Hypoxia and Hypoxia-Inducible Factors in Normal and Malignant Breast Epithelium

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Doctoral Dissertation, to be defended by due permission of the Faculty of Medicine, Lund University, Sweden at the Main Lecture Hall, Department of Pathology, Malmö University Hospital MAS, on the 29th of May 2008 at 09.15 am for the Degree of Doctor of Philosophy, Faculty of Medicine

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Abstract:

Breast cancer treatment is based on tumor and patient related factors such as tumor stage, grade, hormonal status, HER2 status, patient age, and family history to name a few. It is today widely acknowledged that hypoxia and hypoxia-inducible factors (HIF:s) contribute to tumor progression. We therefore set out to explore the impact of hypoxia on mammary epithelial differentiation and what consequences the hypoxic response might have on breast cancer development and behavior. We found that hypoxia induces a less differentiated, estrogen receptor (ER)-negative/cytokeratin (CK) 19-positive phenotype in mammary cancer cells in vivo and in vitro and suggest that hypoxia-induced dedifferentiation is one of the mechanisms behind hypoxia-driven malignant progression. Hypoxia was also found to significantly impair both morphological and functional differentiation of non-transformed, immortalized epithelial mammary cells grown in three-dimensional cultures. Heterogeneous ER expression in breast cancer was additionally found to be related to cyclin D1, a cell cycle regulator frequently over-expressed in breast cancer. As cyclin D1 expression was not affected by hypoxia, our findings suggest two separate mechanisms behind varied ER expression in breast cancer. The two pivotal regulators of hypoxic response, HIF-1α and HIF-2α, were analyzed in two cohorts of breast cancer patients and found to be un-correlated suggesting HIF-α subtype specific mechanisms of induction. Furthermore, HIF-2α was found to be an independent prognostic factor related to distant recurrence.

Key words: Breast, Breast cancer, Differentiation, Hypoxia, HiF-1, HiF-2, 3D-cultures, prognosis

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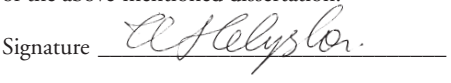
Signature  Date 2008-04-28

To my teachers

Das schönste Erlebnis ist die Begegnung mit dem Geheimnisvollen. Sie ist der Ursprung jeder wahren Kunst und Wissenschaft. Wer nie diese Erfahrung gemacht hat, wer keiner Begeisterung fähig ist und nicht starr vor Staunen dastehen kann, ist so gut wie tot: Seine Augen sind geschlossen.

The most beautiful thing we can experience is the mysterious. It is the source of all true art and all science. He to whom this emotion is a stranger, who can no longer pause to wonder and stand rapt in awe, is as good as dead: his eyes are closed.

Albert Einstein, Mein Weltbild, 1931

List of papers

This thesis is based on the following papers:

Paper I

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

Paper II

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

Paper III

Karolina Helczynska, Anna-Maria Larsson, Linda Holmquist Mengelbier, Erik Fredlund, Signe Borgquist, Göran Landberg, Sven Påhlman, Karin Jirström.
Hypoxia Inducible Factor-2α correlates to distant recurrence and is an independent predictor of poor outcome in invasive breast cancer.
Submitted

Paper IV

Karolina Helczynska, Annika Jögi, Elisabet Johansson, Siv Beckman, Christer Larsson, Sven Påhlman.
Hypoxia impairs morphological and functional organization of immortalized human breast epithelial cells in three-dimensional extra cellular matrix cultures.
Manuscript

Erratum: In Paper II the list of authors includes E. Påhlman. This name is a printing error, as no such person exists.

Contents

The Breast

Development

Human breast development begins in the fourth week of gestation with the formation of the mammary ridges; two parallel thickenings of the epidermis along the ventral sides of the body. In humans, most of the ridge regresses, with only a small residue on each side continuing to develop into a mammary gland. At birth, the male and female breast is a small ductal tree consisting of 15-25 lactiferous ducts with little or no secretory units. Postnatally, proliferation of the skin and subcutaneous connective tissue elevates the nipple and forms the areola. Thereafter, while the male breast remains largely unchanged, the female breast continues to develop and change through defined stages governed by steroid sex hormone levels, the process ending with involution at the onset of menopause [58, 65]. The macroscopic appearance of the developing breast during puberty has been described and classified by Tanner [119]. The system divides mammary maturation into one pre pubertal and four pubertal stages based on the gross appearance of the breast. Specifically, the pre-adolescent stage 1 is defined by isolated elevation of the nipple. Stage 2 is the breast bud stage: elevation of breast and nipple as a small mound, with concomitant enlargement of the areola diameter. During stage 3, further enlargement of breast and areola occurs, with no separation of their contours. Next, at stage 4, the areola and the nipple both project to form a secondary mound above the level of the breast. Stage 5, the mature stage, implies the projection of only the nipple, the areola having regressed to the general contour of the breast. Histologically at puberty the undeveloped ductal tree undergoes extensive branching, and small buds containing epithelial cells appear at the ends and sides of the smallest ducts. These are the future secretory entities [65]. Simultaneously, there is expansion of the stromal component, which reaches its fully developed state already at puberty [58]. However, there is no differentiation of the secretory unit and the gland remains immature until the completion of the first pregnancy when, due to rising levels of circulating estrogens and progesterone, there is rapid proliferation and differentiation of the alveoli [58, 86]. At the termination of lactation, the secretory cells undergo involution and the breast acquires a microscopic appearance largely resembling that of the prepregnant stage.

The breast is a unique tissue as it continues to develop throughout the lifetime of its bearer. The differentiation and maintenance of the resting and active mammary gland is an intricate collaboration between stromal and systemic signals. As all organogenesis,

the development of mammary tissue displays many of the features associated with carcinogenesis, such as the concept of pluripotent cells, growth and development under hypoxic conditions, transition from resting to proliferative state, stromal invasion, and stimulation of angiogenesis. Therefore, unsurprisingly, many of the signals, pathways, and proteins essential for the proper differentiation of the mammary gland are also found to be associated with cancer progression [210].

Mammary Epithelial Stem Cells

The developmental changes occurring in the mammary gland at puberty and pregnancy involve both hypertrophy and hyperplasia of pre-formed structures as well as de novo formation of the ductal and secretory components. As the cycle of proliferation, differentiation, and involution occurs with each recurring pregnancy, these cellular dynamics require the existence of a pool of pluripotent cells whose progeny can give raise to the various components of the mature mammary gland [208]. These tissue-specific stem cells exist in an undifferentiated form throughout the duration of the reproductive phase of a woman's life. Through asymmetric division, which gives rise to one identical stem cell and one progeny with the ability to differentiate, a stable number of pluripotent cells is maintained [42, 113]. Because of their longevity, tissue-specific stem cells are prone to genomic damage, and as cancers are thought to be the result of a series of mutations occurring over years, these cells have therefore been implicated as targets of transformation during carcinogenesis [155]. The concept of cancer stem cells or tumor initiating cells is discussed in more detail in the Present Investigations section.

Mammary Epithelial Differentiation and Cell Markers

The epithelium is a cellular boundary acting as a selective permeability barrier between the external and the internal environments or between compartments within the body. Through epithelial cell polarity i.e. distinction between apical and basal surfaces of the cell, the epithelium secures the influx and efflux of specific substances. Epithelial differentiation is the process of a stepwise acquisition of characteristics and functions specific to each separate cell type, and cellular polarization is a hallmark of functional and morphological differentiation. On the tissue level, differentiation results in a fully developed and performing organ. The mammary gland epithelium contains cell types with two distinct differentiation lineages: the luminal and the myoepithelial. They can be ultrastructurally differentiated from about the 20th week of gestation, however as shall be discussed, their biochemical phenotype is not established until later [62]. These cells form the mammary ductal system with an inner layer of luminal and an outer layer of myoepithelial cells resting on the basal lamina. The two cell types have separate functions when fully differentiated; the luminal cells in the secretory portions of the gland secrete milk and the contractile properties of the myoepithelial cells help expel the milk from the functional unit of the mature breast, the terminal duct lobular unit (TDLU), illustrated in Figure 1. Characterization of the putative mammary stem cell and thereby a common

progenitor for the two cell types has proposed several cell marker proteins with which the specific stage of differentiation of the mammary epithelium can be established.

Cytokeratins (CK:s) are a family of differentially expressed intermediate filament peptides, found in the cytoskeleton of epithelia. The specific subset of CK:s currently expressed by an epithelial cell depends on the type of epithelium it belongs to and on the actual moment in the course of its differentiation [8, 125]. In the clinical setting, CK expression patterns are often used to determine the origin of anaplastic primary or metastatic carcinomas and to establish the differentiation state of a given carcinoma. Fully differentiated breast myoepithelial cells express CK14, α-smooth muscle actin (α-SMA), and vimentin, which are other components of the epithelial cytoskeleton. Together with the common acute lymphoblastic leukemia antigen (CALLA) these proteins are established markers of the differentiated myoepithelial cell [80-82, 163]. The luminal cells on the other hand specifically express CK18, CK19, epithelial specific antigen (ESA) and, due to their secretory role, sialomucin (also known as MUC-1 or epithelial membrane antigen), a large-molecular-weight glycoprotein found in milk [17, 108, 143, 194]. The acquisition of lineage specific cell markers is a stepwise process with sialomucin and α-SMA being considered as hallmarks of terminal differentiation of luminal and myoepithelial cells, respectively. This stepwise process has been elegantly illustrated by Anbazhagan et al. [8] in a study of CK expression patterns in fetal and infant breast tissues. According to the study, fetal tissue expressed CK19 in both luminal and basal (myoepithelial) cells and lacked CK18, CK14, and α-SMA expression. In the infant, there was a gradual increase in the expression of CK14, α-SMA, and vimentin in the myoepithelial cells, and of CK18 in the luminal. At the same time CK19 became confined exclusively to the luminal cells and its expression diminished as the breast matured. In concert with these findings, Stingl et al. [188], Pèchoux et al. [137], and Gudjonsson et al. [79], in their search for mammary stem cells, have shown that the luminal population within the breast contains bipotent cells, that give rise to both differentiated luminal and myoepithelial

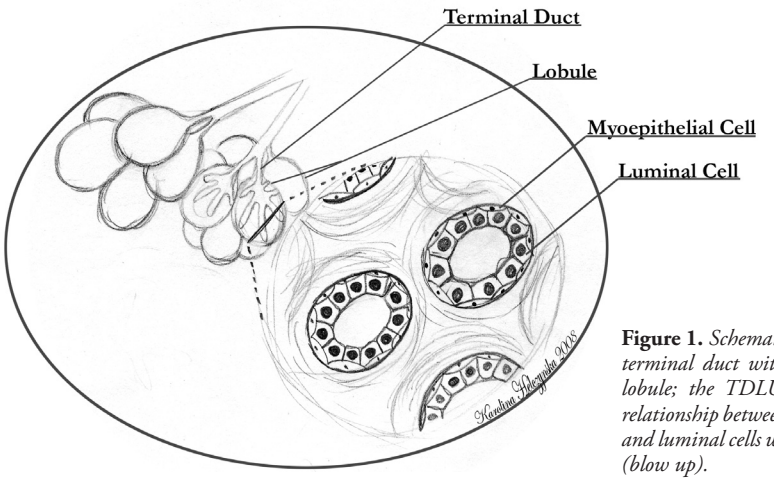


Figure 1. *Schematic drawing of a terminal duct with its associated lobule; the TDLU, showing the relationship between myoepithelial and luminal cells within an acinus (blow up).*

cells. These cells were found to be ESA$^+$, MUC$^-$ and CK19$^+$ [79]. They have a distinct morphology and are physically situated between the myoepithelial and luminal cells; above the myoepithelial but without lumen contact [79, 179]. Hence they have come to be known as intermediate cells.

CK19 holds special interest in this context. It was originally described as a strictly luminal cell marker albeit with a heterogeneous distribution pattern within the ductal luminal epithelium [17]. Based on further observations of CK19 expression, which appeared typical of regions of labile or variable cellular differentiation, Stasiak et al. [185] proposed that CK19 is a neutral keratin, which does not commit the cell to any one of the local differentiation options. Combining current findings, CK19 has come to be considered a putative mammary stem cell marker, but as exemplified in this thesis (Papers I and IV), it is the pattern of co-expression of the various differentiation-associated proteins that best describes the differentiation state of the mammary epithelial cell.

Steroid Hormone Receptors

As discussed previously, breast development and differentiation occur under the influence of local and systemic signals, among which the steroid sex hormones estrogen and progesterone exert the most powerful influence. Clarke et al. [43] found that in a resting, non-lactating, non-malignant breast approximately 10 to 15% of the luminal cells expressed estrogen and progesterone receptors (ER and PgR, respectively) and that the receptors were co-expressed in 96% of these cells. Interestingly, the steroid receptor positive cells were largely distinct from the population of proliferating cells. Subsequent studies by Clarke et al. [44] showed that a majority of the ER$^+$/PgR$^+$ cells had the morphology, localization and cell marker expression pattern of the intermediate cells described above.

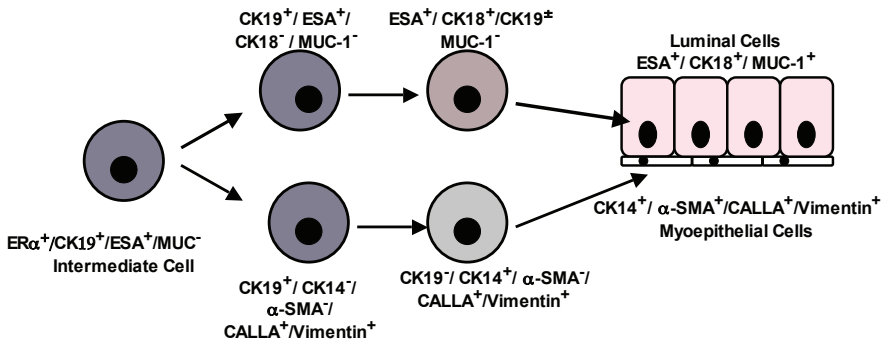


Figure 2. Proposed model of mammary epithelial differentiation based on refs. [8, 41, 44, 79, 137, 188] showing the bipotent intermediate cell giving rise to two progeny which then undergo stepwise differentiation into luminal and myoepithelial cells, respectively.

Together, these findings propose a tentative model of mammary epithelial differentiation, illustrated in Figure 2. Interestingly most human breast carcinomas are CK19 and ERα positive, once again implicating the role of stem cells in carcinogenesis.

The Cell Cycle[131, 160]

Normal cells proliferate in response to extrinsic mitogenic signals. Their presence triggers a myriad of signal cascades, which end in the transcription and/or activation of cell cycle regulatory proteins: cyclins, cyclin dependent kinases (CDKs), and cyclin kinase inhibitors (CKIs) [47]. Together, these proteins control the transition of the cell from one phase of the cell cycle to another. Whereas CDKs are constitutively expressed throughout the cycle, cyclins and CKIs have short half-lives, and are cell cycle phase specific; their expression is rigorously controlled through changing rates of transcription and proteolysis and influenced by various hormones, cytokines, growth factors, and enzymes [131]. D-type cyclins are expressed continuously at a low level throughout the cycle. Upon contact with a mitogen, a G0/G1 cell responds with a surge of cyclin D synthesis as a new cycle is initiated [13].

Cyclins act as activators of the CDK kinase activity. It is the cyclin/CDK complex that regulates transcription of the various genes required for further progression through the cell cycle. Cyclin D1, the predominant D type cyclin in breast, binds specifically to CDK4 and 6; the presence of this complex is considered essential for the completion of G1 [166]. A restriction point exists at the end of G1. Up to this point cell cycle signaling is mitogen-dependent, however once it is passed, the cell becomes committed to enter S phase and initiate DNA replication [136]. Cyclin E, the next cyclin to be expressed, is

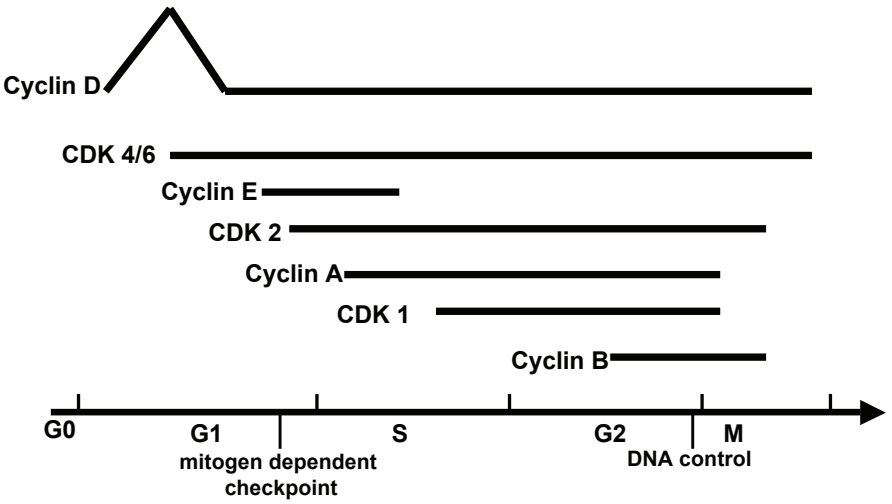


Figure 3. Varying levels of cell cycle regulatory proteins during cell division. Adapted from [131].

required together with CDK2 for the transition from G1 to S phase. Thereafter cyclin A/CDK2 and cyclin A/CDK1 complexes take over and drive the cell through the S phase by controlling DNA replication. Cyclin B and CDK2 are required for G2 to M phase transition when the cell passes yet another checkpoint where the condition of the replicated DNA is assessed. As soon as a cyclin is no longer necessary, it is degraded through the ubiquitin-mediated proteasome system [142]. A schematic representation of the cell cycle with the expression patterns of the associated regulatory proteins is shown in Figure 3.

The CKIs are important regulators of the cyclin/CDK activity. Their expression is up-regulated in response to signals that indicate unfavorable conditions such as DNA damage or lack of nutrients. However, their effect on cell cycle progression is not only negative. The members of the kinase inhibitor protein (KIP) family regulate the cell cycle in a CDK-type-dependent manner [172]. The cyclin E/CDK2 complex for instance is inhibited by CKIs p21 and p27. The same CKIs facilitate the assembly and activity of the cyclin D/CDKs complex [106].

It is easy to see that any aberration of the regulatory mechanisms of the cell cycle will be likely to cause loss of normal growth control, which is a characteristic of malignant tumors. This will be discussed further in the section on Additional Prognostic Factors.

Breast Disorders

Clinical Considerations

The introduction of mammographic screening has led to an increase of asymptomatic cancers. However, in the majority of cases a palpable focal mass, nipple discharge, pain, and/or visible macroscopic changes prompt the patient to seek her physician. All these symptoms may be associated with benign conditions; nevertheless the investigation of each patient is aimed at ruling out cancer. The investigation of a mammary abnormality is carried out according to the triple assessment principle: physical examination, mammography and ultra sound, and biopsy by fine needle aspiration. The next step is based on the overall evaluation of the findings, which should be carried out by a multidisciplinary team consisting of a pathologist, a surgeon, an oncologist, and a radiologist.

Benign disorders

Several benign conditions present as a solitary mass, causing differential diagnostic problems. The most common are cysts, fibroadenomas, papillomas, and abscesses. Cysts are fluid filled, epithelium-lined cavities of varying sizes, influenced by ovarian hormones and therefore displaying cyclic variations in size. Fibroadenomas are tumors consisting of stromal and epithelial components and papillomas are polyps of the ducts. Abscesses are frequently associated with recent or ongoing lactation. A feature common to the benign tumors is that they are generally associated with younger patient age, in contrast to cancer which rarely presents below the age of 40 years [62].

Premalignant Lesions

There exists a variety of morphologic abnormalities collectively referred to as fibrocystic changes. They are clinically relevant in two ways. One is that they present as a palpable mass, thereby creating the need for investigation to rule out malignancy. The second reason is that some of these changes are associated with an increased risk of developing premalignant lesions and invasive carcinoma. The concept of premalignant lesions refers to the idea that cancer develops as a continuous progression from atypical hyperplastic lesions, to low grade carcinoma in situ (CIS), to high grade CIS, and finally to invasive

cancer. Evidence in favor of this view comes from the observed coexistence of these lesions in surgical specimens as well as from studies showing increased incidence of invasive carcinomas in breasts with a former history of these diagnoses [6, 59].

Cancer

Breast cancer, cancer of the colon and rectum, and skin cancer are the most common malignancies in Swedish women, with breast cancer corresponding to approximately 30% of all female cancer [182]. Increased screening rates have directly contributed to the declining mortality rates as the majority of women nowadays present with early-stage i.e. not disseminated disease. So, in spite of increasing incidence rates, early detection and improved treatment have greatly altered the survival rates for breast cancer patients [181, 205]. The multifactorial etiology of breast cancer involves an assortment of various aspects such as reproductive factors, diet, physical exercise, body weight, endogenous and exogenous hormones, environmental exposures, and genetics [205]. Being a heterogeneous disease, breast cancer includes several histological and molecular subtypes, which differ in their biology and natural history and thereby prognosis and response to treatment [60, 183, 205]. It is the goal of ongoing research to provide clinicians with detailed knowledge of each individual subtype so that individually designed specific treatment might improve survival, eliminate over- and under-treatment and help avoid unnecessary side effects of therapy for each individual patient. Today, treatment strategies are largely based on histological subtype, tumor grade, tumor stage, and biological predictive markers, as shall be discussed below.

Non-Invasive Breast Cancer

The epithelial cells of the TDLU are considered to be the site of origin for most mammary cancers, the majority of them being adenocarcinomas [203]. Carcinoma in situ (CIS) designates a malignant tumor growing within spaces surrounded by an intact basal membrane i.e. without invasion of the surrounding stroma. The epithelial cells within the lesion grow towards the center of the duct, away from the basal membrane and hence away from the stromal vasculature. As the distance from the vascular supply increases, the cells become hypoxic and eventually die creating necrotic centers [85]. Though not invasive at the time of diagnosis, CIS is a precursor of invasive cancer and consequently a potentially serious condition. The evidence comes from the observation that ductal CIS treated with limited excision (biopsy) recur at the site of the excision as invasive cancer [135]. Therefore, the value of a predictive indicator for CIS lies in its ability to accurately foresee the risk of recurrence and/or development into invasive cancer. Today, apart from size of the lesion and surgical margin width, the two most important, though far from ideal, prognostic factors are nuclear grade and the presence or absence of central necrosis [174, 175, 198]. Briefly, low nuclear grade designates monomorphous, small nuclei, often ER positive, and none to very few mitoses. High nuclear grade designates polymorphous, large, usually ER negative nuclei with high mitotic rate. Intermediate nuclear grade lies in-between the low and the high. Necrosis is associated with higher tumor grade. Due

to the shortcomings of the prognostic factors at hand, with a limited possibility of foreseeing the risk of developing invasive cancer on individual basis, many CIS patients are forced to undergo unnecessary over-treatment.

Invasive Breast Cancer

According to the WHO Histological Classification of Breast Tumors [205], invasive breast carcinomas are divided into subtypes based on their microscopic appearance and growth pattern. The three major groups are: invasive ductal cancer, which is the largest group, comprising 50-80% of all breast cancers; lobular breast cancer, representing 5-15% of all tumors; and tubular cancer, responsible for 2% of the tumors. Medullary breast cancer, mucinous cancer, invasive papillary breast cancer, and metaplastic cancer are some of the other smaller groups, each accountable for 1-2% of the tumor population. The histopathological characteristics of each group are of prognostic significance. For example, the natural history of tubular cancer has a 10-year survival larger than 80%. Untreated medullary cancer has a 50-60% 10-year survival and ductal carcinoma < 50% [64].

Differentiation and Nottingham Histological Grade (NHG)

Differentiation status of a cancer refers to its ability to morphologically and functionally resemble the tissue it originates from. This can be assessed at the tissue and at the cellular level and the more the architecture of a cancer resembles that of the normal tissue it derives from, the higher its grade of differentiation. The grade of differentiation is a spectrum ranging from a totally anaplastic (barely showing signs of its origin) cancer, to a cancer with retained cellular polarization and thereby ability to form structures such as tubuli or glands. As discussed previously, epithelial differentiation is accompanied by the expression of cell type- and differentiation level- specific proteins such as cytokeratins, which can be studied in order to establish the origin of a tumor as well as its differentiation status. At the intra-cellular level, several distinct morphological features of the cytoplasm and the nucleus of a malignant epithelial cell reveal its level of differentiation. The nuclear characteristics of a malignant epithelial cell are collectively referred to as nuclear pleomorphism. These features are: increased nuclear size, variable nuclear shape and size, and increased number of nucleoli [61].

All invasive breast tumors are graded according to the method first described by Bloom and Richardson [23] and later modified by Elston and Ellis [63]. The method is based on graded assessment of tubule and/or gland formation, nuclear pleomorphism, and mitotic counts within a given tumor. Each entity is scored as 1, 2 or 3, the scores are then totaled and the final count is presented as the NHG index or the histological grade. A sum of 3-5 implies a highly differentiated grade I tumor, 6-7 a moderately differentiated grade II tumor, and 8-9 a poorly differentiated grade III tumor. Histological grade is a recognized significant prognostic factor [87], routinely used as the point of reference in various prognostic studies.

Tumor Stage or the TNM Classification

Staging means that various pieces of prognostic information are gathered in order to foresee the expected course of a specific case of malignancy. The single most important prognostic indicator in breast cancer is the presence or absence of axillary lymph node metastasis. Moreover, the risk of distant recurrence is proportional to the number of nodes involved [39]. The risk of nodal involvement increases with increasing size of the tumor and tumor size on its own is an independent prognostic factor [35]. Tumor size is the most powerful prognostic factor for patients with negative node status. Presence of distant metastases signifies disseminated disease with unfavorable prognosis. The anatomical extent of the disease, called the tumor stage, is often measured according to the T- tumor, N- node, M- metastasis system, where tumor size and extent together with presence or absence of nodal and distant metastases are assessed. Tumor stage is a major determinant for the choice of treatment procedures. Briefly, stage I is a tumor less than 20 mm in greatest dimension with no lymph node (N0) and no distant (M0) metastases. Stage II is a tumor 20 to 50 mm in greatest dimension, maximum 3 axillary nodal metastases (N1) and M0 OR more than 50 mm in greatest dimension and N0 and M0. Stage III is a tumor of any size, with 4 to 9 lymph node metastasis and M0. Stage IV, the most advanced, implies generalized disease, M1.

Additional Prognostic factors

The difference between a prognostic and a predictive factor is that the first is any feature that correlates with the natural history of the disease, while the second is any feature associated with response to a given therapy. Some factors might be both prognostic and predictive. Apart from the previously discussed tumor grade and tumor stage, additional patient or tumor related features provide information about the expected behavior of the cancer.

Heredity.

A family history of breast cancer is one of the most important risk factors for the disease, in most families the increased risk being a combination of environmental factors and genetic input. Hereditary breast cancers account for approximately 5-10% of all cases and the discovered genes responsible for the susceptibility include *BRCA-1* (20-40% of hereditary breast cancers), *BRCA-2* (10-30%), *TP53, PTEN, ATM, CHK2, STK11* (ca. 1% each), and Fanconi's Anemia genes (ca. 1%)[75, 212]. Several clinical features distinguish hereditary breast cancers from the sporadic. As many as 30% of women who present with breast cancer before the age of 35 years have a genetic predisposition for the disease. There is an increased risk of contra lateral disease and a familial association with malignancies of the ovaries, colon, and prostate [212]. BRCA1 cancers are characteristic in that they are often of high grade and ER^-/PgR^- and HER-2 negative (so called triple-negative, see separate section below) with a basal like phenotype [99]. Patients with a hereditary predisposition to develop breast cancer should be enrolled in special follow up programs.

Age.

Women presenting with breast cancer before the age of 35 years have been shown to have poor prognosis [3, 129] and women older than 50 years have been shown to have better prognosis [50, 54]. Cancers in the younger women are more often ER^- and lymph node positive, whereas cancers in the older women are more often associated with positive prognostic factors. In clinical studies, age is sometimes used as an indirect indicator of menopausal status. Nevertheless, although related, these two variables should be considered as separate. Clark et al. [40] investigated the relationships between ER, PgR, and a variety of patient characteristics in a cohort of women with primary breast cancer. Older women were found more likely to have ER^+ tumors than younger women, and age, not menopausal status, was found to be the primary determinant of increased ER concentrations. When patients of the same age were compared, premenopausal women had higher progesterone receptor concentrations than postmenopausal, perhaps a sign of greater estrogen-mediated synthesis of progesterone receptor.

ER and PgR expression.

Even though two distinct estrogen receptors exist, ERα and ERβ, the function and significance of the latter is still unclear and beyond the scope of this thesis. Therefore, unless specified otherwise, ER in this book refers to ERα. Two major differences exist between the normal and the malignant breast tissue with respect to ER expression. One is that in contrast to normal breast tissue where about 10% of the epithelial cells are ER^+, approximately 75% of the malignant epithelial cells in ER positive cancers express the receptor. The second difference is that in the normal tissue, ER^+ cells are separate from the proliferating cells, as seen by Ki67 expression (see section on steroid hormone receptors in the normal breast), while in the malignant tissue, ER^+ cells proliferate in response to circulating estrogen. Hence, estrogen stimulates tumor growth [5, 43]. In spite of these disparities, hormone receptor positive tumors display high level of differentiation i.e. have low tumor grade, and lower proliferation rate [133]. Initially, it was assumed that ER expression status could be used as a favorable prognostic factor and the first clinical trials did in fact report lower rate of recurrence and a more indolent course for the ER positive tumors. However, recent studies with a longer follow up time have shown that ER-positive tumors, though slower growing, do not have a lower metastatic potential and over time are not associated with a much better prognosis [205].

Progesterone is a steroid sex hormone that in normal mammary glands promotes growth and cellular proliferation. Its receptor, a ligand-activated transcription factor, is a downstream target for the ER transcription factor and therefore, its presence in breast cancers may be used as an indirect marker of functional ER. Consequently, reports show that lack of PgR expression in ER^+ tumors may be a marker of aberrant growth factor signaling that could contribute to tamoxifen resistance [10] and that PgR is a stronger predictor of response to endocrine therapy than ER [15, 67, 151, 165, 187]. The exact contribution of each receptor to the response to endocrine therapy is still not entirely understood and under ongoing debate. Clinical evidence does show that ER^+/PgR^- tumors are less

sensitive to tamoxifen than ER^+/PgR^+ tumors [15, 151]. Rakha et al. [147] show in a series of 1,944 consecutive cases of primary invasive breast cancer, that each hormone receptor subclass (ER^+/PgR^+, ER^+/PgR^-, ER^-/PgR^+, and ER^-/PgR^-) is a distinct entity of breast cancer with specific biologic and clinical characteristics. Hence, the overall hormone receptor status, including both ER and PgR, needs to be evaluated for best prognostic and predictive information.

Over-Expression of HER-2 [121].

The human epidermal growth factor receptor-2 gene encodes HER-2, one of the four transmembrane tyrosine kinase receptor proteins HER-1, HER-2, HER-3, and HER-4; all members of the epidermal growth factor receptor (EGFR) or HER family. Receptor activation involves ligand binding to the receptor and dimerization with a dimerization partner. The HER tyrosine kinases provide binding sites for ligands such as transforming growth factor alpha (TGF-α), epidermal growth factor (EGF), heregulin, and amphiregulin which in turn activate downstream signal pathways regulating cell growth, motility, and differentiation. The HER-2 oncogene however, also known as erbB2, is an exception as it is an orphan receptor with no known ligand. Its activation is based on its being the preferred dimerization partner of the other HER receptors [122, 161]. Malignant cells, often containing excessive amounts of the erbB2 protein have a deregulated control of the downstream signal pathways network. ErbB2 protein over-expression/gene amplification is seen in approximately 20-30% of breast cancers and is associated with increased proliferation, inhibition of apoptosis, and enhanced metastatic potential. The vast majority of clinical studies investigating the prognostic significance of HER-2 protein over-expression/gene amplification in breast cancer predict worse patient outcome [9, 122, 161, 177, 200]. Today, the greatest value of HER-2 status comes from its role in predicting the response to therapy with a monoclonal antibody directed at the HER-2. Furthermore, erbB2 over-expression/amplification is specifically associated with tumor responsiveness to various chemostatics as well as to endocrine therapy, where negative and even detrimental effects of tamoxifen have been reported [10, 121, 138, 144].

Molecular Profiles, the Basal-Like, and the Triple-Negative Tumors.

For a long time, breast carcinomas were classified according to histological subtype, grade, and ER and PgR status. Even though there exist strong correlations between these established clinicopathological variables and prognosis and outcome, it is evident that patients presenting with similar clinical and pathological variables may demonstrate varying outcomes and responses to given therapy. The traditional taxonomy of breast cancer was revolutionized by the discovery of the significance of HER2 status and the development of Trastuzumab. Nowadays, characterization of HER2 expression is an integral part of the pathological work-up for all breast cancers. Breast cancer research is continuously producing new promising markers of prognostic and/or predictive relevance in an effort to shed light on the complex biology of this cancer. The ultimate goal is to further improve our classification tools and our ability to provide each patient with an individually tailored therapy. Gene expression studies using various methods

such as DNA microarrays, quantitative real time polymerase chain reaction (QRT-PCR), and immunohistochemistry applied on tissue microarrays have emerged as classification methods that take into account the biological heterogeneity of breast cancer [148]. Based on specific constellations of several groups of genes, four main classes of breast cancer called the basal-like, the HER-2 positive, the normal-like, and the luminal have been identified [140, 184]. Importantly, the various sub-groups have been further shown to be linked to different outcomes and responses to treatment [31, 157, 162]. Currently, clinical randomized trials that apply gene expression profiling to define specific prognostic and predictive profiles have been introduced as well as quality control projects that address the reproducibility of microarray measurements [148]. It is envisaged that this new technology might eventually come to improve the routine clinical management of breast cancer.

The basal-like subtype of breast carcinomas, characterized by expression of genes usually found in normal basal/myoepithelial cells such as CK:s 5/6, CK14, CK17, vimentin, p-cadherin, and often lack of ER, PgR, and HER2 have been known for a long time [118, 167] but received renewed attention with the results of the microarray based gene expression profile analyses. These tumors are characterized by high grade, presence of necrotic zones, and an overall more aggressive clinical behavior with a distinctive metastatic pattern, often affecting younger patients and BRCA-1 gene mutation carriers [152]. The more accurate identification of these tumors has led to clinical trials that specifically test various chemotherapies directed at the basal-like cancers.

Lack of expression of ER, PgR and HER2 is designated triple-negativity and gene profiling has shown that these tumors constitute a specific though heterogeneous subset of breast cancers over-represented among women of African-American origin and BRCA-1 gene mutation carriers. Similar to the basal-like cancers, triple-negative cancers have been reported to be more aggressive and associated with poor prognosis. Through their receptor negativity these tumors represent an oncological challenge, as the only systemic therapy available to these patients is chemotherapy. Even here, several clinical trials are currently testing the efficacy of various agents in the management of triple negative cancers [152].

Aberrations in Cell Cycle Regulation.

Many breast cancers show abnormal G1/S regulation [55, 128, 171]. The most frequent defect, seen in approximately 50% of cancers, is cyclin D1 over-expression [186]. The over-expression is often due to amplification of the cyclin D1 gene, *CCND1*, which is observed in 15-20% of breast cancers [132]. As a cell cycle regulator, cyclin D1 regulates progression through G1 by modifying the activity of CDKs 4 and 6 [131]. However, contrary to what might be expected, the tumorigenic effect of cyclin D1 over-expression does not necessarily originate from CDK-mediated uncontrolled growth and proliferation [128]. Rather, the oncogenic properties of cyclin D1 seem to be derived from its ability to activate various growth promoting receptors, including the ER, in a CDK-independent manner [69, 141]. Clinical studies have linked cyclin D1 over-expression to positive ER status, however its prognostic value is unclear. Better, worse or unaffected prognosis has been reported

[160]. *CCND1* amplification on the other hand, has been more unequivocally linked to impaired survival [20, 127].

Through the years, various methods of measuring the proliferative rate of a breast cancer tumor have been assessed for prognostic relevance. S-phase fraction, mitotic index, thymidine labeling index, and immunohistochemical analyses of Ki-67 are some examples. Several trials have established a positive significant correlation between the fraction of cells in S-phase and prognosis [30, 67, 116] and analysis of S-phase fraction is now recommended as part of the routine clinicopathological work-up of a breast cancer [190].

Treatment

Surgery

Surgical excision

Surgical excision is the cornerstone of primary breast cancer treatment. A majority of patients will be cured by the surgery, in some instances combined with postoperative radiation therapy. Surgical excision of the tumor may be performed either as a breast conserving procedure or as a mastectomy, with or without axillary dissection, the choice of procedure largely depending on the outcome of the preoperative triple-step assessment; the TNM-score. Several randomized trials have shown that in selected patients (taking into account tumor size, location, breast size, and the patient's general health status) breast conservation followed by radiotherapy has survival rates equal to those for mastectomy [12, 68, 204]. In reality, breast conservation therapy usually refers to a multidisciplinary treatment approach including local excision of the tumor, a separate sentinel node excision and/or axillary dissection, and radiation [96].

In case of local or regional recurrence, tumor excision is performed in order to gain local tumor control. Disseminated disease with distant metastases (M1) has long been considered a contraindication to surgical therapy. However, recent publications present results suggesting that women with M1 disease do benefit from ablation of the primary tumor [209].

Sentinel lymph node

Sentinel lymph node was first described for malignant melanoma [126]. Defined as the first lymph node that receives the lymphatic drainage from the tumor, the concept has since been extended to breast cancer [168] . The sentinel node is identified peroperatively following injection of a radioactive colloid and/or a dye and sent off for histopathological evaluation. If found to be devoid of metastases, the patient is spared an axillary dissection, a procedure associated with grave complications such as lymph edema, nerve injury, and shoulder dysfunctions.

Axillary dissection

Axillary dissection involves removal of 10 or more lymph nodes from the ipsilateral axilla and has dual purpose. It protects against future tumor recurrence in the axilla and provides prognostic information about the stage of the disease [96]. It is reported that approximately 30% of patients who have undergone axillary dissection will have procedure related arm symptoms [190].

Radiation Therapy

Postoperative radiation therapy (RT) has been shown to significantly improve overall and recurrence free survival for patients who are operated with breast conserving procedure and those operated with a total mastectomy for a tumor exceeding 50 mm in size or with 3 or more lymph node metastases [75, 190]. Recent studies have shown benefit of post-mastectomy RT even in case of 1 to 3 lymph node metastases [11, 134]. However, the extent of the benefit is still under debate and these cases have not been included by the international guidelines as indications for RT.

Systemic Therapy

Hanahan et al [83] discuss specific essential alterations in normal cell physiology that collectively designate malignant growth: self-sufficiency in growth signals, insensitivity to growth-inhibitory signals, evasion of apoptosis, limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis. Each of these capabilities is therefore a potential goal for systemic anti-cancer therapy. Traditionally this therapy has in breast cancer been seen as either of two modalities: cytotoxic chemotherapy or endocrine therapy, however, recent developments have created a yet another entity, the targeted therapy.

Modern treatment strategies stress the importance of applying targeted therapies whenever possible, even though supplementation with less target specific cytotoxic chemotherapy therapy is often required. Evidence is accumulating that the three therapy modalities are intrinsically influenced by each other. As discussed previously, HER-2 status has been found to predict tumor response to various chemostatics as well as to endocrine therapy [121, 138, 144]. Also, it seems that the effectiveness of chemotherapy might be dependent on ER status as ER-negative tumors are reported to have greater benefit from chemostatics than ER-positive tumors [19, 75]. In addition, Jirström et al. have shown adverse effects of tamoxifen treatment in ER^+ cancers with high cyclin D1 staining intensity and/or gene amplification [102].

Cytotoxic Chemotherapy

Cytotoxic chemotherapy targets rapidly proliferating cells. The agents used act by damaging DNA through cross links and double strand breaks (alkylating agents such as cyclophosphamide and alkylating-like agents such as cisplatin), as antimetabolites, thereby inhibiting DNA and RNA synthesis (methotrexate, fluorouracil), by preventing cell division (doxyrubicin and epirubicin), or by interfering with function of microtubules, thereby disrupting cell division (vinca alkaloids and taxanes). There are ongoing efforts to find markers of responsiveness (predictive factors) to the various agents and thereby increase the specificity of each given treatment. As an example, preliminary results from small studies indicate that basal like cancers, often associated with BRCA1 mutation, might be particularly sensitive to DNA damaging therapy [99].

Systemic treatment with these agents may be given as neoadjuvant, adjuvant or palliative therapy. Neoadjuvant chemotherapy is given prior to surgery in order to reduce tumor size and to allow the patient to be treated with a breast conserving procedure. The rationale behind adjuvant therapy, i.e. systemic anti-cancer therapy given to patients who have undergone breast cancer surgery lies in the reported improved survival for these women. Even though early stage disease accounts for increasing proportion of breast cancer diagnoses, a significant number of these women will still succumb to disseminated disease. This shows that even though the disease might appear contained with our diagnostic instruments, some women present with a clinically undetectable disseminated cancer. These patients will not be cured by surgery alone and adjuvant treatment is therefore prescribed in order to obtain better disease control. Palliative treatment is given in order to relieve the symptoms of overtly disseminated disease.

Endocrine Therapy

Endocrine therapy targets steroid hormone dependence of the ER^+ breast cancer. Initially hormone depletion was achieved by irradiation of the ovaries or by surgical oophorectomy. The discovery of the ER provided oncologists with a predictive marker for the hormone depletion therapy and thereby a possibility to select patients with the greatest benefit of the risky surgical procedures. With the introduction of anti-estrogen therapy in the early 70's, organ ablation procedures were largely abandoned.

ER has two transactivation domains, AF1 and AF2, used differentially in different tissues, which regulate its activity through various co-activators and co-repressors. AF2, the main activation domain in the breast, is hormone dependent and requires estrogen for its activation [133]. AF1 is hormone independent and can be activated by various phosphorylation pathways [202]. Tamoxifen is a competitive estrogen agonist-antagonist that selectively inhibits the activation of AF2 without exerting any negative effect on the AF1. In breast cancer, the antagonistic effect results in inhibition of the ER-mediated transcription of genes essential for tumor growth. However, the uninhibited agonistic activity of tamoxifen, mediated by AF1, is associated with side effects from the tissues where AF1 is the principal transactivator of the receptor e.g. a significantly increased risk of endometrial cancer. Fulvestrant is a pure ER antagonist that impairs receptor dimerization by acting on both AF1 and AF2. Aromatase inhibitors act by inhibiting

aromatase, the enzyme that catalyzes the conversion of testosterone to estradiol, and of androstenedione to estrone, which are the last steps of estrogen biosynthesis. Aromatase inhibition causes profound estrogen deprivation thus preventing ER activation, irrespective of its transactivation domains.

The choice of agent is based on several factors, the patient's menopausal status being the most important one. In a pre-menopausal woman lowering the level of circulating estrogens by aromatase inhibitors starts off a positive feedback mechanism that causes release of gonadotropins from the anterior pituitary gland. These in turn stimulate secretion of estrogen and progesterone from the ovaries, effectively counteracting the object of anti-estrogen therapy. Even in a peri-menopausal woman with sub-active ovaries, this therapy holds a risk of inducing unwanted ovarian activity.

Targeted Therapy

Rather than simply interfering with rapidly dividing cells, targeted therapy inhibits tumor growth by interfering with a protein specific to the cancer and vital to its progression or sustainment.

Trastuzumab is a monoclonal antibody that targets the HER-2, preventing dimerization of the receptor, which is an essential step in its activation. Clinical trials such as that of Slamon et al. [178] for metastatic breast cancer and of Smith et al. [180] and Romond et al. [158] for early-stage breast cancer have shown overwhelmingly positive results and Trastuzumab is now included in standard treatment protocols for HER-2 amplified metastatic and early-stage breast cancer. Lapatinib is a dual tyrosine kinase inhibitor that blocks activation of HER-1 and HER-2. Preliminary reports suggest that Lapatinib might enhance the anti-epidermal growth factor receptor targeting therapy and currently there are several ongoing Phase III trials assessing the efficacy of combination therapy regiments with Lapatinib in both early-stage and metastatic breast cancer [57].

Neovascularization is a prerequisite for continued tumor growth. In the healthy, non-malignant setting, active angiogenesis is necessary only in embryogenesis, endometrial proliferation, wound healing, and pregnancy related changes in the mammary gland. Therefore, tumor associated angiogenesis provides attractive potential targets for anti-cancer therapy with low toxicity. When tumor volume outgrows the capacity of the existent vessels, tumor cells release angiogenic factors such as vascular endothelial growth factor (VEGF), platelet derived growth factor (PDGF), transforming growth factor (TGF), and EGF which all act through specific receptors [153]. Conceptually, the anti-angiogenic therapy can work by directly sequestering the ligand or blocking its receptor with monoclonal antibodies or soluble receptors. Bevacizumab is a monoclonal antibody against VEGF. Prospective randomized clinical trials have already established its role in treatment of metastatic colon and lung cancer [173]. Its benefit in breast cancer treatment has been shown for metastatic disease and is currently being evaluated in various drug combinations as well as planned for assessment in the adjuvant setting [57, 75, 84]. Sunitinib is a tyrosine kinase inhibitor that acts on several receptors with tyrosine kinase activity such as VEGFR and PDGFR. Though still in the early clinical trials, preliminary clinical data have shown the efficacy of sunitinib as monotherapy in metastatic breast cancer [57, 84].

Hypoxia

Physiology and Definitions

Oxygen content in the atmosphere is approximately 21% of the air volume, corresponding to a partial pressure of 20 kPa or 150 mmHg. Blood is oxygenated in the lungs by breathing. The O_2 is then transported bound to hemoglobin to the end capillaries where it diffuses down a pressure gradient into the cells supported by the capillary. The blood oxygen content is reduced during transport within the arterial system so that by the time it reaches the cell, oxygen partial pressure is roughly 7.5 kPa or 56 mmHg as illustrated in Figure 4 [154]. Once inside the cell oxygen is utilized by the mitochondrial respiratory chain for energy synthesis [4]. When oxygen concentration, due to systemic or local factors, falls below a critical threshold, the intricate balance of the oxidative metabolism can no longer be sustained.

From a physiological point of view, the term "hypoxia" cannot be defined as a specific level of oxygen partial pressure. This is due to the fact that organisms, organs, and cells all have individual spans of tolerance for variations in oxygen pressure. Rather, the definition needs to come from the effect a given oxygen pressure has on the organism, organ or cell. Hypoxia will then be defined as insufficient oxygen pressure, low enough to restrict or inhibit normal functions of the biological system that is being studied. The organism, organ or cell is subsequently forced into an adaptive process that will compensate for

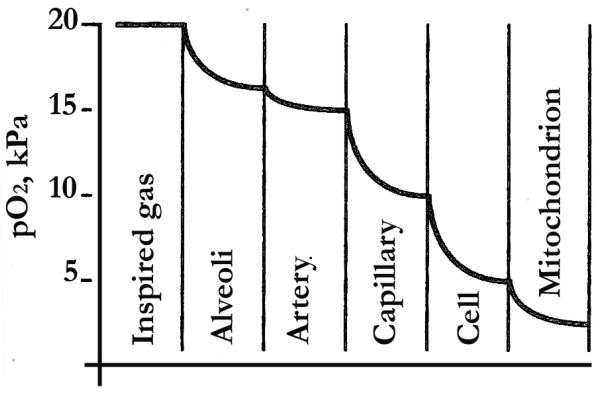


Figure 4. *The various stages of oxygen transport showing corresponding approximate values of oxygen partial pressures. Adapted from [154].*

the diminished oxygen supply and let it survive. Inability to compensate leads to death. At the cellular level inadequate oxygen supply leads to reduced ATP production, which results in compromised membrane transports and thereby inability to sustain electrolyte gradients, depolarization of membranes, and intracellular lactic acid build up due to the shift to anaerobic metabolism [91]. These changes lead to a dramatic increase in transcription of some specific hypoxia responsive genes involved e.g. in erythropoiesis, angiogenesis, glucose transport, and glycolysis.

At the systemic level in the human body, the cellular compensatory reactions lead to acute responses such as increased heart rate, vasodilation, and hyperventilation as well as chronic responses such as increased production of red blood cells, increased renal excretion of hydrogen ions, and local angiogenesis. Hochachka et al. have shown that immediate and adaptive responses, named defense and rescue phases, exist even at the cellular level [89]. Brown et al. [27] suggested and Pigott et al. [146] established the existence of acute and chronic hypoxia within solid tumors by showing frequent microregional fluctuations in perfusion in several primary and metastatic human tumors. Holmquist-Mengelbier et al. [93] showed distinct cellular acute and chronic responses to hypoxia within neuroblastoma tumors and in paper III we show similar results for breast cancer.

As discussed in the following section, tumor hypoxia has important clinical consequences. Therefore, various invasive and non-invasive techniques have been developed to measure the level of oxygenation within normal and malignant tissues [28, 91]. With these measurements, investigators searched for approximations of the critical threshold for oxygen concentration below which adaptive and compensatory mechanisms are initiated. The most widely applied method uses a polarographic oxygen electrode, which is inserted into the tissue. With this method investigators have shown that there exists a Gaussian distribution of oxygen tensions within a normal tissue and that the median lies between 40 and 60 mmHg [28, 201]. Keeping in mind the individual variations of tumors and cell lines in sensitivity to diminished oxygen availability, the general consensus seems to be that O_2 partial pressure of 1.3 kPa or 10 mmHg is the minimum level required for adequate oxidative metabolism [28]. For cell line cultures, which are carried out in incubators flushed with gas, it is easier to handle the various O_2 concentrations as percentage of the atmospheric pressure. Consequently, the median tension in normal tissues of 40 – 60 mmHg corresponds to approximately 6 - 8%, and 10 mmHg equals to 1.3%.

Clinical Consequences of Tumor Hypoxia

It has been shown that the maximum diffusion distance for oxygen is 100 – 150 µm [37, 195]. Malignant tumors are parasitic growths that use the host's vessels for the supply of oxygen and nutrients, and removal of waste products. Through uncontrolled growth the tumor soon outgrows the limit of O_2 diffusion and hypoxic and necrotic areas appear within its bulk. Furthermore, even though tumor induced angiogenesis occurs, the newly formed vessels are leaky, irregular, have arterio-venous shunts and blind ends and

cannot sustain the metabolic demands of the tumor tissue. The presence of necrotic areas within a tumor is generally considered pathognomonic for its rapid, aggressive growth. As discussed previously for DCIS, the presence of central necrosis is one of the strongest prognostic and thereby predictive factors for this malignancy.

Tumor hypoxia has clinical consequences as it has been shown to be a strong selective force in the progression of many cancers. By inducing and/or enhancing preexisting neoplasm-associated genomic instability and heterogeneity as well as proteome changes both within the tumor cells proper and stromal cells, hypoxia promotes tumor progression through local, perifocal, regional, and distant spread [91, 170]. For cancers of the uterine cervix, soft tissue sarcomas, and head and neck cancers, the presence of tumor hypoxia is an independent prognostic factor, clearly associated with significantly shorter recurrence-free and overall survival [25, 26, 90]. Also, it has bees appreciated for many years that hypoxic tumors have decreased sensitivity to radiation therapy [28, 29, 49, 71, 77]. The mechanism for this resistance is explained by the physicochemical oxygen fixation hypothesis [29]. According to this hypothesis radiotherapy kills cells through formation of free radicals within the DNA structure. Molecular oxygen makes this damage permanent by reacting with the free electron of the free DNA-radical. In the absence of oxygen, much of the damage is soon repaired by hydrogen donation from e.g. cellular glutathione [29, 77]. Garcia-Barros et al. [72] suggested that apart from the direct cell killing effect of ionizing radiation, tumor growth is additionally inhibited by radiation induced endothelial cell apoptosis within tumor vasculature. Moeller et al. [124] further linked radio-resistance to tumor vasculature by demonstrating that irradiation induced Hypoxia-Inducible Factor-1α and VEGF secretion by tumor cells, which in turn conferred protection from apoptosis to endothelial cells and thereby prevented secondary tumor cell death. These findings might provide an additional mechanism for hypoxia related resistance to radiation therapy. Preclinical studies have also provided evidence for several mechanisms for hypoxia-associated resistance to chemotherapy [29]. First, hypoxia generally causes cells to arrest or slow their progression through the cell cycle [7, 74], which is a problem since chemotherapy generally targets rapidly proliferating cells. Second, hypoxic cells are located at increasing distances from vessels, which deliver the cytotoxic drugs making it difficult to deposit adequate amounts of the drug [88]. Third, the hypoxic milieu with its lowered pH might affect the efficacy of some agents [28]. Fourth, it has been shown that hypoxia related changes in protein expression include upregulation of proteins that directly confer drug resistance, as exemplified by the MDR-1 gene [45]. Fifth, hypoxia has been shown to select for mutant p53, thereby lessening the efficacy of drugs that act through p53-mediated apoptosis [76].

Hypoxia-Inducible Factors

The major mediators of the hypoxic response leading to resistance to apoptosis, initiation of tumor cell survival mechanisms, angiogenesis, increased migration and invasion, as well as changes in glucose metabolism and glucose transport, are the hypoxia-inducible factors (HIF:s).

HIF is a heterodimer consisting of two proteins; HIF-α and HIF-β (also known as aryl hydrocarbon nuclear receptor translocator, ARNT)-subunits, which together form a transcription factor that recognizes specific, conserved DNA sequences, referred to as the hypoxia-response-elements (HRE:s), located in the promoter or enhancer regions of HIF target genes. HIF activity relies on the availability of the α subunit. Both HIF-α and HIF-β are constitutively expressed in cells at the mRNA level, however, the activity of the α-subunit is rigorously controlled through both oxygen-dependent and oxygen-independent mechanisms [170] [34]. Three different α-subunits exist: HIF-1α, HIF-2α, and HIF-3α. HIF-1α and HIF-2α (also known as endothelial PAS domain protein, EPAS; or hypoxia-like factor, HLF) share almost 50% overall amino acid identity and are regulated through similar mechanisms [207]. HIF-3α has been reported to function primarily as a negative regulator of the HIF system, however, its relevance to breast cancer tumorigenesis is still unresolved [115].

Oxygen-Dependent Regulation of HIF-1/2α.

Intracellular hypoxia leads to an almost immediate increase in HIF-α activity through oxygen-dependent post-translational modifications of the proteins. In the presence of oxygen, three prolyl hydroxylases, the prolyl hydroxylase-domain proteins (PHD) 1-3, hydroxylate two proline residues in HIF-α in a reaction requiring oxygen, 2-oxogluta-

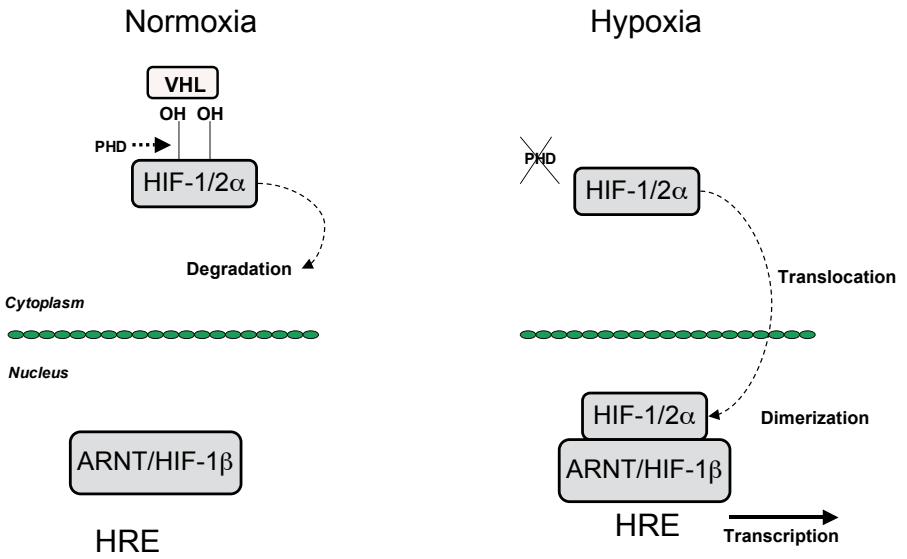


Figure 5. Oxygen dependent regulation of HIF-α activity. At normoxia, HIF-α is hydroxylated by prolyl hydroxylases (PHD). This modification induces conformational changes that render the HIF-α recognizable to the von Hippel-Lindau tumor suppressor protein (VHL), which then targets the HIF-α for ubiquitination and proteasomal degradation thus leading to low HIF-α levels at normoxia. At hypoxia, the oxygen sensing PHDs are inactive and hence, HIF-α is allowed to translocate to the nucleus, dimerize with HIF-1β and exert its role of a hypoxia-induced transcription factor.

rate, Fe^{2+}, and ascorbate. This renders the HIF-α susceptible to recognition by the von Hippel-Lindau tumor suppressor gene product (pVHL), which is a part of a multisubunit ubiquitin ligase complex. The pVHL tags the α-subunit with poly-ubiquitin, thus allowing for recognition by the proteasome and subsequent degradation (Figure 5). At hypoxia, inadequate oxygen levels inactivate the PHD:s. Additional processes add to the oxygen dependent regulation of HIF-α activity, such as acetylation of HIF-α and thereby enhancement of the interaction between pVHL and HIF-α, and hydroxylation of aspargine residues in HIF-α by factor inhibiting HIF (FIH) which blocks the recruitment of HIF-α co-activators p300 and CREB-binding protein (CBP) [34, 170].

Oxygen-Independent Regulation of HIF-1/2α.

Conditions that disrupt the degradation machinery, such as absence of HIF ubiquitination as a result of mutated pVHL, and sequestration of the iron ions needed for the hydroxylation reaction by iron chelators mimic the hypoxia-induced stabilization of HIF-α even at normal oxygen levels, thus leading to non-hypoxic HIF activation. Growth factors and oncogenic signaling through mutated oncogenes such as Ras or Src or through inactivated tumor suppressors such as p53 and pVHL [14, 36, 101] stabilize the HIF-α proteins leading to enhanced transcription of their target genes e.g. VEGF under normoxic conditions. The majority of these factors act through two common cellular kinase pathways: the mitogen-activated protein kinase (MAPK) pathway and the phosphoinositide 3-kinase/Akt (PI3-K) pathway [1, 14, 70, 123]. Several growth factors have been implicated as inducers of HIF responses through these pathways, e.g. EGF, IGF-1, insulin, heregulin, interleukin-1β, androgens, TNF-α, angiotensin II, and thrombin [14]. Interestingly, some of these growth factors have been shown to enter autocrine loops with active HIF, leading to reciprocal positive regulation [66, 114]. Unlike hypoxia, which regulates HIF-α in the same manner in all cell types, growth factor regulation of HIF activity via the MAPK and/or PI3-K pathways is thought to be cell/tumor type specific [21, 95, 109]. However, similar to hypoxia, induction of HIF-α by growth factors appears not to occur at the transcriptional level but via modifications of the translational machinery or enhancement of HIF-α protein stabilization [95, 109].

HIF-1α versus HIF-2α.

The two HIF-α subunits are structurally similar in their DNA binding and dimerization domains, dimerize with the same protein, and are regulated in a similar fashion. Why then, do two different subunits exist?!

Investigation of the induction of HIF target genes in response to hypoxia in different cell-lines, organs, and tumors yields more or less heterogeneous, cell-line, tumor type, and HIF subunit specific results regarding both quality i.e. the specific target genes transcribed, and their quantity, as shown for the kidney, liver, neuroblastoma, renal cell carcinoma, and breast cancer cell lines [22, 33, 94, 103, 150]. This is related to the transcriptional response to hypoxia in a given normal or malignant cell being determined by its developmental and physiological programming. Specifically, regarding HIF:s, this

programming results in: differential transcriptional and post-transcriptional regulation of the two HIF-α subunits leading to varying HIF-1α and HIF-2α expression patterns; differing HIF-α inducers; varying levels of HIF-1α and HIF-2α induction as well as subunit-specific target genes.

Studies of HIF-1α and HIF-2α expression in developing mouse embryos have revealed varying expression patterns for the two subunits. HIF-1α was found to be ubiquitously expressed, whereas HIF-2α expression was restricted to vascular endothelial cells, the lung, and the sympathetic nervous system [94, 98, 196, 197]. Studies of knockout mice showed that Hif-1α $^{-/-}$ and Hif-2α $^{-/-}$ mice both exhibited intrauterine lethality albeit for different reasons. While Hif-1α $^{-/-}$ displayed disturbed vasculogenesis and CNS defects [97, 117, 164], the Hif-2α $^{-/-}$ mice died from aberrant cardiac function caused by insufficient noradrenaline levels [196], displayed normal vasculogenesis but defective vascular remodeling [139], or died from respiratory distress caused by inadequate surfactant production due to reduced VEGF levels [46]. These findings were the first indications of distinct, nonredundant roles of the two HIF-α subunits. The differing expression patterns have been shown to persist in the adult life; in contrast to HIF-1α, which remains ubiquitously expressed in all tissues, HIF-2α expression is restricted to specific organs [206]. At hypoxia, induction of HIF-2α in normal, non-malignant tissues is restricted to a subset of cells within these organs [159, 206]. Interestingly, the tissue and cell-type specificity of HIF-2α vanishes with malignant transformation; HIF-2α is widely expressed in tumors, tumor associated macrophages, and in cancer cell lines [112, 193, 207].

Both HIF-1α and HIF-2α are expressed in breast cancer. In breast cancer cell-lines, both subunits have been shown to be induced by hypoxia [22, 33]. However, as shown in Paper III, HIF subunit expression patterns are cell-line, oxygen level, and time dependent, similar to the results reported for neuroblastoma [93].

Present Investigations

Aim: The object of the studies performed within this thesis was to explore the impact of hypoxia on mammary epithelial differentiation and what consequences the hypoxic response might have on breast cancer development and behavior.

Results and Discussion

The discussion of Papers I-IV has been sectioned into two separate though related parts:

Hypoxia has a Negative Impact on Differentiation in Malignant and Normal Mammary Epithelium, Papers I and IV

In **Paper I**, we take advantage of a naturally occurring phenomenon to investigate the phenotypic changes in malignant mammary epithelial cells caused by hypoxia in vivo. Ductal carcinoma in situ (DCIS) of the breast is a malignant, though non-invasive lesion with an inherent risk of developing into invasive mammary cancer. Structurally, an intact basal membrane surrounding pre-existing spaces, such as ducts or lobules, filled with transformed cells, defines the lesion. Through unheeded proliferation, these malignant cells come further and further away from the vasculature located at the basal membrane. Eventually, the cells furthest away from the vessels go under and areas of central necrosis are formed. The DCIS lesion creates thereby a natural model of a contained oxygen gradient accessible to immunohistochemical analyses using routine clinicopathological procedures.

The hypoxic milieu within the lesion was confirmed by a rising gradient of HIF-1α stabilization. We then analyzed the expression patterns of two proteins associated with mammary epithelial differentiation, cytokeratin 19 (CK19) and ER, and found that the expression of these proteins changed within the areas of high HIF-1α expression. The new ER^-, $CK19^+$ phenotype was additionally connected to morphological features of low differentiation. The in vivo findings were mimicked by in vitro data.

We concluded that hypoxia induced a less differentiated phenotype in the malignant cells and proposed hypoxia-induced dedifferentiation as one of the mechanisms behind hypoxia-driven malignant progression.

The DCIS model provided us with an in vivo model of hypoxic HIF stabilization. Even though a clear correlation between hypoxia and tumor progression exists [25, 26, 29, 90, 91], some authors have voiced doubt as to whether HIF-α expression actually corresponds to hypoxia in tumors. Studies correlating the binding of the chemical hypoxia marker, pimonidazole, to CAIX, GLUT-1, and HIF-1α expressions and tumor oxygen tensions measured by Eppendorf electrodes have produced varying results; some showing significant positive and some non-significant correlations [100, 110, 120]. As discussed for

Paper III, HIF-α expression in tumors is a result of both hypoxic and non-hypoxic induction. Furthermore, HIF-target genes are differentially induced by HIF-1α and HIF-2α [33, 93, 94]. In this DCIS model, where the cells farthest away from the blood supply displayed changes mimicked by hypoxic in vitro conditions, it seems highly plausible that the new phenotype is in fact a product of hypoxia.

In order to investigate the impact hypoxia has on mammary epithelial differentiation, we needed markers that specifically characterize various levels of maturity, similar to the CD proteins in hematopoietic differentiation. At that time, the CK:s offered an overview of the epithelial differentiation status suitable for the intended screening. Cytokeratin expression patterns in mammary development had been detailed for the entire life span of the female breast from embryogenesis to post menopausal adult life [8, 125]. Also, the fact that CK expression is routinely analyzed on paraffin sections in clinical practice made this an easily accessible, practical system with verified antibodies and established evaluation criteria. Furthermore, CK expression in mammary cancer had been analyzed in relation to malignant progression, in transition from benign to malignant lesions, and in relation to clinicopathological parameters, thus creating points of reference from which findings from more basic research can access everyday clinical concepts. Since the publication of Paper I, search for putative mammary stem cells has established several additional markers of the early differentiation stages. ESA, MUC, Lineage, CD24, CD44, and CALLA expression in DCIS and in hypoxic cell lines could and should be analyzed. Additionally, even though the CK:s provide a good overall appraisal of differentiation, the system is rather robust and requires additional markers to achieve detailed analysis of cellular maturity. CK19 had then recently been shown to characterize a pluripotent mammary epithelial cell, thus confirming its status as a marker of early differentiation [79]. Apart from CK19, we analyzed the expression of CK14 and vimentin. Both are markers of the myoepithelial lineage and as such were not expressed by the luminal cells of the DCIS. We found no hypoxia related changes in their expression. According to the accepted view on differentiation markers associated with early stages of mammary epithelial differentiation (Fig. 2), this was not an unexpected finding. By analyzing additional cell markers a more precise definition of the dedifferentiated state could be achieved. However, as shown by Al-Hajj et al. [2], both morphological and functional analyses are required to establish the actual level of epithelial cell maturity. The study by Al-Hajj et al. also established that similar to neoplastic cells of hematopoietic malignancies, breast cancer cells retain remnants of normal differentiational programs.

What is hypoxia driven malignant progression through dedifferentiation? Hypothetically, hypoxia seems to set off programs that evolutionarily have been designed for embryogenesis. The affected cells acquire traits of early developing cells such as ability to survive in hypoxic milieu and to migrate. It seems that these "programs" are by default coupled to lower levels of differentiation, i.e. that in order to survive hypoxia, the fully differentiated cell, through HIF directed/connected pathways, automatically suppresses expressions of proteins correlated to higher level of maturity, such as milk synthesis. Changes in the cytoskeleton seen as changes in CK expression patterns are also explainable in the light of hypoxia-induced metastatic potential. During the past decade, the concept of cancer stem cells or tumor initiating cells has evolved greatly, offering

explanations for the development, progression and metastasis of cancer. How does the concept of cancer stem cells fit into the thesis of hypoxia-induced dedifferentiation and malignant progression?

A stem cell is an undifferentiated cell with the ability to, through asymmetric cell divisions, self-renew and to produce progeny, which are capable of differentiating into the various cell types present within the mature organ [169]. In vivo self-renewal assays have demonstrated within tumors the presence of a subset of self-renewing cells with tumorigenic potential. Only these cells were found to be able to reconstitute a tumor after injection into an immunodeficient mouse [51]. Such cancer stem cells have been identified in a number of neoplasms, including those of hematopoietic origin [107], brain [176], breast [2], and colon [130, 156]. The cancer stem cells are not the same as the normal somatic or embryonic stem cells yet, they do share several similarities, the most pivotal being the mentioned ability to self-renew *ad infinitum*.

Stem cells in general reside in specific locations, "niches", which supply them with signals needed for maintaining the undifferentiated stem cell population. A central factor contributing to the stability of the undifferentiated state is hypoxia. This has been shown in studies of embryogenesis where low oxygen levels were found to be essential to proper development of the placenta and embryo, reviewed in [16]. As an example, by rising oxygen levels, Genbacev et al. [73] induced differentiation and slower proliferation in cytotrophoblasts from a developing placenta. Lee et al. [110] injected pimonidazole hydrochloride into pregnant mice and verified the existence of hypoxic regions that co-localized with angiogenesis in the developing mouse embryos. Ramirez-Bergeron et al. [149] showed defective hematopoiesis and inhibition of vasculogenesis and angiogenesis in ARNT $^{-/-}$ mouse embryos that thus lacked the HIF response to hypoxic stimuli.

The cancer stem cells are hypothesized to arise through either oncogenic mutations in normal stem cells or through alterations within a previously differentiated cell that now acquires the potential of unlimited self-renewal [32]. Recent findings indicate that hypoxia-induced pathways are implicated in the modulation of the transcriptional activity of factors essential to the maintenance of stem cell phenotype, such as Oct-4, c-Myc, and Notch [16]. Interestingly, Takahashi [191, 192] et al. were able to create stem cells from adult fibroblasts by inducing expression of Oct-4, c-Myc, Sox2, and Klf4. Knowing that hypoxia induces dedifferentiation, it is easy to envisage that by potentiating the genomic instability of malignant cells and/or by setting off "embryologic" pathways originally designed to maintain an undifferentiated highly proliferative state, hypoxia contributes to formation of, if not *de facto* creates cancer stem cells.

In **Paper IV**, we investigated whether the observed phenotypic changes in tumor cells were in fact paralleled by hypoxia-induced alterations of differentiational behavior defined as the ability of epithelium-derived cells to form polarized three-dimensional structures. For these experiments we used a non-transformed, immortalized mammary epithelial cell-line, MCF-10A, known to have the capacity to form polarized, acini-like structures in 3D cultures [53]. The MCF-10A cells were allowed to grow in extra-cellular matrix at normoxic and hypoxic conditions and the colonies formed were then investigated by

immunofluorescence. We saw that at hypoxia, the capacity to form polarized structures was significantly diminished. This was paralleled by changes in distribution patterns of proteins associated with epithelial polarization such as α6-integrin, laminin V, ERM-proteins (ezrin/radixin/moesin), and HMFG-1. The hypoxic distribution patterns were similar to those reported to occur during malignant progression in breast cancer.

We concluded that hypoxia impairs both morphological and functional differentiation of the MCF-10A cells. The observed changes in distribution patterns of polarization-associated proteins at hypoxia further support our thesis of hypoxia-induced dedifferentiation as a mechanism behind malignant progression.

The 3D culture method enables easy access for analyses by immunofluorescence. However, we wish to supplement our analyses with more quantitative ones, and seeing as each experiment takes 21 days to complete, ones that allow for a more massive screening. The observed differences are of qualitative rather than quantitative character; therefore, a Western blot might show the same protein levels in normoxic and hypoxic cultures in spite of the observed differences in distribution. As several of the analyzed proteins were located in the cytoplasm of the hypoxic cells as opposed to the cell membrane in the normoxic cells, cytoplasm vs. cell membrane fraction analyses might provide us with a quantitative method. We are currently optimizing isolation of the hypoxic spheroids from the Matrigel.

Our findings raise the question of hypoxia-induced selection; seeing that a few polarized hypoxic organoids have been found, do we select for the few cells that ARE able to polarize at hypoxia? If this is the case, do these cells represent a subpopulation of stem-like cells? Selection would imply increased apoptosis and we do not see any evidence of that. If the organoids are formed only by a selected subpopulation of cells, this should be true also for normoxia, and it does not seem plausible that this subpopulation would expand so greatly at normoxia during the course of the experiment. Furthermore, *per definitionem*, stem-like cells should not be able to polarize in the same way as fully differentiated cells. The morphology of mammospheres formed by mammary stem-like cells is different from that of acini-like structures formed by luminal cells [56]. Our group and others have previously shown that the hypoxia-induced phenotype in neuroblastoma and breast cancer cell lines is reversible [48, 92]. To this end, we are currently conducting time series experiments where the 3D cultures are alternated between hypoxic and normoxic growth conditions. Reversibility would then point in favor of hypoxia-induced dedifferentiation.

Hypoxia-Inducible Factors in Relation to Clinicopathological and Tumor Biological Variables in Invasive Breast Cancer, Papers II and III.

Paper II: Having observed and correlated the changes in ER expression within DCIS to hypoxia (**Paper I**), we next analyzed details of ER expression in invasive breast cancer. We observed heterogeneous ER staining patterns within 24 ER^+ tumors. Staining for HIF-1α in these tumors revealed an inverse relationship between ER and HIF-1α in the HIF-1α positive, i.e. hypoxic, regions thereby confirming our previous findings. Other groups subsequently verified the hypoxia driven down-regulation of ER and attributed it to hypoxia-induced proteasome-dependent degradation of the receptor [189] [38, 48]. However, Kronblad et al. correlated increased phosphorylated-ERK expression to hypoxic HIF-1α stabilization and ER down-regulation in vivo and in vitro and went on to show that inhibition of phosphorylated-ERK resulted in renewed expression of the hypoxia-down-regulated ER through induction of ER transcription [104]. Hence, several mechanisms seem to convey the hypoxia-driven down-regulation of ER.

HIF-1α expression as studied in **Paper II** was observed in 9 out of 24 tumors with heterogeneous ER expression. Therefore, another mode of ER down-regulation accountable for the remaining tumors was sought after. Cyclin D1, over-expressed in approximately 50% of breast cancer, is known to be closely associated to ER. For one, it functions as an ER co-activator in a CDK independent manner and contributes to ligand independent ER transcriptional activity. Second, the mitogenic effect of estrogen is conveyed through cyclin D1 expression. Cyclin D1 over-expression in breast cancer is thus generally closely associated to ER positivity [18]. However, conflicting results have been reported concerning the prognostic relevance of cyclin D1 over-expression. We found within 6 of the tumors with heterogeneous ER expression areas of inverse cyclin D1 and ER expression. The regions of high cyclin D1 and low ER expression exhibited high proliferation and lower level of differentiation. Interestingly, in an additional cohort of ER^+ tumors, we found variable cyclin D1 expression. The cyclin D1-high, ER^+-low tumors were associated with high tumor grade and high proliferation, similar to the findings observed within the tumors with heterogeneous ER expression.

Paper II conveys an important aspect of breast cancer research aimed at finding prognostic and predictive markers, and of current methods of breast cancer classification. By applying general methods, without taking into account the small, statistically non-significant subgroups of differing clinical significance, we miss important biological aspects of the disease and hence create diverse groups with variable prognosis within. As a worst-case scenario, based on a too general classification we might in fact be offering some patients deleterious treatment. Attractive as it might be to publish articles on independent prognostic factors in large patient cohorts, our goal should perhaps be to identify the miniature groups. Then, with time, rather than "merely" prolonging survival in large groups, complete cure could be offered to a growing number of the small subgroups.

In **Paper III**, we investigated the correlation between HIF-1α and HIF-2α and their association to traditional clinicopathological variables and survival in two patient cohorts. We found no correlation between the expression of the two α-subunits. HIF-2α, though uncorrelated to any established clinicopathological variable, correlated significantly to impaired recurrence free- and breast cancer specific- survival. Interestingly, HIF-2α was significantly associated with distant but not local or regional recurrence. HIF-1α on the other hand exhibited variable correlations to tumor variables in the two cohorts, but no significant correlation to survival. These results pointed to differential, subtype specific induction of HIF-α as well as subtype specific functions. We therefore analyzed in vitro the impact of each subunit on the hypoxic induction of VEGF and found subtype specific correlations that were both time and oxygen level dependent.

It is today widely acknowledged that HIF:s contribute to tumor progression [170]. Given the clear correlations between hypoxia and tumor aggressiveness, and between hypoxia and HIF-α stabilization, one would expect HIF-1α and HIF-2α to have clear-cut negative correlations to prognosis. However, in breast cancer, HIF-1α has been repeatedly found to exhibit variable correlations to clinicopathological variables and survival [24, 52, 78, 105, 199, 211]. Regarding HIF-2α, even though findings in neuroblastoma correlate HIF-2α expression to poor prognosis, the correlation seems to be related to its non-hypoxic regulation [93, 145]. Leek et al. found a positive correlation between HIF-2α expressing tumor associated macrophages and bad prognosis in breast cancer [111, 112]. The findings in **Paper III** support the idea of an oncogenic, non-hypoxic role of HIF-2α, separate from HIF-1α. Firstly, an overall hypoxic induction of both subunits would create a positive correlation between them and similar associations between them and clinicopathological parameters. Secondly, patients with a HIF-2α positive tumor had an increased risk of distant recurrence. The fact that HIF-2α did not in any way correlate to established tumor and clinical variables, raises a question as to whether the significant factor was not the overall expression but the presence of specific HIF-2α expressing cells with an enhanced metastatic ability but whose presence is not picked up by the clinicopathological variables such as NHG or overall hormonal status. This would imply a situation similar to that observed in neuroblastoma, where a subpopulation of HIF-2α expressing cells was found to have an immature stem cell-like phenotype [145]. Detailed studies of HIF-2α expression in vivo, preferably in whole sections as opposed to TMA, with simultaneous staining for markers of early differentiation, correlation to the angiogenic status of the tumor and to a bioreductive marker of hypoxia, such as pimonidazole, are warranted. Furthermore, HIF-2α is known to be induced by hypoxia only in selected normal tissues but in most, if not all transformed ones [22, 206]. It was also observed (personal observations, not shown) that HIF-2α was expressed in proliferative, non-malignant breast lesions, devoid of HIF-1α stabilization. Even this finding points to an oncogenic, non-hypoxia dependent function of the HIF-2α.

We further verify that the details of the in vitro hypoxic response are cell line dependent. The cell lines investigated represent some of the different aspects of breast cancer; MCF-7 and T-47D are both ER^+, non-invasive cell lines whereas MDA-468 is ER^- and highly tumorigenic. Others have shown that different cell lines have different basal levels of HIF

target gene expressions and thus different levels of hypoxic induction of those genes [22, 33]. Extending these observations to clinical considerations would suggest that sub-groups of breast cancer (histological or otherwise specified) might differ in their levels of hypoxic vs. non-hypoxic HIF-α induction and thus present with differing prognostics.

The patient based analyses of HIF-2α expression in relation to clinical aspects and survival conducted in **Paper III** were intended as a screening to evaluate the biological significance of HIF-2α. Proper analysis of its prognostic vs. predictive value requires a randomized cohort with an untreated arm. Future studies will show if the observed prognostic significance of HIF-2α expression is general or sub-group specific.

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