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G1-PHASE CYCLIN EXPRESSION IN NEOPLASTIC B CELLS

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STOCKHOLM
2002

SUMMARY

By virtue of the role of G1-phase cyclin proteins as regulators of differentiation, proliferation, and apoptosis, their improper expression can lead to altered cellular homeostasis and tumor formation. Cyclins provide the regulatory partner for cyclin-cdk complexes whose enzymatic activity drives cell cycle progression. Overexpression of cyclins can lead to increased cyclin-cdk activity thereby uncoupling normal growth restraints and leading to uncontrolled cellular proliferation – one of the hallmarks of cancer cells. The aim of this thesis was to clarify the expression patterns of the G1 cyclins and define their relevance in neoplastic B cells.

The D-type cyclins (D1, D2, and D3) display variation in their pattern of expression between cell types, suggesting that they may play a role in cellular differentiation as well as proliferation. To further specify the relationship between cellular phenotype and D-cyclin expression, we studied the D-type cyclins in a variety of B cells, including B cells infected with Epstein-Barr virus (EBV). Cyclin D2 was the predominant D-cyclin expressed in lymphoblastoid cell lines (LCLs) and group III Burkitt's lymphoma lines (BLs) and was associated with an activated B-cell phenotype (CD10-, CD39+). In contrast, EBV(-) BL lines and group I and II BL lines expressed only cyclin D3. Although cyclin D2 expression is often associated with the presence of EBV gene products, our results fail to demonstrate this relationship in EBV(-) BLs infected with EBV, and illustrate that host cell phenotype also influences D-cyclin expression.

We explored the expression of the D-cyclins and cyclin E in human adult B-cell acute lymphoblastic leukemias (B-ALL), B-cell chronic lymphocytic leukemias (B-CLL), and immunocytoma (IC). Our studies revealed the overexpression of cyclin E protein in blast cells from 6/6 B-ALL patients at relapse compared to blasts from the same patients at diagnosis. This change in protein expression was both quantitative and qualitative as up to four additional smaller molecular weight species of the cyclin E protein were observed at relapse. In addition, we observed in B-ALL patients, that low cyclin E protein levels at diagnosis showed a positive association with improved clinical outcome. Our studies suggest that cyclin E protein levels reflect the malignant status of B-ALL blasts, and implicate the potential use of cyclin E as a prognostic marker in adult B-ALL. We also found cyclin E protein overexpression in B-CLL (4/12) and IC (4/4), however, no correlation with advanced disease or prognosis was observed. In contrast to B-ALL, these samples were cyclin D3 negative. However, upon stimulation with *Staphylococcus aureus* and interleukin-2, cyclin D3 expression was induced in B-CLL cells in a manner similar to that observed in normal B cells. Our results suggest that the D-cyclin pathway is maintained intact in B-CLL cells but that the expression of cyclin E, as observed in some B-CLL and IC samples, is pathological.

Cyclins have been shown to play a role in the induction of apoptosis in some cell types. We studied cyclins D3 and E in B-cell lines provoked to enter apoptosis by exposure to nitric oxide (NO). The pre-B-cell line NALM-6 was highly susceptible to NO-induced apoptosis and had a concomitant 8-fold increase in expression of the cyclin E protein. No change in protein levels of cyclin D3, cyclin A, or the anti-apoptotic protein bcl-2 were observed. Our results raise the question of an association between cyclin E expression and apoptotic susceptibility.

KEY WORDS: Cell cycle, G1 phase, cyclin D, cyclin E, EBV, Burkitt's, B-ALL, B-CLL, apoptosis
ISBN: 91-7349-292-2

*In loving memory of
Tommy Axtun
(1944-2002)*

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ABBREVIATIONS

B-ALL	Precursor B-cell acute lymphoblastic leukemia
B-CLL	B-cell chronic lymphocytic leukemia
BL	Burkitt's lymphoma
CD	Cluster of differentiation
CDC	Cell-division-cycle
cdk	Cyclin-dependent kinase
cki	Cyclin-dependent kinase inhibitor
CLL	Chronic lymphocytic leukemia
DNA	Deoxyribonucleic acid
EBV	Epstein-Barr virus
FACS	Flourescence activated cell sorting
G ₀	G zero; A state of quiescence lying outside of the cell cycle
G ₁	First gap phase of the cell cycle
IC	Immunocytoma
Ig	Immunoglobulin
IL	Interleukin
kDa	Kilo Dalton; molecular mass unit for proteins
LCL	Lymphoblastoid cell line
mAb	Monoclonal antibody
mRNA	Messenger ribonucleic acid
NO	Nitric oxide
PBL	Peripheral blood lymphocytes
PBMC	Peripheral blood mononuclear cells
PI	Propidium iodide
pRb	Retinoblastoma protein
PS	Phosphatidyl-serine
R	Restriction point
RT-PCR	Reverse transcription-polymerase chain reaction
S phase	DNA-synthesizing phase of the cell cycle
SAC	<i>Staphylococcus aureus</i> strain Cowan I
SDS-PAGE	Sodium dodecyl sulfide-polyacrylamide gel electrophoresis
SNAP	S-nitroso-N-acetyl-D,L-penicillamine

LIST OF PAPERS

- I. Pokrovskaja, K, Ehlin-Henriksson B, Bartkova J, Bartek J, **Scuderi R**, Szekely L, Wiman KG, Klein G. Phenotype-related differences in the expression of D-type cyclins in human B cell-derived lines. *Cell Growth and Differentiation* 7: 1723-1732, 1996.
- II. **Scuderi R**, Palucka KA, Pokrovskaja K, Björkholm M, Wiman KG, Pisa P. Cyclin E overexpression in relapsed adult acute lymphoblastic leukemias of B-cell lineage. *Blood* 87: 3360-3367, 1996.
- III. **Scuderi R**, Wiman KG, Celsing F, Pokrovskaja K. Aberrant cyclin E expression in chronic B-cell malignancies. *Submitted, 2002*.
- IV. Mozart M*, **Scuderi R***, Celsing F, Aguilar-Santelises M. Nitric oxide induces apoptosis in NALM-6, a leukemia cell line with low cyclin E protein levels. *Cell Proliferation* 34: 369-378, 2001. *co-first authors.

"...we must think unthinkable thoughts and ask unanswerable questions. Yet we must not lose ourselves in abstract vapors of philosophy... never underestimate how much assistance, how much satisfaction, how much comfort, how much soul and transcendence there might be in a well-made taco and a cold bottle of beer."

from Jitterbug Perfume by Tom Robbins

INTRODUCTION

Research on cell cycle regulation has gained considerable popularity within recent times and more than 20,000 articles have been published on this topic within the last ten years. The importance of research in this field was symbolized here at the Karolinska Institute last year as three investigators - Paul Nurse, Leland Hartwell and Timothy Hunt - were awarded the 2001 Nobel Prize in Physiology or Medicine for their "discoveries of key regulators of the cell cycle."

As we continue to learn more about the intricacies of the cell cycle and the biology of neoplastic cells, the hope is that this knowledge can be applied for the benefit of cancer patients. The study of cyclins as prognostic markers for the classification of high-risk patients is one area of interest. Another, is the development of more rational strategies for the treatment of cancer. Clinical trials are already underway using drugs that inhibit cyclin-dependent kinases, and the initial results are promising.

The aim of this thesis was to clarify the expression patterns of G1-phase cyclins and define their relevance in neoplastic B cells. In the following introductory section, an attempt was made to provide a background of the important concepts in this field of research.

1. THE CELL CYCLE AND CELL CYCLE-REGULATORY PROTEINS

The cell cycle model depicts the series of events that occur during cellular replication and division. This process is generally represented as four distinct phases: G1, S, G2 and M.

G₀ (*g zero, quiescence*). A "resting" state. Technically speaking, this phase lies outside of the cell cycle model. In G₀, cells are often smaller in size and have a lower biosynthetic rate. When recruited to enter the cell cycle by growth factors or removal of confluent restraints, the cells enter the cycle in G1.

G1 phase (*gap 1*). High biosynthetic activity is characteristic of this phase as the cells prepare for the DNA replication event. In early G1, cells are under the control of growth factors which are required for normal progression through G1 up until the restriction point (R) (Pardee 1974). After R, the cells become "committed" to complete the cell cycle even if growth factors are removed (Pardee 1974; Zetterberg et al. 1995).

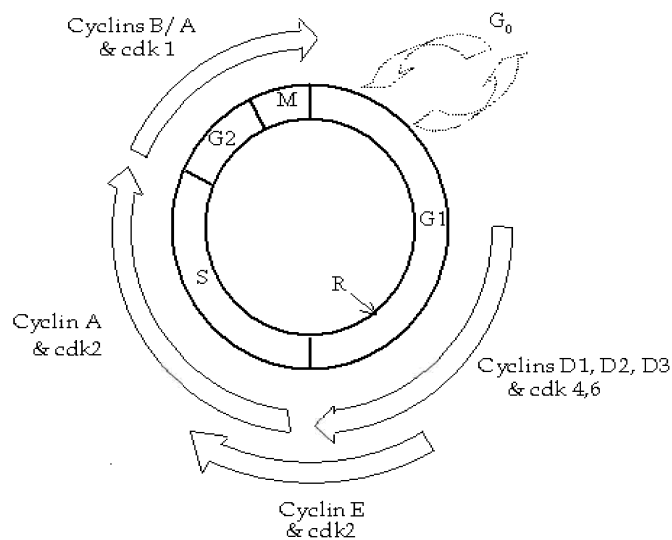


Figure 1. The cell cycle and the key regulators that drive progression through its four phases. Late in G1, at the restriction point "R", cells become committed to complete the cell cycle even in the absence of growth factors.

S Phase. S phase begins as DNA synthesis starts and continues up to the point where the DNA has been replicated and the cell consists of a duplicate set of chromosomes.

G2 phase (*gap 2*). The second gap phase occurring between the conclusion of S phase and the start of mitosis.

M phase (*mitosis*). During mitosis, the replicated chromosomes become condensed, the nuclear envelope breaks down, sister chromatids separate from each other, and the cell divides into two daughter cells ("cytokinesis").

The fundamental components of the cell cycle machinery are the cyclins, cyclin-dependent kinases (cdks), and cdk inhibitor proteins (ckis) (reviewed in Malumbres and Barbacid 2001; Olashaw and Pledger 2002). Normally, expression and destruction of cyclin proteins occurs in a specific, temporal manner with certain cyclins acting only during particular phases of the cell cycle (Fig. 1).

In simple terms, cyclins provide positive growth regulatory signals by combining with and aiding in the activation of cdks, whereas cdk inhibitors provide a negative growth regulatory signal by complexing with and diminishing the activity of cdks. The transitions between cell cycle phases, or "checkpoints," are mediated through cyclin-cdk protein interactions and serve to ensure that all steps of the previous phase are completed prior to continuing to the next phase (Hartwell and Weinert 1989). Deregulation of these "checkpoints" is a common characteristic of cancer cells (Hunter and Pines 1994).

Cyclins

Cyclins were first described as proteins whose levels fluctuate during the cell cycle in marine invertebrates (Rosenthal et al. 1980; Evans et al. 1983; Standart et al. 1987). Further studies revealed that cyclin proteins are evolutionarily conserved among different species (Koff et al. 1991; Leopold and O'Farrell 1991; Motokura et al. 1991). In fact, several of the human cyclin genes were discovered by their ability to rescue yeast cell mutants from cell cycle arrest (Koff et al. 1991; Lew et al. 1991; Xiong et al. 1991). The cyclin proteins are short-lived and their cellular concentration is determined by mRNA transcription and subsequent degradation of the translated protein by ubiquitin-dependent proteolysis (Yew 2001).

The D-cyclins. In mammalian cells, the D-cyclins (D1, D2, D3) are the first cyclins to appear in the G1-phase of the cell cycle and are expressed in response to extracellular stimuli (Matsushime et al. 1991; Ajchenbaum et al. 1993; Solvason et al. 1996; Tanguay and Chiles 1996). D-cyclins are the regulatory subunits of holoenzyme complexes formed by partnering with cyclin-dependent kinases 4 or 6 (cdk 4/6). Active cyclin D-cdk 4/6 complexes mediate progression through the early- to mid-G1 phase of the cell cycle and are thought to act as a link between mitogenic cues and the cell cycle machinery (reviewed in Hunter

and Pines 1994; Sherr 1994). The effects of D-cyclin proteins on the cell cycle are illustrated by experiments whereby overexpression of cyclins D1, D2, or D3 shorten the G1 phase (Ando et al. 1993; Kato and Sherr 1993; Quelle et al. 1993), as well as in studies where antibodies against the D-cyclins prevent entry into S phase (Baldin et al. 1993; Quelle et al. 1993; Bartkova et al. 1994; Lukas et al. 1994; Lukas et al. 1995).

The three human D-type cyclin genes encode small (33-34 kDa) proteins that share an average of 57% identity over the entire coding region and 78% in the cyclin box (Xiong et al. 1992) - a region of approximately 100 amino acids which is responsible for cdk binding and activation (Kobayashi et al. 1992; Lees and Harlow 1993) (Fig. 2).

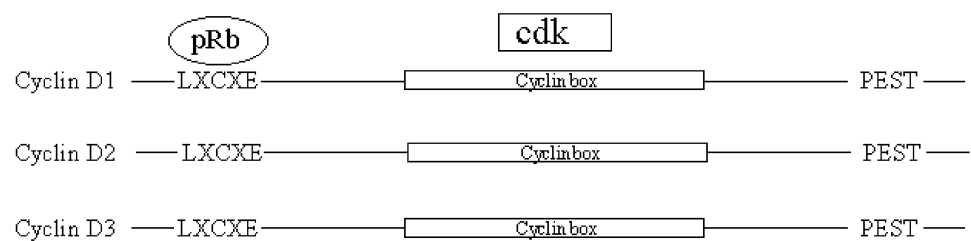


Figure 2. The three D-cyclins share substantial homology in three key regions: the LXCXE-motif which represents the retinoblastoma protein (pRb) binding domain, the cyclin box which forms protein-protein interactions with the cyclin-dependent kinase (cdk) partner, and the PEST domain which is targeted for ubiquitination and degradation by the proteasome complex (adapted from Bates and Peters 1995).

There are two principal mechanisms by which cyclin D-cdk complexes promote G1 progression. First, the enzymatic activity of cyclin D-cdk complexes functions to initiate phosphorylation of the growth repressive retinoblastoma protein (pRb), thus aiding in its inactivation. Second, the D-cyclins and their cdk partners promote proliferation by sequestering cyclin-dependent kinase inhibitors (cki) p27 and p21, thereby relieving cyclin E-cdk2 complexes of this cki inhibition. Interestingly, while complexes containing cyclins D1/D2-cdk4/6-p27/p21 are shown to be enzymatically active (Blain et al. 1997; LaBaer et al. 1997; Mahony et al. 1998; McConnell et al. 1999; Parry et al. 1999), cyclin D3-cdk4 complexes were shown to be mutually exclusive in either sequestering p27 or having catalytic activity (Olashaw and Pledger 2002).

Several lines of investigation have led to the idea that the D-cyclins have non-redundant roles in cellular differentiation and proliferation. For example, inhibition of granulocyte differentiation occurs following forced expression of cyclins D2 and D3 but not D1 (Kato and Sherr 1993). In addition, the temporal expression of the D-cyclins is such that cyclin D2 is expressed several hours (12h.) prior to cyclin D3 in stimulated normal T lymphocytes (Ajchenbaum et al. 1993) suggesting that they perform different roles in early- and late G1, respectively.

Genetically engineered mice lacking either the cyclin D1 or cyclin D2 gene ("knock-outs") have revealed that although these cyclins are not essential for survival, certain developmental defects arise from the respective D-cyclin deficiency. Cyclin D2 knock-out mice develop hypoplastic testes in males and defective ovaries in females (Sicinski et al. 1996). Cyclin D1 knock-out mice are smaller in size than their littermates, have neurological symptoms, hypoplastic retinas, and the adult females exhibit impaired mammary development during pregnancy (Sicinski et al. 1995). In line with this observed relationship between tissue specificity and D-cyclin function, cyclin D2 overexpression is observed in testicular and ovarian cancers (Sarfati et al. 1988; Sicinski et al. 1996; Bartkova et al. 1999) and cyclin D1 plays a role in the development of breast cancer (Wang et al. 1994; Yu et al. 2001).

Whereas, the mechanisms that govern expression of the D-cyclins remain largely unknown, some insight into the promoter regions and the activation of D-cyclins has been revealed. The cyclin D1 and cyclin D2 promoters have been shown to contain considerable homology, whereas the cyclin D3 promoter differs by lacking a serum-inducible box and negative regulatory sequences (Herber et al. 1994; Brooks et al. 1996). In line with these findings, cyclin D3 is the most ubiquitously expressed of the D-cyclins and is found in almost all tissues. In addition to promoter specificity, inhibition of D-cyclin expression may result from promoter methylation as seen for cyclin D2 in Burkitt's lines (Sinclair et al. 1995). The recent findings that a mouse strain deficient in cyclin D1 but with a "knocked-in" cyclin E gene in its place sheds light on the importance of transcriptional control of cyclins by their promoter region. In these knock-in experiments it was discovered that all of the developmental defects described in cyclin D1 knock-out mice (Sicinski et al. 1995) were restored by the knocked-in

cyclin E (Geng et al. 1999). These results illustrate that the timing of cyclin D1 expression - governed by its promoter - is more important than the protein itself, since its role in differentiation can be entirely replaced by cyclin E. Studies on the expression of cyclin D1 have shown that activation of the ras pathway is a required upstream event. Activated ras induces the expression of cyclin D1 (Filmus et al. 1994; Liu et al. 1995), while dominant-negative ras mutants block cyclin D1 expression even in the presence of mitogens which normally induce it (Peeper et al. 1997).

In normal human B cell proliferation, cyclin D3 appears to be the dominant D-cyclin expressed (Blanchard et al. 1997; Wagner et al. 1998). A role for cyclin D2 in normal B cell proliferation and differentiation *in vivo* is questionable, since no cyclin D2 positive B cells were found inside or around germinal centers of secondary follicles by immunocytochemistry on tissue sections (Teramoto et al. 1999). Interestingly, cyclin D2 expression predominates in several types of virally-infected B cells including lymphoblastoid cell lines (LCLs), and group III EBV-positive Burkitt's lymphoma (BL) lines with an LCL-like phenotype (Palmero et al. 1993).

The E-cyclins. Later in G1, near the S-phase boundary, cyclin E protein levels reach a peak, followed by a decrease as cells proceed through S phase (Lew et al. 1991; Koff et al. 1992; Ajchenbaum et al. 1993; Ohtsubo et al. 1995). The cyclin E gene was discovered by the ability of its gene product to restore the entry of budding yeast (*Saccharomyces cerevisiae*) strains devoid of G1 cyclins into the S phase of the cell cycle (Koff et al. 1991; Lew et al. 1991). Activated kinase complexes consisting of cyclin E and cdk2 (Dulic et al. 1992; Koff et al. 1992) are also capable of phosphorylating pRb (Hinds et al. 1992), and appear to be important in progression through G1. Acceleration of the G1- to S-phase transition by overexpression of cyclin E was demonstrated *in vitro* in mammalian fibroblasts and HeLa cells (Ohtsubo and Roberts 1993; Resnitzky et al. 1994; Wimmel et al. 1994), while blocking of cdk2 by antibodies inhibited the entry of mammalian cells into S phase (Pagano et al. 1993; Tsai et al. 1993). Normally, this decrease in cdk2 activity at the G1/S transition is controlled by the ubiquitin-dependent proteolysis of cyclin E (Koepp et al. 2001).

Several isoforms of the cyclin E protein have been described. Sewing and co-workers were the first to describe a shorter variant of cyclin E, termed cyclin Es, which lacks 49 amino acids within the cyclin box (Sewing et al. 1994). Mumberg et al. isolated an additional splice variant lacking 45 amino acids which they termed cyclin ET (third isoform of cyclin E) (Mumberg et al. 1997). Both of these isoforms have a reported molecular weight of 43 kDa as compared to the 50 kDa full length cyclin E protein, and neither one of them is capable of activating cdk2. In contrast, lower molecular weight protein species of cyclin E found in breast cancer samples were shown to possess functional activity at levels even higher than full-length cyclin E when tested in *in vitro* kinase assays and transfection experiments (Porter et al. 2001). Another variant of cyclin E - cyclin E-L - has an additional 15 amino acids, a molecular weight of 52 kDa, and functions similar to cyclin E (Ohtsubo et al. 1995). Whereas, the preceding isoforms of cyclin E represent splice variants from the same gene, a novel G1-phase cyclin with considerable homology to cyclin E was cloned and named cyclin E2; the expression of cyclin E2, like cyclin E, peaks at the G1- to S-phase transition and forms active complexes with cdk2 (Zariwala et al. 1998).

Cyclin A. The cyclin A protein appears later in the cell cycle, closer to the G1/S-phase transition (Sherr 1994). Cyclin A associates with cdk2 and cdk1 and the enzymatic activity of these complexes is required for progression through S phase and into mitosis (Girard et al. 1991; Dulic et al. 1992; Pagano et al. 1992; Heichman and Roberts 1994).

Cyclin B. The Cyclin B protein appears next, during S-phase progression, and the formation of cyclin B/CDC2 (cell-division-cycle 2; also known as cdk1) complexes assists in regulating mitosis. The subsequent degradation of cyclin B allows cells to exit from mitosis (reviewed in King et al. 1994).

Cyclin-dependent kinases

The cyclin-dependent kinases (cdks) are serine-threonine kinases which form holoenzyme complexes with cyclin proteins. The cellular levels of cdks remain in excess throughout the cell cycle, but their activity is post-translationally controlled on several levels. In addition to cyclin binding, cdk activation requires the phosphorylation of a threonine amino acid residue by a cdk-activating kinase

(CAK) composed of cdk7 and cyclin H, and dephosphorylation of a threonine and tyrosine residue by the cdc25 phosphatase (Fig. 3) (Morgan 1995).

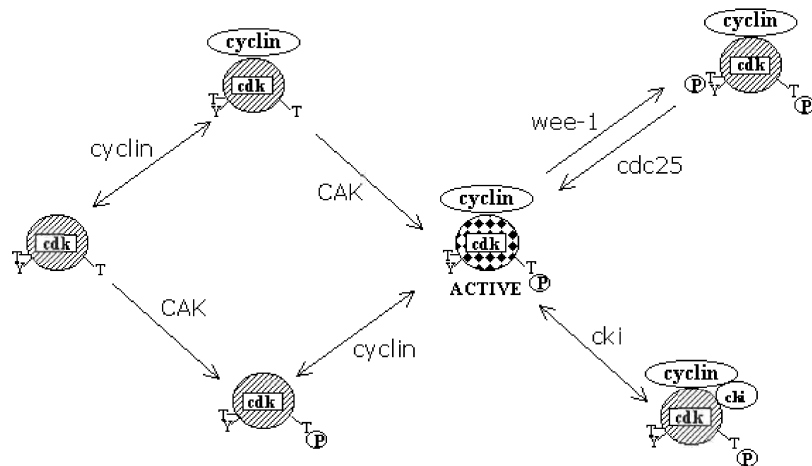


Figure 3. In addition to cyclin binding, cdk activation requires phosphorylation of a threonine (T) residue at position 160 by the CAK kinase, and dephosphorylation of threonine 14 and tyrosine (Y) 15 by cdc25. Inactivation of cdk activity is mediated by cyclin-dependent kinase inhibitor (cki) binding, and phosphorylation of inhibitory amino acid residues threonine and tyrosine by the wee1 kinase (adapted from Morgan 1995).

Once activated, cdks phosphorylate and thereby regulate the function of target proteins involved in cell cycle progression. Examples of such substrates are B-myb, a transcription factor required for S-phase entry, which is phosphorylated by cyclin A-cdk2 complexes (Lane et al. 1997), and pRb, which is discussed below.

Cdk1 (CDC2) is the mitotic cdk that can combine with either cyclin B or cyclin A to initiate mitosis, whereas cdk2 forms complexes with cyclins E and A to promote the entry and completion of S phase (Fig. 1). The role of cdk3 is still unclear. It is expressed at low levels and shows no enzymatic activity (Ye et al. 2001). Cdk5 is only found to be active in brain tissue (Lew et al. 1994; Tsai et al. 1994), and may be involved in building synapses and transmitting nerve signals (Smith and Tsai 2002).

Cdk4 and cdk6 pair with the D-cyclins and their only known substrates are pRb, p107 and p130. cdk4 appears to play an important role in melanocyte proliferation. Mutations in the cdk4 gene which cause over-function of the enzyme have been detected in three separate melanoma-prone families (Zuo et al. 1996; Soufir et al. 1998). Similarly, mice expressing a knocked-in mutant cdk4 develop melanomas at a much higher frequency than their littermates (Sotillo et al. 2001).

Cyclin-dependent kinase inhibitors

The members of the cyclin-dependent kinase inhibitors (ckis) belong to two families, the CIP/KIP family (p21^{CIP1/WAF1}, p27^{KIP1} and p57^{KIP2}), and the INK4 family (p15^{INK4b}, p16^{INK4a}, p18^{INK4c} and p19^{INK4d}). Members of the CIP/KIP family bind complexes containing cyclin D, E, and A and their cdk partners, whereas, the INK family members bind exclusively to cdk4 or cdk6.

p21^{CIP1/WAF1} was the first mammalian cki to be identified (el-Deiry et al. 1993; Gu et al. 1993; Harper et al. 1993; Xiong et al. 1993). The p21 promoter has a p53 binding site and p53-mediated arrest of the cell cycle in response to DNA damage is achieved by transactivation of p21 and its subsequent inhibition of cyclin-cdk complexes (el-Deiry et al. 1993). p21 functions not only as a cyclin-cdk inhibitor but also inhibits the activity of the proliferating cell nuclear antigen (PCNA), a protein involved in DNA-replication (Waga et al. 1994). Interestingly, while high concentrations of p21 exert an inhibitory effect, at lower concentrations the binding of p21 has been shown to enhance cdk4/6 activity (LaBaer et al. 1997).

p27^{KIP1} was first described as a protein that accumulates following TGF- β treatment or contact-inhibition of cultured cells (Polyak et al. 1994; Slingerland et al. 1994). p27 is considered important in inducing and maintaining the quiescent state of cells (reviewed in Yew 2001; Olashaw and Pledger 2002). Addition of mitogens to quiescent cell cultures down-regulates p27 (Firpo et al. 1994; Winston et al. 1996). p27 knockout mice show an increased frequency of tumor formation and are one third larger than their littermates (Fero et al. 1996; Kiyokawa et al. 1996; Nakayama et al. 1996).

One member of the INK4 family, p16, is altered in many transformed cell lines and some primary cancers (Kamb et al. 1994) and supports the notion that ckis may function as tumor suppressor genes. The p16 gene is commonly altered in T-lineage ALLs (Hebert et al. 1994; Cayuela et al. 1996).

The retinoblastoma protein family and E2F/DP transcription factors

The retinoblastoma protein (pRb) and the E2F/DP transcription factors belong to larger families of cell cycle-regulatory proteins which regulate G1 phase progression. pRb, and the related "pocket proteins" p107 and p130, function to repress cellular progression in G1 by binding to and modulating the transcriptional activity of the E2F/DP family members (E2F1-6, DP1 and DP2) through both transcriptional repression and activation mechanisms (Dyson 1998; Trimarchi and Lees 2002).

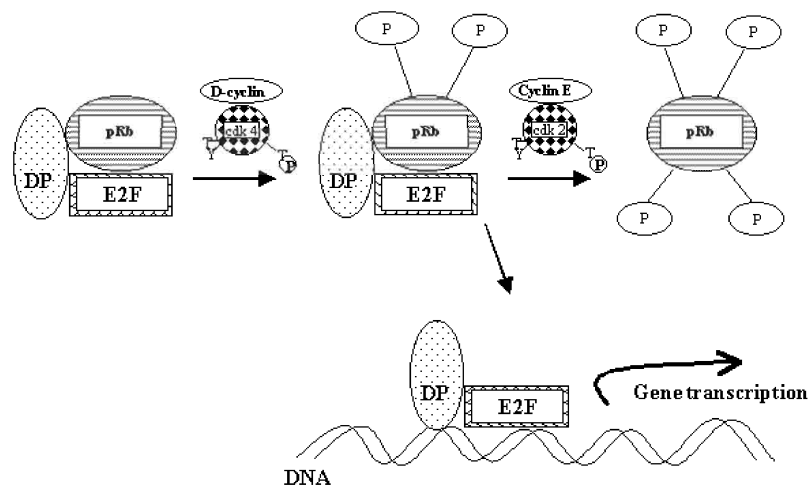


Figure 4. Model of transcriptional activation following hyperphosphorylation of pRb. Sequential phosphorylation of the retinoblastoma protein by D-cyclin-cdk4/6 complexes and cyclin E-cdk2 complexes results in liberation of the DP/E2F transcription factors which turn on gene transcription of S-phase initiators.

pRb is underphosphorylated in the early stages of G1 and is sequentially phosphorylated first by D-cyclin-cdk4/6 complexes in early G1 phase followed by cyclin E-cdk2 in late G1. In the underphosphorylated form, pRb-E2F complexes can bind DNA and actively repress certain promoters. Phosphorylation of pRb by cyclin-cdk complexes results in the liberation of E2F. The E2F proteins, by forming heterodimers with members of the DP family (LaThangue 1994), can

bind DNA and regulate gene transcription of important cell cycle-regulatory genes (e.g. cyclin A, cyclin E, cdc25, thymidine kinase, and dihydrofolate reductase) (Fig. 4) (Nevins 1992; DeGregori et al. 1995; Muller 1995). It has been proposed that phosphorylation of pRb family members may be the key event governing the restriction point (R) (Weinberg 1995; Zetterberg et al. 1995), and studies have shown that ablation of these family members can effectively remove the R constraint (Sage et al. 2000).

Cyclin expression and cancer

Of great interest is the link between the irregular expression of cyclins and a variety of malignancies including some of hematopoietic origin.

Amplification of the cyclin D1 gene locus (11q13) is observed in several human cancers including squamous cell carcinomas of the head and neck, esophageal carcinomas, bladder cancer, breast cancer, small cell lung tumors and hepatocellular carcinomas (Hall and Peters 1996). In addition, inversion of chromosome 11 [inv(11)(p15;q13)] has been reported in parathyroid adenomas (Motokura et al. 1991). Both of these types of genetic lesions give rise to increased cyclin D1 protein levels.

Cyclin D1 is identical to the bcl-1 oncogene (Rosenberg et al. 1991) originally identified as the gene placed under control of the immunoglobulin heavy chain in t(11;14) lymphomas (Tsujimoto et al. 1984). While normally absent in B cells, cyclin D1 is illegitimately activated in mantle cell lymphomas carrying the t(11;14) translocation (Bosch et al. 1994) and in other B-cell neoplasms with chromosome 11 derangements such as atypical B-CLL, hairy cell leukemia, and some multiple myeloma (Sola et al. 2000).

The mechanisms linking the development of breast cancer with irregular cyclin D1 expression continue to be uncovered. Wang and co-workers showed that forced cyclin-D1 overexpression induces mammary adenocarcinomas in mice (Wang et al. 1994). More recent data revealed that mice lacking cyclin D1 are resistant to the formation of breast cancers normally induced by ras and neu oncogenes (Yu et al. 2001). These findings clearly illustrate the pivotal role of cyclin D1 in breast tumor development and suggest that therapeutic targeting against cyclin D1 may prove useful at least in some forms of breast cancer.

Cyclin D2, cloned and localized to the short arm of chromosome 12 (12p13) (Inaba et al. 1992), co-operates with ras to transform rat embryo fibroblasts (Kerkhoff and Ziff 1995), and was discovered to be amplified in one case of Non-Hodgkin's lymphoma and one case of Burkitt's lymphoma (Hoglund et al. 1996). Expression of cyclin D3 (6p21 (Inaba et al. 1992)) appears to be less frequently implicated in tumorigenesis (Bartkova et al. 1996), although a recent article shed light on the potential role of deregulated cyclin D3 expression in B-cell malignancies harboring the rare t(6;14) translocation (Sonoki et al. 2001).

The cyclin E gene is amplified in several human cancers including breast, gastric, colorectal and ovarian (Akama et al. 1995; Kitahara et al. 1995; Courjal et al. 1996; Marone et al. 1998). In other cases, abnormally high levels of the protein are detected despite an intact cyclin E gene, suggesting overactive transcription or some form of post-translational defect. Overexpression of cyclin E is associated with advanced stage of disease and poor prognosis in breast cancer (Keyomarsi et al. 1994; Nielsen et al. 1996; Porter et al. 1997). In addition to its potential as a driving force in neoplastic cell proliferation, overexpression of cyclin E may also play a role in tumorigenesis by leading to chromosome instability (Spruck et al. 1999).

2. NORMAL AND NEOPLASTIC B CELLS

Normal B cell development

The pluripotent stem cell of the bone marrow gives rise to the numerous blood cells of lymphoid and myeloid lineage (Fig. 5).

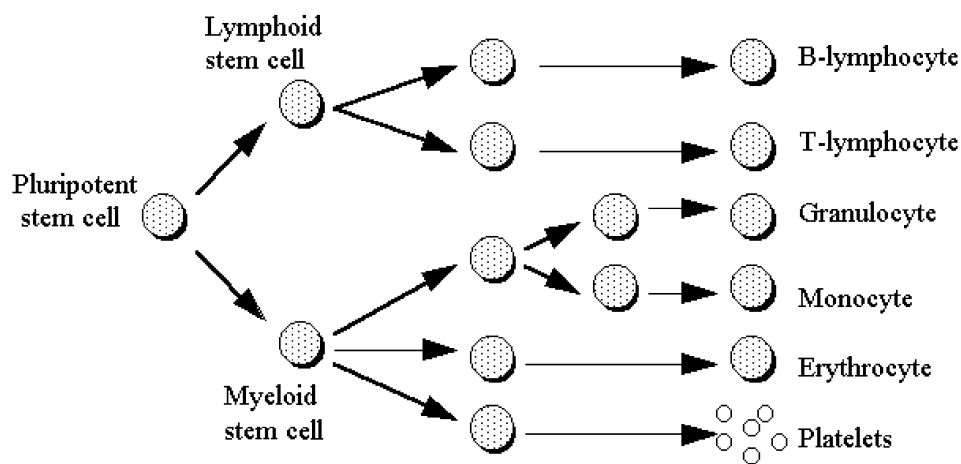


Figure 5. Model of hematopoiesis.

Human B cell differentiation occurs in several orderly steps first in the bone marrow and later in secondary lymphoid organs and includes DNA recombination events for the generation of immunoglobulin (Ig) heavy and light chains (Tonegawa 1983). The progression of B lymphocytes through the various stages of differentiation has been characterized with respect to both cytoplasmic and cell surface antigens (clusters of differentiation, CD) which are detected using monoclonal antibodies (mAbs). In addition, immunoglobulin gene rearrangements for the generation of antibodies and the pre B-cell receptor (BCR) occur at precise stages of development and are used in mapping B-cell development (Fig. 6). Both positive and negative selection of B cells occurs in the bone marrow and through signalling pathways involving the pre-BCR (made up of μ HC, ψ LC, and $Ig\alpha/Ig\beta$) (Craxton et al. 1999; LeBien 2000; Muschen et al. 2002). The remaining B cells migrate to secondary lymphoid organs where they are exposed to antigen and undergo somatic hypermutation in order to produce high affinity antibodies.

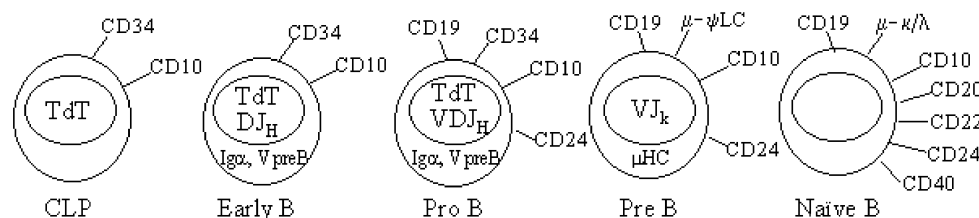


Figure 6. Stages in normal human B-cell development in the bone marrow. CLP represents a common lymphoid progenitor which may become a T cell or a B cell. CD antigens are frequently used to define individual stages. Representative intracellular protein expression, immunoglobulin gene rearrangements (DJ_H , VDJ_H , VJ_k), and components of the pre-BCR ($Ig\alpha$, $VpreB$, μHC , ψLC) are shown. TdT:terminal deoxynucleotidyl transferase (adapted from LeBien 2000).

Epstein-Barr Virus and B cells

The Epstein-Barr virus (EBV) is a double-stranded DNA virus of the Herpesviridae family. EBV infects B cells through the CD21 receptor (also known as C3d or CR2) where it can remain latent throughout an individual's lifetime. 90% of the human population are infected at some point in their life. Infection occurring in early childhood is usually asymptomatic, whereas infection later in life can lead to a benign and self-limited disease with fever, malaise, sore throat, and lymphadenopathy known as infectious mononucleosis. The endemic African form of Burkitt's lymphoma (BL) is associated with EBV infection, and it is thought that the virus triggers B-cell proliferation and is important both in the development and maintenance of the malignant phenotype (Shimizu et al. 1994; Okano 1997).

Table 1. Epstein-Barr virus latencies and EBV-associated diseases (in Okano and Gross 2001).

Latency	EBV gene expression	Association with human disease
I	EBNA-1, EBERs	Burkitt's lymphoma, Gastric carcinoma
II	EBNA-1, EBERs, LMP-1,2A,2B	Nasopharyngeal carcinoma, Hodgkin's lymphoma
III	EBNA-1-6, EBERs, LMP-1,2A,2B	Lymphoproliferative disorders, Infectious mononucleosis
IV	LMP-2A, EBERs	Seropositive healthy individuals

Infection of B cells *in vitro* with EBV leads to the transformation of cells into a lymphoblastoid cell line (LCL). LCLs are immortalized by the EBV infection and express all nine EBV encoded growth transformation-associated proteins (EBV-determined nuclear antigen (EBNA)-1-6, latent membrane protein (LMP)-1, LMP-2A, LMP-2B) and the EBV-encoded RNAs (EBERs), a phenotype referred to as latency III (see Table 1). EBNA-1 is required for the replication and maintenance

of the EBV genome as a circular extrachromosomal episome, whereas, EBNA-2, EBNA-3, EBNA-5, EBNA-6, and LMP-1 have a role in the immortalization of B cells (Farrell 1995; Okano 1998).

Precursor B-cell acute lymphoblastic leukemia (B-ALL)

B-ALL is a heterogeneous disease with patients responding differently to chemotherapy and leukemic blasts expressing a range of phenotypes. The characteristic feature of ALL is an abnormal accumulation of immature lymphocytes in the bone marrow, peripheral blood, lymphatic system, and often can include several other tissues. It is generally believed that leukemic blasts become "frozen" at a particular stage of differentiation - unable to develop normally but retaining their ability to proliferate (Greaves 1986). The incidence of ALL in adults ranges between 0.7 and 1.8/100 000 (Hoelzer and Gokbuget 2000). ALL is more common in children than in adults, and is the most frequent neoplastic disease in children with a peak age of 3-4 years. The majority of adult ALL cases (approximately 75%) are of B-cell lineage rather than T-cell lineage. Congenital chromosomal abnormalities such as Down's Syndrome and Klinefelter's Syndrome as well as environmental factors such as ionizing radiation and exposure to benzene may be associated with a higher incidence of ALL (Botti and Verma 1990). Patients present with bone marrow insufficiency including anemia, thrombocytopenia, and neutropenia. The occurrence of fatigue, infection and bleeding is common at the time of presentation. Without treatment, normal blood precursor cells yield to the expanding leukemic cells and death by hemorrhage or infection will occur within a few months.

The subcategories of ALL which comprise this heterogeneous disease group are defined by means of morphologic, immunologic, and cytogenetic characterizations (for a comprehensive European-American classification of leukemias and lymphomas see Harris et al. 1994). Morphologically, ALL cells are categorized into three groups L1, L2, and L3. L1 cells are small and homogenous. L2 cells are larger in size and more heterogeneous in nature. The L3 group, or "Burkitt-type", consists of cells containing vacuoles in the cytoplasm and belongs to a poor prognosis group as compared to L1 and L2. The level of differentiation (e.g. common-B, pre-pre-B) and the cell lineage, B cell or T cell, can be determined by immunologic criteria. Because the differentiation markers (clusters of differentiation, CD) present in normal B and

T cell ontogeny are often retained in the leukemic cells, the malignant cells can be "immunophenotyped" based on their particular antigen expression as determined by mAbs (Fig. 6). The distinctions of therapeutic importance, however, are those between T-cell, mature B and B-cell precursor immunophenotypes (Pui and Evans 1998).

Factors generally indicative of a worse prognosis among ALL patients commonly include high white blood cell (WBC) counts, older age (> 25-60 years), a late complete remission (> 3-5 weeks), male sex, and karyotypic abnormalities (e.g. the t(9;22) translocation) (Clarkson et al. 1986; Kantarjian 1994).

It is important to note that childhood and adult ALL are different disease entities. From the clinical perspective, childhood ALL is considered chemocurable, whereas in adults treatment remains a much greater challenge (Proctor 1994). Cure rates in children are currently reaching 80%, whereas, despite the high rate of initial remission among adult ALL patients (70-80%) (Clarkson et al. 1986; Kantarjian et al. 1992; Rivera et al. 1993; Proctor 1994), only 15% to 40% obtain a long-term disease-free survival, with the remainder relapsing in their disease (Pui and Evans 1998). Although some reports of evolutionary changes in ALL blasts from the time of diagnosis to the time of relapse have been described, most often the cytogenetic and phenotypic characteristics remain unchanged during the progression of the disease (Raghavachar et al. 1987; Chucrallah et al. 1994).

Studies on the kinetics of blast cells suggest the presence of two pools of leukemic cells, one in the bone marrow and another in the peripheral blood. The leukemic cells in the peripheral blood generally have a lower proliferation rate compared to blasts in the bone marrow; proliferative fractions of 2.9% and 5.7%, respectively, are found following bromodeoxyuridine (BrdU) staining (Cooper 1993). One explanation for this difference could be that the bone marrow provides a more growth permissive environment due to the contribution of soluble growth factors by stromal cells.

B-cell chronic lymphocytic leukemia (B-CLL)

B-CLL is the most common adult leukemia in the Western world. This disease is characterized by the accumulation of small, mature-appearing lymphocytes in

the peripheral blood, bone marrow, and secondary lymphoid organs. The initial clinical findings in B-CLL may be limited to lymphocytosis or may include peripheral lymphadenopathy and/or hepatosplenomegaly. The incidence of CLL in the United States is 2.7/100,000, and the incidence in men is 2.8 times higher than in women (Diehl et al. 1999). The median age at diagnosis is 64 years, and B-CLL is one of the only adult leukemias that is not associated with exposure to chemicals or to ionizing radiation (Caligaris-Cappio 2000). In some patients with B-CLL, the clinical course can be indolent and require no therapy at all with survival not uncommonly reaching decades. Other patients may have a more aggressive form of the disease and require conventional chemotherapy.

The most distinctive marker of B-CLL is surface expression of the CD5 molecule. B-CLL cells also express CD23 and faint surface immunoglobulin, usually of IgM or IgD class (Fournier et al. 1994). The origin of the B-CLL lymphocyte is still controversial, but the normal counterpart cell most similar to the B-CLL lymphocyte is probably the B-1 cell, which, like B-CLL cells, are CD5+, have low CD20, and form rosettes with mouse erythrocytes (Caligaris-Cappio 2000, and references therein).

Most CLL cells in the peripheral blood are resting. Classic kinetic studies reported that more than 99% of peripheral B-CLL lymphocytes are in the G₀ phase of the cell cycle (Zimmerman et al. 1968; Andreeff et al. 1980), and more recent studies using proliferating cell nuclear antigen (PCNA) and bromodeoxyuridine (BrdU) have confirmed these low proliferation rates for B-CLL (Lin et al. 2002).

In line with the observed low fraction of proliferating B-CLL cells, their accumulation is thought to be due to defects in the apoptotic pathway rather than to a proliferative advantage. Alterations in the expression of bcl-2 family members and receptor-mediated signals such as those acting through the Fas/Fas ligand pathway may play a role in inhibiting apoptosis in B-CLL (reviewed in Osorio and Aguilar-Santelises 1998).

Prognostic markers in B-CLL include clinical staging (Rai and Binet, based on clinical features such as B-symptoms, lymphadenopathy, anemia, and thrombocytopenia), white blood cell count, bone marrow infiltration, presence of

atypical lymphoid cells, lymphocyte doubling time, lactate dehydrogenase, thymidine kinase, sb2-microglobulin, sCD23, CD38, cytogenetics, and IgVH status (Montserrat 2002).

3. APOPTOSIS

Apoptosis or “programmed cell death” is a mechanism whereby cells actively participate in their own demise, the purpose of which being the maintenance of tissue homeostasis within an organism. Apoptotic death plays an important role in the development of the body. For example, the most illustrative example of apoptosis is the death occurring in the cells between the fingers of the human fetus that die by apoptosis and change the webbed appearance of the early hand into a hand with distinct fingers. In addition, apoptosis is involved in shaping the immune system by, for example, removing unwanted B cell populations (Green and Scott 1994; Strasser 1995; Van Parijs and Abbas 1998). Defective apoptosis can therefore lead to an undesired accumulation of B cells as seen in B cell neoplasms and autoimmune diseases. Signals that trigger apoptosis are numerous and include hormones, cytokines, antigen-receptor interactions, cell-cell interactions, and a variety of chemical, physical and viral agents (for review see Hengartner 2000). Knowledge about apoptotic-inducing mechanisms can aid in the development of novel drug strategies against human diseases.

General features of apoptosis and basic mechanisms

As a cell undergoes death by apoptosis, certain biochemical and morphological changes occur such as mitochondrial transmembrane potential disruption, membrane phosphatidyl-serine exposure, cell shrinkage, DNA-fragmentation, and blebbing. The culmination of apoptosis is reached as cells fragment into apoptotic bodies and are ingested by phagocytic cells.

A family of cysteine aspartyl proteases, or caspases, are central to the apoptotic pathway. Caspases induce apoptosis by targeting and cleaving specific intracellular proteins. Over 100 such targets are already reported (Earnshaw et al. 1999; Nicholson 1999), and the function of these target proteins can be induced or abolished following caspase cleavage leading to pro-apoptotic signals (e.g. DNases) or loss of essential cellular proteins (e.g. cytoskeletal proteins), respectively. There are two main apoptotic pathways that lead to activation of caspases. One pathway involves transmembrane death receptors known as Fas (or Apo-1 or CD95). Stimulation of these receptors by Fas ligands – produced in

cytotoxic T cells - causes caspase activation (Walczak and Krammer 2000). The second major pathway of apoptosis involves the mitochondrion. Several different death signals converge upon the mitochondrion and can cause release of cytochrome c, which also leads to caspase activation.

The bcl-2 related proteins

The bcl-2 gene was originally identified as the chromosomal breakpoint of t(14;18)-bearing B-cell lymphomas (Bakhshi et al. 1985). Bcl-2 and the related bcl-xL serve to inhibit apoptosis, while other family members like bax and bcl-xS can promote apoptosis. The family members complex with each other to form homo- and heterodimers and it is the cumulative ratio between these players that determines a cell's fate (Reed et al. 1996). The anti-apoptotic function of bcl-2 appears to be its role in stabilizing the mitochondrion and preventing the release of cytochrome c (Kluck et al. 1997; Martinou et al. 2000).

Nitric oxide and apoptosis

Nitric oxide (NO) gas is involved in the regulation of several physiological functions such as vasodilation, nerve signalling, and immune response, and classically mediates its effects through the guanylate cyclase-cGMP pathway. (Ignarro 1990). However, additional effects of NO such as pro-apoptotic and anti-apoptotic signalling have been reported and shown to function through cGMP-independent pathways.

NO is produced in the body by three nitric oxide synthase (NOS) enzymes (Forstermann et al. 1995). Endothelial NOS (eNOS) and neuronal NOS (nNOS) are calcium-dependent enzymes which produce steady, low levels of NO. Inducible NOS (iNOS) is calcium-independent and can produce large quantities of NO in response to the cytokines and endotoxins released through inflammation or infection. The synthesis of NO involves oxidation of a nitrogen atom on the amino acid L-arginine (Mayer and Hemmens 1997).

The cGMP-independent modes of action of NO can occur via direct interactions with proteins, nucleic acids, and heme groups, and result in both anti-apoptotic and pro-apoptotic signals. For example, caspases are inhibited by S-nitrosylation (Dimmeler et al. 1997; Kim et al. 1998), and a reduction of apoptosis is observed through this mechanism in T cells exposed to the Fas ligand (Melino et

al. 1997). Higher levels of NO may serve to promote apoptosis, e.g. by causing direct oxidative damage leading to DNA strand-breaks, or interfering with oxidative respiration in the mitochondrion and causing the release of cytochrome c (Kroncke et al. 1997). Other pro-apoptotic effects of NO may arise through the s-nitrosylation of mitogen-activated protein kinases (MAPKs) which regulate the expression of the bcl-2 family members in a manner favoring cell death (Boyd and Cadenas 2002, and references therein).

Cyclins and apoptosis

It has been proposed that cellular proliferation and cell death may share common pathways (King and Cidlowski 1995). Indeed, cyclin A, which has a role both in S-phase entry and M-phase entry, is also implicated in apoptosis. Cyclin A levels rise in mouse fibroblasts in parallel with myc-induced apoptosis (Jansen-Durr et al. 1993), and transfection experiments with an inducible cyclin A gene demonstrate that cyclin A expression is sufficient to cause apoptosis (Hoang et al. 1994). Similar results were shown for cyclin D1 in neurons (Kranenburg et al. 1996), cyclin D3 in HeLa cells (Janicke et al. 1996), and cyclin E in hematopoietic cells exposed to ionizing radiation (Mazumder et al. 2000). In all cases, the associated kinase activity of a partner cdk was required for the apoptotic effect. The link between cyclins and apoptosis probably involves the convergence of conflicting signals upon the nucleus. For example, the effect of a particular cyclin on apoptosis is enhanced in sub-optimal conditions such as serum starvation where cells are already growth-arrested, and in quiescent post-mitotic neurons. In these settings, the cell is receiving a proliferative signal from cyclin-cdk complexes while at the same time lacking the other normally associated mitogenic cues. This discrepancy in signals could be one of the mechanisms leading to apoptosis.

AIMS OF THE STUDY

By virtue of the role of G1-phase cyclins as regulators of differentiation, proliferation, and apoptosis, their improper expression can lead to altered cellular homeostasis and tumor formation. The aim of this thesis was to clarify the expression pattern of G1-phase cyclins and define their relevance in neoplastic B cells. Specific focus was placed on the following three areas:

1. Differential expression of D-type cyclins in neoplastic B cells (papers I and III):

The D-cyclins (D1,D2,D3) are expressed in a cell lineage- and tissue-specific manner throughout the body. One aim of this thesis was to establish a better characterization of D-type cyclin expression patterns in a variety of neoplastic B cells.

2. Cyclin E expression in acute and chronic B-cell leukemias (papers II and III):

Cyclin E is an important regulatory protein involved in the G1- to S-phase transition of the cell cycle. Is cyclin E expression altered in adult acute lymphoblastic leukemias and chronic B-cell malignancies? Can determining cyclin E levels be useful as a clinical prognostic marker?

3. G1-phase cyclin expression in B cells undergoing apoptosis (paper IV):

It has been shown that cyclins affect the induction of apoptosis in some settings. How are the G1-phase cyclins affected as B cells are provoked to enter into apoptosis?

MATERIALS AND METHODS

Cell lines

All cell lines were grown in flat-bottom culture flasks maintained in a 5% CO₂ environment at 37 degrees Celsius in humidified incubators. In paper I, cell lines were grown in Iscove's Modified Dulbecco's Medium supplemented with 10% fetal bovine serum, 2mM L-glutamine, 100 units/ml penicillin and 100 units/ml streptomycin. The cell lines used in the remaining articles were cultured as above but with RPMI 1640 medium (GIBCO BRL) supplemented with 10% fetal calf serum (FCS), 2mM L-glutamine, and 100 units/mL penicillin.

Patients

Peripheral blood was collected from adult patients with precursor B-cell acute lymphoblastic leukemia (B-ALL), B cell chronic lymphocytic leukemia (B-CLL) and lymphoplasmacytic leukemia or immunocytoma (IC). The diagnosis of leukemia/lymphoma was based upon standard morphologic and immunologic criteria. Each sample was enriched by Ficoll-Paque (Pharmacia, Uppsala, Sweden) gradient centrifugation. Cells were used either after viable freezing in 10% dimethyl sulfoxide and 50% AB serum or fresh after venipuncture for *in vitro* stimulation studies. The viability of frozen leukemic cells after thawing was assessed by trypan blue exclusion and was always above 90%. Healthy donor B cells were further enriched by magnetic isolation using Dynabeads (Dynal A.S., Norway) coated with CD19 antibodies.

In vitro stimulation

Following CD3+ and CD56+ depletion with antibodies and magnetic separation, fresh B-CLL cells were incubated in vitro with either RPMI 1640 medium and 10% FCS alone, or with the addition of 0.005% (v/v) standardized Pansorbin cells [*Staphylococcus aureus* Cowan strain 1 (SAC)] and 1,000 units/ml recombinant human interleukin-2 (IL-2). Cells were harvested daily for up to 6 days. Mitogen stimulation was performed on peripheral blood B cells from a healthy donor separated with anti CD19 coated magnetic beads and cultured for three days in medium containing 5µg/ml of anti-CD40 rabbit serum and 1000 U/ml human recombinant IL-4.

RNA Extraction and Reverse Transcription

Total RNA was phenol-extracted using the Ultraspec-II kit (Biotech Lab, Inc., Houston). 1 µg of RNA in a volume of 11.3 µL dH₂O was denatured for 5 min at 70°C and then reverse-transcribed for 45 min at 40°C with 8.7 µL RT-mix (4 µL 5X First Strand Buffer (Gibco BRL), 2 µL 0.1 M DTT (Gibco BRL), 1 µL dNTP mix (5mM ea. dATP, dTTP, dCTP, dGTP), 0.2 µL 1mM N6 random hexamer primers (Pharmacia), 1 µL 200 units/µL M-MLV reverse transcriptase (Gibco BRL), 0.5 µL 40 units/µL RNasin (Promega). The reaction was completed by incubating at 95°C for 5 min. Prior to PCR, 80 µL of dH₂O was added to each tube of cDNA.

D-cyclin PCR

The three D-cyclins, D1, D2, and D3 were amplified simultaneously in a triple-PCR reaction using the same primer sequences as previously described (Uchimarui et al. 1997). 2.5 µL of cDNA was added to 47.5 µL of PCR-mix (5 µL 10XPCR buffer II (Perkin Elmer), 2 µL dNTP mix (as above), 3 µL 25mM MgCl₂, (Perkin Elmer), 5 µL of each 2 µM primer (D123, D1, D2, D3), 0.25 µL AmpliTaq (Perkin Elmer), and 17.25 µL dH₂O). The reaction was performed in a Perkin Elmer GenAmp 9600 thermal cycler with the following parameters: 94 °C for 3 min, 28 cycles of 94°C for 30 sec, 50 °C for 45 sec, 72 °C for 60 sec. A final extension was performed at 72 °C for 5 min.

Cyclin E PCR

Cyclin E primers p-341 (5' GGG CAA ATA GAG AGG AAG TC 3') and p-742/723 (5' GGG GAC TTA AAC GCC ACT TA 3') were purchased from Eurogentech. 2.6 µL cDNA was added to 22.4 µL of PCR mix (2.5 µL 10XPCR buffer II, 1 µL dNTP mix, 2 µL 25mM MgCl₂, 0.36 µL of both primers p-341 and p-742/723, 0.1 µL AmpliTaq, and 16.1 µL of dH₂O). The parameters used were: 94 °C for 3 min, 35 cycles of 94°C for 30 sec, 53°C for 45 sec, 72 °C for 60 sec. A final extension was performed at 72 °C for 5 min.

Cell cycle analysis

Fixed and permeabilized cells were incubated with DNA staining solution (propidium iodide and RNase A). DNA staining was evaluated by fluorescence 2 intensity in the linear scale using FACScan (Becton & Dickinson) and analyzed using CellFit software.

Determination of apoptosis

Cells were stained with Annexin-V-Fluos (Boehringer-Mannheim) and propidium iodide and analyzed by flow cytometry. Dot plots with mean fluorescence intensity (MFI) of annexin-V (FL1) on the x-axis and MFI of propidium iodide (FL3) on the y-axis enabled the visualization of intact cells, cells with exposed phosphatidyl-serine (representing apoptotic cells), and necrotic cells.

Northern Blotting

Complementary DNA (cDNA) strands of cyclin D2, cyclin D3, and cyclin A radiolabelled with [³²P]dCTP were used as probes. Ten µg of cytoplasmic RNA was electrophoresed on denaturing agarose gels, transferred to nylon filters, and hybridized with the individual probes at 42 degrees Celsius in 50% formamide, 6X SSC, 0.2% SDS, 5X Denhardt's solution, 200 µg of salmon sperm DNA, and 0.1 mM ATA for 16 hours.

Western blotting

Cells were lysed under ice-cold conditions in immunoprecipitation buffer, followed by the addition of Laemmli buffer. Protein content of the lysates was quantitated using the DC protein Assay Kit (Bio-Rad). Equal concentrations of samples were electrophoresed in 10% SDS-polyacrylamide gels, transferred to nitrocellulose membranes, and detection of cyclin proteins was later performed using the following antibodies: cyclin A (Santa Cruz). Cyclin D3 polyclonal antibodies 1:200 (sc182, Santa Cruz Biotechnology, Santa Cruz, CA), cyclin E monoclonal antibodies 1:400 (14591A, Pharmingen, San Diego, CA), cdk2 polyclonal antibodies 1:1000 (558896, Pharmingen, San Diego, CA), p27 polyclonal antibodies 0.75:1000 (Transduction Laboratories, Lexington, KY), actin antibodies 0.75:1000 (I-19, Santa Cruz Biotechnology, Santa Cruz, CA), secondary anti-rabbit, anti-mouse and anti-goat horseradish peroxidase-linked antibodies 1:5000 (Amersham, Amersham Place, UK), and the enhanced-chemiluminescence (ECL) kit from Amersham.

Densitometry

Scanning of ECL-exposed films was performed on an Arcus II scanner (AGFA) using the Fotolook software. Densitometric values were obtained using the NIH Image 1.58 program. Sample values were normalized by calculating the ratio of

the absorbance x area value of each sample to a common standard present on each blot (Reh cell line) and to each sample's own actin value.

Immunostaining

Cytospin slides were prepared from B cells stimulated with anti-CD40 and IL-4, B-ALL cells, and exponentially growing Reh cells. Slides were fixed in cold methanol:acetone (1:1) for 10 min, rehydrated in PBS for 1 hr and incubated for 1 hr at room temperature with the primary antibody (mouse monoclonal DCS-22, from Dr. Jiri Bartek(Bartkova et al. 1996)). After washing 3 times for 10 min in PBS, the slides were incubated for 30 min with 1:100 diluted biotinylated rabbit-anti-mouse Fab2 fragment (DAKO A/S, Denmark), washed for 10 min in PBS, incubated for another 30 min with 1:300 diluted Texas Red conjugated streptavidin (Vector Lab., Inc., Burlingame, CA), and washed and mounted with balanced salt solution:glycerol (1:1) containing 2.5% 1,4-diazabicyclo-(2.2.2.) octane (Sigma). Bisbenzimidazole (Hoechst 33258) was added at a concentration of 0.4 µg/ml to the secondary antibody for DNA staining. The images were recorded on a DAS microscope Leitz DM RB with a Hamamatsu dual mode cooled CCD camera C4880.

Statistics

Simple linear regression, Mann-Whitney U test, and Student's t-test were used. P values of less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Differential expression of D-type cyclins in neoplastic B cells (*Papers I and III*):

In normal B cell proliferation and differentiation, cyclin D3 appears to play the dominant role among the three D-cyclins (D1,D2,D3). Cyclin D1 is absent in normal lymphocytes and cyclin D2 is found only sporadically and at low levels *in vivo*. Interestingly, cyclin D1 expression is activated in neoplastic B cells with chromosome 11 alterations, such as mantle cell lymphomas, and cyclin D2 expression is associated with virally-infected lymphoid cells. These observations suggest a role for aberrantly expressed D-cyclins in the neoplastic transformation of B cells.

In *paper I*, the expression of the D-type cyclins was studied in normal B cells, lymphoblastoid cell lines (LCLs) and Burkitt's lymphoma (BL) lines. Using northern blot, western blot, and immunocytochemistry, D-cyclin expression was analyzed at both the mRNA and protein level. As expected, cyclin D1 expression was absent in all samples. Cyclin D2 was the predominant D-cyclin expressed in EBV-transformed LCLs and group III BLs and was associated with an activated B-cell - or lymphoblastoid - phenotype (CD10-, CD39+), while EBV(-) BL lines and group I and II BL lines expressed only cyclin D3.

To test whether or not EBV infection was sufficient to induce cyclin D2, we studied EBV(-) BL lines and their *in vitro* EBV-converted sublines. In most cases, the EBV-converted sublines maintained their original cyclin D2-negative status and a CD10+/CD39- phenotype, despite the expression of a full set of EBNAs and LMPs. From these findings, we conclude that full EBV genome expression does not always lead to cyclin D2 expression. Rather, the activation of cyclin D2 in EBV-induced transformation may require a specific cellular background or a particular stage of B-cell differentiation.

The expression of cyclin D3 in B-cell chronic lymphocytic leukemia (B-CLL) has been a matter of some debate. Two independent groups reported both a complete lack (0/10) (Delmer et al. 1995) and the presence of cyclin D3 transcripts in the majority (10/11) (Suzuki et al. 1999) of B-CLL samples. Using flow cytometry, Wolowiec et al. reported cyclin D3-positive cell fractions in all of

the B-CLL populations they studied (Wolowiec et al. 2001). Our intention in *paper II* was to dispel the idea that B-CLL cells have a particular cyclin D3 phenotype. Rather, we believe that the reported cyclin D3-negativity in B-CLL was either due to inadequate sensitivity of detection or to the early G0/G1 status of the cells at a point in the cell cycle prior to the position where cyclin D3 is normally expressed. To confirm this idea, we studied cyclin D3 protein expression in both resting and activated cells. We found the protein to be undetectable in resting B lymphocytes and in B-CLL and IC samples. However, we observed that B-CLL cells went from D3-negative to D3-positive following 2 days of *in vitro* stimulation with interleukin-2 (IL-2) and *Staphylococcus aureus* Cowan strain I (SAC) consistent with their progression through G1 (Fig. 2, *paper III*). This induction of cyclin D3 protein in B-CLL was similar to that observed in normal B lymphocytes (Fig. 5, *paper I*).

B-ALL samples from peripheral blood were cyclin D3-positive without any need for stimulation *in vitro*, and had an immunocytochemical cyclin D3 staining pattern similar to stimulated lymphocytes, with 10% strongly stained nuclei (Fig. 3, *paper III*). These results suggest that cyclin D3 is readily detected in B-ALL because the cells have progressed further in G1 than B-CLL and IC to the point where cyclin D3 is normally expressed at high levels. This is supported by the larger size of B-ALL blasts, and the slightly higher fraction of cells in the S-G2/M phases of the cell cycle, which both reflect progression through G1 phase.

These findings suggest to us that the cyclin D3 expression observed in B-ALL and B-CLL is within normal limits. Earlier discrepancies regarding cyclin D3 expression in B-CLL may have arisen because the investigators were looking at a single time point only.

Cyclin E expression in acute and chronic B-cell leukemias (*papers II and III*):

In *papers II and III*, cyclin E expression was studied in peripheral blood lymphocytes from adult patients with B-ALL, B-CLL and IC. The majority of samples tested expressed cyclin E at levels higher than normal peripheral blood mononuclear cells (PBMcs). These findings raise the questions of a possible role for cyclin E in the pathogenesis of leukemia, and the potential use for cyclin E as a prognostic marker.

In B-ALL, we observed a relationship between cyclin E levels and two clinical features. Out of 12 patients in our study, the only long term survivors were the two patients who had the lowest levels of cyclin E at diagnosis. In paired B-ALL samples taken at diagnosis and at relapse, we found an overexpression of the cyclin E protein in blasts from 6/6 patients at relapse, plus the presence of up to four additional lower molecular weight species of cyclin E not seen in blasts from the time of diagnosis. These results support both the value of cyclin E as a prognostic marker in B-ALL and the role of cyclin E levels as a marker of malignant progression.

Whether or not the overexpression of cyclin E and its isoforms reflects a greater proliferation potential of blasts is a matter of speculation at this point. Despite higher levels of cyclin E in relapsed blasts, we observed no associated increase in the S-phase fraction of these cells. However, we have shown a more aggressive engraftment of relapsed B-ALL blasts in SCID mice (Palucka et al. 1996). Because cyclin E plays a role in the progression of cells through G1, its higher levels in relapsed blasts may confer a stoichiometrical advantage when it comes to forming active cyclin-cdk complexes prior to the onset of S phase. In this manner, blasts from relapse would have a "head start" when it comes to forming the catalytic complexes required for S-phase entry under permissive conditions such as in the SCID mouse environment.

The lower molecular weight cyclin E proteins found in relapsed B-ALL blasts may represent splicing variants or post-translationally modified isoforms of the protein. Regardless of their origin, the presence of cyclin E isoforms at relapse appears to reflect the increased malignant status of the blasts, as they are not observed in normal cells and are less apparent in blasts from diagnosis. Detection of cyclin E isoforms was also described in breast cancer samples and was correlated with malignant progression of the disease (Keyomarsi et al. 1994). We did not study the associated kinase activity of cyclin E and its isoforms in our B-ALL samples, but the cyclin E variants described in breast cancer were shown to possess functional activity at levels even higher than full-length cyclin E when tested in *in vitro* kinase assays and transfection experiments (Porter et al. 2001). These results suggest that the presence of lower molecular weight isoforms of cyclin E may also play a role in conferring a proliferative advantage in neoplastic cells.

In the chronic B-cell leukemias B-CLL and IC, we also observed an aberrant expression of cyclin E in some samples. Overexpression of cyclin E was found in 4/12 B-CLL samples and 4/4 IC samples as compared to normal peripheral blood mononuclear cells (PBMCs). In contrast to the normal expression pattern of the D-cyclins that we observed in B-CLL, the expression of cyclin E in B-CLL and IC appears pathological as these cells have extremely low proliferation fractions and are considered to be predominantly in the G₀ phase of the cell cycle where cyclin E is not normally expressed. Unlike our observations in B-ALL, however, we found no correlation between cyclin E levels and survival status or stage of disease in chronic B-cell leukemias. The four B-CLL patients who overexpressed cyclin E had no other distinguishable characteristics (Table 1, *paper III*).

To assess a potential functional role of cyclin E in B-cell leukemias, we looked at the kinase partner of cyclin E, cdk2, and found it to be expressed in B-ALL samples but not in B-CLL or IC. These results suggest that despite the illegitimate expression of cyclin E in B-CLL and IC, the levels of its partner kinase are too low to allow cell-cycle function via e.g. phosphorylation of the retinoblastoma protein (pRb). Therefore, the overexpression of cyclin E, as observed in B-CLL and IC, probably represents a defective accumulation of the protein rather than reflecting enhanced proliferation. In line with this idea, we observed no significant relationship between cyclin E levels and percentage of cells in the S-G2/M phases of the cell cycle. Whether the abnormal accumulation of cyclin E seen in these B-CLL and IC samples results from increased transcription of the cyclin E gene or a decreased degradation of the protein remains to be determined.

To summarize, our results show differences between acute and chronic lymphocytic leukemias with respect to cyclin E and its clinical relevance. Whereas, in a small patient group of B-ALL, low cyclin E levels were associated with better prognosis, no such relationship was found in chronic B cell malignancies. Prospective studies on larger patient populations should be performed in order to evaluate whether or not cyclin E can serve as a prognostic marker in B-cell leukemias.

G1-phase cyclin expression in B cells undergoing apoptosis (*paper IV*):

In *paper IV*, we studied the expression of the G1-phase cyclins D3, E, and A in B-cell lines induced to undergo apoptosis *in vitro*. Using the nitric oxide (NO)-donor S-nitroso-N-acetyl-D,L-penicillamine (SNAP) at various concentrations (10-1000 μ m), we determined the minimal concentrations at which cell survival was affected. At a SNAP concentration of 250 μ m, we found one B-cell line that was sensitive (NALM-6) and one that was resistant (FLEB) to NO-induced apoptosis. Visualization of G1-arrest was studied by propidium iodide (PI) staining, and entry into apoptosis was confirmed by phosphatidyl-serine (PS) exposure and DNA-fragmentation, as measured by flow cytometry.

Following 24 hours of exposure to 250 μ m of SNAP under sub-optimal conditions (0.5% bovine serum albumin), a 7.8-fold induction of the cyclin E protein was observed in NALM-6 in parallel with G1 arrest and apoptosis. FLEB cells showed only a modest 1.6-fold increase in cyclin E. A clear G1 skewing was observed in NALM-6 cells with a nearly 4-fold increase in the G1 population (Fig. 3, *paper IV*). In addition, PS-exposure, reflecting an early stage of apoptosis, was observed in 28% of the NALM-6 cell population compared to 10% in FLEB. DNA-fragmentation, a later event of apoptosis, reached 55% in NALM-6 compared to only 4% in FLEB. Despite the increased expression of cyclin E in NALM-6, the proliferative response was steadily decreased as illustrated by thymidine incorporation (Fig. 1, *paper IV*). We observed no change in protein levels of cyclin D3, cyclin A, or the anti-apoptotic protein bcl-2.

As mentioned in the introduction, cyclins D1, D3 and E have previously been implicated in apoptosis. We have identified cyclin E as a novel target of nitric oxide and found an association between induction of cyclin E and apoptotic susceptibility in the pre-B-cell line NALM-6. We speculate that the rise in cyclin E levels may result from DNA damage caused by exposure to nitric oxide. Increased levels of cyclin E in response to DNA-damage have been reported in human retinoblastoma and lymphoid cells treated with DNA-damaging agents like topoisomerase I inhibitors and ionizing radiation (Lauricella et al. 1998; Mazumder et al. 2000). Other possibilities include the activation of signal transduction pathways by s-nitrosylation and the mobilization of transcription factors which target cyclin E. In order to illustrate a direct role for cyclin E in the apoptotic susceptibility of NALM-6, experiments that abolish cyclin E function -

such as cyclin E anti-sense or dominant-negative cdk2 mutants - should be performed, and the resulting effects on apoptosis monitored.

CONCLUSIONS

- A. EBV-induced transformation of B cells is associated with cyclin D2 expression. However, our results show that EBV infection is not sufficient to induce cyclin D2 expression in EBV(-) Burkitt's lines (BLs). Rather, it seems that a lymphoblastoid phenotype, together with EBV infection, is the consistent feature of cyclin D2-positive cells. Our results are consistent with the idea that D-cyclins are expressed in a cell lineage-specific manner, and that B cells may switch their D-type cyclin phenotype during differentiation.
- B. The expression pattern of cyclin D3 is maintained intact in B-CLL cells and mirrors the pattern observed in normal B cells.
- C. Cyclin E is overexpressed in B-ALL, IC, and in some cases of B-CLL. In B-ALL, increased cyclin E levels are found at relapse, and may reflect progression of the disease into a more aggressive stage. Low cyclin E levels at the time of diagnosis were observed in two long-term survivors of B-ALL. No correlation between cyclin E and advanced disease was observed in B-CLL and IC. The use of cyclin E as a prognostic marker should be studied prospectively in a larger group of patients to assess its value.
- D. We present the novel finding that cyclin E, but not cyclin D3 or cyclin A, is induced following exposure of a pre-B-cell line to nitric oxide (NO). The observed G1 arrest and entry into apoptosis which occur in parallel suggest a potential role for cyclin E in NO-induced apoptosis. Further studies will be required to show a more definitive relationship between cyclin E function and apoptotic susceptibility in this setting.

ACKNOWLEDGEMENTS

Magnus Björkholm, for helpful advice and support from my first day at Karolinska back in 1993 and through it all.

Fredrik Celsing, my advisor, for always taking time out to help me, and for giving me the support I needed to get this thesis done. I'll always have fond memories of our collaboration in the lab and on the football pitch.

Klas Wiman, my co-advisor, for introducing me to cyclins and allowing me to join in group meetings and collaborative projects back in the old days at MTC, and for your continued support throughout the years.

Katja Pokrovskaja, my dear colleague and mentor, for having an enormous amount of patience with me and for always giving me meaningful feedback on my writing. Your help in the preparation of this thesis is deeply appreciated.

Miguel Aguilar-Santelises, mentor and colleague, for finding an overlap in our research interests and for getting new projects started. Thank you for sharing your research experience with me and for always taking time out for me. You gave me the extra push I needed to get this thesis completed.

Pavel Pisa, my first research mentor, for introducing me to the field of hematology and giving me the opportunity to do research in your group. I will always be grateful for your generosity and your help with this thesis.

Stefan Einhorn, for helpful lessons in statistics and cancer biology.

Bengt Fagrell, for introducing me to Karolinska, Stockholm's archipelago, and Idre fjäll.

Members of the Hematology Lab, Anne-Marie "Bebban" Sjögren, Margareta "Meta" Andersson, Ann-Marie Andreasson, Maria Malec, Mi Hou, Margareta "Maggan" Söderquist, Masih Ostad, Doina Anton, Zheng Chengyun and Anne Sundblad, for creating a nice work atmosphere and for always lending a hand.

Others who helped me along the way, Karolina Palucka, Li Xuri, Gudmundur Runarsson, Barbara MacNamara, Malin Müller, Joy Roy, Angelina de Santiago, Britt-Marie Johansson, Pia Hägerman, Monica Intaglietta, Iréne Areström, Yuri Sheikine and Ricardo Giscombe.

Jan "Janne" Sjöberg, for all the good times with the boat, the guitars, and the gäddas. It has been a real treat to know you and to work with you all of these years.

Carlos Garcia, for always bringing a smile to the lab and for helping me with figures, cell culturing, automobile parts, salsa, and Cuban proverbs (but I still don't know what "one hair from the wolf" means???)

Dawei Xu, for your generosity in sharing ideas and suggestions, lab space, and reagents.

Marlene Mozart, for a nice collaboration, with the hope of continuation.

Friends and colleagues from the Microbiology and Tumor Biology Center: Laszlo Szekely, Kristinn Magnusson, Ismail Okan, Yisong Wang, Galina Selivanova, Lotta Asker, Qiang Wang, Shohre Ziaie, Elena Kashuba, Lyda Osorio, and Pedro Otavio de Campos Lima.

Kristian, Stijn, Shahab, and Rutger for stimulating scientific conversations about bowling, and the band (did we decide on Brother Seamus?).

Other sources of support and inspiration: Zheer Tawfique, Ola Hallert, Luigi De Petris, Fausto Labruto, Neil Portwood, Olle Sangfelt, Sven Erickson, Öjar Melefors, Kiet Tran, Miloslav Beran, Jiri Bartek, and Bengt Kallin.

Britt Telander and Britt-Marie Höglund, from the Department of Medicine, for your consistent help over the years.

My parents Dante and Anita and my siblings Steven, Annette and Paul for a lifetime of support, och min familj i Stockholm, Mormor Iris och Morbror Tommy, för hjälp med det svenska språket och för det underbara sällskapet.

Special thanks to: Karolinska Institutet, Karolinska Institutets Stipendiestiftelser, Stiftelsen Bengt Lundqvists Minne, Konung Gustaf V:s Jubileumsfond, Svenska Läkaresällskapets Hennerlöfska fond, Gladys More Thomsons Forskningsfond, and Cancerfonden (Swedish Cancer Society).

Tullfritt Gatukök vid Roslagstull för de schyssta burgarna.

REFERENCES

- Ajchenbaum, F., K. Ando, J. A. DeCaprio and J. D. Griffin (1993). Independent regulation of human D-type cyclin gene expression during G1 phase in primary human T lymphocytes. *J Biol Chem* 268(6): 4113-4119.
- Akama, Y., W. Yasui, H. Yokozaki, H. Kuniyasu, K. Kitahara, T. Ishikawa and E. Tahara (1995). Frequent amplification of the cyclin E gene in human gastric carcinomas. *Jpn J Cancer Res* 86(7): 617-21.
- Ando, K., F. Ajchenbaum-Cymbalista and J. D. Griffin (1993). Regulation of G1/S transition by cyclins D2 and D3 in hematopoietic cells. *Proc Natl Acad Sci* 90: 9571-9575.
- Andreeff, M., Z. Darzynkiewicz, T. K. Sharpless, B. D. Clarkson and M. R. Melamed (1980). Discrimination of human leukemia subtypes by flow cytometric analysis of cellular DNA and RNA. *Blood* 55(2): 282-93.
- Bakhshi, A., J. P. Jensen, P. Goldman, J. J. Wright, O. W. McBride, A. L. Epstein and S. J. Korsmeyer (1985). Cloning the chromosomal breakpoint of t(14;18) human lymphomas: clustering around JH on chromosome 14 and near a transcriptional unit on 18. *Cell* 41(3): 899-906.
- Baldin, V., J. Lukas, M. J. Marcotte, M. Pagano and G. Draetta (1993). Cyclin D1 is a protein required for cell cycle progression in G1. *Genes Dev* 7: 812-821.
- Bartkova, J., J. Lukas, H. Muller, D. Luthjohlt, M. Strauss and J. Bartek (1994). Cyclin D1 protein expression and function in human breast cancer. *Int J Cancer* 57: 353-361.
- Bartkova, J., E. Rajpert-de Meyts, N. E. Skakkebaek and J. Bartek (1999). D-type cyclins in adult human testis and testicular cancer: relation to cell type, proliferation, differentiation, and malignancy. *J Pathol* 187(5): 573-81.
- Bartkova, J., M. Zemanova and J. Bartek (1996). Abundance and subcellular localisation of cyclin D3 in human tumors. *Int J Cancer* 65: 323-327.
- Bates, S. and G. Peters (1995). Cyclin D1 as a cellular proto-oncogene. *Sem Cancer Biol* 6: 73-82.
- Blain, S. W., E. Montalvo and J. Massague (1997). Differential interaction of the cyclin-dependent kinase (Cdk) inhibitor p27Kip1 with cyclin A-Cdk2 and cyclin D2-Cdk4. *J Biol Chem* 272(41): 25863-72.
- Blanchard, D. A., M. T. Affredou and A. Vazquez (1997). Modulation of the p27kip1 cyclin-dependent kinase inhibitor expression during IL-4-mediated human B cell activation. *J Immunol* 158(7): 3054-61.
- Bosch, F., P. Jares, E. Campo, A. Lopez-Guillermo, M. A. Piris, N. Villamor, D. Tassies, E. S. Jaffe, E. Montserrat, C. Rozman and et al. (1994). PRAD-1/cyclin D1 gene overexpression in chronic lymphoproliferative disorders: a highly specific marker of mantle cell lymphoma. *Blood* 84(8): 2726-32.
- Botti, A. C. and R. S. Verma (1990). The molecular biology of acute lymphoblastic leukemia (review). *Anticancer Res* 10: 519-526.

- Boyd, C. S. and E. Cadenas (2002). Nitric oxide and cell signaling pathways in mitochondrial-dependent apoptosis. *Biol Chem* 383(3-4): 411-23.
- Brooks, A. R., D. Shiffman, C. S. Chan, E. E. Brooks and P. G. Milner (1996). Functional analysis of the human cyclin D2 and cyclin D3 promoters. *J Biol Chem* 271(15): 9090-9099.
- Caligaris-Cappio, F. (2000). Biology of chronic lymphocytic leukemia. *Rev Clin Exp Hematol* 4(1): 5-21.
- Cayuela, J.-M., A. Madani, L. Sanhes, M.-H. Stern and F. Sigaux (1996). Multiple tumor-suppressor gene 1 inactivation is the most frequent genetic alteration in T-cell acute lymphoblastic leukemia. *Blood* 87: 2180-2186.
- Chucrallah, A. E., S. A. Stass, Y. O. Huh and H. M. Kantarjian (1994). Adult lymphoblastic leukemia at relapse: cytogenetic, immunophenotypic, and molecular changes. *Blood (suppl.)* 84: 307a.
- Clarkson, B. D., T. Gee, R. Mertelsmann, S. J. Kempin, M. Andreeff, E. Berman, R. J. O'Reilly, Z. A. Arlin, S. Ellis, C. Little, C. Cirincione and R. E. Dinsmore (1986). Current status of treatment of acute leukemia in adults: an overview of the Memorial experience and review of literature. *Crit Rev Oncol Hematol* 4(3): 221-248.
- Cooper, E. H. (1993). Leukemia Kinetics. Cell proliferation in lymphomas. Ed. J. Crocker. Oxford, Blackwell Scientific Publications: 115-130.
- Courjal, F., G. Louason, P. Speiser, D. Katsaros, R. Zeillinger and C. Theillet (1996). Cyclin gene amplification and overexpression in breast and ovarian cancers: evidence for the selection of cyclin D1 in breast and cyclin E in ovarian tumors. *Int J Cancer* 69(4): 247-53.
- Craxton, A., K. L. Otipoby, A. Jiang and E. A. Clark (1999). Signal transduction pathways that regulate the fate of B lymphocytes. *Adv Immunol* 73: 79-152.
- DeGregori, J., T. Kowalik and J. R. Nevins (1995). Cellular targets for activation by the E2F1 transcription factor include DNA synthesis- and G1/S-regulatory genes. *Mol Cell Biol* 15: 4215-4224.
- Delmer, A., F. Ajchenbaum-Cymbalista, R. Tang, S. Ramond, A.-M. Faussat, J.-P. Marie and R. Zittoun (1995). Overexpression of cyclin D2 in chronic B-cell malignancies. *Blood* 85(10): 2870-2876.
- Diehl, L. F., L. H. Karnell and H. R. Menck (1999). The American College of Surgeons Commission on Cancer and the American Cancer Society. The National Cancer Data Base report on age, gender, treatment, and outcomes of patients with chronic lymphocytic leukemia. *Cancer* 86(12): 2684-92.
- Dimmeler, S., J. Haendeler, M. Nehls and A. M. Zeiher (1997). Suppression of apoptosis by nitric oxide via inhibition of interleukin-1 β -converting enzyme (ICE)-like and cysteine protease protein (CPP)-32-like proteases. *J Exp Med* 185(4): 601-7.
- Dulic, V., E. Lees and S. I. Reed (1992). Association of human cyclin E with a periodic G1-S phase protein kinase. *Science* 257: 1958-1961.

- Dyson, N. (1998). The regulation of E2F by pRB-family proteins. *Genes Dev* 12(15): 2245-62.
- Earnshaw, W. C., L. M. Martins and S. H. Kaufmann (1999). Mammalian caspases: structure, activation, substrates, and functions during apoptosis. *Annu Rev Biochem* 68: 383-424.
- el-Deiry, W. S., T. Tokino, V. E. Velculescu, D. B. Levy, R. Parsons, J. M. Trent, D. Lin, W. E. Mercer, K. W. Kinzler and B. Vogelstein (1993). WAF1, a potential mediator of p53 tumor suppression. *Cell* 75(4): 817-25.
- Evans, T., E. T. Rosenthal, J. Youngbloom, D. Distel and T. Hunt (1983). Cyclin: a protein specified by maternal mRNA in sea urchin eggs that is destroyed at each cleavage division. *Cell* 33: 389-396.
- Farrell, P. J. (1995). Epstein-Barr virus immortalizing genes. *Trends Microbiol* 3(3): 105-9.
- Fero, M. L., M. Rivkin, M. Tasch, P. Porter, C. E. Carow, E. Firpo, K. Polyak, L. H. Tsai, V. Broudy, R. M. Perlmutter, K. Kaushansky and J. M. Roberts (1996). A syndrome of multiorgan hyperplasia with features of gigantism, tumorigenesis, and female sterility in p27(Kip1)-deficient mice. *Cell* 85(5): 733-44.
- Filmus, J., A. I. Robles, W. Shi, M. J. Wong, L. L. Colombo and C. J. Conti (1994). Induction of cyclin D1 overexpression by activated ras. *Oncogene* 9(12): 3627-33.
- Firpo, E. J., A. Koff, M. J. Solomon and J. M. Roberts (1994). Inactivation of a Cdk2 inhibitor during interleukin 2-induced proliferation of human T lymphocytes. *Mol Cell Biol* 14(7): 4889-901.
- Forstermann, U., H. Kleinert, I. Gath, P. Schwarz, E. I. Closs and N. J. Dun (1995). Expression and expressional control of nitric oxide synthases in various cell types. *Adv Pharmacol* 34: 171-86.
- Fournier, S., M. Rubio, G. Delespesse and M. Sarfati (1994). Role for low-affinity receptor for IgE (CD23) in normal and leukemic B- cell proliferation. *Blood* 84(6): 1881-6.
- Geng, Y., W. Whoriskey, M. Y. Park, R. T. Bronson, R. H. Medema, T. Li, R. A. Weinberg and P. Sicinski (1999). Rescue of cyclin D1 deficiency by knockin cyclin E. *Cell* 97(6): 767-77.
- Girard, F., U. Strausfeld, A. Fernandez and N. Lamb (1991). Cyclin A is required for the onset of DNA replication in mammalian fibroblasts. *Cell* 67: 1169-1179.
- Greaves, M. F. (1986). Differentiation-linked leukemogenesis in lymphocytes. *Science* 234: 697-704.
- Green, D. R. and D. W. Scott (1994). Activation-induced apoptosis in lymphocytes. *Curr Opin Immunol* 6(3): 476-87.
- Gu, Y., C. W. Turck and D. O. Morgan (1993). Inhibition of CDK2 activity in vivo by an associated 20K regulatory subunit. *Nature* 366(6456): 707-10.

- Hall, M. and G. Peters (1996). Genetic alterations of cyclins, cyclin-dependent kinases, and Cdk inhibitors in human cancer. *Adv Cancer Res* 68: 67-108.
- Harper, J. W., G. R. Adami, N. Wei, K. Keyomarsi and S. J. Elledge (1993). The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* 75(4): 805-16.
- Harris, N. L., E. S. Jaffe, H. Stein, P. M. Banks, J. K. C. Chan, M. L. Cleary, G. Delsol, C. D. Wolf-Peeters, B. Falini, K. C. Gatter, T. M. Grogan, P. G. Isaacson, D. M. Knowles, D. Y. Mason, H.-K. Muller-Hermelink, S. A. Pileri, M. A. Piris, E. Ralfkiaer and R. A. Warnke (1994). A revised European-American classification of lymphoid neoplasms: a proposal from the international lymphoma study group. *Blood* 84(5): 1361-1392.
- Hartwell, L. H. and T. A. Weinert (1989). Checkpoints: controls that ensure the order of cell cycle events. *Science* 246(4930): 629-34.
- Hebert, J., J. M. Cayuela, J. Berkeley and F. Sigaux (1994). Candidate tumor-suppressor genes MTS1 (p16^{INK4A}) and MTS2 (p15^{INK4B}) display frequent homozygous deletions in primary cells from T- but not B-cell lineage acute lymphoblastic leukemias. *Blood* 84(12): 4038-4044.
- Heichman, K. A. and J. M. Roberts (1994). Rules to replicate by. *Cell* 79: 557-562.
- Hengartner, M. O. (2000). The biochemistry of apoptosis. *Nature* 407(6805): 770-6.
- Herber, B., M. Truss, M. Beato and R. Muller (1994). Inducible regulatory elements in the human cyclin D1 promoter. *Oncogene* 9(4): 1295-304.
- Hinds, P. W., S. Mitnacht, V. Dulic, A. Arnold, S. I. Reed and R. A. Weinberg (1992). Regulation of retinoblastoma protein functions by ectopic expression of human cyclins. *Cell* 70: 993-1006.
- Hoang, A. T., K. J. Cohen, J. F. Barrett, D. A. Bergstrom and C. V. Dang (1994). Participation of cyclin A in Myc-induced apoptosis. *Proc Natl Acad Sci U S A* 91(15): 6875-9.
- Hoelzer, D. and N. Gokbuget (2000). New approaches to acute lymphoblastic leukemia in adults: where do we go? *Semin Oncol* 27(5): 540-59.
- Hoglund, M., B. Johansson, J. Pedersen-Bjergaard, P. Marynen and F. Mitelman (1996). Molecular characterization of 12p abnormalities in hematologic malignancies: deletion of KIP1, rearrangement of TEL, and amplification of CCND2. *Blood* 87(1): 324-30.
- Hunter, T. and J. Pines (1994). Cyclins and cancer II: cyclin D and cdk inhibitors come of age. *Cell* 79: 573-582.
- Ignarro, L. J. (1990). Nitric oxide. A novel signal transduction mechanism for transcellular communication. *Hypertension* 16(5): 477-83.
- Inaba, T., H. Matsushima, M. Valentune, M. F. Roussel, C. J. Sherr and A. T. Look (1992). Genomic organization, chromosomal localization, and independent expression of human cyclin D genes. *Genomics* 13: 565-574.

- Janicke, R. U., X. Y. Lin, F. H. Lee and A. G. Porter (1996). Cyclin D3 sensitizes tumor cells to tumor necrosis factor-induced, c- Myc-dependent apoptosis. *Mol Cell Biol* 16(10): 5245-53.
- Jansen-Durr, P., A. Meichle, P. Steiner, M. Pagano, K. Finke, J. Botz, J. Wessbecher, G. Draetta and M. Eilers (1993). Differential modulation of cyclin gene expression by MYC. *Proc Natl Acad Sci U S A* 90(8): 3685-9.
- Kamb, A., N. Gruis, J. Weaver-Feldhaus, Q. Liu, K. Harshman, S. Tavtigian, E. Stockert, R. Day, B. J. III and M. Skolnick (1994). A cell cycle regulator potentially involved in genesis of many tumor types. *Science* 264: 436-440.
- Kantarjian, H. M. (1994). Adult acute lymphocytic leukemia: critical review of current knowledge. *Am J Med* 97: 176-184.
- Kantarjian, H. M., E. H. Estey, S. O'Brien, E. Anaissie, M. Beran, M. B. Rios, M. J. Keating and J. Gutterman (1992). Intensive chemotherapy with mitoxantrone and high-dose cytosine arabinoside followed by granulocyte-macrophage colony stimulating factor in the treatment of patients with acute lymphocytic leukemia. *Blood* 79: 876-881.
- Kato, J. and C. J. Sherr (1993). Inhibition of granulocyte differentiation by G1 cyclins, D2 and D3, but not D1. *Proc Natl Acad Sci* 90: 11513-11517.
- Kerkhoff, E. and E. B. Ziff (1995). Cyclin D2 and Ha-Ras transformed rat embryo fibroblasts exhibit a novel deregulation of cell size control and early S phase arrest in low serum. *Embo J* 14(9): 1892-903.
- Keyomarsi, K., N. O'Leary, G. Molnar, E. Lees, H. L. Fingert and A. B. Pardee (1994). Cyclin E, a potential prognostic marker for breast cancer. *Cancer Res.* 54: 380-385.
- Kim, Y. M., R. V. Talanian, J. Li and T. R. Billiar (1998). Nitric oxide prevents IL-1beta and IFN-gamma-inducing factor (IL-18) release from macrophages by inhibiting caspase-1 (IL-1beta-converting enzyme). *J Immunol* 161(8): 4122-8.
- King, K. L. and J. A. Cidlowski (1995). Cell cycle and apoptosis: common pathways to life and death. *J Cell Biochem* 58(2): 175-80.
- King, R. W., P. K. Jackson and M. W. Kirschner (1994). Mitosis in transition. *Cell* 79: 563-571.
- Kitahara, K., W. Yasui, H. Kuniyasu, H. Yokozaki, Y. Akama, S. Yunotani, T. Hisatsugu and E. Tahara (1995). Concurrent amplification of cyclin E and CDK2 genes in colorectal carcinomas. *Int J Cancer* 62(1): 25-8.
- Kiyokawa, H., R. D. Kineman, K. O. Manova-Todorova, V. C. Soares, E. S. Hoffman, M. Ono, D. Khanam, A. C. Hayday, L. A. Frohman and A. Koff (1996). Enhanced growth of mice lacking the cyclin-dependent kinase inhibitor function of p27(Kip1). *Cell* 85(5): 721-32.
- Kluck, R. M., E. Bossy-Wetzel, D. R. Green and D. D. Newmeyer (1997). The release of cytochrome c from mitochondria: a primary site for Bcl-2 regulation of apoptosis. *Science* 275(5303): 1132-6.

- Kobayashi, H., E. Stewart, R. Poon, J. P. Adamczewski, J. Gannon and T. Hunt (1992). Identification of the domains in cyclin A required for binding to, and activation of, p34cdc2 and p32cdk2 protein kinase subunits. *Mol Biol Cell* 3(11): 1279-94.
- Koepp, D. M., L. K. Schaefer, X. Ye, K. Keyomarsi, C. Chu, J. W. Harper and S. J. Elledge (2001). Phosphorylation-dependent ubiquitination of cyclin E by the SCFFbw7 ubiquitin ligase. *Science* 294(5540): 173-7.
- Koff, A., F. Cross, A. Fisher, J. Schumacher, K. Leguellec, M. Philippe and J. M. Roberts (1991). Human cyclin E, a new cyclin that interacts with two members of the cdc2 gene family. *Cell* 66: 1217-1228.
- Koff, A., A. Giordano, D. Desai, K. Yamashita, J. W. Harper, S. Elledge, T. Nishimoto, D. O. Morgan, B. R. Franza and J. M. Roberts (1992). Formation and activation of a cyclin E-cdk2 complex during G1 phase of the human cell cycle. *Science* 257: 1689-1694.
- Kranenburg, O., A. J. van der Eb and A. Zantema (1996). Cyclin D1 is an essential mediator of apoptotic neuronal cell death. *Embo J* 15(1): 46-54.
- Kroncke, K. D., K. Fehsel and V. Kolb-Bachofen (1997). Nitric oxide: cytotoxicity versus cytoprotection--how, why, when, and where? *Nitric Oxide* 1(2): 107-20.
- LaBaer, J., M. D. Garrett, L. F. Stevenson, J. M. Slingerland, C. Sandhu, H. S. Chou, A. Fattaey and E. Harlow (1997). New functional activities for the p21 family of CDK inhibitors. *Genes Dev* 11(7): 847-62.
- Lane, S., P. Farlie and R. Watson (1997). B-Myb function can be markedly enhanced by cyclin A-dependent kinase and protein truncation. *Oncogene* 14(20): 2445-53.
- LaThangue, N. B. (1994). DP and E2F proteins: components of a heterodimeric transcription factor implicated in cell cycle control. *Curr Opin Cell Biol* 6: 443-450.
- Lauricella, M., M. Giuliano, S. Emanuele, M. Carabillo, R. Vento and G. Tesoriere (1998). Increased cyclin E level in retinoblastoma cells during programmed cell death. *Cell Mol Biol (Noisy-le-grand)* 44(8): 1229-35.
- LeBien, T. W. (2000). Fates of human B-cell precursors. *Blood* 96(1): 9-23.
- Lees, E. M. and E. Harlow (1993). Sequences within the conserved cyclin box of human cyclin A are sufficient for binding to and activation of cdc2 kinase. *Mol Cell Biol* 13(2): 1194-201.
- Leopold, P. and P. H. O'Farrell (1991). An evolutionarily conserved cyclin homolog from *Drosophila* rescues yeast deficient in G1 cyclins. *Cell* 66: 1207-1216.
- Lew, D. J., V. Dulic and S. I. Reed (1991). Isolation of three novel human cyclins by rescue of G1 cyclin (cln) function in yeast. *Cell* 66: 1197-1206.
- Lew, J., Q. Q. Huang, Z. Qi, R. J. Winkfein, R. Aebersold, T. Hunt and J. H. Wang (1994). A brain-specific activator of cyclin-dependent kinase 5. *Nature* 371(6496): 423-6.

- Lin, C. W., T. Manshouri, I. Jilani, D. Neuberg, K. Patel, H. Kantarjian, M. Andreeff, Z. Estrov, M. Beran, M. Keating, E. Estey and M. Albitar (2002). Proliferation and apoptosis in acute and chronic leukemias and myelodysplastic syndrome. *Leuk Res* 26(6): 551-9.
- Liu, J. J., J. R. Chao, M. C. Jiang, S. Y. Ng, J. J. Yen and H. F. Yang-Yen (1995). Ras transformation results in an elevated level of cyclin D1 and acceleration of G1 progression in NIH 3T3 cells. *Mol Cell Biol* 15(7): 3654-63.
- Lukas, J., J. Bartkova, M. Welcker, O. W. Petersen, G. Peters, M. Strauss and J. Bartek (1995). Cyclin D2 is a moderately oscillating nucleoprotein required for G1 phase progression in specific cell types. *Oncogene* 10: 2125-2134.
- Lukas, J., M. Pagano, Z. Staskova, G. Draetta and J. Bartek (1994). Cyclin D1 protein oscillates and is essential for cell cycle progression in human tumor cells. *Oncogene* 9: 707-718.
- Mahony, D., D. A. Parry and E. Lees (1998). Active cdk6 complexes are predominantly nuclear and represent only a minority of the cdk6 in T cells. *Oncogene* 16(5): 603-11.
- Malumbres, M. and M. Barbacid (2001). To cycle or not to cycle: a critical decision in cancer. *Nature Rev Cancer* 1(3): 222-31.
- Marone, M., G. Scambia, C. Giannitelli, G. Ferrandina, V. Masciullo, A. Bellacosa, P. Benedetti-Panici and S. Mancuso (1998). Analysis of cyclin E and CDK2 in ovarian cancer: gene amplification and RNA overexpression. *Int J Cancer* 75(1): 34-9.
- Martinou, J. C., S. Desagher and B. Antonsson (2000). Cytochrome c release from mitochondria: all or nothing. *Nat Cell Biol* 2(3): E41-3.
- Matsushime, H., M. F. Roussel, R. A. Ashmun and C. J. Sherr (1991). Colony-stimulating factor 1 regulates novel cyclins during the G1 phase of the cell cycle. *Cell* 65: 701-713.
- Mayer, B. and B. Hemmens (1997). Biosynthesis and action of nitric oxide in mammalian cells. *Trends Biochem Sci* 22(12): 477-81.
- Mazumder, S., B. Gong and A. Almasan (2000). Cyclin E induction by genotoxic stress leads to apoptosis of hematopoietic cells. *Oncogene* 19(24): 2828-35.
- McConnell, B. B., F. J. Gregory, F. J. Stott, E. Hara and G. Peters (1999). Induced expression of p16(INK4a) inhibits both CDK4- and CDK2- associated kinase activity by reassortment of cyclin-CDK-inhibitor complexes. *Mol Cell Biol* 19(3): 1981-9.
- Melino, G., F. Bernassola, R. A. Knight, M. T. Corasaniti, G. Nistico and A. Finazzi-Agro (1997). S-nitrosylation regulates apoptosis. *Nature* 388(6641): 432-3.
- Montserrat, E. (2002). Prognosis in chronic lymphocytic leukemia: let's have a look! *Haematologica* 87(6): 561-2.
- Morgan, D. O. (1995). Principles of CDK regulation. *Nature* 374: 131-134.

- Motokura, T., T. Bloom, H. G. Kim, H. Juppner, J. V. Ruderman, H. M. Kronenberg and A. Arnold (1991). A novel cyclin encoded by a bcl-linked candidate oncogene. *Nature* 350: 512-515.
- Muller, R. (1995). Transcriptional regulation during the mammalian cell cycle. *Trends Genet* 11: 173-178.
- Mumberg, D., M. Wick, C. Burger, K. Haas, M. Funk and R. Muller (1997). Cyclin ET, a new splice variant of human cyclin E with a unique expression pattern during cell cycle progression and differentiation. *Nucleic Acids Res* 25(11): 2098-105.
- Muschen, M., S. Lee, G. Zhou, N. Feldhahn, V. S. Barath, J. Chen, C. Moers, M. Kronke, J. D. Rowley and S. M. Wang (2002). Molecular portraits of B cell lineage commitment. *Proc Natl Acad Sci U S A* 99(15): 10014-9.
- Nakayama, K., N. Ishida, M. Shirane, A. Inomata, T. Inoue, N. Shishido, I. Horii and D. Y. Loh (1996). Mice lacking p27(Kip1) display increased body size, multiple organ hyperplasia, retinal dysplasia, and pituitary tumors. *Cell* 85(5): 707-20.
- Nevins, J. R. (1992). E2F: a link between the Rb tumor suppressor protein and viral oncoproteins. *Science* 258: 424-429.
- Nicholson, D. W. (1999). Caspase structure, proteolytic substrates, and function during apoptotic cell death. *Cell Death Differ* 6(11): 1028-42.
- Nielsen, N. H., C. Arnerlov, S. O. Emdin and G. Landberg (1996). Cyclin E overexpression, a negative prognostic factor in breast cancer with strong correlation to oestrogen receptor status. *Br J Cancer* 74(6): 874-80.
- Ohtsubo, M. and J. M. Roberts (1993). Cyclin-dependent regulation of G1 in mammalian fibroblasts. *Science* 259: 1908-1912.
- Ohtsubo, M., A. M. Theodoras, J. Schumacher, J. M. Roberts and M. Pagano (1995). Human cyclin E, a nuclear protein essential for the G1-to-S phase transition. *Mol Cell Biol* 15(5): 2612-2624.
- Okano, M. (1997). High susceptibility of an Epstein-Barr virus-converted Burkitt's lymphoma cell line to cytotoxic drugs. *Leuk Res* 21(5): 469-71.
- Okano, M. (1998). Epstein-Barr virus infection and its role in the expanding spectrum of human diseases. *Acta Paediatr* 87(1): 11-8.
- Okano, M. and T. G. Gross (2001). From Burkitt's lymphoma to chronic active Epstein-Barr virus (EBV) infection: an expanding spectrum of EBV-associated diseases. *Pediatr Hematol Oncol* 18(7): 427-42.
- Olashaw, N. and W. J. Pledger (2002). Paradigms of growth control: relation to Cdk activation. *Sci STKE* 2002(134): RE7.
- Osorio, L. M. and M. Aguilar-Santelises (1998). Apoptosis in B-chronic lymphocytic leukaemia. *Med Oncol* 15(4): 234-40.
- Pagano, M., R. Pepperkok, J. Lukas, V. Baldin, W. Ansorge, J. Bartek and G. Draetta (1993). Regulation of the cell cycle by the cdk2 protein kinase in cultured human fibroblasts. *The Journal of Cell Biology* 121(1): 101-111.

- Pagano, M., R. Pepperkok, F. Verde, W. Ansorge and G. Draetta (1992). Cyclin A is required at two points in the human cell cycle. *EMBO J.* 11: 961-971.
- Palmero, I., A. Holder, A. J. Sinclair, C. Dickson and G. Peters (1993). Cyclins D1 and D2 are differentially expressed in human B-lymphoid cell lines. *Oncogene* 8: 1049-1054.
- Palucka, A. K., R. Scuderi, A. Porwit, S. Jeha, A. Gruber, M. Björkholm, M. Beran and P. Posa (1996). Acute lymphoblastic leukemias from relapse engraft more rapidly in SCID mice. *Leukemia* 10: 558-563.
- Pardee, A. B. (1974). A restriction point for control of normal animal cell proliferation. *Proc Natl Acad Sci USA* 71: 1286-1290.
- Parry, D., D. Mahony, K. Wills and E. Lees (1999). Cyclin D-CDK subunit arrangement is dependent on the availability of competing INK4 and p21 class inhibitors. *Mol Cell Biol* 19(3): 1775-83.
- Peeper, D. S., T. M. Upton, M. H. Ladha, E. Neuman, J. Zalvide, R. Bernards, J. A. DeCaprio and M. E. Ewen (1997). Ras signalling linked to the cell-cycle machinery by the retinoblastoma protein. *Nature* 386(6621): 177-81.
- Polyak, K., M. H. Lee, H. Erdjument-Bromage, A. Koff, J. M. Roberts, P. Tempst and J. Massague (1994). Cloning of p27Kip1, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* 78(1): 59-66.
- Porter, D. C., N. Zhang, C. Danes, M. J. McGahren, R. M. Harwell, S. Faruki and K. Keyomarsi (2001). Tumor-specific proteolytic processing of cyclin E generates hyperactive lower-molecular-weight forms. *Mol Cell Biol* 21(18): 6254-69.
- Porter, P. L., K. E. Malone, P. J. Heagerty, G. M. Alexander, L. A. Gatti, E. J. Firpo, J. R. Daling and J. M. Roberts (1997). Expression of cell-cycle regulators p27Kip1 and cyclin E, alone and in combination, correlate with survival in young breast cancer patients. *Nat Med* 3(2): 222-5.
- Proctor, S. J. (1994). Acute lymphoblastic leukaemia in adults: the case for a strategic shift in study approach. *Br J Haematol* 88: 229-233.
- Pui, C. H. and W. E. Evans (1998). Acute lymphoblastic leukemia. *N Engl J Med* 339(9): 605-15.
- Quelle, D. E., R. A. Ashmun, S. A. Shurtleff, J. Kato, D. Bar-Sagi, M. F. Roussel and C. J. Sherr (1993). Overexpression of mouse D-type cyclins accelerates G1 phase in rodent fibroblasts. *Genes Dev* 7: 1559-1571.
- Raghavachar, A., E. Thiel and C. R. Bartram (1987). Analyses of phenotype and genotype in acute lymphoblastic leukemias at first presentation and in relapse. *Blood* 70(4): 1079-1083.
- Reed, J. C., T. Miyashita, S. Takayama, H. G. Wang, T. Sato, S. Krajewski, C. Aime-Sempe, S. Bodrug, S. Kitada and M. Hanada (1996). BCL-2 family proteins: regulators of cell death involved in the pathogenesis of cancer and resistance to therapy. *J Cell Biochem* 60(1): 23-32.

- Resnitzky, D., M. Gossen, H. Bujard and S. I. Reed (1994). Acceleration of the G1/S phase transition by expression of cyclins D1 and E with an inducible system. *Mol. Cell. Biol.* 14(3): 1669-1679.
- Rivera, G. K., D. Pinkel, J. V. Simone, M. L. Hancock and W. M. Crist (1993). Treatment of acute lymphoblastic leukemia: 30 years' experience at St. Jude Children's Research Hospital. *N Engl J Med* 329: 1289-1295.
- Rosenberg, C. L., E. Wong, E. M. Petty, A. E. Bale, Y. Tsujimoto, N. L. Harris and A. Arnold (1991). PRAD1, a candidate BCL1 oncogene: mapping and expression in centrocytic lymphoma. *Proc Natl Acad Sci USA* 88: 9638-9642.
- Rosenthal, E. T., T. Hunt and J. V. Ruderman (1980). Selective translation of mRNA controls the pattern of protein synthesis during early development of the surf clam, *Spisula solidissima*. *Cell* 20: 487-494.
- Sage, J., G. J. Mulligan, L. D. Attardi, A. Miller, S. Chen, B. Williams, E. Theodorou and T. Jacks (2000). Targeted disruption of the three Rb-related genes leads to loss of G(1) control and immortalization. *Genes Dev* 14(23): 3037-50.
- Sarfati, M., D. Bron, L. Lagneaux, C. Fonteyn, H. Frost and G. Delespesse (1988). Elevation of IgE binding factors in serum of patients with B cell derived chronic lymphocytic leukemia. *Blood* 71: 94.
- Sewing, A., V. Röncke, C. Bürger, M. Funk and R. Müller (1994). Alternative splicing of human cyclin E. *Journal of Cell Science* 107: 581-588.
- Sherr, C. J. (1994). G1 phase progression: cycling on cue. *Cell* 79: 551-555.
- Shimizu, N., A. Tanabe-Tochikura, Y. Kuroiwa and K. Takada (1994). Isolation of Epstein-Barr virus (EBV)-negative cell clones from the EBV-positive Burkitt's lymphoma (BL) line Akata: malignant phenotypes of BL cells are dependent on EBV. *J. Virol.* 68(9): 6069-73.
- Sicinski, P., J. L. Donaher, Y. Geng, S. B. Parker, H. Gardner, M. Y. Park, R. L. Robker, J. S. Richards, L. K. McGinnis, J. D. Biggers, J. J. Eppig, R. T. Bronson, S. J. Elledge and R. A. Weinberg (1996). Cyclin D2 is an FSH-responsive gene involved in gonadal cell proliferation and oncogenesis. *Nature* 384(6608): 470-4.
- Sicinski, P., J. L. Donaher, S. B. Parker, T. Li, A. Fazeli, H. Gardner, S. Z. Haslam, R. T. Bronson, S. J. Elledge and R. A. Weinberg (1995). Cyclin D1 provides a link between development and oncogenesis in the retina and breast. *Cell* 82: 621-630.
- Sinclair, A. J., I. Palmero, A. Holder, G. Peters and P. J. Farrell (1995). Expression of cyclin D2 in Epstein-Barr virus-positive Burkitt's lymphoma cell lines is related to methylation status of the gene. *J Virol* 69: 1292-1295.
- Slingerland, J. M., L. Hengst, C. H. Pan, D. Alexander, M. R. Stampfer and S. I. Reed (1994). A novel inhibitor of cyclin-Cdk activity detected in transforming growth factor beta-arrested epithelial cells. *Mol Cell Biol* 14(6): 3683-94.
- Smith, D. S. and L. H. Tsai (2002). Cdk5 behind the wheel: a role in trafficking and transport? *Trends Cell Biol* 12(1): 28-36.

- Sola, B., G. Roue, F. Duquesne, H. Avet-Loiseau, M. Macro, V. Salaun and X. Troussard (2000). Expression of cyclins D-type in B-chronic lymphoproliferative disorders. *Leukemia* 14(7): 1318-9.
- Solvason, N., W. W. Wu, N. Kabra, X. Wu, E. Lees and M. C. Howard (1996). Induction of cell cycle regulatory proteins in anti-immunoglobulin-stimulated mature B lymphocytes. *J Exp Med* 184: 407-417.
- Sonoki, T., L. Harder, D. E. Horsman, L. Karran, I. Taniguchi, T. G. Willis, S. Gesk, D. Steinemann, E. Zucca, B. Schlegelberger, F. Sole, A. J. Mungall, R. D. Gascoyne, R. Siebert and M. J. Dyer (2001). Cyclin D3 is a target gene of t(6;14)(p21.1;q32.3) of mature B-cell malignancies. *Blood* 98(9): 2837-44.
- Sotillo, R., J. F. Garcia, S. Ortega, J. Martin, P. Dubus, M. Barbacid and M. Malumbres (2001). Invasive melanoma in Cdk4-targeted mice. *Proc Natl Acad Sci U S A* 98(23): 13312-7.
- Soufir, N., M. F. Avril, A. Chompret, F. Demenais, J. Bombléd, A. Spatz, D. Stoppa-Lyonnet, J. Benard and B. Bressac-de Paillerets (1998). Prevalence of p16 and CDK4 germline mutations in 48 melanoma-prone families in France. The French Familial Melanoma Study Group. *Hum Mol Genet* 7(2): 209-16.
- Spruck, C. H., K. A. Won and S. I. Reed (1999). Deregulated cyclin E induces chromosome instability. *Nature* 401(6750): 297-300.
- Standart, N., J. Minshull, J. Pines and T. Hunt (1987). Cyclin synthesis, modification and destruction during meiotic maturation of the starfish oocyte. *Dev Biol* 124: 248-258.
- Strasser, A. (1995). Life and death during lymphocyte development and function: evidence for two distinct killing mechanisms. *Curr Opin Immunol* 7(2): 228-34.
- Suzuki, R., H. Kuroda, H. Komatsu, Y. Hosokawa, Y. Kagami, M. Ogura, S. Nakamura, Y. Kodaera, Y. Morishima, R. Ueda and M. Seto (1999). Selective usage of D-type cyclins in lymphoid malignancies. *Leukemia* 13(9): 1335-42.
- Tanguay, D. A. and T. C. Chiles (1996). Regulation of the catalytic subunit (p34^{PSK-33}/cdk4) for the major D-type cyclin in mature B lymphocytes. *J Immunol* 156: 539-548.
- Teramoto, N., K. Pokrovskaja, L. Szekely, A. Polack, T. Yoshino, T. Akagi and G. Klein (1999). Expression of cyclin D2 and D3 in lymphoid lesions. *Int J Cancer* 81(4): 543-50.
- Tonegawa, S. (1983). Somatic generation of antibody diversity. *Nature* 302: 575-581.
- Trimarchi, J. M. and J. A. Lees (2002). Sibling rivalry in the E2F family. *Nat Rev Mol Cell Biol* 3(1): 11-20.
- Tsai, L. H., I. Delalle, V. S. Caviness, Jr., T. Chae and E. Harlow (1994). p35 is a neural-specific regulatory subunit of cyclin-dependent kinase 5. *Nature* 371(6496): 419-23.
- Tsai, L.-H., E. Lees, B. Faha, E. Harlow and K. Riabowol (1993). The cdk2 kinase is required for the G1-to-S transition in mammalian cells. *Oncogene* 8: 1593-1602.

- Tsujimoto, Y., L. R. Finger, J. Yunis, P. C. Nowell and C. M. Croce (1984). Cloning of the chromosome breakpoint of neoplastic B cells with the t(14;18) chromosome translocation. *Science* 226(4678): 1097-9.
- Uchimaru, K., T. Taniguchi, M. Yoshikawa, S. Asano, A. Arnold, T. Fujita and T. Motokura (1997). Detection of cyclin D1 (bcl-1, PRAD1) overexpression by a simple competitive reverse transcription-polymerase chain reaction assay in t(11;14)(q13;q32)-bearing B-cell malignancies and/or mantle cell lymphoma. *Blood* 89(3): 965-74.
- Waga, S., G. J. Hannon, D. Beach and B. Stillman (1994). The p21 inhibitor of cyclin-dependent kinases controls DNA replication by interaction with PCNA. *Nature* 369(6481): 574-8.
- Wagner, E. F., M. Hleb, N. Hanna and S. Sharma (1998). A pivotal role of cyclin D3 and cyclin-dependent kinase inhibitor p27 in the regulation of IL-2-, IL-4-, or IL-10-mediated human B cell proliferation. *J Immunol* 161(3): 1123-31.
- Walczak, H. and P. H. Krammer (2000). The CD95 (APO-1/Fas) and the TRAIL (APO-2L) apoptosis systems. *Exp Cell Res* 256(1): 58-66.
- Van Parijs, L. and A. K. Abbas (1998). Homeostasis and self-tolerance in the immune system: turning lymphocytes off. *Science* 280(5361): 243-8.
- Wang, T. C., R. D. Cardiff, L. Zukerberg, E. Lees, A. Arnold and E. V. Schmidt (1994). Mammary hyperplasia and carcinoma in MMTV-cyclin D1 transgenic mice. *Nature* 369(6482): 669-71.
- Weinberg, R. A. (1995). The retinoblastoma protein and cell cycle control. *Cell* 81: 323-330.
- Wimmel, A., F. C. Lucibello, A. Sewing, S. Adolph and R. Müller (1994). Inducible acceleration of G1 progression through tetracycline-regulated expression of human cyclin E. *Oncogene* 9: 995-997.
- Winston, J., F. Dong and W. J. Pledger (1996). Differential modulation of G1 cyclins and the Cdk inhibitor p27kip1 by platelet-derived growth factor and plasma factors in density-arrested fibroblasts. *J Biol Chem* 271(19): 11253-60.
- Wolowiec, D., L. Ciszak, A. Kosmaczewska, D. Bocko, R. Teodorowska, I. Frydecka and K. Kuliczowski (2001). Cell cycle regulatory proteins and apoptosis in B-cell chronic lymphocytic leukemia. *Haematologica* 86(12): 1296-304.
- Xiong, Y., T. Connolly, B. Futcher and D. Beach (1991). Human D-type cyclins. *Cell* 65: 691-699.
- Xiong, Y., G. J. Hannon, H. Zhang, D. Casso, R. Kobayashi and D. Beach (1993). p21 is a universal inhibitor of cyclin kinases. *Nature* 366(6456): 701-4.
- Xiong, Y., J. Menninger, D. Beach and D. C. Ward (1992). Molecular cloning and chromosomal mapping of CCND genes encoding human D-type cyclins. *Genomics* 13: 575-584.
- Ye, X., C. Zhu and J. W. Harper (2001). A premature-termination mutation in the Mus musculus cyclin-dependent kinase 3 gene. *Proc Natl Acad Sci U S A* 98(4): 1682-6.

- Yew, P. R. (2001). Ubiquitin-mediated proteolysis of vertebrate G1- and S-phase regulators. *J Cell Physiol* 187(1): 1-10.
- Yu, Q., Y. Geng and P. Sicinski (2001). Specific protection against breast cancers by cyclin D1 ablation. *Nature* 411(6841): 1017-21.
- Zariwala, M., J. Liu and Y. Xiong (1998). Cyclin E2, a novel human G1 cyclin and activating partner of CDK2 and CDK3, is induced by viral oncoproteins. *Oncogene* 17(21): 2787-98.
- Zetterberg, A., O. Larsson and K. G. Wiman (1995). What is the restriction point? *Curr Opin Cell Biol* 7: 835-842.
- Zimmerman, T. S., H. A. Godwin and S. Perry (1968). Studies of leukocyte kinetics in chronic lymphocytic leukemia. *Blood* 31(3): 277-91.
- Zuo, L., J. Weger, Q. Yang, A. M. Goldstein, M. A. Tucker, G. J. Walker, N. Hayward and N. C. Dracopoli (1996). Germline mutations in the p16INK4a binding domain of CDK4 in familial melanoma. *Nat Genet* 12(1): 97-9.