Invasion and Proliferation in Malignant Cells

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Abstract

Two key events in the oncogenic process of tumor cells are to acquire uncontrolled proliferation and invasive properties. This allows the tumor to grow and invade beyond the tissue from which the tumor cells originate. We here specifically studied p16 and ERK1/2 with special focus on and the relation to proliferation and invasion in non-melanoma skin cancer and in breast cancer.

In a model system of basal cell carcinoma, we observed that tumor cells changed phenotype from a highly proliferative type in the centre of the tumor to an invasive type with low proliferation and a marked upregulation of p16 at the invasive front. The expression of p16 was transcriptionally regulated and possible p16 activators such as ERK1/2 or Ets were not the sole contributors. Similar findings were observed in squamous cell carcinoma of the skin, despite a non functional Rb pathway, which might indicate a proliferation independent role for p16 in invasive behaviors.

In primary breast cancer, a signaling cascade from VEGFR2 via ERK1/2 phosphorylation to Ets-2 phosphorylation and cyclin D1, could be outlined and ERK1/2 phosphorylation was linked to small tumors with good prognosis. We also observed a Notch1 independent activation of Hes1 in breast cancer. Further, postmenopausal patients with ERK1/2 phosphorylated tumors had an impaired tamoxifen response, but ERK1/2 phosphorylation was not linked to tamoxifen resistance in premenopausal women.

Taken together, our results implicate that there is a general inverse association between invasion and proliferation in some malignancies and this novel finding could be important when designing new treatment strategies for cancer patients.

Key words:

p16, ERK1/2, proliferation, invasion, basal cell carcinoma, squamous cell carcinoma, breast cancer

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To my beloved family

TABLE OF CONTENTS

LIST OF PAPERS

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

I **Svensson S., Nilsson K., Ringberg A. and Landberg G.** Invade or proliferate? Two contrasting events in malignant behavior governed by $p16^{INK4a}$ and an intact Rb pathway illustrated by a model system of basal cell carcinoma. *Cancer Research: 63, 1737-1742 (2003)*

II **Nilsson K.,* Svensson S.,* and Landberg G.** Retinoblastoma protein function and p16INK4a expression in actinic keratosis, squamous cell carcinoma *in situ* and invasive squamous cell carcinoma of the skin and links between p16INK4a expression and infiltrative behavior. *Modern Pathology: 17, 1464-1474 (2004)*

III **Svensson Månsson S., Reis Filho J. S. and Landberg G.** Transcriptional upregulation and unmethylation of the promoter region of p16 in invasive basal cell carcinoma cells and partial co-localisation with the γ2 subunit of laminin-5. *Manuscript*

IV **Svensson S., Jirström K., Ryden L., Roos G., Emdin S., Ostrowski MC. and Landberg G**. ERK1/2 phosphorylation is linked to VEGFR2 expression and Ets-2 phosphorylation in breast cancer and is associated with tamoxifen treatment resistance and small tumours with good prognosis. *Oncogene: 24, 4370-4379 (2005)*

V **Svensson Månsson S.,* Manetopoulos C.,* Johansson M. E., Ryden L., Jirström K., Landberg G. and Axelsson H.** Association between the ERK pathway and the Notch target gene Hes1 in primary breast cancer and in breast cancer cell lines. *Manuscript*

* Authors contributed equally.

ABBREVIATIONS

3´-UTR	3´-untranslated region
AK	Actinic keratosis
AP-1	Activating protein 1
ARF	Alternative reading frame
BCC	Basal cell carcinoma
CAK	Cyclin dependent kinase activating complex
CDC	Cell division cycle
CDK	Cyclin dependent kinase
CINK4I	Cyclin dependent kinase 4 inhibitor
CIS	Squamous cell carcinoma *in situ*
CSL	CBF-1, suppressor of hairless, LAG-1
DCIS	Ductal carcinoma *in situ*
DFS	Disease free survival
DNA	Deoxyribonucleic acid
E2F	E2 promoting binding factor
ECM	Extracellular matrix
EGF	Epidermal growth factor
ER	Estrogen receptor
ERK	Extracellular signal-regulated kinase
FAK	Focal adhesion kinase
FGFR	Fibroblast growth factor receptor
GSK	Glycogen synthase complex
HDAC	Histone deacetylase
HePTP	Haematopoetic protein tyrosine phosphatase
Hh	Hedgehog
HPV	Human papillomavirus
IGF	Insulin growth factor
INK4	Inhibitor of kinase 4
LCIS	Lobular carcinoma *in situ*
LEF	Lymphocyte enhancer complex
LHRH	Luteinizing hormone-releasing hormone
LOH	Loss of heterocygozity
JNK	C-Jun N-terminal kinase
MAPK	Mitogen activated protein kinase
MAPKK	Mitogen activated protein kinase kinase
MAPKKK	Mitogen activated protein kinase kinase kinase
MEF	Mouse embryo fibroblast
MKP	Mitogen activated protein kinase phosphatase
MLC	Myosin light chain
MLCK	Myosin light chain kinase
MMP	Matrix metalloproteinase
MT-MMP1	Membrane type matrix metalloproteinase 1
MTS1	Multiple tumor suppressor 1
NHG	Nottingham histological grade
PCNA	Proliferating cell nuclear antigen
PI3K	Phophoinositide 3-kinase
PKC	Protein kinase C
PLC	Phospholipase C
PP2A	Type 2 protein serine/threonine phosphatase
RNA	Ribonucleic acid
RB	*Retinoblastoma* gene
Rb	Retinoblastoma protein
RSK1	p90 ribosomal S6 kinase 1
PR	Progesterone receptor
PTCH	*Patched* gene
RTK	Receptor tyrosine kinase
SCC	Squamous cell carcinoma
SCF	Skp1/Cullin/F-box protein complex
SFK	SRC-family of tyrosine kinases
SMO	*Smoothened* gene
STAT	Signal transducer and activator of transcription
SV40	Simian virus 40
TACE	Tumor necrosis factor-α-converting enzyme/ metalloproteinase
TCF	T-cell factor
TGF	Transforming growth factor
uPAR	Urokinase plasminogen activator
VEGF	Vascular endothelial growth factor
VEGFR2	Vascular endothelial growth factor receptor 2
WHO	World Health Organization

INTRODUCTION

Human Cancer

General aspects

Cancer is a very common disease with uncontrolled cell growth and spread which may affect almost any tissue in the body. It can be divided into benign and malignant tumors where the benign tumors are well differentiated and does not invade the surrounding tissue nor metastasize. The malignant tumors can be both differentiated and undifferentiated and have the property to invade surrounding tissue and to metastasize to distant sites. Worldwide, men are most commonly affected by lung and stomach cancer and women by breast and cervical cancer. Eleven million people are diagnosed with cancer and 7 million people die of cancer every year according to the World Health Organization (WHO).

Tumor genesis is a multistep process and genetic alterations drive the progressive transformation of normal human cells to highly malignant derivatives. These genetic alterations affect the regulatory mechanisms that control normal cell proliferation and homeostasis. According to Hanahan and Weinberg (1), there are six acquired capabilities during tumor development that are commonly shared by most cancer cells. The cells become self sufficient in growth signals, insensitive to antigrowth signals, resistant to apoptosis, the replicative potential becomes limitidless, the tumors sustain angiogenesis and start invasion.

Skin cancer

The anatomy of the skin

The skin is the largest organ in the body. It functions as a protective barrier against microorganisms such as bacteria and fungi. The skin also protects the body from UV light, dehydration and helps to regulate the body temperature. Nerve endings located in the skin makes it possible to detect warmth, coldness and touch. In addition, the epidermal keratinocytes (squamous cells) secrete both cytokines and eicosanoids contributing to an inflammatory response and the antigen presenting cells of the skin, the Langerhans cells, plays an important role in development of for example contact hypersensitivity.

The skin consists of three major layers, the epidermis, the dermis and the subcutis (Figure 1). The epidermis consists of a multilayered sheet of keratinocytes and it can be divided into five different layers starting from the bottom to the top, the stratum basale with basal cells that undergo division and push already existing cells into the layers above, stratum spinosum, stratum granulosum, stratum licidum and the stratum corneum. There is a progressive renewal of the skin and cells are frequently lost from the stratum corneum which is made of dead flat skin cells. Apart from the keratinocytes and the Langerhans cells, there are two other specialized cell types in the epidermis, the melanocytes that produce melanin, the pigment in the skin which protects us from UV light, and the Merkel cells with an unknown function. The primary function of the dermis is to sustain and support the epidermis. The dermis can be divided into two different areas, the papillary dermis, closest to the epidermis, and the reticular dermis. The papillary dermis contains delicate collagen fibrils that extend as a sheet around blood capillaries and nerves. The reticular dermis contains mostly collagen organized into bundles associated with elastic fibers. The dermis contains many specialized

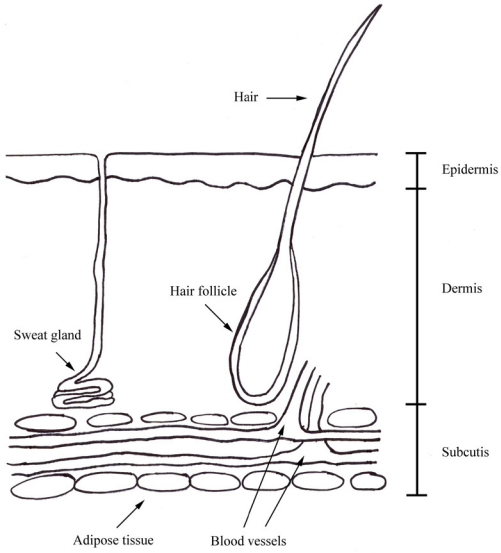

Figure 1. Schematic illustration of skin anatomy.

cells and structures, for example hair follicles, sebaceous, apocrine and sweat glands, blood vessels and nerves. The subcutaneous layer consists of loose connective tissue or adipose tissue that harbors larger blood vessels and nerves (2).

Basal cell carcinoma

Non-melanoma skin cancer comprises mainly basal cell carcinoma (BCC) and squamous cell carcinoma (SCC) and BCC is the most common malignancy within the fair-skinned population with increasing incidence worldwide (3). It is generally believed that most BCCs arise from hair follicle keratinocytes (4, 5). The lesion usually develops on sun damaged skin in people with fair skin and freckles, but it can also arise in areas not exposed to intense sunlight. BCC is sometimes locally very aggressive but does rarely metastasize (6).

BCC can develop in a both sporadic and hereditary fashion. Nevoid BCC syndrome or Gorlin's syndrome is an autosomal dominant inherited disorder and one characteristic feature of these patients is that they develop multiple BCC at an early age. Studies have shown that individuals affected by nevoid BCC syndrome harbor mutations and/or loss of heterozygosity in the *patched* (*PTCH*) gene (7-10). LOH and/or mutations in *PTCH* gene, in addition induction of the Gli1 protein (11) as well as activating mutations in the *smoothened* (*SMO*) gene (12) are also common in sporadic BCCs. Alterations in the *PTCH* and genes associated to the hedgehog (Hh)/patched/smoothened pathway results in constitutively active hedgehog signaling and thereby increased proliferation instead of differentiation. In addition, *P53* gene mutations are also frequent in both hereditary and sporadic basal cell carcinoma (9, 13).

There is no formal grading system for BCCs. In Sweden however, a proposal to classify BCCs into four different groups according to the "Sabbatsbergsmodellen" has been suggested. In this model, BCC classification is dependent on growth pattern and histological invasive

appearance (Figure 2) because the growth pattern can predict the recurrence of BCC (6). The different groups are called IA, IB, II and III. Group IA consists of nodular tumors that have round tumor masses growing within the dermis and peripheral palisading of tumor cell nuclei is prominent. Large tumor clusters are dominating. The group IA tumors have an even ”pushing” border between the tumor and the surrounding tissue. The tumor masses might be solid or with a central necrosis. Some of the tumors have an ulcer at the skin surface. This tumor growth pattern is the most common with around 50 % of the tumors (14). Group IB, the superficial tumors, have multiple buds of proliferating basal cells growing inwards from the epidermis into the superficial dermis whilst maintaining attached to the dermis. Group II and III are the infiltrative tumors of which group III are the most aggressive. Group II display small micro nodular and irregularly tumor clusters with an uneven border towards close tissue and group III display groups of tumor cells of varying size, often with small spiky irregularly branching strands. Both groups can be found in subcutaneous tissue such as bone, muscles and cartilage. In the morphoeic type, which is a subgroup of infiltrative tumors (≈5%), are all tumor cell clusters small and grow in irregular strands of tumor cells that infiltrate into the dense sclerotic eosinophilic fibrous stroma.
BCC is an easily treated disease, which is dependent on the location and size of the tumor, and the most common treatment of BCC is surgery.

Squamous cell carcinoma
SCC of the skin is derived from keratinocytes in the epidermal layer of the skin. It is a locally invasive malignancy that rarely metastasizes but can form lethal metastasis (15). Unlike BCC that is thought to develop de novo, it is generally believed that SCC can emerge from the premalignant lesions called actinic keratosis (AK) (16) and squamous cell carcinoma *in situ* (CIS). It has been estimated that 10% of the AK lesions develop into SCC of the skin (7), but it has been argued whether AK progress to CIS before further progression into SCC. SCC mainly occurs on sun exposed sites such as the back of the hands or in the face. The lips and the ears are also common sites for SCC. SCC is treated in the same way as BCC and surgery is the most common method used.
AK can be recognized by the presence of atypical keratinocytes in the deeper portions of the epidermis, by a variably thickened epidermis and that the loose and basket-weaved stratum corneum is replaced by a dense parakeratotic and or hyperkeratotic area. There are often some acantholytic cells and also some acanthosis. In addition, infiltration of lymphocytes is frequent (17). In the preinvasive stage of SCC, CIS, the entire epidermal layer is replaced by

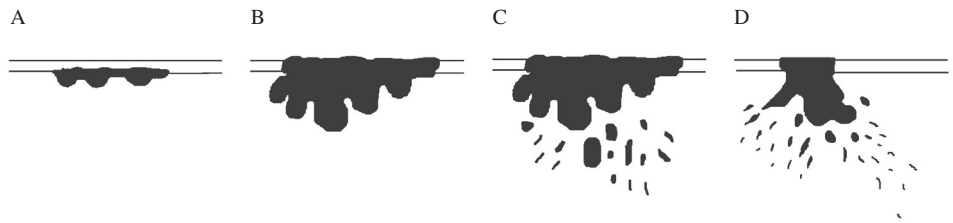

Figure 2. Basal cell carcinoma classification according to “Sabbatsbergsmodellen” (A) IB = superficial basal cell carcinoma, (B) IA = nodular basal cell carcinoma, (C and D) II and III = infiltrative basal cell carcinomas.

atypical keratinocytes, but the atypical keratinocytes have not invaded trough the basement membrane. SCC is composed of tumor cells that mimic the epidermal stratum spinosum and the tumor cells infiltrate into the adjacent dermis with irregularly shaped clusters of tumor cells.
Inactivation of the tumor suppressor gene *P53* encoding the p53 protein seems to play a central role in the development of SCC. Approximately 50% of all SCCs harbor mutations in the gene with many of the alterations being typically UV induced such as CC→TT and C→T mutations (7, 18, 19). In addition, mice deficient in p53 develop SCC resembling tumors if they are subjected to UV-B radiation (20). In most SCCs, Ras is activated, either biochemically in its GTP bound form or by direct mutation (15). The *INK4A* is another locus commonly altered in cancer. It has been found that up to 24% of the SCCs harbor mutations in the gene (21, 22) and loss of heterozygosity of the 9p21 region (where the *INK4A* locus is situated) has also been observed in SCCs (16, 23).
The role of human papillomavirus (HPV) in skin SCC is still being debated. In one study HPV deoxyribonucleic acid (DNA) was found in 27% of SCCs from immunocompetent patients (24). HPV 38 has been detected in approximately 50% of SCC whereas in normal skin 10% harbored the virus (25). Another study showed that HPV has been found in 43% of actinic keratosis and 13% of the SCC (26). On the contrary, HPV DNA was found in samples collected in the superficial layers of SCC, whereas deeper into the SCC HPV DNA was found only small fraction of tumors (27).

Breast cancer
Breast cancer is the most common malignancy diagnosed in women in the Western World with approximately one in eight women affected and today breast cancer is one of the leading causes of death globally (28). The worldwide incidence of breast cancer is about a million affected women per year (29). In Sweden the breast cancer incidence was more than 6500 in 2004 and there has been an annual increase of 1.5% in incidence for the last 20 years (Socialstyrelsen, Statistics, Health and Diseases, 2005:9). The increasing incidence is thought to be due to more advanced screening techniques (mammography) as well as increased exposure to risk factors (e.g. hormones and environmental factors). Fortunately, the prognosis for a patient developing breast cancer has improved. In Sweden, despite the increase in incidence, the number of breast cancer deaths has remained relatively constant, about 1500 per year (Socialstyrelsen, Statistics, Causes of Death 2003), and the progress is believed to be due to both screening resulting in an earlier diagnosis as well as improved patient treatment strategies over the last years.

The anatomy of the breast
Already as early as embryonic week 6, can the developing human breast be recognized and in week 12-16 small clusters of cells start to branch that lay the foundation for the future ducts and milk producing glands. During the reproductive phase in life the female breast tissue will continually change. The breast mature with lobule, ducts, fat and connective tissue at the onset of menarche due to the release of hormones (estrogen) from the ovaries (Figure 3), but it is not until pregnancy that the breast becomes fully developed. During pregnancy the pituitary gland and placenta releases hormones with the end result of an increased number of terminal ducts. In addition, the lobular epithelium increases to become the major component of the breast tissue. During lactation the breast epithelial cells become vacuolated and the

lumina distended with breast milk. When lactation ceases the epithelium is eliminated by apoptosis and the glands returns to a more virgin like anatomy. After the age of thirty, the breast tissue starts to degenerate and the terminal ducts atrophy and the fibrous connective tissue is gradually replaced with fat. The terminal duct lobular unit consists of two different epithelial cells, the luminal cells and the myoepithelial cells. The luminal cells have milk secreting capacity and the myoepithelial cells have contractile capacity as a response to oxytocin stimulation (2).

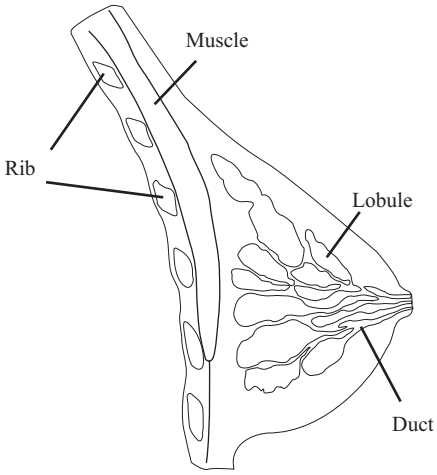

Figure 3. Schematic illustration of breast anatomy.

Pathology

The majority of breast cancers are of the luminal phenotype. If a breast cancer is classified as non-invasive or in situ the cancer cells have a restricted growth inside the basement membrane of the ducts or the lobules. There are two large groups of *in situ* cancers, ductal carcinoma *in situ* (DCIS) and lobular carcinoma *in situ* (LCIS). For prognostic determination, considering nuclear grade, architectural differentiation and the presence of necrosis has proven important (30). If left untreated, many of the *in situ* carcinomas progress into invasive carcinomas (31).

The invasive cancer cells invade, passing the basement membrane, into the surrounding tissue. The invasive carcinomas are classified according to tumor type, histological grade and tumor stage to predict prognosis. Another important factor predicting prognosis is age at diagnosis.

According to the WHO classification system of invasive breast cancer, invasive ductal carcinoma makes up the vast majority (80-85%) of invasive breast cancers. Microscopically, invasive ductal carcinoma grows as irregular cords and nests of epithelial cells mainly within the dense fibrous stroma and the cells show a variable degree of differentiation. The less differentiated grows as solid sheets of neoplastic cells and the well-differentiated tumor cells may form abortive glands. The second most common type of invasive carcinoma (5-15%) is lobular carcinoma, which may occur alone or mixed with ductal carcinoma. The most common growth pattern of lobular carcinoma consists of single or two cell strands

of malignant cells infiltrating between stromal fillings often encircling ducts and lobules. Medullary carcinoma comprises up to 7% of all breast carcinomas. It is an invasive cancer but the tumor cells grow in a solid mass with lymphoid infiltrate in the periphery of the tumor. Tubular carcinoma is composed of infiltrating well-formed small ducts that resemble the normal breast. The tumor cells are small only with slight nuclear atypia. Colloid or mucinous carcinoma makes up of about 2% of the breast cancers. Histologically, mucous is found within and around clusters of epithelial cells. Two uncommon types of invasive cancer with lower incidence than mucinous carcinoma are invasive papillary carcinoma and metaplastic carcinoma (2, 31).

Nottingham histological grade (NHG) is the formal tumor grading classification system introduced in the 1950s by Bloom and Richardson (32) further modified in 1991 by Elston and Ellis (33). NHG can be performed on all subtypes of tumors and evaluates the resemblance between the tumor cell and the normal cell from which it arose. The grading is based on morphological appearance of the tumor and tumor cells and three parameters are evaluated, tubular formation, nuclear atypia and mitotic count. The tumor grade is based on the sum of the scores of these parameters NHG I: well differentiated (score 3-5), NHG II: moderately differentiated (score 6-7) and NHG III: poorly differentiated (score 8-9).

Staging of breast cancer takes into account the size and describes the extent of the breast cancer disease. The TNM classification system was accepted in 1992 by American Joint Committee on Cancer and involves the size of the primary tumor (T), lymph node status (N) and distant metastasis (M). Stage I patients have tumors less than 2 cm in size and are negative for lymph node and distant metastasis. Stage II tumors are between 2-5 cm in size and do not involve skin and chest wall, but can be lymph node positive. Stage III disease are locally advanced attached to the skin or the chest and are lymph node positive and stage IV tumors display positive lymph nodes above the collarbone, distant metastases beyond the breast axilla or to distant organs (34, 35).

Patient age is also an important prognostic marker. Patient younger than 35 and older than 75 seems to have worse prognosis that patients between 36 and 74 years of age (36, 37).

Molecular markers in invasive breast cancer

The estrogen receptor (ER) and the progesterone receptor (PR) are routinely evaluated in all primary invasive tumors using immunohistochemistry. The percentage of positive tumor cell nuclei (regardless of intensity) is scored and tumors with more than 10% positive nuclei of either receptor are considered positive. Approximately 70-80% of all invasive breast tumors are considered ER positive and subjected to endocrine treatment, for example tamoxifen. Around 60% of tumors are PR positive and a few percent of the PR positive tumors are ER negative, but they still seem to respond to endocrine treatment and PR has shown to be an independent predictive factor that might more accurately predict the response to endocrine treatment than ER (38, 39).

The *c-erbB2* proto-oncogene encodes a tyrosine kinase receptor called ErbB2 (HER2/Neu). The receptor is signaling via the phosphoinositide-3 kinase (PI3K) and the ERK1/2 kinase cascades which results in many diverse effects related to cell growth and differentiation (40). The *c-erbB2* gene is overexpressed/amplified in approximately 20% of all breast cancers and most commonly overexpressed in low differentiated breast cancers (41, 42). Trastuzumab (Herceptin) is a recombinant monoclonal antibody which targets the extracellular domain of ErbB2 protein and reduces signaling via the PI3K and the extracellular signal regulated

kinase1/2 (ERK1/2) kinase cascades and induces apoptosis and growth arrest. Patients with tumors that overexpress the ErbB2 protein may benefit from adjuvant treatment with this antibody and up to 52% reduction in recurrence rate has been reported (43-45).

Treatment

Surgery

Due to the introduction of mammography, breast tumors are discovered at an earlier stage in tumor progression and are generally smaller in size than before the introduction of mammography. Therefore, breast conserving surgery has become the treatment of choice and the advantages of breast conserving surgery are that good cosmetic result can be achieved which leads to a better self image and a more rapid recovery of the patient. Breast conserving surgery is often combined with postoperative radiation. If the tumor is large (>4 cm), multifocal/multicentric or if the patient does not want postoperative radiation then mastectomy is preferred. In terms of survival, both methods are equally effective (46). In addition to surgical removal of the primary tumor, dissection of up to ten axillary lymph nodes is recommended for all patients with invasive breast cancer. It is becoming increasingly common that the lymph node(s), sentinel node(s), which the lymphatic drainage passes from the breast to the axillary lymph nodes, is removed and examined as an indicator of axillary lymph node status, especially for patients with tumors less than 2.5 cm in size and with clinically negative axillary lymph nodes (47).

Radiotherapy

Postoperative radiotherapy is given to patients with breast conserving surgery to remove possible residual breast cancer cells. The radiation reduces the risk of recurrent disease and is a standard procedure for patients with invasive breast cancer. Post-mastectomy radiotherapy is offered to patients with high risk of recurrence, patients with insufficient operation margins and patients with locally aggressive tumors and/or axillary lymph node metastasis. These patients may receive radiation not only to the area of surgery, but also to axillary lymph nodes, around the collarbone and to the chest wall (48).

Chemotherapy

Chemotherapy can be given to patients both before and after surgery. Neo-adjuvant chemotherapy is used to shrink the size of a tumor prior to surgery. Adjuvant chemotherapy is given after surgery to reduce the risk of recurrence as a standard treatment for patients with hormone receptor negative tumors irrespective of lymph node status. Chemotherapy is most commonly given as a cocktail of drugs and the idea is to eliminate rapidly dividing tumor cells. Unfortunately, other rapidly dividing cells are also affected resulting in unwanted side effects such as hair loss, nausea and immunodeficiency. Treatment with combinations of anthracyclins seems to be more effective than non-anthracyclin combinations for example CMF (cyclophosamide, methotrexate and fluoracil) (49). Anthracyclins (doxorubicin, epirubicin) functions by interchalating into the minor groove of DNA and by free radical damage to ribose DNA. The main effect of cyclophosphamide is due to its metabolite phosphoramide mustard, which forms DNA cross links between and within DNA strands and thereby causes cell death. Methotrexate and fluotracil are both antimetabolite drugs, methotrexate inhibits the synthesis folic acid, necessary for thymidine formation and hence DNA synthesis and fluoracil is an pyrimidine analogue, that is transformed inside the cell into different cytotoxic metabolites which are then incorporated into DNA and ribonucleic

acid (RNA), finally inducing cell cycle arrest and apoptosis. Taxanes (paclitaxel and docetaxel) bind to and stabilizes the microtubules which cause inhibition of cell division and induction of apoptosis. In randomized trials, taxanes have proven effective in metastatic disease but also in high risk early breast cancer disease (50). In some settings, hormone receptor positive patients are given a combination of adjuvant endocrine therapy, adjuvant chemotherapy and radiotherapy (49).

Endocrine therapy
Historically, hormonal treatment of breast cancer patients has been used for over 100 years. The idea is to either block the interaction of estrogen with its receptor or to deprive the ER positive cell of estrogen. In premenopausal women, the majority of estrogen is produced in the ovary and the minority of estrogen in the peripheral tissue such as adipose tissue by aromatase conversion of androstenedione and testosterone to estrone and estradiol. The primary source of estrogen in postmenopausal women is produced by aromatization. In premenopausal women, effective treatment against breast cancer is deprivation of serum and tissue estradiol by either irreversible ovary ablation or by reversible treatment with luteinizing hormone-releasing hormone (LHRH) agonists (51). Tamoxifen has proven effective in both premenopausal and postmenopausal women. Tamoxifen is a selective estrogen receptor modulator that stabilizes the receptor in the nucleus. Today, five years of treatment with tamoxifen is the current guidelines (51, 52). In postmenopausal women, were the vast majority of estrogen production is catalyzed by the enzyme aromatase, aromatase inhibitors reduce the levels of estrogens in the circulation and have shown effective in improving disease free survival (DFS), but since the follow-up time is short, no significant difference was found between tamoxifen and aromatase inhibitors (53). Therefore tamoxifen is still the primary choice of treatment for ER positive postmenopausal women but in unresponsive patients, the aromatase inhibitor Anastrozole is the second-hand choice. Fulvestrant is a pure ER antagonist which blocks and degrades ER that is currently only used in advanced breast cancer and not in the adjuvant setting (54, 55).

The Cell Cycle

Overview
The cell cycle is the process by which cells multiply by division. In multicellular organisms it plays an essential role during early development, but most cells are quiescent in an adult organism. Only specialized cells such as cells that populate the hematopoietic system or epithelial cells in the skin or the gastrointestinal tract continue to proliferate actively. Quiescent cells, except cells that are terminally differentiated e.g. senescent cells, have the capacity to re-enter the cell cycle, for example during wound healing. Therefore, the cell cycle must be tightly regulated and loss of cell cycle control ultimately leads to cancer.
The basic cell cycle is divided into four different phases. During two of these phases the cell generates a copy of the genomic material, the S or synthetic phase, and divides into two identical daughter cells, the M or mitosis phase (Figure 4). The S-phase and the M-phase are separated by two different gap phases, the G1- and G2-phases. In G1 the cell checks the integrity of its genomic content and prepares for multiplying its genome. In G2 the duplication of the genome has been completed and the cell synthesizes proteins needed for mitosis. It is essential for the cell that S- and M-phases are not initialized before the previous

is completed and therefore certain criteria have to be meet which is controlled by different checkpoints. The two most critical checkpoints are the R1 and the R2 checkpoints which occur at the G1/S and the G2/M transitions, respectively. These are points of no return that commit the cell to a new round of cell division. During the G1-phase the cell makes most of the decisions between quiescence and growth. Before the restriction point (R1), the cell responds to mitogenic and anti-mitogenic external signaling. If a cell division stimulatory signal binds to a receptor on the cell surface, the signal is transmitted to the nucleus via a cascade of cellular signaling pathways. This makes the cell complete G1-phase and starts the S-phase. On the other hand if such signal is absent or anti-mitogenic, the cell exits the cell cycle into a G0-phase (56).

The cell cycle machinery

All mammalian cells share the same molecular machinery to regulate the cell cycle. The proteins that drive the cell cycle progression are called cyclin dependent kinases (CDKs) (57). The CDKs are serine threonine kinases, highly homologous, have a molecular weight of approximately 34 kDa and the expression levels of CDKs remain at a rather constant level throughout the cell cycle. Cyclins consists of a family of proteins that share a 100 amino acid region of structural homology called the cyclin box which binds to and forms a heterodimeric complex with CDKs and thereby regulate the catalytic activity of the CDKs (58). In addition to the necessary complex formation, the CDKs must be phosphorylated by CDK activating kinase (CAK) for proper catalytic activity (59) CDKs are also regulated by inhibitory phosphorylations that can be removed by cell division cycle (CDC) 25A, B and C (57). The active cyclin-CDK complex drives the cell cycle by phosphorylating cell cycle phase specific substrates needed for induction of critical enzymes involved in for example DNA replication, nuclear envelope breakdown and chromosome segregation (60). In contrast to CDKs, cyclins oscillate throughout the cell cycle enabling different complexes to be active in specific phases of the cell cycle. When the role of the cyclin-CDK complex has been completed, the cyclins are rapidly ubiquitinated and degraded (61).

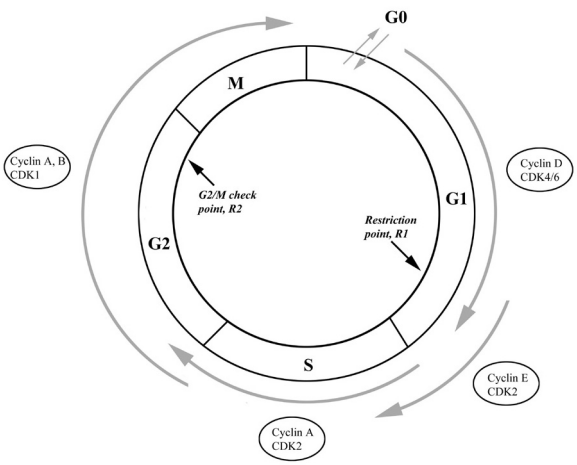

Figure 4. The cell cycle. Quiescent cells (G0-phase) have the capacity to reenter the cell cycle. Upon mitogenic stimulation D-type cyclins are upregulated and form a complex with CDK4/6 which drives the cell trough G1-phase. Together with the cyclin E-CDK2 complex the cyclin D-CDK4/6 enable the cell to pass the restriction point R1 which is the point of no return and the cell is committed to one round of cell division. Cyclin E-CDK2 together with cyclin A-CDK2 push the cell trough the S-phase where the DNA is replicated and cyclin A/B-CDK1 complexes are active in G2- and M-phase in which the cell prepares for and undergoes mitosis. Modified from (61).

G1/S checkpoint cyclins and cyclin dependent kinases

Two different cyclins in complex with CDKs function and drive the cell trough the G1-S transition, cyclin D and cyclin E. The expression of D-type cyclins (D1, D2 and D3) are directly affected by mitogenic stimuli, for example as an end point in the ERK1/2 signaling (62-65). The different D-type cyclins are expressed in a tissue specific manner but all of them interact with CDK4 and CDK6 (66). D-type cyclins are rather unstable molecules and are targeted for degradation by the Skp1/Cullin/F-boxprotein (SCF) ubiquitin ligase complex and exported out of the nucleus by the glycogen synthase kinase-β (GSK3β), a kinase inhibited by the RAS/PI3K/AKT pathway (67). Upon mitogenic stimulation, the cyclin D-CDK4/6 complex is formed, with the help of p21 and/or p27, enters the nucleus and becomes activated by CAK mediated phosphorylation (59). The active cyclin D-CDK4/6 complex phosphorylates the retinoblastoma protein (Rb) and initiates the inactivation of Rb and subsequent displacement of for example histone deacetylases (HDACs) from Rb. This results in chromatin remodeling and thereby transcription of early E2 promoting binding factor (E2F) dependent genes necessary for S-phase entry, for example cyclin E (Figure 5) (68). In active state, Rb binds to and inhibits proteins such as the E2F family of transcription factors. The Rb/E2F complex also participates in an active repression of some promoters. Cyclin E-CDK2 further inactivates Rb by phosphorylation on additional sites and transcription of genes essential for S-phase entry occur, enhanced by free E2F transcription factors. This shift in Rb phosphorylation from the mitogen dependent cyclin D-CDK4/6 phosphorylation to the mitogen independent cyclin E-CDK2 phosphorylation coincides with passing trough the restriction point (R1) after which the cell are destined divide into two new daughter cells. Beyond the G1-S restriction point Rb is kept hyperphosphorylated by the cyclin A- and the cyclin B-CDK complexes. When the cell exit mitosis and enters the G1-phase Rb returns to its hypophosphorylated state (56). It has been a general assumption that the cyclin D-CDK4/6 and the cyclin E-CDK2 complexes are necessary for S-phase entry, however, data are now present showing that embryonic mice lacking all D-type cyclins (69) or both CDK4 and 6 (70) are viable at embryonic week 17.5 or until the end of pregnancy, respectively, and yet the developing embryos proliferate in a cyclin D-CDK4/6 independent fashion. In addition, targeting both cyclin E1 and E2 in mice also results in embryonic lethality but the embryos are found alive until E10.5 (71) and CDK2 knockouts are viable but sterile (72). These data indicate that cyclins and CDKs are redundant and that these proteins are required for proliferation only in selected cell types.

Rb pocket proteins

The *retinoblastoma* gene (*RB*), encoding a protein called Rb, was the first tumor suppressor gene to be cloned (73). In the hereditary disease retinoblastoma, the affected patients have an inherited mutation in the *RB* gene and when the other *RB* allele is lost, as the rate-limiting step, the patients develop retinoblastoma tumors (74). Rb plays a very important role in the cell cycle, by interacting with E2F family of transcription factors (E2F in a dimeric complex with DP), acting as a check in the progression of cells from G1- to S-phase by repressing genes necessary for DNA synthesis (75-77). The Rb mediated repression of transcription occurs in two different ways, Rb blocks the interaction between E2F transcription factors (76) and co-activators as well as other transcriptionfactors for example Elf-1(78). In addition, Rb recruits chromatin remodeling enzymes e.g. HDACs, SWI/SNF chromatin remodeling factors (68, 79), polycomb proteins (80) and methyl transferases to further repress transcription (81).

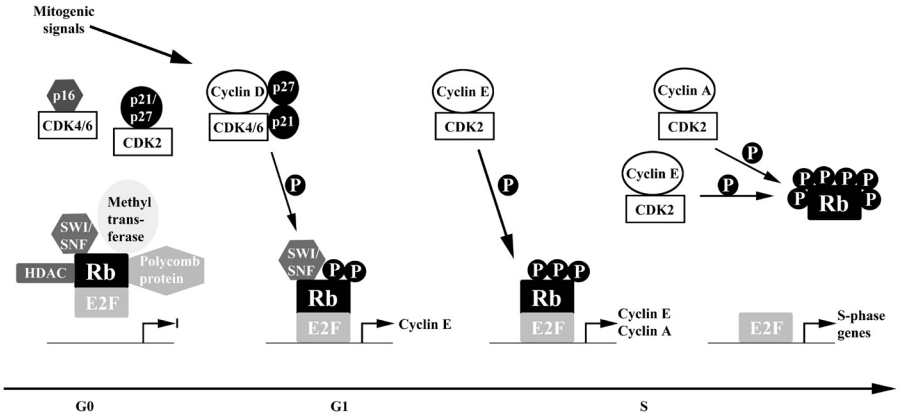

Figure 5. The G1/S transition. In G0 cells are inactive due to high levels of CDK inhibitors for example p16, p21 and p27 inhibiting the kinase activity of CDKs and thereby Rb is kept in its hypophosphorylated state. Upon mitogenic stimulation, the cyclin D-CDK4/6 complex is formed, with the help of p21 and/or p27 sequestered from the cyclin E-CDK2 complex. The active cyclin D-CDK4/6 complex phosphorylates the retinoblastoma protein (Rb) on multiple sites and initiates the inactivation of Rb and subsequent displacement HDACs, methyl transferases and polycomb proteins from Rb, resulting in chromatin remodeling and transcription of early E2F dependent genes necessary for S-phase entry, for example cyclin E. Cyclin E-CDK2 further inactivates Rb by phosphorylation on additional sites. Cyclin A is synthesized and transcription of early S-phase genes essential for S-phase entry occurs, enhanced by free E2F transcription factors. Beyond the G1-S restriction point Rb is kept hyperphosphorylated by the cyclin A-CDK2 complex.

In addition, more than 100 proteins have been shown to bind to Rb (82). Inactivation of Rb ultimately results in increased proliferation, but Rb has also shown to be associated with apoptosis (83).

Inactivation of Rb can occur by different mechanisms; (I) genetic mutations in the RB gene as observed in retinoblastoma, (II) Rb is targeted by many tumor related viruses that sequester Rb from its physiological partners, such as the HPV (84), the simian virus 40 (SV40) large T antigen (85) and adenovirus E1A (86), (III) during the cell cycle progression Rb is phosphorylated on different residues and eventually becomes hyperphosphorylated and is thereby inactivated by disabling its transcription factor binding abilities (87) and finally, (IV) Rb can also be cleaved by caspases and degraded in response to apoptotic stimuli (88).

The regulation of Rb is very complex and there are 16 serine/threonine CDK phosphorylation sites on Rb and different phosphorylated forms of Rb exists which might affect transcription factor binding differently (89-91). Cyclin D1 and cyclin E that form complexes with CDKs and other proteins that have an LXCXE motif can bind to Rb by interaction with the LXCXE binding domain within the Rb pocket (92, 93) and there is also a CDK4 docking site in the C-terminus of Rb (94, 95).

The Rb together with the Rb-related p107 and p130 comprise the ”pocket protein” family and are central participants in a gene regulatory network that governs antimitogenic signals. They have a similar overall structure with a highly conserved ”pocket” domain. The pocket domain is used for interaction with the E2F family of transcription factors (E2F1-6) and viral oncoproteins. However, p107 and p130 bind a different set of E2Fs from those bound by Rb (87) and Rb is expressed at a constant level throughout the cell cycle, p107 increases

in cells entering the cell cycle and is low in terminally differentiated cells whereas p130 is low in dividing cells but is high in quiescent and differentiated cells (96-98).

CDK inhibitors

One of the primary mechanisms used to regulate the CDKs that drive the cell cycle involves CDK inhibitory proteins. Two families have been described based on the function and on structural homology. The CIP/KIP family that comprises $p21^{CIP1/Waf1/Sdi1}$ (99, 100), $p27^{KIP1}$ (101, 102) and $p57^{KIP2}$ (103, 104) (henceforth referred to as p21, p27 and p57 or collectively as CIP/KIP) and the INK4 family that consists of $p15^{INK4b}$ (105), $p16^{INK4a}$ (106), $p18^{INK4c}$ (107) and $p19^{INK4d}$ (107, 108) (henceforth referred to as p15, p16, p18 and p19 or collectively as INK4). The first member of the CIP/KIP family to be cloned was p21 and p21 was identified as a p53-dependent CDK inhibitor upregulated as a response to DNA damage (109). However, p21 can be regulated in a p53 independent manner for example by transcriptional activation of the signal transducer and activator of transcription 1 (STAT1) protein (110). The p27 protein is upregulated as a response to for example transforming growth factor-β (TGF-β) stimulation (101) or serum starvation and is targeted for degradation by cyclin E-CDK2 phosphorylation (111). The proteins in the CIP/KIP family bind and inhibit a broad range of the cyclin-CDK complexes. Binding of CIP/KIP proteins to cyclin E-CDK2 or cyclin A-CDK2 inhibit the kinase activity of the protein complexes and thereby the phosphorylation of the Rb, but p21 or p27 binding to cyclin D-CDK4/6 is necessary for assembling the complex and for the serine/threonine phosphorylation function (59). p21 also bind to proliferating cell nuclear antigen (PCNA) a subunit of DNA polymerase-δ which disrupts its function of DNA replication but not DNA repair (112).

INK4 proteins have higher specificity than the CIP/KIP proteins and bind only CDK4 or CDK6 and inhibit the interaction with the D-type cyclins as well as the CIP/KIP proteins. When an INK4 protein binds to CDK4/6 the CIP/KIP proteins are translocated to the cyclin E-CDK2 complex and thereby the INK4 proteins indirectly inhibit CDK2 activity. The INK4 proteins are transcriptionally differently regulated and do not seem to be redundant (113). P15 expression remains at a constant level throughout the cell cycle and can be induced by TGF-β stimulation (105) and p18 and p19 levels are low in G1-phase and elevated in S-phase cells (59, 113).

p16

The p16 protein was originally discovered when human diploid fibroblasts were transformed by SV40 (114) and in a yeast-two hybrid screen it became clear that the protein acted as a CDK4 inhibitor (115). At a similar time point two other groups cloned the melanoma susceptibility gene on chromosome 9p21 (116, 117). Due to the simultaneous research, the p16 gene has a number of alternative names; INhibitor of cyclin dependent Kinase 4 (INK4a), Multiple tumor suppressor (MTS1), CDK4-inhibitor (CINK4I) and CDKN2a (from the HUGO project). The p16 protein is encoded by three different exons, exon 1α, exon 2 and exon 3 on the *INK4A* locus (Figure 6). This locus also encodes another protein from exon 1β and exon 2 called $p14^{ARF}$ ($p19^{ARF}$ in mouse). The two proteins are completely distinct with different amino acid sequences due to that the $p14^{ARF}$ protein is encoded from an Alternative Reading Frame (ARF). The gene encoding the p15 protein is located 30 kb away on the same chromosome as p16 and $p14^{ARF}$, whereas the human p18 and p19 genes are situated on the chromosomes 1p32 and 19p13, respectively.

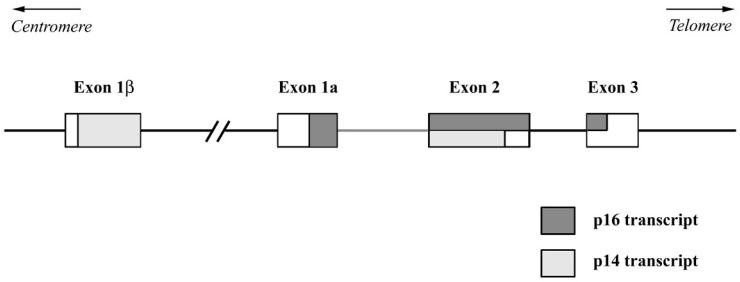

Figure 6. The *INK4a* locus. The *INK4a* locus contains 4 different exons termed 1β, 1α, 2 and 3. The exons are spliced into two different transcripts. The exons encoding p16 are shown in dark grey and the exons encoding the $p14^{ARF}$ transcript are light grey. Modified from (113).

The first mice generated that affected p16 expression was deleted in both exon 2 and exon 3 termed $INK4a^{\Delta 2,3}$ and showed a loss of expression not only for the p16 protein but also the $p19^{ARF}$ protein (118). The mice were viable, fertile, spontaneously developed carcinomas and were highly susceptible to carcinogens. Mouse embryo fibroblasts (MEFs) from the $INK4a^{\Delta 2,3}$ mice bypassed replicative senescence and could be transformed by H-Ras alone. One year later a pure $ARF^{-/-}$ mouse expressing the p16 protein were generated by targeting exon 1β (119). These mice and MEFs displayed all the main features of the $INK4a^{\Delta 2,3}$ mice and MEFs indicating that the features of the $INK4a^{\Delta 2,3}$ were due to loss of $p19^{ARF}$. In 2001, pure p16 knock-out mice were generated by two independent research groups (120, 121). In one of the reports the mice did not show any significant predisposition to spontaneous tumors during their first 17 months (120), but in the other report, 25% of the mice over 1 year spontaneously developed tumors, for example sarcomas and lymphomas (121). MEFs from these animals proliferated normally, could enter senescence and were not transformed by H-ras, but when introducing both Myc and Ras the MEFs were easily transformed.
p16 is inactivated in a large number of cancers by loss of heterozygosity (LOH) of the 9p21 locus, point mutations and small deletion as well as DNA promoter methylation (reviewed by Ruas and Peters (113)). LOH targeting the 9p21 locus has been found in for example acute lymphoblastic leukemia, BCC, glioma and non small cell lung carcinoma. Mutations are common in for example pancreatic adenocarcinoma and esophageal carcinomas. In kindred with familiar melanoma, germline mutations have been detected and the majority of mutations affect both p16 and the $p14^{ARF}$ proteins. Mutations specifically targeting p16 has also been detected (113). DNA methylation aberrations are the most common epigenetic lesion in cancer cells and are commonly found in breast and colon carcinoma. Methylation of CpG islands, which are CpG dinucleotide rich regions located mainly in the promoter regions of many genes, serves as a mechanism for inactivation of tumour suppressor genes in cancer (122). Methylation of the CpG rich region of the p16 promoter, in addition to point mutations of the p16 locus or gene deletion, has also been identified as a mechanism to abrogate p16 expression in a variety of human neoplasms (113, 123).

Regulation and expression of p16
In normal tissue, the expression of p16 is relatively low or even undetectable (113). The regulation of the p16 protein is not well understood but when p16 is activated it remains at a relatively constant level throughout the cell cycle (124). In humans, there also seems

to be a feedback loop between Rb and p16 indicating that Rb can indirectly influence the transcription of p16 (125, 126). Activation of the Ets-1 and Ets-2 transcription factors via ERK1/2 induce the expression of p16 that accumulates as cells in culture approach senescence (127-130). The effect of Ets-1 and Ets-2 on p16 expression in senescent cells can be counteracted by the basic helix-loop-helix protein called Id-1 (130). Other transcription factors that have been shown to modulate p16 expression are SP1, SP3 (131, 132) and Bmi1 (133) and β-catenin (134). JunB is reported to suppress cell proliferation by transcriptional activation of p16 in mouse embryo fibroblasts and 3T3 cells (135). In addition, the basic helix-loop-helix proteins E47, E12, E2-2 and HEB decrease proliferation and activate a luciferase reporter gene containing the p16 promoter (136, 137). Furthermore, a regulatory domain element with the capacity to regulate the *INK4/ARF* locus was recently discovered. The replication protein cdc6 binds to the regulatory domain and by recruitment of HDACs this results in heterochromatinization and repression of the locus (138). In addition to transcriptional regulation of p16 expression, it has recently been reported that there is an instability determinant in the 3′-untranslated region (UTR) of the p16 mRNA which is a specific target for AUF1, a protein that promotes p16 mRNA decay (139).

p16 and senescence

Cellular senescence is a mechanism to suppress proliferation and for cells grown in culture it is the state cells enter when they have reached their maximum number of population doublings. Senescence has been linked to tumor suppression both by genetic and biochemical data (140). For example, inactivation of in vitro inducers of senescence in mice renders the mice tumor prone and human cancer which divides indefinitely and bypasses senescence, requires telomere maintenance. Generally, a senescent cell are irreversible growth arrested in G1-phase and cannot initiate DNA replication (141) some cells are resistant to apoptotic stimuli (142), are metabolically active months after growth arrest, have increased lysosomal and β-galactosidase activity (143), and are large and flat in cell size (144). There are also cell-type specific changes, for example fibroblast in senescence have increased expression of collagenase (141). Senescence has been proposed to be induced by decreased telomere length, oncogene activation of for example Ras and some forms of DNA damage such as oxidative stress by reactive oxygen species (145). Senescence can be both p16 dependent and independent and for example p53 driven (stabilization of p53 is mediated by $p14^{ARF}$) where p21 is induced. In p16 dependent senescence, p16 induced which results in hypophosphorylated Rb. In cultured fibroblasts, both p16 and p21 accumulates as the cells approach senescence. p21 levels are highest at the time point when the cells stop proliferating then the levels decrease whereas p16 peaks at a time point after proliferative arrest and is thought to maintain the senescence associated proliferative arrest (146). In oncogene associated senescence (eg Ras) both the p16/Rb and the $p14^{ARF}$/p53 seems to be essential (147).

Additional functions of p16

There are emerging evidence of additional functions of p16 besides regulating Rb phosphorylation. p16 has been demonstrated to inhibit glioma cell (148) and HUVEC (149) invasion/migration on vitronectin (150) by blocking the $\alpha_v b_3$-integrin (148) and reducing the levels of matrix metalloproteinase-2 (MMP-2) (151). In addition, p16 has also shown to inhibit $\alpha_v b_3$-integrin localization to focal contacts and cell spreading on vitronectin (152). On the contrary, in migrating keratinocytes p16 is upregulated compared to non-migrating keratinocytes (153-155). In addition to migration, there are some implications of p16 being

involved in angiogenesis and apoptosis as well, thus restoration of p16 expression in glioma cells has shown to result in reduced VEGF expression and thereby negatively affects tumor angiogenesis (156) and introduction of p16 into colon cancer cells has shown to induce apoptosis as well as tumor regression (156, 157).

The Extracellular Signal-regulated Kinase Pathway

The ERK pathway transmits extracellular signals from outside the membrane to the appropriate site of action, for example the nucleus, and regulates cell fate that can alter proliferation, differentiation, migration, invasion and cell death. ERK1/2 are responsive to a wide variety of extracellular signals such as growth factors, hormones and neurotransmitters and the end effectors can be transcription factors, protein kinases and phosphatases, cytoskeletal elements, regulators of apoptosis and a variety of signaling molecules. Other pathways that function in parallel to the ERK pathway are the c-Jun N-terminal kinase (JNK), p38 mitogen activated protein kinase (MAPK) and ERK5 kinase pathways.

Regulation of the ERK1/2 kinase pathway

The isoezymes ERK1 and ERK2 (ERK1/2) show approximately 90% homology and phosphorylation on one threonine and one tyrosine residue is necessary for activation (158). ERK1 and ERK2 are activated under the same circumstances, however, there seems to be non-redundant functions between the two, thus knocking out ERK1 results in viable, fertile normal sized mice (159) whereas knocking out ERK2 is embryonically lethal (160, 161).

ERK1/2 are activated as downstream targets of a variety of receptor tyrosine kinases (RTK) and the major regulators of ERK1/2 are peptide growth factors for example epidermal growth factor (EGF), insulin growth factor-1 (IGF-1), TGF-α and TGF-β that bind to RTKs. As a general concept, when a growth factor binds to a RTK the binding leads to dimerization of two receptor subunits and trans-autophosphorylation of intracellular tyrosine residues (Figure 7). This results in recruitment of other phospho-tyrosine signaling molecules such as phopholipase Cγ (PLCγ). Grb2 binds to the tyrosine kinases directly or via Shc and recruits Sos, which is a guanine nucleotide exchange factor. Sos catalyses the conversion of GDP to GTP on Ras. Activated Ras bind to Raf and initiates a phosphorylation cascade from Raf (MAPKKK) to MEK1/2 (MAPKK) and further to ERK1/2 (MAPK). RTK can also activate ERK1/2 independently of Ras, for example, in primary endothelial cells where activation of the RTK, vascular endothelial growth factor receptor 2 (VEGFR2), activates ERK1/2 via PLCγ (162, 163).

G-protein coupled receptors are the other major group of receptors that activate ERK1/2 either via transactivation of RTKs or in a Ras independent manner (reviewed by Wetzker and Böhmer 2003 (164)). The G-protein coupled receptors activate PLCβ and protein kinase C (PKC) and subsequently stimulate and phosphorylate Raf. Another candidate protein identified to activate the cascade is phophoinositide 3-kinase-γ (PI3Kγ).

Emerging evidence indicate cross-talk between the Notch1 pathway and the ERK1/2 pathway in mammals. Upon Notch activation, the membrane bound receptor is cleaved by tumor-necrosis factor-α-converting enzyme/metalloproteinase (TACE) and by the γ-sectretase protease complex. The cleavage results in release and translocation of the cytoplasmic domain of Notch ($Notch^{IC}$) into the nucleus. In the nucleus $Notch^{IC}$ binds to the DNA-binding transcriptional repressors CSL (CBF-1, suppressor of hairless, LAG-1) and

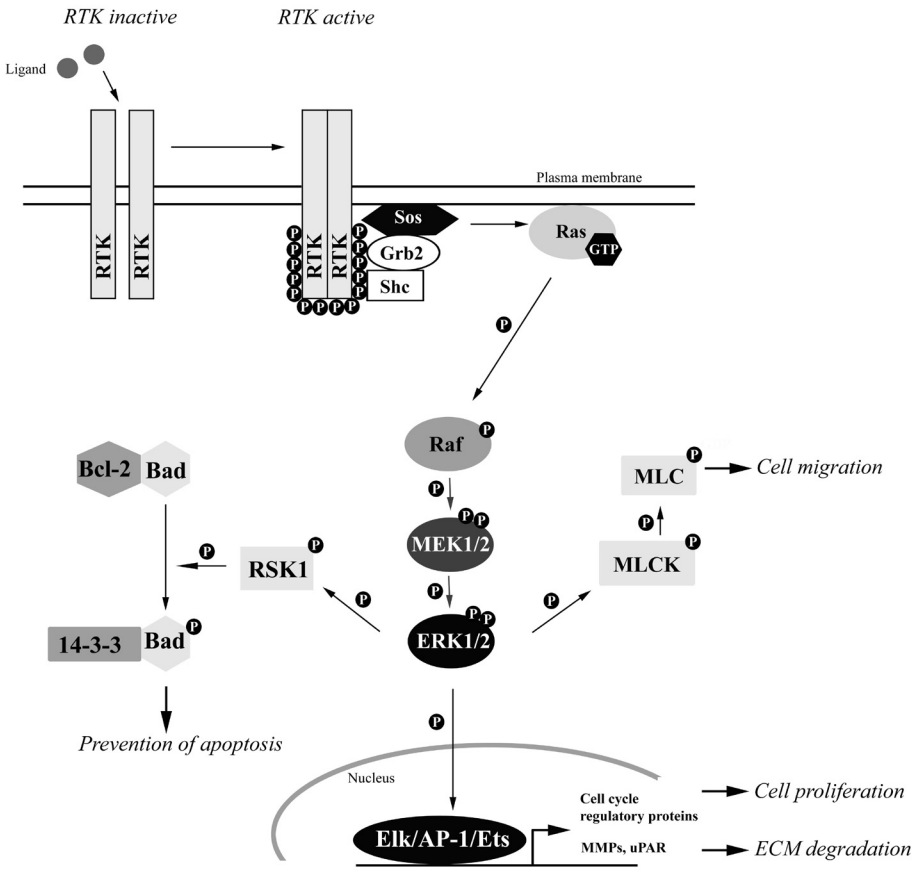

Figure 7. The ERK1/2 pathway. Growth factors bind to RTKs resulting in subsequent dimerization of two receptor subunits and trans-autophosphorylation of intracellular tyrosine. Grb2 binds to the tyrosine kinases directly or via Shc and recruits Sos, which is a guanine nucleotide exchange factor. Sos catalyses the conversion of GDP to GTP on Ras. Activated Ras bind to Raf and initiates a phosphorylation cascade from Raf to MEK1/2 and further to ERK1/2. The ERK1/2 then phosphorylates nuclear proteins such as Elk-1 members of the AP-1 family as well as Ets transcription factors and. ERK1/2 also phosphorylates cytosolic proteins such as RSK1 and MLCK, ultimately resulting in altered cell proliferation, ECM degradation, prevention of apoptosis and increased cell migration. Modified from (176).

Mastermind as well as other co-activators (165). The complex formed induces transcription of target genes such as the hairy/enhancer of split (Hes) and the Hes related repressor protein (HERP) families of transcription factors which mediate many of the Notch signaling effects (166). Depending on the celltype, activation of Notch has shown to either increase or decrease the activation of the ERK1/2 pathway. In HEK fibroblasts, in melanoma cells and in small cell lung cancer cells stable expression of Notch1 increased the activation of ERK1/2 (167-169). On the contrary, active Notch1 repressed ERK1/2 phosphorylation in endothelial cells (168). However, the mechanism behind the crosstalk is still unclear and remains to be elucidated.

Downregulation of ERK1/2 activity is so far not fully understood. However, it is evident

that de-phosphorylation plays a key role in the inactivation of ERK1/2 proteins and can be achieved by removing phosphates from either one or both of the regulatory residues. This process and is carried out by a variety of different enzymes and is tightly regulated. Three groups of phosphatases have been implicated in the inactivation of ERK1/2 proteins, protein Tyr phosphatases e.g. haematopoietic protein tyrosine phosphatase (HePTP) (170), protein Ser/Thr phosphatases e.g. type 2 protein tyrosine phosphatase (PP2A) (171) and dual specificity phosphatases e.g. mitogen activated protein kinase phosphatase-3 (MKP-3) (172).

Downstream targets and effects of ERK1/2

Activated ERK1/2 execute their function through downstream substrates and about 160 has so far been discovered (173). The substrates may vary under different settings and in different cell types. Many substrates are localized to the nucleus and seem to be involved in transcriptional regulation, while others are contained in the cytosolic and other cellular compartments. One of the best studied transcription factors activated by the ERK1/2 is Elk-1 which is dually phosphorylated upon mitogen stimulation (174). The phosphorylation enhances the DNA binding capacity of Elk-1 which induces transcription of, for example, the c-fos gene (175). The c-fos transcription factor and the c-jun transcription factor are both phosphorylated by ERK1/2 and the phosphorylation enhances the dimerization of the two transcription factors to form the activating protein 1 (AP-1) transcription factor complex. Binding of AP-1 to AP-1 consensus sequence results in transcription of for example cell cycle regulatory proteins such as cyclin D1, p53, p21 and MMPs which alter proliferation and degradation of extracellular matrix (176). Other transcription factors that also are activated by ERK1/2 phosphorylation are Ets-1 and Ets-2. Activation of Ets-1 and Ets-2 induces p16 expression resulting in decreased proliferation (130). In addition, depending on the interaction partner Ets-2 might activate or repress transcription of many target genes involved in invasion such as urokinase plasminogen activator (uPAR) and MMP-9 (177). ERK1/2 activation can also enhance cell motility (178, 179) potentially leading to metastasis in tumor cells. By directly phosphorylating the myosin light chain kinase (MLCK) leading to phosphorylation of the myosin light chain (MLC) which enhances cell migration (180) as well as activation of calpain resulting in focal adhesion turnover and cell detachment (181). Activation of ERK1/2 pathway is also associated with cell survival through phosphorylation of the p90 ribosomal S6 kinase 1 (RSK1) protein which directly phosphorylates the proapoptotic protein Bad (182, 183). Phosphorylation of Bad causes dissociation from the anti-apoptotic proteins Bcl-x_L and Bcl-2 (176, 184) and association with the 14-3-3 protein that sequester the protein away from the mitochondria (185).

There is also evidence that ERK1/2 can affect the activity of the ER receptor by phosphorylation (186, 187) resulting in ligand independent activation of ER. Downstream targets of ERK1/2 such as the RSK1 can also phosphorylate ER (188), potentially leading to resistance of tamoxifen treatment in breast cancer.

Cell Migration and Invasion

A key event in the oncogenic development is to acquire motile and invasive properties and invade beyond the tissue from which the cells originate into an ectopic site. Generally, the neoplastic cells need to detach from the neighboring tumor cells in the primary tumor and

break trough the basement membrane. Tumor cells that have broken trough the basement membrane can then migrate and infiltrate into the surrounding tissue. The invasively growing cells eventually reach the blood stream or the lymphatic vessels. Since lymphatic vessels contain no basement membrane or tight junctions are these easier to penetrate which makes lymph node metastasis close to the origin tumor an early event in the malignant process. The tumor cells then need to break into the blood vessel, intravasate, either originating from the primary tumor or the lymph node metastasis, survive in the circulation and travel to a distant site, extravasate and attach and start proliferating in a distant organ.

Molecular mechanisms

A tumor cell use migration and must modify its shape and interact with the extracellular matrix (ECM) during migration. The migration process of tumor cells is similar to the migration process of normal cells at for example wound healing (189). Migration and invasion can be divided into different steps, protrusion of the leading edge, attachment to the ECM, ECM degradation, cell contraction and rear end detachment. At the leading edge, lamellipodia and filipodia is extended and the leading end adheres to the ECM via multiple receptors such as integrins (190), and CD44 (191). Ligand induced integrin dimerization and focal adhesions are formed (Figure 8). The intracellular parts of the integrins interact directly with proteins such as focal adhesion kinase (FAK), α-actin and talin which bind adaptor proteins leading to recruitment of vinculin, talin, more α-actin and the SRC-family of tyrosine kinases (SFKs). The assembly of focal adhesion contacts induces the PI3K/Akt/PKB signaling pathway that can promote cell survival and resistance to anoikis (192, 193). The active complex also promotes activation of Rho-GTPases (RhoA, Rac and Cdc42) that are needed for actin remodeling, necessary for cell motility (194, 195). Before cell contraction, proteases such as MMPs are recruited to the ECM binding sites and are released to cleave ECM components such as collagen, laminin and fibronectin (196-198). Integrins can also active the ERK1/2 pathway via FAK (199) resulting in MLCK phosphorylation (180). In turn MLCK phosphorylates MLC which activate actomyosin (myosin II binds to actin filaments) and generate contraction of the cell. In addition, activation of ERK1/2 also results in calpain activation and altered gene expression (176). Following cell contraction, the focal adhesions at the rear of the cell is resolved and the rear of the cell loses attachment to the ECM.

Cellular patterns of tumor cell migration

Histological observations of migration have revealed at least two different patterns of tumor invasion, single cell and tumor cluster invasion. The individually migrating neoplastic cells can either migrate in a ”mesenchymal (fibroblast-like)” (essentially as described above) or in an ”ameboid-like” fashion without any proteolytic remodeling of the ECM. Epithelial tumors, fibrosarcoma or glioblastoma migrate in a mesenchymal fasion and lymphoma, small cell lung carcinoma and small cell prostate cancer cells migrate in an ”ameboid-like” fashion. The single neoplastic cells migrate after loosing their adhesion to neighboring cells by downregulation of receptors mediating cell-cell contacts. Groups of cells that retain cell-cell adhesion migrate into the tissue and migrate in either clusters or as multicellular strands or sheets. The sheets or strands remain in contact with the primary site and can be detected in invasive cancers such as breast cancer and BCC (nodular pushing growth or nodular invasive front). Clusters of cells are detached for the tumor bulk and can also be seen in

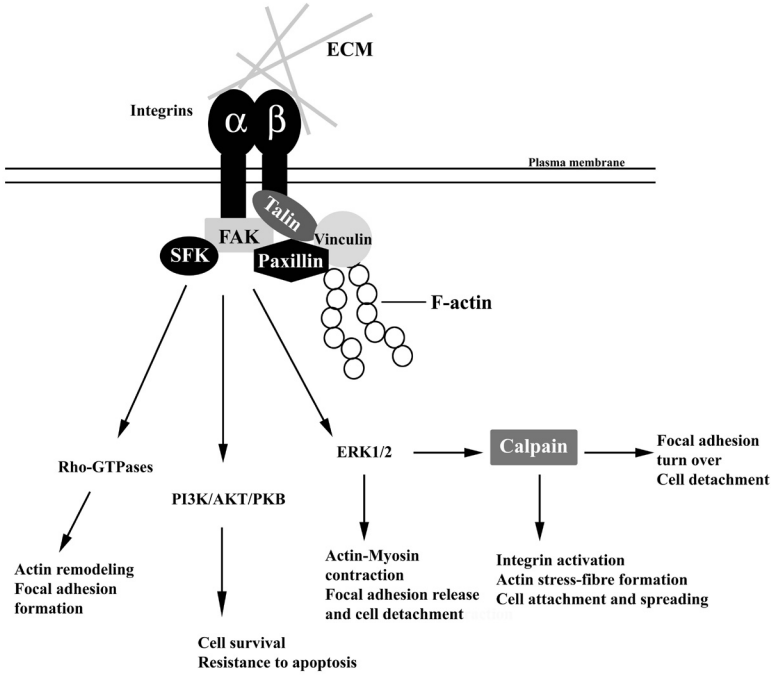

Figure 8. Focal adhesion signaling. Integrin assembly leads to the recruitment of FAK, α-actin and talin which bind adaptor proteins leading to recruitment of vinculin, talin, more α-actin and SFKs. The assembly of focal adhesion contacts induces the PI3K/Akt/PKB signaling pathway that promote cell survival and resistance to anoikis, activation of Rho-GTPases needed for actin remodeling and active the ERK1/2 pathway resulting actin-myosin mediated contraction of the cell and activation of calpain modulating adhesion and actin dynamics. Modified from (258).

breast cancer and BCC (irregulary branching strands infiltrative tumor cells). In the cellular formations cortical actin filaments assembly among the cell-cell junctions and allows joint contraction of all the cells. The front of the tumor clusters consists of path finding cells and the tailing edge is essentially passive. Epithelial cancer cells can undergo the transition from a collective invasion pattern to a single cell mesenchymal like invasion pattern called epithelial-mesenchymal transition (EMT) and a single neoplastic cell can undergo conversion from a mesenchymal to amoeboid invasion pattern (MAT) (189, 200).

Invasion promoter/suppressor genes in cancer

There are many genes implicated in tumor cell invasion for example E-cadherin, N-cadherin and β-catenin contributing to enhanced motility and ECM degradation .The cell-cell adhesion receptor E-cadherin is linked to the actin cytoskeleton by binding to α-catenin, β-catenin and plakoglobulin (γ-catenin). E-cadherin is frequently lost during advanced stages of epithelial cancer progression as a result of reduced levels of gene transcription of more rarely gene mutations (201). In addition, integrin signaling and RTKs can induce phosphorylation of the E-cadherin complex which might mediate the degradation via endocytosis of the complex (202). Loss of E-cadherin is considered to be an important step in invasion of epithelial tumor cells (203, 204). β-catenin binds not only to E-cadherin bind also to the APC multicomplex

where it is targeted for degradation by phosphorylation by GSK-3β. Mutation in β-catenin or APC, renders β-catenin in the nucleus bound to the lymphocyte enhancer factor (LEF)/T-cell factor (TCF) which affects transcription (205) of for example MMP-7 (206), laminin-5 γ2 and membrane type matrix metalloproteinase1 (MT-MMP1) (207). Gain of N-cadherin in cancer cells results in increased motility and invasion (208). The expression of N- and E-cadherin seems somewhat interrelated. For example, in SCC cells transfected with N-cadherin, E-cadherin was decreased and in antisense transfections of N-cadherin, E-cadherin expression was increased (209). Other proteins that have been implicated in tumor invasion are SRC, the RTKs c-Met and fibroblast growth factor receptor (FGFR), Ras and PTEN (reviewed by Mareel and Leroy (200)).

Laminin-5

The basement membrane consists of dense sheets of ECM that separates the epithelium from the surrounding connective tissue. A class of proteins commonly found in the basement membrane are laminins. There are more than 15 different forms of laminin consisting of three different chains or subunits (α, β, and γ) linked to each other by disulfide bonds. The laminins affects cell behavior such as cell adhesion and migration and the interaction between the normal/tumor cell and the ECM is primarily mediated by integrins (210).

Laminin-5 consists of the α3, β3 and γ2 subunits and the β3 and γ2 are specific to laminin-5. Laminin-5 interacts specifically with integrins α3β1, α6β1 and α6β4 and is the major adhesive component of epidermal basement membrane which stably anchorage basal keratinocytes to the connective tissue in the epidermal/dermal junction by binding to integrin $\alpha_6\beta_4$ (210, 211). In normal tissue, laminin-5 is overexpressed by keratinocytes at the wound edge of injured skin and the migratory-promoting activity of laminin-5 is thought to contribute to the wound healing process (212). Since laminin-5 has shown to regulate both adhesion and migration it remains to be elucidated which processes that contribute to determining the mode of action of laminin-5, even though proteolytic cleavage of the protein seems to be one process determining the effect of laminin-5 (211). In tumors, laminin-5 or its subunits has been suggested to be involved in tumor cell invasion. For example, there are reports showing that the γ2 subunit of laminin-5 is highly expressed in invading gastric carcinoma cells (213), lung adenocarcinoma cells (214), at the invasive front of head and neck squamous cell carcinomas (215), esophageal squamous cell carcinoma (216), as well as at the site of microinvasion in squamous cell carcinoma of the skin (155). Further, exogenous laminin-5 has been shown to either promote or inhibit keratinocyte migration in culture (217-219) and in addition, the γ2 subunit of laminin-5 is accumulated in the cytoplasm of migrating keratinocytes (153-155, 220). Despite these evidence of upregulation of the γ2 subunit in invading cells the significance of the γ2 subunit still unclear. Speculatively, the overexpression of the γ2 subunit in combination with lower or impaired expression of the other laminin-5 subunits might result in the ability to form basement membrane structures (211). It is also likely that some of the overexpressed γ2 subunit could promote tumor invasion due to loss of cellular adhesion, since there is no integrin binding site on the γ2 subunit. It is also possible that the γ2 subunit is secreted and cleaved by MMP-2 and thereby promotes tumor cell invasion (221) since migrating keratinocytes secrete the γ2 chain when migrating (155).

THE PRESENT INVESTIGATION

Aims

The general aim of thesis was to investigate key features of malignant growth such as proliferation and invasion in non-melanoma skin cancer and breast cancer with emphasis on the cell cycle inhibitor p16 and the growth factor mediating enzymes ERK1/2.

The specific aims of this study were to:

- Delineate the relation between invasion and proliferation using a model system of basal cell carcinoma with clear invasive growth pattern by investigating the expression of the cell cycle inhibitor p16, phosphorylated Rb, and the proliferative markers Ki-67 and cyclin A2 in relation to invasive behavior.

- Evaluate the expression of p16, phosphorylated Rb, Ki-67 and cyclin A2 in premalignant forms of skin squamous cell carcinoma and in invasive skin squamous cell carcinoma and specifically study the relationship between p16 and invasion.

- Investigate the regulatory mechanisms controlling p16 expression in basal cell carcinoma.

- Characterize the expression of ERK1/2 phosphorylation in relation to upstream signaling activators and downstream target effectors.

- Examine the impact of ERK1/2 activation as a prognostic and predictive marker in two randomized trials including 564 premenopausal and 248 postmenopausal breast cancer patients.

- Explore the link between the Notch1 receptor and the Notch1 target gene Hes1, and the relation to ERK1/2 phosphorylation, cyclin D1 and proliferation in both primary breast cancer and breast cancer cell lines.

Results and Discussion

Invade or proliferate? Two contrasting events in malignant behavior governed by p16^{INK4a} and an intact Rb pathway illustrated by a model system of basal cell carcinoma. (Paper I)

Invasion and proliferation are regulated by very complex cell signaling pathways and many different target proteins, which are often deregulated in cancer. Despite the fact that some of the pathways and target proteins are active in both invasion and proliferation there are studies suggesting that invasion/migration and proliferation or at least mitosis are inversely related in some tumors and cell lines (223-226) whereas others have stated the opposite (227).

BCC is a tumor type which possesses characteristic invasive features and is sometimes highly proliferative and therefore BCCs are suitable to use as a model when studying invasion and proliferation *in vivo*.

The aim of this study was to investigate the relationship between invasion and proliferation with special reference to the cell cycle inhibitory protein p16 in a model system of BCC including the subgroups superficial, nodular and BCC with invasive growth pattern. The tumor cells with characteristic invasive features of BCC cells that were investigated in this paper were tumor cells at the nodular invasive front (pushing growth pattern), BCC cells near ulceration and infiltrative BCC cells growing in irregularly branching strands (Figure 9).

We determined the localization pattern of p16 using immunohistochemical analysis in 47 BCC of with 8 were superficial, 27 were nodular and 12 were tumors with infiltrative growth pattern. In all BCCs p16 positive tumor cells were observed and 42/47 displayed a p16 staining that were both nuclear and cytoplasmic. The remaining 5/47 tumors displayed only cytoplasmic staining. Interestingly, p16 was most prominently localized to obvious infiltrative parts, such as irregularly branching strands of tumor cells, ulceration and at the nodular invasive front in the majority of tumors (Figure 10), which was unanticipated since p16 is a tumor suppressor and inactivation of the protein is associated with a more malignant feature in many tumors (61). In addition it has been reported that restoration of p16 in human glioma cells inhibits proliferation and invasion (150, 151) and glioma (148) as

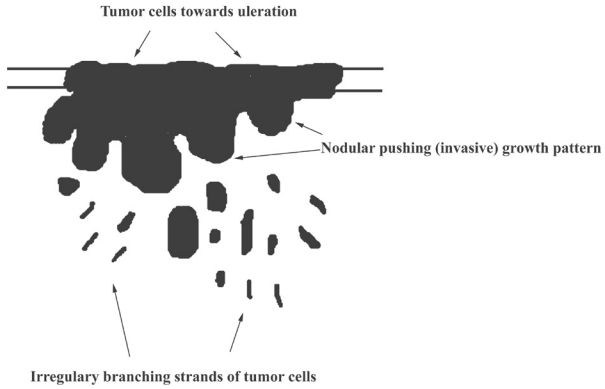

Figure 9. Schematic illustration showing invasive growth patterns in basal cell carcinoma.

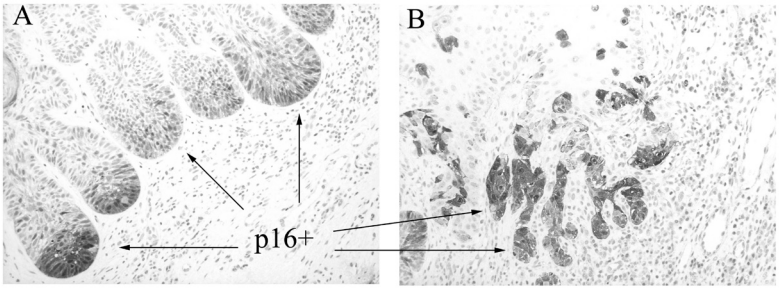

Figure 10. Basal cell carcinoma stained with p16. (A) p16 upregulation at the nodular invasive front and (B) in infiltrative branching strands of tumor cells.

well as HUVEC (149) migration. The p16 upregulation could therefore be a mechanism to block invasion and this hypothesis is further supported by the fact that BCC is an essentially non-metastatic and a very slowly growing tumor type. However, arguing against being an inhibitor of invasion in BCC, if the tumor is left untreated it continues to grow invasively into subcutaneous tissue such as bone, muscles and cartilage. In addition, the upregulation of p16 was linked to BCC subgroup and the infiltrative tumors showed a more prominent upregulation of p16 at the invasive front than the nodular and superficial tumors. BCC tumor cells are histologically similar to basal keratinocytes and it has been shown that migrating keratinocytes upregulate p16 and block proliferation when migrating (153-155).

Ki-67 is a proliferative marker, that is present in all cell cycle phases (G1, S, G2 and M-phase) except for the G0-phase, and was assessed by immunohistochemistry to investigate if the p16 positive cells were proliferating or not. In addition, we investigated Rb phosphorylation status using a phospho-specific Rb antibody. Both Ki-67 and phosphorylated Rb were present in the p16 low areas in the centre of the tumors, but were clearly decreased in p16 high areas indicating that p16 induced a proliferative arrest in BCC cells at the invasive front by inhibiting CDK4/6-cyclin D dependent Rb phosphorylation. These results are similar to results obtained in colorectal cancers with a functional Rb pathway, small clusters at the invasive front had higher p16 expression and lower proliferation than larger clusters with less p16 expression and higher proliferation (228, 229). The results were further verified using microdissection. The BCCs were microdissected into different areas depending on the histological invasive appearance. p16 as well as proliferation (cyclin A2) was monitored using Western blotting and S-phase using flow cytometry. We observed an inverse correlation between p16 and cyclin A2 and in addition, the S-phase analysis mirrored the cyclin A2 expression, confirming the immunohistochemical results.

Taken together, these observations suggests that invasion and proliferation are inversely linked in BCC and that BCC cells can change phenotype form a proliferating cell in the centre of the tumor to a non-proliferating cell at the invasive front of BCC, an effect most likely mediated by p16 and an intact Rb pathway.

Retinoblastoma protein function and $p16^{INK4a}$ expression in actinic keratosis, squamous cell carcinoma *in situ* and invasive squamous cell carcinoma of the skin and links between $p16^{INK4a}$ expression and infiltrative behavior. (Paper II)

Non-melanoma skin cancers include both BCC and SCC. Skin SCC is a locally invasive

malignancy and in general, it is believed that the pre-malignant form AK can develop into SCC, but it is still unclear whether AK develop into CIS and then to SCC (7, 16). In BCC we found a prominent upregulation of p16 and decrease in proliferation at the invasive front (paper I). Therefore, the aim of this study was to investigate the expression pattern of p16, phosphorylated Rb and Ki-67 in squamous cell carcinomas and the pre-malignant forms CIS and AK. We also aimed to delineate the expression pattern at the invasive front of SCC.

p16 expression was assessed using immunohistochemistry of 35 paraffin embedded invasive SCC and premalignant lesions. In general, a weak cytoplasmic staining was observed in all AK whereas in all CIS we observed strong nuclear and cytoplasmic staining except for one tumor with mixed staining pattern. In the 13 invasive SCCs the p16 staining pattern varied. In nine tumors only cytoplasmic staining was observed whereas four displayed combined nuclear and cytoplasmic staining. These results are in agreement with others stating that p16 expression is common in AK (16, 19) and in CIS (19, 155). However, using immunoshistochemistry p16 expression has been reported to be rare and observed in a few invasive SCC only (16, 155) whereas others have stated that is commonly expressed in all SCCs (19). Cytoplasmic staining of p16 has been considered unspecific (230) and this could possibly explain the discrepancies between the different studies. Given that we further validated the immunohistochemical results using Western blotting on frozen sections of SCC, CIS and AK and could correlate the intensity of the band to immunohistochemical p16 data and that additional results using cell lines indicate a true p16 staining in the cytoplasm (231, 232), we conclude that the p16 staining observed in the cytoplasm was accurate and that p16 is commonly expressed in invasive SCC.

We next analyzed the Rb phosphorylation and Ki-67 status in the tumors to investigate if p16 inhibited the inactivation of Rb by phosphorylation and thereby proliferation in invasive SCC and the pre-malignant lesions. In AK with cytoplasmic p16 expression only few phospho-Rb positive cells were observed and the lesions were generally low in proliferation. It is therefore unclear if the Rb-pathway was functional or not in this premalignant lesion. CIS were completely negative for phosphorylated Rb but were positive for Ki-67 despite the high levels of p16, indicating that p16 could not block proliferation in CIS due to a non-functional Rb protein. In all SCCs Ki-67 were present regardless of p16 localization, but the tumors with p16 expression in both the nucleus and the cytoplasm were negative for phosphorylated Rb in a similar manner to the pre-invasive lesion CIS. The invasive SCCs with p16 staining restricted to the cytoplasm displayed co-localization between p16 and Rb phosphorylation, suggesting that Rb pathway was disrupted due to the cytoplasmic localization of p16 and that the cytoplasmic p16 could affect neither Rb phosphorylation nor proliferation. The mechanism underlying the localization pattern of p16 is not clear, but mutations have been found in the *INK4* locus in SCC lesions (21, 23) and in an immunofluorescence study, inactivating mutations within *INK4A* lead to accumulation of the mutant forms of p16 to the cytoplasm (233).

Further, we investigated if p16 was linked to invasive growth pattern and in 10 out of 13 invasive SCCs, p16 protein expression was increased at the invasive front (Figure 11) as well as in smaller tumor clusters compared to the centre of tumors, but the expression pattern was not as clear as in BCC (paper I). The increase was seen regardless of subcellular localization of p16 which might possibly indicate proliferation independent role for p16 in invasion both in SCC and in BCC. Indeed, p16 has been shown be upregulated in migrating keratinocytes (153-155), but on the contrary shown to inhibit invasion and migration in glioma cells (148,

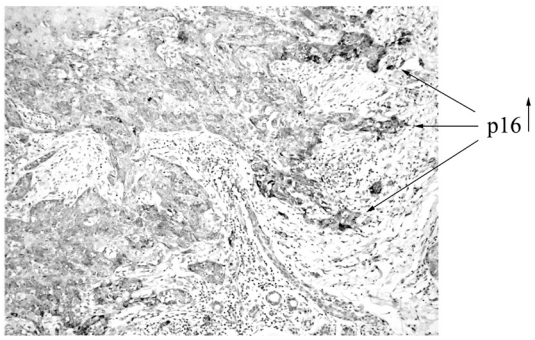

Figure 11. The expression of p16 was incerased at the invasive front in the majority of squamous cell carinomas.

150, 151). In addition, the cytoplasmic p16 could possibly bind and inhibit Cdk6 which has been shown to localize to the ruffling edge of spreading cells (152). Whether the expression of p16 promotes or inhibits invasion in BCC and SCC skin lesions is still unclear and remains to be elucidated.

Immunohistochemical staining of p16 were also reduced in differentiating SCC cells. On the contrary to our results, p16 has been shown to be upregulated in differentiation of normal human epidermal keratinocytes (234) and in trophoblasts (235), but overexpression of p16 could not induce differentiation in epidermal keratinocytes (234). However, in K5262 cells p16 overexpression induced erythroid differentiation (236) suggesting a role for p16 in some differentiating cells.

In summary, p16 is commonly expressed in invasive SCC and the pre-malignant lesions AK and CIS and from our work we conclude that both CIS and invasive SCC have non-functional Rb pathways. In addition, p16 was upregulated at the invasive front in the majority of invasive SCC suggesting that p16 could possibly be involved in invasion of SCC tumor cells.

Transcriptional upregulation and unmethylation of the promoter region of p16 in invasive basal cell carcinoma cells and partial co-localisation with the γ2 subunit of laminin-5. (Paper III)

p16 is a cell cycle inhibitor that has been extensively studied and despite this, little is known about mechanisms regulating p16 expression *in vivo.* There is, however, a number of different transcription factors for example Ets (130), SP1 (131, 132), and the polycomb protein transcriptional repressor Bmi1 (133, 237) known to regulate p16 expression. In addition, genetic events such as mutation, loss of heterozygozity (LOH) (113) and hypermethylation of the p16 promoter region are common in cancer and can abrogate the expression of p16 (113, 123).

In this study we focused on investigating the mechanisms promoting the p16 upregulation observed at the invasive front of BCC (paper I), by analyzing relative p16 mRNA levels in comparison to protein levels, p16 promoter methylation and proteins potentially affecting the expression of p16.

Microdissection was preformed to compare p16 mRNA and protein levels in different areas (more invasive vs. less invasive) of frozen BCCs. The p16 protein levels analyzed by Western blotting corresponded to the immunohistochemical staining of the same tumor ($p = 0.025$) and in agreement with paper I, an increased level of p16 was observed in the

more invasive areas compared to the less invasive areas. Relative p16 mRNA and protein level changes were similar in different areas of the BCC ($p = 0.031$) (Figure 12) and both p16 protein and mRNA level was increased in areas with more invasive tumor cells. On the contrary, lower levels of p16 protein and mRNA was observed in less invasive areas, suggesting that p16 is regulated on mRNA level either by transcriptional activation/ repression or increased/decreased mRNA stabilization in tumor cells. Recently, it has been reported that AUF1 targets the instability determinant in the 3′-untranslated region of p16 mRNA and promotes p16 mRNA decay (139) and it is possible that AUF1 can influence the levels of p16 mRNA in BCC.

LCM followed by methylation specific PCR revealed that the p16 promoter was unmethylated at the invasive front, whereas in central parts of BCC the p16 promoter was found to be both methylated and unmethylated. This suggests that promoter methylation can affect the transcription of p16 in BCC *in vivo* and that unmethylation of the p16 promoter at the invasive front of BCC could enable p16 transcription, given that methylation of the p16 promoter has been has been identified as a mechanism to eliminate p16 expression in a variety of human tumors (113, 123). Contradictory to our results, others have shown that p16 promoter methylations are infrequent in BCC (22, 238). However, due to us using the nested PCR approach it is possible that increased sensitivity could account for the discrepancies observed. In addition to promoter methylation, mutations and LOH are other genetic events that could contribute to p16 gene silencing in the centre of BCCs. However, mutations in the p16 gene are not common in BCC (239), but LOH in chromosome 9 is common (239, 240). Therefore, LOH of the in combination with methylation of the promoter might contribute to the heterogeneous p16 pattern observed in BCC.

ERK1/2 has been linked to p16 upregulation via the two transcriptionfactors Ets-1 and Ets-2 (130). From the immunohistochemical staining we concluded that there was occasional co-localization but no complete overlap between p16 and ERK1/2 phosphorylation as well as between p16 and Ets-2 phosphorylation. These data suggest that neither the ERK1/2 signaling pathway nor Ets-2 are directly or alone involved in the activation of p16 expression even though a ”hit and run” activation affecting the p16 expression cannot be excluded from our results. Ets-1 was only observed in a few scattered cells indicating

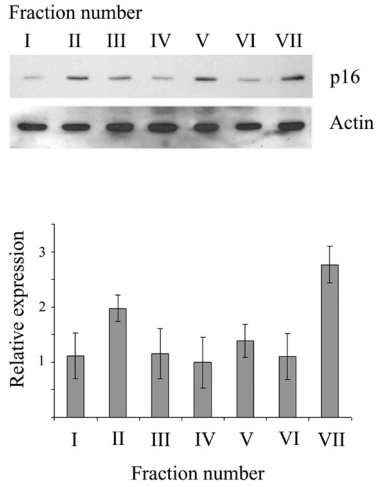

Figure 12. Protein and relative mRNA levels in areas of a basal cell carcinoma with different histological invasive growth patterns. Fractions II, V ans VII were prepared from more invasive areas and fractions I, III, IV and VI from less invasive areas of BCC. Note that tumor cells from the more invasive areas harbour an increased amount of both p16 protein and mRNA.

that Ets-1 is not commonly expressed in BCC. We also tested the transcription factor SP1 and the polycomb protein Bmi1 and these proteins were evenly expressed throughout the BCC tumors. Altered expression levels of these proteins does not seem to be mediating the p16 upregulation observed at the invasive front, however, inactivation or activation and binding to co-regulatory proteins could possibly be involved in regulating the levels of p16 in BCC.

From these observations we conclude that p16 is indeed regulated on mRNA level and that methylation of the p16 promoter may be involved in the regulation. Regarding transcription factors and additional proteins known to affect p16 expression, a candidate protein could not be established. However, the regulation of genes is a very complex process and it is possible that multiple transcription factors in combination with co-activators and repressors as well as the correct chromatin structure are needed for p16 expression and therefore remains undetectable in this study.

The γ2 subunit of laminin-5 has been shown to co-localize with p16 at the site of microinvasion of skin SCC and, in addition, to be upregulated in migrating keratinocytes as observed for p16 (153-155). We therefore aimed to investigate the p16 expression in relation to invasive growth pattern and to the γ2 subunit. In agreement with our previous results (paper I), nuclear and cytoplasmic staining of p16 was found in all BCCs tested and the positive cells were mainly observed in tumor areas with nodular pushing growth pattern, in tumor cells with irregularly branching infiltrative growth pattern and in tumor cells towards ulceration. In the majority (14/15) of BCCs a distinct cytoplasmic γ2 staining pattern was observed in tumor cells with irregularly branching infiltrative growth and in tumor cells near ulceration. On the contrary, tumor cells at the edge of nodular pushing growth pattern lacked γ2 staining, but were p16 positive. In some samples, tumor cells arranged in irregularly branching infiltrative strands near the tumor bulk were positive for p16 but lacked γ2 subunit expression. In agreement with our results, other studies have shown preferential expression of the γ2 subunit in invading carcinoma cells (213-216), but the role of the accumulated γ2 expression in the cytoplasm of invasive cancer cells is unclear and remains to be elucidated. However, it is possible that expression of the γ2 chain aid the invasion of BCC cells by affecting the association of other laminin isoforms and thereby the ability to form basement membranes (211). It is also likely that some of the overexpressed γ2 chain is secreted and cleaved by MMP-2 and thereby promotes tumor cell invasion (221) since migrating keratinocytes secrete the γ2 chain when migrating (155) and MMP-2 is mainly found in the stromal cells of BCC (222).

In conclusion, we observed that the γ2 subunit is upregulated in one type of invasive growth pattern, irregularly branching infiltrative growth pattern and in tumor cells with infiltrative growth pattern located close to an ulcer, and the proliferative arrest as a result of p16 upregulation was observed as a more general phenomenon in different types of BCC invasion.

ERK phosphorylation is linked to VEGFR2 expression and Ets-2 phosphorylation in breast cancer and is associated with tamoxifen treatment resistance and small tumours with good prognosis. (Paper IV)

The main function of the ERK1/2 kinase signaling pathway is to mediate extracellular signals from outside the cell to target proteins inside the cell. ERK1/2 has been linked to several cellular processes such as proliferation, migration and differentiation. There is only

a few reports investigating ERK1/2 in breast cancer, but ERK1/2 has nevertheless been linked to lymph node metastasis (241), breast cancer relapse (242), decreased response to anti-hormonal treatment for patients with advanced primary breast carcinoma or metastatic disease (243) as well as good prognosis (244). All these reports focused on the relationship between ERK1/2 and prognostic as well as treatment predictive information.

Therefore, in order to better understand the importance of ERK1/2 signaling in breast cancer and clarify the relation between ERK1/2 phosphorylation and upstream activators and downstream targets such as VEGFR2, ErbB2, Ets-2 and Cyclin D1 in breast cancer, as well as the relation to clinico-pathological parameters, three different patient cohorts including a total of 886 patients were analyzed. I addition, patient outcome without interference of treatment and the effect of ERK1/2 phosphorylation on the response to tamoxifen comparing a treated group with an untreated control group were assessed in a patient cohort of 248 postmenopausal women.

ERK1/2 phosphorylation immunoreactivity was scored as percent positive nuclei in four different groups and was initially evaluated in cohort I and II (a total of 362 patients). It was possible to evaluate ERK1/2 phosphorylation in 293 tumors of which 46% was scored as positive.

In relation to clinico-pathological parameters ERK1/2-phosphorylation correlated inversely to tumor size in both cohorts ($p = 0.001$), but not to any other clinico-pathological parameters. By analyzing the relevance of ERK1/2 phosphorylation to clinical outcome for the untreated patients in cohort II we could separate prognostic information from treatment predictive information. Tumor specific ERK1/2 phosphorylation correlated significantly with overall survival. Patients with tumors expressing high or intermediate levels of ERK1/2 phosphorylation had the best prognosis, whereas patients with tumors negative for ERK1/2 phosphorylation significantly poorer prognosis ($p = 0.017$). In a Cox multivariate analysis including ERK1/2 phosphorylation, age, lymph node status and tumor size, ERK1/2 phosphorylation was independently associated to survival ($p = 0.012$). An earlier publication (242) showed that increased ERK1/2 activity in the lysates of the cytosolic fraction of primary breast cancers was liked to increased risk of relapse. However, the patients included in this study received different adjuvant therapy in the form of radiotherapy and/or systemic therapy and in addition, the methods used for analyzing ERK1/2 activity were different, which might explain the differences observed. Even so, our data analyzing only untreated patients suggests that ERK1/2 phosphorylation is linked to small tumors with good prognosis for postmenopausal breast cancer patients.

Postmenopausal women with ER positive and ERK1/2 phosphorylation positive tumors did not respond to tamoxifen ($p = 0.662$), but patients with ER positive and ERK1/2 phosphorylation negative tumors responded to the treatment ($p = 0.042$). The lack of tamoxifen response in postmenopausal women could possibly be explained by ER phosphorylation and hence ligand independent activation of ER. Thus, there is evidence that ERK1/2 can affect the activity of the ER receptor by phosphorylation (186, 187) and in addition downstream targets of ERK1/2 such as RSK can also phosphorylate ER (188). The lack of tamoxifen response in patients with ERK1/2 phosphorylation positive tumors is supported by a study by Gee et al (243). However, we could not confirm the results using a patient cohort consisting of 500 premenopausal women (paper VI) and in another small study ERK1/2 activation could not be liked to tamoxifen response (241) Therefore, we conclude that further studies are needed to investigate the true role of ERK1/2 phosphorylation, its impact on tamoxifen response and

also if a selected group of patients could possibly benefit from treatment with tamoxifen in combination with a drug targeting ERK1/2.

In both analyzed cohorts, immunohistochemical ERK1/2 phosphorylation reactivity correlated strongly and significantly to Ets-2 phosphorylation ($p < 0.001$ in both cohorts). Ets-2 is a key nuclear mediator of ERK1/2 signaling (245) and has been shown to phosphorylate Ets-2 on threonine 72 (246). In addition, ERK1/2 is the major mediator of Ets-2 phosphorylation in macrophages (247). In macrophages, Akt in complex with JNK can also activate Ets-2 by phosphorylation (248). However, the strong significant association between Ets-2 and ERK1/2 phosphorylation suggests that ERK1/2 is an activator of Ets-2 in breast cancer.

We also investigated a limited number of upstream regulators of the ERK1/2 pathway such as VEGF-A, VEGFR2 and ErbB2 as well as downstream targets of ERK1/2 for example cyclin D1, p21 and p16. In cohort I no associations were found between the above listed proteins and ERK1/2 phosphorylation. In cohort II we observed a strong correlations between ERK1/2 phosphorylation and VEGFR2 ($p < 0.001$) and a significant correlation to VEGF-A ($p = 0.017$). In primary endothelial cells and in NIH3T3 fibroblasts it has been reported that VEGFR2 can activate ERK1/2 via PKC (162, 163) In addition, ERK1/2 phosphorylation correlated to nuclear intensity of cyclin D1 ($p = 0.004$). These results are in agreement with previous studies showing that cyclin D1 mRNA is increased by sustained activation of ERK1/2 activity (249-251). The discrepancies between the cyclin D1 observations are probably due to the different detection methods used. In cohort I Western blotting were preformed on tumor extracts probably consisting of a mixture of tumor and normal cells and cyclin D1 content of the tumors in cohort II was evaluated using immunohistochemistry.

To validate our results we analyzed a third cohort (due to the inconsistencies between cohort I and cohort II). The third cohort consisted of 524 tumors of which 365 tumors could be evaluated for ERK1/2 phosphorylation. In cohort III we confirmed the correlations between ERK1/2 phosphorylation and tumor size ($p < 0.001$) and positively to VEGFR2 ($p = 0.046$), cyclin D1 ($p = 0.029$) and Ets-2 phosphorylation ($p < 0.001$).

Our data indicate that there is possibly an active signaling cascade *in vitro* from VEGFR2 via ERK1/2 to Ets2 and cyclin D1 in breast cancer and in addition, our results suggests that there is a link between this pathway and tumor size (Figure 13).

Association between the ERK pathway and the Notch target gene Hes1 in primary breast cancer and in breast cancer cell lines. (Paper V)

The Notch receptors are activated by different ligands (Delta-like or Jagged) that are expressed by neighboring cells. Upon activation of Notch, the receptor is cleaved and the intracellular part of the receptor translocates to the nucleus and induces transcription of for example hairy enhancer of split (Hes1). In mammals cross-talk between the Notch and the ERK1/2 pathways has been reported. In melanoma cells, for example, stable expression of Notch1 increased the phosphorylation of ERK1/2 (168) and, on the contrary, endothelial cells with active Notch1 repressed ERK1/2 phosphorylation (252).

The purpose of this study was to investigate the relationship between Notch1 and ERK1/2 phosphorylation and the relation to Hes1, cyclin D1 and proliferation in both primary breast cancer and in breast cancer cell lines. In addition, we investigated Notch1, ERK1/2 phosphorylation and Hes1 in relation to clinico-pathological parameters, relapse free survival (RFS) and tamoxifen response.

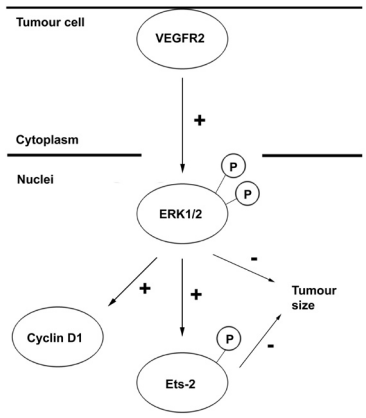

Figure 13. Schematic illustration of links between ERK1/2 phosphorylation and other parameters observed in primary breast cancer.

The primary breast cancer cohort consisted of 564 patents with stage II disease randomized to either adjuvant tamoxifen treatment or no treatment for two years. It was possible to array 500 tumors into tissue microarrays and evaluate ERK1/2 phosphorylation in 439 (78%), tumor specific Notch1 in 387 (69%) and Hes1 in 380 (67%). Surprisingly, in this cohort there was no significant correlation between Notch1 and Hes1, even though Hes1 is considered to be a primary Notch target (166). Interestingly, there was a strong significant correlation between ERK1/2 phosphorylation and Hes1 ($p < 0.001$) suggesting that the Hes1 expression was driven by ERK1/2 signaling independently of Notch1 in primary breast cancer. In addition, there was an inverse correlation between ERK1/2 phosphorylation and Notch1 ($p = 0.009$). Recent evidence indicates that Notch indirectly can suppress ERK1/2 phosphorylation (252), which might explain the inverse correlation found in the patient cohort.

By experiments using breast cancer cell lines and inhibitors designed to inhibit either the ERK1/2 or the Notch pathway, we investigated the effects on Hes1 expression. The γ-secretase inhibitor DAPT that inhibits the activating cleavage of Notch had a potent effect reducing the relative Hes1 mRNA levels by 60% in the MDA468 cell line. On the contrary, the MEK1/2 inhibitor U0126 led to downregulation of Hes1 mRNA levels in MCF7 by 20% and in CAMA1 30%. These data indicate that Notch was the major driving force of Hes1 expression in MDA468 and that Hes1 expression was maintained in a Notch-receptor independent way and that ERK1/2 contributed to the basal Hes1 mRNA expression levels in MCF7 and CAMA1. This is in line with a previous study using neuroblastoma cells showing an ERK1/2 dependent regulation of Hes1 (253). However, the mechanism behind the induction of Hes1 by ERK1/2 is currently unknown and requires further studies.

In paper IV we showed that ERK1/2 phosphorylation correlated to the upstream receptor VEGFR2 and the downstream target cyclin D1 but not to ErbB2 in primary breast cancer. In agreement with previous results, ERK1/2 phosphorylation correlated to VEGFR2 ($p < 0.001$) and cyclin D1 ($p = 0.008$), but neither to ErbB2 nor to *c-erbB2*. Notch1 did not correlate to any, but Hes1 were significantly linked to, all of the above mentioned proteins. Using cell lines, we also investigated the changes in cyclin D1 expression levels. Applying U0126 inhibitor resulted in a signifficant reduction in cyclin D1 mRNA expression levels

(65-50%) in all cell lines investigated except for T47D. The exact mechanism resulting in the downregulation of cyclin D1 mRNA is unknown, but the transcription factors Ets-2 (65, 254) and Egr-1 (255) regulated by ERK1/2 can induce cyclin D1 expression. DAPT treatment also reduced the levels of cyclin D1 in MCF7 (25%) and T47D (40%), which could possibly be a downstream target of Notch signaling (256).

In the primary breast cancer cohort there was there was a positive correlation between Hes1 levels and the proliferative marker Ki-67. Interestingly, the MDA468 cell line, which has an active Notch signaling, showed a decrease in proliferation monitored by [^{3}H]-thymidine incorporation assays and growth curves upon DAPT treatment. Treatment with the MEK1/2 inhibitor decreased proliferation with about 50% in MCF7, T47D and MDA468.

Notch1, ERK1/2 phosphorylation and Hes1 were examined in relation to known prognostic variables such as patient age, tumor size, grade, or the presence of lymph-node metastases. In addition, correlations to ER and PR status were also investigated. No associations between Notch1 and the above stated parameters were found, except for a negative correlation to ER status ($p = 0.014$), which is in line previous report showing that the mRNA levels of Notch1 was inversely correlated to ER (257). In agreement with paper IV and a study by Bergqvist et al (244), ERK1/2 phosphorylation correlated inversely to tumor size ($p = 0.007$) and, in addition, inversely to NHG status ($p = 0.011$) suggesting that tumors showing high fraction ERK1/2 phosphorylation are rather small and well differentiated. Hes1 also correlated inversely to tumor size ($p = 0.011$) as well as to nodal status ($p = 0.030$). Further, it has been reported that ERK1/2 can phosphorylate the PR leading to nuclear translocation and inhibition of cytoplasmic degradation and in this tumor material we observed a significant positive link between ERK1/2 phosphorylation and PR expression ($p = 0.042$).

Untreated patients with hormone receptor positive (ER or PR) and ERK1/2 phosphorylation positive tumors, had a significantly better RFS than the patients with ERK1/2 phosphorylation negative tumors, but the effect of ERK1/2 phosphorylation on RFS was not statistically significant in a multivariate analysis including NGH, tumor size, age at diagnosis and lymph node status. These results are somewhat in agreement with our previously reported results (paper IV) indicating a better prognosis for postmenopausal untreated women with tumors positive for phosphorylated ERK1/2, whereas others have stated the opposite (242). However, the enrolled patients had received different adjuvant therapies and that the methods used for analyses were different in the studies which could account for the observed discrepancies.

In paper IV we demonstrated a link between ERK1/2 phosphorylation and an impaired response to tamoxifen in a patient cohort of postmenopausal women, results further supported by Gee et al (243), whilst other have shown that ERK1/2 is not a marker for lack of endocrine responsiveness (241). In this study including only premenopausal women, we could not confirm any association between ERK1/2 phosphorylation and tamoxifen response and therefore, further studies are needed to investigate the relationship between ERK1/2 phosphorylation and tamoxifen response.

In conclusion, in this paper we show that there is a connection between the ERK1/2 signaling pathway and Hes1 expression in both primary breast cancer and in breast cancer cell lines and in addition, as in paper IV a strong significant correlation was observed between ERK1/2 phosphorylation and VEGFR2 as well as cyclin D1.

Conclusions

- p16 was upregulated and the proliferation was decreased in different invasive growth patterns of basal cell carcinoma as in infiltrative branching strands, in tumor cells towards ulceration and at the nodular pushing invasive front, which was in contrast to the high proliferation in the central parts of the tumor. This suggests that, basal cell carcinoma cells change phenotype form a highly proliferative type in the centre of the tumor to an invasive type with low proliferation at the invasive front. The proliferative change at the invasive front was mediated by p16 inhibiting proliferation via an intact Rb pathway.

- p16 expression was observed in invasive squamous cell carcinoma of the skin and the precancerous lesions actinic keratosis and squamous cell carcinoma *in situ*. Invasive squamous cell carcinoma and squamous cell carcinoma *in situ* tumor cells proliferated despite the expression of p16 indicating that the Rb pathway was non-functional.

- p16 was upregulated at the invasive front in the majority of invasive squamous cell carcinomas, possibly indicating a proliferation independent role for p16 at invasion.

- p16 mRNA levels was elevated and the promoter region was unmethylated at the invasive front of basal cell carcinoma indicating a transcriptional regulation of p16 in invasive tumor cells.

- In basal cell carcinoma, the γ2 subunit of laminin-5 was upregulated in infiltrative branching strands of tumor cells and in tumor cells close to ulceration, but not in tumor cells at the nodular pushing invasive front whereas p16 was upregulated in all mentioned invasive growth patterns. This suggests that the γ2 subunit levels are increased in one type of invasive growth pattern and that the proliferative arrest induced by p16 was a more general event in different types of invasive behavior.

- A signaling cascade from VEGFR2 via ERK1/2 to Ets-2 phosphorylation and cyclin D1 could be outlined in primary breast cancer and ERK1/2 phosphorylation was linked to small tumors with good prognosis.

- For postmenopausal patients, ERK1/2 phosphorylation was associated with impaired tamoxifen response, but ERK1/2 phosphorylation was not linked to tamoxifen resistance for premenopausal women.

- ERK1/2 phosphorylation was linked to the primary Notch target Hes1 both in primary breast cancer and in breast cancer cell lines indicating a Notch independent regulation of Hes1.

POPULÄRVETENSKAPLIG SAMMANFATTNING PÅ SVENSKA

Cancer tillhör våra vanligaste sjukdomar. Enligt WHO drabbas 11 miljoner människor av cancer varje år och 7 miljoner personer avlider årligen med cancer som dödsorsak. Det finns ca 200 olika typer av cancer och vissa former kan vara livshotande medan andra är ofarliga. Huden är kroppens största organ och de två vanligaste typerna av hudcancer kallas basalcellscancer och skivepitelcancer. Dessa tumörformer kan vara lokalt väldigt aggressiva, men metastaser förekommer sällan. Både basalcellscancer och skivepitelcancer behandlas vanligtvis med ett enkelt kirurgiskt ingrepp. Bröstcancer är en av de vanligaste cancerformerna som drabbar kvinnor och i Sverige insjuknar ca 6500 personer årligen i bröstcancer. Antalet diagnostiserade fall ökar årligen, men trots ökningen håller sig antalet dödsfall relativt konstant vilket troligtvis beror på förbättrade behandlingsmetoder och rutinmässig mammografiscreening. Bröstcancer behandlas med antingen bröst bevarande kirurgi eller radikal mastektomi och strålning. För att minska risken för återfall ges cytostatika till patienter med tumörer som är negativa för östrogenreceptorn alternativt hormonell terapi (ofta Tamoxifen) till de patienter som har tumörer som är positiva för östrogenreceptorn.

Celldelning (proliferation) kan delas in i olika faser, G1, S, G2 och M, och är den process där en cell duplicerar sin arvsmassa (DNA) för att generera två identiska dotterceller (Figur 4). I G1 förbereder sig cellen för att duplicera sin arvsmassa och i S-fasen sker själva dupliceringen av DNAt. I G2 förbereder sig cellen för att dela sig till två identiska celler i M-fasen. Cellen kan också vara vilande i G0-fasen. Celldelningen i normala celler är hårt reglerad men kan aktiveras av olika stimuli via olika signalkaskadvägar t.ex. via extracellulärt signal-reglerade kinaser 1 och 2 (ERK1/2). Signalering via ERK1/2 leder till en uppreglering av olika proteiner t.ex. cyklin D som är ett viktigt protein i cellens G1-fas. Utöver att inducera celldelning har ERK1/2 flera olika funktioner. Aktivering av ERK1/2 kan öka cellens migration, inhibera programmerad död (apoptos), öka uttrycket av proteiner som bryter ner extracellulär matrix och kan även leda till minskad proliferation.

Retinoblastomaproteinet (Rb) fungerar som en cellcykel broms och för att inaktivera Rb och driva cellcykeln framåt måste Rb fosforyleras. De proteiner som driver cellcykeln bildar komplex och kallas cyklinberoende kinaser (CDK) och cykliner. Under cellcykeln agerar olika cyklin-CDK komplex. I G1 och i G1/S övergången fosforyleras Rb av CDK4/6-cyclin D komplexet och i G1/S övergången är också cyklin E-CDK2 aktivt. Cyklin-CDK komplexen regleras genom att cyklinerna uppregleras och bryts ned i de olika cellcykelfaserna (t.ex. genom olika stimuli som beskrevs ovan). Cyklin-CDK komplexen kan också regleras av cyklinberoende kinas inhibitorer. Proteinet p16 inhiberar specifikt CDK4/6-cyclin D komplexet och hindrar på så sätt proliferationen genom att stoppa cellen i G1 fas.

Cancer utvecklas i flera olika steg genom en rad genetiska förändringar och tumörceller kan karaktäriseras av bland annat okontrollerad proliferation och spriding i den vävnad som cancern uppstod i (invasion). Målsättningen med detta avhandlingsarbete har varit att undersöka tumörcellernas proliferations kontroll i relation till invasion, både i primära tumörer och i cellinje system med tyngdpunkt på cellcykel inhibitorn p16 och signaleringsproteinet ERK1/2.

Basalcellscancer har ett distinkt histologiskt invasions mönster som gör tumör typen lämplig att använda som ett modellsystem för att studera förhållandet mellan invasion

och proliferation. Immunohistokemiska analyser av p16, Ki-67 (proliferationsmarkör) och fosforylerat Rb visade att tumörceller vid den invasiva fronten, både vid den nodulära puttande fronten, vid ulceration samt i spetsiga och kilformade utlöpare av tumörceller (Figur 9), hade ökat p16 uttryck (Figur 10), låg proliferation (Ki-67) och inget fosforylerat Rb till motsats från centrala delar av basalcellscancern som proliferarade och hade fosforylerat Rb. Detta tyder på att invasion och proliferation är omvänt kopplade i basalcellscancer och att tumör cellerna byter fenotyp från en cell med hög proliferation centralt i tumören till en cell med låg proliferation vid den invasiva fronten. Denna förändring i proliferation (och möjligen invasion) var troligtvis en effekt av p16 som inhiberade cyklin D-CDK4/6, därmed fosforylering av Rb och proliferationen. Genom att anlysera RNA nivåerna i förhållande till proteinnivåerna vid den invasiva fronten kunde vi också visa att p16 reglerades på RNA nivå i basalcellscancer. Vidare kunde vi genom att immunohistokemiskt analysera olika faktorer som kan påverka RNA nivåerna av p16 utesluta att kända faktorer som reglerar p16, så som Ets, SP, Bmi och ERK1/2, ensamt reglerade p16 nivåerna i basalcellscancer.

Vi ville också undersöka en annan typ av hudcancer, invasiv skivepitelcancer, om Rb regleringen var normal och om p16 uppregelarades vid den invasiva fronten. Immunohistokemiska analyser av p16 visade två olika scenarier i skivepitelcancer. I vissa skivepitelcancrar observerade vi bara cytoplasmatisk p16 färgning och i de andra både cytoplasmatisk och nukleär färgning. Gemensamt var att tumörcellerna prolifererade trots att de var p16 positiva vilket tyder på att cellcykel regleringen via Rb inte fungerade i skivepitelcancer. Vi fann dock att p16 uppreglerades vid den invasiva fronten även i skivepitelcancer på liknande sätt som i basalcellscancer (Figur 11). Detta skulle kunna tyda på att p16 har en speciell funktion vid invasion av tumörceller som är oberoende av dess proliferations kontroll.

För att ytterligare studera en faktor som är viktig vid proliferation och invasion, studerade vi ERK1/2 aktivering i bröstcancer. I en studie som inkluderande totalt ca 800 bröstcancer patienter fann vi att (vascular endothelial growth factor receptor 2) VEGFR2 och proteinerna cyklin D samt Ets-2 var signifikant kopplade till ERK1/2 i bröstcancer och utgör troligtvis en aktiv signalkaskad väg i små, mindre aggressiva tumörer (Figur 13). Vi kunde också visa med hjälp av en annan studie (ca 500 patienter) att ERK1/2 korrelerade till Hes1. För postmenopausala kvinnor randomiserade till tamoxifen eller ingen adjuvant behandling, observerade vi ett samband mellan ERK1/2 och försämrad tamoxifen effekt, men för randomiserade premenopausala kvinnor påverkade inte ERK1/2 effekten av tamoxifen. Detta tyder på att fler och större studier behövs för att utröna den verkliga effekten av ERK1/2 på patienters svar på tamoxifen.

Idag verkar det finnas en generell uppfattning om att tumörceller invaderar och prolifererar samtidigt. Våra resultat visar att tumörceller i vissa tumörtyper inte invaderar och prolifererar samtidigt vilket kan vara viktigt när man utformar nya behandlingsstrategier.

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