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Cell cycle perspectives on breast cancer cell behaviour

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Faculty of Medicine

Academic dissertation
By due permission of the Faculty of Medicine, Lund University, Sweden,
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Abstract

Uncontrolled proliferation and the capacity to infiltrate surrounding tissues are two important characteristics of aggressive tumour cells. Previous observations in both colorectal cancer and basal cell carcinoma indicated that infiltrative tumour cell behaviour might be counteracted by a high proliferative activity, suggesting a coordination of these two activities at the cellular level. Here we studied the potential relation between proliferative activity and migratory behaviour in breast cancer, by focusing on the cell cycle regulatory proteins cyclin E and cyclin D1.

By expressing cyclin E in a breast cancer cell line we obtained experimental results indicating that increased proliferative activity obstructed migratory and invasive capacity. When validating these results in a large set of primary breast cancers, we observed that increasing cyclin E levels correlated with a less infiltrative tumour growth appearance – a finding in line with our experimental results.

Several studies have proposed that cyclin E is strongly associated with poorer disease outcome in breast cancer. Therefore, we continued to investigate the potential prognostic relevance of the inverse relation between cyclin E and infiltrative tumour growth. We revealed a distinct subgroup of less infiltrative, cyclin E high breast cancers with a relatively favourable prognosis. This subgroup was an important exception compared to the majority of tumours where cyclin E indeed correlated to a poorer outcome.

Furthermore, we delineated in more detail, how the migratory capacities of tumour cells related to cell cycle activities. Synchronised G0/early G1 cells displayed an increased migratory potential compared to both late G1/S-phase cells as well as unsynchronised, actively cycling cells. In addition, silencing of cyclin D1 indicated a novel CDK- and cell cycle independent function of cyclin D1 in restraining migratory capacity. This novel role of cyclin D1 seemed to influence the behaviour of ER positive breast tumours, where cyclin D1 high tumours were in general of smaller size and, further, exhibited a somewhat less infiltrative growth pattern. In addition, increased cyclin D1 levels correlated to a more favourable prognosis.

Key words: cyclin E, cyclin D1, proliferation, migration, invasion, breast cancer

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

I **Berglund P, Stighall M, Jirström K, Borgquist S, Sjölander A, Hedenfalk I, Landberg G.** Cyclin E overexpression obstructs infiltrative behavior in breast cancer: a novel role reflected in the growth pattern of medullary breast cancer. *Cancer Research: 65 (21), 9727-9734 (2005).*

II **Berglund P*, Stighall M*, Jirström K, Rydén L, Fernö M, Nordenskjöld B, Landberg G.** Cyclin E confers a prognostic value in premenopausal breast cancer patients with tumours exhibiting an infiltrative growth pattern. *Journal of Clinical Pathology: 61, 184-191 (2008).*

III **Berglund P, Nilsson K, Lehn S, Tobin N, Härkönen P, Landberg G.** The oncogene cyclin D1 inhibits migratory capacity in breast cancer and is linked to favourable prognostic features. *Manuscript*

* Authors contributed equally.

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ABBREVIATIONS

AP-1	Activating Protein-1
BRCA1/2	Breast Cancer 1/2, early onset
Cdc6	Cell division cycle 6 homolog
CDK	Cyclin-Dependent Kinase
C/EBPβ	CCAAT/Enhancer Binding Protein Beta
Cip/Kip	CDK interacting protein/ Kinase inhibitory protein
CREB	cAMP Responsive Element Binding Protein
DMP1	cyclin D-interacting Myb-like Protein 1 (alias DMTF1)
ECM	ExtraCellular Matrix
EGFP	Enhanced Green Fluorescence Protein
EGFR	Epidermal Growth Factor Receptor
Egr-1	Early growth response -1
ER	Estrogen Receptor
ERK	Extracellular-signal-Regulated protein Kinase
FAK	Focal Adhesion Kinase
FISH	Fluorescence In Situ Hybridisation
GSK3β	Glycogen Synthase Kinase 3β
GTPase	Guanine-Tri-Phosphatase
HDAC	Histone Deacetylase
HER	Human Epidermal growth factor Receptor
HiNF-P	Histone Nuclear Factor - P
INK4	Inhibitor of cyclin dependent Kinase 4
JNK	c-Jun N-terminal Kinase
KLF8	Kruppel-Like Factor 8
MAPK	Mitogen-Activated Protein Kinase
MCM	Mini-Chromosome Maintenance
MEF	Mouse Embryo Fibroblast
MEK	Mitogen-activated protein kinase/ Extracellular signal-regulated kinase Kinase
MMP	Matrix MetalloProtease
NFAT	Nuclear Factor of Activated T-cells
NFκB	Nuclear Factor of kappa light polypeptide gene enhancer in B-cells
NHG	Nottingham Histological Grade
NPAT	Nuclear Protein, Ataxia-Telangiectasia locus
MRCK	Myotonic dystrophy kinase-Related CDC42-binding Kinase
PI3K	PhosphatidylInositol 3-Kinase
PKB/Akt	Protein Kinase B/v-akt murine thymoma viral oncogene homolog
pRb	Retinoblastoma protein
PR	Progesterone Receptor
ROCK	Rho Kinase
SCF	Skp1-Cullin-F-box complex
SP1	Specificity Protein 1 transcription factor
STAT	Signal Transducer and Activator of Transcription

INTRODUCTION

The cell cycle

Overview

Cell reproduction requires duplication of the DNA followed by partitioning of the nucleus and division of the cytoplasm and plasma membrane in order to produce two daughter cells. This process is called the mitotic cell cycle and its sequential steps are normally accomplished under stringent control. With the exception of early embryonic cells, the cell cycle can be divided into four distinct phases: G1, S, G2 and M. The G1-phase constitutes a gap between the preceding cell division and the onset of a new round of DNA replication. During this phase diverse metabolic, stress and environmental cues are being integrated and interpreted, and based on these signals the cell decides whether to divide or not. If the proper mitogenic signals are received the cell enters the DNA synthesis phase, S-phase, in which the DNA is replicated, leading to the duplication of chromosomes. The second gap phase, G2, then ensues allowing the cell to prepare for mitosis. During mitosis, M-phase, the duplicated chromosomes segregate towards opposite poles to form two new nuclei and the plasma membrane of the mother cell divides in-between, yielding two daughter cells. The absence of sufficient mitogenic growth factors or the presence of growth-inhibitory signals will trigger the cell to exit the active cell cycle and enter a resting state, called G0. Depending on the cell's state and environmental cues, it can either remain in quiescence with the capacity to re-enter the cell cycle, or become permanently incapable of dividing by undergoing terminal differentiation or senescence. Defects in DNA replication during S-phase or in the proper allocation of chromosomes during M-phase can have serious consequences for the survival and behaviour of a cell. Such defects are the ultimate cause behind cancer. The cell cycle machinery is endowed with several control mechanisms ascertaining that the S-phase and M-phase are executed flawlessly. If genomic damage is detected the cell cycle is halted at certain checkpoints and progress is allowed only if the damage has been properly repaired. G1 checkpoints make sure that cells do not enter the S-phase with DNA damage and S-phase checkpoints halt the replication process when copying errors are detected. Further on, checkpoints in G2 block the progression into mitosis if replication is incomplete and M-phase checkpoints take care of defects in chromosome alignment, thereby preventing unequal segregation. Still other mechanisms monitor that each step of the cell cycle is performed once and only once, ensuring for example that the DNA is replicated only once each cycle.

As soon as the cell has taken the decision to divide in late G1 phase, the following series of steps will proceed through mitosis according to a more or less fixed schedule, independently of external influences (Weinberg, 2007). The period in G1 where the cell is susceptible to pro- and anti-proliferative signals from the extracellular environment and neighbouring cells is therefore of crucial importance for controlling appropriate cell division. Incipient tumour cells have acquired genetic defects that deregulate the integration and interpretation of these signals. This enables them to grow and divide in a way that initially threatens the architecture and functions

of the tissue and organ harbouring the tumour cells, and could eventually be disastrous for the organism. The cell cycle machinery that regulates the important progression from G1- to S-phase will be discussed in the next sections, followed by a description of some of the upstream signalling networks that affect the expression and function of these cell cycle regulators.

The G1- to S-phase progression

A group of serine/threonine kinases, called cyclin-dependent kinases (CDKs), play an important role in regulating the progression from G1- to S-phase. These kinases form active heterodimeric complexes with their regulatory subunits, the cyclins. By phosphorylating target proteins in a temporally ordered fashion they drive and initiate the entry into S-phase. The timing and extent of CDK activity is regulated by the presence of cyclins, activating phosphorylations and dephosphorylations on specific CDK residues and by the binding of modulating partners called CDK inhibitors (Malumbres and Barbacid, 2001). In order for mitogenic stimuli to trigger S-phase entry, brakes on the cell cycle progression have to be inactivated and removed. Perhaps the most important cell cycle suppressors are proteins of the retinoblastoma family, pRb (p105), p107 and p130, collectively referred to as the pocket proteins (Giacinti and Giordano, 2006). These proteins block cell cycle progression by suppressing transcription factors of the E2F family (E2F1-5). When active, the E2Fs regulate the expression of genes required for S-phase entry including genes encoding DNA replication proteins, enzymes involved in nucleotide synthesis and components of the origin recognition complex (Dyson, 1998). The pocket proteins are thought to inhibit E2F activity by binding and blocking their transcriptional activation domain and by recruiting histone deacetylases (HDACs), SWI/SNF factors, Polycomb group proteins and methyltransferases to the promoter sites where the E2Fs are situated. Recruitment of these latter proteins induces a chromatin structure that prevents transcription (Stevaux and Dyson, 2002). The interaction between the retinoblastoma proteins and E2F family members is a complex matter, as specific combinations appear at different time points during G1 and S-phase, and exert different transcriptional effects. In G0- early G1, E2F4 and –5 are bound to promoter sites forming transcriptional repressor complexes together with p107 and p130 (Gaubatz et al., 2000). Later in G1, the activating E2F1, -2 and –3 associate with S-phase target genes, but are inhibited in their transcriptional function by the repressing activity of pRb. Regulating the activity of pRb is therefore of importance since it will decide whether or not a cell is allowed to progress into S-phase and to divide, and it has been shown that inactivation of pRb is a common theme in human tumours. Loss of the normal retinoblastoma protein functions leads to inappropriate liberation of E2F activity and results in deregulated cell cycle control (Sage et al., 2000). A germline mutation in the *Rb* gene, coding for pRb, is inherited in familial cases of retinoblastoma and pRb is also frequently lost or inactivated in various other types of cancer. Mutations of p107 and p130 do not seem to occur in primary tumours, indicating a specific role for pRb in tumorigenesis (Bartek et al., 1996; Classon and Harlow, 2002). Viral oncoproteins such as the human papilloma virus E7, the adenovirus E1A and the simian virus 40 large T antigen are able to inactivate pRb by sequestering it from its normal binding partners (Dyson et al., 1989). The fundamental role of the CDKs in G1- and S-phase is mainly attributable to their

ability to regulate pRb by means of phosphorylation. The first CDK complexes to appear in the G1-phase are cyclin D-CDK4/6 followed by cyclin E-CDK2. These complexes are normally strictly regulated but tumours often exploit them in various ways to deregulate the cell cycle control (Malumbres and Barbacid, 2001). Figure 1 presents a simplified picture over some of the regulating events that take place during the progression through G1 to S-phase.

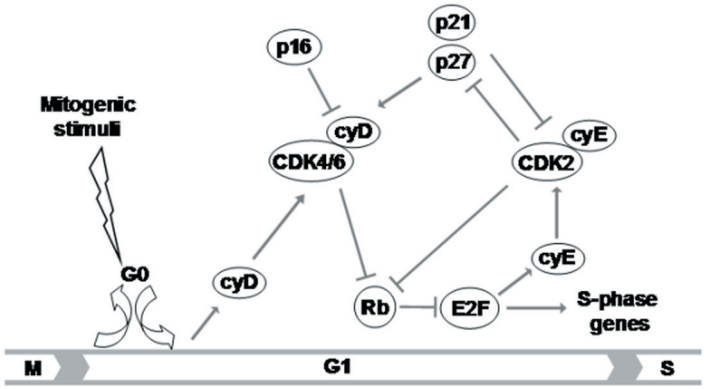


Figure 1. *Inactivation of the retinoblastoma protein during G1. Mitogenic stimuli, e.g. growth factors, induce the expression of the D-type cyclins that bind to and activate their CDK partners, CDK4 and CDK6. Cyclin D-CDK4/6 initiates a series of inactivating phosphorylations on pRb, which enable E2F-mediated transcription. The emergence of active cyclin E-CDK2 complexes reinforce the phosphorylation of pRb resulting in the expression of genes involved in DNA synthesis.*

Cyclin D

Under normal conditions the D-type cyclins act as growth factor sensors that integrate mitogenic signals with the cell cycle machinery, thereby enforcing the decision of cells to enter the S-phase (Sherr, 1995). *CCND1*, *-2* and *–3* comprise a family of related genes that are situated at different chromosome loci and code for proteins approximately 33-34 kD in size. They share an average of 57% identity over the entire coding region and 78% in the cyclin box (Inaba et al., 1992; Xiong et al., 1992). The D-type cyclins are probably regulated by different transcription factors, resulting in a tissue specific expression pattern during development and in adult tissues, but they all seem to exert similar functions in driving cell cycle progression (Ciemerych et al., 2002). Cyclin D1 is rapidly induced upon mitogen stimulation and declines when these factors are withdrawn. Many transcription factors transactivate the *CCND1* promoter, such as AP-1, Egr-1, STAT proteins, CREB, β-catenin and NF-κB (Coqueret, 2002). Cyclin D1 is an unstable protein with a half-life of approximately 20 minutes and its degradation is mediated by the ubiquitin-dependent 26S proteasome. Phosphorylation by GSK3β redirects cyclin D1 from the nucleus to the cytoplasm where the protein becomes bound to the E3 ubiquitin ligase $SCF^{FBX4\ \alpha\beta\ crystalline}$, targeting it for proteasomal degradation (Diehl et al., 1998; Lin et al., 2006). Via the cyclin box, all D-type cyclins are able to bind and activate their kinase partners CDK4 and CDK6 (Matsushime et al., 1992; Meyerson and Harlow, 1994), and the most recognised function of these complexes is to phosphorylate pRb (Sherr and Roberts, 1999). When activated during mid G1, the cyclin D-CDK4/6 complexes are responsible for the initial phosphoryla-

tion of pRb that disrupts the pRb-HDAC interactions, resulting in a more open chromatin structure. This remodelling facilitates a partial transcriptional activity from the E2F-bound promoters. Subsequent phosphorylations by cyclin E-CDK2 complexes fully inactivate pRb and prevent pRb from binding and repressing E2F (Harbour et al., 1999). In order to become enzymatically active, CDK4/6 needs to be phosphorylated and dephosphorylated by CAK (CDK activating kinase) and Cdc25A, respectively (Iavarone and Massague, 1997; Kato et al., 1994). In addition, the assembly and stabilisation of cyclin D-CDK4/6 complexes are promoted by the interaction with the Cip/Kip family members p21 and p27. These latter proteins belong to a group of proteins that are referred to as CDK inhibitors owing to their ability to inhibit other CDK complexes such as cyclin E-CDK2. Thus, when stable ternary complexes are formed between p21/p27 and cyclin D-CDK4/6, this will indirectly facilitate the activation of cyclin E-CDK2 complexes that, in turn, are important for the subsequent cell cycle progression (Sherr and Roberts, 1999). CDK4/6 is further negatively regulated by the INK4 family members p15, p16, p18 and p19. By specifically competing with the D-type cyclins in binding to CDK4/6, the INK4 inhibitors form binary inactive complexes that block the cyclin D-dependent pRb-phosphorylation (Sherr and Roberts, 1995). The role of cyclin D in regulating the cell cycle has been illustrated in various experiments. Overexpression of cyclin D1 resulted in both an accelerated G1 progression and in a reduced requirement for growth factor stimulation to exit G0 (Musgrove et al., 1994; Quelle et al., 1993; Resnitzky et al., 1994). Further, blocking cyclin D1 by injection of inhibiting antibodies could prevent G1 progression (Baldin et al., 1993; Quelle et al., 1993). The classical cell cycle function of cyclin D1 is probably dependent on pRb since cells lacking pRb are independent of cyclin D1 for cell cycle progression (Lukas et al., 1995). Although the D-type cyclins and their associated kinases undoubtedly play an important role in regulating the cell cycle, the generation of knock-out mice lacking different combinations of the cyclins and CDKs has generated quite surprising results. For example, mice lacking individual D-cyclins, CDK4 or CDK6 are viable and present narrow, tissue-specific defects (Kozar and Sicinski, 2005). Knocking out all three D-cyclins was shown to be embryonic lethal due to severe anemia and cardiac abnormalities but, still, many organs were able to develop normally. Further, fibroblasts derived from these triple knockout mice proliferated relatively normally in culture, although they required higher mitogen stimulation to exit G0 (Kozar et al., 2004). Similar results were observed in mice lacking both CDK4 and CDK6 (Malumbres et al., 2004). These experiments collectively indicate that the cyclin D-CDK complexes are not essential for cell cycle progression, at least not during embryonic development. Despite the devaluation of the importance of the D-type cyclins in G1 progression, cyclin D1 expression is clearly relevant in tumour development. Increased levels of cyclin D1 protein have been shown in a number of primary human tumours, and in a fraction of these tumours the increased protein expression correlated with gene amplification (Donnellan and Chetty, 1998). A causative role for cyclin D1 in breast cancer formation was suggested because transgenic mice engineered to overexpress cyclin D1 in the mammary glands developed hyperplasia and breast tumours (Wang et al., 1994). Cyclin D1 probably plays a key role in the initiation of certain types of malignancies,

as several oncogenic pathways target and are dependent on cyclin D1 for inducing transformation. In the mouse, mammary tumour formation triggered by the Ras and Her2/Neu pathway requires cyclin D1, whereas the *Myc* and *Wnt-1* oncogenes are able to elicit malignant transformation in the absence of cyclin D1 (Yu et al., 2001). In addition to the well-established CDK-dependent role, the D-type cyclins have been shown to have CDK-independent functions by binding and activating or repressing several transcription factors such as the oestrogen and androgen receptors, DMP1, STAT3, SP1 and C/EBPβ (Coqueret, 2002; Lamb et al., 2003). Cyclin D1 has also been suggested to modulate cell migration by controlling Rho/ROCK signalling and expression of thrombospondin 1 (Li et al., 2006c). Cyclin $D1^{-/-}$ mouse embryo fibroblasts presented increased ROCK II activity and increased thrombospondin 1 expression, properties that were linked to their decreased migratory ability.

Cyclin E

The promoter regions of the genes coding for the E-type cyclins, *CCNE1* and *-2*, contain E2F binding sites. Expression of cyclin E1 and -2 is therefore induced when the pRb-mediated repression of E2F is alleviated (Ohtani et al., 1995). Cyclin E1 and –2 share 47% overall amino acid similarity and 70% similarity within the cyclin box, and they also share the same expression patterns and affinities to binding partners (Lauper et al., 1998). Cyclin E1 has been more extensively studied and will be referred to as cyclin E below. Being an E2F target, cyclin E levels peak during late G1-phase and as the S-phase progresses cyclin E becomes degraded. Two distinct pathways that involve the ubiquitin-proteasome machinery mediate the degradation of cyclin E. One pathway exclusively targets free, monomeric cyclin E and involves the Cul-3 protein (Singer et al., 1999), whereas the other pathway involves the SCF-Fbw7 ubiquitin ligase. In order for the F-box protein Fbw7 to target cyclin E for destruction, cyclin E needs to be phosphorylated on specific residues. Both CDK2 and GSK3β have been shown to carry out these phosphorylations that in turn induce cyclin E turnover (Welcker et al., 2003). When expressed, cyclin E binds to and activates CDK2 and the activated complex phosphorylates several target proteins that are involved in initiating DNA replication (Hwang and Clurman, 2005). Apart from cyclin E binding, the activity of CDK2 is regulated by the interactions of Cip/Kip CDK inhibitors and by inhibitory and activating phosphorylations. Through binding to and phosphorylating pRb, cyclin E-CDK2 complexes complete the inactivation of pRb that was initiated by cyclin D-CDK4/6, resulting in an unrestrained E2F transcriptional activity. This event is often regarded as the point where further cell cycle progression becomes independent of mitogenic stimulation (Malumbres and Barbacid, 2001). Cyclin E not only stimulates its own expression by promoting E2F activity, the cyclin E-CDK2 complexes are also able to reinforce their activity by targeting the CDK inhibitor p27 for degradation (Sheaff et al., 1997). In addition to pRb and p27, cyclin E-CDK2 phosphorylates proteins that are directly involved in S-phase activities, such as NPAT/p220, Cdc6 and the centrosome regulating proteins nucleophosmin and CP110 (Chen et al., 2002; Ma et al., 2000; Mailand and Diffley, 2005; Okuda et al., 2000). Phosphorylating NPAT/p220 enables NPAT/p220 to interact with and activate the transcription factor HiNF-P that in turn induces transcription of histone H4 genes. The cyclin E-CDK2/NPAT/HiNF-P

pathway coordinates DNA replication with histone synthesis so that the newly replicated DNA immediately becomes packaged as chromatin (Miele et al., 2005). Cyclin E-CDK2 also directly phosphorylates Cdc6, thereby promoting the assembly of pre-replication complexes, which in turn licences the DNA for replication (Mailand and Diffley, 2005). Further, cyclin E exerts a kinase-independent function by binding to MCM helicases and facilitating their loading into the pre-replication complexes, a necessary step for the initiation of DNA replication (Geng et al., 2007). By phosphorylating nucleophosmin, cyclin E-CDK2 initiates centrosome duplication during the S-phase. Several lines of evidence have shown that cyclin E-CDK2 activity plays a critical role in G1-S-phase progression. Overexpression of cyclin E in cell lines causes decreased requirements for mitogens, a more rapid G1-progression as well as a prolonged S-phase. Further, inhibition of cyclin E-CDK2 activity was shown to prevent S-phase entry (Ohtsubo and Roberts, 1993; Ohtsubo et al., 1995; Tsai et al., 1993). In contrast to cyclin D-CDK4/6, the cell cycle regulatory function of cyclin E-CDK2 is not dependent on the pRb-E2F pathway, since excess cyclin E stimulates S-phase entry in the presence of mutated pRb (Lukas et al., 1997). Similar to the experience from genetically modified mice lacking the D-type cyclins and their associated kinases, studies utilising cyclin E- and CDK2-null mice have enforced a re-evaluation of the importance of cyclin E-CDK2 in cell cycle regulation. Mice lacking cyclin E1, cyclin E2 or CDK2 are viable although the CDK2$^{-/-}$ mice are sterile (Sherr and Roberts, 2004). Knocking out both cyclin E genes caused embryonic lethality due to trophoblastic failure and placental defects (Parisi et al., 2003). These results indicated that normal proliferation and development was independent of cyclin E expression, whereas cyclin E seemed to be critical in the process of endoreplication, i.e. multiple rounds of DNA synthesis without cell division. The role for cyclin E in endoreplication has been suggested to involve defective MCM loading (Hwang and Clurman, 2005). Cyclin E1- and E2-null mouse embryo fibroblasts (MEFs) were able to continue normal asynchronous cell division but they were unable to re-enter the cell cycle from a G0-state. These cells were further resistant to transformation induced by different combinations of oncogenic stimuli, such as *c-Myc*, *H-Ras*+*c-Myc*/dominant negative p53/*E1A* (Geng et al., 2003). In contrast to the cyclin E knockout mice, CDK2$^{-/-}$ mice did not present any placental defects; CDK2-null MEFs were able to enter the S-phase after serum stimulation and were further transformed by oncogenes (Berthet et al., 2003). These results demonstrate that CDK2 is dispensable for cell division and further stress the CDK-independent functions of cyclin E in cell cycle regulation. In addition, the absence of phenotypes in the CDK2$^{-/-}$ mice and MEFs might partially be explained by the overlap between CDK2 and CDK1. It has been shown that cyclin E can bind and activate CDK1, implying that this redundancy might compensate for the loss of CDK2 function (Aleem et al., 2005). The fact that cells lacking cyclin E are resistant to oncogenic transformation, indicates that cyclin E could be functionally involved in tumorigenesis. Many cancers present elevated levels of cyclin E protein and/or mRNA. This is most likely due to mutations in upstream pathways that regulate cyclin E expression, although gene amplifications do occur in primary tumours, albeit infrequently (Hwang and Clurman, 2005). Defects in cyclin E proteolysis might also contribute to excess protein levels and Fbw7 mutations have

been reported both in cancer cell lines and in primary tumours (Moberg et al., 2001; Spruck et al., 2002). Although mouse models have failed to show that cyclin E overexpression is sufficient to induce tumour formation (Bortner and Rosenberg, 1997), cyclin E deregulation might contribute to the tumorigenic process in conjunction with additional mutations. The current view favours that increased genetic instability might be the mechanism by which deregulated cyclin E expression promotes tumour formation. Cyclin E overexpression, alone or in combination with loss of the p53 checkpoint, has been shown to induce genetic instability and aneuploidy in cancer cell lines and in primary cells (Minella et al., 2002; Spruck et al., 1999). Both defects in S-phase progression due to impaired MCM loading, and centrosome amplifications (Kawamura et al., 2004) have been proposed as possible explanations for the cyclin E-induced genetic instability, but this issue is still not settled.

Upstream signalling networks involved in cell cycle regulation

A plethora of signals can potentially affect cells in their decision to divide. The availability of nutrients, growth factors, hormones, cytostatic factors, cell-cell communications as well as the interaction between cells and their surrounding extracellular matrix (ECM), collectively dictate whether a cell is allowed to progress into S-phase or not. Signals of different kinds are sensed and received by receptor molecules that relay the input information into an intracellular signalling network. This network can be viewed as separate pathways that communicate with each other upon signalling events and coordinate the information to yield a cellular response. Several pathways have been mapped that influence the expression and activity of cell cycle regulators involved in the G1- and S-phase progression, such as the MAP-kinase pathway and the PI3-kinase pathway (Liang and Slingerland, 2003; Meloche and Pouyssegur, 2007). Anchoring membrane-bound complexes, such as the E-cadherin-β-catenin complex and integrin based focal adhesions (Walker and Assoian, 2005), connect cytoskeletal structures to the extracellular environment and link information from neighbouring cells and the ECM to the cell cycle machinery. The actin and microtubule cytoskeletons play a role in the execution of physical cell division events and, in addition, are involved in the signalling transduction that regulates cell cycle progression. A schematic illustration depicting some of the pathways in the network that transduce signals to the cell cycle machinery is shown in figure 2.

Mitogen-activated protein kinase (MAPK) pathways

MAP kinases are a family of serine/threonine protein kinases that are involved in the regulation of a variety of cellular activities such as cell proliferation, differentiation, cell death and movement. MAPK signalling cascades are organised hierarchically into three levels where the MAPKs are phosphorylated and activated by MAPK kinases, which in turn are phosphorylated by MAPKK kinases. These latter kinases interact with small GTPases, connecting the MAPK pathway to cell surface receptors and external stimuli. One MAPK pathway that is extensively involved in cell cycle regulation signals through Ras-Raf-MEK1/2-ERK1/2, where ERK1/2 constitutes the final effector MAPK (Pearson et al., 2001). ERK1/2 are multifunctional kinases that phosphorylate a vast array of proteins including other protein kinases, signalling effectors, receptors, cytoskel-

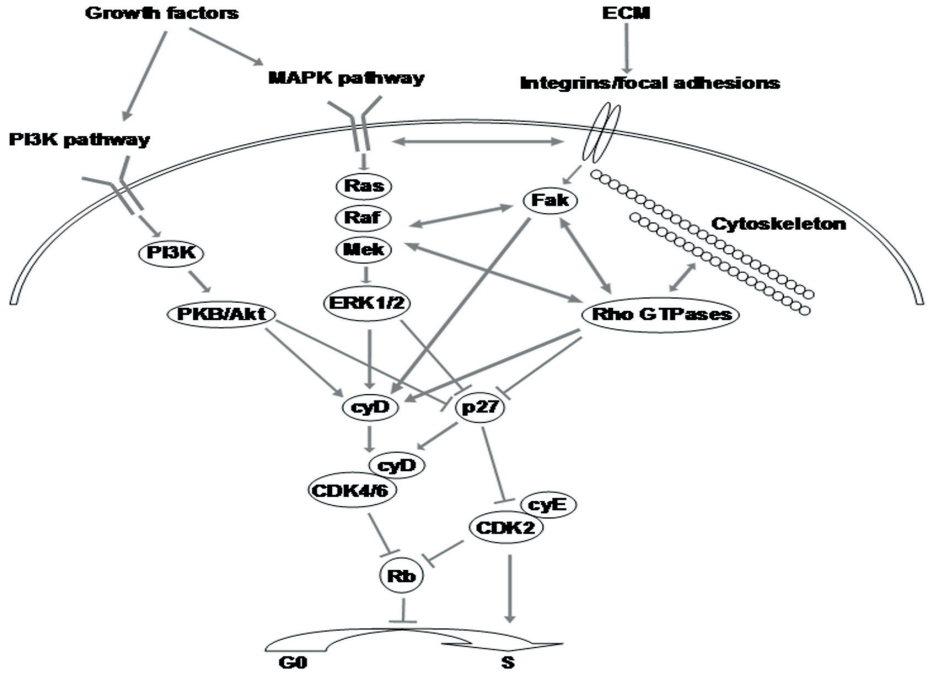


Figure 2. *Extracellular signals are transduced through several pathways that impinge on the cell cycle regulators cyclin D1 and p27. The net result on the cell cycle progression into S-phase is influenced by the complex crosstalk between the different pathways. Signalling interactions in this network are indicated in a very simplfied way.*

etal proteins and nuclear transcriptional regulators. ERK1/2 become rapidly phosphorylated and activated in response to mitogenic stimulation, and sustained ERK1/2 activity until late G1 is required for successful S-phase entry (Yamamoto et al., 2006). The activity declines at the G1-S-phase transition and is not necessary at the actual S-phase entry (Meloche, 1995). ERK1/2 stimulate cell growth by inducing global protein synthesis through increased ribosome activity. This impact is partially mediated through ERK1/2-dependent enhancement of mTOR signalling (Wullschleger et al., 2006). ERK1/2 probably use several mechanisms to promote G1 progression where induction of cyclin D1 expression is one important function (Meloche and Pouyssegur, 2007). The *CCND1* promoter contains binding sites for the transcriptional complex AP-1, and ERK1/2 induce the expression of AP-1 components (Treinies et al., 1999). ERK1/2 further regulate cyclin D1 expression through phosphorylation and inactivation of Tob, which acts as a transcriptional co-repressor that negatively regulates cyclin D1 transcription (Suzuki et al., 2002). In addition, activation of ERK1/2 in G1 regulates the CDK inhibitors p21 and p27. Transient activation of ERK1/2 induces expression of p21, which likely contributes to the stabilisation of cyclin D1-CDK4/6 complexes (Liu et al., 1996). As mentioned earlier, the MAPK pathways impinge on many cellular activities besides proliferation. For example, the MAPK family members ERK1/2, JNK and p38 have all been shown to play a role in regulating cell migration. The different MAPK members phosphorylate target proteins that are involved

in focal adhesion dynamics, actino-myosin contractility and actin and microtubule cytoskeletal organisation (Huang et al., 2004). Exactly how the different downstream responses, which are induced by active MAPK signalling, relate to each other has not yet been extensively studied. However, it seems likely that a certain degree of coordination is necessary to ensure an efficient execution of the functional responses.

The PI3K pathway

A variety of stimuli, many of which also induce MAPK pathways, activate phosphatidylinositol 3-kinase (PI3K). PI3K activity results in the synthesis of the important lipid second messenger PIP_3 (phosphatidylinositol 3,4,5 triphosphate), which in turn recruits PKB/Akt and other effectors to the plasma membrane, leading to their activation (Woodgett, 2005). PKB/Akt is a serine/threonine kinase that is best known to promote cell survival by phosphorylating several pro-apoptotic target proteins, but it is also involved in regulating proliferation, angiogenesis and cell invasion and migration (Yoeli-Lerner and Toker, 2006). The induction of cyclin D1 upon growth factor stimulation is not exclusively dependent on the MAPK pathway. Cyclin D1 accumulation is also modulated by protein degradation through the activity of GSK3β, which in turn is a well-known target of PKB/Akt (Cross et al., 1995). PKB/Akt mediated phosphorylation inactivates GSK3β, leading to stabilisation of the cyclin D1 protein. It has been shown that MAPK activity fails to increase cyclin D1 levels in the presence of PI3K inhibitors (Treinies et al., 1999). PKB/Akt has also been suggested to promote cyclin D1 transcription and translation (Liang and Slingerland, 2003). In addition to supporting cyclin D1 accumulation, the PI3K pathway has been shown to be involved in regulating p21 and p27. PKB/Akt directly phosphorylates p27, leading to an impaired nuclear localisation, and thereby preventing p27 from inhibiting nuclear cyclin E-CDK2 complexes (Liang et al., 2002). Similarly, p21 is suggested to accumulate in the cytoplasm upon PKB/Akt phosphorylation (Zhou et al., 2001). The multitude and complexity of downstream effects triggered by PI3K-PKB/Akt activity is stressed by findings that indicate a novel role for a specific isoform of PKB/Akt in suppressing tumour cell migration and invasion (Yoeli-Lerner and Toker, 2006). In one study, Akt1 (PKBα) was shown to repress breast cancer cell motility by inhibiting the transcriptional activity of NFAT (Yoeli-Lerner et al., 2005). In an *in vivo* mouse model of breast cancer, expression of Akt1 accelerated tumorigenesis through increased cellular proliferation but also interfered with the metastatic progression, resulting in fewer metastatic lesions (Hutchinson et al., 2004).

The Integrin-FAK pathway

The ECM affects the behaviour of cells through interactions with integrins, a large family of cell surface receptors. Integrins consist of two transmembrane subunits, α and β, and different combinations of these subunits dictate the binding specificity to various ECM constituents. Integrins transduce signals from the ECM by binding to the cytoskeleton, cytoplasmic kinases and membrane bound growth factor receptors (Giancotti and Ruoslahti, 1999). Larger aggregates consisting of integrins, associated signalling mediators and actin filaments make up structures known as focal adhesions. Activated focal adhesion complexes induce intracellular

signalling that affects cell cycle progression, survival, differentiation, polarity and migration. By integrating ECM adhesion and signalling transduction with cytoskeletal structures, the integrins add a positional and architectural dimension to the signalling mechanisms that determines the G1-phase progression. The involvement of integrin-mediated adhesion in cell cycle control is reflected in the anchorage dependent proliferation of normal cells. Tumour cells display a reduced adhesion dependency for their proliferation and survival, but they still utilise and benefit from certain types of integrin signalling (Guo and Giancotti, 2004). The signalling through ECM-integrin complexes and growth factor receptors is tightly interwoven and regulates cell cycle progression in a cooperative manner (Assoian and Schwartz, 2001). Focal adhesion kinase, FAK, has been extensively studied as being one of the cytosolic integrin partners that is important in modulating receptor signalling and cell cycle control. Overexpression of FAK in mouse fibroblasts accelerated G1 progression and promoted tumour cell proliferation *in vivo* (Wang et al., 2000; Zhao et al., 1998). Conversely, expression of a dominant negative FAK mutant inhibited cell cycle progression. FAK affects the cell cycle indirectly by regulating the activity of the MAP kinases JNK and ERK1/2, which in turn positively regulate cyclin D1 expression (Oktay et al., 1999; Zhao et al., 1998). FAK has also been shown to directly contribute to cyclin D1 expression by inducing the transcription factor KLF8 that binds to and activates the *CCND1* promoter (Zhao et al., 2003). Other downstream effector molecules of integrin signalling are PI3K and the Rho-family of small GTPases, that both play a role in regulating cyclin D1 levels. Membrane localisation and subsequent activation of Rac, a member of the Rho-family involved in many cellular activities, has been shown to be dependent on integrin-mediated adhesion (del Pozo et al., 2000).

The actin cytoskeleton and Rho GTPases

In addition to the regulatory involvement of growth factors and cell adhesion, the organisation of the actin cytoskeleton has been implicated in G1-phase progression. Disruption of the cytoskeleton integrity has been shown to prevent quiescent (G0) cells from progressing through G1 by affecting both ERK1/2-dependent and independent mechanisms (Bohmer et al., 1996; Huang and Ingber, 2002; Lohez et al., 2003; Reshetnikova et al., 2000). Studies using several different cell types have indicated that actin reorganisation in early G1 is important for proper cyclin D1 and p27 expression. However, an integrated actin cytoskeleton does not seem to be as important for G1 progression in cells that enter G1 directly from mitosis, probably due to the fact that these cells do not need to upregulate cyclin D1 and downregulate CDK inhibitors to the same extent as quiescent cells (Margadant et al., 2007). The small GTPase proteins RhoA, Rac1 and Cdc42 have been suggested as candidates for linking cytoskeletal organisation with cell cycle progression. The role of the Rho GTPases in modelling the actin cytoskeleton is well established and RhoA, Rac1 and Cdc42 are important regulators of cell migration through the control of different modes of actin reorganisation (Raftopoulou and Hall, 2004). In fibroblasts, inhibition of these small GTPases was shown to block mitogen-stimulated G1-S-phase progression, and conversely, microinjection of active RhoA, Rac1 and Cdc42 was sufficient to induce quiescent cells to progress into S-phase (Olson et al., 1995). The downstream effectors of RhoA,

ROCKI and ROCKII, are able to affect cell cycle proteins by distinct mechanisms. ROCK signalling acts partially via the MAPK pathway to elevate cyclin D1 levels and through MAPK-independent mechanisms to increase and decrease cyclin A and p27 expression, respectively (Croft and Olson, 2006). In endothelial cells, p27 was downregulated through a RhoA-mediated increase of the F-box protein Skp2, which is required for degradation of p27 (Mammoto et al., 2004). In addition to RhoA, Rac1 and Cdc42 have been shown to stimulate cyclin D1 expression independently of MAPK signalling, possibly by direct interactions with the transcription factor NF-κB (Coleman et al., 2004; Joyce et al., 1999).

A cell cycle perspective on tumour cell behaviour

As indicated in the above sections regarding the signalling networks that regulate the cell cycle, it is clear that distinct pathways are able to induce many different cellular responses. Which response a certain type of signalling evokes is most likely dependent on several factors, including the state of specific members of the triggered pathway and the general intra- and extracellular context in which the signalling takes place. The end result of signalling through one pathway could be modulated by the state of other, interconnected pathways. Furthermore, neighbouring cells and the ECM environment might influence whether the response will be A or B, or alternatively no response at all. Cell type and differentiation state are other factors that clearly are involved in deciding cellular behaviour in terms of signalling evoked responses. In addition, the proliferative activity of a cell could potentially be a factor that affects the range of possible cellular responses, including migratory behaviour. Theoretically, there could be different reasons for assuming that cell cycle activities would modulate cellular responses that are unrelated to proliferation, such as cell migration. Firstly, the signal-transducing, information-processing network that mediates cell cycle progression is the same that integrates migration-stimulating cues into a coordinated migratory response. A successful triggering of the G1-S-transition might evoke feedback mechanisms that negatively affect the same signalling pathways that mediated the cell cycle progression. Since these pathways are involved in the implementation of migratory responses, cell cycle activities would have a restraining impact on migration. Secondly, as a cell traverses the point in G1 where it becomes committed to complete the cell cycle, it might need to focus its abilities in order to optimally execute the division. If this is the case, then it could be assumed that a cell is unlikely to commit its cytoskeletal and genetic machinery to both cell division and migration concurrently. This would imply that there is a window spanning from the end of M-phase to late G1 where a cell's freedom to migrate is maximal. This reasoning might be relevant when trying to understand the complex behaviour of tumour cells. By definition, tumour cells belong to a proliferative population and will therefore spend time in the active cell cycle phases where they either prepare themselves to divide (G1- or G2-phase) or are occupied in the acts of division (S- or M-phase). Thus, in principle, the migratory ability of a tumour cell could depend on the frequency at which it enters the active cell cycle. However, the plastic nature of tumour cells will most likely enable the development of capabilities that could bypass the proliferative constraints on migration. Nevertheless, assuming that such capabilities have not been acquired, the hypothesised coordinated relation between migration and active cell

cycling at the cellular level could impact the overall invasive behaviour of a tumour. Supposing two tumours with all other parameters equal except for proliferative activity, the tumour that consists of highly proliferative cells would be predicted to exhibit a less invasive growth compared to the tumour that consists of low-proliferative cells. Despite the obvious simplification of such theoretical reasoning, there are observations and experimental results that seem to support a coordinated regulation of cell proliferation and motility. For example, tumour cells localised at the invasive front of basal cell carcinomas were shown to express high levels of p16 and to be low-proliferative (Svensson et al., 2003). *In vivo* models of tumour cell motility and invasion in mouse and rat where actively invading cells were isolated and directly analysed, showed that the invasive cells were less proliferative compared to the general population of cells from the primary tumour (Wang et al., 2007). Further, studies using glioma and astrocytoma cell lines led to similar conclusions, i.e. that migrating cells presented a reduced proliferative activity (Giese et al., 1996; Mariani et al., 2001). However, another study failed to show that the migratory activity of medulloblastoma cells was influenced by their mitotic activity (Corcoran and Del Maestro, 2003). Undoubtedly, divergent results might be due to the use of different experimental settings and cell lines. To gain more knowledge about the relation between proliferative activity and the migratory/invasive capacity of tumour cells is not just of theoretical interest, it might also contribute to a deeper understanding of tumour behaviour in general. The process of cell migration and invasion is in it self very complicated and will be briefly addressed in the following section.

Tumour cell migration and invasion

In order for a locally established primary epithelial tumour to become malignant and thereby constitute a threat to the host, the tumour cells have to break loose from their origin tissue context and invade into the surrounding tissue, enter the vasculature and circulate to distant sites, and finally extravasate and establish metastatic foci. Invasion is performed either by single cells or by clusters of cells. Loss of expression of E-cadherin and certain cytokeratins, as well as upregulation of N-cadherin and vimentin are changes that are frequently involved in single cell invasion. This change of gene expression is often referred to as epithelial-to-mesenchymal transition, which is a naturally occurring process in specific cell types, that leads to loss of cell-cell contacts and the gain of cell motility (Guarino et al., 2007). In addition, expression of matrix metalloproteinases (MMPs) enables the breakdown of the basement membrane that separates the epithelial cell layer from the tissue stroma. The activity of MMPs also generates proteolytic fragments that further enhance tumour cell migration (Stamenkovic, 2003). When the basement membrane barrier is penetrated, tumour cells utilise the process of directional movement to invade the surrounding stroma (Condeelis et al., 2005). The directionality is governed by gradients of either ECM binding sites or soluble chemoattractants such as chemokines and growth factors. Concentration gradients of chemoattractants are due either to passive diffusion from blood vessels or active secretion from stromal cells. Carcinoma cells can recruit different host cells to support their migration through the stroma towards the vasculature (Karnoub et al., 2007; Kedrin et al., 2007). Paracrine communications between tumour cells and cancer-associated fibroblasts and macrophages have been shown to enhance tumour cell invasiveness.

For example, secretion of CSF-1 (colony stimulating factor 1) by tumour cells can trigger macrophage secretion of EGF (epidermal growth factor), which in turn stimulates the migratory behaviour of tumour cells (Wyckoff et al., 2004). The migratory response involves regulation of pathways that control cell-ECM adherence and actin cytoskeleton remodelling. Two modes of single cell migration have been described, an elongated mesenchymal type and a rounded amoeboid type that utilise different mechanisms of actin cytoskeleton remodelling and are variably dependent on integrin-mediated adhesion (Friedl and Wolf, 2003; Sahai and Marshall, 2003). The elongated mode of migration in a three-dimensional matrix resembles migration over a 2D surface and could be described as a multistep cycle. This migration cycle starts with membrane protrusion of the leading edge driven by gradient stimulated local actin polymerisation. Formation of cell-ECM focal adhesions at the leading edge and the recruitment of actin binding proteins to these adhesion sites serve to anchor newly formed actin stress fibres. This anchorage is followed by cell contraction where activated myosin II binds to the actin filaments and generates actino-myosin contractility that translocates the cell body in the direction of the gradient. The last step in this migration cycle consists of focal contact disassembly at the trailing edge leading to its detachment. In addition to these steps, 3D movement requires proteolytic remodelling of the ECM (Friedl and Wolf, 2003). The amoeboid mode of migration is characterised by weaker ECM interactions and movement is mediated by cortical filamentous actin instead of stress fibres. Higher cell plasticity in combination with less adhesion enables the amoeboid tumour cells to penetrate the ECM barrier through pre-existing pores without the need for proteolytic cleavage (Condeelis et al., 1992; Friedl and Wolf, 2003). Many tumour cells seem to be able to adapt their mode of invasion in response to changing conditions. For example, inhibition of the proteolytic activity of elongated tumour cells does not block the migratory capacity. Instead, these elongated cells are able to maintain their migration through converting to the proteolytic-independent amoeboid mode of motility (Wolf et al., 2003). A requirement for cell movement, irrespective of mode, is the generation of actino-myosin contractility. Both RhoA and Cdc42 are involved in this process by activating downstream effector kinases, such as ROCK and MRCK, leading to increased levels of myosin phosphorylation, which then can cross-link actin filaments and generate contractile force (Raftopoulou and Hall, 2004; Wilkinson et al., 2005). As mentioned above, clusters of cells as well as individual cells can perform tumour cell invasion. Clustered cell migration requires the maintenance of homophilic cell-cell interactions and it has been shown that cells in these clusters express different kinds of cadherins and gap-junction proteins (Friedl et al., 1995; Friedl and Wolf, 2003). Multicellular contractile bodies are allowed to form due to a specific structure of cortical actin filament assembly along cell junctions implying some kind of supracellular cytoskeletal organization (Hegerfeldt et al., 2002). In histological samples of tumours, different variants of collective migration can be observed. Invasive growth can be generated either by protruding sheets of tumour cells that maintain contact with the primary tumour or by detached groups of cells. A specific example of collective migration is the chains of single tumour cells aligned between stromal fibres, called 'Indian files', that are a typical histological feature of invasive lobular breast carcinoma (Martinez and Azzopardi, 1979).

Breast cancer

Breast cancer is the most frequently diagnosed form of cancer in women in the so-called western world. As for most of cancer diseases, the risk of developing breast cancer is related to age (WHO, 2006). In Sweden, breast cancer makes up about 30% of all diagnosed female cancer and the lifetime risk of developing this disease up to the age of 75 is estimated to be 9.6%. The incidence rate has increased over the last 40 years but the five-year survival rate has improved from 65% in the sixties to about 85% in the nineties. The improved survival among breast cancer patients is probably due to both earlier detection and better treatment (Socialstyrelsen, 2007; Talback et al., 2003). In addition to the general cancer risk factors age and radiation, the likelihood of developing breast cancer is associated with hormone exposure. Life history factors such as early menarche, late menopause, late first birth or nulliparity increase the lifetime exposure of endogenous oestrogen peaks during the menstrual cycle. Oral contraceptives and hormonal replacement therapy add exogenous oestrogen to the overall hormone exposure and further increase the risk of breast cancer. The majority of breast cancer cases arise sporadically, although approximately 20% of breast cancer patients have a known family history of the disease. 5-10% of all cases are considered to be hereditary, mainly due to mutations in the major breast cancer susceptibility genes *BRCA1* and *BRCA2* (Kumar, 2007).

Adult breast glands undergo major developmental changes during pregnancy, lactation and involution. Breast glands consist of a branched ductal network encompassed by a basement membrane and surrounded by connective- and fat tissue. The ductal network terminates in lobular units that secrete milk during lactation. Ducts are bilayered structures comprised of an outer basal layer of myoepithelial cells that surround an inner layer of polarised luminal epithelial cells (Moinfar, 2007). Basal myoepithelial cells resemble smooth muscle cells and exhibit contractile properties and they form adhesive interactions with luminal cells, other myoepithelial cells and the basement membrane (Adriance et al., 2005). The epithelial cells lining the lobular units differentiate during pregnancy and lactation to secrete milk.

Current evidence supports the belief that both luminal epithelial cells and myoepithelial cells are derived from common breast epithelial precursor cells located within the luminal compartment (Gudjonsson et al., 2002; Pechoux et al., 1999). This distinct population of mammary precursor/stem cells has been shown to have the exclusive ability to form tumours in mice and are suggested to be the cells that drive tumorigenesis (Al-Hajj et al., 2003; Liu et al., 2005). The cancer stem cell hypothesis is based on the fact that stem cells are long-lived, slowly dividing cells with the capacity for self-renewal. Due to their longevity, stem cells could be exposed to damaging agents for long periods of time and might therefore accumulate mutations that result in tumorigenic transformation. Aberrant differentiation of these transformed progenitor/stem cells is further suggested to generate the phenotypic heterogeneity found in human breast cancers (Dontu et al., 2003).

The heterogeneity of this disease is reflected in the different morphology exhibited by the clinically recognised forms of breast cancer. Non-invasive breast carcinomas that have not penetrated the basement membrane are classified as ductal carcinoma *in situ* (DCIS) or lobular

carcinoma *in situ* (LCIS). The invasive carcinomas are classified into several different subtypes, for example invasive ductal carcinoma, invasive lobular carcinoma, tubular carcinoma and medullary carcinoma. Approximately 80% of all breast cancers are classified as invasive ductal carcinoma. This subtype is defined by exclusion as not falling into any of the other categories of invasive carcinomas. It is a heterogeneous category ranging from well-differentiated tumours with tubule formation to poorly differentiated tumours consisting of sheets of anaplastic cells. The tumour margins are usually irregular and the carcinomas often elicit a strong host reaction composed of fibroblasts, lymphocytes and ECM (Kumar, 2007). Invasive lobular carcinomas make up 10-15% of all invasive carcinomas and present a distinct morphological appearance with aligned strands ('Indian file' pattern) of invading tumour cells (Martinez and Azzopardi, 1979). Tubular carcinomas are rare and account for only a few percent of the invasive breast carcinomas. This subtype is recognised by differentiated cells that grow in well-formed tubule structures (Kumar, 2007). Medullary carcinomas account for roughly 5% of invasive carcinomas and are characterised by large anaplastic, highly proliferative cells that grow in a pushing, well-delimited pattern with a pronounced lymphoid infiltration (Pedersen, 1997). Despite the low differentiation grade of this subtype, patients with medullary carcinomas have a relatively better prognosis and are on average diagnosed at a younger age (Pedersen et al., 1995). The frequency of medullary carcinomas seems to be higher in patients with *BRCA1* mutations and *BRCA1* tumours display many of the medullary characteristics such as low differentiation grade, p53 mutations and a negative oestrogen receptor (ER) status (BCLC, 1997; Eisinger et al., 1998; Lakhani et al., 2002). Efforts have been made to describe the diversity of breast tumours in terms of differences in gene expression patterns. Based on a subset of genes with significantly greater variation in expression between different tumours than between successive samples from the same tumour, five distinct subtypes of breast tumours have been defined (Sorlie et al., 2001). The two most distinguishable subtypes identified when gene expression profiles were analysed in three independent breast cancer data sets, were the so-called luminal-like and basal-like breast cancer subtypes. One suggested hypothesis to the consistent differences in gene expression patterns between the identified subtypes is that they originate from different breast epithelial cell lineages. Patients with basal-like tumours were further shown to have a significantly poorer clinical outcome compared to patients with luminal-like tumours (Sorlie et al., 2003).

The prognosis of breast cancer patients is routinely estimated on the basis of several parameters. Tumour size at the time of diagnosis is correlated to disease outcome where a larger tumour size predicts a worse prognosis. The presence of lymph node metastases is further linked to more aggressive disease. If the tumour has spread to other organs and established distant metastases, patients are rarely curable although chemotherapy may prolong survival. These three parameters are included in the tumour-node-metastases (TNM) staging system that is used to establish the clinical stage of the disease (Singletary et al., 2002). Morphological assessment of the degree of tumour differentiation has also been shown to provide important prognostic information in breast cancer (Elston and Ellis, 1991). In the currently established grading system, termed Nottingham Histological Grade (NHG), tumour differentiation grade is based on three parameters:

presence of tubular structures within the invasive tumour, nuclear atypia (accounting for nuclear size, nuclear morphologic variability and number of nucleoli), and mitotic count (a measure of tumour proliferative activity). A score from 1-3 is given to the parameters individually and the total sum of these scores defines the three NHG differentiation grades. Studies have shown that tumour grade is strongly correlated with prognosis; patients with grade I tumours have a significantly better survival than those with grade II and III tumours (Elston and Ellis, 1991; Sundquist et al., 1999). These clinical and histological parameters are being used to establish prognostic estimations that in turn guide the choice of therapy regime. Despite their correlations to prognosis, the current clinical criteria fail to accurately predict disease outcome and a significant number of patients are being misclassified, leading to either over-treatment or under-treatment of these patients. Several attempts have been made to find new and better prognostic and treatment predictive markers. One approach has been to analyse the gene expression profiles of breast tumours. In one study, supervised classification was used to identify a gene expression signature that strongly correlated with development of distant metastases (van 't Veer et al., 2002). Based on the expression of 70 genes, a 'good prognosis' signature and a 'poor prognosis' signature were defined. The power of this gene set to predict patients at risk of developing distant metastases was further validated in other breast tumour data sets, and it was shown that the prognostic signature had a lower degree of misclassification compared to the clinically used criteria (van de Vijver et al., 2002). Work is in progress to develop assays that could implement these encouraging results in a clinically suitable setting.

To date, three treatment predictive molecular markers: oestrogen receptor (ER), progesterone receptor (PR) and HER2, are routinely assessed in the clinical management of breast cancer patients. The ER and PR function as ligand activated transcription factors and regulate expression of several genes, many of which are involved in proliferation. These steroid hormone receptors are normally expressed in only a fraction of breast epithelial cells where they play a role in oestrogen induced paracrine signalling that regulates the proliferation of ER- and PR-negative cells (Clarke, 2003; Clarke et al., 1997; Laidlaw et al., 1995). However, ER and PR expression is significantly increased in a majority of breast cancers, and many of the receptor positive tumour cells are themselves dependent on oestrogen for their proliferation (Clarke et al., 2004). The selective oestrogen receptor-modulating drug tamoxifen, which inhibits oestrogen activation of the ER, has been proven successful in the treatment of ER-positive breast cancer (EBCTCG, 1998). Hormone receptor status is therefore routinely evaluated by immunohistochemical staining of formalin-fixed tumour tissue sections, and patients with hormone receptor positive disease are considered likely to respond to endocrine therapies. The human epidermal growth factor receptor family consists of EGFR, HER2, HER3 and HER4. Upon ligand activation and receptor dimerisation, these receptors activate several downstream pathways that in turn regulate diverse processes involved in tumour cell behaviour, including differentiation, proliferation, survival and migration (Hsieh and Moasser, 2007). Mutational activation of these receptors is observed in many malignancies and HER2 is amplified and overexpressed in up to 25-30% of breast cancers (Slamon et al., 1989). HER2 amplified tumours have further been

linked to a poorer prognosis (Borg et al., 1990; Paterson et al., 1991). The monoclonal antibody trastuzumab directed against HER2, has been shown to induce tumour regression in a fraction of patients with HER2-amplified tumours (Tokunaga et al., 2006). In addition to ER and PR evaluations, immunohistochemical staining of HER2 protein is performed routinely together with HER2 amplification detection by FISH analysis; tumours with strong staining and over-amplification are considered eligible for treatment with trastuzumab.

G1-cyclins in breast cancer

As mentioned earlier, deregulation of cell cycle control is a prerequisite in tumour formation and several cell cycle related changes have been observed in breast cancer. Inactivating mutations of *Rb*, overexpression of cyclin D1 and cyclin E, and downregulation of p27 and p16 are examples of alterations that occur in breast cancer (Malumbres and Barbacid, 2001). Furthermore, some of these changes have been assigned prognostic or predictive relevance. Depending on tumour material and method of detection, cyclin D1 protein overexpression is reported in 25-60% of invasive breast carcinomas and *CCND1* gene amplification occurs with a frequency ranging from 10-30% (Courjal et al., 1996; Gillett et al., 1994; Gillett et al., 1996; Jirstrom et al., 2005; McIntosh et al., 1995; Nielsen et al., 1997; Pelosio et al., 1996; Seshadri et al., 1996; Stendahl et al., 2004; Umekita et al., 2002; van Diest et al., 1997; Zukerberg et al., 1995). Generally, the gene amplified tumours seem to express somewhat higher levels of protein, but the discrepancy between the frequencies of protein overexpression and gene amplification implies that cyclin D1 protein could be elevated by means other than gene amplification (Gillett et al., 1994; Jirstrom et al., 2005). All studies to date on cyclin D1 in breast cancer report that cyclin D1 overexpression is significantly more common in hormone receptor positive tumours. It has been shown that cyclin D1 can activate the oestrogen receptor in a ligand-independent manner (Zwijsen et al., 1998), and cyclin D1 seems to have an important impact on anti-oestrogen treatment response. In two studies, it was shown that strong nuclear staining intensity for cyclin D1 was associated with an abrogated tamoxifen response in both pre- and postmenopausal breast cancer patients, and *CCND1* amplification was even linked to an adverse effect in tamoxifen treated premenopausal patients (Jirstrom et al., 2005; Stendahl et al., 2004). There is a major discrepancy between different studies regarding the prognostic information of cyclin D1. However, the majority of studies report either no correlation or a negative correlation, such that patients with cyclin D1 overexpressing tumours experienced a relatively better outcome (Gillett et al., 1996; Nielsen et al., 1997; Seshadri et al., 1996; Stendahl et al., 2004; van Diest et al., 1997). Recalling the role of cyclin D1 in cell cycle regulation and breast cancer formation in mice, this might seem counterintuitive, but cyclin D1 overexpression in human breast cancer is generally associated with less aggressive tumour characteristics such as a lower histological grade, normal p53 and a retained pRb function (Loden et al., 2002; van Diest et al., 1997). In addition to the less aggressive background, it is possible that cyclin D1 overexpression in established invasive breast carcinomas could result in gain of as yet unknown tumour suppressive properties. When comparing cyclin D1- and cyclin E overexpression, it is clear that these events have distinct consequences and take place in different subsets of breast tumours. Increased levels of cyclin E

protein vary in frequency between 26-46%, again depending on patient selection and method of detection (Chappuis et al., 2005; Donnellan et al., 2001; Han et al., 2003; Keyomarsi et al., 2002; Kuhling et al., 2003; Nielsen et al., 1996; Porter et al., 2006; Porter et al., 1997; Rudolph et al., 2003; Span et al., 2003), and *CCNE1* amplifications are rarely observed (Callagy et al., 2005; Schraml et al., 2003). In contrast to cyclin D1 overexpression, increased cyclin E levels are strongly correlated with a hormone receptor negative status and a higher histological grade (Nielsen et al., 1996; Porter et al., 2006; Rudolph et al., 2003). In addition, cyclin E overexpression has been related to a basal-like tumour phenotype and *BRCA1*-associated breast cancer (Chappuis et al., 2005; Foulkes et al., 2004). There is no consensus in the literature regarding the prognostic information of cyclin E. However, the majority of studies report a significant correlation between high cyclin E protein or mRNA levels and a poorer outcome (Chappuis et al., 2005; Donnellan et al., 2001; Han et al., 2003; Keyomarsi et al., 2002; Kuhling et al., 2003; Nielsen et al., 1996; Porter et al., 1997; Sieuwerts et al., 2006). In one study, the negative impact on outcome was restricted to postmenopausal patients (Rudolph et al., 2003). Two studies did not find any prognostic value of cyclin E (Porter et al., 2006; Span et al., 2003). A confounding factor in many of these studies is that the different prognostic analyses were based on patient subsets including both post-operatively untreated and treated patients. Due to the mixed patient cohorts, it is difficult to conclude whether cyclin E correlates with a worse prognosis or if cyclin E affects the response to certain therapies. It is likewise hard to draw any general conclusion regarding whether cyclin E confers independent prognostic information or not. The discrepancy between these studies could probably be explained by the use of heterogeneous patient cohorts, different detection methods and choice of cut-off and differences in statistical calculations. However, it might also reflect the limited power of one isolated gene product to predict the aggressive behaviour and clinical outcome of a tremendously complex disease. Maybe the most important result to come from the attempts to find isolated prognostic factors in breast cancer is the overall tumour biological information that these studies have generated. For example, the dichotomy between ER-positive/cyclin D1-high and ER-negative/cyclin E-high breast tumours adds molecular information to the observed heterogeneity.

THE PRESENT INVESTIGATION

Aim

The main objective of this thesis has been to study the relation between proliferative activity and migratory/invasive capacity in breast cancer cells, and to further explore the relevance of this relationship in tumour behaviour.

The specific aims were:

- To analyse the consequences of cyclin E overexpression for breast cancer cell behaviour, with specific focus on effects not directly involved in cell cycle regulation.
- To study how cyclin E overexpression relates to clinical and histopathological parameters in breast cancer.
- To determine the prognostic relevance of cyclin E in premenopausal breast cancer.
- To determine the association between cyclin E protein levels and tamoxifen response in premenopausal breast cancer patients.
- To test the influence of cell cycle activity on migratory capacity.
- To analyse the potential role of cyclin D1 in tumour cell migration.

Results and discussion

Increased S-phase activity and decreased invasiveness as a result of cyclin E overexpression in breast cancer cells – experimental results in agreement with in vivo observations (paper I)

It is well established that cyclin E regulates important steps during the G1-S-phase transition and that cyclin E overexpression leads to deregulated cell cycle control (Hwang and Clurman, 2005). Based on these observations, cyclin E has been considered to have tumour promoting properties and it has also been found that many malignancies express elevated cyclin E protein levels. Furthermore, there are studies that suggest cyclin E overexpression to be a prognostic marker in breast cancer (Keyomarsi et al., 2002; Sieuwerts et al., 2006). Previous studies have however mainly focused on the cell cycle aspects of cyclin E expression. In paper I, we analysed the functional consequences of cyclin E overexpression in the breast cancer cell line MDA-MB-468, with particular emphasis on potential consequences not directly associated with the cell cycle. Initially, stable cyclin E-EGFP overexpressing clones were generated and analysed together with control clones expressing EGFP only. The cyclin E-high clones exhibited a permanently increased S-phase fraction compared to the control clones, indicating that the cyclin E-EGFP construct influenced the entry into and/or progression through the S-phase. In order to extend the investigation of cyclin E-mediated consequences, we conducted a microarray assay comparing the overall gene expression patterns of cyclin E-high clones and control clones. Approximately 430 genes were finally assigned to be differentially expressed between the clones, of which a majority were downregulated. Due to varying quality of the performed hybridisations, the filtering and selection criteria applied reduced the total number of differentially expressed genes, which most likely decreased the power of this analysis. Nevertheless, by classifying the approved changed genes into functional categories (using the ontological software GoMiner), we could obtain rough indications of functional consequences resulting from the differential gene expression. First of all, the functional category 'cell proliferation' contained a significant number of altered genes, illustrating that the ontological approach was able to capture a known functional difference. 'Cell adhesion' was another category that contained a significant number of altered genes, and this led us to speculate whether adhesion-related properties differed between the control and cyclin E-high clones. To test this hypothesis, we performed three functional assays measuring the adhesive, migratory and invasive capacity of the tumour cells, respectively. In a simple attachment assay, the cyclin E-high clones showed, on average, a higher capacity to adhere to an ordinary cell culturing substratum. Further, the cyclin E-high clones presented a reduced ability to migrate in scratch assays. Perhaps the most tumour biologically relevant difference observed, was the reduced invasiveness exhibited by the cyclin E-high clones. In agreement with this result, transiently cyclin E-transfected cells were also shown to have a decreased invasive ability, in addition to an increased S-phase fraction. The similarity between the stably and transiently transfected cells strengthened the probability that the decreased invasive phenotype of the stable clones was a result of cyclin E-induced changes, and not due to random clonal variation.

The actin cytoskeleton is involved in both adhesive and motility functions (Kodama et al., 2004; Vasioukhin et al., 2000); thus, to elucidate possible mechanisms behind the decreased invasiveness, we initially studied potential differences in actin cytoskeleton organisation. In contrast to the control clones, the cyclin E-high clones exhibited a membrane-associated actin organisation indicative of more rigid cell-cell contacts, which may potentially be involved in their low-invasive phenotype. Unfortunately, our efforts to further explore the underlying reasons for the cytoskeletal differences did not yield any conclusive results. For example, we did not find any difference in the membrane associated E-cadherin-β-catenin interactions, which are known to be important for cell-cell adhesions (Kovacs et al., 2002). In addition, we were unable to detect differences in the activity of the small GTPases that regulate actin cytoskeleton organisation (Raftopoulou and Hall, 2004). Nevertheless, the list of altered genes contained interesting candidates that, speculatively, could contribute to the low-invasive phenotype. *THBS1* (thrombospondin 1), *PXN* (paxilin) and *MMP7* (matrix metalloproteinase 7) were all differentially expressed, and their respective protein products have been shown to function in cell-ECM interactions, focal adhesion assembly/integrin signalling and ECM degradation, respectively (Adams and Lawler, 2004; Hagel et al., 2002; Shiomi and Okada, 2003).

To test the *in vivo* relevance of the main finding that cyclin E overexpression obstructed invasive capacity, we analysed cyclin E levels in relation to tumour growth patterns in a large set of primary breast carcinomas. Tumour biopsies were arranged in tissue microarrays and cyclin E immunoreactivity was scored as fractions of positive nuclei. Growth patterns were assessed using whole tumour sections and characterised by the mode of infiltration, ranging from a "sieving" and exclusively infiltrative pattern to a clearly pushing pattern with well-defined tumour margins. The degree of local infiltrative growth was used as an *in vivo* equivalent to the experimentally measured invasive capacity. Interestingly, a majority of the tumours that expressed the highest levels of cyclin E presented a predominantly pushing growth pattern. With decreasing levels of cyclin E expression, the proportion of infiltrative tumours increased, such that almost all cyclin E negative tumours exhibited an exclusively infiltrative growth pattern. This inverse association between cyclin E expression and infiltrative growth was clearly in agreement with our experimental results. Another aspect of this association was that the frequency of cyclin E deregulation differed between specific breast cancer subtypes. Tumours of the medullary subtype consistently expressed high levels of cyclin E whereas the majority of the other main subtypes (i.e. ductal, lobular and tubular) expressed very low levels.

In conclusion, cyclin E overexpression induced an increased S-phase activity and obstructed the invasive capacity of breast cancer cells. From these experiments, it is not possible to determine whether it was cyclin E *per se* or the elevated S-phase activity that impeded the invasive behaviour. Furthermore, the association between cyclin E expression and low-infiltrative tumour growth challenges the current understanding of how cyclin E relates to aggressive tumour behaviour. Deregulated cell cycle control and increased proliferation are obviously traits associated with aggressive tumours, whereas decreased invasive behaviour is not. Hence, these results imply a more complex function of cyclin E in breast cancer biology.

Prognostic implications of the association between cyclin E expression and tumour growth pattern in premenopausal breast cancer (paper II)

Various conclusions have been reached concerning the prognostic relevance of cyclin E expression in breast cancer (Porter et al., 2006; Sieuwerts et al., 2006), and a major reason underlying the discrepancies might be the differences between the tumour samples studied. To obtain prognostic information one needs to analyse tumours from patients that have not received adjuvant treatment after surgery. Such patient cohorts are not obtainable today, since a majority of breast cancer patients receive adjuvant endocrine- and/or chemotherapy. Therefore, retrospective studies of well-characterised patient cohorts with tumours from systemically untreated patients are the only means to determine whether cyclin E confers pure prognostic information rather than treatment predictive information. In light of our findings in paper I, we proceeded to investigate whether the association between cyclin E protein expression and tumour growth pattern in breast cancer could contribute to a more detailed understanding of the prognostic relevance of cyclin E. For this purpose, we analysed a patient cohort consisting of 288 stage II primary breast cancers from premenopausal women not subjected to any adjuvant treatment. The entire cohort consisted of 564 patients that were enrolled in a clinical trial of adjuvant tamoxifen treatment; hence in addition to the prognostic analysis, we evaluated the relevance of cyclin E as a predictor of tamoxifen response.

Cyclin E protein expression was determined immunohistochemically, scoring the fraction of positive nuclei. In this cohort, 33.5% of the tumours were negative for cyclin E, 26.5% expressed low levels (<10% positive nuclei) and 40% expressed high levels of cyclin E (>10%). Regarding the distribution of growth patterns, 74% exhibited an exclusively infiltrative growth, 13% were predominately infiltrative, 10% were predominately pushing and 3% of the tumours presented an exclusively pushing growth pattern. 83% (44/53) of the predominately/exclusively pushing tumours expressed high levels of cyclin E, reiterating in detail the general observation in paper I. These low-infiltrative, pushing tumours were further characterised by larger tumour size, negative lymph node status, histological grade 3, and hormone receptor negativity. Approximately 40% of these tumours had been classified as medullary carcinomas. In addition, the pushing tumours exhibited a better prognosis as estimated by the rate of recurrence free survival (RFS), compared to tumours with an infiltrative growth pattern. Recently, it has been shown that there is a clear overlap between medullary breast carcinomas, *BRCA1*-mutation dependent tumours and tumours with a basal-like gene expression profile (Bertucci et al., 2006; Chappuis et al., 2005; Foulkes et al., 2004). Our observations suggest that a high cyclin E protein level is another feature shared by tumours belonging to this putative subgroup.

When analysing the association between cyclin E and RFS in the total cohort of untreated patients, we did not find any significant correlation. However, considering the high frequency of cyclin E overexpression among the less aggressive tumours with pushing growth features, this might not be surprising. Indeed, when focusing on the patients whose tumours exhibited an

infiltrative growth pattern, a majority of all patients, we found a significant correlation between increased cyclin E protein levels and poor outcome. Furthermore, in this major subgroup of patients, cyclin E protein levels contributed prognostic information independently of histological grade, lymph node status and tumour size. In the total cohort, cyclin E overexpression was almost exclusively associated with a high rate of proliferation (reflected by a high fraction of Ki67 staining, unpublished result), but apparently this did not always translate into more aggressive tumour behaviour. Speculatively, this might be explained by the inability of certain tumours to simultaneously combine a high level of proliferation with the process of invasion. The experimental results from paper I, where ectopic cyclin E expression induced both increased S-phase activity and decreased invasive capacity, support the notion that exaggerated proliferation could result in a decreased ability to invade, leading to a less infiltrative tumour growth. Following this line of reasoning, the most aggressive tumours would have acquired abilities that enable them to avoid or override the proliferative constraints on infiltrative behaviour.

The second part of this study dealt with the association between cyclin E expression and anti-oestrogen treatment response. There are experimental results indicating that cyclin E overexpression has a negative effect on tamoxifen response, and in one study high levels of cyclin E mRNA were reported to correlate with a poorer response to treatment (Dhillon and Mudryj, 2002; Span et al., 2003). An inherent problem of studying cyclin E and anti-oestrogen response is the fact that cyclin E overexpression in breast tumours is strongly correlated with hormone receptor negativity. Since it is the hormone receptor positive patients that are given endocrine therapy, the group of tamoxifen-treated patients with cyclin E-high tumours will generally be relatively small. When analysing the relation between cyclin E expression and tamoxifen response in the ER positive group in this cohort, we did not observe any correlation between increasing levels of cyclin E expression and a poorer treatment response.

Detailing the relation between cell cycle activities and migration – a specific role for cyclin D1 in modulating migratory capacity (paper III)

Based on our previous observations of a negative association between proliferative activity and motility/invasion, we set out to investigate in more detail how the migratory capacity relates to cell cycle activities. Initially, by culturing MDA-MB-231 breast cancer cells in serum free medium followed by serum stimulation, we established populations synchronised at successive cell cycle phases, ranging from a resting G0 population to a population dominated by actively cycling S-phase cells. In order to assess whether the different synchronised populations differed in regard to migratory capacity, we needed a method that could quantify migratory potential without significantly affecting cell cycle characteristics. Boyden migration chambers were found to be suitable for this purpose, since the highly motile MDA-MB-231 cells only required 3h in the migration chambers to generate quantifiable migration data. We reasoned that 3h in the migration chambers should not cause a major change in the cell cycle status of the different populations. The migration assays clearly showed a cell cycle specific variation, where cells from the G0- and early-G1 populations migrated significantly faster than cells from the successive populations. When interpreting the migration results, we used an unsynchronised population

as a reference. Asynchronously growing populations contains a mixture of cells in G1, S, G2 and M-phase. Cells corresponding to the resting G0 and early G1-phase cells, present in the synchronised setting, are most likely not found in a continuously cycling population. This difference enabled us to compare the migratory capacity of resting G0/early G1 cells and unsynchronised cells.

As mentioned in Paper III, stimulating starved cells with serum will trigger signalling through several pathways, where the MAP kinase pathway is but one. The activated signalling cascades could induce different phenotypic responses that do not necessarily have to be related. This fact made it somewhat problematic to derive a simple causal connection between the observed concomitant changes in cell cycle progression and migratory capacity. However, some observations are in favour of an explicit connection between cell cycle aspects and migration. First, serum starved G0 cells not exposed to serum stimulation prior to the migration assays displayed significantly increased migration compared to actively cycling, unsynchronised cells. Further, when inhibiting ERK1/2 in synchronised early G1 cells, these cells still migrated relatively more rapidly compared to unsynchronised cells (compare U0126 treated cells in Figure 2C with the reference level). Finally, the main finding in paper I, where cyclin E overexpression induced an increased S-phase fraction and decreased motility and invasiveness, supports the notion that the poorest migratory capacity displayed by the S-phase dominated populations was causally affected by the S-phase activities.

Cyclin D1 is important for cells to progress through G1 and later to enter into S-phase. One could speculate that silencing of cyclin D1 using siRNA in an asynchronously growing population would induce a cell cycle shift towards the G1-phase, thereby potentially affecting the overall migratory capacity of the population. It seems unlikely, though, that removal of cyclin D1 would result in an accumulation of G0/early G1 cells. By using this approach we aimed, primarily, to study if G1 cells in an unsynchronised population displayed a relatively higher migratory capacity. In addition, we would obtain information regarding the specific impact of cyclin D1 on migratory behaviour. Knocking down cyclin D1 in MDA-MB-231 cells not only caused a G1 accumulation, but also increased ERK1/2 phosphorylation and, interestingly, induced an increased migratory potential. The results from the migration assays varied substantially but, nonetheless, upon repeating the experiment we obtained a significant difference. Importantly, we also observed an increased migratory capacity when silencing cyclin D1 in an independent cell line, MDA-MB-435. However, in MDA-MB-435 cells, neither the cell cycle phase distribution nor ERK1/2 activity was substantially affected by cyclin D1 knock-down. Furthermore, CDK4/6 silencing in MDA-MB-231 did not result in increased migration, despite a G1-phase accumulation and increased ERK1/2 phosphorylation. Taken together, these results indicated that increasing the fraction of G1 cells did not affect the general migratory capacity of the population. The results also indicated that the increased migration upon cyclin D1 knock-down was likely mediated via mechanisms unrelated to both cell cycle regulation and ERK1/2 activity. The lack of a migratory increase upon CDK4/6 silencing further suggested that cyclin D1 influenced the migratory capacity in a CDK independent manner.

The finding, obtained from two independent cell lines, of a novel and CDK-independent function of cyclin D1 in restraining tumour cell migration, is interesting. How cyclin D1 mechanistically restrains cell migration needs to be further investigated. Regarding this question, the transcriptional functions of cyclin D1 might be of importance and will be addressed in future studies. Our results are opposite to the findings of two reports showing that *cyclin D1-/-* mouse embryo fibroblasts (MEFs) and mouse mammary epithelial cells (MECs) have a reduced capacity to migrate (Li et al., 2006a; Li et al., 2006b). Expression of cyclin D1 in these cells resulted in an increased movement velocity. There could be several reasons for the discrepancy between these results and ours. First of all, genetically modified MEFs and MECs that are adapted to the absence of cyclin D1 most likely present an overall different behaviour compared to established, cyclin D1 expressing human breast cancer cells. This makes a comparison of the two experimental systems problematic. Secondly, the conflicting effects of cyclin D1 on migration are probably mediated by distinct mechanisms. Cyclin D1 modulated the migratory capacity in the mouse cells through CDK4 and p27, whereas in MDA-MB-231 cells, both CDK4/6 and p27 were of no obvious relevance.

The main findings in this study indicated that cyclin D1 is able to affect both migratory capacity and proliferative activity. To investigate possible *in vivo* characteristics that would reflect this dual function of cyclin D1, we performed an immunohistochemical analysis of cyclin D1 in a large breast cancer cohort consisting of tumours from premenopausal patients (the same material as in paper II). Cyclin D1 expression was scored as nuclear intensity, and in this material, increasing cyclin D1 staining intensity not only correlated with higher proliferation, but also with smaller tumour size. Since tumours size reflects several tumour biological properties, including local tumour infiltration, the inverse link between cyclin D1 and tumour size was considered interesting. Extrapolation of our experimental results would hence imply that high levels of cyclin D1 would confer hampered local tumour spread. Theoretically, this interpretation might then explain why cyclin D1-high tumours tended to be smaller. In addition, tumours with the highest cyclin D1 expression seemed to be overrepresented among tumours with a less infiltrative growth appearance, supporting the notion that cyclin D1 influences infiltrative behaviour in vivo.

When we analysed the prognostic information of cyclin D1 protein levels in untreated ER+ premenopausal breast cancer patients, strong cyclin D1 staining intensity correlated with a better prognosis. Cyclin D1 intensity together with Ki67 expression could successfully define subgroups of patients with significantly different outcome, as measured by the rate of recurrence free survival. Intermediate-strong cyclin D1/low Ki67 expression was the most favourable combination while absent-low cyclin D1/high Ki67 expression correlated with the poorest outcome. In other words, tumours consisting of highly proliferative cells lacking the putative migratory constraints of stronger cyclin D1 expression appeared to be the most aggressive.

Supplementary background to Paper III

For the sake of this thesis, I would finally like to present a series of performed experiments that initially showed truly interesting results. At the last minute, unfortunately, these results had

to be omitted from the manuscript (Paper III) due to serious problems with off-target effects related to the pool of siRNA oligos originally used to silence cyclin D1. This experience necessitated a revision of the siRNA experiments, some of which could be repeated with a modified setting (presented in Paper III). To show the full extent of my work and to understand the circumstances that led to the discovery of the off-target effects, I will here go through the original experiments and briefly discuss some general considerations regarding RNAi.

One objection to Figure 1 in Paper III was that the progression from G0/G1 to S-phase and the concomitant decrease in migratory ability could be independent consequences of serum stimulation. In an attempt to show a causal link between the changes in migratory ability and cell cycle activities, I attempted to exclusively interfere with the cell cycle progression by knocking down cyclin D1 and E in starved cells prior to serum stimulation. In these original experiments, pools consisting of four different siRNA oligos were used to target the respective cyclins. The changes in migratory capacity after serum stimulation were then monitored in control cells and cells with silenced cyclin expression. As expected, the migratory ability of the synchronised control cells was reduced substantially as they progressed into S-phase. Importantly, the cyclin D1 and E silenced cells with an obstructed S-phase entry presented a significantly smaller reduction in migratory ability (Supplementary Figure 1A-C). This partial rescue in migratory capacity did, at least, provide support for a true cell cycle component contributing to the net migratory capacity.

The partial migratory rescue observed warranted us to test whether the cyclins might be directly involved in modulating the migratory capacity. Cyclin D1 and E were therefore knocked down in unsynchronised cells and the effect on migratory capacity was assessed. Transfections with both siRNA pools reduced the cyclins very efficiently and caused a three-fold increase in migratory ability. Seemingly, this effect was mainly cyclin D1 dependent, since silencing of cyclin E alone only resulted in a minor migratory increase (Supplementary Figure 2A-C). In addition, the original cyclin D1 siRNA pool showed an even greater impact on MDA-MB-435 melanoma cell migration, although the effect varied substantially between experiments (Supplementary Figure 2D-E).

As presented in Paper III, we addressed the involvement of ERK1/2 activity in both cell cycle regulation and migration. In conformity with the result presented in Figure 3A in Paper III, knock-down of cyclin D1 with the original siRNA pool increased ERK1/2 phosphorylation. However, when we initially analysed the possibility that ERK1/2 mediated the link between cyclin D1 and migratory capacity, we obtained different results (Supplementary Figure 3A-B). Clearly, the absolute level of migration was decreased when ERK1/2 were inhibited, but the cyclin D1 silenced cells still presented significantly increased migration compared to control siRNA treated cells. Seemingly, this indicated more directly that the assumed impact of cyclin D1 on migration was not dependent on ERK1/2 activity.

Knock-down of the cyclin D1 associated CDKs further suggested that cyclin D1 modulated migration through kinase-independent mechanisms. To proceed in our investigation of how the original cyclin D1 siRNA pool affected MDA-MB-231 cell migration, we conducted a microar-

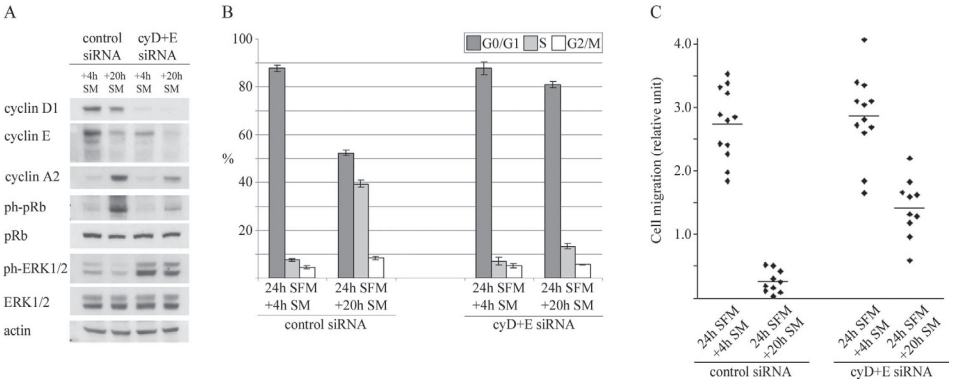
ray experiment. The possibility that cyclin D1 could affect cellular behaviour through altering gene expression is supported by the fact that cyclin D1 interacts with several transcription factors (Coqueret, 2002). The most prominent transcriptional difference between control and cyclin D1 siRNA treated cells was, as expected, related to cell cycle associated functions, but no specific gene category coupled to the processes of migration or motility was significantly affected. However, many genes involved in microtubule-based processes were significantly down regulated as a result of cyclin D1 knock-down (Supplementary Figure 4B). The microtubule cytoskeleton is involved in various cellular processes, including cytoskeletal rearrangements implicated in migration (Kodama et al., 2004). In addition, we identified specific, differentially expressed genes whose protein products are involved in focal adhesion turnover, actin polymerisation and membrane cytoskeleton contacts; genes considered relevant in the context of migration (Supplementary Figure 4C). Taken together, the gene expression analysis did not provide us with any obvious answer to why the original cyclin D1 siRNA pool affected migration. Nonetheless, the altered expression of microtubule-related genes and the list of migration associated genes were considered interesting and worth further investigations.

To establish the assumed role of cyclin D1 in modulating migratory capacity, we tried to overexpress cyclin D1 in MDA-MB-231 cells. Cyclin D1 was successfully overexpressed but, contrary to our expectations, no decrease in the migratory capacity was observed. We therefore planned an alternative approach in order to study the supposed negative impact of cyclin D1 on migration. In principle, the increased migration induced by silencing endogenous cyclin D1 should be reverted if cyclin D1 was simultaneously overexpressed. This would be possible to test since two of the oligos in the siRNA pool (#17 and 18) targeted the 3' untranslated region. This region is present in the endogenous cyclin D1 mRNA but is not part of the cyclin D1 mRNA transcribed from the expression vector. As a starting point, we analysed if these two oligos had the same phenotypic effect as the original pool of four (#15, 16, 17, 18). Surprisingly, even though the two oligos knocked down cyclin D1 with a comparable efficiency as the pool of four, they did not induce the same strong increase in migration. It later turned out that the three-fold increase seen in the initial experiments was mainly attributable to one oligo (#15). Because all four oligos knocked down cyclin D1 equally well, but only one single oligo induced a major increase in migration, our previous conclusions regarding the ability of cyclin D1 to modulate the migratory capacity could not be entirely correct. In an attempt to ascertain whether cyclin D1 still could affect migratory behaviour, we decided to repeat the siRNA experiments without oligo #15. The results and conclusions from these efforts are presented in Paper III and have been discussed above.

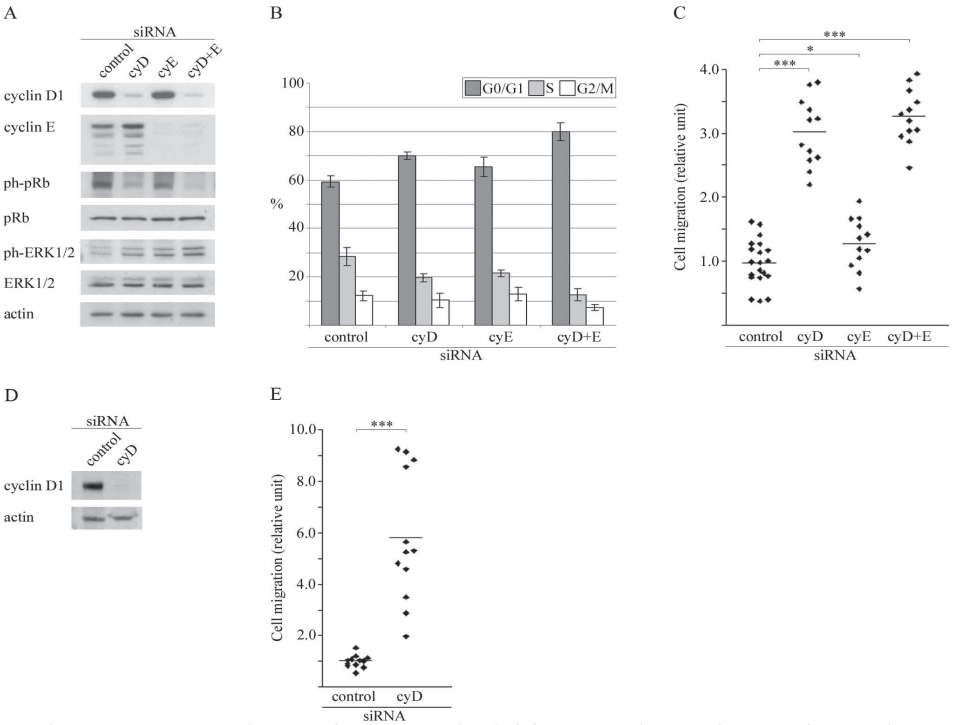
Our experience reflects the general problem with off-target effects that is inevitably attached to the present state of the RNAi methodology. Determining the specificity of an siRNA oligo is a very complex matter that is affected by several parameters, many of which are still unknown. Currently, there is no algorithm that can be used to design siRNA oligos without undesired off-targets. With the exception of near-perfect sequence identity, overall sequence identity between an siRNA and any given mRNA seems to be a poor predictor of the number and identity of

off-target genes. One parameter that has recently been shown to be associated with off-targeting is the siRNA seed region-3'UTR match (Birmingham et al., 2006). The seed region consists of six or seven nucleotides at the 5' end of the siRNA guide strand (positions 2-7/8). This region is known to be important for endogenous microRNA-target mRNA recognition (Bartel, 2004) and therefore microRNA-mediated gene silencing and siRNA off-targeting might take place through similar mechanisms. In a preliminary search for potential off-target genes in our gene expression analysis, we looked for down-regulated genes with sequences matching oligo #15. One of the down-regulated genes, USP24 (ubiquitin-specific protease 24), actually presented a perfect oligo #15 seed region-3'UTR match. Theoretically, USP24 might therefore be an interesting candidate involved in the elevated migratory capacity observed in the original siRNA experiments. To date, not much is known about the function of this gene and the potential role of USP24 in modulating migration warrants further investigation.

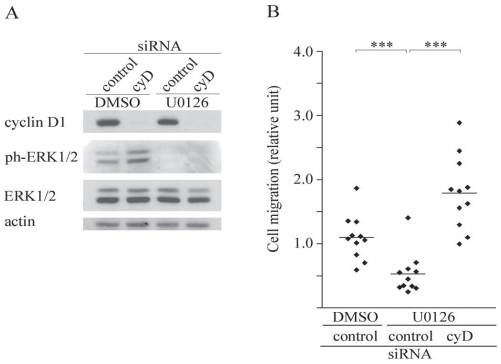
Due to the risk of off-targets, gene functions and phenotypes resulting from siRNA experiments should generally be interpreted with caution. There are some general measures that can be taken in order to mitigate off-targeting (Jackson and Linsley, 2004). It has been shown that the off-target effect is dependent on the concentration of siRNA oligos applied (Persengiev et al., 2004). One should therefore try to reduce the concentration to the lowest possible. Along this line, one suggested approach to reduce the off-target effect is to use pools of siRNAs for the same gene. Combining different oligos, each at a lower concentration, could silence the target gene as efficiently as one oligo at a higher concentration, but with a lower off-target effect. Our experience with siRNA pools clearly demonstrates that, despite the benefits, the risk of off-targeting is still present and should not be underestimated. Maybe the best way to increase the confidence with which a certain phenotype can be ascribed to silencing of a particular target gene is to use different siRNA oligos, or pools of oligos, separately. If the same phenotype is observed with independent oligos it is most likely caused by silencing of their common target gene, since each oligo presents a specific off-target profile.



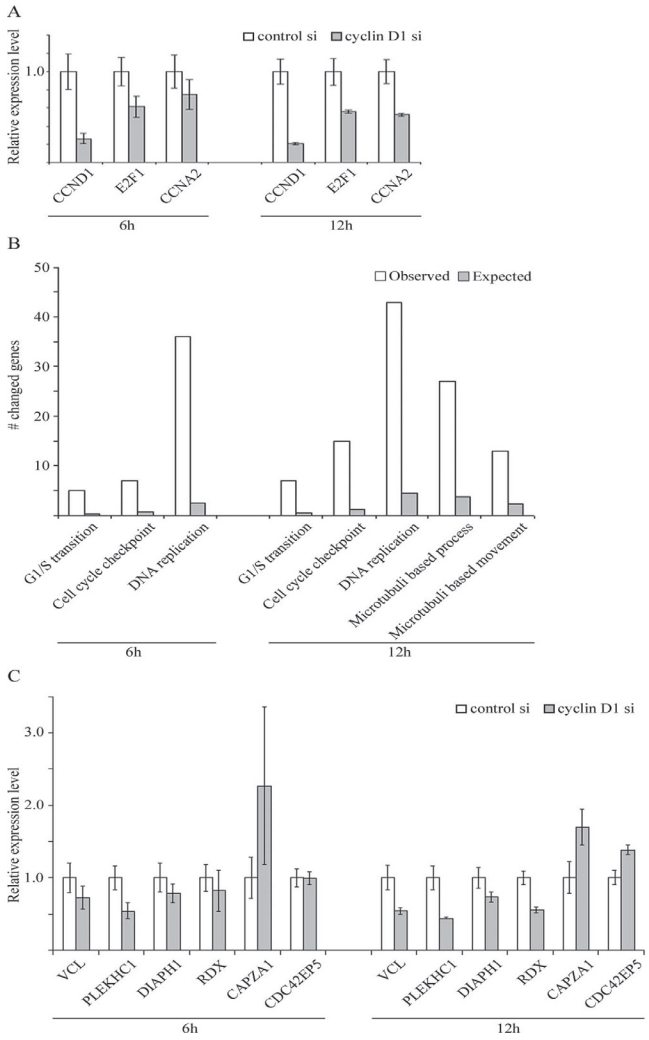
Supplementary Figure 1. *Cyclin D1 and cyclin E were knocked down using the original siRNA pools in starved MDA-MB-231 cells. Protein expression (**A**), cell cycle phase distributions (**B**) and migratory capacity (**C**) were measured after 4h and 20h of serum stimulation. The cells were allowed to migrate for 3h towards a serum gradient. The migration assays were performed at least three times in triplicates. Each spot represents the number of migrating cells from one chamber. The reference level (1.0) represents the normalised mean value of migrating unsynchronised cells.*



Supplementary Figure 2. *Cyclin D1 and cyclin E were knocked down separately or together, using the original siRNA pools in unsynchronised cells. The first panel shows protein expression (**A**), cell cycle phase distributions (**B**) and migratory capacity (**C**) of MDA-MB-231 cells, analysed 24h post siRNA treatment. The second panel shows cyclin D1 protein expression (**D**) and migratory capacity (**E**) of MDA-MB-435 cells 24h post siRNA treatment. The cells were allowed to migrate for 3h towards a serum gradient. The migration assays were performed at least three times in triplicates. Each spot represents the number of migrating cells from one chamber. The reference level (1.0) represents the normalised mean value of migrating unsynchronised cells.*



Supplementary Figure 3. *The MEK inhibitor U0126 was used to study the role of ERK1/2 activity in the elevated migratory capacity caused by the original cyclin D1 siRNA pool. 20 µM U0126 or solvent (DMSO) was added to the transfected cells 4h before protein harvest and migration assays. (**A**) ERK1/2 activity was analysed with a phospho-specific ERK1/2 antibody. (**B**) U0126 was also added to the medium in the migration chambers to make sure that ERK1/2 was inactivated when cells migrated. Migration assays were conducted as in Figure 1 and 2.*



Supplementary Figure 4. *In order to study early transcriptional effects induced by the original cyclin D1 siRNA pool, mRNA was harvested 6h and 12h after siRNA treatment. The gene expression profiles at these time points were analysed using the Illumina platform comparing mRNA levels from cyclin D1 silenced cells and control treated cells in triplicates. The analysis was conducted by AROS Applied Biotechnology AS, Århus, Denmark. (**A**) Successful down-regulation of cyclin D1 mRNA at both time points was validated in the gene expression analysis. E2F1 and CCNA2, two important cell cycle regulatory genes, was also significantly downregulated indicating an early effect on cell cycle activities. (**B**) Classification of the differentially expressed genes into biologically coherent categories, using the web-based software Gene Ontology Tree Machine, further stressed the impact on cell cycle regulatory functions. The changes are exemplified by the GO-categories 'G1/S transition of mitotic cell cycle', 'cell cycle checkpoint' and 'DNA replication' which all contained a significant number of down regulated genes. In addition, genes related to microtubuli based processes were significantly down regulated at the 12h time point, raising the possibility that the migratory response induced by the cyclin D1 siRNA pool could be mediated by changes in the microtubuli cytoskeleton. (**C**) From the list of differentially expressed genes, 6 were of specific interest due to their documented connection to migration.*

Conclusions

The general conclusion from the conducted experiments is that there exist a link between proliferative activity and migratory capacity. Although this relation is not generally applicable to all malignancies, it might reflect relevant tumour biological characteristics of certain tumours and may be of potential clinical significance.

The more specific conclusions are:

• Cyclin E overexpression in MDA-MB-468 breast cancer cells induces an elevated S-phase fraction and hampers cell motility and invasiveness.

• High levels of cyclin E protein correlate with a low-infiltrative, pushing tumour growth pattern in primary breast cancer.

• High levels of cyclin E protein is significantely more common among medullary breast carcinomas compared to other subtypes.

• Medullary breast carcinomas belong to a subgroup of tumours characterised by a pushing growth pattern, high cyclin E expression, histological grade 3, hormone receptor negativity, larger tumour size and fewer lymph node metastases. These tumours present a relatively favourable prognosis.

• High cyclin E protein levels correlate with a worse prognosis and confer independent prognostic information in the major subgroup of premenopausal patients with tumours exhibiting an infiltrative growth pattern.

• Elevated levels of cyclin E are not associated with a poorer tamoxifen response in premenopausal patients with ER positive disease.

• The migratory capacity of MDA-MB-231 breast cancer cells is influenced by their cell cycle position: 1) specific properties of resting G0/early G1 cells render them relatively more migratory; 2) S-phase activities impede migration.

• Silencing of cyclin D1 increases the migratory capacity of MDA-MB-231 and MDA-MB-435 cells, implying a dual function of cyclin D1 in regulating both cell cycle progression and migratory behaviour.

• High cyclin D1 protein levels correlate with a more favourable prognosis in premenopausal breast cancer patients with ER positive disease.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Bröstcancer är ett samlingsnamn för ett stort antal sjukdomar som uppvisar väldigt olika egenskaper. Hur allvarlig en viss typ av bröstcancer är bestäms av de specifika genetiska förändringar, mutationer, som tumörcellerna av olika anledningar förvärvar. Generellt för tumörceller är att de drabbats av mutationer som medför att de inte längre beter sig som normala celler. De börjar föröka sig i en alltför snabb takt och slutar svara på signaler som normalt sätt motverkar onödig celldelning. En lokal och begränsad tillväxt utgör oftast inte ett problem i sig. Det är först när tumörcellerna förändras ytterliggare och börjar växa in i omkringliggande vävnad och sprida sig till ställen i kroppen där de inte hör hemma, som det kan bli allvarligt. Denna process kallas metastasering. Nu är det lyckligtvis så att det finns väldigt många naturliga kontrollsystem, både inne i cellen och utanför, som bromsar uppkomsten av okontrollerbart förökande och metastaserande tumörceller. Att cancer huvudsakligen utvecklas först i livets senare skede beror på att dessa kontrollmekanismer, trots allt, fungerar ganska väl. När tumörceller väl har uppkommit avgör, som sagt, deras specifika förändringar hur sjukdomen kommer att utvecklas. Olika tumörers tillväxttakt och spridningssätt kan variera kraftigt och att förstå de genetiska och cellbiologiska orsakerna till dessa variationer, är en utmaning av astronomiska mått.

Min forskning har gått ut på att försöka förstå hur bröstcancerceller lyckas kombinera en hög celldelningstakt med förmågan att migrera, dvs aktivt förflytta sig. Att många aggressiva tumörceller på ett eller annat sätt lyckas med detta är klart. Trots detta så har tidigare observationer i bla tjocktarmscancer och vissa former av hudcancer, pekat på att i vissa lägen motverkar en alltför hög delningstakt tumörcellernas migrationsförmåga. Båda aktiviteter är ytterst komplicerade och är dessutom beroende av liknande intracellulära strukturer. Det är alltså inte otänkbart att en del tumörceller måste koordinera dessa processer och därför kan ha svårigheter att utföra dem samtidigt. Jag har med olika experimentella metoder försökt påvisa att bröstcancercellers delningsaktivitet faktiskt påverkar deras migrationsförmåga. Vidare så har jag analyserat om mina försöksresultat kan användas för att bättre förstå riktiga brösttumörers egenskaper och beteende.

I mitt första arbete stimulerade jag bröstcancerceller att gå in i delningsprocessen genom att höja cellernas uttryck av ett protein som heter cyklin E. Det finns flera varianter av dessa sk cykliner, och gemensamt för många av dem är att de ökar cellernas delningstakt. Cyklin E är ett intressant protein och många studier har visat att höga nivåer av cyklin E är kopplat till aggressivare brösttumörer med sämre sjukdomsprognos. Jag försökte studera om ökade cyklin E nivåer, och därmed ökad celldelningsaktivitet, förändrade cellernas migrationsegenskaper. Det visade sig att jämfört med kontrollbehandlade celler, så hade de cyklin E-överuttryckande cellerna en minskad förmåga att aktivt förflytta sig. Dessa resultat verkade alltså stödja idén om att någonting i celldelningsprocessen motverkar cellernas rörelseförmåga. Detta gjorde oss nyfikna på om det kunde finnas samvariationer mellan riktiga brösttumörers lokala spridningssätt och deras cyklin E uttryck. Vi spekulerade att om höga nivåer av cyklin E och en hög celldelningsaktivitet faktiskt motverkar tumörcellernas migrationsförmåga, då borde brösttumörer med ett högt cyklin E-uttryck uppvisa en mindre lokal spridning. Vi analyserade flera hundra brösttumörer

och försökte gradera varje tumörs ”infiltrativa” växtsätt. Intressant nog så fann vi ett tydligt samband mellan ett mindre infiltrativt växtsätt, dvs en mer lokalt begränsad tumörspridning, och ökade cyklin E nivåer. Dessa observationer pekade på att cyklin E var både kopplat till en ökad celldelningstakt och ett mer begränsat växtsätt i riktiga brösttumörer.

I det andra arbetet undersökte vi hur kopplingen mellan cyklin E-nivåerna och tumörväxtsättet påverkade brösttumörernas aggressivitet. Som jag nämnde ovan, så har flera studier visat att höga nivåer av cyklin E i tumörerna generellt sätt leder till en sämre prognos. I det tumörmaterial som vi analyserade så fanns det som sagt en klar koppling mellan höga cyklin E nivåer och ett mindre infiltrativt växtsätt. Det visade sig dock att det fanns en hel del cyklin E-höga tumörer som trots allt växte väldigt infiltrativt. Det viktiga med denna studie var att vi kunde visa på att inte alla patienter med cyklin E-höga tumörer hade en dålig prognos. Den grupp av patienter vars tumörer uttryckte mycket cyklin E och samtidigt växte mer begränsat, uppvisade en relativt sätt bättre prognos.

I mitt sista arbete så ville vi mer detaljerat studera hur tumörcellers delningsaktivitet relaterade till deras migrationsförmåga. För att kunna göra detta behandlade jag först tumörceller så att de gick in i ett sk vilande stadium, med väldigt låg delningsaktivitet. Dessa celler stimulerades därefter så att de samordnat började dela sig mer aktivt. Jag försökte sedan mäta om migrationsförmågan skiljde sig mellan de vilande cellerna och de aktivt delande cellerna. Genom detta försöksupplägg så kunde vi konstatera att de vilande, icke delande cellerna hade en högre migrationskapacitet jämfört med de aktivt delande cellerna. För att studera detta fenomen vidare så hämmade vi uttrycket av proteiner som är viktiga i celldelningsprocessen och analyserade om cellernas migrationskapacitet ändrades. Vi lyckades sakta ner celldelningstakten till viss del men vi kunde inte mäta någon större migrationsskillnad. Detta skulle kunna bero på att celldelningstakten inte bromsades upp tillräckligt mycket. Ett av de proteiner som vi hämmade var cyklin D1. Både cyklin D1 och cyklin E är viktiga för att reglera cellernas delning. När uttrycket av cyklin D1 trycktes ner så gjorde vi en mycket oväntad och intressant observation. Med lägre nivåer av cyklin D1 fick cellerna en högre förmåga att migrera. Det visade sig att cyklin D1 verkade kunna påverka cellernas migrationskapacitet på ett sätt som inte hade att göra med proteinets roll i celldelningsprocessen. Vi följde upp detta fynd genom att återigen studera om det var skillnader i riktiga brösttumörer beroende på deras cyklin D1 nivåer. Cyklin D1-höga tumörer hade generellt en fortare tillväxttakt men verkade samtidigt bli något mindre. En tolkning av denna något motsägelsefulla observation, var att trots att tumörcellerna delade sig fortare så gjorde de höga cyklin D1-nivåerna att de samtidigt spred sig långsammare. Sammantaget skulle detta kunna förklara varför dessa tumörer som helhet blev mindre. Vidare kunde vi se att patienter med cyklin D1-höga tumörer faktiskt hade en bättre sjukdomsutveckling jämfört med patienter med cyklin D1-låga tumörer.

I mitt arbete så har jag alltså påvisat att tumörcellers delningsaktivitet kan hämma deras migrationsförmåga. Jag har vidare sett att detta förhållande skulle kunna förklara vissa brösttumörers beteende. Mina resultat visar också på en ny intressant funktion hos cyklin D1. Pusselbitar som förhoppningsvis ökar kunskapen om tumörers skrämmande men fascinerande biologi.

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