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Translational Aspects of Erythropoietin Receptor and Hypoxia-Inducible Factors in Breast Cancer

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2012

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1

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Faculty of Medicine

Academic dissertation

By due permission of the Faculty of Medicine, Lund University, Sweden to be defended at the main lecture hall, Dept of Pathology , Skåne University Hospital, Malmö, on Friday 3rd of February, 2012, at 09.15 for the degree of Doctor of Philosophy, Faculty of Medicine.

Faculty opponent:
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Organization LUND UNIVERSITY	Document name DOCTORAL DISSERTATION
Department of Laboratory Medicine, Malmö Division of Molecular Medicine	Date of issue 2012-02-03
	Sponsoring organization
Author(s) Anna-Maria Larsson	

Title and subtitle

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Abstract

The main function of erythropoietin (EPO) is in hematopoiesis where EPO stimulates increased proliferation, survival and differentiation of erythrocytic precursors in response to hypoxia. The EPO effect is mediated via binding to the EPO receptor (EPOR), which induces activation of different intracellular signaling pathways. Recombinant human EPO (rhEPO) is also used in treatment of cancer patients with anemia but some studies have reported negative effects on patient survival. Here we demonstrate a correlation between increased Hb levels and tumor response in patients with metastatic breast cancer and anemia, treated with rhEPO. The improved tumor response seen in patients with increased Hb levels might be due to improved oxygenation in tumors counteracting negative effects of hypoxia.

EPOR has also been found in non-hematopoietic tissues and in tumors of various origins. We have evaluated EPOR expression in breast tumors from a clinical trial evaluating tamoxifen treatment versus no adjuvant treatment. We found that high EPOR expression correlates to impaired tamoxifen response in patients with estrogen receptor (ER) positive tumors. EPOR expression also correlated to survival in these patients. When further investigating EPOR function we found that EPOR knockdown impaired proliferation in ER positive, but not ER negative breast cancer cells, supposedly via modulating effects of ER activity. EPOR knockdown also improved tamoxifen response in ER positive breast cancer cells. These effects were not dependent on EPO. Our results suggest an EPO-independent but ER-dependent function of EPOR in breast cancer cells.

Hypoxia is a common feature of solid tumors and is believed to be a consequence of tumors outgrowing their vasculature. Tumor hypoxia is associated with a more aggressive phenotype and treatment resistance. The main regulators of the hypoxic response are the hypoxia-inducible factors (HIFs) 1 & 2. We have investigated HIF-1α and HIF-2α expression in breast cancer and found a correlation between HIF-2α expression and distant metastasis and impaired prognosis, suggesting that HIF-2α has an important role in tumor progression. We also show differential time and oxygen dependent regulation of the two different HIF-α subunits and differences in their contribution to inducing VEGF expression.

Key words:	Breast Cancer, Anemia, Hypoxia, Erythropoietin (EPO), EPO receptor (EPOR), Estrogen Receptor (ER), Hypoxia-Inducible Factors (HIFs), Tamoxifen Response, Proliferation

Classification system and/or index termes (if any):

Supplementary bibliographical information:	Language English
ISSN and key title: 1652-8220	ISBN 978-91-86871-69-7

Recipient's notes	Number of pages 74	Price
	Security classification	

DOKUMENTDATABLAD enl SIS 61 41 21

In memory of

Kristina, Inga and Elisabet...

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I   Erythropoietin Enhances Response to Treatment in Patients with Advanced
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       Acta Oncol 2004;43(6):594-597

II   Erythropoietin Receptor Expression and Correlation to Tamoxifen Response
       and Prognosis in Breast Cancer
       Anna-Maria Larsson*, Karin Jirström, Erik Fredlund, Sofie Nilsson,*
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       Clin Cancer Res 2009;15(17);5552-9

III   EPO-independent Functional EPO Receptor in Breast Cancer Enhances
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IV   Hypoxia-Inducible Factor-2α Correlates to Distant Recurrence and Poor
       Outcome in Invasive Breast Cancer
       *Karolina Helczynska, **Anna-Maria Larsson**, Linda Holmquist Mengelbier,*
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       Sven Påhlman and Karin Jirström
       Cancer Res 2008; 68(22);9212-20

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List of abbreviations

AF	activating function domain
ARNT	aryl hydrocarbon receptor nuclear translocator
ASCO	American Society of Clinical Oncology
ASH	American Society of Hematology
bHLH	basic helix-loop-helix
CBP	CREB binding protein
CISH	chromogenic in situ hybridization
CRA	cancer-related anemia
CREB	cAMP response element-binding
DBD	DNA binding domain
EBCTCG	Early Breast Cancer Trialists Collaborative Group
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
ELISA	enzyme-linked immunosorbent assay
EMT	epithelial-mesenchymal transition
EPO	erythropoietin
EPOR	erythropoietin receptor
ER	estrogen receptor
ERE	estrogen response element
ERK	extracellular signal-regulated kinase
ESA	erythropoiesis-stimulating agents
FCM	flow cytometry
FIH	factor inhibiting HIF
FISH	fluorescence in situ hybridization
Hb	hemoglobin
HER2	human epidermal growth factor receptor 2
HIF	hypoxia-inducible factor
HNF	hepatocyte nuclear factor
HRE	hypoxia-response element
IHC	immunohistochemistry
IGFR	insulin-like growth factor receptor
JAK	Janus kinase
LHRH	luteinizing-hormone-releasing hormone
MAPK	mitogen-activated protein kinase
mTOR	mammalian target of rapamycin
NHG	Nottingham histological grade
OS	overall survival
PFS	progression-free survival
PHD	prolyl hydroxylase domain
PI3K	phosphatidylinositol 3-kinase
PKC	protein kinase C
PR	progesterone receptor

QOL	quality of life
QPCR	quantitative polymerase chain reaction
RBC	red blood cell
RFS	recurrence-free survival
rhEPO	recombinant human erythropoietin
RTK	receptor tyrosine kinase
siRNA	small interfering RNA
STAT	signal transducer and activator of transcription
SweBCG	Swedish Breast Cancer Group
TAM	tamoxifen
TMA	tissue microarray
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
VHL	von Hippel Lindau
WB	western blot

Background

Breast Cancer

Epidemiology and Etiology

Breast cancer is the most common cancer among women in the western world and represents almost 30% of all cancer cases in women in Sweden. The incidence is slowly increasing leading to approximately 7400 Swedish women being diagnosed with breast cancer every year (Socialstyrelsen, 2010) and it has been estimated that the life time risk for a woman being diagnosed with breast cancer is one in nine. However, improvements in diagnostics and treatments of breast cancer have led to improvements in survival rates and today the 5-year survival is approximately 87% and the 10-year survival approximately 78% (NORDCAN, 2011; Socialstyrelsen, 2010). The mortality rate has decreased in the last two decades (Coleman et al., 2011), despite the increasing incidence.

The etiology of primary breast cancer is multifactorial and there are several known risk factors where the most established ones are age, geographical location, exposure to ionizing radiation, early menarche, late menopause, late age at first pregnancy, nulliparity, intake of exogenous hormones (oral contraceptives and hormone replacement therapy), familial history and previous breast cancer or atypical hyperplasia (McPherson et al., 2000). Of these known risk factors age, familial history and previous breast cancer are most important for increased risk, whereas the others can be seen as more modest risk factors (Singletary, 2003). In recent years there has been an increased focus on evaluating effects of diet, weight, alcohol intake, smoking and physical activity on breast cancer risk and it seems that physical activity has a positive impact on reducing breast cancer risk whereas excessive alcohol intake and weight gain in postmenopausal women contribute to increasing the risk (McPherson et al., 2000; Tobias and Hochhauser, 2010).

Among families of women with increased risk of developing breast cancer, research is focusing on genetic factors. In the 1990's the discovery of *BRCA1* and *BRCA2* was a breakthrough but these inherited genetic mutations are only detected in 15-20% of families with increased breast cancer risk and approximately 5 % of breast cancer overall (Nathanson et al., 2001). The search for other high-penetrance mutations as well as combinations of low-penetrance mutations continues but so far no gene as dominant as *BRCA1* and *BRCA2* has been found.

Pathology and Diagnostics

Histological classification: Breast cancers are categorized into different subtypes based on morphology. According to the World Health Organization (WHO) classification of breast tumors 2003, there are 17 different histological types that

represent approximately 25% of all breast cancers. The remaining 75% belong to the most dominant subtype, referred to as "invasive ductal carcinoma, not otherwise specified" (Gruver et al., 2011). Of the specified histologic subtypes invasive lobular cancer represents 5-15% of breast tumors, medullary carcinoma up to 7%, mucinous carcinoma 2%, tubular carcinoma up to 2 % and neuroendocrine carcinoma 2-5% (Weigelt et al., 2010). The other specified histological subtypes are rare and represent less than 2% of all invasive breast carcinoma. Histological subtype has a rather small impact on prognosis compared to other prognostic factors but mucinous, tubular and medullary carcinomas have a more favorable prognosis compared to ductal and lobular carcinoma (Li, 2010; Yerushalmi et al., 2009).

Nottingham Histological Grade: Histological grade has long been used in diagnostic pathology of breast cancer and has a stronger impact on survival than histological subtype. It is based on the knowledge that the morphological appearance of tumors can be correlated with the degree of malignancy, and a grading of tumor dedifferentiation. The currently used system is based on Elston & Ellis grading system, Nottingham Histological Grade, introduced in 1991 (Elston and Ellis, 1991), involving a modification of the former most commonly used method described by Bloom and Richardson (Bloom and Richardson, 1957). The grading involves evaluation of three morphological features: tubule formation, nuclear polymorphism and mitotic counts. Each of these features can be scored from one to three and then the scores are summarized, and depending on the total sum tumors are divided into grade I, II or III. Survival analyses, in several independent large cohorts, have shown a significant correlation to prognosis

with well-differentiated tumors (grade I) having an improved survival compared to moderately (grade II) and poorly (grade III) differentiated tumors (Sundquist et al.,1999). In order to calculate an index predicting survival that also include the important prognostic factors lymph node status and tumor size the Nottingham prognostic index was introduced and evaluated (Galea et al., 1992; Haybittle et al., 1982). This index is calculated as follows: 0,2 x size + grade (1-3) + node status (1-3).

TNM: TNM is a staging system based on primary tumor size (T), lymph node involvement (N) and distant metastases (M) that is continuously revised and updated as diagnostics improve (Singletary and Greene, 2003). It is used to estimate the clinical stage of the disease and combines three important prognostic factors, size, nodal status and distant metastasis.

Hormone receptor status: In diagnostic pathology of breast cancer all invasive breast tumors are evaluated for Estrogen Receptor (ER) and Progesteron Receptor (PR) expression. Traditionally ER was quantified in cytosol-based ligand-binding assays but today immunohistochemical (IHC) evaluation are the standard analysis (Harvey et al., 1999), using a cutoff at 10% of positive cells to predict whether a tumor is hormone-responsive or not. At the previous European expert meeting in St Gallen a 50% cut-off was proposed as a "cut-off" value for a new category of highly endocrine-responsive tumors (Beckmann et al., 2009) but this has not been implemented in Swedish guidelines for breast cancer treatment (SweBCG, 2011). The values of ER and PR as prognostic factors have been evaluated in several studies. ER has historically been considered a positive prognostic factor (Crowe et al., 1991) but its impact might not be significant, independently of other

prognostic factors (Fisher et al., 1988). PR has been proposed to have prognostic impact in node negative breast cancer (Banerjee et al., 2004) but its value independent of ER is debatable (Fisher et al., 1988). Recently a large study evaluating ER/PR status as prognostic factors found that patients with ER+/PR+ tumors had an improved prognosis compared to patients with ER+/PR-, ER-/PR+ and ER-/PR- tumors, largely independent of other demographic and clinical tumor characteristics (Dunnwald et al., 2007). However the most important roles of ER/PR evaluation are as treatment predictive factors for antihormonal treatment.

HER2: HER2 is the protein product of the gene *ERBB2/NEU* and has been found to be overexpressed in 20-30% of all breast cancers (Borg et al., 1990; Slamon et al., 1987). HER2 is a transmembrane receptor belonging to the epithelial growth factor receptor (EGFR) family and the type I group of receptor tyrosine kinases (RTKs) (Barros et al., 2010; Harari and Yarden, 2000). This RTK family has important roles in cell proliferation, differentiation, adhesion, survival and migration (Barros et al., 2010). HER2 has no known ligand-binding extracellular domain but exerts its action as a co-receptor, forming heterodimers with other receptors of the EGFR family (Barros et al., 2010; Harari and Yarden, 2000). Overexpression/gene amplification of HER2 is associated with impaired prognosis (Borg et al., 1990; Slamon et al., 1987; Witton et al., 2003) and is also a treatment predictive marker for treatment targeting the HER2 pathway, i.e. the monoclonal antibody trastuzumab (Barros et al., 2010) and the tyrosine kinase inhibitor lapatinib (Baselga et al., 2002; Giampaglia et al., 2010). In diagnostics, HER2 is first evaluated by IHC and scored from 0 to 3+. If the expression is 0 or 1+ the tumor is considered HER2 negative. If the expression is 2+ or 3+ the tumor is further evaluated for *ERBB2* gene amplification using *in situ* hybridization techniques FISH or CISH (Payne et al., 2008; Wolff et al., 2007). There is currently a discussion regarding how to achieve a more validated and reliable evaluation of HER2 expression since the interlaboratory results are inconsistent (Wolff et al., 2007). However, a recent Swedish reproducibility survey confirmed a good reproducibility between different pathology departments (Ryden et al., 2009).

Ki67: Ki67 is a proliferation associated protein expressed during all active phases of the cell cycle but absent in resting cells (that have entered G0 arrest) and evaluates the growth fraction (percentage) of tumor cells (Gerdes et al., 1983; Scholzen and Gerdes, 2000). Ki67 is evaluated in breast cancer and has been shown to carry prognostic information (Sahin et al., 1991; Veronese et al., 1993), although it is not yet included as a prognostic factor in the current treatment guidelines (Yerushalmi et al., 2010). The variation in Ki67 expression scores, differ greatly between different laboratories why its role as a prognostic factor is difficult to evaluate. There are still methodological issues to be solved, i.e. lack of standardization and lack of a clearly defined cut-off value. The fact that Ki67 has not been confirmed to be an independent prognostic factor in prospective trials (Beckmann et al., 2009) has led to increasing scientific efforts aiming to evolve techniques for Ki67 evaluation that are more precise and repeatable. Ki67 has also been proposed to carry treatment predictive information in endocrine treatment and chemotherapy (Dowsett and Dunbier, 2008).

Molecular Subclassification: As a complement to the traditional pathology diagnostics of breast cancer, molecular

subclassification is emerging to provide improved information about prognostic and treatment predictive factors as well as an improved knowledge about the different genotypes of breast cancer. In the beginning of this century gene expression profiling could define subgroups of breast cancer that correlated to prognosis and these subgroups were confirmed and validated in larger tumor materials and by different research groups (Sorlie et al., 2001; Sotiriou et al., 2003; van de Vijver et al., 2002). One of the greatest utilities of these gene-clustering analyses is that it can be used to divide the large group of ER positive breast cancers into subgroups with different prognosis (Loi et al., 2007; Wirapati et al., 2008). Eventually four different subgroups have been established: the basal like-, HER2-, luminal A-, and luminal B breast cancer types. There is a relatively high concordance (75-90%) between molecular subtypes and IHC phenotype (Kaufmann and Pusztai, 2011) and the four major molecular subtypes correlates to traditional IHC markers as follows: Basal like (ER-/PR-/HER2-), HER2 (HER2+/ER-), Luminal A (ER+/low grade/proliferation) and Luminal B (ER+/high grade/proliferation). These molecular subtypes can also differ regarding chemotherapy sensitivity, which has been shown in neoadjuvant clinical trials (Rody et al., 2007; Rouzier et al., 2005). There are commercially available genomic prognostic signatures, where of Mammaprint® and Oncotype DX® are the most established (O'Toole et al., 2011), but up until now they are not being used in the Swedish treatment guidelines (SweBCG, 2011).

Treatment

Principles of treatment: Breast cancer is, as stated above, a very heterogeneous disease. In Sweden regional treatment guidelines apply in order to guide clinicians and try to certify that all patients are being offered the most efficient treatment available (SweBCG, 2011). Different prognostic and treatment predictive factors are evaluated in order to make sure that the patients get the best available treatment. A major dividing line in the treatment options is the presence or absence of distant metastasis. As long as breast cancer is locoregional it is considered curable, but once it becomes metastatic there is no longer a cure and treatment is aimed at palliation, improving quality of life and survival prolongation (Beslija et al., 2009). The following treatment section will focus on treatment with a curable intention, i.e. treatment of locoregional disease.

Treatment predictive and prognostic factors: Breast cancer prognosis has improved dramatically in the last fifty years due to improvements in early diagnostics and adjuvant systemic treatments. Still the tools of predicting treatment response and survival are not sensitive enough and therefore some patients receive treatment that they will not benefit from, whereas others do not get sufficient treatment. Prognostic factors intend to predict patient outcome independent of adjuvant treatment, whereas treatment predictive factors aim to predict the response of a patient to a specific therapy (Weigel and Dowsett, 2010). Some factors can have mixed prognostic/predictive significance.

The most important prognostic factors used in the clinical setting are patient and tumor characteristics previously described, i.e. age, histological type, TNM, grade, HER2 overexpression/gene amplification, ER and PR status. The most important treatment predictive factors today are ER and HER2 expression to determine which patients can benefit from endocrine and HER2 targeted therapies respectively. Molecular

subclassification techniques are emerging and will probably be more important for treatment decisions in the near future.

In the breast cancer research field, thousands of potentially useful prognostic and predictive markers have been suggested but since they are not validated in the clinical setting they will not be addressed here. One interesting and potentially useful biomarker though, is the detection of circulating tumor cells that have been shown to carry prognostic information in the metastatic setting (Cristofanilli et al., 2004) and potentially also in the adjuvant setting (Saloustros et al., 2011).

Surgery: Historically modified radical mastectomy has been the surgery of choice in breast cancer patients since the mid of last century. In the 1980's partial mastectomy followed by radiotherapy was introduced and large, independent prospective randomized trials, with long-term follow-up, have confirmed that there are no differences in survival between the two different methods (Fisher et al., 1995; van Dongen et al., 2000; Veronesi et al., 1990). Today, breast-conserving surgery followed by radiotherapy is considered the standard procedure for most patients, whereas modified radical mastectomy can be used in some patients to ensure radicality.

Regarding axillary lymph node diagnostics, historically axillary dissection has been used but in the end of last century, the sentinel node technique was introduced (Frisell et al., 2001; Veronesi et al., 2003), a technique that accurately could provide diagnostic information regarding nodal status by excising only a few lymph nodes. The procedure saves patients with node negative (N0) disease from the side effects of axillary dissection.

Radiotherapy: Postoperative radiotherapy of the breast is standard treatment after breast conserving surgery and has been shown to decrease the risk of locoregional recurrence (Fisher et al., 1995; Liljegren et al., 1999). Postmastectomy radiotherapy is offered to patients with increased risk of locoregional recurrence based on tumor size and number of affected lymph nodes. Although locoregional recurrences are decreased no effect on survival was initially shown since the beneficial effect of radiotherapy on survival is counteracted by the late cardiovascular side effects (EBCTCG, 2000). However, long term follow up data show that avoidance of locoregional recurrences was relevant to 15-year breast cancer mortality (Clarke et al., 2005). Further studies show that the effects of locoregional control on survival are heterogeneous (Kyndi et al., 2009).

Systemic therapy: The aim of systemic therapy in the adjuvant setting is eradicating potential micrometastatic disease. Depending on different prognostic and treatment predictive factors patients are offered systemic treatment with chemotherapy, endocrine therapy and/or targeted therapies.

Chemotherapy: Adjuvant chemotherapy in breast cancer has been evaluated since the mid of last century. Since then polychemotherapy has been introduced and new drugs evaluated (Gianni et al., 2001). The most efficient chemotherapy used in the clinical setting today is anthracyclin-based polychemotherapy in combination with taxanes (SweBCG, 2011). Anthracyclin-based polychemotherapy was found superior in the EBCTCG metaanalysis of 2005 (EBCTCG, 2005) and survival has been further improved by adding taxanes in the treatment (Goldhirsch et al., 2009).

Chemotherapy is also used in the metastatic setting but much more individualized than in the adjuvant setting since the goal of treatment is different in this group of patients.

Endocrine therapy: Endocrine treatment is used to treat hormone responsive breast cancer and affects the estrogen induced signaling pathways in different ways, either by blocking or modulating the receptor (tamoxifen, fulvestrant) or by reducing the amount of estrogen produced in the body (ovarian suppression or aromatase inhibitors).

Tamoxifen is a selective estrogen receptor modulator (SERM) that has an antagonistic effect on ER in breast cancer tissue but an agonistic effect in other tissues (Ali and Coombes, 2002). It has been used in the treatment of breast cancer since the 1970's, first in the metastatic setting (Cole et al., 1971) and then in the adjuvant setting (Stoll, 1976) to improve survival (Osborne, 1998; EBCTCG, 1998). Traditionally tamoxifen has been the standard treatment for both pre- and postmenopausal patients but today aromatase inhibitors have shown superior effect in subgroups of postmenopausal patients.

Fulvestrant is an ER antagonist with no agonistic effects, mainly used in postmenopausal patients with advanced breast cancer (Vergote and Robertson, 2004).

Ovarian suppression can be achieved using the luteinizing hormone-releasing hormone (LHRH)-analogue *goserelin* and has been shown to be effective in premenopausal patients with ER+ breast cancer. However, the value of adding goserelin to standard treatment with chemotherapy and tamoxifen has not been clearly evaluated (Goel et al., 2009; Sverrisdottir et al., 2011).

Aromatase Inhibitors (AIs) block the conversion of androgens to estrogens in peripheral tissue and thereby reduce the estrogen levels even further in postmenopausal women (Chumsri et al., 2011). Today there are three different AIs used (anastrozole, letrozole and exemestane) and different clinical trials have shown that AIs are superior to tamoxifen in postmenopausal women (Cuzick et al., 2010; Mouridsen et al., 2009). The current treatment guidelines recommend use of AI upfront in postmenopausal high risk ER+ breast cancer, whereas the effect of AI in low risk patients has not been that convincing (Goldhirsch et al., 2009).

Targeted therapies: Breast cancer research is today focused on finding new targeted therapies to further improve prognosis in specific subgroups of breast cancer.

In the last decade HER2 targeting therapy has evolved and today the monoclonal antibody *trastuzumab* is being used in the adjuvant treatment of HER2 positive breast cancer (Guarneri et al., 2010) after prospective randomized trials have proven the beneficial effect in these patients (Piccart-Gebhart et al., 2005; Romond et al., 2005). In the HERA trial, one year of treatment with trastuzumab after adjuvant chemotherapy significantly improved disease-free survival among women with HER2-positive breast cancer (Gianni et al., 2011). Trastuzumab is also used in the metastatic setting and in patients that develop trastuzumab resistance lapatinib is an alternative. *Lapatinib* is a HER1/HER2 tyrosine kinase inhibitor and it has been proposed that treatment combinations of trastuzumab and lapatinib can be used in progressive disease (Scaltriti et al., 2009; Spector and Blackwell, 2009), but more studies are needed to verify this treatment regimen.

Bevacizumab is a monoclonal antibody targeting the vascular endothelial growth factor (VEGF). It has been evaluated in the metastatic setting (Miles et al., 2010; Miller et al., 2005) with an effect on progression free survival in HER2 negative metastatic breast cancer, but no effect has been shown on overall survival. Bevacizumab is currently also being evaluated in the neoadjuvant setting in the Swedish PROMIX trial.

ER and Tamoxifen Resistance

ER is important in breast cancer progression and is expressed in approximately 75% of all invasive breast cancers (Allred et al., 2004). Its function in the tumor cell context differs from the normal breast where only approximately 10% of the cells express the ER. These cells are non-proliferating and seem to regulate proliferation in adjacent ER- cells in a paracrine fashion (Clarke et al., 1997). However, in the tumor context the ER+ cells will proliferate in response to estrogen (Allred et al., 2004; Clarke et al., 1997; Doisneau-Sixou et al., 2003). The differences in regulation of ER induced proliferation in normal versus malignant breast tissue are not known in detail.

There are two different forms of ERs reported in breast cancer: ERα and ERβ, which are coded by different genes (*ESR1* and *ESR2* respectively) on different chromosomes. The function of ERβ has previously not been fully understood and although recent research have elucidated more information on its function this section will focus primarily on ERα, further referred to as ER.

ER is a member of the nuclear receptor superfamily of transcription factors and the classical action of ER is estrogen (estradiol; E2) binding to ER, inducing a conformational change enabling the binding of ER to estrogen response elements (EREs) in target genes enabling recruitment of co-activator or co-repressor multiprotein complexes (Ali and Coombes, 2002). These complexes influence the activity of the receptors, which will lead to activation or repression of target gene transcription. It has been reported that ER action affects up to 1000 different genes in breast cancer (Kok and Linn, 2010) and hence it is a very potent transcription factor. ER can also be activated in a ligand-independent way from phosphorylation by tyrosine kinases activated by different growth receptor pathways (Britton et al., 2006; Nicholson et al., 2004). Non-genomic actions of ER have also been reported and ER expression has been found in cytoplasm and in connection with the cell surface membrane (Levin and Pietras, 2008). The three most important domains of the ER are two activating function domains (AF-1 and AF-2) and the DNA-binding domain (DBD). AF-2 is activated upon ligand binding to its ligand-binding domain, whereas AF-1 can be activated in a ligand-independent way by phosphorylation (Lannigan, 2003). The DBD mediates specific binding to ER target genes.

Tamoxifen is a selective estrogen receptor modulator (SERM) that can have both agonistic and antagonistic effects on the ER, dependent of the tissue and cellular context (McDonnell, 1999). Hence its importance in breast cancer treatment is due to its ability to act as an antagonist in breast, whereas it has agonistic effects on bone, uterus and in the cardiovascular system. The mechanisms of these differences in SERM activity depending on tissue context are not fully known. In breast, tamoxifen acts as an antagonist by competitive binding of ER and inducing a conformational change different to that induced by estrogen, which leads to the recruitment of transcriptional co-repressors instead of co-activators. It seems

that in tissues dependent on AF-1 activation tamoxifen has an agonistic effect versus its antagonistic effects in tissues dependent on AF-2 activation (McDonnell, 1999).

Tamoxifen resistance is unfortunately common in breast cancer and can be divided into two categories: *de novo* resistance and acquired resistance (Sengupta and Jordan, 2008). *De novo* resistance if defined as ER+ breast cancers which are non-responsive to tamoxifen from the very beginning and can be illustrated in the experimental setting by HER2 overexpressing ER+ breast cancer cells that can form tumors even despite tamoxifen treatment. Although a majority of ER+ breast cancers are initially responsive to tamoxifen most of them will acquire resistance over time (Riggins et al., 2007). However, the molecular mechanisms for tamoxifen resistance are not fully understood, but several genomic and extra-genomic factors are being reported to be involved (Sengupta and Jordan, 2008) and

increasing research efforts are focused in this area with the main goal of overcoming tamoxifen resistance in patients. All reported mechanisms will not be discussed in this thesis.

However, one important mechanism in acquired tamoxifen resistence is the increased expression/modulation of different growth receptor signaling pathways (McDonnell, 1999). Elevated expression of HER-2, EGFR and IGF-1R have been reported in non-responsive tumors (Nicholson et al., 2004) and also activity of kinases downstream of these signaling pathways have been reported elevated such as ERK1/2, MAPK and PI3K pathways (Kurokawa and Arteaga, 2003; Osborne and Schiff, 2003). The increased cross-talk between ER and different growth factor pathways and modulations of ER interactions with different co-regulatory proteins seem to be responsible for inducing tamoxifen resistance in this setting.

Anemia in Cancer

Definition and Epidemiology

Anemia is defined in the medical literature as a reduction below normal in the number of erythrocytes/mm3, in the quantity of hemoglobin (Hb), or in the volume of packed red cells per 100 ml of blood, which occurs when the equilibrium of blood loss (through bleeding and destruction) and blood production is disturbed (Anderson, 1994). A more physiologic definition is that anemia is a reduction in red blood cell mass below that required to secure the oxygen demand of tissues (Clarke and Pallister, 2005), but in clinical practice Hb concentration is used as a surrogate marker.

Anemia is common in cancer patients but estimates of incidence and prevalence differ considerably, probably due to differences in patient populations and study methodologies (Birgegard et al., 2005). It has been reported that between 30 and 50% of cancer patients develop anemia at some point during the course of the disease (Mercadante et al., 2000). The incidence of anemia differs between different malignant diagnoses and is in general more common in hematological malignancies than in solid tumors and also more common in advanced rather than in earlier disease (Clarke and Pallister, 2005). One problem in evaluating the incidence of anemia is that different studies use different cut-offs in Hb levels as criteria for anemia (Caro et al., 2001). The National Cancer Institute Common Terminology for Adverse Events (NCI CTCAE) defines anemia as grade 1 (mild, Hb: 10-12g/dl), grade 2 (moderate, Hb: 8.0-9.9 g/dl), grade 3 (severe, Hb: 6.5-7.9 g/dl) and grade 4 (life-threatening, Hb < 6.5 g/dl) (Clarke and Pallister, 2005).

A few years ago a large European Cancer Anemia Survey (ECAS) was published, defining the incidence, prevalence and treatment of anemia in cancer patients with different malignant diagnoses (Ludwig et al., 2004). According to this multinational, prospective survey, the prevalence of anemia was 39% at enrollment and 67% during the study (follow up time was 6 months). Anemia in this study was defined as Hb levels < 12g/dl and also included patients with mild anemia. The incidence of anemia varied according to type of malignancy, stage of disease and treatment type. In breast cancer specifically, 30% of the included patients were anemic at enrollment, and overall 62% during the study.

Anemia has been shown to be a strong negative prognostic factor for survival in different cancer forms (Caro et al., 2001; Clarke and Pallister, 2005). One problem in the published clinical studies has been to evaluate if anemia is an independent prognostic factor, regardless of disease severity or if it modifies the relation between disease severity and survival. However, using Cox proportional hazards models adjusted for disease stage and/or severity it has been found to be an independent prognostic factor (Caro et al., 2001). The strongest association between anemia and poor outcome is found in cancer patients receiving radiotherapy (Harrison et al., 2002; Kumar, 2000; Lee et al., 1998), but this association has also been shown in patients receiving radio-chemotherapy and chemotherapy (Bacci et al., 2000; Boehm et al., 2007; Clarke and Pallister, 2005; Dubsky et al., 2008; Van Belle and Cocquyt, 2003).

Pathophysiology

Pathogenesis: The pathogenesis of cancer-related anemia (CRA) is complex and multifactorial and can be due to both the disease itself and to tumor treatment (Ludwig and Fritz, 1998; Mercadante et al., 2000). Factors contributing to anemia in cancer patients include tumor-associated bleeding, hemolysis, hypersplenism with hemophagocytosis, nutritional deficiencies, marrow damage from metastases or myelodysplasia, impaired erythropoietin (EPO) production, treatment toxicity (chemotherapy and radiotherapy) etc (Birgegard et al., 2005). In most cases complex interactions between tumor cells and the immune system are also involved. CRA describes a form of anemia of chronic disease that is specific to cancer patients and secondary to tumor-induced activation of the inflammatory and immune systems. CRA is a hyporegenerative anemia characterized by normocytic, normochromic anemia with a relative reticulocytopenia, reduced red blood cell survival, impaired iron utilization, suppression of erythropoiesis, enhancement of erythroid apoptosis and inadequate erythropoietin synthesis (Clarke and Pallister, 2005).

Radiotherapy and chemotherapy also contribute to anemia in cancer patients, mostly due to myelosupression via direct injury to hematopoietic stem cells, or structural or functional damage to the stroma or microcirculation, or injury to accessory cells that have regulatory functions in the bonemarrow (Birgegard et al., 2005; Mauch et al., 1995; Mercadante et al., 2000). Chemotherapy, especially platinum based regimes, can also cause nephrotoxicity and thereby an impaired EPO production.

Symtoms and effects: The symtomatology of anemia in cancer is diverse and ranges from asymtomatic to life threatening. In response to anemia several compensatory mechanisms are activated. The cardiovascular system increases pulse rate, peripheral vasodilatation and blood flow. There is also a shift of the oxygen dissociation curve, enhancing release of oxygen to the tissues (Birgegard et al., 2005; Jones, 1995; Mercadante et al., 2000). The respiratory rate is increased in an attempt to improve blood oxygenation and blood is also shunted to oxygen-sensitive vital organs (Birgegard et al., 2005). In response to hypoxia, EPO production is increased, although this requires a significant reduction in Hb levels.

When these physiologic compensatory mechanisms fail to compensate for decreased oxygen transport different symptoms occur. Some of them are due to the compensatory mechanisms themselves, exemplified by tachycardia, pallor of the skin and mucous membranes, dyspnea, gastrointestinal symptoms due to blood shunting etc. Anemia can also cause cerebral hypoxia, which may generate symptoms such as fatigue, dizziness, vertigo, depression and impaired cognitive functions (Birgegard et al., 2005; Mercadante et al., 2000). Also the immune system can be affected and impaired.

Fatigue is a complex disorder in cancer patients and anemia is believed to be a key factor in development of fatigue (Blohmer et al., 2005). Anemia can contribute to fatigue in various ways, for example by causing dyspnea, reduced physical work capacity and exercise tolerance due to effects on the pulmonary and cardiovascular systems and skeletal muscles. The complex interactions between CRA and the immune system with overexpression of inflammatory cytokines have also been shown to be involved in

causing fatigue and cachexia (Kurzrock, 2001).

Evaluating Quality of Life (QOL) in cancer patients has become a growing priority in oncology and has also become a widely accepted outcome measure in clinical trials of cancer therapies (Blohmer et al., 2005). QOL focuses on evaluating the effect of an illness on a patient's physical, psychological and social well-being as perceived by the patient (Soni and Cella, 2002). Many of the symptoms caused by anemia have a negative impact on QOL and a significant correlation between Hb levels and QOL has been established by several independent studies (Cella et al., 2003; Holzner et al., 2002; Lind et al., 2002).

Treatment

Transfusion: The traditional treatment of anemia in cancer patients is red blood cell (RBC) transfusions, but few studies have evaluated the impact of blood transfusion on outcome in cancer patients. Some of these studies show an improved prognosis in anemic patients with cervical cancer receiving transfusion compared to controls (Henke, 2001; Kapp et al., 2002). However, in a recently published trial, evaluating patients with head and neck cancer receiving radiotherapy, transfusion did not improve outcome but instead showed a trend towards impaired prognosis in these patients compared to controls (Hoff et al., 2011). There might also be a risk of transfusion-dependent immunosupression that might impact tumor progression in cancer patients (Varlotto and Stevenson, 2005).

Erythropoietin: Erythropoietin (EPO) is a 30,4 kDa glycoprotein hormone produced mainly in the adult kidney and fetal liver in response to hypoxia (Koury and Bondurant, 1991; Zanjani et al., 1981). Its main function is in erythropoiesis where it stimulates proliferation, survival and differentiation of erythrocytic precursor cells (see background, EPO/EPOR section). In 1985 the *EPO* gene was cloned (Jacobs et al., 1985; Lin et al., 1985) and shortly thereafter, recombinant human EPO (rhEPO) was introduced in the treatment of anemia due to chronic renal failure (Eschbach et al., 1987). Since then rhEPO has been used in the treatment of anemia associated with acquired immunodeficiency syndrome, rheumatoid arthritis, myelodysplasia, aplastic anemia, neonatal prematurity, and in hematological and non-hematological malignancies (Samol and Littlewood, 2003).

rhEPO treatment: There are two main variants of rhEPO used in the pharmacological setting: epoetin-α and epoetin-β. The difference between these compounds is basically a difference in glycosylation status. In addition to these compunds there is also another substance used in the clinic called darbepoetin-α. The epoetins have the same amino acid sequence as endogenous EPO, whereas darbepoetin has two additional oligosaccharide chains as a result of five amino acids substitution in the protein backbone (Seidenfeld et al., 2006). These modifications were introduced in order to prolong the proteins half-life (Kiss et al., 2010). However, both the epoetins and darbepoetin have the same pharmacological actions identical to those of endogenous EPO (Seidenfeld et al., 2006). In the following section the term rhEPO refers to epoetins, whereas the term erythropoiesis-stimulating agents (ESAs) will be used when both epoetins and darbepoetin are referred to.

rhEPO treatment in Cancer-related Anemia

In Cancer-related anemia (CRA), rhEPO was introduced in order to elevate Hb levels, increase QOL and decrease needs for blood transfusion. In the year 1990 the first pilot study of rhEPO treament in anemic myeloma patients showed an increase in Hb levels after rhEPO treatment concluding that rhEPO was a promising therapeutic tool for correcting myeloma-associated anemia (Ludwig et al., 1990). This study was followed by several trials of rhEPO treatment in patients with hematological malignancies and anemia, showing an increase in Hb levels in a majority of patients treated with rhEPO and concluding that rhEPO was a safe treatment (Cazzola et al., 1995; Osterborg et al., 1996). Further trials also evaluated effects of rhEPO treament on QOL and found that different QOL parameters improved (Dammacco et al., 2001; Osterborg et al., 2002).

To confirm previous important findings regarding effects of rhEPO treament in CRA, two community-based larger studies were conducted in the USA (Demetri et al., 1998; Glaspy et al., 1997). More than four thousand patients with non-myeloid malignancies and anemia receiving chemotherapy were recruited to receive rhEPO treatment and the studies concluded that rhEPO treatment raised Hb levels, decreased transfusion needs and improved QOL in these patients. Since these studies were not randomized no placebo group was used as control and therefore rhEPO treatment could not be compared to placebo. Several later studies have evaluated effects of rhEPO on QOL in anemic patients with malignancies of different origins and confirmed the improvements in QOL (Cella et al., 2003; Littlewood et al., 2001; Patrick et al., 2003), but not all of these trials will be discussed here.

To conclude one can state that in the beginning of this century rhEPO was mostly considered a safe and effective alternative to blood transfusions in anemic cancer patients. In the evidence-based clinical practice guidelines of Epoetin use in patients with cancer, from the American Society of Clinical Oncology and Hematology (ASCO/ASH) respectively in 2002, rhEPO was recommended for anemia treatment in patients with chemotherapy-induced anemia. Worth to mention is also that an estimate of 300,000 patients in the USA was using rhEPO each year and that the turnover rates for rhEPO sales were billions of dollars per year (Brower, 2003).

A possible association between rhEPO treatment and survival has also been evaluated in different studies. Two randomized placebo-controlled rhEPO trials reported a trend towards improved survival in rhEPO treated patients with non-myeloid malignancies and anemia compared to controls (Littlewood et al., 2001; Witzig et al., 2005). However these trials were not designed to evaluate survival as a primary end point and therefore did not have power to significantly detect differences in survival (Steensma and Loprinzi, 2005). A meta-analysis evaluating rhEPO trials before 2001 could not consistently conclude whether or not rhEPO had an effect on survival, although a trend towards improved survival was seen (Bohlius et al., 2005).

In 2003 the first reports on possible detrimental effects of rhEPO treatment in cancer patients were published. The Breast Cancer Erythropoietin Trial (BEST) evaluating rhEPO treatment to prevent anemia in patients with metastatic breast cancer receiving chemotherapy, was terminated early due to impaired survival

in the rhEPO treated group compared to controls (Leyland-Jones, 2003). The reason for the differences in survival could not be determined from subsequent analysis after the study but might be related to imbalances in treatment groups, a relatively high target Hb, or that rhEPO effects in non-anemic patients might affect survival in a negative way (Leyland-Jones et al., 2005). In the BEST publication it is speculated that the relationship between Hb and survival is a U-shaped curve with increased risks at more extreme Hb levels.

At the same time another large randomized trial evaluating rhEPO treatment of head and neck cancer patients with anemia undergoing radiotherapy reported impaired locoregional progression free survival (PFS) in rhEPO treated patients (Henke et al., 2003). Both these studies were designed to evaluate survival as a primary endpoint and they both reported rhEPO treatment associated with impaired survival. Another large randomized trial evaluating darbepoetin-α in patients with head and neck cancer receiving radiotherapy was terminated early because of an interim analysis showing poorer outcome in the treatment group compared to controls (Overgaard et al., 2009). There are further trials reporting negative effects on survival by ESA treatment but all will not be discussed here.

Several systematic reviews and meta-analyses have been evaluating ESA treatment and effects on survival. Interestingly the results of these meta-analyses changed over time with the early studies reporting a survival benefit in ESA treated patients whereas the later studies are showing the opposite (Bohlius et al., 2011). Explanations for this surprising result could be found both at the level of individual trials and at the meta-analysis level. One speculation is that in the early trials more positive results

were published early whereas the negative or neutral results were not published. When a recent meta-analysis included both published and unpublished data there was no longer a survival advantage in the ESA treated patients from early trials indicating that maybe all results had not been reported from the beginning (Bohlius et al., 2011).

Over the years more than 20 systematic reviews and meta-analyses have been published and all will not be discussed here. However, in the updated ASCO/ASH ESA guidelines from 2010 (Rizzo et al., 2010), one large meta-analysis using individual patient data from 53 randomized controlled trials, reported that ESA treatment was found to increase on study mortality and worsened overall survival (Bohlius et al., 2009). Further analyses trying to find subgroups with increased or decreased risk of ESA induced survival impairment was unsuccessful. Three literature based meta-analyses (Bennett et al., 2008; Lambin et al., 2009; Tonelli et al., 2009) showed similar results, i.e. reporting on detrimental effects of ESA on survival. However, one other meta-analysis did not find a significant difference in mortality between ESA treated patients compared to controls (Glaspy et al., 2010). None of three new randomized clinical trials published since 2007 demonstrated a significant increased risk in mortality with ESA treatment compared to placebo (Hoskin et al., 2009; Schouwink et al., 2008; Tsuboi et al., 2009), but according to the ASCO/ASH guidelines this might be due to study limitations and small sample size.

In conclusion, the updated guidelines from ASCO/ASH 2010 concerning the use of ESAs in cancer patients recommend that ESAs are only used as an alternative to RBC transfusion, in patients with chemotherapy-associated anemia with a Hb level <10 g/dL, after discussions concerning potential

harms and benefits (Rizzo et al., 2010). If used, ESAs should be administered at the lowest doses possible and should increase Hb to the lowest levels possible to avoid transfusions. Clinicians are urged to exercise caution in considering ESA use in patiens with malignancy being treated with curative intent.

Regarding mechanisms of how ESA can affect tumor progression and patient survival it is not conclusively known. Intensive research is focusing on understanding the biology of EPO and EPO receptor (EPOR) in tumors and tumor progression (discussed in background, EPO and EPOR section). In the unabridged guidelines from ASCO/ASH it is discussed whether ESA effects on survival can be explained by adverse effects of ESA treatment including increased venous thromboembolic events, increased inflammatory cytokine production etc, or if it is an actual effect of ESA on EPOR expressing tumor or surrounding cells.

EPO and EPOR

EPO and EPOR function

EPO: The human *EPO* gene is located on chromosome 7 and the EPO protein is mainly produced in the fetal liver and adult kidney in response to hypoxia (Koury and Bondurant, 1991; Zanjani et al., 1981), and is secreted into circulation as a 165 amino acid glycoprotein hormone. Thirty-nine percent of EPO consists of carbohydrate compound, 3 N-linked and one O-linked glycans and the N-linked glycosylation appears to be important for proper biosynthesis and/ or secretion and for stability in circulation (Sasaki et al., 2000).

EPOR: The human *EPOR* gene is located on chromosome 19 and consists of eight exons and seven introns. Different splice variants have been reported in murine cell lines and bone marrow (Barron et al., 1994) as well as in tumor cells (Arcasoy et al., 2003). The EPOR protein (66kDa) is a 508 amino acid peptide (Jones et al., 1990) that belongs to the cytokine receptor superfamily and consists of an extracellular (ligand-binding), a transmembrane and an intracellular domain (Lacombe and Mayeux, 1998; Lombardero et al., 2011). The number of EPORs expressed at the cell surface of normal or transformed erythroid cells is low, approximately up to one thousand receptors per cell (D'Andrea and Zon, 1990). The EPORs are mainly expressed at the colony-forming unit erythroid (CFU-E) stage and then receptor expression decreases with erythroid maturation (Mayeux et al., 1987).

EPO/EPOR signaling: The main function of EPO is stimulation of proliferation, survival and differentiation of erythrocytic precursor cells in response to hypoxia. The classical and well characterized EPO-induced signaling in erythropoiesis mediated via EPOR is described in Figure 1. One single molecule of EPO binds to two adjacent EPORs on the cell surface and the receptors are activated via homodimerization (Lacombe and Mayeux, 1998). The EPOR does not have any endogenous tyrosine kinase activity but is activated via phosphorylation by JAK2, a tyrosine kinase that is constitutively associated with the EPOR. The specific tyrosines in the intracellular domain of the EPOR that have been phosphorylated serve as docking sites for intracellular proteins containing src homology 2 (SH2) domains including STAT5, which is a potent signal transducer and transcription activator. After binding, these proteins can be phosphorylated and activated. Once activated STAT5 is translocated to the nucleus and initiates transcription of different erythroid genes (Lacombe and Mayeux, 1998; Lombardero et al., 2011). A direct association of PI3K and EPOR leading to EPO-induced signal transmission has been shown (Damen et al., 1993; He et al., 1993; Mayeux et al., 1993; Miura et al., 1994a) and an alternative pathway for PI3K activation via the adaptor protein IRS2 has also been described (Verdier et al., 1997). Downstream of PI3K, AKT is activated leading to increased survival in erythroid precursor cells. The RAS/MAPK pathway is also activated via EPO stimulation (Carroll et al., 1991; Gobert et al., 1995; Miura et al., 1994b) and leads to increased proliferation in erythrocytic precursors. EPO signaling has also been shown to upregulate c-myc mRNA expression via a PKC dependent pathway (Spangler et al., 1991).
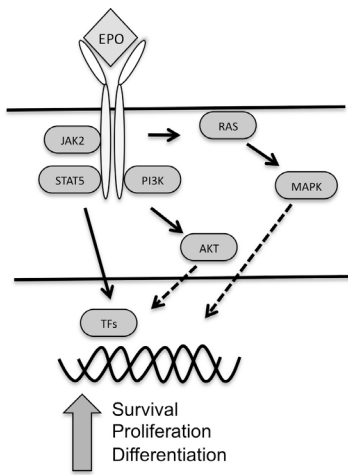
Previously, the EPO induced signaling was

Figure 1. Classical EPO/EPOR signaling in erythropoiesis
EPO binding to EPOR induces homodimerization and the receptor is activated via JAK2 phosphorylation of EPOR tyrosine residues that serves as docking sites for other proteins. By binding EPOR STAT5 and other proteins become phosphorylated via JAK2 and different down stream signaling cascades are activated, i.e. STAT5, PI3K/AKT and RAS/MAPK pathways, leading to increased proliferation, survival and differentiation.

believed to be dependent on phosphorylation of the eight intracellular tyrosine residues of the EPOR. However recent results show that JAK2-mediated EPOR phospho-tyrosine-independent signals for erythroblast formation are associated with activation of ERK independent of STAT activation (Menon et al., 2006). This suggests that EPOR might activate proliferative pathways (via ERK) despite lacking its intracellular phosphorylated tyrosine sites.

EPO/EPOR in fetal development: EPO is essential for normal fetal development as shown in *EPO* and *EPOR* knockout mice where homozygous mice (*EPO*$^{-/-}$ and *EPOR*$^{-/-}$) die at embryonic day 13-15 with severe anemia. However heterozygous mice (*EPO*$^{+/-}$ and *EPOR*$^{+/-}$) were viable and fertile and had normal leukocyte and erythrocyte counts (Wu et al., 1995). *EPO* and *EPOR* knockout embryos also exhibit defects in angiogenesis and cardiac morphogenesis (Kertesz et al., 2004; Wu et al., 1999).

In the human fetus the primary site for EPO production is the liver, whereas the kidney is the major site for EPO production in adults (Moritz et al., 1997; Zanjani et al., 1981). Although the renal cell type responsible for EPO production has not been consistently proved (Lombardero et al., 2011) most data support that the peritubular fibroblast-like interstitial cells are the EPO producing cells (Weidemann and Johnson, 2009).

EPO/EPOR in non-hematopoietic tissues: EPO and EPOR expression has also been found in other organs of developing fetuses. In humans EPO expression was found in liver, kidney, endothelial cells, neural retina of the eye and adrenal cortex, whereas EPOR expression had a more wide-spread distribution including liver, kidney, myocardium, lung, neural retina, adrenal gland and endothelial cells (Juul et al., 1998). EPO and EPOR have been reported to be required for normal brain development (Yu et al., 2002). EPO protein expression in human milk has also been reported (Juul et al., 2000), suggesting that it might have a function in erythropoiesis, neurodevelopment and gut maturation of neonates (Lombardero et al., 2011).

As EPO was originally believed to only be involved in regulating erythropoiesis, recent research has focused on evaluating other potential functions of EPO in humans. In the brain a paracrine EPO/EPOR system has been suggested since EPO production is found in astrocytes and EPOR in neurons (Marti et al., 1996; Masuda et al., 1993;

Masuda et al., 1994; Morishita et al., 1997). In the cardiovascular system EPO and EPOR are expressed in endothelial cells (Anagnostou et al., 1994; Beleslin-Cokic et al., 2004), smooth muscle cells (Morakkabati et al., 1996) and cardiac myocytes (Cai et al., 2003; Tramontano et al., 2003). In both brain and cardiovascular systems EPO has been shown to have a tissue-protective role in rescuing ischemic cells from apoptosis (Genc et al., 2004; Santhanam et al., 2010).

EPOR expression has also been found in the gastrointestinal tract although its function there is not as well characterized as in the brain (Juul et al., 2001; Sereno et al., 2006). In the endocrine system EPOR expression has recently been found in pancreatic (Fenjves et al., 2003) and parathyroid cells (Ozturk et al., 2007), but its function has not been completely unraveled. Also in maturing lungs EPOR expression has been found suggesting a paracrine role for EPO signaling (Zhang et al., 2008).

In the female reproductive system EPO/EPOR signaling has an important angiogenic role with estrogen (E2) regulated EPO production in the oviduct and uterus stimulating EPOR expressed in vascular endothelial cells of the endometrium (Masuda et al., 2000; Yasuda et al., 1998). The finding that EPO production in the female reproductive system is regulated by E2 (besides hypoxia) highlights a difference from the other systems in the body, where EPO is primarily regulated in response to hypoxia.

EPO and EPOR in Cancer

In the beginning of this century EPO and EPOR expression in tumor cells was reported for solid tumors of various origins, for example breast cancer (Acs et al., 2001;

Arcasoy et al., 2002); cervical cancer (Acs et al., 2003), head and neck cancer (Arcasoy et al., 2005a; Winter et al., 2005), prostate cancer (Arcasoy et al., 2005b), renal cancer (Westenfelder and Baranowski, 2000) gastric cancer (Ribatti et al., 2003) endometrial cancer (Acs et al., 2004b), lung cancer (Dagnon et al., 2005) and in melanoma (Kumar et al., 2005; Selzer et al., 2000). For an overview of all studies reporting EPOR expression in breast cancer see Table 1.

Many of the early studies used anti-EPOR-antibodies that were later claimed to be unspecific and therefore the results of these studies have been questioned (Elliott et al., 2006). This illustrates the importance of using adequate positive and negative controls when analyzing molecular markers with different methods.

However EPOR mRNA is expressed in tumors of different origins (Arcasoy et al., 2005b; Dagnon et al., 2005; Sinclair et al., 2008; Winter et al., 2005) but the *EPOR* gene does not seem to be overexpressed or amplified in tumors (Sinclair et al., 2008). However, a recent study of EPO and EPOR mRNA expression in melanoma cells showed increased copy number of the EPO and EPOR loci in 30% and 24% of the tumors respectively (Kumar et al., 2011). Due to the controversies regarding anti-EPOR antibodies, one study using a newly produced antibody evaluated EPOR expression in 66 tumor cell lines and found low expression in 54 of them (lower than 100 EPOR dimers/cell) compared to the positive control (expressing 1000-10000 EPOR dimers/cell), whereas the remaining 12 cell lines had a higher expression (400-3200 dimers/cell). In further functional analyzes using radio-labeled EPO only one cell line was able to bind EPO to the cell surface (Swift et al., 2010). It should be mentioned that this study was performed

industries. This makes the investigations of EPOR expression and function in cancer even more complex, since the financial interest from the pharmaceutical industry might be a potential bias.

In one of the clinical trials reporting reduced survival in patients with head and neck cancer treated with rhEPO (Henke et al., 2003) (discussed in background, Anemia in Cancer section) a substantial effort was made to further elucidate if this effect was dependent on EPOR expression in tumor cells. First analysis reported that in patients with EPOR expressing tumors, rhEPO treatment impaired prognosis whereas in patients with EPOR negative tumors rhEPO did not affect survival (Henke et al., 2006). However in this study the antibody used was claimed to be unspecific and the results questioned (Elliott et al., 2006). Then a new analysis of EPOR mRNA expression levels was performed but were not able to show the same correlation to survival in the whole patient material but during subgroup analysis an increased EPOR mRNA expression correlated to impaired survival in rhEPO treated patients with unresected tumors (Miller et al., 2009). However in this subgroup only 14 tumors were analyzed rendering a very small group in the survival analysis. This study is although illustrative of the difficulty of persuing mechanisms behind clinical findings when available evaluation markers are suboptimal or not thoroughly validated.

rhEPO effects on tumor cells: rhEPO effects on tumor cells have been investigated in various tumors and show discordant results. Most studies have been performed in vitro using different cell lines and in breast cancer cells rhEPO has been shown to have a stimulatory effect on proliferation (Acs et al., 2001) and also in xenografts (Arcasoy et al., 2002), whereas we and other investigators

have reported no effect on proliferation (Paper III; LaMontagne et al., 2006). rhEPO stimulation has also been evaluated in tumors of other origins but all will not be discussed in detail here. Briefly in other tumor contexts the reported results from rhEPO stimulation studies are contradictory where some studies show effects on proliferation whereas other show no effect at all (Osterborg et al., 2007). One common problem is the rhEPO doses used in *in vitro* experiments and most studies reporting effects on proliferation have used supra-physiological doses (Fandrey and Dicato, 2009). The contradictory results of rhEPO experiments suggest that exogenous EPO does not consistently provide a proliferative stimulus for cancer cells and whether the contrasting data is a result of differences in cancer cell contexts or in experimental procedures is not clear (Hardee et al., 2006).

A possible capacity of rhEPO to rescue tumor cells after exposure to different toxic treatments has also been explored. rhEPO increased tumor cell resistance to dakarbazin and cisplatin in melanoma cells (Kumar et al., 2005; Mirmohammadsadegh et al., 2010) and resistance to cisplatin in cervical cancer cells (Acs et al., 2003). In another study increased resistance to both radiotherapy and cisplatin was reported in glioma and cervical cancer cells (Belenkov et al., 2004). Again other studies have reported contradictory results where rhEPO instead increased chemosensitization in renal carcinoma and leukemia cells treated with daunorubicin and vinblastin (Carvalho et al., 2005). Two studies reported no effects of rhEPO on cytotoxic effects of paclitaxel (LaMontagne et al., 2006) adriamycin and tamoxifen in breast cancer cell lines (Gewirtz et al., 2006). Another study evaluating rhEPO effects in squamous carcinoma and colorectal carcinoma xenografts, reported increased sensitivity to 5-FU, due to

remodeling of tumoral microvessels (Tovari et al., 2005).

Regarding functional EPOR in breast cancer a recent study has shown that rhEPO treatment impaired response to trastuzumab via EPOR/JAK2 mediated activation of Src2 and inhibition of PTEN (Liang et al., 2010). Another recent study evaluated rhEPO effects in breast tumor xenograft models and reported increased lung metastases in the immunocompromised mice treated with rhEPO in adjunct to Paclitaxel (Hedley et al., 2011). In that study there was no effect of rhEPO on the primary tumor suggesting that the rhEPO response was not due to direct effects on tumor cells but rather to effects on the microenvironment or systemic effects. Another study supporting this conclusion used a xenograft model with EPOR negative lung cancer cells. rhEPO stimulation increased tumor growth and the mechanism suggested was that rhEPO promoted angiogenesis through recruiting and stimulating proliferation of endothelial cells (Okazaki et al., 2008).

There is still no consensus regarding what effects rhEPO treatment might have on tumor progression. A number of questions lack answers; whether rhEPO has a direct effect on tumor cells via a functional EPOR, if rhEPO effects are more important for intratumoral endothelial cells or other stromal cells or if rhEPO works through some other, unknown mechanism. Of course one problem is methodological. It is difficult to mimic the complexity of the *in vivo* environment in experimental settings and initial *in vitro* experiments using cancer cell lines to study potential mechanisms should be followed by xenograft models, evaluating EPO effects on both tumor cells, stroma as well as systemic responses.

EPO/EPOR autocrine/paracrine loop:

Several studies that reported EPOR expression in tumor cells also found EPO expression and an autocrine loop has been suggested in tumors (Hardee et al., 2006). Under non-malignant conditions, a paracrine loop has been reported in brain where astrocytes produce endogenous EPO that rescue EPOR expressing neurons from hypoxia-induced apoptosis (Genc et al., 2004). Also in the female reproductive system endogenous EPO production in the uterus stimulates proliferation of EPOR expressing endothelial cells in the endometrium during the menstrual cycle (Masuda et al., 2000). In different cancer cell lines EPO mRNA levels have been reported and shown to increase in response to hypoxia (Acs et al., 2001; Batra et al., 2003; Paper II). The EPO protein however is more difficult to detect. Previous studies have analyzed EPO protein expression in breast cancer using IHC (Acs et al., 2001; Arcasoy et al., 2002; Arcasoy et al., 2003) but without adequate positive and negative controls it is difficult to interpret the results.

In our lab we have tried several different antibodies and a sensitive ELISA but not been able to detect any EPO production in different breast cancer cell lines (Paper II). Another study analyzing 24 different cancer cell lines reported increased EPO production in response to hypoxia using a very sensitive ELISA (Yasuda et al., 2003), although the EPO amounts reported were very low and measured in fg/ml (100fg/ml ≈ 0,012mU/ml). Worth to mention is however that *in vivo* circulating endogenous EPO will be present to the tumors via the circulation in concentrations of around 5-150 mU/ml (Fandrey and Dicato, 2009; Lappin et al., 2002).

To further elucidate the presence of a functional endogenous EPO/EPOR signaling in tumors, strategies to block EPO

signaling pathways have been used (Hardee et al., 2006). Blocking EPO signaling using soluble EPOR or anti-EPO antibodies resulted in tumor cell destruction and reduced vascularity in ovarian and uterine xenografts (Yasuda et al., 2001). Use of EPO signaling blocking substances also delayed tumor growth in a breast tumor chamber model (Arcasoy et al., 2002). Further studies are needed in order to elucidate the mechanisms of EPO/EPOR signaling in different tumors.

EPOR function in tumor cells: EPOR protein expression in different tumor cells is today quite established but the main question is whether the receptor is functional or not and if so through what mechanisms. Until today most research has been focusing on evaluating EPOR functionality in tumors as equivalent to its function in erythropoiesis. In different *in vitro* models rhEPO has been shown to stimulate the "classical" downstream targets of EPOR in cervical cancer cells (Lopez et al., 2011), in prostate cancer (Feldman et al., 2006), breast cancer (Lester et al., 2005; Liang et al., 2010), neuroblastoma (Um et al., 2007), melanoma (Kumar et al., 2005; Kumar et al., 2011; Mirmohammadsadegh et al., 2010), head and neck cancer (Lai et al., 2005) but this stimulatory capacity is not always coupled to an increase in proliferation, although in some studies coupled to increased invasion and migration.

To further evaluate a potential function of EPOR in tumors EPOR knockdown experiments have been performed. In melanoma xenografts EPOR knockdown resulted in reduced tumor growth in response to rhEPO (Kumar et al., 2011). In cervical cancer EPOR knockdown impaired proliferation and invasion in vitro and also impaired tumor growth in xenograft models, independent of EPO, suggesting a functional EPO-independent role of EPOR expression (Paragh et al., 2009). In cervical cancer cells inhibition of either EPO or EPOR expression using RNA-interference impaired growth (Lopez et al., 2011), and similar results have been reported in prostate cancer (Jeong et al., 2009).

Tissue protection: The tissue protective effect of EPO signaling has been proposed to be dependent on a different receptor complex as opposed to the classical EPOR heterodimer in erythropoiesis. RhEPO has been shown to exert a tissue protective effect in preclinical models of normal neuronal and cardiac cells in response to ischemic injuries (Grasso et al., 2004). The common β receptor (βcR) has been proposed as a co-factor and an EPOR/βcR heteroreceptor has been reported that mediates the tissue protective effect of EPO in neurons and cardiomyocytes (Brines et al., 2004). This heteroreceptor complex has recently been reported in endothelial cells (Su et al., 2011). Also in the tumor context this receptor has been proposed (Arcasoy, 2008) but there are today no conclusive data to verify the expression of such a heteroreceptor complex in tumor cells.

EPO effects on angiogenesis: EPOR expression has been found in normal endothelial cells (Anagnostou et al., 1994) and also in endothelial cells from tumors (Miller et al., 2011). There are data suggesting that EPO can induce recruitment of circulating endothelial cells and also stimulate endothelial cell proliferation (Anagnostou et al., 1990; Okazaki et al., 2008). Furthermore, increased lung metastasis in breast cancer xenograft models in mice treated with rhEPO without affecting primary tumor growth was reported, and one proposed mechanism behind this finding was EPO induced increased endothelial progenitor mobilization and angiogenesis (Hedley et al., 2011). rhEPO treatment

also increased lymph node angiogenesis and metastasis in xenograft models from breast cancer and melanoma in a manner associated with increased migration and proliferation of lymphatic endothelial cells (Lee et al., 2011). EPO also induced angiogenesis in human glioma (Nico et al., 2011). These results indicate that EPO might have important effects on angiogenesis in tumors and participate in the hypoxia driven vascularization of solid tumors.

EPO regulation

EPO is primarily produced in the fetal liver and adult kidney in response to hypoxia. A complex and delicate sensing system in the kidneys detect differences in oxygen pressure and induces increased EPO production. The main transcription factors to induce this response are hypoxia-inducible factors (HIFs). Although HIF-1 was originally detected in EPO studies, later investigations have proven HIF-2 to be the primary transcription factor inducing EPO expression (Kapitsinou et al., 2010; Warnecke et al., 2004). In hypoxia-induced EPO production the HIFs cooperate with hepatocyte nuclear factor 4 (HNF4) and other transcriptional co-activators (Jelkmann, 2011).

The hypoxia-induced regulation of EPO seems to be tissue-specific since differences have been shown between regulation in kidney versus hepatic cells. It seems that in the liver EPO production can be upregulated at a cellular level, whereas in the kidney upregulation is achieved through recruitment of larger number of cells with a fixed amount of EPO mRNA (Koury et al., 1988; Koury et al., 1991; Weidemann and Johnson, 2009). Also differences in locations of important

regulatory elements for hypoxic induction between liver and kidney cells have been reported (Kochling et al., 1998). It should be noted that most *in vitro* studies on EPO regulation are performed using hepatoma cell lines (Jelkmann, 2011).

EPO is of course also regulated by other important transcription factors and different GATA transcription factors have been reported as important repressors (Obara et al., 2008). Both GATA-2 and NFxB have also been reported to mediate the suppression of *EPO* gene expression in hepatoma cells treated with proinflammatory cytokines (La Ferla et al., 2002).

Methylation is another way in which *EPO* expression can be suppressed (Wenger et al., 1998; Yin and Blanchard, 2000) and *EPO* hypermethylation has recently been reported in different tumor cells (Steinmann et al., 2011).

Under normal conditions low plasma levels of EPO are needed to sustain a steady state in erythropoiesis. However during hypoxia EPO plasma levels can increase dynamically to very high levels, and then decrease towards normal levels before Hb levels are normalized (Jelkmann, 2011). The mechanisms of this rapid decrease are not fully understood but might be caused by lowered HIF levels during long-term hypoxia (Stiehl et al., 2006).

Estrogen (Masuda et al., 2000; Yasuda et al., 1998), thyroid hormone (Fandrey et al., 1994) and retinoic acid (Kambe et al., 2000) have also been reported to induce EPO expression in a tissue-dependent but hypoxia-independent way.

Hypoxia

Definition and physiology

Hypoxia occurs when the demand of oxygen is not balanced by the oxygen supply and can be defined as a state of reduced O_2 availability restricting the function of organs, tissues or cells (Hockel and Vaupel, 2001). The mechanisms responsible for the onset of hypoxia *in vivo* can be reduced O_2 levels in the circulation, reduced tissue perfusion or diffusion capacity, or inability of cells to utilize O_2. The partial oxygen pressure (pO_2) in the body differs depending on the context, ranging from high arterial pO_2 (approx. 100 mmHg) gradually decreasing to approx. 50 mm Hg in the end-capillary structures. Due to its definition the threshold for hypoxia differs depending on the tissue context but an onset of metabolic hypoxia has been estimated to occur at approx. 8-10 mmHg (Hockel and Vaupel, 2001). In the laboratory setting, trying to mimic in vivo conditions using in vitro experiment settings, 8-10 mmHg corresponds to approx. 1% O_2.

Tumor hypoxia

Hypoxia is common in solid tumors and it has been proposed that a majority of locally advanced tumors exhibit hypoxic areas heterogeneously distributed within the tumors (Vaupel et al., 2005). In solid tumors, hypoxia is mainly caused by a combination of the tumor outgrowing its vasculature and the abnormal formation of intra-tumoral angiogenesis. The perfusion-limited oxygen delivery can be due to structural and functional abnormalities in microvessels, leading to microvessel shut down and this process is often transient and referred to as acute hypoxia. The diffusion-limited hypoxia is caused by increases in diffusion distances from blood vessels to the tumor cells and can be referred to as a more chronic hypoxia (Harris, 2002; Vaupel et al., 2005).

Another contributing factor in tumor hypoxia is anemia. In normal tissues of anemic patients the reduced O_2-carrying capacity of the circulation is compensated by a rise in local blood flow and an increased O_2 extraction from the blood. In the tumor tissue these adaptation mechanisms do not work sufficiently well. In a comparison of non-anemic versus anemic breast cancer patients, the hypoxic fraction of O_2 levels ($pO_2 \leq 5mmHg$) in the tumors significantly increased from 53% in non-anemic patients to 77% in anemic patients (Vaupel et al., 2003).

Although hypoxia is toxic to both normal and malignant cells, the latter can undergo genetic and adaptive changes allowing them to survive and proliferate in spite of the low oxygen levels (Harris, 2002). This adaptation will affect several processes important in malignant progression. Exposure of tumor cells to hypoxia promotes the expression of genes that are important in the regulation of angiogenesis, genetic instability, immune evasion, metabolism, invasion and metastasis, treatment resistance and stem-cell maintenance (Semenza, 2011). Hypoxia has also been proposed to promote de-differentiation in tumors (Helczynska et al., 2003; Jogi et al., 2002; Lofstedt et al., 2007).

Hypoxia-Inducible Factors

The Hypoxia-Inducible Factors (HIFs) are important transcription factors in mediating

the effects of hypoxia. HIF-1 was originally discovered as an important transcription factor inducing *EPO* transcription in response to hypoxia (Semenza and Wang, 1992). Later HIF-2 (also named EPAS-1 or HLF-1) was discovered showing close sequence similarity to HIF-1 (Ema et al., 1997; Tian et al., 1997; Wiesener et al., 1998) and eventually HIF-3 was reported as a negative regulator of HIF induced transcription (Makino et al., 2001). HIF-1 is still the most studied HIF and increasing research is focusing on HIF-2, but relatively little is still known about HIF-3.

The HIFs belong to the basic helix-loop-helix PAS (bHLH-PAS) domain family of transcription factors and are active as heterodimers consisting of one HIF-α subunit and one HIF-β subunit. Under hypoxic conditions HIF-1α and HIF-2α rapidly accumulate and bind HIF-1β (also called ARNT), recruits the co-activators p300 and CREB-binding protein (CBP) and

binds to hypoxia-response elements (HREs), activating the transcription of hundreds of different hypoxia-responsive genes. HIF-1 binding is only detected at genes with increased expression but can also regulate transcription of as many genes in a negative way indirectly by regulating transcriptional repressors and microRNAs (Mole et al., 2009)

HIF regulation: HIF-α subunits are continuously transcribed and translated and the regulation is primarily post-translational (Gordan and Simon, 2007). The expression of HIF-α proteins is tightly regulated in an oxygen dependent way, illustrated in Figure 2. In the presence of oxygen HIF-α is hydroxylated by prolyl hydroxylase domain proteins (PHDs) generating a binding site for the von Hippel-Lindau (VHL) protein, which is a component of an ubiqutin ligase complex. HIF-α is then polyubiquitinylated and subjected to proteasomal degradation. Another way in which oxygen regulates
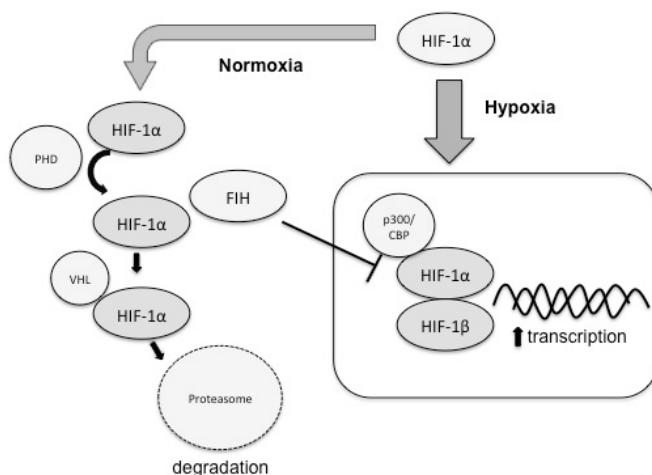
Figure 2. Oxygen dependent regulation of HIF-1α
In normoxia, HIF1-α is hydroxylated by PHDs, generating a binding site for the VHL protein. HIF1-α is then polyubiqutinylated and subjected to proteasomal degradation. HIF1-α can also be hydroxylated by FIH-1, thereby preventing binding of the co-activators p300 and CBP and inhibiting activation of gene transcription.
During hypoxia, HIF1-α is stabilized and translocates to the nucleus where it forms a heterodimer with HIF-1β. Co-regulators p300 and CBP are recruited and the complex binds to HREs in target genes and activates gene transcription.

HIF-α is via the factor inhibiting HIF-1 (FIH-1) protein that can hydroxylate HIF-α, thereby preventing binding of the co-activators p300 and CBP and inhibiting activation of gene transcription (Kaelin and Ratcliffe, 2008; Semenza, 2011).

HIF-α can also be regulated in oxygen-independent ways, for example by mutations in the regulating proteins. The most obvious example is *VHL* mutations that impair the ability of pVHL to ubiquitinate HIF-α leading to HIF-α accumulation in non-hypoxic cells. These mutations are most common in renal cell cancer and hemangioblastoma (Kaelin and Ratcliffe, 2008).

HIF-α can also be accumulated due to increased transcription and translation. Several different important signaling pathways in tumor cells have been associated with increased HIF-α expression. Different growth factor signaling systems that have been reported to increase HIF-α protein levels include EGFR in prostate cancer (Zhong et al., 2001), HER2 (Laughner et al., 2001) and RAS in breast cancer (Blancher et al., 2001), and VEGF in colon cancer (Calvani et al., 2008). There are several different growth factors reported to upregulate or stabilize HIF-1α expression (Bardos and Ashcroft, 2004) but all will not be discussed here. The effects on HIF-α expression modulated by these different growth factors seem to be dependent on signaling pathways central to the tumor cells, i.e. PI3K/AKT, mTOR and RAS/MAPK pathways (Bardos and Ashcroft, 2004; Semenza, 2003).

HIF-1 versus HIF-2: Differences in HIF-1 and HIF-2 function can be discussed in the context of embryonal development. *HIF-1A*^{-/-} mouse embryos die in mid gestation due to decreased erythropoiesis and cardiovascular malformations and neural tube defects, indicating that HIF-1 is needed for normal development (Iyer et al., 1998; Yoon et al., 2006). However, the developmental function of HIF-2 is not as clearcut. *HIF-2A* knockout mice models have generated differing phenotypes. Most *HIF-2A*^{-/-} mice die in utero (Compernolle et al., 2002; Peng et al., 2000; Scortegagna et al., 2003; Tian et al., 1998) and the few mouse strains that survived showed multiple organs pathology (Scortegagna et al., 2003) or died neonatally due to severe respiratory distress syndrome (Compernolle et al., 2002).

Although to generalize these results it seemed that most of the *HIF-2A* knockout mice experienced defects in vascular development of different organs. The different phenotypes may be due to different genetic backgrounds. The discrepancies of HIF-1 and HIF-2 involvement in embryogenesis can also be illustrated by the difference in expression. HIF-1α mRNA was more abundantly expressed and especially high in the myocardium, primitive gut and neuroepithelium in mouse embryonic development whereas HIF-2α mRNA was mostly expressed in the developing vasculature, but also found in lung, kidney, olfactory epithelium and adrenal gland (Jain et al., 1998). HIF-1α expression is expressed ubiquitously in human tissues, whereas HIF-2α expression has been restricted to endothelium, kidney, heart, lung, liver, pancreas, intestine and brain (Ema et al., 1997; Tian et al., 1997; Wiesener et al., 2003).

The differences in gene regulation by HIF-1 and HIF-2 respectively, have been investigated in different studies using gene expression microarrays and chromatin immunoprecipitation (ChIP). HIF-1 seems to be the most dominant of the two in most tissues but the regulation is tissue and cell type specific (Lofstedt et al., 2007). Erythropoiesis, angiogenesis and glycolytic

metabolism is regulated by several genes and is differently regulated by HIF-α subtypes in different cell types (Gordan and Simon, 2007). It seems that HIF-1α is needed for inducing glycolytic enzymes in response to hypoxia whereas HIF-2α can mediate many other hypoxia-responses in angiogenesis, growth and metastasis.

Previous studies have not found selective binding of HIF-1α or HIF-2α to specific loci but rather the binding to different loci seemed to be cell type specific. Recently differences in HIF-1α and HIF-2α regulated gene transcription in the breast cancer cell line MCF-7 was reported, stating that despite a large overlap in HIF-α isoform binding there were substantial differences in gene regulation with HIF-2α contributing very little to the overall HIF response (Mole et al., 2009). It seemed that HIF-1α was the most important regulator for adaptation to acute hypoxia.

Differences in HIF-α subunit regulation by time and oxygen levels in neuroblastoma has been reported (Holmquist-Mengelbier et al., 2006), suggesting that HIF-1α is more important in acute hypoxia whereas HIF-2α is more important in response to chronic hypoxia and that HIF-2α is also stabilized at "normal" end-capillary O$_2$ levels of 5%. The time- and oxygen dependent regulation of HIF-1α and HIF-2α seem to be relevant in breast cancer as well as reported in this thesis (paper IV). HIF-1α and HIF-2α expression patterns in tumors also differ. HIF-1α is most often found in perinecrotic zones, representing areas of hypoxia (Helczynska et al., 2003) whereas HIF-2α is also found in perivascular areas that are non-hypoxic (Holmquist-Mengelbier et al., 2006). These findings also imply different roles of HIF1α and HIF-2α in tumor progression.

Clinical Impact of Tumor Hypoxia

Assessment of tumor hypoxia *in vivo* can be performed using a computerized pO$_2$-histography system with O$_2$-sensing electrodes (Vaupel et al., 1991) and has been used to measure pO$_2$ in solid tumors of various origins. Using these techniques tumor oxygenation has been shown to correlate to poor prognosis in patients with soft tissue sarcoma (Brizel et al., 1996), cervical cancer (Dunst et al., 2003) and head and neck cancer (Brizel et al., 1997; Nordsmark and Overgaard, 2004). When evaluating hypoxia using HIF-1α and HIF-2α expression as a substitute marker for hypoxia, correlations to impaired patient survival has been reported for several different tumor forms (Semenza, 2010).

In breast cancer the correlation between HIF-1α expression and prognosis is not clearcut. Several independent studies have reported that high HIF-1α expression correlates to impaired survival (Dales et al., 2005; Trastour et al., 2007; Vleugel et al., 2005; Yamamoto et al., 2008) but correlations to important tumor characteristics like size, grade, stage, nodal status, hormone receptor status and HER2 status differ. Some studies have found a correlation only in subgroups, i.e. HER2 positive (Giatromanolaki et al., 2004), ER postive (Generali et al., 2006), lymph node positive (Kronblad et al., 2006; Schindl et al., 2002) and lymph node negative (Bos et al., 2003) tumors. The impact of HIF-1α expression on prognosis is complex which is illustrated in paper IV of this thesis where two different breast cancer cohorts were evaluated for HIF-1α expression and correlation to survival was inconsistent.

HIF-2α has also been evaluated in relation to survival in different tumors and high

expression of HIF-2α has been reported to correlate to poor prognosis in non-small cell lung cancer (Giatromanolaki et al., 2001), colorectal cancer (Yoshimura et al., 2004), head and neck cancer (Koukourakis et al., 2006) and neuroblastoma (Holmquist-Mengelbier et al., 2006). In breast cancer HIF-2α expression in tumor-associated macrophages was associated to increasing tumor grade, high tumor vascularity and poor overall survival (Leek et al., 2002). High HIF-2α expression in tumor cells of breast cancer was also reported to be associated with increased vascular density, increased HER2 membrane expression and extensive nodal metastases (Giatromanolaki et al., 2006). Recently another study evaluating HIF-2α in breast cancer reported improved survival in patients with tumors with high HIF-2α expression and association with luminal tumor differentiation (Stiehl et al., 2011).

To conclude HIF1α and HIF-2α expression and associations to prognosis in cancer patients is far from fully elucidated and since the regulation of HIFs and their target genes is differently regulated in a tissue- and cell specific manner, further research should address these questions.

Tumor hypoxia and treatment resistance: Hypoxia is also associated with resistance to radiotherapy and chemotherapy. In chemotherapy resistance, direct and indirect effects have been proposed. The direct effects reassemble resistance to radiotherapy (discussed in the next section) in the sense that it is the lack of O2 in the tissue that prevents the cytotoxic effects of the cytostatics. The indirect effects of hypoxia can be due to abnormal vasculature impairing drug delivery and reducing drug diffusion, cell cycle arrest (most cytotoxic drugs target proliferating cells), clonal selection of resistant cells, and changes in

pH (Shannon et al., 2003).

The fact that hypoxic cells are more resistant to radiotherapy than normoxic cells has been known for a long time and several studies have reported impaired survival after radiotherapy treatment in patients with hypoxic tumors of various origins (Moeller et al., 2007). The mechanisms behind hypoxia-induced radiotherapy resistance have since long been considered a result of the oxygen enhancement effect. DNA damage is created by direct ionization from radiation or is induced by interaction of oxygen radicals formed by ionization of water surrounding the DNA. DNA strand breaks result and can lead to fatal chromosomal aberrations, if not repaired. In the presence of oxygen DNA breakage can be stabilized and repair mechanisms are not as efficient (Moeller et al., 2007). Hence that the lack of oxygen in hypoxia contributes to that the radiation-induced damage to the cells is not as extensive as in the presence of oxygen. Studies from mid of last century have shown that oxygenated cells are 2,5- to 3 times more sensitive to radiotherapy than hypoxic cells (Moeller et al., 2007).

To counteract the effects of hypoxia-induced resistance different approaches to increasing tumor oxygenation have been evaluated in the clinical setting, i.e. increasing oxygen delivery by hyperbaric oxygen treatment, blood transfusions or rhEPO, or by using different hypoxia-sensitizers or hypoxic cytotoxins (Moeller et al., 2007; Overgaard, 2007). Recently a meta-analysis of clinical trials using hypoxic modification in radiotherapy of head and neck cancer reported improved therapy response and survival (Overgaard, 2011). An experimental study reported that hyperbaric oxygen induced differentiation and less aggressive tumors in a rat adenocarcinoma model (Moen et al., 2009).

With increasing knowledge of the biological effects hypoxia has on tumors and the importance of HIFs in mediating these effects, the mechanisms of hypoxia-induced resistance have proven to be more complex than initially believed. HIF induced resistance can be due to activation of drug resistance genes, suppression of apoptosis and drug-induced senescence, changes in cell cycle regulation, induction of autophagy, inhibition of DNA damage and mediating metabolic reprogramming. Today several compounds that inhibit HIF pathways in different ways are being evaluated in clinical trials (Rohwer and Cramer, 2011).

HIFs in tumor progression and metastasis: As mentioned above hypoxia has impact on many central cellular processes in malignant progression, i.e. proliferation, apoptosis, angiogenesis, metabolism, genetic instability etc. Recent research has focused on HIF roles in metastasis, tumor stem cell characteristics and epithelial to mesenchymal transition (EMT). HIF has been reported to regulate several genes important for EMT, which is a process where cells lose there epithelial phenotype to aquire mesenchymal features rendering the cells ability to invade and migrate away from the primary site (Majmundar et al., 2010). Hypoxia has also been reported to increase the tumor stem cell fraction in different tumors and promote acquisition of a stem-like state (Heddleston et al., 2010).

Different roles of HIF-1α and HIF-2α has been proposed and in gliomas HIF-2α was only expressed in the tumor stem cell population whereas HIF-1α was present in both stem and non-stem cell tumor populations and stabilized at lower O_2 levels than HIF-2α. Several genes associated with the hypoxic response in normal cells were shown to have higher expression in the stem like sub-population and that HIFs were required for tumor stem cell survival and tumor propagation (Li et al., 2009).

It has been suggested that tumor stem cells might reside in hypoxic niches within a tumor in a similar way that normal stem cells reside in the hypoxic niche in bone marrow in order to keep their non-differentiated state (Gilbertson and Rich, 2007). In neuroblastoma and breast cancer hypoxia has been reported to induce a de-differentiated state (Jogi et al., 2002; Helczynska et al., 2003) and that HIF-2α is associated with a more immature phenotype and correlates to poor patient survival (Holmquist-Mengelbier et al., 2006; Pietras et al., 2009). Hence it has been proposed that HIF-2α may alter genetic activity of cancer cells to a more stem-like phenotype (Heddleston et al., 2010). Tumors that contain large regions of hypoxia are more likely to metastasize and increasing evidence for linking hypoxia to metastasis via HIF regulated gene transcription has been reported (Chaudary and Hill, 2006).

Present Investigations

Aims

To unravel a putative correlation between Hb levels and tumor response in patients with metastatic breast cancer and anemia, treated with rhEPO (paper I)

To investigate potential roles of EPO and EPOR in breast cancer and possible correlations to treatment prediction and prognosis (paper II)

To unravel effects of rhEPO on EPOR expressing breast cancer cells and to investigate EPOR functionality (papers II & III)

To investigate roles of hypoxia-inducible factors in breast cancer and possible correlations to prognosis (paper IV)

Results and Discussion

Paper I

Increasing Hb levels correlate to tumor response

To investigate a possible correlation between increasing Hb levels and response to anti-tumoral treatment we used data from a prospective randomized multi center trial evaluating two different doses (LD: low dose; 1000U versus HD: high dose; 5000U, three times per week) of rhEPO (Neorecormon®, epoetin-β) treatment in 180 patients with metastatic breast cancer and anemia (Hb < 110 g/L). Effects on Hb levels and Quality of Life (QOL) had been evaluated and reported elsewhere (Olsson et al., 2002). During the study, response to tumor treatment was also evaluated and tumor response and Hb levels (base line and after 8-12 weeks of rhEPO treatment) data were available for 109 patients.

When evaluating tumor response in relation to increases in Hb levels we found that the patient group that responded to anti-tumoral treatment also had a significant mean increase in Hb levels (99,8-115,3 g/L) after 8-12 weeks of rhEPO treatment, whereas the patient group with no tumor response did not have a significant mean increase (97,9-100,2 g/L). The increase in Hb levels in the tumor response group is probably due to rhEPO treatment but might also be affected by the positive tumor response, as such. Patients with anemia who respond to their anti-tumoral treatment often gain an increase in Hb levels due to the treatment counteracting negative effects of the tumor disease on erythropoiesis. This observation is supported by the finding that in the historic controls of this study, the tumor response group also had a significant increase in Hb levels (104,7-112,1 g/L) even though they had not received any rhEPO treatment. However blood transfusions were not registered in the control group and might also have contributed to increases in Hb levels. Interestingly the study patient group that did not respond to antitumoral treatment did not have an increase in Hb levels during rhEPO treatment. Either these patients were not able to increase their erythropoiesis in response to rhEPO treatment or a potential beneficial effect of rhEPO treatment on Hb levels was counteracted by tumor progression repressing erythropoiesis.

Further subanalyzes of changes in Hb levels showed that in the patient group with the highest increase in Hb levels, a larger proportion of patients also responded to their antitumoral treatment, and it was a significant linear association between increases in Hb levels and tumor response. This further strengthens the association between increasing Hb levels and tumor response. It is difficult to evaluate how much of this effect that is due to rhEPO induced increases in Hb levels and how much of the Hb increase can be due to effects of antitumoral treatment.

However, sub-analyses of the two dose groups (HD and LD) showed that there was a larger proportion of patients with tumor response in the HD group and also a larger increase in Hb levels, suggesting that increasing Hb levels with rhEPO also has a positive effect on tumor response. Theoretically this effect can be due to that increasing Hb levels positively affects tumor oxygenation and counteracts negative effects of hypoxia. Hypoxia is associated

with a more aggressive phenotype and tumor resistance, and by reversing these phenomena a positive effect of tumor treatment might be enhanced. Since breast cancer cells also express EPOR it is possible that rhEPO can have a direct effect on tumor cells, although this has not been supported by our *in vitro* experiments (Paper III).

However, since survival data has not been registered for this rhEPO study group it is not known whether the increased tumor response in the rhEPO treated group is also accompanied by an improved survival. Survival data from a large trial evaluating rhEPO treatment in patients with metastatic breast cancer, having survival as a primary endpoint showed impaired survival in rhEPO treated patients compared to controls (Leyland-Jones, 2003). This might be contradictory to our results but since we do not have comparable data a conclusive answer cannot be obtained. Another difference in these two studies is that the patients in our study had lower Hb levels at baseline and it has been proposed that rhEPO might be more beneficial in patients with lower Hb levels, but more detrimental in patients with higher Hb levels. There might also be imbalances in patient and treatment characteristics between treatment groups in the two studies that potentially could affect outcome.

One major problem in evaluating effects of rhEPO in our study was that no placebo group was included in the original study design. When the study was designed it was considered unethical to have a placebo group since rhEPO had been documented to have a beneficial effect on Hb levels and QOL, and therefore a low dose (LD) of rhEPO was evaluated compared to the standard dose (HD). For comparison a control group was evaluated after the study inclusion was completed. Therefore a strict comparison between the control and the rhEPO treated groups cannot be performed although the control group could serve as a reference when evaluating tumor response in the original study group.

To conclude our results support a positive correlation between increasing Hb levels and tumor response in patients with metastatic breast cancer and anemia. Potential effects on survival was however not evaluated and since other studies have reported detrimental effects of rhEPO treatment on survival in patients with metastatic breast cancer there is no clear consensus on what effects rhEPO might have in this setting. In the literature there are conflicting data on rhEPO effects on survival in cancer patients (discussed in background, Anemia in Cancer section) and future research will continue to address this important question. One potential explanation of the contrasting data could be due to subgroups of patients having a beneficial effect of rhEPO treatment whereas in other subgroups, rhEPO treatment is detrimental. Therefore it is of great importance to further unravel potential effects of rhEPO in cancer patients.

Furthermore, EPOR expression in tumor cells as well as other cells in the tumor microenvironment should be evaluated and possible effects of rhEPO treament on these cells as well as systemic effects should be investigated.

Papers II and III

EPOR expression in breast cancer

In light of several clinical trials reporting adverse effects of rhEPO on survival in cancer patients and accumulating evidence of EPOR expression outside the hematopoietc context, we set out to investigate EPOR expression and function in breast cancer.

In the beginning of this century, a few studies reported EPOR expression in breast cancer (Acs et al., 2001; Arcasoy et al., 2002) but at this point no possible correlations to prognostic or treatment predictive information were evaluated. We found EPOR mRNA expression in breast cancer cell lines, tumor specimens and also in normal breast tissue. The mRNA expression levels differed between samples and were further only slightly increased in response to hypoxia (paper II). To evaluate protein expression we used the commercially available C-20 antibody (sc-695, Santa Cruz) that was most commonly used in previous studies evaluating EPOR expression in tumors. The specificity of this antibody had been questioned (Elliott et al., 2006) but we validated the sensitivity of the antibody using si-RNA targeting EPOR to knock down EPOR expression in control cells as a negative control. Using our assays we could show the sensitivity of the antibody to detect EPOR protein in breast cancer cells by Western Blot and IHC techniques (paper II).

As the specificity of the C-20 antibody had been questioned and to further improve EPOR identification we produced an in-house anti-EPOR antibody, detecting the full-length EPOR protein (paper III). In Western Blots this antibody was more specific compared to the C-20 antibody

rendering cleaner blots. However, in IHC stainings the background staining was not sufficiently reduced in negative controls (si-EPOR knockdown cells) and we are currently working on further improving the antibody to increase the specificity in IHC stainings. Using the new in-house antibody in Western Blot techniques, we verified EPOR protein expression in five different breast cancer cell lines and in five tumor samples. We found EPOR expression of varying levels in both ER+ and ER- cancer cells (paper III).

EPOR expression correlates to tamoxifen response and prognosis in breast cancer

Having established that EPOR is expressed in breast cancer we went on to evaluate possible correlations to important tumor characteristics and outcome in breast cancer patients (paper II). We therefore evaluated EPOR expression in breast tumors using a tissue micro array (TMA) from a randomized trial evaluating two years of adjuvant tamoxifen treatment versus no treatment, in premenopausal patients with stage II breast cancer. EPOR expression correlated to age, tumor size and tumor type, but there were no correlations to the important prognostic factors NHG and nodal status.

Interestingly there was a strong correlation between EPOR expression and tamoxifen response in hormone receptor positive tumors. Patients with tumors with low expression of EPOR had a beneficial effect of tamoxifen on recurrence-free survival (RFS), but in patients with tumors with high EPOR expression tamoxifen had no significant effect on RFS. Using a Cox proportional hazards model including a treatment interaction variable there was a

significant association between tamoxifen treatment response and EPOR expression that was still significant in multivariate analysis. This finding that high EPOR expression correlates to impaired tamoxifen response suggests that EPOR might be involved in tamoxifen resistance which will be further discussed below.

When evaluating EPOR as a prognostic variable the untreated control group was used. In the ER+ group, patients with tumors with high EPOR expression had an improved RFS compared to patients with tumors with low EPOR expression. This correlation between EPOR expression and prognosis was still significant in multivariate analysis adjusted for other important prognostic factors, suggesting that EPOR expression is an independent prognostic factor in this patient group. However, in the ER- group there was no association between EPOR expression and RFS. This finding together with the finding of EPOR expression correlating to tamoxifen response suggest a link between ER and EPOR, which might consist of a cross-talk between the respective signaling pathways of these receptors. Our findings that EPOR expression yielded prognostic information in patients with ER+ tumors, were further supported by data from a clinical breast cancer micro array study, in which high EPOR mRNA levels correlated to favorable outcome.

Whether EPOR expression is only a marker of tumors with a less aggressive phenotype or whether EPOR has a significant impact on tumor behavior remains to be seen. However, the fact that EPOR expression correlates to prognosis as well as tamoxifen response implies that it has a function in breast cancer that needs to be further investigated. In contrast to our data another study evaluating EPOR expression in breast cancer has found that high EPOR expression correlates to impaired prognosis (Pelekanou et al., 2007). However in this study EPOR expression was evaluated in a heterogenous population of breast cancer patients with varying stages and a majority of patients had received pre- and post-operative chemotherapy. No sub-analyses were made with respect to hormone receptor status and therefore the results are difficult to compare with our results in paper II. It is also difficult to evaluate prognostic information in a patient group that is heterogeneous and has received antitumoral treatment since this will have significant effects on the prognosis. The contrasting results of these studies illustrate that EPOR can have different prognostic and predictive value in different subgroups. In the search for new and improved cancer therapies it is of great importance to find subgroups of patients where a specific marker or combinations of markers can serve as prognostic and treatment predictive factors and eventually also represent a treatment target.

Undetectable EPO protein in EPOR expressing breast cancer cells and no effect of rhEPO stimulation

EPO has been shown to have an autocrine/paracrine function in CNS and the cardiovascular system (Genc et al., 2004; Santhanam et al., 2010) and a potential similar function has been suggested for tumor cells (Hardee et al., 2006). We therefore analyzed EPO mRNA levels in different breast cancer cell lines and found very low levels in normoxia that increased during hypoxia (Paper II). However when using a sensitive ELISA to evaluate protein expression, no EPO protein could be detected in the supernatant or intracellularly in the different breast cancer cells analyzed. This suggests that the EPO protein either is not produced at all or in very low amounts. Another study has reported EPO levels

in different tumor cell lines at very low amounts (Yasuda et al., 2003) and the question whether these extremely low EPO protein levels has a biological impact on tumor cell behavior remains to be elucidated. As discussed in the background, EPO and EPOR section, several studies have reported effects on tumor cell proliferation by rhEPO stimulation. We further investigated effects of rhEPO stimulation on cell proliferation and also rhEPO stimulation in combination with doxorubicin to evaluate a possible effect of rhEPO rescuing cells from cytotoxic cell death. To summarize, no substantial effect on proliferation or cytotoxic cell death was seen when using different doses of rhEPO under different time points (paper III and data not shown). To further evaluate possible effects of rhEPO stimulation on EPOR signaling we evaluated activation of the classical EPOR signaling proteins JAK2, STAT5, AKT and ERK1/2 (paper III). In general, no significant effect on downstream signaling was seen as compared to rhEPO-stimulated erythroleukemic UT7 cells used as a positive control. Exceptions were slight increases in pERK1/2 in two cell lines and a decrease in pAKT in one cell line. The control cells were untreated and there is a theoretical possibility that rhEPO vehicle components are responsible for these minute changes in signaling protein modifications. Interestingly high baseline levels of pAKT and pERK were seen despite serum deprivation suggesting high growth promoting signaling in the breast cancer cell lines used.

Regarding rhEPO stimulation *in vitro*, contrasting results have been reported (discussed in background, EPO and EPOR section) and it is difficult to draw any definitive conclusions from these studies. However, our negative data show that effects of rhEPO stimulation cannot be generalized and does not occur in a

cell- and experiment independent way since it cannot be repeated under different experimental settings. Furthermore, the rhEPO doses used in *in vitro* experiments to increase proliferation have been very high and at supraphysiological levels why the physiological relevance of these doses can be questioned. A recent study reported effects of rhEPO treatment in breast cancer xenograft transplants and showed increased metastasis frequency in rhEPO treated mice compared to controls (Hedley et al., 2011). Interestingly, rhEPO treatment had no effect on the primary tumor suggesting that the important effects of rhEPO on metastasis might not be due to direct proliferation stimulating effects on tumor cells. Potential effects of rhEPO are much more complex in *in vivo* models since rhEPO also might have effects on other cells in tumors, i.e. endothelial cells, stromal cells, immune cells and possibly also systemic effects on the immune system.

Functional EPOR expressed in breast cancer

The correlations of EPOR expression to tamoxifen response and prognosis suggest that EPOR is functional in breast cancer cells. However, since we could not find any signs of EPOR activation during rhEPO stimulation in our *in vitro* assays, we went on to further investigate possible EPOR functions by knocking down the EPOR. Using si-RNA targeting the EPOR we analyzed effects of EPOR knockdown on cell proliferation in vitro. Interestingly in ER+ cell lines EPOR knockdown had a pronounced effect on proliferation, evaluated by MTT assays and Ki67 stainings, whereas in ER- cell lines EPOR knockdown did not affect proliferation (paper III). It seemed that the effect of EPOR knockdown in ER+ breast cancer cells was related to basal levels of EPOR expression, since a more pronounced effect on proliferation was seen

in the cancer cells with the highest EPOR expression. These results support a function of EPOR in ER+ breast cancer cells.

To further investigate a possible relation between EPOR and ER we analyzed ER activity in ER+ breast cancer cells and found that EPOR knockdown substantially reduced ER activity (paper III). Furthermore EPOR knockdown also improved the effect of tamoxifen on ER activity by reducing ER activity more, in relative terms, in the EPOR knockdown cells compared to controls.

To conclude we found that EPOR expression in breast cancer is associated with impaired tamoxifen response in patients with hormone receptor positive tumors. A relation between EPOR and ER was further strengthened by the fact that EPOR expression only generated prognostic information in ER+ tumors. Furthermore EPOR knockdown impaired proliferation only in ER+ breast cancer cells and also impaired ER activity and improved tamoxifen response. Taken together these results imply a crosstalk between EPOR and ER signaling pathways of which the molecular mechanisms remain to be elucidated. In our *in vitro* experiments we did not find any phosphorylation of the EPOR and no activation of classical down stream signaling proteins (JAK2, STAT5, MAPK) involved in the classical EPOR pathway, after rhEPO stimulation. EPO knockdown did not affect proliferation in ER+ breast cancer cells, further strengthening an EPO-independent function of EPOR in these cells.

These results suggest that EPOR activity in tumor cells might differ from the classical EPOR signaling in erythropoiesis. Another finding that supports differences in EPOR function in the different cell contexts is that in the knockdown experiments EPOR protein was rapidly reduced in the UT-7 cells whereas EPOR protein levels were considerably more stable in breast cancer cells, also indicated by the less efficient knock down of EPOR in breast cancer cells. This implies that the turnover mechanisms of EPOR differ depending on the cell context.

The effect of EPOR knockdown on proliferation suggests that EPOR drives proliferation in ER+ breast cancer cells. However high EPOR expression correlates to improved prognosis in ER+ tumors from untreated breast cancer patients and this might seem contradictory since proliferation is associated with enhanced tumor growth and thereby also a worse prognosis. As an example high Ki67 expression has been reported to be associated with impaired prognosis in ER+ tumors (Klintman et al., 2010). However, proliferation is a complex process, and the most fundamental trait of cancer cells involves their ability to sustain chronic proliferation (Hanahan and Weinberg, 2011). The control of growth promoting signals is substantially deregulated in cancer compared to normal tissues, and the regulation of proliferation is multifactorial. *In vitro* experiments cannot reflect the complexity of a tumor microenvironment, which besides tumor cells consists of stromal cells, extracellular matrix, immune cells, and also is dependent on systemic factors. Therefore it is difficult to speculate in what implications the *in vitro* finding of EPOR knockdown effects on proliferation might have in the *in vivo* tumor environment. To further address this issue, effects of EPOR knockdown should be evaluated in relevant *in vivo* models, for example xenograft mouse models. Another way to try to elucidate EPOR involvement in proliferation of ER+ breast cancer cells is by microarray data analysis of gene expression in EPOR knockdown cells, which we will perform in a near future.

Since our results further imply a possible role for EPOR in tamoxifen resistance future research should focus on investigating possible molecular mechanisms behind this observation with the aim to target the EPOR pathway in order to improve tamoxifen effects in breast cancer.

Paper IV

Increasing research efforts are focusing on the roles of HIFs in cancer and most recent reports discuss different roles of the two HIF-α subunits. In paper IV we set out to investigate the expression of HIF-1α and HIF-2α in breast cancer.

HIF-2α correlates to poor prognosis and distant recurrence

To investigate potential correlations between HIF-1α, HIF-2α and important clinical variables we evaluated the protein expression of these subunits in two consecutive cohorts of breast tumors. Interestingly high HIF-2α expression correlated to impaired prognosis in cohort I, and this correlation was independent of other important prognostic factors, as shown in the Cox regression multivariate analysis. This correlation was supported by a trend towards an impaired survival in patients with tumors with high HIF-2α expression in cohort II. On the contrary a recent study reported a correlation of high HIF-2α and improved survival in breast cancer patients and an association with luminal tumor differentiation (Stiehl et al., 2011). Since the patient and tumor characteristics were not described in that study it is difficult to directly compare it to our results. However the contradictory results further imply that the correlation of HIF-2 to prognosis is complex and may be tumor and cell type specific.

In our study there was also a correlation between high HIF-2α expression and distant recurrence in cohort I, indicating a role of HIF-2α in the metastatic process. This observation is further supported by the findings of HIF-2α correlation to tumor stem cells/tumor-initiating cells of neurally derived tumors (Heddleston et al., 2010;

Pietras et al., 2009) and upregulation of genes important for invasion and metastasis (Majmundar et al., 2010).

To further elucidate potential roles of HIFs in metastasis we will analyze HIF-2α expression in circulating tumor cells (CTCs) from patients with metastatic breast cancer. Circulating tumor cells are tumor cells captured and enriched from the circulation and thereby have the potential of metastasis. Expression of HIF-1 and VEGF has recently been reported in CTCs from breast cancer patients (Kallergi et al., 2009) but so far no correlations to prognosis or treatment prediction have been reported and as for HIF-2α expression no data has been reported.

HIF-1α correlations to prognosis

The impact of HIF-1α expression on prognosis is complex which is illustrated in paper IV, where the two different breast cancer cohorts were evaluated for HIF-1α expression and correlation to survival was inconsistent. In cohort I HIF-1α expression correlated negatively to NHG, which was rather surprising, since most HIF-1α data reported indicate a relation to more aggressive tumors. There was also a trend towards a negative correlation to node status. These two correlations to NHG and node status, which are two of the strongest prognostic factors in breast cancer, might explain the lack of correlation to survival. In fact there was a trend towards an improved survival in patients with high HIF-1α expression, however the correlation was not significant in statistical analysis. On the contrary, in cohort II HIF-1α expression correlated positively to NHG status and had a negative association to survival.

Since HIF-1α and prognostic relevance is not clear-cut in the context of breast cancer (discussed in background, hypoxia section) the contradictory findings in this study as well as for other studies is not completely unexpected. The differing results of HIF-1α correlations to prognosis in different studies can be due to differences in patient and tumor materials but also due to different IHC stainings and evaluation methods or small factual differences. Rather small differences of a protein expression on the cellular level can potentially have impact on prognostic analyses if the tumor cohort studied is small. To overcome this problem larger tumor materials should be investigated. The HIF proteins are relatively unstable and needs optimized experimental procedures as well as proper positive and negative staining controls. The fact that in our study the same antibody and experimental procedures were used, indicate that the prognostic relevance of HIF-1α expression in breast cancer is complex and variable due to different tumor materials, i. e. its prognostic relevance is not as substantial as was initially believed. One can of course argue that the two different cohorts used in our study were collected ten years apart and that differences in time from surgery to fixation and other technical circumstances might have changed during these years. Since the HIF proteins are relatively unstable the "real" HIF-expression in viable tumor tissue at time of surgery might fade during following procedures and therefore not conclusively be representative.

Different roles of HIF-1α and HIF-2α in breast cancer

The observation that there was no correlation between HIF-1α and HIF-2α protein expression in tumors from two different breast cancer cohorts suggests that the HIF-α subunits are regulated in different ways and that hypoxia might not be the dominant factor in regulating both these subunits.

HIF-2α has been proposed to drive a pseudo-hypoxic phenotype under normoxic conditions (Pietras et al., 2010), correlated to aggressive behavior that may in part explain these findings. The differences in time- and oxygen level dependent regulation of HIF-1α versus HIF-2α reported in paper IV, further supports that these two proteins are differentially regulated. It seems that HIF-1α is more important in the response to acute hypoxia whereas the role of HIF-2α is more pronounced at chronic hypoxia. This observation is also supported by data in neuroblastoma from our group as well as others (Holmquist-Mengelbier et al., 2006; Lin et al., 2011).

Our findings also suggest that both subunits are involved in VEGF regulation but also in that context the roles of the two different HIF-α subunits seem to differ. HIF-1α knockdown at acute hypoxia (4h) had a greater impact on VEGF levels than knocking down HIF-2α, further supporting a more dominant role of HIF-1α in the acute hypoxic response where as HIF-2α is more important in the chronic response. In the evaluation of protein expression in the breast tumor material we also found a stronger correlation between HIF-1α and VEGF, and although HIF-2α was also correlated to VEGF this correlation was not significant, supporting a role of HIF-1α as the more dominant HIF in VEGF regulation in breast cancer.

The results from this study suggest that the regulation of HIF-1α and HIF-2α is more complex than what was initially believed, and in addition to the differences in the hypoxic regulation of the proteins, other factors than hypoxia seem to have a strong impact. Recent data also suggest that HIF-2α is more

dependent on transcriptional regulation than HIF-1α (Holmquist-Mengelbier et al., 2006; Lin et al., 2011) which further supports differences in regulation.

To conclude, HIF-1α and HIF-2α have different roles in breast cancer, both in relation to hypoxia, but also in relation to tumor progression and prognosis. To further evaluate the interrelationship between hypoxia and HIF-α subunits *in vivo* in breast cancer additional markers for physiologic hypoxia could be used. HIF-1α and HIF-2α protein expression needs to be evaluated in larger breast tumor cohorts to verify the potential prognostic value of these factors.

With extensive adjuvant therapies it is difficult to discriminate between prognostic and treatment predictive values of different factors in relation to survival. Since HIF-1α and HIF-2α seem to have both independent and overlapping functions in breast cancer it is important to evaluate them separately in order to find potential subgroups where either subunit might have prognostic impact. Since hypoxia is associated with aggressive tumor behavior, tumor resistance and impaired prognosis and the HIF proteins are the most important mediators of the hypoxic response it is tempting to assume that both HIF-α subunits should be negative prognostic markers in general. However recent research underline the differences in regulation of these subunits, and HIF-2α association with tumor stem cell characteristics and distant metastasis imply that the functions of the different HIFs are more complex. Therefore it is of great importance to further elucidate HIF-1 and HIF-2 function in breast cancer with the overall aim of finding subgroups of patients that could benefit from HIF-targeted therapies.

Conclusions

Increasing Hb levels correlate to tumor response in rhEPO treated patients with metastatic breast cancer and anemia (paper I)

EPOR protein is expressed in breast cancer (papers II & III)

EPOR expression correlates to prognosis and tamoxifen response in patients with hormone receptor positive breast cancer (paper II)

Functional EPO-independent but ER-dependent EPOR in breast cancer, modulates ER activity and tamoxifen effects (paper III)

rhEPO stimulation has no effect on breast cancer cell proliferation, survival or classical EPOR down stream signaling *in vitro* (paper III)

HIF-2α expression correlates to impaired prognosis and distant recurrence in breast cancer (paper IV)

HIF-1α and HIF-2α are differently regulated in a time- and oxygen dependent manner in breast cancer and have different roles in VEGF regulation (paper IV)

General Discussion

Anemia, Hypoxia and EPO

Since anemia has been associated with impaired prognosis in cancer patients it is tempting to believe that by treating anemia one can potentially also affect prognosis. This hypothesis has been tested in various ways, both by using blood transfusions and rhEPO (see background, Anemia in Cancer section). Our results show that there is a positive correlation between increasing Hb levels and tumor response (paper I) suggesting that raising Hb levels might improve outcome. However, recent reports from other transfusion and rhEPO trials seem to point in another direction, that these treatments might in fact be detrimental to patients and increase mortality and tumor progression.

It seems that the biology of cancer and correlations to anemia and hypoxia are more complex than initially believed. There is still no consensus on whether hypoxia is a consequence of rapid progression of aggressive tumors or if tumor aggressiveness is a consequence of hypoxia. The truth probably lies somewhere in between. Hypoxia can be the result of rapid tumor growth and can also impede a more aggressive tumor phenotype and potentially the tumor will have advantage of the hypoxia by increasing features important for progression and metastasis and treatment resistance. There is a risk that by treating anemia, the increased oxygenation will induce survival and proliferation of tumor cells that have already acquired a more aggressive phenotype. On the other hand if hypoxia is in fact promoting a tumor stem cell phenotype, increasing O_2 levels could reverse this feature, leading to increased differentiation of tumor cells.

However, there seem to be different mechanisms of the hypoxia-induced response. One part of the hypoxic response is induced by the reduced levels of O_2, and that is responsible for the adaptation of tumor cells to the more hostile environment in order to survive. Another part of the system seems to be regulated by other factors, for instance increased cellular signaling in response to different growth factors. Since HIF-2 can be stabilized even at "normoxic" end-capillary O_2 levels in perivascular areas of tumors, it has the potential to induce tumor progression independent of oxygen regulation, hence that anemia treatment in order to improve tumor oxygenation will not affect these phenomena.

Using HIF-1α and HIF-2α as markers for hypoxia is not uncomplicated since it seems that they have different roles in the hypoxic response and probably also different roles in tumor development and progression that are more complex and may involve other features than modulating effects of impaired oxygenation. The different prognostic impact of these respective HIF-α subunits reported from different studies (discussed in paper IV section) also highlights the importance of further characterization of their functions and relevance in breast cancer. Eventually increased knowledge of their respective roles in tumor development and progression will provide a platform for development of successful treatments targeting the HIF pathways.

When considering aspects of anemia and hypoxia in cancer from a broader perspective, one has to take into account

various systemic effects as well. Interactions with the tumor microenvironment, immune system and other components of the host environment, are not easily assessed in either clinical or pre-clinical investigations. The question whether anemia is an independent prognostic factor and if reversal of anemia can improve prognosis, or whether anemia is a consequence of a more advanced disease in the individual remains to be seen.

With regard to rhEPO treatment, EPO was initially proposed to be a hormone with one function only, to increase erythropoiesis. However, as discussed in this thesis EPO has additional functions, which still are not completely understood. Further investigations are needed in order to find out more about these complex functions, and in the future potentially produce modified rhEPO substances that exclusively affect erythropoiesis or other specific functions.

The ideal way of trying to counteract the negative effects of anemia and hypoxia in the cancer context would be to prevent them from occurring. However, that is problematic since they often occur as a consequence of the tumor disease and treatment and as a result of interactions of different systems in the organism. Recent trials using rhEPO treatment to prevent anemia in cancer patients receiving chemotherapy also reported increased tumor progression and mortality in rhEPO treated patients compared to controls. However it is not possible to evaluate potential effects of raising Hb levels independent of other non-hematopoietic effects of rhEPO. Therefore we will have to focus our efforts on increasing our understanding of the complexity of the interactions of tumor biology, anemia and hypoxia in order to find new and improved ways to counteract the negative effects.

EPOR in Cancer

Initially the EPOR was identified on erythrocytic precursor cells and believed to only play a part in erythropoiesis. However, increasing research efforts are focused on uncovering the function of the EPOR in different organs and also in various tumor forms. Since EPOR expression has been found in many different organs it is tempting to believe that it also has a function in these different tissue contexts, which has also been reported (discussed in background, EPO and EPOR section).

We initially started to investigate EPOR expression in breast cancer due to conflicting data of rhEPO treatment effects on survival. Since the function of EPO/EPOR signaling in erythropoiesis was established the obvious hypothesis was that the same signaling effects would be seen in breast cancer. However last years research in this field suggest that EPO and EPOR functions in the cancer context seem to differ from that in erythropoiesis. Our results suggest that EPOR can have an EPO-independent function in breast cancer cells (paper III), which further complicates unraveling the potential relevance of EPOR expression in tumor cells. Since ER status is an important feature in breast cancer dividing the heterogeneous breast cancer disease into two groups (ER+ and ER-) with different characteristics it is not difficult to believe that EPOR signaling pathways could be differently regulated in the context of ER status. The finding of estrogen regulated EPO/EPOR signaling in the female reproductive organs also suggest an interaction between ER and EPO/EPOR signaling respectively.

It is of great importance to further elucidate potential EPO-dependent and independent EPOR functions in the cancer context, and how these functions might contribute to tumor development and progression, not only in tumor cells but also in other important components of the tumor microenvironment. Our findings that EPOR correlates to tamoxifen response in hormone receptor positive breast cancer will be further addressed in future studies with the overall aim of trying to find potential treatment targets that can improve tamoxifen effects in breast cancer patients.

To summarize this thesis I will highlight the complexity of anemia and hypoxia in the cancer context and that we need to carefully validate new treatment agents before using them outside of clinical trials. Regarding hypoxia the different roles of the HIFs need further characterization to improve our understanding of their respective function in cancer. When it comes to potential EPOR functions, these are only beginning to be unraveled and increased research is needed to further comprehend their relevance in cancer.

As for the entire field of future cancer research, the main challenge will be to increase our knowledge of tumor biology and interactions with the host, with the general aim of finding new treatment targets and defining subgroups of patients that will benefit from different treatment combinations in personalized medicine.

Populärvetenskaplig Samman-fattning

Bröstcancer är den vanligaste cancerformen bland kvinnor i Sverige och varje år insjuknar ca 7000 kvinnor i denna sjukdom. Tack vare förbättrad diagnostik och behandling överlever en majoritet av dessa kvinnor men det finns fortfarande en andel där sjukdomen ej är botbar och därmed dödlig. Därför finns fortfarande ett stort behov av nya behandlingsmetoder.

Anemi kallas i dagligt tal för "blodbrist" och uppstår när de röda blodkropparna i blodet inte kan transportera tillräckligt med syre till kroppens olika organ. Graden av anemi brukar värderas genom mätning av hemoglobinnivåer (Hb) i blodet, eftersom det är hemoglobinets uppgift att binda syre i röda blodkroppar. Många cancerpatienter utvecklar anemi, dels p.g.a. att cancersjukdomen i sig kan påverka bildningen av röda blodkroppar men även för att en vanlig biverkan av cancerbehandling (både cellgifts- och strålbehandling) är att benmärgen skadas och därför inte kan producera tillräckligt många blodkroppar. Anemi ger upphov till olika symtom såsom trötthet, yrsel och försämrad livskvalitet. Det finns också studier som visar att cancerpatienter med anemi har en sämre prognos jämfört med patienter som inte har anemi.

Anemi behandlas traditionellt med blodtransfusioner men sedan ett tjugotal år tillbaka används även rekombinant humant erythropoietin (rhEPO). Erythropoietin (EPO) är ett hormon som framför allt produceras av njurarna då syrenivån i kroppen blir för låg och dess främsta funktion är att stimulera produktionen av röda blodkroppar i benmärgen. Studier har visat att rhEPO-behandling kan höja Hb-nivåer och förbättra livskvaliteten hos cancerpatienter med anemi, varför detta läkemedel introducerades under 90-talet som ett alternativ till blodtransfusioner. Initialt rapporterades i studier att även överlevnaden hos cancerpatienter som behandlades med rhEPO kunde förbättras men senare studier har visat motsatt effekt d.v.s. att överlevnaden hos cancerpatienter som behandlats med rhEPO är sämre än för obehandlade patienter.

Vi har studerat sambandet mellan ökande Hb-nivåer och effekten av patientens tumörbehandling hos patienter med metastaserad bröstcancer och anemi som erhållit rhEPO-behandling. Våra resultat visar att det finns ett samband mellan ökande Hb-nivåer och förbättrat behandlingssvar avseende tumörbehandlingen (tumörrespons) hos dessa patienter. Teoretiskt skulle dessa resultat kunna förklaras av att man genom att öka bildningen av nya röda blodkroppar förbättrar syresättningen i kroppen och därigenom motverkar negativa effekter av syrebrist i tumören (se hypoxi-stycke nedan).

Effekter av rhEPO-behandling vid cancer kompliceras dock ytterligare av att studier rapporterat att det även finns EPO-receptorer (EPOR) i tumörceller samt på s.k. endotel-celler som bygger upp kärlväggen i kroppens blodkärl. Vid bildningen av röda blodkroppar utövar EPO sin effekt genom att binda till EPOR på cellytan på förstadier till röda blodkroppar vilket leder till aktivering av olika processer inuti cellen

på att HIF-2 är viktigt för tumörspridning och dessa fynd kommer att undersökas vidare genom att studera HIF-2- uttryck i cirkulerande tumörceller som kan återfinnas i blodbanan.

Att undersöka olika faktorer som kan ge information om prognos och förväntad behandlingseffekt (prediktion) är mycket viktigt inom cancerforskning. Genom att vidare karaktärisera funktionen hos prognostiska och prediktiva markörer kan man hitta nya mål för riktad cancerbehandling för att i en framtid kunna hitta nya läkemedel som kan angripa tumörcellerna på ett effektivt sätt.

Vad gäller effekter av rhEPO är det av yttersta vikt att vidare klargöra potentiella effekter av rhEPO-behandling på tumörer för att veta hur denna behandling påverkar tumörsjukdomen hos cancerpatienter med anemi.

Sambanden mellan anemi, hypoxi och cancer är komplexa och inte helt klarlagda varför det är viktigt att fortsatt utreda dessa frågor för att på sikt kunna förbättra behandlingen vid olika cancersjukdomar.

Acknowledgements

I would like to extend my gratitude to the following people…

Sven Påhlman, my supervisor, for believing in me, for support during all these years, and for introducing me to the never-ending fascination of tumor biology. I especially appreciate that you are a man of your word, your enthusiasm and optimism and ability to always find the positive aspect of things… and I'm glad I made it before you retire...

Lisa Rydén, my co-supervisor, for teaching me clinical and practical aspects of cancer research, for believing in me and giving me the opportunity to start the CTC-MBC-project.

My colleagues at CMP: *Siv* for invaluable help with IHC staining etc, and for creating such welcoming atmosphere in the lab and in our room. *Elisabeth*, for practical help and *Kristin* for invaluable help with administration…
Karolina H, Helen P, Annika J and Karin L for being my "senior" CMP friends and colleagues and for enjoyable company inside as well as outside the lab.
Susann for invaluable work in the EPOR-project and *Marika* for joining the project. We will unravel the EPO/EPOR mystery…
All former and present members of "Mol Med" and CMP for contributing to creating a stimulating, inspiring and enjoyable work environment at the lab.

My co-authors for fruitful collaborations. Especially *Karin Jirström* for help with TMA analysis and for contributing to bringing female power into the field of cancer research… and *Signe Borgquist* for preparing the way…

My colleagues at SOK for contributing to a friendly and creative atmosphere at the clinic. The *ST after work team* for great social activities. My clinical supervisor *Jakob Eberhard* for always finding time to support me.

Carsten Rose and *Mona Ridderheim*, my former and present head of department, for supporting the integration of cancer research and clinical oncology and for giving me the opportunity to combine the fascinating worlds of research and clinical work.

The CTC-crew: *Lisa, Lotta, Sara, Anette, Monika, Emma, Cia* and everyone else involved in the CTC-MBC trial at KFE and SOK. Thank you for your enthusiasm and devotion to this project. It has been great working on this project in parallel with finishing my thesis and I look forward to the continuation…

Eva and Pelle, my parents, for endless love and support in all different ways, for taking care of grandchildren and going on amazing travels together. For teaching your children that you can do anything you set your mind to…

Måns and Ling, my brother and his wife, for bringing an international atmosphere and lots of fun into our lives… and for introducing the "groggveranda"…

Christina and Sven, Caroline with family, my "in-laws", for providing a second family where I am always welcome and for all support.

My friends, for fun, laughter and support, for making life so enjoyable…

Theo and Elina, my wonderful children, for enrichening life... for being you and for always keeping me in the present, reminding me of what's really important in life. You are truly amazing…

Peter, my husband, for endless love and support. For being the love of my life and my best friend at the same time. I really appreciate your support of my work and for joining me on this rollercoaster ride of research…

All patients included in the studies for participation, and all patients at SOK, for always reminding me of the remarkable abilities of human nature.

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