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# ESTROGEN RECEPTOR β SIGNALLING IN MAMMARY EPITHELIAL AND BREAST CANCER CELLS

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Stockholm 2010

Published by Karolinska Institutet. Printed by Universitetsservice US-AB


ISBN 978-91-7457-171-4

*To My Family*

# ABSTRACT

Estrogens are key players in the etiology and progression of breast cancer, and mediate their effects through the estrogen receptors (ERα and ERβ). ERα plays important roles in proliferation and progression of breast cancer, whereas a distinct function of ERβ in the initiation and development of breast cancer is not yet clearly established. The general aim of this thesis was to increase our understanding of the molecular and cellular mechanisms of estrogen signalling in the normal and cancerous breast, focusing on the potential anti-tumourigenic effect of ERβ. Using cell lines with endogenous expression or inducible expression of ERβ we have characterised possible pathways of how ERβ could mediate its anti-tumourigenic effects.

The role of ERβ in cell proliferation and cell cycle regulation has been characterised, mainly *in vitro*. In **paper I** we investigated how ERβ re-expression would affect breast cancer cells in *in vivo*. Presence of ERβ in breast cancer xenografts reduced tumour growth and the number of intratumoural blood vessels. Expression of the pro-angiogenic growth factors vascular endothelial growth factor and platelet-derived growth factor β were also reduced upon ERβ expression, both *in vitro* and *in vivo*. These findings suggested an anti-tumourigenic role for ERβ by inhibiting growth and angiogenesis.

Studies in ERβ$^{-/-}$ mice have suggested a role for ERβ in the regulation of cell adhesion. In **paper II** we looked at cell-cell adhesion with a focus on E-cadherin. We reported that decrease of ERβ in mammary epithelial cells was associated with a decrease of E-cadherin protein levels through different posttranscriptional regulatory mechanisms, including protein shedding, internalisation and degradation. This correlated with an increase in β-catenin transcriptional activity and impaired morphogenesis on Engelbreth-Holm-Swarm matrix. This study suggests that ERβ has an important role in maintaining cell adhesion and a differentiated phenotype.

In **paper III** we analysed the effects of ERβ on cell-extracellular matrix adhesion. We found that integrin α1 and integrin β1 levels increased in breast cancer cells following ERβ expression. Also, the formation of vinculin containing focal complexes and actin filaments was enhanced, correlating to a more adhesive potential as seen by adhesion to ECM proteins. Furthermore, the migratory potential of the breast cancer cells was decreased upon ERβ expression. This study indicates that ERβ affects integrin expression and clustering and consequently adhesion and migration of breast cancer cells.

ERβ has been implicated as an indicator of endocrine response in breast cancer. In **paper IV** we investigated if ERβ could modulate pathways implicated in endocrine resistance development. Expression of ERβ in human breast cancer cells resulted in a decrease in both active Akt, as well as its upstream regulator, the epidermal growth factor receptor 2 and 3 (HER2/HER3) dimer. Expression of the tumour suppressor and important inhibitor of Akt signalling, PTEN was increased upon expression of ERβ. Further, ERβ expressing breast cancer cells had also an increased sensitivity to tamoxifen. In all, these data provide a possible mechanistic insight into how ERβ may contribute to endocrine sensitivity.

In conclusion, the studies presented in this thesis contribute to the knowledge of ERβ function in normal and cancerous breast, and highlight several possible anti-tumourigenic mechanisms for ERβ. Although the mechanisms have not yet been fully characterised, in breast cancer, ERβ seems to affect growth, adhesion, angiogenesis and sensitivity to endocrine therapy. These studies highlight the importance of ERβ as a prospective prognostic marker with potential as a target in the treatment of breast cancer.

# LIST OF PUBLICATIONS

I. Johan Hartman, **Karolina Lindberg**, Andrea Morani, José Inzunza, Anders Ström and Jan-Åke Gustafsson. *Estrogen receptor β inhibits angiogenesis and growth of T47D breast cancer xenografts*. Cancer Res. 2006 Dec 1;66(23):11207-13

II. Luisa A. Helguero, **Karolina Lindberg**, Cissi Gardmo, Thomas Schwend, Jan-Åke Gustafsson and Lars-Arne Haldosén. *Different roles of estrogen receptors α and β in the regulation of E-cadherin protein levels in a mouse mammary epithelial cell line.* Cancer Res. 2008 Nov 1;68(21):8695-704

III. **Karolina Lindberg**, Anders Ström, John G. Lock, Jan-Åke Gustafsson, Lars-Arne Haldosén and Luisa A. Helguero. *Expression of estrogen receptor β increases integrin α1 and integrin β1 levels and enhances adhesion of breast cancer cells.* J Cell Physiol. 2010 Jan;222(1):156-67

IV. **Karolina Lindberg**, Luisa A. Helguero, Yoko Omoto, Jan-Åke Gustafsson and Lars-Arne Haldosén. *Estrogen receptor β represses Akt signaling in breast cancer cells via downregulation of HER2/HER3 and upregulation of PTEN – implications for tamoxifen sensitivity.* Manuscript

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| | |
|---|---|
| AF | Activator function |
| AI | Aromatase inhibitor |
| AP-1 | Activator protein-1 |
| bFGF | Basic fibroblast growth factor |
| BM | Basement membrane |
| CAM | Cell adhesion molecule |
| CHD | Coronary heart disease |
| DBD | DNA binding domain |
| DME | Drug metabolising enzymes |
| ECM | Extracellular matrix |
| EMT | Epithelial-mesenchymal transition |
| eNOS | Endothelial nitric oxide synthase |
| ER | Estrogen receptor |
| EGF | Epidermal growth factor |
| EGFR | Epidermal growth factor receptor |
| ERE | Estrogen response element |
| E2 | 17β-estradiol |
| FAK | Focal adhesion kinase |
| FGFR | Fibroblast growth factor receptor |
| GF | Growth factor |
| HAT | Histone acetylases |
| HDAC | Histone deacetylases |
| HER | Human epidermal growth factor receptor |
| LBD | Ligand binding domain |
| LDL | Low-density lipoprotein |
| MAPK | Mitogen-activated protein kinase |
| MMP | Matrix metalloproteinase |
| MRP | Multidrug resistance-associated protein |
| NFκB | Nuclear factor κB |
| NO | Nitric oxide |
| PDK | Phosphoinositide-dependent kinase |
| $PIP_3$ | Phosphatidylinositol 3,4,5 triphosphate |
| $PIP_2$ | Phosphatidylinositol 4,5 diphosphate |
| PI3K | Phosphatidylinositol 3-kinase |
| PR | Progesterone receptor |
| PTEN | Phosphatase and tensin homologue deleted on chromosome 10 |
| RE | Response element |
| RTK | Receptor tyrosine kinase |
| SERD | Selective estrogen receptor downregulator |
| SERM | Selective estrogen receptor modulator |
| SFKs | Src-family of kinases |
| SP-1 | Specificity protein-1 |
| TF | Transcription factor |
| TKI | Tyrosine kinase inhibitor |
| VEGF | Vascular endothelial growth factor |
| VEGFR | Vascular endothelial growth factor receptor |
| VSMC | Vascular smooth muscle cell |

# 1 INTRODUCTION

## 1.1 ESTROGEN RECEPTOR SIGNALLING

### 1.1.1 Estrogen

Estrogens are a class of sex steroid hormones, synthesized from the common precursor cholesterol [1]. The three major estrogens include 17β-estradiol (E2), which is the most potent hormone, and its metabolites estrone (E1) and estriol (E3). The enzyme cytochrome P-450 aromatase catalyses the last step in the biosynthesis of androgens to estrogens. Aromatase is located in the endoplasmic reticulum of estrogen-producing cells. The main estrogen production occurs in the ovaries, and to a lesser extent in the adrenal glands, adipose tissue, brain and testis. After menopause the ovarian production of estrogen declines, and the adrenal cortex and ovaries secrete mostly androgens, which are converted to estrogens in the peripheral tissues, such as adipose tissue and muscle [2]. Estrogens are involved in many physiological processes such as growth, differentiation, and functioning of reproductive tissues, but they also have important actions on other systems, such as bone, liver, cardiovascular system and brain. Loss of estrogen is usually associated with a rise in low-density lipoprotein (LDL), hot flushes and night sweats, and an increase in bone loss [3]. Aberrant estrogen signalling is associated with many human diseases such as cancer, osteoporosis, Parkinson's and schizophrenia [4-6]. Estrogens biological action is mediated through the estrogen receptors (ERs).

### 1.1.2 Estrogen receptors

Estrogen receptors are members of the steroid receptor gene superfamily of nuclear receptors. Two ERs, ERα and ERβ have been identified. ERα was identified in the late 1950s, however it was not until 1986 that ERα was cloned and sequenced from MCF-7 human breast cancer cells [7]. Ten years later ERβ was identified and cloned [8]. ERα and ERβ are present in many of the same tissues, but differences in organ and tissue distribution, as well as in levels of expression, have been reported. ERα is mainly expressed in uterus, ovary (theca cells), bone, prostate (stroma), white adipose tissue, kidney, testis (Leydig cells), epididymis, breast, liver, skeletal muscle and various regions of the brain, whereas ERβ is mainly expressed in prostate (epithelium), ovary (granulosa cells), lung, testis, epididymis, bone marrow, salivary gland, regions of the brain, urogenital tract and intestinal epithelium [9, 10].

ERα and ERβ have five distinguishable domains, A/B, C, D, E and F (Fig. 1) [11]. The N-terminal **A/B domain** contains the activator function 1 (AF-1) domain, which is constitutively active and mediates ligand independent transcriptional activation. Comparison of ERα and ERβ AF-1 domain has revealed that ERβ has a weak AF-1 function compared to ERα [12]. In addition, the N-terminal region is involved in interactions with co-regulators. Also, phosphorylation sites for different kinases are present in the AF-1 domain of both ERα and ERβ, resulting in stimulation of AF-1 activity [13-19]. The A/B domain shares the least homology between the

receptors, suggesting that this domain contributes to ER specific subtype actions on target genes.

The **C domain** harbours the DNA binding domain, mediating sequence specific DNA binding. Although ERα and ERβ are products of distinct genes - ERα is located on chromosome 6, while ERβ is located on chromosome 14 [20, 21] - they share relatively high degrees of homology in the DNA binding domain, approximately 97%. ChIP-on-chip analysis has shown that there is a high degree of overlap between DNA regions bound by ERα and ERβ respectively. However, there are also DNA regions preferentially bound by ERα or ERβ, as well as regions in which ERα binds only in the presence of ERβ. ERα binding sites have overrepresentation of TA-rich motifs and forkhead transcription factor binding sites, whereas ERβ binding regions are more closely located to transcription start sites and have a predominance of classical estrogen response elements (EREs) and GC-rich motifs, thereby explaining the differences in gene expression and effects by each respective ER [22].

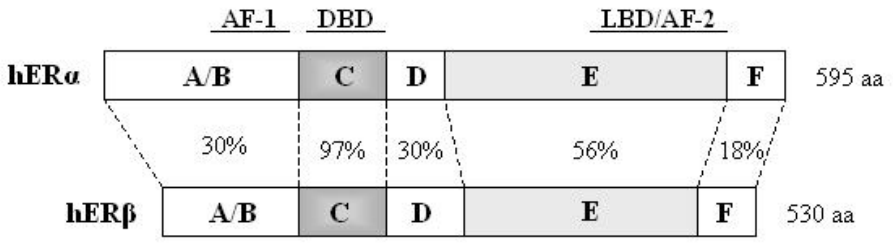


***Figure 1.*** *Structure and degree of homology between ERα and ERβ. The A/B domain contains the ligand-independent transcription activation function-1 (AF-1), and sites for phosphorylation. The C domain mediates DNA binding. The D domain joins the C and E domain and contains nuclear localisation sequences. The E domain contains the ligand-dependent transcription activation function-2 (AF-2) and is involved in ligand binding. The F-domain is involved in co-regulator recruitment. Full-length ERα is 595 amino acids long, whereas ERβ contains 530 amino acids. Homology at the amino acid level is indicated in %.*

The **D (hinge) domain** joins the E/F-domain with the C-domain, and contains nuclear localisation sequences [23].

The **E domain** contains the ligand binding domain and the ligand-dependent transactivation AF-2 domain. This domain is also involved in nuclear translocation, co-factor binding, and receptor homo/hetero dimerisation, as well as interactions with heat shock proteins. Although both ERα and ERβ bind to 17β-estradiol with the same affinity, their ligand binding domains only have 56% amino acid identity [9], suggesting that ligands may be developed which have different affinities for the two ERs.

The carboxy terminus **F domain** seems to have a modulatory role for receptor transcription activity and recruitment of co-regulators [24, 25].

### 1.1.3 Estrogen receptor signalling

In their unliganded state, ERs are associated with inhibitory protein complexes of heat shock proteins, repressing their function [26]. Upon ligand binding, the ER conformation is altered and the receptors dissociate from the inhibitory complex, triggering receptor dimerisation, recruitment of co-regulators, and binding of the receptor complex to promoter regions of target genes. The co-regulators can either stimulate (co-activators) or inhibit transcription (co-repressors). The co-activators, the SRC family and p300/CBP-associated factor, have intrinsic histone acetylase (HAT) activity, whereas the co-repressors, such as SMRT and NCoR, recruit histone deacetylases (HDACs). These result in the alteration of chromatin structure, thereby facilitating or blocking, respectively, the recruitment of the RNA-polymerase machinery to the promoter [27-29].

Various mechanisms for ER transcriptional regulation have been described (Fig. 2). *The classical mechanism* involves direct binding of ER dimers to DNA with classical EREs, either as homo- or heterodimers. *Non-classical mechanisms* involve regulatory interactions with other transcriptional complexes, such as activator protein-1 (AP-1) and specificity protein-1 (SP-1) [30, 31], and thereby resulting in an indirect binding to DNA. ERα and ERβ signal in opposite ways on AP-1 driven promoters in the presence of E2, where ERα activates transcription and ERβ inhibits transcription. In contrast, anti-estrogens all increase AP-1 activity of both ERs [32].

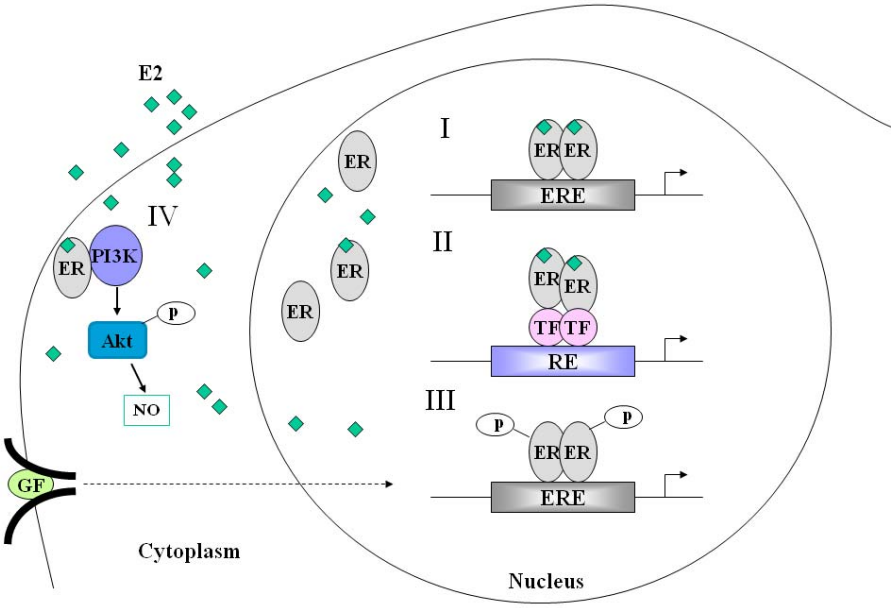


***Figure 2.*** *ER signalling mechanisms.* ***I*** *The classical pathway involves ligand binding of 17β-estradiol (E2), and activation of the receptor, followed by direct binding to estrogen response elements (EREs).* ***II*** *The non-classical pathway involves protein-protein interactions with other transcription factors (TF), followed by indirect DNA binding to response elements (RE).* ***III*** *Ligand-independent ER signalling involves growth factor (GF) signalling and phosphorylation and subsequent activation of the receptor.* ***IV*** *The non-genomic pathway involves rapid estrogenic effects involving membrane localised ER or other distinct receptors. The example shows ER binding to phosphatidylinositol 3-kinase (PI3K) resulting in an increase of nitric oxide (NO) levels in the cytoplasm.*

In addition, E2 have so-called *non-genomic effects* outside the nucleus. These are very rapid responses, occurring within seconds or minutes after addition of E2, and therefore do not involve new synthesis of mRNA or protein [33]. These actions involve membrane located ERs [34] or other distinct receptors [35], where ligand activation results in signalling cascades including activation of kinases, phosphatases and increases in ion fluxes across membranes, consequently leading to physiological responses. Examples include changes in intracellular calcium [36], increases in cAMP [37], and activation of membrane-associated enzymes, as described in Fig. 2 – section IV, where ERα has been shown to bind to the p85α regulatory subunit of phosphatidylinositol 3-kinase (PI3K), leading to activation of Akt and endothelial nitric oxide synthase (eNOS). This results in an increase of nitric oxide (NO) levels in the cytoplasm [38]. Posttranslational modifications such as phosphorylation, acetylation, SUMOylation, ubiquitinylation and methylation can also modulate the transcriptional activity of ERs [1].

ER activities have been presumed to require ligands, but several growth factors have been shown to stimulate ER activity in absence of ligand, mediated by the phosphorylation of the receptor by different kinases resulting in its activation [13-19]. *Ligand independent* activation of ER was first suggested in the early 1990s, where nuclear translocation and transcriptional activation of ER was observed in ovariectomized mice treated with epidermal growth factor (EGF) [39]. Since then, pathways and mechanisms involved in ligand independent activation have emerged but are not fully characterized.

### 1.1.4 Estrogen receptor ligands

The discovery of the second estrogen receptor isoform (ERβ) encouraged the search for ligands selective for either ERα or ERβ to allow investigation of the biological function of each ER *in vitro* and *in vivo*. Selectivity of ligand action has been possible due to differences in ERα and ERβ tissue distribution and since the ligand binding domains only have 56% amino acid sequence identity. Altogether, this has made it possible to develop agonists and antagonists with different affinities and potencies for each ER, which is of therapeutic value for treatment of estrogen-linked diseases. Today several selective agonists/antagonists with isoform selective affinity (Fig. 3) exist.

ERα and ERβ bind to E2 with comparable affinity [10]. The most used selective ligand for ERα is tetrasubstituted propylpyrazole (PPT). It has a 410-fold binding selectivity for ERα versus ERβ and activates gene transcription only through ERα [40]. PPT has also been shown to have similar effects as E2 in many tissues [41].

Known used ERβ agonists include diarylpropionitril (DPN), 7-bromo-2-(4-hydroxyphenyl)-1,3-benzoxazol-5-ol (WAY 200070), and the phytoestrogens coumestrol, and genistein, which have ERβ binding selectivies of 70-, 68-, 3-, and 19-fold, respectively [10, 42, 43]. Phytoestrogens are plant-derived chemicals with estrogenic activity and are classified according to their chemical structure: isoflavones, flavones, flavanones, coumestans, stilbenes, and lignans. Isoflavones are the most studied phytoestrogens, present in high concentrations in soy products and red clover [44]. In countries with a high intake of phytoestrogens there is a lower incidence of

breast cancer, suggesting that phytoestrogens may have a protective role in breast carcinogenesis through its preference for ERβ [45-48].

A variety of synthetic estrogen antagonists have been developed. These include selective estrogen receptor modulators (SERMs), including tamoxifen and raloxifene. Tamoxifen is metabolised in the liver to its active metabolite 4-hydroxytamoxifen. Unlike estrogens which are uniformly agonists, SERMs function as ER antagonists in some tissues, like breast and brain, agonists in other tissues, such as the heart and bone, and mixed ER agonists/antagonists in the uterus [49]. Selective estrogen receptor downregulators (SERDs) like ICI 182,780 (ICI) are pure antagonists and exhibit no agonist activity in any tissue yet measured.

The response of the ERs to the synthetic anti-estrogens tamoxifen and raloxifene as measured with ERE reporters are different. Together with ERα these ligands are partial E2 agonists, whereas with ERβ they are pure ER antagonists [50-52]. The difference in the N-terminal region is a possible explanation for these differences. Furthermore, when ERα and ERβ are co-expressed under experimental conditions, it has been shown that they form heterodimers preferentially over homodimers [53], and with an increase in the relative ERβ/ERα ratio in a cell, repression of ERα-mediated partial agonistic activity in response to tamoxifen is seen [12, 54]. Thus, depending on heterodimerisation or homodimerisation, differences in receptor activities and selection of target genes can be seen [55-58].

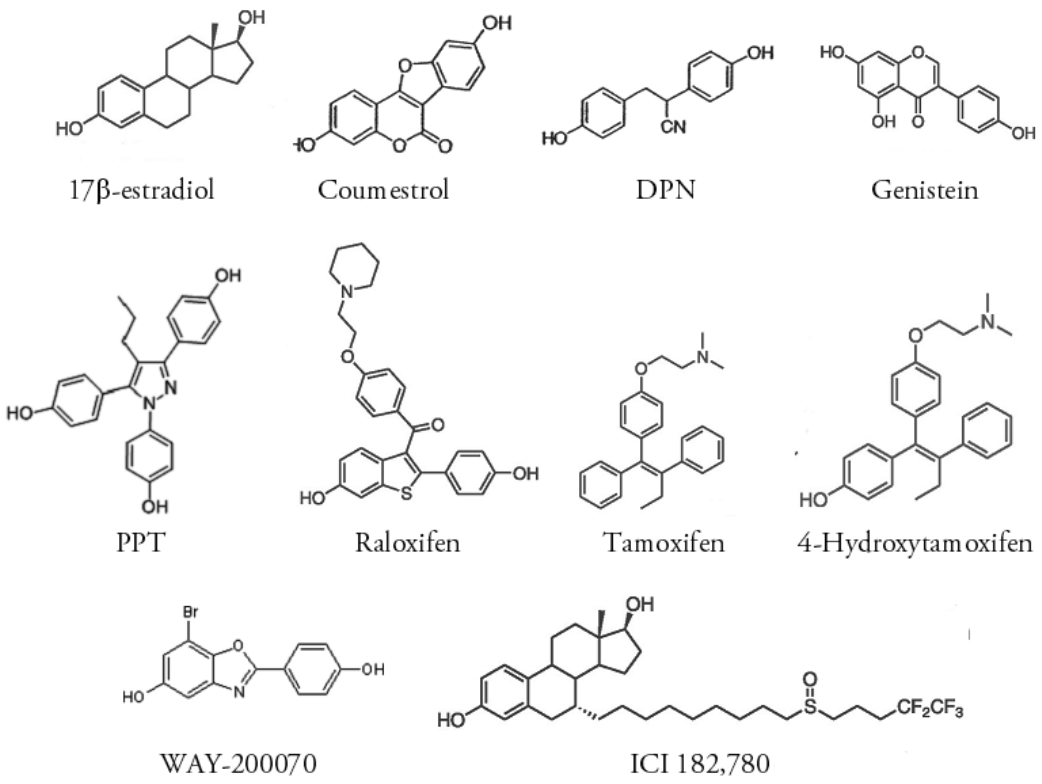


***Figure 3.*** *Estrogen receptor ligands*

## 1.2 ESTROGEN RECEPTORS IN HEALTH AND DISEASE

### 1.2.1 Breast Cancer

Breast cancer is the most frequently diagnosed cancer in women. Women of the Western world have 12% lifetime risk of developing breast cancer, where approximately 25% of women diagnosed will die from the disease due to metastases. In Sweden, approximately 15-20 women are diagnosed every day. Approximately two-thirds of breast cancers occur when ovarian estrogens are no longer produced, that is during the postmenopausal period. Although much less common, men can also develop breast cancer with approximately 40 men in Sweden diagnosed every year. The treatment and prognosis is the same as for women [59].

Breast cancer arises in the ductal and lobular epithelium of the mammary gland. As most breast cancers, at least initially, are dependent on estrogen – approximately 60% in premenopausal women, and 75% in postmenopausal women [60, 61], blockers of estrogen action are important drugs in its treatment. Estrogen-independent breast cancers follow a more aggressive clinical course than the estrogen-dependent breast cancers.

Breast cancer is a heterogeneous disease, and can be divided into four molecular classes on the basis of ER status, where luminal A and B are ER positive subtypes, whereas ER negative breast tumours include HER2 overexpressing and basal-like subtypes. Basal-like subtype is the most aggressive type of the disease, and has the worst outcome. This subtype is negative for ER, progesterone receptor (PR) and HER2. Tumour grade can discriminate luminal A from luminal B tumours, where luminal A cancers have low histological grade, whereas luminal B tumours have high histological grade.

The incidence of breast cancer has increased during the last 50 years. The reasons behind this are unclear, however, improved diagnostic methods could contribute to this. Other reasons could be increased carcinogenic factors in the diet, as well as environmental pollutants, some of which have shown affinity for ERs. Since estrogen plays a crucial role in most breast cancers in women, early menarche, late menopause and a low number of pregnancies are also correlated to an increased risk for breast cancer, due to greater number of menstrual cycles, and therefore larger amounts of circulating sex hormones during the life time. Obesity is also correlated to increased risk due to estrogen synthesis in the adipose tissue, whereas early pregnancy and breast feeding decreases the risk of breast cancer, due to lower levels of hormones and due to development of a fully differentiated mammary gland [62]. About 5-10% of breast cancers are thought to be hereditary, where the most common cause is inherited mutations in BRCA1 and BRCA2 genes. The risk may be as high as 80% in carriers of BRCA mutations. Women who inherit these mutations are usually young when breast cancer develops and often both breasts are affected. These mutations also increase the risk for developing other cancers, particularly ovarian cancer [63].

The management of breast cancer has improved dramatically over the recent decades due to earlier detection, and new therapeutic strategies. But still there are challenges in breast cancer treatment and thus it is important to explore new possible therapeutic strategies, find better prognostic markers, and circumvent endocrine resistant tumour growth.

### 1.2.2 Estrogen receptors in the normal and cancerous breast

Estrogen is a critical regulator of breast epithelial cell proliferation, differentiation and apoptosis. Females deficient in aromatase cannot convert androgens to estrogens, and do not develop breasts at puberty. However, estrogen replacement therapy in these females results in normal breast development [64]. Knockout mice for the two ERs have revealed the importance of these receptors *in vivo*, where ERα has been shown to be essential for normal mammary gland development and function, whereas ERβ is important for the terminal differentiation of the mammary gland [65]. ERα is expressed in 7-10% of luminal epithelial cells in the normal human breast, and its expression fluctuates with the menstrual cycle [66-68]. In contrast, ERβ expression is high in the normal breast, 80-85% of cells and does not fluctuate during the menstrual cycle [69, 70].

Estrogen plays an important role not only in development of normal mammary glands but also in the cancerous breast, where aberrant signalling leads to proliferation and progression of breast cancer. E2 treatment of breast cancer cells stimulates proliferation *in vitro,* and growth of tumour cell xenografts in mice [33]. The ERs are crucial mediators of estrogen function and have important roles in carcinogenesis. Therefore, both estrogen synthesis and ER actions have been targeted by therapies to control hormone dependent breast cancers. ERβ is often lost during carcinogenesis, whereas ERα expression in increased, suggesting a possible tumour suppressor role for ERβ. ERα promotes proliferation, whereas ERβ has been shown to suppress ERα transcriptional activity and lower expression of ERα target genes, e. g. pS2, cyclin D1 and PR [71, 72], and to promote anti-proliferative and pro-apoptotic functions as well as decrease motility [73-76]. Due to its decreased expression, ERβ:s ability to modulate ERα is altered during tumourigenesis [69, 77], and this may contribute to the pathogenesis of breast cancer.

The majority of breast cancers express ERα, approximately 70%, providing a predictive value for endocrine therapy, such as treatment with tamoxifen and aromatase inhibitors. Those tumours expressing both ERα and PR have the greatest benefit from endocrine therapy [78]. In general, ERα expression is associated with low tumour grade, long disease-free survival and high overall survival. ERα positive tumours are also less invasive. In contrast, loss of ERα is associated with poor prognosis, elevated incidence of metastasis and increased recurrence [79]. The significance of ERβ is still under debate. Loss of ERβ has been associated with a more invasive phenotype, poor survival and tamoxifen resistance, and its expression is associated with a better disease outcome and tamoxifen sensitivity [80]. In addition, when ERβ is phosphorylated at serine 105, it is associated with better survival, and phosphorylated ERβ could therefore be used as a prognostic marker [81]. This phosphorylation could implicate ligand-independent activation of the receptor. Altered expression of ER co-factors has also been reported during breast tumourigenesis, where SRC-3 has been reported to be overexpressed in breast cancer and contribute to endocrine resistance, by reducing the antagonist activity of tamoxifen [82, 83].

Interestingly, in breast cancer cells, ERβ promoter region has been found to be hypermethylated compared to normal breast epithelial cells. This hypermethylation was inversely correlated to ERβ mRNA levels. Treatment with a demethylating agent reactivated ERβ expression, suggesting one possible mechanism for the described disappearance of ERβ in breast cancer. These observations from cell

lines were also reflected in primary breast cancer tumours [84]. Increased promoter methylation has also been noted in premalignant lesions and in approximately 60% of invasive breast cancers, corresponding to poor clinical prognosis [85]. Regarding ERα, its expression levels have also been shown to be regulated by DNA methylation. Hypermethylation of ERα promoter is associated with a decrease in mRNA levels and inhibition of DNA methyltransferases reactivates ERα expression [86].

Crosstalk between ER and growth factor pathways may also contribute to breast cancer since growth factors can activate ERs independently of hormone, e.g. EGF has been shown to activate both ERβ and ERα via MAPK phosphorylations [17, 87]. Constitutive activation of the PI3K pathway and overexpression of HER2 also activates ERα [15, 88]. These crosstalks may provide alternative growth pathways for tumours.

### 1.2.3 Endocrine therapy

During recent years, new cancer therapies have emerged that inhibit specific pathways important for tumour growth, however endocrine therapy is the most efficient treatment in estrogen receptor positive breast cancers. Endocrine therapy blocks the action of estrogen on tumour cells by either inhibiting estrogen binding to its receptors or its synthesis. Mortality from breast cancer is decreased when using endocrine therapies [89], but their efficacy is limited by intrinsic and acquired therapeutic resistance.

Early breast cancer therapies included surgical removal of the ovaries, but during the 1970s, the synthesis of competitive inhibitors of estrogen-ER binding led to targeted cancer therapy, especially the SERM tamoxifen [90]. Later, development of other effective endocrine therapies involved targeting estrogen synthesis, such as aromatase inhibitors (AIs) [91], or ER signalling, such as other SERMs (raloxifene) and anti-estrogens (ICI) [92, 93].

ERα positive breast cancers represent the majority of breast cancer cases, therefore, ERα has been the major therapy target for decades. Tamoxifen has been used in endocrine therapy for the last 25 years, and is therefore the most well studied anti-estrogen. It binds to ER, thereby preventing estrogen binding, and alters the receptor conformation, leading to co-repressor recruitment, inhibition of AF-2 activation, and as a result, transcriptional activation is blocked. However, the AF-1 domain remains active in the presence of tamoxifen [94]. Tamoxifen has also been used as preventive treatment in patients with high risk of developing breast cancer [95]. Tamoxifen displays partial agonist-antagonist activities in different tissues and cells, related to the environment of ER co-activators and co-repressors in these tissues. It acts as an antagonist in the breast whereas in the bone and cardiovascular system it functions as an agonist. Therefore, tamoxifen promotes beneficial effects such as reducing the risk of osteoporosis. The agonistic properties of tamoxifen is enhanced in cells with increased levels of co-activators such as SRC3 or SRC1 [96]. However, long-term treatment with tamoxifen is associated with an increased risk of endometrial cancer, blood clots and stroke [95].

Raloxifene, the second generation SERM, is similar to tamoxifen, in that it acts as an estrogen antagonist in breast tissue by competitive binding to ER.

Raloxifene is approved for breast cancer prevention, but not for treatment [97]. Raloxifene is also used to prevent and treat osteoporosis in women after menopause.

The SERD ICI binds and promotes degradation of the receptor through the ubiquitin-proteasome pathway [98]. The downregulation of ER in the presence of SERDs, completely inhibits ER-mediated gene transcription by inactivating both AF-1 and AF-2 domains [99]. ICI is used to treat ER-positive breast cancer patients who fail to respond to tamoxifen. No agonistic effects are observed with ICI [100].

Aromatase inhibitors (AIs), block the production of estrogens by inhibiting the enzyme aromatase that converts androgens to estrogens [101], thereby suppressing both tumour and plasma estrogen levels. AIs offer some benefits over tamoxifen in postmenopausal women with estrogen-dependent breast cancers [102, 103], however acquired resistance after an initial response commonly occurs. In tumours with ER, SRC3 and HER2 expression, tamoxifen has been shown to act as an agonist and stimulates tumour growth [104], suggesting that aromatase inhibitors might be more effective in such patients, which is also supported by clinical studies [105, 106].

Despite the development of new therapeutic treatments, resistance to all forms of endocrine therapy exists, and therefore limits our ability to take full advantage of endocrine treatments for breast cancer. Thus important challenges within breast cancer research include characterising the factors and pathways responsible and identifying solutions how to overcome this resistance.

### 1.2.4 Endocrine resistance

Approximately 30% of ERα-positive tumours fail to respond to endocrine therapy [107], and therefore it is of great importance to develop more specific biomarkers that predict the therapeutic response to endocrine therapy and to identify new therapeutic targets for endocrine resistant disease. Resistance to endocrine therapy in ER-positive tumours can be classified as either intrinsic, i.e. resistance occurs *de novo* at the initial exposure to endocrine therapy, or as acquired, i.e. the resistance manifests over time.

The lack of expression of ERα is the primary mechanism behind intrinsic resistance to tamoxifen, but it has also been documented recently that patients carrying inactive alleles of cytochrome P450 2D6 (CYP2D6), fail to convert tamoxifen to its active metabolite, endoxifen, and as a consequence, are less responsive to tamoxifen. This is seen in approximately 8% of Caucasian women [108]. Regarding acquired resistance, several mechanisms have been postulated, as described below.

I. **Estrogen receptors:** Initially, loss of ERα expression or occurrence of ERα mutations was suggested to be implicated in acquired resistance. However, loss of ERα expression occurs only in a minority (15-20%) of breast cancers [109], and less than 1% of ER positive tumours have mutations in ERα [110]. More recently, truncated isoforms of ERα and ERβ [111, 112], as well as post-translational modifications of ERα, have been suggested to contribute to endocrine resistance, where phosphorylation of the receptor has been shown to change the pharmacology of the receptor, resulting in ligand-independent or tamoxifen mediated activation of the receptor [15, 113]. Increased transcriptional activity of activator protein 1 (AP-

1), and nuclear factor κB (NFκB) is also associated with endocrine resistance [114]. Furthermore, overexpression and increased phosphorylation of co-activators, such as SRC3, leads to constitutive ER-activated transcription and is associated with reduced responsiveness to tamoxifen in patients [83]. Not much is known about ERβ function in endocrine resistance, but recent studies have shown that reduced levels of ERβ is associated with resistance to tamoxifen therapy [115], and therefore insight into ERβ relation to endocrine resistance is of importance.

II. **Growth factor signalling pathways:** Crosstalk between receptor tyrosine kinases (RTKs) and ER signalling has been shown to promote resistance to endocrine therapy. The RTKs are responsible for persistent ER activity by activation of MAPK and Akt, with subsequent phosphorylation and activation of the ERs. This activation results in increased expression of ER responsive genes, such as amphiregulin, an epidermal growth factor receptor (EGFR) ligand, thereby creating an ability to maintain cell growth and proliferation despite endocrine therapies, (by activation of RTK pathways) [116]. Furthermore, HER2 signalling can be activated by membrane ER, and the kinase cascade downstream of HER2 can activate ER and its coregulatory proteins [104]. In all, activated RTKs result in limited efficacy to endocrine therapy, explaining why tamoxifen is less effective in HER2 and EGFR positive tumours [83, 117-120]. The importance of HER3 in the development of the hormone resistant phenotype is recently beginning to be understood. In MCF-7 cells transfected with heregulin cDNA, the ligand for HER3, the cells become estrogen independent and resistant to anti-estrogens, both *in vitro* and *in vivo* [121]. In addition, siRNA against HER3, abrogates HER2 mediated tamoxifen resistance in breast cancer cells [122]. HER2 and HER3 positive breast cancer patients are also more prone to relapse on tamoxifen [123], suggesting that HER3 and its ligands are good potential targets for modulating tamoxifen resistance. Increased activity of Akt signalling pathway, one of the downstream effectors of EGFR family, is also associated with tamoxifen resistance [124-126], and knockdown of phosphatase and tensin homologue deleted on chromosome 10 (PTEN), the negative regulator of Akt, in estrogen-positive breast cancer cell lines, results in anti-estrogen resistance and hormone independent growth [127]. This further strengthens the connection between RTK/PI3K/Akt signalling pathway and estrogen receptors in resistance to endocrine therapy. Recent observations also suggest a reversible phenotype of breast cancer cells, where some ER-negative tumours that overexpress HER2, become ER-positive after anti-HER2 therapy [128, 129].

III. **Alterations in the intracellular pharmacology:** Drug-metabolising enzymes (DME) play key roles in the activation and deactivation of drugs. Therefore, increased/defective metabolism of a drug will influence its toxic and therapeutic effects on the tumour. The use of serotonin reuptake inhibitors, to treat hot flushes in women, are known to inhibit CYP2D6 [130], thereby resulting in a reduced response to tamoxifen. Overexpression of co-activators and/or downregulation of co-repressors can also abrogate the effect of SERMs [96]. Furthermore, the tumour can develop resistance by active drug efflux by membrane transporters, such as multidrug resistance-associated proteins (MRPs) [131].

The knowledge behind the mechanisms of why breast cancer cells become resistant to endocrine therapy is increasing and several potential good targets have been discovered. But still the picture is not complete and therefore more research is needed to treat these therapeutic resistant diseases.

### 1.2.5 Estrogens and angiogenesis

The formation of new blood vessels, known as angiogenesis, is essential for many physiological processes like wound healing, female reproduction, and embryogenesis. However an imbalance in the growth of blood vessels contributes to the pathogenesis of numerous disorders, including cancer. Signals that trigger angiogenesis include metabolic stress (for example low oxygen or low pH), mechanical stress, immune/inflammatory response and genetic mutations. Small blood vessels consist of endothelial cells, whereas larger vessels also are surrounded by mural cells (pericytes and vascular smooth muscle cells (VSMCs)). ERα and ERβ are both expressed in endothelial cells and VSMCs [132, 133].

In contrast to normal vessels, tumour vessels are structurally and functionally abnormal. They are highly disorganized, having an uneven diameter, being repeatedly turned and bend, dilated, and having excessive branching and shunts. Tumour cells cannot grow or metastasise to another organ without blood vessels, therefore targeting angiogenesis is a promising therapy in cancer treatment. But without an efficient blood supply the delivery of anti-cancer drugs to the tumour will not be as effective. Agents blocking the VEGF pathway, such as Avastin, have shown good results in preclinical tumour models, however in clinical trials it has not prolonged overall survival [134].

Endothelial cells, stromal cells, blood cells, extracellular matrix and tumour cells all release both pro- and anti-angiogenic molecules. Basic fibroblast growth factor (bFGF) was the first angiogenic factor identified [135], followed by the identification of a large number of angiogenic factors such as vascular endothelial growth factor (VEGF), one of the most potent endothelial mitogens. VEGF induces rapid angiogenic responses, and is commonly overexpressed in breast cancers. Several studies have also found an inverse correlation between VEGF expression and overall survival in breast cancer, and that increased VEGF or VEGF receptor (VEGFR) is associated with resistance to endocrine therapy [136-138].

Estrogens have been shown to stimulate angiogenesis through different mechanisms, but primarily through the release of VEGF [139]. In nude mice implanted with MCF-7 human breast cancer cells, estrogens stimulated angiogenesis within developed tumours, whereas the anti-estrogen tamoxifen inhibited angiogenesis in the tumours [140]. Induced angiogenesis by bFGF or VEGF in the chick chorioallantoic membrane, was also inhibited by several ER antagonists, including 4-hydroxytamoxifen, nafoxidene, clomiphene and ICI 182,780 [141, 142]. Furthermore, the ER antagonist ICI 182,780 inhibited estrogen-induced cell proliferation, migration and tube formation of endothelial cells [143-145]. The SERM tamoxifen has been shown to reduce microvessel density in primary breast carcinomas [146], thereby causing tumour regression. Interestingly, tamoxifen also reduces angiogenesis in ER negative tumours [147].

The role of the two ERs in the cardiovascular systems has been studied in various mouse models. In ERα knockout mice, loss of ERα prevented estrogen stimulated angiogenesis [148], supporting a role of ERα in mediating the primary vasculoprotective effects of estrogen. As concluded from studies of ERβ deficient mice, ERβ seems to have an essential role in regulation of vascular function and blood pressure [149]. However, ERs physiological relevance and precise role in the vasculature, mediating the biological effects of estrogen, are still incompletely understood and remain to be determined.

## 1.3 CELL ADHESION

### 1.3.1 Introduction to cell adhesion

Cell adhesion is required for the survival of normal cells, providing the cell with an instrument to communicate with its surroundings. The interaction of cells with neighbouring cells and with the extracellular matrix (ECM) influences a variety of signalling events involved in many important cellular processes, such as survival, differentiation, mitogenesis, maintenance of three-dimensional structures and normal function of tissues. These mechanisms, in response to extracellular signals must be tightly controlled to maintain the integrity of multicellular organisms [150].

Cell interaction with neighbouring cells and the extracellular environment is mediated by different classes of cell adhesion molecules (CAMs), including integrins, cadherins, and members of the immunoglobulin and selectin families. The formation of organized structures by CAMs permits an efficient flow of information in signalling pathways. The creation of new tissues and organs, wound closure, extravasation of immune cells, are a few examples of processes all dependent on cell adhesion, with defective function in CAMs being involved in the pathological development of many diseases, including cancer, cardiovascular disease, and immune disorders [150].

Focal complexes are small matrix adhesions seen within spreading or migrating cells and usually found in membrane protrusions of the cell. These adhesions are very dynamic structures and either turn over or mature into larger, more elongated structures, called focal contacts (or focal adhesions). Focal adhesions are found in resting cells with low motility and are usually the attachment points of actin stress fibers to the cell membrane. Interestingly, at intermediate levels of adhesiveness a maximum rate of cell migration occurs, since weakly attached cells cannot generate traction to move efficiently, and at too high levels of adhesiveness cells cannot break contacts and are therefore immobile [151].

### 1.3.2 Extracellular matrix

A large part of tissue volume, besides cells, is filled by a network of proteins and polysaccharides, namely the ECM. The matrix is secreted, assembled and remodelled by surrounding cells, and is divided into two types; interstitial/stromal ECM, that surrounds the connective tissues, and basement membrane (BM), that is present at the

basolateral surface of different cell types in many tissues [152, 153]. The BM is composed mainly of laminin, collagen type IV, entactin/nidogen, and proteoglycans. Besides providing an organizational role of the cells in the tissues, the ECM also controls the behaviour of cells, deciding whether they will proliferate, stop growing, migrate, or undergo apoptosis. Disruption of cell adhesion to the ECM has deleterious effects on cell survival, leading to anoikis, a specific type of apoptosis [154]. The effects of the ECM on cells are mainly mediated by the integrins.

### 1.3.3 Integrin family of cell adhesion receptors

Integrins mediate attachment of cells to the ECM and take part in specialized cell-cell interactions. Integrins are cell adhesion receptors, transmitting both mechanical and chemical signals, regulating the polarity of cells, and organising and remodelling the cytoskeleton during adhesion and migration. In addition to their role in adhesion, migration, and invasion, integrins can also regulate proliferation, survival, and differentiation. The integrin family consists of 18 α-subunits and 8 β-subunits, together forming 24 different heterodimers. Each heterodimer is non-covalently linked and grouped into subgroups depending on ligand-binding properties and subunit composition. The structures of the α- and β-subunits are similar, consisting of a long extracellular domain, a single transmembrane spanning domain and a short, noncatalytic cytoplasmic domain. Some integrins are ubiquitously expressed, while others are expressed in a stage- or tissue-restricted manner [155].

The name "integrin" was given to symbolise its importance in maintaining the integrity of the cytoskeletal-ECM linkage. Apart from binding to ECM components, some integrins mediate cell-cell adhesion by binding to cellular counter receptors on other cells. They can also bind soluble proteolytic fragments and a number of pathogens use them as an entry into the cell. Many integrins recognize specific motifs present in their cognate ligands; the most common motifs are Arg-Gly-Asp (RGD), and Leu-Asp-Val (LDV) [156]. Each heterodimer can bind one or several ligands, which sometimes overlap between each heterodimer (Fig. 4). For example, integrin α5β1 selectively binds fibronectin, whereas integrin αVβ3 binds a wide range of ECM molecules, including fibronectin, fibrinogen, von Willebrand factor, vitronectin, and also proteolysed forms of collagen and laminin. The affinity for the ligands can also vary depending on the cell type in which the integrins are expressed. Furthermore, the function of some integrins can be compensated by others, such as α6β1 by α3β1, whereas other integrins cannot be replaced and their absence may lead to embryonic or perinatal lethality [157, 158].

Integrins are bi-directional signalling receptors involved in outside-in and inside-out signalling. Upon ligand binding integrins undergo conformational changes, clustering together into focal contacts where they associate with actin-binding proteins such as talin, vinculin, and paxillin. This links the integrins to the cytoskeleton, and results in recruitment of enzymes leading to outside-in signalling. Integrins signal mainly by activating focal adhesion kinase (FAK) and subsequently Src-family of kinases (SFKs), but also through the PI3K/Akt pathway. Another way in which integrins transmit outside-in signalling is through their ability to influence growth factor receptors such as EGFR, VEGF and fibroblast growth factor receptor (FGFR).

The inside-out signalling mainly acts to bring integrins into its active conformation, enabling the integrin to bind its ligand [159].

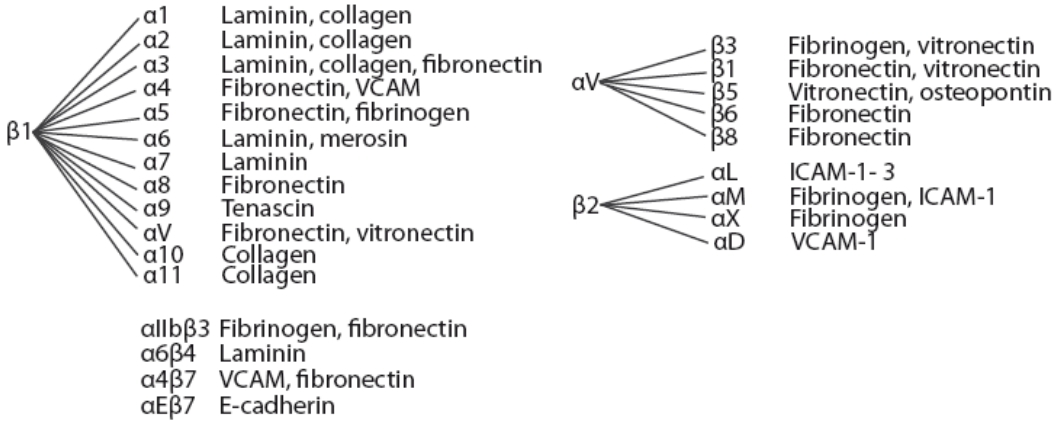


***Figure 4.*** *Integrin heterodimers and some of their ligands.*

### 1.3.4 Integrins in cancer

Deregulated integrin function contributes to the pathogenesis of many diseases including cancer. Even though neoplastic cells are much less dependent on ECM adhesions for their survival and proliferation [160], they still benefit from integrin signals during tumour initiation and progression. Integrin signalling contributes to diverse functions in the tumour cell including migration, invasion, proliferation, survival and tumour angiogenesis. When comparing integrin expression between normal and tumour tissue, expression levels of integrins can vary considerably, where expression of integrins that favour proliferation, survival, and migration are enhanced in the tumour. However, these changes in integrin expression are complex and depend on the tissue of origin, stage of progression and histological type of the tumour. Although cell-type dependent changes in integrin signalling make it impossible to assign each integrin as pro- or anti-tumourigenic, several studies have indicated that integrin α6β4 and αVβ3 are often upregulated in many cancers, whereas integrin α2β1 and α5β1 often are downregulated.

**Integrin α6β4** is shown to be highly expressed in tumours with strong activation of the PI3K/Akt pathway, in cell lines with HER2 expression [161-165] and in tumours expressing high levels of HER3 [166]. The β4 integrin is also expressed in tumour blood vessels, and shown to promote tumour angiogenesis [167]. Expression of integrin β4 is linked to poor prognosis in breast cancer [168]. **Integrin αVβ3** is associated with cell invasion and metastasis [169]. **Integrin α2β1** is expressed at high levels in normal differentiated epithelial cells, including those of the normal breast, however, in adenocarcinomas of the breast its expression is decreased [170]. It has also been observed that re-expression of integrin α2β1 in poorly differentiated breast carcinoma cells reverts the malignant phenotype to a differentiated epithelial phenotype [171]. Integrin **α5β1** has been shown to reduce tumourigenesis [172].

Integrins are also involved in regulating the activities of the matrix degrading enzymes, matrix metalloproteinases (MMPs). Cancer cells produce, secrete

and activate MMPs, with the disruption of the surrounding BM being a key marker of a tumours transition to an invasive carcinoma. MMPs are secreted as inactive zymogens (pro-MMPs), which require proteolytic activation by proteases at the extracellular space, allowing specific localized regulation of the activation of the soluble MMPs [173]. The active form of MMP-2 has been shown to be able to bind integrin αVβ3 on the surface of invasive cells [174], thereby localising proteolytic activity to the invasive front. Furthermore, integrin αVβ3 also cooperates with MMP-9 and thereby enhances the migratory potential of breast cancer cells, which could be inhibited by tissue inhibitors of MMPs [175]. In the invasive breast cancer cell line MDA-MB-231, blocking of integrin α3 subunit reduced MMP-9 activity and inhibited migration and invasion [176].

### 1.3.5 Integrins and therapeutic opportunities

In recent years, progress has been made towards targeting integrins in cancer, due to the fact that integrins have been shown to play a direct role in cancer progression, and since tumour resistance to cytotoxic therapies often involves complex cell-ECM and microenvironment interactions. Several monoclonal antibodies and small molecules interfering with the adhesive function of integrins have been developed to treat a variety of conditions such as thrombosis, inflammation and neoangiogenesis [177]. In recent years progress has been made towards targeting integrins in cancer. Two major targets involve:

**Integrin β4**, due to its angiogenetic effects, upregulation in tumour cells and angiogenic endothelial cells, and enhancement of tyrosine kinase receptor signalling. Inactivation of integrin α6β4 is seen to inhibit tumour growth both *in vitro* and *in vivo* [178, 179].

**Integrin β1** is aberrantly expressed in approximately 30-50% of breast cancers and correlates with more poorly differentiated tumours, presence of axillary node metastasis, and a decrease in overall and recurrence-free survival [180-182]. Inhibition of integrin β1 with an inhibitory antibody has been shown to decrease proliferation and increase apoptosis of breast cancer cells, resulting in changes in the composition of tumour colonies, whereas non-malignant cells are unaffected. This was also seen *in vivo*, with no discernible toxicity to the animals making integrin β1 a promising therapeutic target and predictive factor [183]. In addition, emerging evidence indicates that integrin β1 has an important role in mediating resistance to cytotoxic chemotherapies [184-189]. Therefore it has been suggested that integrin β1 should be a prognostic marker to identify patients with high-risk disease, and who therefore may benefit from a more aggressive targeted therapy, such as radiation therapy.

These studies highlight the importance of continued research in the role integrins have in tumour progression, the development of new agents as cancer therapeutics, and the identification of factors that influence their effectiveness. Integrins are also found in tumour-associated host cells, such as fibroblasts, bone marrow-derived cells, vascular endothelium and platelets. Thus, integrin antagonists may also inhibit tumour progression both in the tumour cells themselves and in the tumour microenvironment.

### 1.3.6 E-cadherin

Cadherins are the major CAMs responsible for calcium dependent cell-cell adhesion. The first three cadherins discovered were named accordingly to which tissue they were found in; E-cadherin in epithelial cells, N-cadherin on nerve cells, muscle and lens cells, and P cadherin on cells in the placenta and epidermis. E-cadherin is essential for epithelial cell shape and in maintenance of the epithelial phenotype. It is expressed ubiquitously by epithelial cells, and inhibition of its function leads to loss of the epithelial characteristics. E-cadherin also plays important roles in proliferation, survival, differentiation, and migration. Knockout of the gene results in embryonic lethality [190].

Cadherins share an extracellular domain consisting of multiple repeats of a cadherin-specific motif [191] and mediate calcium-dependent homotypic cell-cell adhesion at specialized sited called adherens junctions. Here they establish linkages with the actin-cytoskeleton through the catenins, α-, β-, γ-catenins, and p120. These catenins are essential for the function of E-cadherin, where deletion of the catenin binding sites leads to loss of cadherin mediated adhesion. Loss of membrane E-cadherin results in free β-catenin which may enhance transcription of TCF/Lef-1 regulated genes [192]. E-cadherin is trafficked to and from the cell surface by exocytic and multiple endocytic pathways and subsequently recycled to sites of new cell-cell contacts or sorted to the lysosome for degradation.

### 1.3.7 E-cadherin in cancer

*In vitro* and *in vivo* studies have implicated E-cadherin as both an invasion and tumour suppressor. Loss or mutation of E-cadherin, or disruption of cadherin-catenin complexes in tumours, are associated with an invasive malignant phenotype. In breast cancers, partial or total loss of E-cadherin correlates with loss of differentiation characteristics, increased tumour grade, metastatic behaviour and poor prognosis. Moreover, reduced E-cadherin expression is often associated with inappropriate expression of N-cadherin and cadherin-11, proteins usually expressed in mesenchymal cells. In mice, forced downregulation of E-cadherin promotes tumour invasion and metastasis [193], whereas, forced- or over-expression of E-cadherin results in inhibition of breast cancer growth, invasion and metastasis, both *in vitro* and *in vivo* [194, 195].

About 80% of the mutations that have been identified in the E-cadherin gene result in a truncated protein, whereas the remaining 20% are missense mutations. Various mechanisms, both genetic and epigenetic, contribute to the disruption of E-cadherin dependent junctions in cancer cells. Some carcinomas acquire loss-of-function mutations in the E-cadherin gene, whereas in others, members of the SNAIL/SLUG family of transcription factors suppress transcription of the E-cadherin gene. Even RTKs and SFKs can induce the disruption of adherens junctions in neoplastic cells containing E-cadherin expression [196, 197], where tyrosine phosphorylation of the E-cadherin/β-catenin complex by RTKs results in ubiquitinylation and subsequent endocytosis and degradation. Hypermethylation around the promoter region of E-cadherin is also a common silencing mechanism of the gene. Another mechanism involves cleavage by MMPs, which disrupts cadherin mediated cell-cell contacts.

One of the hallmarks for the transition from a benign tumour to a metastatic tumour is epithelial-mesenchymal transition (EMT). EMT is a process where epithelial cells lose their epithelial characteristics and acquire a mesenchymal-like phenotype. This change involves the loss of E-cadherin-mediated cell-cell adhesion, gain of expression of mesenchymal genes, loss of apical-basal polarity, acquisition of a more migratory phenotype and a reorganization of the cytoskeleton [197]. EMT has been described as an important process in tumour progression, metastatic processes and in therapeutic resistance. However, not all tumour types require EMT to initiate metastatic invasion [198]. Several environmental factors and signalling pathways have been described to induce EMT, including receptor tyrosine kinase receptors.

## 1.4 RECEPTOR TYROSINE KINASES

### 1.4.1 Epidermal growth factor receptor family

Cell growth is tightly regulated through the activation of cellular signal transduction pathways, with growth factors and their receptors playing important roles in communication between the outside and inside of the cell. The human EGFR family consists of four closely related receptors: EGFR, HER2, HER3 and HER4, containing an extracellular ligand binding domain and an intracellular RTK domain. HER3, however, is different from the other members in that it has a deficient tyrosine kinase domain and therefore relies on heterodimerisation with other members of the EGFR family for transduction of its signals. These receptors activate numerous downstream pathways in response to extracellular ligands, resulting in effects on differentiation, migration, proliferation and survival. There are no conventional ligands for HER2, which instead acts as a dimerisation partner for the other members of the EGFR family, but the other receptors are each activated by binding a subset of EGF-related growth factors [199]. The EGFR family can also be activated through ligand-independent mechanisms such as irradiation and high receptor density due to overexpression [200, 201]. Upon ligand binding, the receptors undergo a conformational change, form active homodimers or heterodimers, activate their intracellular kinase activity by autophosphorylation and associate with specific signalling molecules to subsequently initiate numerous downstream signalling events such as mitogen-activated protein kinase (MAPK) and Akt signalling pathways. The activated receptor is then internalised and either destroyed, attenuating the signal, or recycled to the outer membrane to be reactivated.

### 1.4.2 EGFR family and cancer

At least three of the four receptors, EGFR, HER2, and HER3 have important roles in human mammary carcinogenesis and resistance to endocrine therapy. In contrast to the other family members, HER4 seems to mediate anti-proliferative effects and does not appear to play a major role in cancer pathogenesis [202, 203]. Instead HER4 expression has been associated with favourable prognostic factors and a more positive outcome [204-207]. EGFR and HER2 are commonly amplified in breast cancer. Abnormal

receptor activation is due to several mechanisms including receptor overexpression, mutations and ligand-dependent and independent mechanisms. Each alteration leading to phosphorylation of the receptor and activation of downstream events such as MAPK and PI3K/Akt signalling.

The HER2 gene is amplified in 20-30% of breast carcinomas contributing to a more aggressive disease, poor survival and resistance to tamoxifen [118, 208-210]. HER2 overexpressing breast cancers are more likely to be ER negative, and in tumours that express both EGFR and HER2, ER is absent or expressed at low levels, inversely correlated with EGFR and HER2 [120, 211]. Patients with breast tumours expressing high levels of HER2 and the ER co-activator SRC3 often develop resistance to tamoxifen [83], possibly as high levels of SRC3 in breast cancer cells have been shown to increase HER2 mRNA and protein upon tamoxifen treatment [212]. HER3 is also overexpressed in many breast tumours and associated with poor prognosis [204, 213, 214]. HER3 has six consensus binding sites for p85 SH2 adapter subunit of PI3K, therefore having the unique ability to channel the signalling to the PI3K/Akt signalling pathway, thereby favouring tumour growth and progression [215]. In contrast, other studies have reported a positive prognostic value of HER3 receptor status, where its expression is correlated with positive ER and PR status and inversely associated with histological grade and longer disease free survival [216, 217].

The importance of the HER2/HER3 heterodimer has emerged, where co-expression of HER2 and HER3 has been associated with decreased patient survival and associated with resistance to endocrine therapy and EGFR family tyrosine kinase inhibitors [104, 123, 218-220]. HER2 tumours usually also express HER3 [218] and downregulation of either HER3 or HER2 correlates with reduced phosphorylation of both HER2 and HER3 [122, 221]. In MCF-7 cells that overexpress of HER2, thereby are resistant to tamoxifen, downregulation of HER3 enhances tamoxifen-induced apoptosis [122]. The HER2/HER3 heterodimer is believed to be the most biologically active and pro-tumourigenic form of the EGFR family complexes [222, 223].

### 1.4.3 Targeting EGFR family proteins in cancer

As evidence for a role of the EGFR family in breast cancer pathogenesis has increased, in recent years, significant efforts have been made to target these receptors for cancer treatment. The current successful approaches include antibodies that bind the extracellular domain of HER2 and EGFR, and small molecule tyrosine kinase inhibitors (TKIs) that inhibit their intracellular kinase activity.

Monoclonal antibodies against HER2 and EGFR have shown great promise in cancer therapy. Trastuzumab is a monoclonal antibody that inhibits HER2 signalling by binding to its extracellular domain [224]. When used in combination with chemotherapy, trastuzumab is effective in approximately 1/3 of the patients with HER2 positive tumours [225]. Many patients with HER2 positive breast tumours fail to respond or develop resistance to trastuzumab, even when combined with chemotherapy [225]. Therefore an idea has emerged that tumours develop alternative mechanisms to activate the PI3K/Akt pathway, which most likely contributes to resistance, including loss of PTEN [226, 227], activation of insulin-like growth factor-1 receptor (IGF-IR) [228] and PI3KCA-activating mutations [226]. Resistance to HER2 targeted therapies

has also been implicated in HER3-PI3K axis dysregulation, where ligands of HER3 rescue cells from the anti-proliferative effects of trastuzumab *in vitro* [229].

TKIs, such as lapatinib, block the kinase domain of both HER2 and EGFR, but show only limited activity as single agents in the treatment of HER2 overexpressing breast cancers [224, 230]. The activity of EGFR and HER2 is clearly repressed in tumours treated with TKIs [231]. However, it was shown that the downstream target Akt was not clearly inactivated. Recent studies also show that TKI effectively prevents auto-phosphorylation of EGFR and HER2, but as the transphosphorylation of HER3 was only transiently suppressed, and thereby HER3 escaped the inhibition of the TKIs [220]. Targeting HER3 may therefore enhance the efficacy of RTK inhibitors. This creates a challenge in drug therapy since in this situation HER3 is the principle mediator of resistance, and since HER3 cannot be a target for TKIs since it does not have catalytic kinase activity. A monoclonal antibody, pertuzumab, which inhibits the dimerisation of HER2/HER3 is currently undergoing a phase II clinical trial as a possible way to overcome this [232]. It may also be possible to target HER3 downstream targets, PI3K/Akt, thereby abrogate HER3 signalling and associated resistance.

Endocrine resistance is often associated with enhanced expression of the EGFR family [166, 233, 234], as mentioned above in the chapter "Endocrine resistance". With the importance of the EGFR family and ER as therapeutic targets in breast cancer, there is increased interest in interactions between estrogen antagonists and EGFR family, where the appropriate combination of endocrine therapy and signal transduction inhibitor is a new area of intensive clinical investigation. In light of this, combined therapy with both tamoxifen/AIs and trastuzumab has been found to offer more clinical benefits than with tamoxifen/AI alone in ER and HER2 positive breast cancers [104, 235, 236].

## 1.5 PI3K/AKT SIGNALLING

### 1.5.1 Introduction to PI3K/Akt signalling

The serine/threonine protein kinase Akt, also known as protein kinase B (PKB), is a downstream target for PI3K [237]. The PI3K family is involved in signalling transduction events downstream of cytokines, integrins and growth factors, and is involved in cellular processes such as cell proliferation, growth, motility, survival and angiogenesis (Fig. 5) [238]. PI3Ks are dimeric enzymes composed of a separate regulatory (p85) and a catalytic (p110) subunit. Upon activation, PI3K phosphorylates phosphatidylinositol 4,5 diphosphate ($PIP_2$) to generate phosphatidylinositol 3,4,5 triphosphate ($PIP_3$), resulting in Akt and phosphoinositide-dependent kinase (PDK) translocation to the cell membrane. PDK and other kinases then phosphorylate Akt, leading to its activation and initiation of downstream signalling cascades. $PIP_3$ levels are tightly regulated by the lipid phosphatase PTEN.

Activation of Akt at the plasma membrane requires phosphorylation on at least two regulatory sites for its maximal activity. Initially it is phosphorylated at Thr308, however, additional phosphorylation at Ser473 is necessary for its full activation [239]. Once activated, Akt translocates to the cytoplasm and nucleus where it

phosphorylates a number of key substrates that promote cell survival, cell cycle progression and motility.

Given the importance of the PI3K/Akt pathway in regulating apoptosis, proliferation and differentiation it is not surprising that inappropriate activation of this pathway results in tumourigenesis.

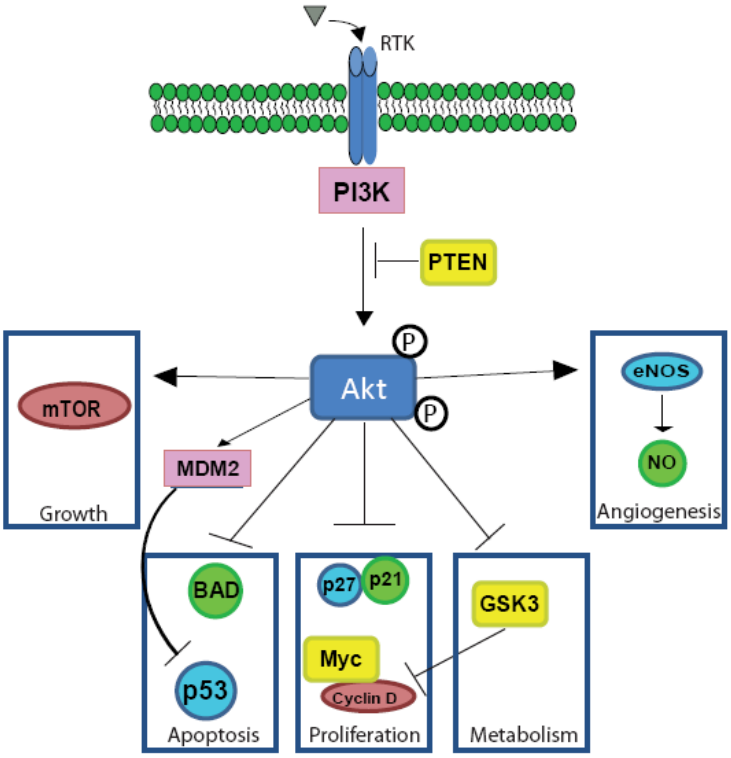


***Figure 5.*** *Overview of the PI3K/Akt signalling pathway (not all involved components are shown).* ***Growth:*** *One of the best conserved functions of Akt is its role in promoting cell growth, the primary mechanism being through the activation of mTOR.* ***Apoptosis;*** *Akt can promote growth factor mediated survival by several mechanisms. Akt can phosphorylate BAD, thereby inhibiting its pro-apoptotic function. Akt can also activate MDM2 and the subsequent downregulation of p53 results in inhibition of apoptosis.* ***Proliferation;*** *Akt is known to play a role in the cell cycle. Akt can phosphorylate p27, thereby preventing p27 localisation to the nucleus, and thus attenuates its cell-cycle inhibitory effects. Similar effects are also seen with p21. GSK3 targets cyclin D and c-myc for proteasomal degradation. Phosphorylation and inhibition of GSK by Akt enhances the stability of these proteins involved in G1-to-S-phase cell cycle transition.* ***Metabolism;*** *Akt stimulates glucose uptake in response to insulin. Akt phosphorylates GSK3, thereby preventing GSK3 from phosphorylating and inhibiting its substrate glycogen synthase, thus stimulating glycogen synthesis.* ***Angiogenesis;*** *Akt plays important roles in angiogenesis. Akt activates endothelial nictric oxide synthase (eNOS). The release of NO produced by eNOS can stimulate vasodilation, vascular remodelling and angiogenesis.*

### 1.5.2 PI3K/Akt signalling pathway in cancer

The PI3K/Akt pathway is often dysregulated in human cancer. Many genomic alterations have been found to activate this pathway, which contributes to tumour progression, metastases and resistance to treatment [238].

Akt deregulation in breast cancer is not due to changed protein levels but due to alterations in signals that control its activation status. Thus mice overexpressing Akt do not show increased tumour incidence, whereas expression of constitutively active versions of Akt leads to tumourigenesis [240]. Constitutively active Akt is found in many types of cancers and is an indication of worse prognosis in breast cancer patients [241, 242]. Akt has been shown to activate ERα by phosphorylation [15], thereby promoting tamoxifen resistance. Further, in MCF-7 cells constitutively active Akt leads to proliferation on low levels of estrogen and resistance to growth inhibitory effects of tamoxifen [124].

In addition to mutational activation of Akt itself, alterations in upstream signalling components, such as mutational activation of PI3K, loss of PTEN, and overexpression of growth factor receptors, have been observed leading to constitutively active Akt. Mutations of the PIK3CA gene, encoding the p110α catalytic subunit of PI3K, occur at significant frequency in several cancers, such as breast, ovarian and colon [243]. These activate Akt and induce oncogenic transformation *in vitro* and *in vivo* [244-246]. PI3K also regulates integrin dependent cell motility by adjusting integrin responses in both normal and cancerous tissues. In breast cancer cells, PI3K is activated upon integrin ligation, promoting formation of lamellae and invasion [162]. Moreover, the negative regulator of PI3K signalling and the tumour suppressor PTEN is commonly inactivated in numerous tumour types, resulting in constitutive upregulation of PI3K activity [247]. Due to these recent discoveries, interest in developing small molecule inhibitors of PI3K isoforms and their effectors have emerged [248]. These may provide a highly effective approach since this pathway is so frequently mutated in cancer.

Strong activation of PI3K/Akt signalling has also been seen in tumour cells expressing high levels of α6β4 integrin, which has been implicated in tumour progression and invasion, and associated with HER2 [162-164, 249]. Inactivation of integrin α6β4 leads to reduced PI3K activity [178] and depletion of the integrin β4 subunit causes reduction in HER3 and Akt phosphorylation [166]. Interestingly, no ERβ expression was found in those tumours expressing high HER3 and integrin β4 levels. Furthermore, overexpression of HER2 is also linked to elevated Akt activities [250-252].

## 1.6 PTEN

### 1.6.1 Introduction to PTEN

The tumour suppressor PTEN is a lipid phosphatase that inhibits cellular proliferation, growth and survival by inactivating PI3K dependent signalling by dephosphorylating its product, $PIP_3$, thereby negatively regulating Akt signalling. PTEN also possesses other biological functions independent of its lipid phosphatase activity, such as regulation of cell migration by dephosphorylating FAK [253]. As an important cellular

regulator, PTEN itself is subjected to careful regulation to ensure its proper function, since defects in PTEN regulation have profound impact on carcinogenesis.

PTEN has been reported to be modified in multiple ways, including phosphorylation [254, 255]. Most cellular PTEN appears to be maintained in a phosphorylated and inactive state, and its transient activation is mediated though dephosphorylation, probably through autodephosphorylation by its own protein phosphatase activity [256]. Phosphorylation of PTEN causes it to remain inactive in the cytosol with decreased catalytic activity, whereas dephosphorylation translocates PTEN to the plasma membrane. The unphosphorylated form is more unstable and subject to ubiquitin-mediated proteasomal degradation. However some sites of phosphorylation makes PTEN more prone to degradation [254].

PTEN can also be regulated transcriptionally, for instance PPARγ and p53 [257, 258] have been shown to upregulate PTEN transcription, whereas TGFβ [259], downregulates transcription. Furthermore, the microRNA miR-21, which is frequently upregulated in cancer [260, 261], has also been shown to repress PTEN [262].

### 1.6.2 PTEN function in breast cancer

Loss of expression of PTEN is a common event in breast cancer. Reduced PTEN protein levels, due to promoter methylation, loss of heterozygosity and posttranslational modifications are found in 31%-48% of breast cancers. On the other hand, PTEN mutations, which results in total loss of PTEN function, are relatively uncommon in breast cancer (<5%) [263-265]. Patients with PTEN loss show poor disease outcome [264-267], and low expression of PTEN correlates with unresponsiveness to trastuzumab [227], tamoxifen [268], and gefitinib [269].

PTEN mediates its main tumour suppressive effects by dephosphorylating $PIP_3$, thereby inhibiting Akt signalling. PTEN loss correlates with activation of Akt in tumour cell lines and its re-expression leads to downregulation of phosphorylated Akt [270-272]. Exogenous addition of PTEN suppresses growth of tumour cells [273-275], with knockdown of PTEN in ER positive breast cancer cell lines resulting in anti-estrogen resistance and hormone-independent growth [127].

In mice, a negligible decrease in PTEN expression is sufficient to promote cancer susceptibility, where 80% expression of normal levels of PTEN caused the development of a spectrum of tumours, with breast tumours occurring at the highest penetrance [276].

These obvious biological effects caused by a modest change of PTEN expression levels emphasises that PTEN needs to be precisely controlled. The fact that PTEN is the only defined regulator of PI3K/Akt signalling defines why it is such a dominant tumour suppressor.

# 2 AIMS OF THE STUDY

The overall aim of this thesis was to increase our understanding of the molecular and cellular mechanisms of estrogen signalling in progression of breast cancer focusing on the anti-tumourigenic effect of ERβ. Specifically, our objectives were:

- To explore ERβ function in tumour growth, focusing on angiogenesis and growth factor expression **(Paper I)**.

- To study how estrogens affect cell-cell adhesion by focusing on ERβ regulation of E-cadherin **(Paper II)**.

- To analyse estrogen effects on integrin expression and the modulation of adhesive and migratory properties of breast cancer cells expressing ERα and ERβ **(Paper III)**.

- To investigate if ERβ can modulate pathways implicated in endocrine resistance development, including PI3K/Akt and HER2/HER3 signaling, as well as PTEN expression. **(Paper IV)**.

# 3 METHODOLOGICAL CONSIDERATIONS

## 3.1 CELL LINES

Immortalised cell lines derived from various tissues and species are useful tools in research and are important *in vitro* models in cancer research. Cell lines have numerous advantages, they are easy to handle, can be propagated infinitely, and exhibit a relatively high degree of homogeneity. Furthermore, cell lines can be grown as xenografts, allowing investigation of effects both *in vitro* and *in vivo*. However, due to their continual culturing, cell lines are prone to genotypic and phenotypic changes, therefore subpopulations may arise, which was highlighted by Osborne and colleagues [277] for MCF-7 cells. Therefore such caveats need to be considered when using cell lines, and one must consider if the cell lines used are valid models for breast cancer.

Breast cancer is a heterogeneous disease, with diverse histopathological features, genetic and genomic variations, and clinical outcomes. Therefore no individual experimental model system fits all aspects of the disease. Most of the cell lines used in breast cancer research are derived from advanced-stage tumours, rather than primary lesions, and therefore represent the most malignant variants. Thus breast cancer research does not necessarily rely on the best representative cell lines. It would be more clinically relevant to use cells that are derived directly from primary tumours, especially since most drug therapies are directed against these. An alternative would be to use primary cultures derived directly from tumours. However there are disadvantages with primary cells; for instance, they grow slowly, have a finite lifespan, and often they only survive two or three passages. When large amounts of cells are needed this is a limitation.

Cells grown *in vitro* are routinely cultured on a two-dimensional (2D) substratum, plastic, and most of the studies done so far have characterized proliferation, differentiation, survival, and molecular and biochemical pathways on 2D substrate. This is a huge limitation, since this environment differs markedly from the breast microenvironment. Cells in 2D lack exposure of components of the ECM, they do not recapitulate three-dimensional (3D) tissue architecture and lack heterotypic cell-cell interactions [278, 279]. Furthermore, *in vivo* acquired resistance to cytotoxic therapies often involves complex cell-ECM and microenvironment interactions. These interactions are not functional in a standard 2D tissue culture, with studies using 3-dimensional cultures showing how these interactions significantly affect the tumour cell response to chemotherapy and radiation [280]. Altogether these aspects need to be considered.

Considerations of limitations and disadvantages have been taken into account when designing experiments and interpreting results in cell lines used in this thesis. The following cell lines were used:

- **T47-D**; human luminal epithelial ductal breast cancer cell line with high endogenous expression of ERα. These cells also express PR and are responsive to estrogen. T47D cells have a PIK3CA mutation (H1047R) in its catalytic domain [281]. T47-DERβ cells, expressing ERβ

(ERβ485), were obtained from Dr. Anders Ström. These cells are under the control of an inducible tet-off system.

- **MCF-7**; human luminal epithelial ductal breast cancer cell line with high endogenous expression of ERα. These cells also express PR and are responsive to estrogen. MCF-7 cells also have a PIK3CA mutation (E545K) in its helical domain [281]. MCF-7ERβ cells, expressing ERβ (ERβ485), were obtained from Dr. Anders Ström. These cells are under the control of an inducible tet-off system.

- **HC11**; undifferentiated mouse mammary epithelial cell line. These cells have stem cell properties and can be induced to differentiate, (through EGF removal and addition of lactogenic hormones), into different lineages according to hormonal conditions [282-285]. They can also grow on reconstituted basement membrane, such as Engelbreth-Holm-Swarm (EHS), and form polarised acini. The formation of acini occurs through a period of ten days of active proliferation, followed by arrest and apoptosis to form the lumen [286]. This type of growth recapitulates mammary morphogenesis *in vivo*.

## 3.2 XENOGRAFTS

Cell lines can be grown as xenografts thus allowing investigation of the *in vivo* environment. However, there are some caveats to consider when using xenograft models. As mentioned above, the cell lines used may not represent the most common types of cancer observed in the clinic. Furthermore, xenografts must also be established in immunocompromised mice, thus the absence of an intact immune system may affect tumour development and progression [287]. The microenvironment may differ between human and mouse, depending on where the cells are injected/transplanted thereby altering the growth of the cells. However, it is still major benefit to grow the cells inside a host and in a three dimensional environment.

## 3.3 REAL-TIME PCR ANALYSIS

Real-time PCR analysis is a very sensitive method for quantification of mRNA. Conventional PCR uses endpoint measurements and relies on either the size or sequence of the amplicon. Whereas with real-time PCR, as the name implies, each transcript is detected in "real time", making the quantitation easier and more precise. For real-time PCR, total RNA from tissues or cells was extracted and reversely transcribed into cDNA and analyzed using gene-specific primers. Compared to conventional PCR, smaller amounts of samples can be used to quantify mRNA.

The method is based on the detection of fluorescence produced by a reporter molecule, DNA binding SYBR green or gene-specific TaqMan probes. TaqMan probes are sequence specific probes consisting of oligonucleotides that are labeled with a fluorescent reporter and a quencher moiety, which emits fluorescence upon hybridization to the right template. However, these probes are very expensive.

SYBR green, a non-specific fluorescent dye, binds to the minor groove of the DNA double helix, and emits fluorescence upon DNA binding. SYBR Green is a simple and cheap option for real-time PCR, but the drawback is that both specific and nonspecific products generate signals, since SYBR green will bind to all dsDNA products. Therefore the specificity of the primers must be thoroughly tested by melting curve analysis to confirm one single PCR product.

SYBR Green based assays were used in the work described in this thesis, and melting curves have been made for each new primer pair. As reference gene 18S rRNA was used as an internal control for differences in input material.

# 4 RESULTS AND DISCUSSION

## *4.1 ESTROGEN RECEPTOR β INHIBITS ANGIOGENESIS AND GROWTH OF T47D BREAST CANCER XENOGRAFTS (PAPER I)*

In the mammary gland, estrogens are potent mitogens, regulating the growth, development and function of normal as well as cancerous cells. ERα mediates the proliferative effect of estrogen in breast cancer, whereas ERβ seems to be anti-proliferative. E2 treatment of ERα-positive T47-D and MCF-7 cells implanted into mice has been shown to increase proliferation and tumour growth [288, 289]. Since ERβ expression decreases or disappears during tumour development [290, 291], the question arised whether reintroduction of ERβ into ERα-positive breast cancer cells could influence the tumour inhibitory properties.

To investigate this hypothesis, T47-D breast cancer cells with inducible expression of ERβ (T47-DERβ) and normal T47-D cells, used as control, were implanted into the mammary fat pad of immunodeficient SCID/beige mice. Mice were also implanted with E2 containing pellets. At the endpoint of 30 days, tumour growth was reduced up to 80% in mice implanted with cells expressing ERβ, compared to the T47-D control cells. Further in three out of eight animals implanted with T47-DERβ cells, no tumour tissue could be found, indicating that regression was complete. Furthermore, the Ki67 proliferation index was decreased in the ERβ expressing tumours, with Ki67 inversely associated with ERβ expression at all time points measured. These results clearly showed that expression of ERβ in T47-D breast cancer xenografts reduced tumour volume, giving further support to the notion that ERβ is anti-proliferative.

For successful tumour growth, increased proliferation must be accompanied by supply of oxygen and nutrients provided by increased blood supply. This is achieved by increased angiogenesis and blood microvessel density [292-294]. Increased microvessel density is a strong predictor for aggressive disease. Microvessel density was examined in the tumours by visualising endothelial cells with an antibody against CD31. Interestingly, ERβ expression correlated with reduced microvessel density, suggesting that ERβ may influence angiogenesis in tumours. However, since oxygen tension is a major stimulator of angiogenesis, it was also possible that the lower microvessel density in the ERβ expressing tumours was simply due to reduced tumour volume and thereby less hypoxic milieu.

To address this question, levels of VEGF and PDGFβ growth factors, important for regulating angiogenesis, were investigated in the xenografts. Both PDGFβ protein and mRNA levels were reduced in the ERβ expressing tumours. No reduction of VEGF mRNA levels was seen, whereas VEGF protein showed a nonsignificant trend to be decreased in the ERβ expressing tumours. Based on these findings, we concluded that the tumour suppressive effects of ERβ *in vivo*, are most likely a consequence of the combined action of reduced angiogenesis and proliferation. Still, induction of these growth factors may be due to hypoxic conditions independent of ERβ.

To investigate if ERβ or hypoxia were involved in regulating the production of VEGF and PDGFβ, T47-DERβ cells were incubated in normoxic and

hypoxic conditions *in vitro*. In both conditions, VEGF and PDGFβ mRNA and secreted protein were reduced upon ERβ expression. To further investigate a possible transcriptional regulation, T47-D cells were transiently transfected with promoter constructs of VEGF and PDGFβ. It has earlier been reported that ERα increases expression of PDGFβ and VEGF, and as expected, estrogen treatment increased VEGF and PDGFβ promoter activity, whereas co-transfection with an ERβ expression vector together with estrogen decreased both VEGF and PDGFβ promoter activities. Accordingly, ERβ might be a transcriptional inhibitor of VEGF and PDGFβ, opposing the effect of ERα. However more detailed studies are needed to confirm this regulation.

There was only a minor upregulation of VEGF promoter activity in response to E2. However, earlier studies have reported that longer constructs show strong activation by estrogen [295]. Buteau-Lozano also showed that deletion of 1.2-2.3 kb upstream of the transcription start site in the VEGF promoter abrogates E2-dependent transcription. This may give an explanation to our lower response to E2, as our promoter construct did not contain this upstream sequence.

Reduction of growth factor expression and secretion could provide one explanation as to how ERβ inhibits tumour growth, since besides being pro-angiogenic, PDGF family members also stimulate proliferative and invasive properties of cancer cells [296, 297]. Furthermore, besides activating endothelial cells, which results in proliferation and migration, VEGF also influences breast cancer cell survival and growth through direct effects on cancer cell surface receptor VEGFR-2 in an autocrine fashion [298].

The main targets for angiogenic factors are the endothelial cells. Estrogens have been shown to be important for angiogenesis and can also affect endothelial cells directly. Endothelial cells express both ERα and ERβ. ERα has been shown to stimulate VEGFR-2 expression of endothelial cells, resulting in increased sensitivity for VEGF, whereas endothelial cells only expressing ERβ do not increase proliferation upon E2 treatment [144, 299, 300]. In MCF-7 xenografts, estrogen was shown to stimulate angiogenesis, whereas tamoxifen inhibited endothelium growth [140]. Tamoxifen has also been shown to reduce vascular density within a tumour [146].

The immune system has been shown to play important roles in the prevention and repression of cancer. As this study used immunodeficient mice, a complete picture of the possible anti-tumourigenic effects of ERβ cannot be obtained due to the lack of functional lymphocytes.

Altogether these results show the importance of ERβ in the tumourigenesis of breast cancer, further supporting its anti-proliferative role, and also highlight a possible role in regulating angiogenesis. This makes ERβ an interesting clinical target, used in combination with both anti-proliferative and anti-angiogenic therapies.

## 4.2 DIFFERENT ROLES OF ESTROGEN RECEPTORS α AND β IN THE REGULATION OF E-CADHERIN PROTEIN LEVELS IN A MOUSE MAMMARY EPITHELIAL CELL LINE (PAPER II)

Studies in ERβ knockout mice have shown reduced expression of several adhesion proteins in the lactating mammary gland, including E-cadherin [65]. Furthermore, E-cadherin mRNA in the mouse ovary has been shown to be upregulated by E2 administration [301]. Previously it has also been shown that ERβ opposes ERα induced proliferation, and that its expression increases apoptosis whereas its inactivation results in loss of growth contact inhibition [73]. This suggests an important role for ERβ in tumourigenesis, as it seems to have an important role in regulating cell-cell adhesion. However, the mechanisms behind this regulation remain to be elucidated. Therefore the focus of this study was to elucidate ERβs role in cell-cell adhesion, with a focus on E-cadherin.

To address the question above we used HC11 mammary epithelial cells with stable expression of short inhibitory hairpin RNAs (shRNA) towards ERα or ERβ (siERα and siERβ, respectively). Using the siRNA approach, we could see that E-cadherin protein levels were upregulated in response to E2 or DPN in ERβ expressing cells. No effect was seen with the ERα agonist PPT in these cells. Furthermore, E-cadherin protein levels were downregulated in response to PPT or E2 only when ERβ expression was reduced. The observed effects could be reversed by the anti-estrogen ICI. These effects were specific for E-cadherin, since β-catenin, α-catenin and p120 catenin did not show any differential regulation by E2 treatment. All together, this data suggests that ligand activation of ERβ upregulates E-cadherin levels and protects it from ERα-induced downregulation, whereas ligand activation of ERα in the absence of ERβ downregulates E-cadherin levels. This suggests that the different ratios of ERα/ERβ are important in the regulation of E-cadherin in ER positive cells.

E-cadherin has been shown to be regulated by estrogens [302-304]. In MCF-7 breast cancer cells, ERα has been correlated with increased E-cadherin expression by upregulation of the Snail/Slug inhibitor, MTA3 [302], however, no correlation was seen with MTA3 and E-cadherin expression in our HC11 cells. This indicated that in these cells, E2 regulation of E-cadherin did not involve the MTA3 pathway. Others have also shown in MCF-7 cells that ERα directly regulates E-cadherin by binding to the E-cadherin promoter with associated co-repressors, resulting in repression of E-cadherin expression [304]. Using real-time PCR, mRNA levels of E-cadherin were measured at different time points in the HC11 cells. Treatment of cells with DPN, E2 or PPT induced a slight increase in E-cadherin mRNA after 2 hours. To study if the slight mRNA induction was translated into increased protein levels, immunoblots were performed at 3 and 6 hours incubation with respective ligands, but no significant tendencies were observed at the protein level. At the same time, reporter assay was performed with candidate regulatory elements (AP1 and 2 putative EREs) from the E-cadherin gene. No ER-mediated activation of transcription was found. This suggests that in HC11 cells, ER regulation of E-cadherin protein levels cannot be explained by changes in gene expression or MTA3 levels, instead it seems that the opposing activities of the ERs occur posttranscriptionally. It is noteworthy to mention that MCF-7 cells do not express ERβ, which may explain the discrepancies between our studies and the studies with MCF-7 cells. Furthermore, since Fujita et al [302] used

10% FBS instead of E2 to stimulate cells, it is not clear whether the effect seen by them was through ligand-dependent or independent activation of ERα. On the other hand, in ERβ positive, ERα and AR negative DU145 prostate cancer cells, E-cadherin levels are increased upon ligand activation of ERβ [303], consistent with our results.

Regulation of E-cadherin expression can occur at several levels from gene methylation [305, 306], transcriptional repression (as mentioned above), to external shedding by MMPs [307-309]. To further investigate the possible mechanisms behind E2 induced downregulation of E-cadherin protein levels in siERβ cells, extracellular shedding was investigated. Immunoblotting was used to analyse E-cadherin fragments in the conditioned media. An 88 kDa E-cadherin protein fragment was seen at higher levels in E2 stimulated siERβ cells. This was further confirmed by immunofluorescence, where less membrane staining and more cytoplasmic staining was observed. These results suggest that one possible mechanism of how E-cadherin is downregulated by ERα in the absence of ERβ is through E2/ERα-induced shedding. Furthermore, the increase in E-cadherin fragments may also inhibit cell adhesion in a paracrine way [310, 311]. Unfortunately, we were not successful in identifying the MMPs associated with this increase in shedding. In addition, immunofluorescence stainings of cell-cell contacts in siERβ cells showed less attachment and increased cytoplasmic granular patterns in siERβ cells. These cells were also larger and flatter than the controls. However, ICI did not reverse the effect seen in the siERβ cells, indicating that the observations are not ERα mediated, but instead a result of reduced levels of ERβ. In summary, these results show that loss of ERβ induces differential E-cadherin cellular localization.

E-cadherin levels can be modulated at the cell surface by endocytosis, endosomal sorting and lysosomal degradation. To further investigate if the E-cadherin granular cytoplasmic staining was related to internalisation, biotinylated (extracellular) protein was investigated. Internalised biotinylated proteins were seen in siERβ cells, whereas in the wild type (wt) or control-siRNA HC11 cells the staining was still associated with the cell membrane. However this internalisation was not further enhanced by E2. Since the fate of the internalised protein can be towards recycling or degradation we decided to further study if E2 could influence the fate of the internalised E-cadherin.

Clathrin-dependent and clathrin independent mechanisms have been postulated for E-cadherin internalisation, therefore double immunofluorescence stainings with clathrin and E-cadherin antibodies were performed in E2-treated and untreated si-ERβ cells. The overall co-localisation was higher in E2 treated siERβ cells than in untreated cells. To further investigate the fate of the intracellular granules observed in siERβ cells, cells were stained with markers for early endosomes (Rab5), late endosomes (Rab7), recycling vesicles (Rab4), exocytic vesicles (Rab11), and lysosomal vesicles (LAMP-1). E-cadherin partially co-localised with early endosomes and lysosomal vesicles, which was further enhanced by E2. All together, E2 induces E-cadherin internalisation through clathrin-dependent pathway. This suggests that when ERβ levels are low, ERα induces shedding and influences E-cadherin fate towards degradation in the lysosome.

Another possible mechanism is the caveolin-1-mediated endocytosis of E-cadherin. Co-localisation studies showed that in siERβ cells, E-cadherin positive granules co-localised with caveolin. However this co-localisation was not enhanced by E2 treatment, suggesting another mechanism of internalisation of E-cadherin upon loss

of ERβ. To further confirm this, a disruptor of caveolae, nystatin, was used to see if E-cadherin could be restored in the siERβ cells. Nystatin partially restored membrane E-cadherin under basal conditions but not in E2 treated siERβ cells, suggesting that the downregulation of E-cadherin protein levels induced by E2/ERα happens independently of the formation of caveolae and is instead related to extracellular shedding and lysosomal degradation.

Ubiquitination of E-cadherin leads to destabilization of adherens junctions, internalisation and further degradation [312]. Ubiquitin mediated internalisation is generally associated with lysosomal degradation [313], however some reports show that the proteasome also might be involved in this regulation [314, 315]. An increase in E-cadherin membrane localisation was seen when treating the siERβ cells with two different proteasome inhibitors, however E-cadherin protein levels were not affected. Taken together, these observations indicate that inhibition of the proteasome favours membrane localisation, but as it does not prevent E2 induced decrease of E-cadherin levels, E2-induced shedding is not influenced. One possibility is that proteasome inhibition depletes the cells from free pools of ubiquitin [316], leading to a shorter fragment of E-cadherin in the membrane. The role of the proteasome in this system remains to be studied.

Loss of E-cadherin is also associated with activation of beta-catenin, transactivation of invasion-associated genes and acquisition of a migratory phenotype, all characteristics of EMT. To further support our data we found that loss of ERβ was associated with an E2-induced activation of β-catenin reporter activity, resulting in increased cellular proliferation [73]. An increase of migration in wt cells was also seen in cells treated with PPT, suggesting that ERα may increase the migratory potential of cells. However the expression of mesenchymal markers was not increased in response to loss of ERβ. Thus the phenotypic changes seen in siERβ cells did not correlate with these EMT characteristics.

Finally, E-cadherin is a significant player in the formation of adherens junctions and necessary for maintaining cell polarity. Growth of siERβ cells on reconstituted basement membrane was disorganized, as the cells did not arrest but instead continued growing, thus giving origin to larger structures. This suggests that ERs regulation of E-cadherin cellular localization and increased β-catenin transcriptional activity might be related to these effects.

In summary, these results show the importance of ERβ in regulating cell-cell adhesion and in maintaining the differentiated phenotype of the cell.

## *4.3 EXPRESSION OF ESTROGEN RECEPTOR β INCREASES INTEGRIN α1 AND INTEGRIN β1 LEVELS AND ENHANCES ADHESION OF BREAST CANCER CELLS (PAPER III)*

Cell migration plays an important role in many pathological and biological processes, including cancer, leading to invasion and metastasis, which in turn promotes the spread of tumours, thereby leading to mortality. Integrins, cell surface receptors for the ECM, play key roles in the regulation of normal and tumour cell migration and survival. Interestingly, integrins undergo dramatic alterations in their levels of expression and affinity when comparing preneoplastic tumours with invasive tumours. Earlier studies

have indicated that estrogens can affect integrin expression [317, 318]. Since previous results in mice and cell lines suggest a role for ERβ in regulating cell adhesion [65, 319] as well as being associated with less invasive tumours [320], and inhibiting the invasion of breast cancer cells [74], we aimed to investigate the function of ERβ in cell-ECM adhesion and migration in breast cancer cells.

To investigate this, we used ERα positive T47-D breast cancer cells with inducible expression of ERβ and screened for ERβ regulation of different integrin alpha subunits (α1, α 2, α 3, α 4, α 5, α V, and α Vβ3) and beta subunits (β1, β2, β3, β4, β6, α Vβ5, and α 5β1) using an ELISA-based method. An upregulation of integrin α1 and integrin β1 was found, which was confirmed by FACS. This upregulation was further enhanced by the treatment with the ERβ agonist DPN, and repressed by the antagonist ICI. Since integrin α1 only exists as a heterodimer with integrin β1, these results indicated that expression of ERβ resulted in a specific increase of the cell surface ECM receptor integrin α1β1.

Next, the regulatory mechanisms behind the increase in integrin α1 and β1 were investigated. Using quantitative real-time PCR, we showed that integrin α1 mRNA was upregulated upon ERβ expression and partially reversed upon co-incubation with the antagonist ICI, whereas integrin β1 mRNA was unaffected. To determine that this was not a cell specific effect, these results were confirmed in a second breast cancer cell line with inducible expression of ERβ, MCF-7ERβ, as well in SW480 colon cancer cells with constitutive expression of ERβ. These results altogether indicated that ERβ regulates integrin α1 at the mRNA level. Screening of the integrin α1 promoter region spanning 10kb revealed one ERE, one SP1 and six AP1 sites, which could be possible sites for ER regulation. Whether ERβ itself affects any of these regulatory elements or acts by inhibiting ERα repressive function on AP-1 sites remains to be studied. However, since ICI treatment, which downregulates ERα, decreased integrin α1 mRNA levels, this suggests that the upregulation is most likely to be achieved by ERβ.

To further corroborate ERβ induction of integrin α1β1, total protein levels were also analysed by immunoblot or immunofluorescence. Both integrin α1 and integrin β1 protein levels were increased upon ERβ expression, thereby confirming the FACS data. Since integrin β1 mRNA was not increased upon ERβ expression, we assume that integrin β1 becomes stabilised upon heterodimerisation with integrin α1 and accumulates, since integrin α1 only heterodimerises with integrin β1. It is also possible that ERβ affects other pathways, such as proteins that bind to the intracellular domain of integrin β1 and thereby activates and stabilises integrin β1. In both cases integrin β1 is less prone to degradation.

Since integrins are essential for cells to adhere we further investigated adhesion and formation of adhesion complexes. ERβ expression caused a four-fold increase in number of adhesions detected per cell, as well as enhanced the integrin-clustering density. Furthermore, ERβ also induced the formation of vinculin-containing focal complexes and actin filaments, all together suggesting a more adhesive potential of these cells. This was confirmed in an adhesion assay to different matrix proteins, namely collagen type I, collagen type IV, fibronectin, laminin, and vitronectin, where the expression of ERβ enhanced the adhesive potential to all different matrix proteins. Similar results were also observed in MCF-7ERβ and HT29ERβ cells. In addition to integrins α1 and β1, ERβ may also influence expression and activation of other integrins and proteins involved in the formation of adhesion complexes and thereby

influence adhesion to ECM. Adhesion to fibronectin and vitronectin increased upon ERβ expression, none of which is a ligand for integrin α1β1. This indicates that ERβ could have an effect on early signalling events involved in cell adhesion. Interestingly PRA usually predominates in breast cancer and in T47-D cells; an increase in PRA/PRB ratios (due to increased expression of PRA) to levels higher than 4:1, results in decreased adhesion and focal contact formation [321]. We observed upon ERβ expression a decrease in PRA/PRB ratios of 25% and therefore, some long-term effects of ERβ expression such as increased integrin β1 levels and increased adhesive potential, may be the result of changes in PR isoform ratio as well. However we did not incubate with progestins as was done in the study referred to above. Furthermore, the idea that the relative ratio of ERα/ERβ in cells dictates the response to estrogens, indicates that some effects we have observed may be influenced by this parameter, since we found that ERβ expression negatively influenced the ERα levels.

The rate of cell migration is determined by the level of adhesiveness of the cells to their substrates. In view of ERβ-induced changes in integrin expression, formation of adhesion complexes and effects on cell-ECM adhesion, we examined influence of ERβ on migration. The migratory capacity of the cells was clearly reduced upon ERβ expression as seen with a wound assay and a chemotactic assay, which is in agreement with previous observations.

In conclusion, these results indicate that ERβ affects integrin expression and clustering and consequently modulates adhesion and migration of breast cancer cells. Thus, ERβ may be a potential target in future therapies, where its expression and/or activation could stop or prevent the invasion and migration of breast cancer cells.

## *4.4 ESTROGEN RECEPTOR β REPRESSES AKT SIGNALING IN BREAST CANCER CELLS VIA DOWNREGULATION OF HER2/HER3 AND UPREGULATION OF PTEN – IMPLICATIONS FOR TAMOXIFEN SENSITIVITY (PAPER IV)*

Dysregulation of the cell cycle and the apoptotic pathway are fundamental events in cancer development. The effect of ERβ expression on cell proliferation has been widely studied and shown to be related to regulation of cell cycle proteins and apoptosis. However, ERβ effects on survival pathways like PI3K/Akt have not been extensively investigated. Therefore we were interested to investigate if ERβ expression had any effects on RTKs/Akt signalling, and as this pathway also is involved in regulating sensitivity to tamoxifen, if ERβ therefore could represent a putative player in regulating tamoxifen sensitivity.

The PI3K/Akt pathway plays important roles in regulating cell proliferation, growth, apoptosis and motility, and deregulation of this pathway is frequently seen in breast cancer, resulting in tumour progression, metastases and resistance to endocrine therapy [238, 241, 322, 323]. To investigate ERβ effect on Akt signalling, ERα positive T47-D and MCF-7 cells with inducible expression of ERβ were used. Upon 4 days of ERβ expression, levels of phospho-Akt (Ser473) were clearly downregulated in both cell lines. Total Akt levels remained unchanged, indicating that reduced phospho-Akt levels were due to less phosphorylation. No

changes in the mock cell line were seen, indicating that the changes in phospho-Akt were not due to changes in doxycycline concentration, but to ERβ expression. Also a downstream target of phospho-Akt, phospho-GSK3β (Ser9), was downregulated. Treatment with ER antagonists, ICI and tamoxifen, did not prevent ERβ-induced decrease of Akt phosphorylation. In summary, expression of ERβ in two different ERα positive breast cancer cell lines, clearly reduced activation of the Akt signalling pathway.

Receptors belonging to EGFR family are known for their role in breast tumour initiation and progression and for the development of endocrine resistance. Members of the EGFR family are also known activators of the PI3K/Akt pathway, therefore effect of ERβ on expression of EGFR, HER2 and HER3 was investigated. Levels of EGFR remained unchanged upon ERβ expression, whereas HER2 protein levels became upregulated and HER3 protein levels downregulated in both T47DERβ and MCF-7ERβ cells. This also correlated with changes at the mRNA level where ERβ expression upregulated HER2 mRNA and downregulated HER3 mRNA. ICI treatment increased both mRNA and protein levels of HER3, but did not inhibit ERβ-induced downregulation of HER3. However, with tamoxifen, there was a distinct difference between protein and mRNA levels, where tamoxifen abolished ERβ downregulatory effect at the mRNA level, which was not seen at the protein level. Interestingly, both antagonists ICI and tamoxifen increased HER2 protein and mRNA levels and abolished ERβ upregulation of HER2 protein levels, whereas mRNA was decreased. This was not clearly related to HER2 protein levels at 4 days, but at 7 days of ERβ expression. Since ERα has been shown to act as a transcriptional repressor of HER2 mRNA [212], we speculate that this change is due to the relative ERα/ERβ ratios in the cells, where upon heterodimerisation of ERα/ERβ, ERα homodimer-mediated repression of HER2 is relieved. Furthermore, in our cell systems, we have seen that ERβ reduces ERα levels, which provides another explanation of less ERα homodimer-mediated repression of HER2. In the light of these results, we also postulate that ERβ homodimers mediate HER2 repression, since removal of ERα makes ERβ act like a repressor not an activator.

HER3 activation results in strong activation of the PI3K/Akt signalling pathway, therefore we investigated if ERβ expression could decrease active HER2/HER3 and Akt. Addition of the HER3 ligand HRG-β1 clearly induced active HER2/HER3 and Akt, an activation that ERβ expression was able to reduce. This suggests that ERβ downregulates the PI3K/Akt pathway through decreased expression of active HER2/HER3. Since HER2 and HER3 have been linked to endocrine resistance, these findings suggest that ERβ agonists together with ERα and RTK inhibitors could be an approach to overcome endocrine resistance.

PTEN is a known downregulator of the PI3K/Akt pathway and its expression is often lost in tumours and associated with endocrine resistance. We thus found it important to investigate if ERβ had any effect on PTEN expression. PTEN mRNA and protein levels were clearly increased upon ERβ expression, further suggesting another possible mechanism of how ERβ could regulate Akt signalling.

Both downregulation of PTEN, increased HER2/HER3 and Akt signalling, have all been associated with endocrine resistance in breast tumours. Furthermore, ERβ has been implicated as an indicator of endocrine response, where low expression is found in tamoxifen resistant tumours, and high levels are associated with a better clinical outcome [111, 115, 324, 325]. With our above described results in

mind we investigated if ERβ expression would sensitise MCF-7 and T47-D cells to tamoxifen. ERβ increased the sensitivity of both MCF-7 and T47-D cells to tamoxifen, thereby providing a mechanistic insight into how ERβ may contribute to endocrine sensitivity. Current endocrine therapies in breast tumours aim to impair ERα activity. Our results show that introduction of ERβ detection may allow a more precise selection of patients that will benefit from endocrine therapy. Furthermore, ERβ also seems to be a useful target in breast cancer by enhancing endocrine sensitivity.

# 5 CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Approximately fifteen years after its identification, dramatic progress has been made in defining the function of ERβ. The availability of knockout mice and the development of ERβ selective agonists have revealed its importance in physiological processes, diseases and signalling pathways. However, we still have a long way to go before we fully understand its multiple roles.

In this thesis the aim was to gain more insight into the molecular and cellular mechanisms of estrogen signalling in breast cancer, focusing on the anti-tumourigenic effects of ERβ in the breast. Several studies have reported that during tumour development in the breast epithelium, the expression of ERα increases, whereas that of ERβ decreases. The clinical significance of ERβ in breast cancer is still under debate, but the studies in this thesis have provided some insights into the functional role of ERβ in breast cancer and its possible role in defining clinical outcome. Although the mechanisms have not yet been fully characterised, ERβ seems to have several important inhibitory functions in breast cancer, such as affecting growth, adhesion, angiogenesis and sensitivity to endocrine therapy.

In the 1970s, Folkman started the era of tumour angiogenesis, which today is one of the most intensively studied areas in cancer research, with several anti-angiogenic drugs developed. In **paper I** we found that ERβ not only inhibited growth of tumour xenografts, but might also have a role in anti-angiogenesis by reducing expression of pro-angiogenic factors, which could have important medical implications. These results are intriguing; however, since the mice used are immunodeficient the complete picture of ERβ in angiogenesis is unclear. It would be interesting to see what effects the different ER selective ligands and antagonists would have on tumour growth and angiogenesis *in vivo*, as well as to look at anti-angiogenic factors and other possible targets such as those reported in **papers II-IV**. Furthermore, whether ERβ directly or indirectly influences angiogenesis through crosstalks with other signalling pathways needs to be investigated. The PI3K/Akt pathway is also activated by VEGF in endothelial cells, and since Akt can activate eNOS leading to angiogenesis, downregulation of Akt signalling by ERβ expression, seen in **paper IV**, could also be one explanation for these results. It is also possible that ERβ directly influences endothelial cells since these cells express both ERs. ERβ ligands used in combination with both anti-proliferative and anti-angiogenic therapies may increase the efficacy of these targeted therapies, however, ERβ physiological relevance and precise role in the vasculature is still incompletely understood and remains to be determined.

Inactivation of E-cadherin is important in the progression of sporadic breast cancer, where its loss is a hallmark of the transition from a normal epithelium to poorly differentiated carcinoma. In **paper II**, we found that ERβ was important for maintaining cell-cell adhesion and for the differentiated phenotype - through E-cadherin. However, the process seems complex; some effects were ligand independent, as well as depended on the relative ratio of ERβ/ERα. There also seemed to be a cell type specific regulation, since different reports have shown different regulatory mechanisms of E-cadherin. It would also be interesting to test these cells with knockdown of ERα or ERβ *in vivo.* Furthermore, the proteolytic enzymes responsible

for fragmentation of E-cadherin need to be identified. Interestingly, integrins are involved in regulating MMPs, and in **paper III** we found changes of integrin expression upon ERβ expression, thereby providing a possible pathway for the fragmentation of E-cadherin.

Few studies have addressed estrogen effects on integrin expression in breast cancer cells. In **paper III** we found that ERβ affects integrin expression, and increases the adhesiveness and decreases the migratory potential of breast cancer cells. Since changes in adhesion between cancer cells and ECM cause progression of metastasis, these results further strengthen ERβ anti-tumourigenic effects. However, ERβ can affect other pathways and proteins involved in cell adhesion and migration. As shown in **paper II**, loss of E-cadherin also correlated with increased cell migration. Therefore a full screen of all integrins, as well as adhesion related proteins needs to be completed to obtain the full picture of ERβ function in cell-ECM adhesion.

Resistance to endocrine therapy remains a major problem. The effects of therapies and the resistance that arises clearly show and reflect the complex biology of the cell. The knowledge of the molecular mechanisms behind resistance to each endocrine agent is crucial, as well as the knowledge of mechanisms that would increase the sensitivity to endocrine therapy. It is interesting to note that in **paper IV** we found that the presence of ERβ increased the response to tamoxifen in two ERα positive cell lines, which is in concordance with clinical studies where ERβ seems to be a marker for endocrine sensitivity. One interesting correlation is that high PRA levels have been associated to tamoxifen resistance [326], and in **paper III** we found lower levels of PRA upon ERβ expression in T47-D cells, suggesting an alternative pathway for ERβ positive effects. We also described a possible mechanism behind this effect, where ERβ by decreasing PI3K/Akt and HER2/HER3 signalling and increasing PTEN levels sensitise breast cancer cells to tamoxifen. However, the picture is complex. So far we have not proven that these signalling pathways are directly linked to ERβ positive effects in response to tamoxifen in breast cancer cells. Therefore more studies are needed. Possible approaches would be using siRNA against PTEN, to see if the response of ERβ is affected, thereby targeting the responsible pathway. Are ERβ effects seen at the mRNA level direct transcriptional events? Are ratios of ERβ and ERα determinants for HER2 transcriptional regulation? These questions may be investigated using ChIP technology. Furthermore, how would ERβ plus tamoxifen affect cell viability upon HER2/HER3 activation (i.e. heregulin treatment)? Integrin linked kinase (ILK) is known to phosphorylate Akt at ser 473, providing another possible regulatory mechanism which could be investigated. Furthermore, HER3 binding proteins which negatively regulate HER3 may also be part of ERβ regulation of HER3. These proteins include NRDP1, LRIG-1 and Ebp1. Since expression of ERβ increased the potency of tamoxifen, it would also be interesting to investigate if ERβ expression would increase the potencies of tyrosine kinase inhibitors and monoclonal antibodies against the EGFR family. Interestingly, in **paper III**, we report that ERβ reduced the migratory potential of T47-D breast cancer cells. PTEN has also been shown to reduce cell migration [253], thereby suggesting another possible mechanism how ERβ affects migration by increasing PTEN levels. Furthermore, E-cadherin has been shown to regulate PTEN protein levels, thereby having a role in cell adhesion [327], and in **paper II**, we found that ERβ upregulates E-cadherin protein levels, further suggesting a possible mechanism how ERβ indirectly increases PTEN levels.

The emergence of endocrine resistance has fuelled the search for alternative therapies and for targets that can interfere with signalling pathways involved in endocrine resistance. These may lead to new strategies for combating ER positive breast cancers. In this respect, ERβ seems to be a good alternative candidate. To our knowledge, no studies have reported these molecular effects of ERβ, making it not only an intriguing target, but also a possible marker of choice for endocrine therapy. Therefore, measurement of not only ERα and PR, but also HER2, HER3, SRC-3, EGFR and ERβ in breast tumours could provide important information for predicting therapeutic responses. Further, since breast cancer is a heterogeneous disease, multiple markers would increase the potential for choosing the most optimal treatment.

There were some major difficulties during the progress of my thesis. The first one was the lack of cell lines, specifically breast cancer cell lines, expressing endogenous ERβ. However, the use of different cell lines overexpressing ERβ allowed us to discover pathways regulated by ERβ in breast cancer. Ovexpression of a protein is not the most optimal approach to study an effect, not only due to its artificial nature, but also since possible unspecific effects, such as squelching, can emerge. However, in order to avoid unspecific effects, titration of the amounts of ERβ was done. Further, it is important to note that the cell line used in **paper II** (HC11) does express endogenous ERα and ERβ and results obtained with this cell line also point out an anti-proliferative role of ERβ [73] as well as its importance in maintaining cell adhesion. Secondly, the lack of specific ERβ antibodies limited the potential to study ERβ, since measurement of mRNA does not always correlate with protein levels. Finally, the lack of ligands with high selectivity for ERβ was also an obstacle. The ligands for ERβ commercially available today do not have the highest selectivity, and when the selectivity is high, it usually favours ERα. Therefore improvement of design of ERβ specific ligands, as well as their general availability, is an important step to further allow us to investigate ERβ function.

A better understanding of the role of ERβ in development and progression of breast cancer is emerging. Major questions regarding ERβ still remain unanswered. For instance, will therapy against ERβ apply to the clinic since levels of ERβ varies in patients? Another approach would be to reactivate ERβ in breast cancer. Since ERβ is thought to be silenced through DNA methylation, demethylating agents could be one approach to achieve its re-expression. In clinical trials, demethylating agents (azacitidine and decitabine) have been used on solid tumours, and given the success of these agents in treating myelodysplastic syndromes, further studies of their *in vivo* action is highly warranted. These agents need actively dividing cells in order to be incorporated, thus due to the short half-lifes of these drugs, slow-growing tumours may require a longer treatment, which would likely increase the toxic effects. Furthermore, these drugs are not very specific, thus may therefore result in expression of oncogenes. Another intriguing question concerns the role of unliganded ERβ in cellular function. In many of our studies ligand-independent effects were seen, however the mechanism behind this has not been investigated. Is it possible that ERβ is phosphorylated in our systems, thereby generating unliganded effects? I anticipate that targeting ERβ in breast cancer increases the therapeutic effects; therefore it would be exciting to try to understand the mechanism behind ERβ positive effects, however, what are the negative aspects in targeting ERβ? The cellular context also plays a role for ERβ function, with distinct functions in different cell types. The ratio of both ERs is

also important, therefore ligands would need to be not only tissue specific, but if possible also cell type specific.

Many of the proteins and processes thought to be modulated by ERβ are subject to regulation by other pathways. Therefore it is of importance to consider that the response to ERβ may depend on the activity of these other signals, a situation that is important during tumour progression and therapy. The work presented in this thesis highlights the possibility of using ERβ as a prognostic marker with potential as a target in treatment of breast cancer. Although these results are encouraging, more work remains to be done.

# 6 ACKNOWLEDGEMENTS

This thesis work was performed at the Department of Biosciences and Nutrition, Karolinska Institutet, Stockholm, Sweden. I would like to express my deepest gratitude to all the colleagues and friends who helped out and made this thesis possible. I would especially like to thank:

My supervisor **Lars-Arne Haldosén**, for taking me on as a PhD student. Thanks for being such a great supervisor with your endless enthusiasm and optimism, and for your guidance and support. For always standing up for me and for always having time for discussions and for challenging me in a scientific aspect.

Professor **Jan-Åke Gustafsson**, my co-supervisor, for you inspiring energy, enthusiasm, and passion for science. For believing in me and supporting me for all these years. Thank you also for providing excellent research facilities.

**Luisa Helguero**, for being an excellent co-supervisor and friend. I will never forget your help and patience during this painful process of creating a new scientist!

**Anders Ström**, my fomer supervisor and now co-supervisor, for taking me as a summer student a long time ago, teaching me about science, and for your never ending stream of ideas.

**Michel Tujague**, my ex co-supervisor, for listening to my confusing ideas and for your helpfulness. Miss you!

To my fellow graduate students and colleagues at Novum, these years would not have been the same without you! Many of you have become really good friends!
**Agneta** for your support and encouragement. **Amena** for all the fun in the lab and office. I really appreciate our daily chats, dinners and fun. I appreciate you warmth and caring and for always thinking of me and supporting me! Big thanks for all lovely white chocolates you have giving me during these years! **Anna E** for your intelligence and for always being there. Wouldn't have survived these last couple of months without your support and our endless discussions ☺. Big thanks for dinners and fun, and for showing me how to climb! I wish you all luck! **Bea**, for your care and nice company at work! **Christina** for the Segeltorp spirit, and fun chats in the lab and during the lunches. **Chunyan** for nice talks and for providing with help when needed. **Eckardt** for your great scientific knowledge, and humour. **Fredrik**, for nice company during lunches and fikas, and fun party memories! **Hui** for nice lunch company. **Lotta** for your generosity and care. For being a lovely friend and colleague and for feeding me with sweets. I still think we should build that cable railway behind our apartments. **Karin E**, for bringing sunshine into the hard days of life at Novum. Miss you in the lab and in Sweden! **Kimberly** for all laughs. **Kirsten**, for making the life in the lab more fun, and for everyday chats in the lab about life and western blots. Thanks for your tremendous support and help. You inspire me! **Knut** for all laughs during the years. **Malin N**, for all our talks about work, family and life in general. For all the lovely

after-noon teas and laughs ☺. Thank you for all your help and support, I appreciate us becoming such good friends. **Malin H** for nice lunch company**. Marco** for nice lunch chats. **Marion**, thanks for fun chats in the office, and for all your help and support at these stressful times. **Milica** for all nice talks and fikas during the years. **Patricia** for being such a sweet person, and for all the nice conversations. **Patrick**, for all your jokes and fun during lunches, fikas and travels. **Per** for nice company and talks. **Peter S** for all fun times and for teaching me how the womens brain function. ATM is now in my dictionary! **Rezin** for your generosity and care. For fun times during fikas at work and outside work, and for making me eat in the "dark"! I will never forget that! **Myriam** for your support, care and waffles at your place. **Nina G**, for fun times, loads of cinnamon buns and fikas. **Pauliina** for always being so happy and enthusiastic! Thanks for all the fun times exercising at the gym together, although you almost beat me up at the boxing class! **Tassos** for your happiness and inspiring energy. For fun times at and outside work. Big thanks for your amazing good cakes. **Tomas**, for all discussions and fun in and outside the lab. For your support and care. I wish you all luck! (We didn't end up last) **Treska,** you are new to the group, wish you all the best. **Wen** for all smiles. **Yoko** for all care and help. Not mentioned, not forgotten, all the rest of the people at Novum, for creating a nice atmosphere and making the department a pleasant place to work at.

To all past colleagues and friends at Novum:
**Christophoros** for your endless support. Big thanks for showing me Greece, and for fun times in LA, Djurö and Greece. **Elin** and **Maria** for your care and support, for fun chats during lunches and fikas, and for cheering me up when I needed it! **Jingwen**, for support, laughs, friendship and crazy times. You fill me with so much happy and positive energy! **Johan** for being such a good friend and giving me support and care in all times. I really enjoyed sharing office with you! You gave me many laughs during these years. Wish you all the best with your career and family! **Karolin** for being fun and enthusiastic about everything. **Lovisa** for your care and for always smiling. **Marcella** for all laughs and fun. **Nicolas**, although we started off in a bad way when you took all my reagents and material in the lab ☺, we ended up with a long-lasting friendship. Thanks for all fun times at work and outside work and for all travels in Sweden, Greece and France. **Nina H** for being such an enthusiastic and nice person. I really enjoyed sharing the office with you. **Zoi**, for your Greek spirit and all fun in Paris and Spetses. Maria N, Cecilia, Jason, Gudrun, Ivan, Tova, Sanyal, Björn, Anne B, Anette, Didier, Kenichi, Jinghong, Sebastian, Paula, Eva, Jane, Ivan, Elin H, Pia, Malahat, Joëlle, David, Otabek and all of you other past colleagues that made Novum a great place to work at.

All administrative staff **Lena**, **Marie** and **Monica**, for always being friendly and helpful. **Inger**, **Lars** and **Gunnel** for help with ordering and fun lunch chats.

To my friends outside science:
**Andreas**, **Ulrika**, **Sasa**, **Magnus**, and **Annica** Thanks for all the good times with dinners, rock boats, movies and parties!!! My life is colourful and bright thanks to all of you! **Maja**, my lovely and sweet Maja. For being one of my best friends and for your never ending support and encouragement. Wish you all the best with your future career and family. **Zlatko** for your crazy ideas and stories. You always put a smile on my face! **Cecile** for all nice dinners and fun together. For guiding me and Claes in the south

and north of France. **Rebecca** and **Marku**s for all nice and fun dinners together during the years. And for cheering me up when I really needed it. **Claudia** for your warmth and care, and sharing the same interest in sweets as me. **Piotr**, for your generosity and care, and for introducing me to the loveliest dogs in the world.

**Gunilla, Daniel** and **Leif** at the finance course. Although late evenings and hard work, we had a lot of fun.

**Gunilla, Björn, Camilla, Jörgen, Catarina, David, Alexander, Mikaela and Josefine**, for all fun times together and for all help and endless care during these years!

To my **parents**, for believing in me, and for all your love and encouragement during these years. To my lovely sister and best friend **Helena**. I miss you so much here in Sweden! Thanks for all your support and for always putting a smile on my face! My brother **Henrik**, for always making me smile and for those crazy calls now and then when you need to be updated in science. Without the support from all of you this would have been impossible.

Finally, the most important person in my life, **Claes**! For your endless love and support, during all these years. I wouldn't survived this time without you ♥

This work was financially supported by the Swedish Cancer Fund

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