bilayer through lipid raft proteins, such as caveolin-1, or in a complex with MNAR/PELP, EGF or HER2 receptor. Alternatively, some ERs could be localised to isolated caveolae rafts that are spread throughout the plasma membrane. This type of signalling is referred to as membrane-initiated steroid signalling (MISS) and it is mediated by G-proteins, calcium and several protein kinases such as MAPKs, PI3K and PKC. This can in turn activate gene transcription both dependently and independently of nuclear ER. A fraction of ER is also localised to the cytoplasm and mitochondria. In breast cancer cells, mitochondrial ER (especially ERβ) is able to prevent radiation-induced cell death [112-114].

The regulation of ER expression is tissue-specific but certain transcription factors and promoter-hypermethylation has been identified as regulators of ERα expression levels, while factors regulating

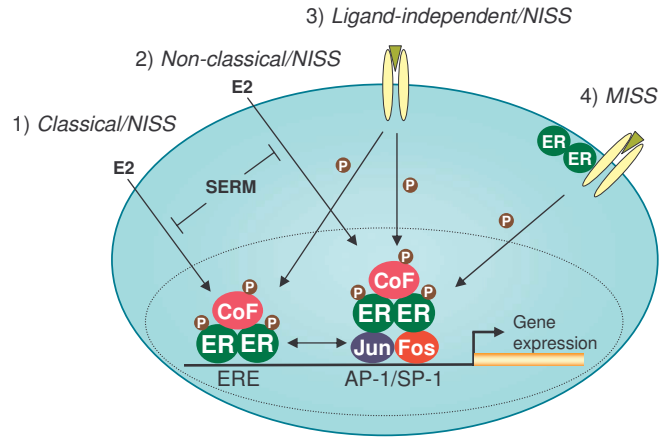

Figure 5. Mechanisms of ER actions. Nuclear-initiated steroid signalling (NISS) can be induced through both ligand-dependent and ligand-independent mechanisms. Ligand-dependent signalling requires binding of oestrogen (E2) to ER, which then activates transcription either through direct binding to oestrogen response elements (EREs) in target genes (classical pathway) or by tethering to other DNA-bound transcription factors (non-classical pathway). Ligand-independent activation of ER is mediated by phosphorylation of ER through activation of different kinase signalling pathways. Membrane- initiated steroid signalling (MISS) can be induced by ERs situated near the plasma membrane, which then signal through different cellular pathways to activate transcription. Modified from [127].

ERβ is less known [95]. Also, E2-signalling has emerged as a regulator of ER expression in an autocrine fashion.

Tamoxifen and tamoxifen resistance mechanisms

For over 30 years tamoxifen has been the predominantly prescribed anti-oestrogen in breast cancer treatment. It was first approved for treating advanced breast cancer in postmenopausal women but later on it was also approved as adjuvant treatment for both post- and premenopausal women with node-negative ER-positive breast cancer [115]. Oral administration of 20 mg daily keeps the steady state serum levels of tamoxifen constant and can even be detected several months after discontinuing treatment [116]. Tamoxifen is best described as a selective endocrine receptor modulator (SERM) since it has both agonist and antagonist effects depending on the cell- and tissue type [81, 117]. In the breast, tamoxifen is an anti-oestrogen, i.e. it inhibits the growth stimulatory effects of oestrogen by competitive binding to the ER. Also, instead of recruiting co-activators like the oestrogen bound ER does, tamoxifen favours ER interaction with co-repressors that inhibit gene transcription. However, in bone, uterus and the cardiovascular system, tamoxifen functions in an oestrogen-like manner. The effects of tamoxifen in bone and cardiovascular system is beneficial, but the unfavourable stimulatory effects it has in the uterus has led to an increase of endometrial cancer in women who take tamoxifen [117]. The mechanisms behind SERMs' tissue-selective agonist/antagonist effect are not fully elucidated. It has been found that tamoxifen has partial agonist effects on genes that are regulated by the AF-1 domain, while genes that are exclusively dependent on AF-2 activity, are repressed by tamoxifen [81, 118]. In breast, ER activity is mainly AF-2 dependent and thus tamoxifen has antagonist effect, while cells in bone and uterus have more AF-1 active transcription and hence tamoxifen has agonist effects in those tissues. It is also possible that the levels of co-activators and co-repressors have an effect on the differential activity of tamoxifen in different cell types. Also, while tamoxifen is a partial agonist for ERα, it is a pure

antagonist for ERβ, probably due to receptor differences in the AF-1 domain, adding another possible reason for the tissue-selective effect.

Meta-analyses or overviews that combine the results from several parallel studies, have revealed the beneficial effects of tamoxifen as adjuvant treatment in ER-positive breast cancer [74]. Five years of tamoxifen treatment is the optimal treatment duration, reducing the annual breast cancer deaths by a third, irrespective of patient age or any other tumour characteristics, and decreasing the recurrences by half. Also, the use of tamoxifen reduces the incidence of a new contralateral cancer, which has prompted the use of tamoxifen as preventative treatment for breast cancer [119, 120]. A major complication in endocrine treatment is resistance to tamoxifen, which can be either *de novo* (at the beginning of treatment) or acquired (after prolonged use). Approximately 40 % of all patients recieving adjuvant tamoxifen experience tumour relapse [121]. This strongly advocates the identification of treatment predictive factors and the development of novel anti-oestrogen treatments.

There are several different mechanisms suggested to be involved in tamoxifen resistance, and owing to the enormous amount of references that describe associations with tamoxifen insensitivity, a complete review in this thesis is simply not possible. However, the main mechanisms will be discussed to some extent and a summarised overview is made in table 1.
The dominant mechanism of *de novo* tamoxifen resistance is the actual lack of ER expression, which accounts for ~30 % of all breast cancers, making ER the strongest predictor for tamoxifen response. Resistance due to loss of ER expression during treatment is not common, which can explain why many patients respond to other endocrine treatment after developing resistance to tamoxifen [75, 122]. Mutations in the *ER* gene are rare, however a mutated ER that is activated without a ligand has been discovered, which potentially could cause tamoxifen resistance [123 135]. Immunohistochemical analysis of ER is routinely used for deciding which patients should recieve endocrine treatment. The definite cut-off for what is considered as ER-positive varies, where many laboratories consider tumors with a low amount of ER-positive cells, i.e. less than 10 %, as ER negative, while others would include tumors with any detectable level of ER as positive. This might explain why some ER negative tumors seem to respond to tamoxifen.

Although not as strong a predictor as ER, another helpful measurement in selecting patients for tamoxifen treatment is PgR, which is regulated by ER. The presence of PgR is indicative of a functioning ER pathway and therefore suggested to add predictive information in an ER-positive subgroup. Moreover, women with PgR-positive tumors might benefit from tamoxifen even if they are classified as ER-negative [124, 125]. Recent studies suggest that the link between PgR expression and ER functionality is oversimplified and it does not explain the fact that there are patients with ER-positive/PgR-negative tumors that respond tamoxifen and moreover, tamoxifen resistant ER-positive/PgR-negative tumors might respond to AIs. A new proposal as to why PgR is predictive of tamoxifen response is that excessive growth factor signalling leads to down-regulation of PgR, and in contrast to ER, loss of PgR expression is much more common in resistant tumours [126, 127].

Large amount of evidence supports that cross-talk between ER and growth factor signalling mediates tamoxifen resistance, supposedly through improper activation of the receptor by phosphorylation. Numerous growth factors are suggested to enhance E2-signalling including epidermal growth factor (EGF), heregulin, insulin, insulin-like growth factor-1 (IGF-1), transforming growth factor α (TGF-α), as well as dopamine, cyclic AMP (cAMP) and phorbol esters [75, 121, 122, 124, 128, 129]. Activation of growth factor receptors can lead to ER phosphorylation through different pathways including the ERK1/2 MAPK and PI3K pathways. The corresponding kinases that actually phosphorylate ER are ERK1/2, RSK and AKT (mentioned in the previous section about ER). Ligands that activate adenylyl cyclase (AC) can lead to ER phosphorylation via activation of PKA. An increased activity in any of the above mentioned pathways could possibly be associated

Table 1. An overview of mechanisms involved in tamoxifen resistance.

Factor	Comment	Mechanism	Ref
ER expression	A prerequisite for endocrine treatment Loss of ER confers *de novo* but not acquired resistance ER mutations are rare	A mutated ER could possibly be constitutively active or hypersensitive to E2	[75]
PgR expression	Indicative of functional ER pathway Loss of PgR is associated with poor tamoxifen response	Non-functional ER or increased growth factor signalling	[126] [127]
ER co-factors	Important for ER regulated transcription activity High AIB1 (co-activator) expression is associated with poor tamoxifen response Low NCoR1 (co-repressor) expression is associated with poor tamoxifen response	Enhance the agonist activity of tamoxifen-bound ER Reduce the antagonist activity of tamoxifen-bound ER	[134] [135] [136]
Cross-talk with ER	Increased activation of signalling pathways that crosstalk with ER is associated with poor tamoxifen response	Ligand-independent ER activation through phosphorylation	[129] [229] [230]
Hypersensitivity to E2	Long-term tamoxifen exposure and/or long-term estrogen deprivation induce hypersensitivity to estrogen and tamoxifen stimulated growth.	Increased growth factor signalling. Agonist effects of tamoxifen.	[121] [226] [231]
Genetic polymorphism	Different variants of cytochrome P450 enzymes exists	Altered metabolism of tamoxifen	[121] [131]
ERβ	High levels are associated with improved tamoxifen response	?	[142] [143] [144]
PKA	Experimental studies show conformational arrest associated with tamoxifen resistance	Phosphorylates ERS305 → tamoxifen has agonist effects	[108]
Pak1	Overexpression and nuclear localisation is associated with poor tamoxifen response	Phosphorylates ERS305 [?]	[110]
COX-2	Elevated COX-2 is associated with poor survival in ER positive tumours	Prostaglandins enhance stromal cell aromatase expression	[232]
Cyclin D1	Overexpression is associated with poor tamoxifen response and gene amplification is associated with potential agonist effect of tamoxifen treatment	Functions as co-factor and recruits co-activators to ER complex	[139] [140]
Bcl-2	Expression is suggested to be associated with better tamoxifen response	?	[232]
"Molecular signatures" *- Two-gene ratio (HOXB13/IL17BR)* *- Oncotype DX™* *- DNA methylated markers*	Microarrays are used for the identification of patients who are responsive to tamoxifen High *HOXB13-to-IL17BR* expression is associated with tamoxifen failure A recurrence score was able identify patients with a high rate of recurrence despite tamoxifen treatment By combining classical markers with DNA methylation, patients with excellent tamoxifen response were identified		[131] [232]

with poor tamoxifen response, which will be discussed further in the results and discussion part. The relevance of HER2 overexpression and its implication in tamoxifen resistance has been studied extensively. An *in vitro* model of tamoxifen resistant breast cancer cells showed increased levels of HER2 expression and increased signalling in ERK1/2 MAPK and PI3K pathways resulting in increased ER phosphorylation [130]. In the clinical adjuvant setting, contradicting results have been reported about the predictive role of HER2, with some reports supporting HER2 as a predictive factor for tamoxifen response while others do not [122, 131].

The importance of co-activators and co-repressors in the regulation of ER activity has implicated a role for them in tamoxifen resistance [132, 133]. Both up-regulation of co-activators as well as down-regulation of co-repressors have been observed in endocrine resistant breast cancer cells [134-136]. Another protein that can be considered as an ER co-factor is cyclin D1, which directly interacts with ER independently of CDKs and also recruits co-activator SRC-1, stimulating ER mediated transcription [137, 138]. Overexpression of cyclin D1 is detected in as much as half of all breast cancers, and the gene located in the 11q13 region is frequently amplified [54]. Overexpression of cyclin D1 protein has been associated with tamoxifen resistance and amplification of the gene has even been suggested to confer agonist effects of tamoxifen treatment [139, 140].

The prognostic and treatment predictive value of ERβ in breast cancer is still unclear, with some studies observing a beneficial prognosis in ERβ positive patients, whereas other found no significant prognostic effect [141-143]. Relatively new findings suggest that high expression of ERβ in the ERα positive subgroup of patients predicts improved response to endocrine treatment [142-144]. Yet, at this time there is no consensus about the role of ERβ in breast cancer prognosis or treatment. Other biomarkers that can predict tamoxifen response, such as specific ER phosphorylations, will be further discussed in the results and discussion part.

Extracellular and intracellular signalling

In normal tissue, cells are constantly communicating with each other through direct cell-cell contacts and through the release of signalling growth factors and cytokines. Each signal has a message for the receiving cell, and thus the decision to grow or divide is not depending on the cell itself, rather it is an integration of juxtracrine and paracrine signalling cues. This type of interaction between cells and their surrounding environment is necessary for maintaining cellular differentiation, proliferation and tissue homeostasis, while a deregulated balance of these signals is central in cancer cell transformation.

There is an immense number of signalling pathways in the cell and they often impinge on one-another, something that is referred to as cross-talk. In response to extracellular signals, intracellular proteins are often phosphorylated by protein kinases. Protein phosphorylation is a common modification for controlling enzyme activity, interaction with other proteins, cellular localisation and protein degradation [145]. In that sense, these signalling systems provide regulatory networks for cells to switch on or off many diverse processes [146]. Protein kinases are the largest family of enzyme proteins described in humans, estimated to include ~2000 members [147]. This thesis will deal with three protein kinases, described below and illustrated in figure 6. They have all shown to play a role in breast cancer and also in tamoxifen treatment response.

Pak1

Pak1 is short for p21-activated kinase 1, a name derived from its ability to become activated by small GTPases of the Rho (p21) family [148, 149]. It belongs to a family of serine/threonine protein kinases composed of six members, which can be subgrouped into group A (Pak1-3) and group B (Pak4-6) [150]. Group A Paks bind Cdc42 and Rac, and are strongly activated upon binding of these GTPases, whereas group B Paks are able to bind the same GTPases but are not activated by them, suggesting that the two groups are differently regulated [151]. The structural features of Pak kinases include an N-terminal regulatory domain and a highly conserved C-terminal catalytic domain [152]. Tissue specificity and differences in the N-terminal region are thought to contribute to the diversity of downstream signalling pathways of different Paks [150]. The regulatory domain of Pak1 is comprised of three SH-3 binding sites, a PBD (p21-binding domain, also called GBD for GTPase-binding domain or CRIB for Cdc42/Rac1 interactive binding), an autoinhibitory segment that overlaps with the PBD, and a Cool/Pix-binding region [152]. In an

unactivated “off-state”, Pak1 exists as a trans-inhibited homodimer, where the regulatory domain of one Pak1 molecule inhibits the catalytic domain of the other [153]. GTPase binding disrupts the dimerization and reverses the negative regulation by conformational changes that facilitate phosphorylation of Thr423, which is required for full activity of Pak1. Although the Thr423 residue can be phosphorylated by Pak1 itself upon activation, the involvement of other kinases at this site seems to be more important. Additional sites are also phosphorylated and may contribute to Pak1’s activity [152]. Other GTPase-independent activation mechanisms of Pak1 also exist. Interaction with SH3-containing adapter proteins, e.g. Nck and Grb2, recruits Pak1 to the plasma membrane where sphingolipids can activate Pak1 through similar mechanisms as GTPases [154]. Also, Pak1 is directly phosphorylated and activated by a number of protein kinases such as AKT, PDK1, and PI3K [152].

The biological effects of Paks are plenty, reflected by the increasing number of interacting proteins and substrates. Signalling by Rac and Cdc42 mediates cytoskeletal rearrangements such as formation of membrane ruffles, lamellipodia, peripheral filopodia, and actin microspikes through Paks’ ability to phosphorylate a plethora of cytoskeletal proteins [155]. This strongly implicates Paks in the control of cell motility, and several studies have shown a correlation between the expression and activity of Pak1 and the invasiveness of cancer cells [156, 157]. In addition to cytoskeletal regulation, Pak1 stimulates several kinase pathways, coupling Pak1 to nuclear signalling and subsequent gene transcription. The JNK/SAPK and p38 MAPK pathways are stimulated by GTPase-activated Pak1 [158, 159], and the ERK1/2 MAPK pathway is targeted by Pak1-mediated phosphorylation of both MEK1 and Raf1, which are upstream activators of ERK1/2 [151]. Pak1 also influences NF-κB activity [160]. Other important mechanisms regulated by Pak1 are cell survival, angiogenesis, cell cycle, migration and mitosis [155, 161].

All of the above mentioned mechanisms are often deregulated in cancer suggesting that Pak1 plays an important role in cancer progression. In human breast cancer, Pak1 expression correlates with high tumour grade and hyperactivation of Pak1 in the mouse mammary gland is sufficient for tumour formation [162-164]. The increased expression of Pak1 in breast cancer cells stimulates expression of cyclin D1, possibly through activation of NF-κB [165]. Interestingly, it has also been found that Pak1 phosphorylates ER at serine 305 and thereby enhance its transcriptional activity. Pak1-mediated phosphorylation of this residue leads to ligand-independent stimulation of the AF-2 domain and also up-regulation cyclin D1 expression in breast cancer cells [166, 167]. This could possibly have an important impact on patients’ response to tamoxifen treatment, supported by the finding that overexpression and particularly nuclear localisation of Pak1 is associated with tamoxifen resistance in breast cancer patients (paper I) [162].

ERK1/2

ERK1 and ERK2 (ERK1/2) are mitogen-activated protein kinases (MAP kinases or MAPKs) regulated by a phosphorylation cascade with two other upstream kinases, and all together they make up the core of what is defined as a MAPK cascade. In humans, three such MAPK cascades are well defined; the ERK1/2, JNK/SAPK and the p38 MAPK pathways. In these pathways, ERK1/2, JNK/SAPK, and p38 are the MAPKs, and they are phosphorylated, and hence activated, by MAPK kinases (MAPKKs, MKKs, or MEKs). MAPKKs are highly specific and each MAPKK only phosphorylate one or few of the MAPKs. The MAPKKs are in turn phosphorylated by MAPKK kinases (MAPKKKs, MKKKs or MEKKs), which are themselves activated by other upstream kinases or by interaction with small GTPases such as Ras [147, 168, 169]. Kinases that are activated by phosphorylations can be inactivated by proteins that remove the phosphate, i.e. protein phosphatases. A subgroup known as dual specificity phosphatases has emerged as selective MAPK phosphatases due to their ability to dephosphorylate the critical phosphothreonine and phosphotyrosine residues required for MAPK activity [170].

ERK refers to extracellular signal-regulated kinase and there are many different stimuli that can activate the ERK1/2 pathway, including growth factors, cytokines, transforming growth factors, hormones and ligands for G protein-coupled receptors [145, 169]. The target substrates are other protein kinases, transcription factors and cytoskeletal proteins, which regulate mechanisms such as cell motility, proliferation, apoptosis and differentiation [145]. In breast cancer, growth factor receptor activation and E2-signalling, both NISS and MISS, activate the ERK1/2 pathway and approximately half of all breast tumours express a more active ERK1/2 pathway compared to surrounding benign tissue [171]. Higher ERK1/2 activity has also been reported in tumours from patients with shorter disease-free survival, proposing that ERK1/2 has prognostic value in breast cancer [172]. Breast cancer cells that are grown for a long time in low oestrogen levels, i.e. long-term oestrogen deprived cells, adapt through an up-regulation of ERK1/2 activity which also leads to higher sensitivity to oestrogen [173, 174]. Thus, cross-talk between the ERK1/2 pathway and ER, resulting in ligand-independent activation and enhanced ER signalling, suggests a role for ERK1/2 in anti-oestrogen resistance. However, some reports have failed to associate ERK1/2 activity with tamoxifen resistance [175].

PKA

PKA, or protein kinase A, is a cAMP-dependent protein kinase and also the most thoroughly described member of the serine/threonine protein kinase family [176]. PKA consists of two regulatory subunits and two catalytic subunits that dissociate upon activation by cAMP. The catalytic subunits are responsible for phosphorylating PKA substrates, while the regulatory subunits serve as inhibitors of the catalytic domain. Increased level of intracellular cAMP, through

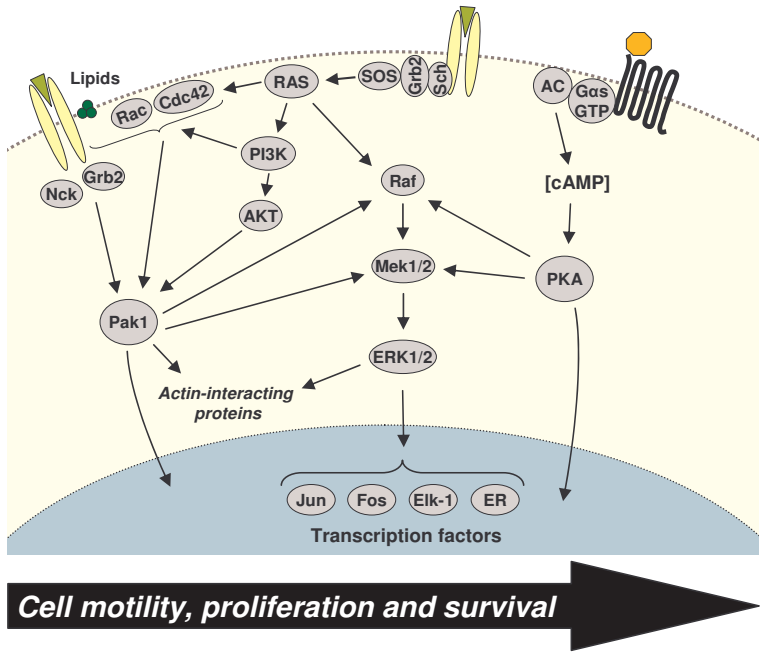

Figure 6. Pak1, ERK1/2 and PKA signalling pathways. The figure illustrates activation mechanisms of three important kinases; Pak1, ERK1/2 and PKA. An activated kinase pathway often impinges on another, demonstrating the complex cross-talk that exists in cells. Protein phosphorylation is an important mechanism by which kinases regulate the function of their substrates. Pak1, ERK1/2 and PKA have several substrates that are not depicted in the figure, and they will not be addressed in the main text since it is not relevant to the discussion of this thesis. The take-home message is that all three pathways are involved in important cellular processes such as cellular motility, proliferation and survival.

G protein-coupled receptors and via activation of AC, leads to the binding of cAMP molecules to the regulatory subunits, thus freeing the catalytic subunits. In addition, the regulatory subunits localise PKA to specific sites near its substrates through binding to A kinase-anchoring proteins (AKAPs) [176-178].

There are increasing numbers of PKA substrates and they play a key role in almost every major cellular pathway, including proliferation, apoptosis, growth and differentiation [179]. Furthermore, G protein-coupled receptors can either activate or inhibit the ERK1/2 pathway via PKA in a cell-type specific manner [180]. Two distinct isoforms of PKA exist, PKA-I and PKA-II, distinguished by their regulatory subunits RI and RII, respectively. The difference in regulatory subunits has an effect on the affinity for cAMP, subcellular localisation and ultimately the functional outcome of PKA-I and PKA-II [181]. In normal cells, both isoforms are expressed in a balance and it is suggested that a loss of this balance may play a role in cancer development and progression [182]. Overexpression of the RI regulatory subunit of PKA-I is associated with tumour formation and elevated levels in breast cancer is associated with worse prognosis [183, 184]. Also, tumours that are resistant to anti-oestrogen often have increased expression of RI [183]. Evidence from *in vitro* models has shown that PKA-mediated phosphorylation of ER of serine 305 is able to induce complete agonist effects of tamoxifen [108], and breast cancer patients with tumours expressing serine 305 phosphorylation have a worse response to tamoxifen compared to those with no phosphorylation (paper II) [110].

Tumour microenvironment and hypoxia

The cellular microenvironment is of great importance both in the normal and tumour condition. Normal cells are dependent on signals from their surroundings in order to achieve appropriate tissue function and structure. In solid tumours, the cancer cells are dependent on interactions with the extracellular environment for their metastatic potential [185]. For a primary tumour to shed metastatic lesions, the cells need to detach from the primary site, invade the surrounding tissue, migrate into the blood or lymph vasculature and then move from the circulation and survive at a secondary site. This whole process is a complex procedure, facilitated by alterations in gene expression that promote cell migration, degradation of the extracellular matrix, formation of new blood vessels and cell survival.

Several genes that are implicated in tumour metastasis are induced by hypoxia – low oxygen levels [186]. Furthermore, it has been proposed that a hypoxic environment can drive the selection of a metastatic tumour phenotype by inducing genomic instability and promoting different genetic aberrations such as point mutations, deletions, and amplifications [187]. Hypoxia is inevitable during solid tumor growth as cellular proliferation exceeds the rate of oxygen supply from existing blood vessels. Cells that are situated distantly from capillaries are subjected to chronic hypoxia (also called diffusion-limited hypoxia), whereas cells more adjacent to vessels can experience acute hypoxia (perfusion-limited) due to variations in blood flow. Also, low glucose levels and low pH are the result of inadequate delivery of nutrients and removal of catabolic waste products. Tumour cells initiate formation of new blood vessels, however these are often abnormal, immature and consequently leaky. The oxygen partial pressure in normal tissue ranges between 40-60 mmHg, corresponding to oxygen levels of approximately 5-6 %, while the median oxygen tension in tumours is somewhere around 10 mmHg, corresponding to 1.3 % oxygen level [188], although it is reasonable to believe that the oxygen levels in tumours are fluctuating over time.

Tumour hypoxia is an independent prognostic factor in several cancer types and it is also associated with increased resistance to different adjuvant treatments [185, 189, 190]. In radiation therapy, hypoxia causes lower amount of DNA damaging oxygen radicals, and the apoptotic

pathway induced by radiation is frequently inhibited in hypoxic cells. Chemotherapeutic drugs are also less efficient in hypoxic areas due to insufficient vascularization, oxygen dependency, non-proliferating cells and apoptosis resistance.

The harsh milieu triggers an adaptive response and several biological processes are regulated by hypoxia. The induction of angiogenesis has already been mentioned, where tumour cells coordinate the expression of pro-angiogenic factors and suppression of anti-angiogenic factors in order to form new blood vessels necessary for sustained tumour growth. Also, during hypoxia, the process of glucose metabolism is altered from an oxygen-dependent (citric acid cycle) to an oxygen-independent method – glycolysis [191]. Many genes involved in glucose metabolism are regulated by hypoxia including glucose transporters and glycolytic enzymes. An effect of glycolysis is accumulation of lactic acid and consequently low intracellular pH (acidosis), however, tumour cells up-regulate enzymes (e.g. CAIX), transporters and pumps that control their pH homeostasis.

As previously described, the hypoxic condition promotes the migratory potential of tumour cells. This is mediated through the process of EMT where loss of cell adhesion is mainly due to repression of E-cadherin, and it has been observed that transcriptional repressors of E-cadherin, i.e. Snail, Slug and Twist, are up-regulated during hypoxia [185, 186]. After detaching from each other, cells need to degrade the BM and extracellular matrix (ECM) in order to invade the stroma. Hypoxia-regulated proteins responsible for ECM degradation are urokinase-type plasminogen activator (uPA) and matrix-metalloproteases (MMPs). In addition, there are other molecules induced during hypoxia that promotes cell motility, such as c-MET, a receptor that mediates hepatocyte growth factor (HGF) signals, CXCR4, a chemokine receptor for CXCL12, and the ECM proteins lysyl oxidase (LOX) and osteopontin (OPN) [185, 186].

The mechanism by which hypoxia regulates apoptosis is truly complex, as both pro-apoptotic and anti-apoptotic genes can be induced [192]. Pro-apoptotic genes are up-regulated through both p53-dependent and independent mechanisms. Hypoxia leads to stabilization of p53, which in turn activates transcription of pro-apoptotic proteins that mediate release of cytochrome c from the mitochondria, while induction of other pro-apoptotic genes, such as BNIP3, is regulated by p53-independent mechanisms. However, the role of BNIP3 in hypoxia-induced apoptosis is not clear, since it has also been shown that BNIP3 can trigger cell survival by autophagy [193, 194]. Resistance to hypoxia-mediated apoptosis through up-regulation of anti-apoptotic genes, such as IAP2, has also been described and tumours with mutated p53 are resistant to p53-mediated apoptosis [195]. Prolonged exposure of tumour cells to hypoxia may lead to a selective pressure, resulting in apoptosis resistant cells and thus a more aggressive tumour.

It is estimated that 1-1.5 % of the genome is transcriptionally regulated by hypoxia. The majority of hypoxia-induced genes are under direct control of a transcription factor called hypoxia-inducible factor 1 (HIF-1), which is only active in low oxygen conditions. Other transcription factors also respond to hypoxia, including NF-κB, CREB, p53, AP-1 and SP-1, although HIF-1 has been shown to play the major role in hypoxia-mediated transcription and is therefore known as the master-regulator of the hypoxic response [185]. HIF-1 is a heterodimeric transcription factor, consisting of a hypoxia-inducible α-subunit and a stable β-subunit. They are both members of the basic-helix-loop-helix PerArntSIM (bHLH-PAS) family of proteins [196], where the basic region confers DNA binding and the HLH part and PAS domains confer protein-protein interactions. Three α-subunits are identified; HIF-1α, HIF-2α, and HIF-3α, of which HIF-1α is by far the most studied and will be discussed in further detail below.

HIF-1α

Under normoxic conditions, the levels of HIF-1α are very low, resulting in basically no activity of HIF-1. This is regulated by oxygen-sensitising enzymes, known as prolyl-4-hydroxylases or PHD's [197]. In the presence of oxygen, the PHD's hydroxylate two proline residues (P402 and

P564) within a specific domain of HIF-1α, referred to the oxygen-dependent-degradation domain (ODDD) (figure 7). The hydroxylated HIF-1α interacts with the E3 ubiquitin ligase complex, consisting of the von Hippel Lindau protein (pVHL), elongin C, elongin B, cullin 2 and Rbx1 protein [198]. This leads to ubiquitination and subsequent degradation of HIF-1α by the 26S proteasome [199]. Besides oxygen, the PHD enzymes are also dependent on iron, 2-oxoglutarate and ascorbate as co-factors and consequently, HIF-1α can be stabilised with iron chelators during normoxia [197]. There are three known isoforms of PHD's; PHD1, PHD2 and PHD3, and although all isoforms are capable of hydroxylating HIF-1α, it is PHD2 that seems to be the rate limiting enzyme for HIF-1α degradation during normoxia [200]. At hypoxia, there is no hydroxylation of HIF-1α by the PHD's, which leads to HIF-1α stabilisation. This is then followed by nuclear translocation and dimerisation with its partner HIF-1β (also known as ARNT, aryl hydrocarbon nuclear translocator). The two subunits bind to distinct hypoxic response elements (HREs) in the promoters of target genes and interact with general transcription activators, such as CBP/p300. The ability to induce transcription is mediated through two transactivation domains in HIF-1α, one referred to as the N-TAD (N-terminal activation domain) and the other as the C-TAD (C-terminal activation domain) [201]. HIF-1α has been shown to induce transcription of PHD2, which serves as a regulatory mechanism for rapid HIF-1α degradation once oxygen is presented [200]. Another oxygen-sensitive regulatory enzyme involved in HIF-1α regulation is factor inhibiting HIF-1 (FIH-1) [202]. FIH-1 hydroxylates HIF-1α at an asparagine residue (N803) in the C-TAD, inhibiting the transcriptional activation by blocking HIF-1α interaction with co-activator CBP/p300 [203]. Both PHD's and FIH-1 are dependent on oxygen, however, PHD's have a lower affinity for

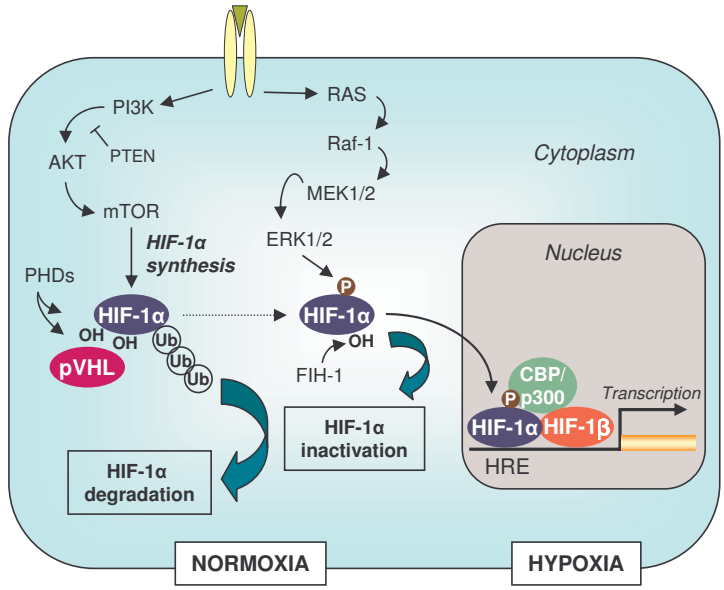

Figure 7. Regulation of HIF-1α. Synthesised HIF-1α protein is constantly degraded under normoxic conditions. This is regulated by hydroxylation of the HIF-1α protein at specific residues, mediated by PHD enzymes. This leads to interaction with the VHL E3 ligase complex, which in turn ubiquitinates HIF-1α and targets it for destruction by the proteasome. Another hydroxylation is mediated by FIH-1, which inhibits the transcriptional activation of HIF-1α. During hypoxia, the PHD's and FIH-1 are non-functional, leading to HIF-1α stabilisation. This will then lead to nuclear translocation followed by interaction with the β-subunit and other transcription factors, enhancing expression of target genes, typically through binding to hypoxia response elements (HRE's). In addition, growth factor stimulation can enhance HIF-1α activity, both through PI3K signalling that will increase levels of HIF-1α, and through ERK1/2 signalling which will phosphorylate HIF-1α and enhance its activity. Modified from Löfstedt, 2007.

oxygen, suggesting that PHD's are inactivated during moderately low levels of hypoxia, leading to activation of N-TAD regulated genes, while full inactivation of FIH-1 requires even lower oxygen levels, and that would lead to activation of genes regulated by C-TAD [194].

As described above, posttranslational modification of HIF-1α is of great importance for regulating its stability and activity. In addition to being stabilised due to non-functional oxygen-dependent enzymes, HIF-1α is phosphorylated and stabilised through different kinase signalling pathways both under hypoxic and normoxic conditions. Activation of the ERK1/2 pathway leads to HIF-1α phosphorylation and enhanced transcriptional activity, supposedly without affecting protein stability [204]. Recently, two ERK1/2 phosphorylation sites in HIF-1α was identified (S641 and S643) and phosphorylation of these residues led to an increased nuclear localisation of HIF-1α through blocked nuclear export [205]. Also, active p38 MAPK pathway is suggested to promote HIF-1α phosphorylation, although the sites have not been identified [204]. Mutations in *PTEN*, an inhibitor of the PI3K pathway, have been associated with increased HIF-1α activity, suggesting that PI3K activation leads to phosphorylation of HIF-1α. In fact, during normoxia, growth factor receptor activation by PDGF, EGF, FGF2, TGF-β, heregulin, insulin, insulin-like growth factor and different cytokines, stimulate the PI3K pathway and leads to increased HIF-1α protein synthesis via mTOR (mammalian target of rapamycin) [194, 206].

In addition, other posttranslational modifications such as acetylation of a lysine in the ODDD (L532) by the acetyltransferase ARD1 (arrest-defective-1), are suggested to promote HIF-1α degradation through an increased interaction between HIF-1α and pVHL [207]. However, the role of ARD-1 in HIF-1α degradation has been opposed by others [208]. Also, MTA-1 (metastasis-associated protein 1), a component of the nucleosome remodelling histone deacetylation (NuRD) complex, increases HIF-1α stability through deactylation [209]. Finally, SUMOylation and S-nitrosation of HIF-1α has been reported to repress or enhance transcriptional activity, respectively [204].

As mentioned previously, hypoxia and the resulting necrotic process are often associated with clinically aggressive behaviour, and markers for hypoxia, such as HIF-1α, have also been linked to poor prognosis and therapeutic resistance in several types of cancer, including breast cancer [210, 211]. In terms of endocrine response in breast cancer, hypoxia leads to down-regulation of ER, which might provoke hormone-independent cellular growth and consequently tamoxifen resistance [211].

THE PRESENT INVESTIGATION

Aims

The general aim of this thesis was to identify different biomarkers in breast cancer and to analyse their prognostic and treatment predictive value. A second main objective was to study the regulation of hypoxia in breast cancer.

The specific aims were:

- To investigate the expression of Pak1 and its association with clinico-pathological parameters in breast cancer.
- To determine whether Pak1 is related to prognosis or tamoxifen response in breast cancer.
- To investigate the expression of ERS305-P and to determine whether it is associated with tamoxifen response.
- To investigate the expression of ERS118-P and to determine whether it is associated with tamoxifen response.
- To delineate the *in vivo* associations between the kinases pERK1/2, pPKA, Pak1 and ERS305-P and ERS118-P, and determine their relation to tamoxifen response.
- To investigate the role of Pak1 in the regulation of HIF-1α and hypoxia.

Results and Discussion

Identifying predictive markers of tamoxifen response in premenopausal breast cancer patients (Paper I-IV)

More than two thirds of all breast cancer patients are eligible for endocrine treatment, and for over 30 years tamoxifen has been the mainstay of anti-oestrogens, both in pre- and postmenopausal breast cancer. A major caveat with tamoxifen is resistance, something that has forced researchers and the drug manufacturing to develop better and more specific treatments. Nonetheless, it should be noted that tamoxifen is beneficial for several patients and overall, has led to substantial decrease in breast cancer recurrences and deaths. At present, one of the challenges is to identify those patients who are less likely to respond to tamoxifen. If they could be recognised, at least then they could be considered for other endocrine treatment options. In postmenopausal women, a major study has shown that anastrozole is more effective compared to tamoxifen or even the combination of anastrozole and tamoxifen. This has caused (or is about to cause) a shift in the standard treatment of postmenopausal women from tamoxifen to AIs as first endocrine treatment choice. Also, the side-effects of anastrozole compared to tamoxifen in these women were less, further endorsing the use of AIs in this patient group [212].

In papers I-IV in this thesis, we have used a randomised trial of adjuvant tamoxifen therapy in premenopausal breast cancer patients and our purpose was to identify treatment predictive factors. The trial included 564 stage II breast cancer patients irrespective of hormone status, who were randomly assigned to two years of adjuvant tamoxifen treatment or no treatment. This trial is unique in the sense that it only consists of premenopausal patients and also by the inclusion of a non-treated control group. Tumour material from 500 of these patients has been collected in tissue microarrays (TMAs), and by immunohistochemical analysis, we have identified biomarkers that are significantly associated with tamoxifen response. The TMA technology is very useful as it promotes high throughput analysis of several biomarkers in exceedingly valuable clinical material, and compared to whole slides, TMA cores haves shown to be representative in up to 95 % [213]. Even though our studies have been done retrospectively, we have used the material from a prospective cohort study, which limits potential bias. Considering the amount of research and publications in the area of tamoxifen resistance mechanisms, the number of predictive factors that have actually reached the clinic is surprisingly low. Studies of a potential prognostic and/or predictive marker often show ambiguous results in various research groups, which can be due to methodological differences, bias in available materials (more often in retrospective studies), poor study design and unreliable statistical analyses. Recently, recommendations on how to report tumour marker studies (REMARK) was published [214]. The offered guidelines help researchers to present their data in a comprehensive manner which makes it easier to draw accurate conclusions and to compare similar studies.

Pak1 is overexpressed in a subset of breast cancers and is associated with tamoxifen resistance (Paper I)

Recent work has demonstrated a role for Pak1 in breast cancer development. In transgenic mice, constitutively active Pak1 expression in mammary epithelium led to hyperplasia and stimulation of oestrogen-inducible genes [166]. Also, active Pak1 signalling has been linked to increased invasiveness and anchorage-independent growth of different breast cancer cell lines [157]. Pak1 was first identified due to its effects on cell motility but over the years, Pak1 has been reported play a role in many cellular processes both in normal and transformed cells. Certainly, its ability to phosphorylate the ER and induce ligand-independent activity has generated great interest in the field of anti-oestrogen research.

In paper I, we determined the protein expression of Pak1 in tumours collected from the patient trial describe above. Before staining the TMAs, we assessed the specificity of the antibody by

comparing immunohistochemical staining and western blot analysis of six different breast cancer cell lines. Also, transient Pak1 overexpression in one cell line was confirmed with the antibody, both with immunohistochemistry and western blot. Initial examination of the stained TMAs led us to divide tumours into low and high expressing tumours, respectively, based on the intensity of Pak1 staining (figure 8). We then further subcategorized tumours into six groups based on their cytoplasmic intensity of Pak1, and we also assessed whether nuclear staining of Pak1 was present or absent. A total of 403 tumours could be analysed for Pak1 staining, where 19 % were categorised as having high cytoplasmic staining and 13 % were positive for nuclear Pak1 staining. The correlation between cytoplasmic and nuclear staining was significant, and all tumours with the highest intensity of Pak1 in the cytoplasm also had nuclear staining. We also observed a significant association between cytoplasmic Pak1 staining and tumour type, where a higher percentage of lobular breast cancers were Pak1 negative compared to ductal and medullary tumours. Furthermore, we observed Pak1 to be significantly associated with both tumour grade and proliferation. This is of particular interest since these attributes are generally coupled to a more aggressive phenotype in cancer, and Pak1 has a well-characterised role in promoting cell motility and proliferation. One mechanism by which Pak1 stimulates proliferation is through upregulation of the cell cycle regulatory protein cyclin D1, and in our analyses, we could identify a significant association between Pak1 and cyclin D1 expression. Another appealing cause as to why Pak1 and cyclin D1 expression correlate in breast cancer could be their simultaneous gene amplification. The *CCND1* gene and *PAK1* gene are located in the same chromosomal region; a region that is amplified in approximately 15 % of all breast cancers. Amplification of *CCND1* in this material was recently examined [140] and therefore also included in our analyses. We were excited to notice a significant association between Pak1 and *CCND1* gene amplification, supporting that *PAK1* might be co-amplified together with *CCND1*.

The previous finding that ER is a substrate for Pak1 lead us to speculate that high expression of Pak1 could compromise the effect of tamoxifen treatment. To examine this possibility, we compared recurrence-free survival among tamoxifen treated and untreated ER-positive patients in relation to Pak1 status. The results showed that patients, whose tumours had low expression of Pak1 and/or no nuclear localisation, had a better recurrence-free survival when treated with tamoxifen compared to no treatment. Conversely, tamoxifen treated and untreated patients, whose tumour had high expression of Pak1 and/or nuclear Pak1, did not show a difference in recurrence-free survival. This observation indicates that Pak1 expression and/or its nuclear localisation can

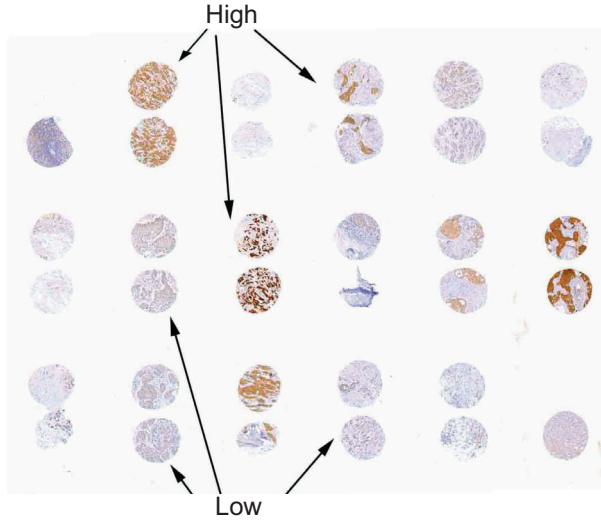

Figure 8. An example of different staining intensity of Pak1 in TMA.

predict tamoxifen response in premenopausal breast cancer patients. To further strengthen our data, we performed a Cox proportional hazards regression model to test whether Pak1 is an independent predictive factor. In this model, we also included an interaction variable in order to explicitly test whether the treatment response is significantly different in relation to Pak1 status. This statistical test revealed that nuclear Pak1 was significantly coupled to tamoxifen response, independent of age, tumour grade, proliferation and node status.

Our previous observation that Pak1 was associated with high tumour grade and proliferation suggests a potential involvement of Pak1 in breast cancer prognosis. However, by analysing recurrence-free survival among untreated patients we concluded that Pak1 does not confer any prognostic information in breast cancer.

In parallel with our clinical studies, we further studied the influence of Pak1 in ER signalling in breast cancer cell lines. We used an MCF-7 cell line with inducible active Pak1 and observed that in the induced state, tamoxifen stimulation led to increased nuclear localisation of Pak1. Whether this is due to membrane initiated or nuclear initiated signalling through ER is not clear, however, the stimulation with tamoxifen also led to increased cyclin D1 expression, suggesting some kind of genomic response. This increase in cyclin D1 was most apparent in cells that had induced active Pak1, indicating that nuclear localisation of Pak1 is important for mediating tamoxifen resistance by increasing cyclin D1. We further tested the ability of Pak1 to induce ER activation in the presence of tamoxifen by measuring the cyclin D1 promoter activity, given that cyclin D1 is a well-known target of ER. Overexpression of wild-type Pak1 led to increased promoter activity when tamoxifen was added. On the contrary, a Pak1 mutant with deficient nuclear localisation signals was not able to induce cyclin D1 promoter activity in the presence of tamoxifen, further supporting the importance of nuclear localisation of Pak1. The enhanced activity of cyclin D1 promoter and the increased protein expression might be interpreted as an agonist response to tamoxifen. In view of that, tamoxifen treatment of an endometrial cell line increased both kinase activity of Pak1 and nuclear expression of Pak1. In cells from endometrium, tamoxifen signals through ER just like oestrogen, that is to say tamoxifen works like an agonist. Hence, these cells can be considered "naturally resistant" to tamoxifen, since tamoxifen is unable to induce anti-oestrogenic effects. All together, the results from our experimental studies show that there is an intimate link between Pak1 expression and ER signalling, and our *in vivo* findings raise the possibility that premenopausal breast cancer patients with high expression of Pak1 and particularly nuclear Pak1 expression are less responsive to tamoxifen.

Phosphorylation of ERαS305 and tamoxifen resistance (Paper II)

Experimental studies have shown a direct link between ER modulation and tamoxifen resistance, where phosphorylation of ER at serine 305 (ERS305-P) by PKA induces agonist effects upon tamoxifen binding. This is explained by a tamoxifen-induced conformational change of the receptor that promotes binding of the co-activator SRC-1 instead of recruitment of co-repressors, resulting in activation of ER-mediated transcription [109]. This implies that serine 305 is a crucial site in ER and phosphorylation of this site might induce resistance to tamoxifen in breast cancer patients.

In paper II, we examined the relationship between ERS305-P and tamoxifen resistance in two breast cancer patient materials; the premenopausal randomized trial described above and a second material representing more advanced metastatic disease. Tamoxifen response in the adjuvant setting was measured as recurrence-free survival, and in the metastatic setting as time to tumour progression. The antibody specific for ER phosphorylated at serine 305 was characterised and validated in several experiments. First, cells were transfected with either wild-type ER or a mutant ER (not able to be phosphorylated at serine 305) and then subjected to PKA activation or inactivation. Only cells transfected a wild-type ER and stimulated with PKA activator showed positive staining with the antibody. Also, pre-absorption experiments with the phospho-peptide

used for immunisation or the non-phosphorylated peptide, showed that the antibody was specific for the phosphorylated ER. The antibody was able to detect the phosphorylated ER in tumour samples using both western blot and immunohistochemistry, while pre-incubation of slides with either phospho-peptide or λ-phosphatase resulted in the absence of staining. In the two materials, the proportion of tumours considered positive for ERS305-P was comparable: 19 % and 17 %, respectively. It should be noted that we included tumours as positive if there was any tumour nuclei with positive ERS305-P staining. Hence, in some tumours the majority of the cells were negative for ERS305-P, and this could of course affect our results. However, since this is the first report on ERS305-P *in vivo* we decided on a cut-off at any positive tumour cells. In the adjuvant cohort, ERS305-P positivity correlated with smaller tumours, while in the metastatic cohort, ERS305-P correlated with histological grade and mitotic index.

Next, we wanted to analyse if ERS305-P in tumours could predict the outcome of tamoxifen treatment. In the adjuvant setting, patients with ERS305-P negative tumours benefitted from tamoxifen while patients with ERS305-P positive tumours did not. However, the treatment effect was not significantly different between the negative and positive subgroups. In the metastatic setting, ERS305-P positive patients had a shorter time to tumour progression, although not reaching significance. Furthermore, ERS305-P was not associate with prognosis in the untreated patient group from the randomised material, which indicates that ERS305-P is a marker for tamoxifen response and not tumour progression.

Our different observations made in the two materials have several possible explanations. An obvious reason is that they represent two clinically different diseases; one of earlier stage and one of advanced disease. Another reason could be due to differences in resistance mechanisms for pre- and postmenopausal patients (the second material included both, whereas the first material only included premenopausal). Also, in the second material, tamoxifen response was measured in a metastatic disease setting, while the expression of ERS305-P was assessed in the primary tumour. We did however examine changes in expression of ERS305-P from primary tumours with their corresponding metastases, although changes in ERS305-P during tumour progression seemed unusual. At the same time we looked at changes in ER and PgR expression as well, and interestingly, we observed a loss of PgR expression in 6 out of 16 tumours during tumour progression (provided in supplementary data).

This is the first study describing ERS305-P in breast cancer and our observation that patients with ERS305-P positive tumours do not respond to tamoxifen extends the compelling evidence obtained by functional experiments that phosphorylation of ER at serine 305 leads to tamoxifen resistance.

Phosphorylation of ERαS118 and tamoxifen sensitivity (Paper III)

In the absence of ligand, ER is phosphorylated at a basal level, while binding of oestrogen or anti-oestrogens, leads to enhanced phosphorylation of the receptor. Several major phosphorylation sites have been mapped to the N-terminal A/B region of ER where the AF-1 domain is situated, including S104, S106, S118 and S167 [102]. Numerous reports have shown a link between activated kinase signalling and increased ER phosphorylation, and more specifically, S118 has been identified as a direct ERK1/2 target residue by several research groups [100, 101, 215, 216]. Experimental studies, where the S118 residue has been mutated either into an alanine (not able to be phosphorylated) or a glutamic acid (mimics phosphorylated form), shows that S118 phosphorylation is very important for ER-mediated transcriptional activity [100, 101]. This has led many to speculate that ligand-independent activation of ER by S118 phosphorylation may contribute to tamoxifen resistance [215, 216]. However, other reports have shown that S118 phosphorylation is required for down-regulation of ER target genes by tamoxifen and that increased ER activity due to ERK1/2-mediated S118 phosphorylation can be inhibited by tamoxifen [217, 218]. This would

imply that tumours with S118 phosphorylation are sensitive to tamoxifen. Several studies have evaluated the expression of ER phosphorylated at S118 (ERS118-P) in breast tumours in relation to prognosis and whether it confers any treatment predictive value. The results, however, have been somewhat inconsistent. Some reports show an association between ERS118-P and better outcome after endocrine treatment [219, 220], while others show no association [221-223]. This discrepancy can be explained by the fact that most studies have been done in relatively small non-randomised studies, where patients have received different types of endocrine treatment (either aromatase inhibitors or tamoxifen) and sometimes chemotherapy. Also, the studies have not been done exclusively in a pre- or postmenopausal setting and some studies have analysed metastatic tumours. Furthermore, differences in staining procedures and scoring systems may contribute to divergences between the different studies.

In paper III, we wanted to determine whether ERS118-P was associated with the outcome of tamoxifen treatment. For this purpose, we stained the same material as used in paper I and II with an antibody that specifically recognise ER only when it is phosphorylated on serine 118. This antibody has been used and validated in other clinical studies and in addition, we performed control experiments to ensure phospho-specificity of the antibody using λ-phosphatase treatment of a tumour sample. The immunohistochemical staining of ERS118-P was evaluated according to the Allred scoring system, which takes both the intensity of the staining and the fraction of positive tumour cells into account. Furthermore, we made extensive specifications of the study following REMARK recommendations (shown in supplementary data). In statistical correlation studies, we found ERS118-P to be correlated with increasing levels of ER but not with PgR. Other studies have also shown the same correlations [224], however, there are also studies showing no correlation with ER [222] and conversely, a positive association with PgR [219]. We did not observe any other associations between ERS118-P and other clinico-pathological parameters. This is in contrast to some other reports were an association has been seen between ERS118-P and smaller tumours and tumours of low grade [222, 224, 225]. Next, we analysed the predictive value of ERS118-P by comparing recurrence-free survival between treated and untreated patients according to the expression of ERS118-P. Before that, we made a cut-off between low expressing tumours and high expressing tumours. In survival analyses, patient with low expressing tumours did not benefit from tamoxifen, while patients with high expressing tumours did. This difference in treatment effect was significant in a multivariable analysis adjusted for age, tumour grade, proliferation, lymph node status and ER.

An *in vitro* model that resembles the *in vivo* situation of oestrogen-independent growth is the long-term oestrogen-deprived (LTED) MCF-7 cells [226]. When LTED cells are grown in oestrogen-deficient medium for a long time, they first become hypersensitive to oestrogens and after a while they become oestrogen-independent. Interestingly, a large increase of ERS118-P levels was noted during the hypersensitive phase. The levels then decreased and stabilized at about two-fold in the oestrogen-independent cells compared to WT. This was accompanied with a steady increase of the ER, and when considering the proportion of phosphorylated ER, the levels of ERS118-P had actually decreased in oestrogen-independent cells [226]. Another study reported elevated levels of ERS118-P in tumours taken from patients who had relapsed after tamoxifen, compared to primary tumours taken before tamoxifen treatment [227], indicating a complex role of ERS118-P in mediating endocrine resistance. Previous reports have observed an association between ERS118-P and factors that are characteristic for good prognosis, such as low grade and smaller tumours [224, 225], suggesting that ERS118-P correlates with improved survival. However, we observed no association between ERS118-P and recurrence-free survival in the untreated control group, suggesting that ERS118-P is not a prognostic factor in premenopausal breast cancer patients.

Finally, we analysed what the result would be if ERS118-P would be implemented as a biomarker of eligibility for tamoxifen treatment. If only patients with tumours expressing high levels of

ERS118-P (52 %) were treated with tamoxifen, it would result in an estimated 10-year survival of 64 % which is the same result as if all patients would be treated with tamoxifen, irrespective of ERS118-P status. Consequently, treatment guided by ERS118-P may save unnecessary treatment for half of the ER-positive premenopausal patients, while not affecting the 10-year recurrence-free survival.

Associations between Pak1, pERK1/2, PKA and ERα phosphorylations – links to tamoxifen response (Paper IV)

The importance of cross-talk between signal transduction pathways and ER in endocrine resistance has been recognised for a long time. Up-regulation of key proteins or enhanced activity in pathways, such as the EGFR/HER2, ERK1/2 and PI3K pathway, has been reported in both *de novo* and acquire tamoxifen resistance [130].

In paper IV, we have made an effort to understand the *in vivo* associations between three relevant kinases: Pak1, ERK1/2 and PKA and their respective association to ERS118-P and ERS305-P as well as the tamoxifen response. The expression of Pak1 had been assessed previously in paper I, as well as the two different phosphorylated forms of ER in paper II and III, respectively. In this study, we extended our previous analyses by adding examination of the activated forms of ERK1/2 (pERK1/2) and PKA (pPKA) in order to present a detailed view of the *in vivo* situation. Both pERK1/2 (nuclear) and pPKA (cytoplasmic) was evaluated using the Allred scoring system. In some tumour samples that often, but not always, were negative for cytoplasmic pPKA, we also observed a clear nuclear expression of pPKA. Therefore we also divided tumours into either negative/low or high expressing depending on the fraction of tumour cells with nuclear pPKA staining. We observed a significant link between pERK1/2 and ERS118-P, which is in line with other reports [223-225]. It is also well-established that ERS118 *in vitro* is directly phosphorylated by ERK1/2 in an oestrogen-independent manner [216]. Furthermore, we detected an association between nuclear pPKA and ERS305-P, supporting the *in vitro* experiments that have shown direct phosphorylation of ERS305 by PKA [108]. In addition, there was an association between pERK1/2 and ERS305-P and also between cytoplasmic pPKA and ERS118-P. Although S305 and S118 residues are not targets for pERK1/2 and pPKA, respectively, these associations might be due to cross-talk. In support of this, there was an association between cytoplasmic pPKA and pERK1/2. To our surprise, there was no correlation between Pak1 and any of the ER phosphorylations, which has been suggested from experimental studies [111]. This might be explained by the fact that we did not analyse that phosphorylated form of Pak1 as oppose to the other kinases.

Since active kinase signalling has been implemented in resistance to endocrine treatments, we analysed whether tamoxifen response differed according to pERK1/2 or pPKA status. Tamoxifen treated patients whose tumour had low expression of pERK1/2 had a better recurrence-free survival compared to control. In all the other subgroups, defined by pERK1/2 or pPKA status, there was a trend towards a beneficial effect of tamoxifen, however not significant in any patient subgroup.

As mentioned earlier, pERK1/2 phosphorylation of S118 has been associated with a ligand-independent activation of ER. However, in paper III we observed that patients whose tumours express high levels of ERS118-P responded well to tamoxifen. To further explore this interplay, we analysed the predictive value of ERS118-P in a subgroup of tumours with low pERK1/2 expression. Interestingly, in this subgroup, a significant beneficial effect of tamoxifen was only observed in patients with high tumour levels of ERS118-P. In this particular subgroup, tumour cell proliferation is probably dependent on E2-mediated ER activation, in which ER is phosphorylated at S118 in response to E2-signalling. Next, we performed the same analysis in the subgroups of low pPKA, both cytoplasmic and nuclear, separately. In the subgroup of low cytoplasmic pPKA tumours, high levels of ERS118-P did not confer a significant beneficial effect of tamoxifen. This might be explained by the increased ratio of pPKA nuclear positivity in this subgroup. Nuclear pPKA

was significantly associated with ERS305-P, which in turn is coupled to tamoxifen resistance, as shown in paper II. Furthermore, in the subgroup of low nuclear pPKA, high levels of ERS118-P was associated with a beneficial tamoxifen response, probably at least to some extent due to lower levels of ERS305-P positive tumors in that subgroup. Our analyses have led us to propose that ERS118-P is more predictive of tamoxifen response than the kinases that phosphorylate the site, i.e. pERK1/2 and possibly pPKA through cross-talk.

In addition to these sets of analyses, we explored the possibility of combining the two ER phosphorylations analysed, to see whether a combination of ERS118-P and ERS305-P would have more predictive value than ERS118-P alone. Although it is a valid assumption that tumours with ERS305-P together with low levels of ERS118-P would have the least tamoxifen response out of the four possible subgroups, the analysis was limited due to few tumors with ERS305-P. Nevertheless, a forest plot showing the effect of tamoxifen in the subgroups and the respective survival plots (provided in supplementary figures) indicate that our postulation is acceptable.

Pak1 phosphorylation of HIF-1α and regulation of the hypoxic response (Paper V)

Hypoxia is a common trait of solid tumours and is highly associated with increased resistance to anti-cancer therapy [190]. The cellular response to a hypoxic environment is mediated by the HIF-1 transcription factor, which induces gene expression that governs multiple processes that increase the survival capacity of the tumour cell. Factors that can modulate the activity of HIF-1 are therefore important in a general tumour biological perspective.

In paper V, we have identified Pak1 as a novel kinase that phosphorylate the hypoxia inducible α-subunit of HIF-1, HIF-1α, which leads to stabilisation and increased transcriptional activation. The fact that HIF-1α is a protein that undergoes several posttranslational modifications, including phosphorylations, has been known for a long time, however, the consequences of HIF-1α phosphorylation has remained elusive. Previous reports on HIF-1α phosphorylation has implemented the ERK1/2 and p38 MAPKs [204], and in a recent report it was shown that ERK1/2 phosphorylation of HIF-1α led to increased nuclear localisation and hence increased transcriptional activity [205].

Pak1 is a serine/threonine protein kinase with an increasing number of described substrates, and in this paper, we identified several Pak1 consensus sites in the HIF-1α protein sequence. By generating GST-fused constructs of full-length HIF-1α and five deleted versions of HIF-1α, we were able to detect *in vitro* phosphorylation of full-length HIF-1α and of one deletion construct (aa 531-826). The fact that other deletion constructs containing aa 531-826 were not phosphorylated in our assays is puzzling. It can be due to folding problems or hidden phospho-sites. Nonetheless, phosphorylation of the full-length construct indicates that Pak1 recognise the HIF-1α protein as a substrate *in vitro*. Next, we studied the *in vivo* situation, by stimulating HeLa cells with growth factor or sphingosine during hypoxia. An increased level of phosphorylated HIF-1α was noticed, together with a total increase of the HIF-1α protein level. A simple explanation for the increased phosphorylation could be increased total protein levels, although, the observed increase of phosphorylated HIF-1α is quite robust and the amount of phosphorylated HIF-1α would only correspond to a fraction of the massive increase of HIF-1α during hypoxia if it was not a direct phosphorylation effect. To ensure that the phosphorylation of HIF-1α was Pak1 specific, we silenced Pak1 in two breast cancer cell lines and exposed them to hypoxia. This led to a decrease in the levels of both phosphorylated HIF-1α and total HIF-1α. Together, these results indicate that Pak1 phosphorylation stabilise HIF-1α protein levels. Moreover, we detected a Pak1-mediated effect on HIF-1α transcriptional activity. The effect was abolished when a kinase-dead Pak1 was used, which further indicates that phosphorylation contributes to increased stabilisation of HIF-1α. The effect on HIF-1α downstream target gene expression was analysed by qPCR and western blot, and silencing of Pak1 followed by hypoxic treatment led to a decreased induction of several

de novo HIF-1α target genes.

The proposed stabilisation of HIF-1α after Pak1 phosphorylation could possibly be due to less pVHL-mediated degradation. In a renal carcinoma cell line, where HIF-1α is constitutively expressed due to lost pVHL expression, Pak1 silencing did not affect the levels of HIF-1α, indicating that the mechanism behind Pak1-mediated HIF-1α stabilisation is dependent on an intact pVHL. Interestingly, it has been suggested that even during hypoxia, HIF-1α and pVHL are able to interact to some extent and it was demonstrated that phosphorylation of HIF-1α by p38 MAPK hindered this interaction and thus stabilised hypoxia induced HIF-1α [228]. It is tempting to speculate that Pak1 phosphorylation is functioning in a similar manner as p38 MAPK to stabilise HIF-1α.

Conclusions

In this thesis we have identified tamoxifen treatment predictive factors in premenopausal breast cancer and also identified a new regulatory mechanism of the hypoxic response in breast cancer cells.

More specific conclusions are:

- Pak1 is overexpressed in breast cancer and it is associated with proliferation and high tumour grade (Paper I).
- High expression of Pak1 and particularly nuclear localisation is associated with poor tamoxifen response but does not affect breast cancer prognosis (Paper I).
- Pak1 and ER signalling are intimately linked in breast cancer cells (Paper I).
- ERS305-P positive tumours are less responsive to tamoxifen (Paper II).
- High expression of ERS118-P is associated with good tamoxifen response (Paper III).
- pERK1/2 and pPKA are associated with ERS118-P and ERS305-P *in vivo* (Paper VI).
- ERS118-P and ERS305-P are better treatment predictive factors than their respective phosphorylating kinases (Paper IV).
- A combination of ERS118-P and ERS305-P might have superior treatment predictive value compared to the respective phosphorylations alone (Paper IV).
- Pak1 phosphorylates HIF-1α and enhance its transcriptional effect through stabilisation of HIF-1α (Paper V).

POPULÄRVETENSKAPLIG SAMMANFATTNING

Bröstcancern blir allt vanligare men tack vare möjligheterna att diagnostisera sjukdomen i ett tidigt skede samt bättre behandlingsmetoder så ökar inte dödligheten i sjukdomen, snarare sjunker den. Till skillnad från vad man kan tro av benämningen så är inte bröstcancer *en* sjukdom utan ett samlingsbegrepp som innefattar *flera* olika typer av tumörer som uppstår i bröstet. Huvudsakligen behandlas bröstcancerpatienter med kirurgi. Beroende på storlek och hur pass utspridd tumören är i bröstet väljer man antingen att ta bort endast en del av eller hela bröstet. Även om tumören tas bort så kan det trots allt finnas cancerceller kvar. Därför ger man patienter även en kompletterande (adjuvant) behandling. Denna kan vara i form av strålning av bröstet eller cytostatika (kemoterapi). I de flesta av fallen kan man även använda endokrin behandling, vilket är detsamma som anti-östrogen. Ca 70 % av alla bröstcancrar är beroende av hormonet östrogen och anti-östrogen motverkar dess effekt. Det finns olika typer av anti-östrogena behandlingar: vissa läkemedel hämmar produktionen av östrogen och andra verkar genom att blockera inbindningen av östrogen till dess mottagande receptor. När östrogen binder till sin receptor leder det till att en mängd gener antingen slås av eller på. Resultatet blir att cellen fortsätter att dela på sig och ge upphov till fler tumörceller.

Det mest använda anti-östrogen är tamoxifen, som verkar genom att binda till östrogenreceptorn precis som östrogen. Därmed finns det färre obundna receptorer för östrogen att binda till. Dessutom har tamoxifen en egen effekt på receptorn som i de flesta fall, beroende på celltyp och vävnad, är den motsatta jämfört med östrogen. I över 20 år har tamoxifen använts i bröstcancerbehandling och det har definitivt bidragit till den observerade minskningen av återfall och dödsfall. Av förklarliga skäl är patienter som saknar östrogenreceptorn i tumören inte ”lämpliga” för behandling med tamoxifen. Men även patienter som *har* östrogenreceptorn, hos vilka tamoxifen bör ha en fungerande effekt, får återfall antingen efter en kort tid eller efter en längre tids behandling. Tumörerna hos dessa patienter har blivit okänsliga (resistenta) mot tamoxifen. Tamoxifenresistens är ett stort kliniskt problem, men om vi skulle kunna förutsäga i vilka fall tamoxifen med stor sannolikhet inte fungerar, så kan en annan sorts anti-östrogen övervägas för dom patienterna. Orsaken till tamoxifenresistens är inte helt klarlagd och endast ett fåtal markörer används kliniskt för att avgöra om en patient är lämplig för tamoxifenbehandling. Markörer som ger information huruvida en patient är känslig för en viss sorts behandling kallas för prediktiva markörer och i bröstcancer är således östrogenreceptorn ett exempel på en prediktiv markör för tamoxifenbehandling.

I delarbeten I-IV har vi identifierat prediktiva markörer för tamoxifenkänslighet utöver de som redan används idag. Detta har vi gjort genom mikroskopisk undersökning av tumörer tagna från patienter vid operationstillfälle. Samtliga tumörer kom från premenopausala patienter som efter operation antingen fick adjuvant tamoxifen i två år eller placebo (där placebo motsvarar obehandlade patienter). Denna kliniska studie gjordes i slutet av 80-talet då betydelsen av tamoxifen inte var helt fastställd. Med de kunskaper vi besitter idag hade en liknande studie, d.v.s. där en patientgrupp inte får *någon* adjuvant behandling, inte kunnat genomföras. För vårt ändamål passar detta samlade material av tumörer väldigt bra, eftersom vi kan jämföra effekten av tamoxifen *vs.* ingen behandling i en homogen patientgrupp, baserat på t.ex. närvaro eller mängden av ett visst protein i tumörcellerna.

I det första delarbetet, identifierade vi ett protein; Pak1, vars höga nivåer och specifika lokalisering till cellkärnan medförde tamoxifenresistens. Vi kunde nämligen med statistiska analyser konstatera att hos patienter med låga nivåer eller frånvaro av kärnlokalisering av Pak1 i tumörcellerna fanns det en signifikant skillnad i antalet återfall mellan obehandlade och tamoxifenbehandlade patienter, där tamoxifengruppen hade färre återfall. Däremot så fanns det *ingen* skillnad i antalet återfall mellan tamoxifenbehandlade och obehandlade hos patienter med höga nivåer eller kärnlokalisering av Pak1. Följaktligen drar vi slutsatsen att Pak1 är en möjlig prediktiv markör för tamoxifenresistens

i premenopausal bröstcancer.

På liknande sätt har vi i delarbete två identifierat ytterligare en markör som indikerar tamoxifenresistens. I detta fall rör det sig om en viss förändring av själva östrogenreceptorn, en fosforylering av en specifik aminosyra. Om tumörcellerna hade just den fosforyleringen så var det en indikation på tamoxifenresistens. I delarbete tre har vi undersökt ytterligare en fosforylering på östrogenreceptorn, fast denna fosforylering visade sig tvärtom vara en markör för bra effekt av tamoxifen. Delarbete fyra är ett sammanfattande arbete där vi har undersökt sambandet mellan olika protein som ger upphov till de olika fosforyleringarna som undersöktes i delarbete två och tre. I detta arbete drar vi slutsatsen att fosforylering av östrogenreceptorn ger en bättre indikation på tamoxifeneffekt jämfört med proteinerna som är associerade med fosforyleringarna. Genom att identifiera prediktiva behandlingsmarkörer så kan vi mer och mer börja skräddarsy behandling efter varje patient, vilket kommer att rädda fler liv i slutändan.

I sista delarbetet har vi igen studerat Pak1 fast med inriktning på hypoxi. Hypoxi innebär låg syrenivå och det är mycket vanligt under tumörbildning eftersom blodkärlen, som förser celler och vävnader med syre och näringsämnen, trängs undan av den snabbt växande tumören. Effekten blir att tumörcellerna måste försvara sig för att kunna klara sig och detta genomförs via aktivering av gener som på olika sätt bidrar till cellernas överlevnad. Detta leder i sin tur till mer aggressiva och svårbehandlade tumörer. Genaktiveringen sker via ett protein; HIF-1α, som bara är aktivt när syrenivån är tillräckligt låg. HIF-1α påverkas av andra proteiner vilket leder antingen till ökad eller minskad HIF-1α aktivitet. I delarbete fem visar vi att Pak1 fosforylerar HIF-1α. Detta påverkar HIF-1α's stabilitet samt aktivitet och därmed ökar också aktiveringen av generna som styrs av HIF-1α. Ur ett syrefattigt tumörcellsperspektiv skulle alltså mer Pak1 protein leda till en bättre anpassning till en tuff miljö och så småningom leda till ökad aggressivitet hos tumörcellerna.

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