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REVIEW ARTICLE

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Cell cycle and no end

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Abstract Our knowledge about the molecular circuits regulating the duplication of the genetic material and the subsequent division of a cell into two daughter cells has exploded over the last decade. Aberrations in the regulation of the cell cycle belong to the hallmarks of malignant transformation, leading, in turn, to the development of tumours. After introducing the basics of eukaryotic cell-cycle regulation and describing the four phases of the cell cycle (namely, G1, S, G2 and M) in more detail, alterations of key components of the cell-cycle machinery in human malignancies and their functional consequences are presented. Principally, deregulation of the cell cycle can be caused by unrestricted activity of cell-cycle promoting factors (many oncogenes fall into this class) or by inactivation of inhibitory factors (many tumour suppressor genes belong to this class). Both types of deregulation have been described in human tumours and are discussed in detail. Perspectives concerning the translation of this knowledge into daily routine practice and future applications are discussed at the end. The molecular mechanisms of actual cell division (sister chromatid segregation and cytokinesis) are mentioned only briefly.

Keywords Cancer cell cycle · Cyclin · Cyclin-dependent kinases · CDK-inhibitors · Cell-cycle checkpoints · Cell-cycle deregulation

Introduction

A closer look at the cell cycle was suggested 10 years ago, as the importance of defects in the cell-cycle regulatory machinery accomplishing DNA replication and cell division for the development of malignant neoplasia was just emerging [43]. Until then, deregulation of proliferation-stimulating signals and the molecules transducing these signals from the cell surface to the nucleus were primarily in the spotlight. Since then, our knowledge regarding mechanisms regulating the onset of DNA replication and subsequent cell division has exploded in such a way that comprehensive coverage is no longer possible. Whole books and conferences are now dedicated to a topic not so long ago considered to be a somewhat obscure field, primarily dealing with yeast cells and mutant yeast strains having defects in cell-cycle regulation [63]. In 2001, the Nobel prize for physiology and medicine was jointly awarded to Leland H. Hartwell, R. Timothy Hunt and Paul M. Nurse for their discoveries of "key regulators of the cell cycle", acknowledging the importance of molecular insights into the cell cycle to molecular biology and medicine in general.

The growth in knowledge during the past 10 years is best illustrated by the fact that several key molecules now in the focus of cell-cycle research and also at the heart of molecular cancer research were not yet discovered 10 years ago. To name just a few very prominent examples: the $p15^{INK4b}$ and $p16^{INK4a}$ polypeptides and the corresponding genes were first described in 1993 and 1994, respectively [27, 86]. The $p21^{CIP1}$ gene was also identified in 1993 [28, 107], followed by $p27^{KIP1}$ the next year [69, 99]. The $p57^{KIP2}$ gene was cloned in 1995 [47]. The peculiar structure of the $p14^{ARF}/p16^{INK4a}$ locus was also elucidated during 1995 [54, 70, 96].

The importance of deregulated cell-cycle control in the development and progression of malignant neoplasia became so obvious and the examples of affected genes so numerous that cancer was sometimes called a "cell-cycle disease" [4]. But the scenario is more complex, since a transformed cell has to overcome several built-in control mechanisms, such as apoptosis and immune surveillance and has to be supported by neo-angiogenesis [26]. The ability to evade apoptosis is particularly important, as continuous stimulation of proliferation usually activates the apoptotic program and eliminates

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these cells from the body [26]. The mechanisms inducing apoptosis and the pathways regulating cell cycle entry and progression are closely interconnected via key regulatory molecules, such as p53, which are involved in regulating both processes [51, 102].

The cell-cycle regulatory machinery is remarkably conserved during evolution from yeast to humans. This allows the substitution of proteins in yeast by their human counterpart and vice versa in experimental settings. Nevertheless, there exist important species-specific differences in the regulation of the cell-cycle entry and progression. These differences have to be taken into account if results from animal models are to be transferred to human pathology [23].

Overview of the normal cell cycle

"Cell cycle" or "cell division cycle" denotes the orderly sequence of events by which a cell duplicates its genetic material (the chromosomes) and divides into two identical daughter cells. It is divided into four distinct phases: in the S phase ("synthesis phase"), the genetic material is duplicated faithfully once and only once, and, in the M phase ("mitosis phase"), the duplicated chromosomes are distributed equally to the two daughter cells. The phases in between were simply named G1 ("gap-1") and G2 ("gap-2"), G1 preceding the S phase and G2 the M phase. Far from being just "gaps", important regulatory mechanisms act during G1 and G2. When a cell does not reach its homeostatic size, does not get the necessary signal for proliferation and is protected by a specific anti-mitogenic signal or terminally differentiated, then it withdraws from the cycle at the early G1-stage into a non-dividing, quiescent or resting stage termed as G0. Most cells in the body of adults are maintained in this G0 stage [52, 57].

The transition from one cell-cycle phase to the next is executed by different classes of cellular proteins (Fig. 1). Cyclin dependent kinases (CDKs), a family of serine/threonine protein kinases that are activated in a cell-cycle stage-specific manner, are the main engines that drive the cell cycle forward [52, 58, 103]. As indicated by the name, association with a regulatory subunit, called cyclin, is an absolute requirement for the kinase activity of the CDKs [16]. Cyclins form a family of closely related proteins that appear and disappear during the cell-cycle phases in a strictly controlled "cyclic" pattern (from which their name was derived) [89]. In mammals, 16 cyclins and 9 CDKs have been identified thus far, but not all of them have a proven function in cell-cycle regulation [33]. Cyclin C, H, K and T, for example, are all structurally similar to the "bona fide cell-cycle cyclins" A, B, D and E, but function primarily in the regulation of basal transcription as components of the RNA polymerase II holoenzyme [33]. The cyclic appearance and disappearance is mediated by transcriptional activation of the cyclin genes and ubiquitin-mediated degradation of the proteins, respectively. In addition to the availability of a particular cyclin, the activities of the different CDKs are also regulated by binding of CDK-inhibitors (CKI), as well as phosphorylation dephosphorylation events [16]. The functional consequences of phosphorylation depend on the particular residue targeted by the kinase. Phosphorylation of the T-loop threonine (T174 in CDK4, T161 in CDK1, T160 in CDK2) by CDK-activating kinase (CAK) activates the CDKs while dephosphorylation of these amino acids by the CDK-associated protein phosphatase has an inhibitory effect. In contrast, phosphorylation of threonine 14/tyrosine 15 residues by WEE1-like kinases inactivate CDKs while dephosphorylation of these amino acids by CDC25 activates the CDKs [45, 52].

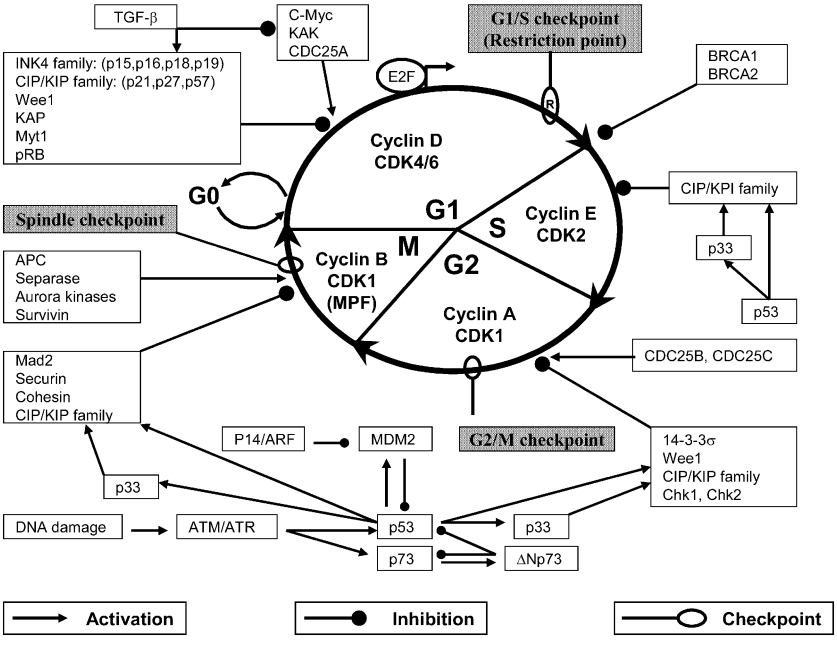

Fig. 1 Negative and positive regulators of the normal cell cycle. Signals promoting and inhibiting the different phases of the cell cycle as well as checkpoints monitoring the proper completion of every phase of the cell cycle are indicated. In the centre of the cycle, the CDK/cyclin complexes driving the respective phase are shown. For details, see the text

In order to strictly control the proper progression of the cell cycle, mammalian cells have also developed a number of regulatory pathways, collectively termed as "cell-cycle checkpoints" (Fig. 1). Checkpoints control the order and timing of cell-cycle transition and ensure that critical events, such as DNA replication and chromosome segregation, are completed accurately. They serve as a brake to pause the cycle in case of DNA damage or errors made in the process [16, 29, 52, 68]. When one cell cycle event has not been successfully completed, checkpoints will delay progression until the step is correctly accomplished, and only then they will relieve the arrest to allow the cell to move to the next phase. In addition to arresting defective cell cycles, checkpoints also mediate repair of DNA damage [64]. Defects in cell-cycle checkpoint pathways result in genomic instability and have been implicated in the transformation of normal cells into cancer cells [16].

Gap-1 phase

Cells respond to extracellular proliferative stimulation only during the G1 phase (and also in G0). Therefore, G1 is the most common target of mitogenic (cell-cycle entry or progression) or anti-proliferative (cell-cycle arrest or exit) signals. Reacting to signals from the extra- or intracellular environment, cells decide either to start a new round of cell division or withdraw from the cell cycle to become quiescent or terminally differentiated [15, 71]. The final commitment to proceed with the cell cycle is made near the end of the G1 phase and is termed the "G1/S transition checkpoint" or "restriction point" (R-point). This represents a "point of no return" because, beyond this checkpoint, cells no longer respond to external signals and proceed with the cycle until completion [90].

In G0 and early G1, the activity of essentially all CDKs (the main cell-cycle engines) is suppressed by the combined action of high CKI activity and low cyclin levels [90]. In the absence of active CDK, the retinoblastoma protein (pRb) stays bound to the E2F, which activates the transcription of genes important for DNA replication only when free from the inhibitory pRb protein [57]. To repeat an already familiar theme: "E2F" is also a family of closely related proteins.

Upon the appropriate extracellular signal stimulating proliferation, D-type cyclins start to accumulate, both due to increased expression as well as reduced proteolysis, indicating a direct link between extracellular stimuli and the cell-cycle machinery [57]. Binding of Cyclin D to CDK4 and CDK6 forms the partially active CDK4/6–Cyclin D complex that later becomes fully active through phosphorylation by CAK [103]. The fully active CDK4/6–Cyclin D holoenzyme phosphorylates pRb and leads to the release of E2F transcription factors, which then, in turn, transcribe many genes that encode proteins required for S-phase entry. Active CDK2/Cyclin E and CDK4/6–Cyclin D holoenzymes together inactivate pRb completely and allow the induction of more E2F-responsive genes that are needed to drive cells through the G1/S transition and to initiate DNA replication [52, 91]. The increase in E2F transcriptional activity further induces more CDK2/Cyclin E as a positive feedback loop. Furthermore, CDK2/Cyclin E facilitates cell-cycle progression through induction of the degradation of inhibitory factors like Hct1 and p27 [57, 90].

In the absence of the appropriate mitogenic signals as well as in the presence of anti-proliferative signals (like transforming growth factor-β) or defective DNA, the G1/S checkpoint is activated and prevents cell-cycle progression. The two families of CKI that serve as effectors of the G1 checkpoint are the INK4 family ($p15^{INK4b}$, $p16^{INK4a}$, $p18^{INK4c}$ and $p19^{INK4d}$) and the CIP/KIP family ($p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$). The INK4 family members function only at G1 to inhibit CDK4/6, but the CIP/KIP family serves as CKI in all the four cell-cycle phases [91].

Synthesis phase

S phase is the stage of the cell cycle in which DNA replication and precise duplication of chromosome occurs. It starts when the proteins required for DNA replication reach a sufficient level. Most importantly, during S phase, the cell has to ensure that the chromosomes are replicated once and only once and that restart of DNA replication does not commence before cell division is finished properly. In order to enable replication of the entire genome in a reasonable timeframe, replication in eukaryotes is initiated at multiple (several hundreds to thousands) sites of the chromosomes simultaneously [39]. The re-replication of DNA before proper completion of cell division is prevented by the so-called "replication licensing system". This regulatory system initiates the formation of a pre-replicative complex at every start point of replication. At first, an "origin recognition complex" is established, which is subsequently joined by cdc6/18, cdt1 and the Mcm proteins ("minichromosome maintenance"). Mcm2–7 function as helicases that unwind the DNA ahead of each replication fork. This interaction between Mcm proteins and the origins of replication is a prerequisite for initiation of DNA synthesis, and displacement of the Mcm2–7 during DNA replication prevents re-start of replication [7]. Like in G1, the licensing process and progression during S phase is strictly regulated by CDK activities. Loading of the MCM complex on to chromatin is only allowed when CDK activity is at very low levels, that is, at the end of the M and beginning of the G1 phase [39, 60]. Phosphorylation of components of the DNA replication machinery by CDK2/Cyclin A is important for initiation of DNA replication. Activation of protein kinases is believed to result in changes in the pre-replication complex (pre-RC) that lead to binding of Cdc45 to the Mcm complex and unwinding of the origin of replication [39, 60]. Cyclin A starts to accumulate during S phase and is abruptly destroyed via ubiquitin-mediated proteolysis before meta-

phase [108]. The synthesis of Cyclin A is activated by E2F, but as a negative feedback loop, E2F activity is inhibited by CDK2/Cyclin A via phosphorylation of the E2F heterodimerisation partner DP1 [33]. After complete duplication of all the chromosomes, the cell cycle enters the second gap phase.

Gap-2 phase

Cells at G2 contain replicated chromosomes consisting of two sister chromatids. At this stage of the cell cycle, cells check if all the genetic material and cellular structures, such as centrosomes, are properly duplicated before the actual process of cell division starts. Damage to the DNA and/or incomplete duplication during the synthesis phase triggers checkpoint pathways that initiate cell-cycle arrest in the G2 phase. In case of DNA damage, ATM (ataxia telangiectasia mutated)- and ATR ("ATM and Rad3-related")-dependent signals induce cell-cycle arrest via inhibition of CDK1 (also called CDC2). In response to genotoxic stress caused by ultraviolet light or ionising radiation, the ATR and ATM signalling pathways are activated, which then leads to activating phosphorylation of human checkpoint kinases (Chk1 and Chk2) [12, 76]. Chk1 and Chk2 induce inhibitory phosphorylation of the phosphates CDC25. This phosphorylation event also creates a binding site for a protein called 14–3-3σ, thereby further inhibiting the function. (The name "14–3-3" is derived from the numbering of the fractions in which this protein was originally discovered). This activity of Chk1 and Chk2 keeps CDK1 in an inactive state and prevents entry into mitosis [103].

In addition to the already familiar theme of regulation by phosphorylation/dephosphorylation and synthesis/degradation, the activity of certain molecules is regulated by their intracellular localisation. A prominent example is the CDC25C phosphatase, which normally resides in the nucleus driving the cell cycle forward by dephosphorylation of the inactive CDK1/Cyclin B holoenzyme (as discussed below). Inhibition of CDC25C activity is a key target and occurs in two ways as described above: by phosphorylation and by binding to 14–3-3σ. Formation of the CDC25C/14–3-3σ complex leads to the export of CDC25C into the cytoplasm, where it cannot exert its normal function [1, 20, 95]. Similarly, 14–3-3σ also binds to CDK1/Cyclin B complex and sequesters it in the cytoplasm to maintain a G2 arrest [64].

In addition to its role in G1 arrest, $p21^{CIP1}$ also plays an important role inducing G2 arrest via blocking the interaction between CDC25C and proliferating cell nuclear antigen (PCNA) [1, 38, 95]. *14–3-3σ* and *$p21^{CIP1}$* are direct target genes of transcriptional activation by p53, thereby representing molecular links between the "p53 pathway" (see below) and the cell-cycle machinery [45, 95].

Mitotic phase

The M phase, which combines mitosis (segregation of the cellular components) and cytokinesis (the final division of the cell into two) is the most dynamic phase of the cell cycle. Entry into mitosis is induced by increased activity of CDK1/Cyclin B holoenzyme, also known as MPF ("mitosis promoting factor"). MPF is regulated through inactivating phosphorylation by two other kinases named Myt1 and Wee1. Dephosphorylation of MPF is the rate-limiting step for entry into mitosis and is achieved by at least two phosphatases, CDC25B and CDC25C [45]. Activated MPF phosphorylates numerous substrates, including motor and microtubule-binding proteins that are important for chromosome condensation, nuclear envelop breakdown, spindle assembly and centrosome separation [59].

Sister chromatid separation and exit from mitosis are controlled by anaphase-promoting complex ("APC"), a ubiquitin-protein ligase that targets key proteins for proteolysis [79]. Sister chromatids are bound together at the kinetochore by a protein called "cohesin" and separation occurs only when "separase" (a protease) cleaves the cohesin off. For this to happen, separase must first be liberated from its inhibitor, "securin", which is destined to degradation by active APC [59]. Another checkpoint, the so-called "mitotic checkpoint" or "spindle-assembly checkpoint", prevents entry into anaphase (segregation of sister chromatids) until both kinetochores of every duplicated chromatid pair have attached correctly to spindle microtubules [59, 79]. The presence of even a single unattached kinetochore leads to the activation of Mad2, which binds transiently to loose unattached kinetochores. This binding event activates Mad2 in order to inhibit APC, thereby preventing the transition from metaphase to anaphase [59, 79]. Proper bipolar attachment of the kinetochores leads to dephosphorylation and relocalisation of Mad2, reiterating the theme of "regulation by localisation". Once the inhibition of APC is abolished, mitosis can resume and the cell cycle can be finished [79].

Cell cycle in cancer

Aberrant activation of the cell cycle can be achieved by induction of positive regulators (often encoded by proto-oncogenes) or through inactivation of negative regulators (often encoded by tumour suppressor genes). Induction of positive regulators is caused by overexpression or mutations leading to permanent protein activity. Inactivation of repressors is caused by deletion, mutation or promoter hypermethylation. All mechanisms can be found in human cancer (Fig. 2).

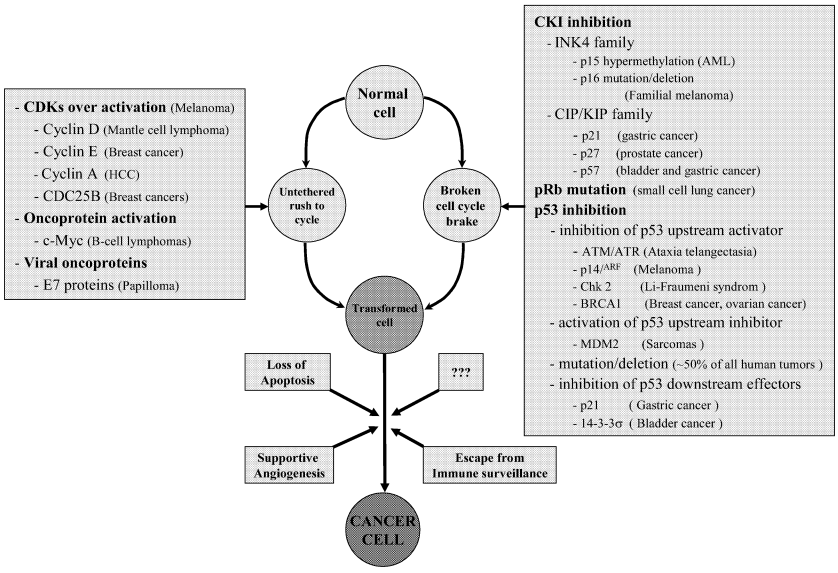

Fig. 2 Deregulation of the cell cycle in cancer. Upregulation of cell-cycle activators and downregulation of cell-cycle inhibitors are both involved in promoting the transformation of a normal cell into a continuously proliferating cell, which is independent of growth-promoting signals and resistant to growth-inhibiting signals. When this transformation is supported by other mechanisms, such as angiogenesis as well as evasion of apoptosis and immune surveillance, it will create the clonogenic malignant cell. For every alteration of cell-cycle regulators, only one example of an associated human malignancy is given. The two-dimensional representation is clearly simplifying the complex interdependence of all participating factors. For further details, see the text and references therein

Activation of cell-cycle promoters

Overexpression of Cyclin D1 caused by gene amplification or aberrant activation of protein synthesis is frequently found in several human tumours. It occurs in more than 90% of mantle cell lymphoma, 60% of breast carcinomas, 40% of squamous cell carcinomas of the head and neck, 40% of colorectal cancers, 20% of prostate cancers and also frequently in lung cancer [24, 67, 94]. Aberrant activation of the *cyclin D1* gene can be induced by chromosomal rearrangement, such as translocation and inversion, which could bring this gene under the influence of a strong promoter or enhancer. t(11;14)(q13;32) and inv(11)(p15;q13), for example, lead to *cyclin D1* activation and are associated with B-cell lymphomas and parathyroid adenomas, respectively [24, 33]. Although not that common, overexpression of Cyclin D2 and D3 has also been reported in some tumours [33]. Deregulation of another G1/S cyclin, Cyclin E, has also been associated with different tumours, and its overexpression is demonstrated to be a powerful predictor of breast cancer outcome [10, 25, 82]. A hyperactive lower molecular weight isoform of Cyclin E that is capable of driving the G1/S transition more efficiently and is overexpressed in breast cancer cells has also been recently identified [32, 46]. Deregulated expression of Cyclin E also induces chromosomal instability, thereby contributing to tumourigenesis [93]. Increased expression of Cyclin A has also been detected in many types of human cancers. In hepatocellular carcinoma, for instance, higher expression of Cyclin A has been found in up to 80% of cases [108]. Despite the strong association between overexpression of several cyclins with the development and progression of tumours, molecular alterations of their binding partners (the CDKs) have been found, thus far, only rarely in cancer. Overexpression of CDK4 and CDK6 as well as constitutively active mutants of these kinases has been reported in some human tumours [46, 95, 104].

c-*Myc* is a potent proto-oncogene that encodes a transcription factor and can promote cell proliferation. It responds to growth-promoting signals in G1 phase by activating the transcription of genes that induce cell-cycle progression, such as *cdc25A*, *cyclin D1*, *D2*, *E*, *A*, *CDK1*, *CDK2*, *CDK4* and *E2F* [32, 45, 46, 103]. Elevated c-Myc expression due to translocation juxtaposing the c-*myc* gene with the immunoglobulin gene enhancer results in B-cell tumours [34]. Overexpression of the c-Myc protein can also be achieved by gene amplification, an alteration frequently seen in breast cancer, which could also serve as a prognostic marker in this malignancy [78].

Overexpression of MDM 2 acts as a positive stimulation of the cell cycle because it antagonises the action of the cell-cycle "brake" p53 (discussed below). Increased expression has been reported in haematological and epithelial malignancies and represents an alternative mechanism to p53 inactivation [103]. Again, this overexpression is mainly caused by an increase in gene copy number.

p73 belongs together with p63 to the family of p53-related proteins, which all are involved in cell-cycle arrest and induction of apoptosis (discussed below). Interest-

ingly, the *p73* gene (first identified in 1997) [35] encodes an N-terminally truncated isoform, called ΔNp73, which acts as a p53 and p73 antagonist. Since the expression of ΔNp73 is induced by p53 and p73, this creates a peculiar negative feedback-loop. Overexpression of ΔNp73 in neuroblastoma turned out to be a new marker for poor prognosis, independent of established prognostic factors, such as tumour stage or N-Myc amplification [56].

The family of CDC25 phosphatases represents another promoter of cell-cycle progression, mainly acting through activation of the CDKs. Overexpression of these phosphatases, particularly of CDC25B, has been observed in 32% of primary breast cancers [103].

Potentially, tumourigenic DNA viruses encode proteins, which also target activation of the cell cycle via pRb hyperphosphorylation [13, 15]. Examples are the adenovirus E1A protein, SV40 T-antigen and HPV protein E7. Although activation of the E2F family of transcription factors is the ultimate target in both normal as well as malignant transformed cells, thus far only circumstantial evidences for molecular alterations of *E2F* genes in human tumours have been described [33].

Inactivation of cell-cycle checkpoints

Similar to the activation of cell-cycle promoters, defects in cell-cycle checkpoints lead to uncontrolled proliferation and could result in malignancy (Fig. 2). In general, two pathways are involved in the negative regulation of the cell cycle: the "Rb pathway" and "p53 pathway" [77]. The "Rb pathway" includes the pRb protein and the two families of CKI (the INK4-family and the CIP/KIP-family). All CKIs inhibit activation of the various CDKs and prevent phosphorylation of *pRb*. The rate of G1/S transition and, therefore, the cell cycle depends on the rate of *pRb* phosphorylation by the CDK4/6–Cyclin D and CDK2/Cyclin E complexes [14, 24]. It is, therefore, not surprising that approximately 90% of human cancers have abnormalities in at least one component of the Rb pathway [24].

Abnormalities in the $p16^{INK4a}$ gene, such as inactivating mutations and deletions, are the second most frequent genetic aberration in human cancers next to defects of the *p53* gene. They occur in a wide range of haematological and epithelial malignancies [14, 45, 87]. The gene encoding the second member of the INK4 family of CKI, namely $p15^{INK4b}$, is located adjacent to the $p16^{INK4a}$ gene at 9p21 and, therefore, a deletion at this region sometimes affects both genes [45, 73]. However, genetic alterations, such as mutations and deletions affecting the $p15^{INK4b}$ gene alone, are rare. Instead, inactivation of $p15^{INK4b}$ occurs mainly through an epigenetic abnormality: promoter hypermethylation has been observed in many haematological malignancies and occurs in up to 75% of acute myeloid leukaemia cases, representing the most frequent abnormality in this malignancy [2, 98, 105]. In acute promyelocytic leukaemia, hypermethylation of the $p15^{INK4b}$ gene has been identified as new prognostic marker for disease-free survival. Also, the $p16^{INK4a}$ gene is epigenetically inactivated in several human tumours [31, 87].

Members of the second CKI family, $p21^{CIP1}$, $p27^{KIP1}$ and $p57^{KIP2}$, share a common N-terminal domain for binding and inhibition of the different CDKs. Therefore, unlike the INK4 family, their effect is not limited to G1/S transition [14]. Downregulation of $p27^{KIP1}$ protein has been observed in human tumours, such as breast, prostate, gastric, lung, skin, colon and ovarian cancers [25, 45], and is also an important marker for cancer progression and poor survival in several malignancies [25, 50]. The $p21^{CIP1}$ gene is a transcriptional target of the p53 protein, with a prominent role in G1/S as well as G2/M arrest in response to DNA damage [45]. Although the critical cell-cycle regulatory role of $p21^{CIP1}$ is not yet strongly supported by a long list of tumour-related abnormalities (as is the case for, e.g. $p16^{INK4a}$ or *cyclin D1*), its key role as a downstream effector of p53 makes it a hot spot of current investigations. As a CKI and as a target gene of p53, $p21^{CIP}$ represents also a direct molecular link between the Rb pathway and the p53 pathway.

Significantly higher survival rate is reported in p53-negative gastric cancer patients with $p21^{CIP1}$ expression than those without $p21^{CIP1}$ [45]. Similarly, a recent study indicated significant correlation between downregulation of $p21^{CIP1}$ with poor prognosis in human gastric cancer [85]. Whether hypermethylation of the $p21^{CIP1}$ gene is a prognostic marker in certain haematological malignancies is still under debate [8, 74, 88].

The above-mentioned transcriptional activation of the $p21^{CIP}$ gene is modulated by $p33^{ING1b}$, which directly interacts with the p53 protein. $p33^{ING1b}$ is encoded by one of several splice-isoforms produced by the *ING1* gene, founding member of the newly discovered "inhibitors of growth" (ING) family. These genes have been implicated in restricting cell growth and proliferation, induction of apoptosis, maintenance of genomic stability and modulation of cell-cycle checkpoints, all classical features of tumour suppressor genes [61].

The third member of the CIP/KIP family of CKIs, $p57^{KIP2}$, is encoded by an imprinted gene that is only expressed from the maternal allele. Decreased expression of $p57^{KIP2}$ has been observed in bladder carcinoma and gastric cancer [45, 65]. Together with the other genes located in the imprinted domain at 11p15.5, *IGF-2* and *H19*, loss of $p57^{KIP2}$ has also been associated with two familial cancer syndromes (Beckwith Wiederman syndrome and Wilms tumor) [33, 45]. In addition to blocking CDK activity, $p21^{CIP1}$ and $p57^{KIP2}$ inhibit proliferation through binding and inactivation of PCNA, which is an auxiliary factor of DNA polymerase [14, 66].

In addition to pRb and its interaction partners, the p53 pathway is of uppermost importance for cell-cycle regulation. It consists of several regulator and effector molecules of the "leading actor", p53. The *p53* gene is mutated in more than 50% of human cancers and is the most frequent genetic alteration associated with malignancy [77]. Furthermore, inactivation of this pathway can

occur via defects in upstream or downstream regulators as seen in several human cancers (Fig. 2). In general, tumours with intact *p53* have a better prognosis and a better response to therapy as compared with those with defective *p53* [77, 103].

The long-sought-after relatives of p53 are p63 and p73 [109]. Despite striking sequence similarities and conserved functional domains, these three proteins exert most probably quite different functions in the cell. The picture is complicated by the fact that p63 and p73 encode several splice-isoforms with partially opposing functions (e.g. ΔNp73, discussed above). Full-length p73 induces cell-cycle arrest and apoptosis, as does p53, but has additional functions in inflammatory response and neurogenesis [56], whereas p63 seems to be most important for proper function of epithelial stem cells [100].

$p14^{ARF}$ is one of the upstream regulators of *p53,* encoded by a gene that shares two common exons with $p16^{INK4a}$ but is translated in an alternative reading frame, creating a completely different protein (therefore the suffix "ARF") [70]. As an upstream activator of *p53* and sharing exons with the $p16^{INK4a}$ gene, the $p14^{ARF}$ gene represents a unique molecular link between the above-mentioned p53 and Rb pathways [87, 90]. It is, therefore, at the centre of intense research activities, and many alterations in human tumours, alone or in concert with $p16^{INK4a}$ or both $p15^{INK4b}$ and $p16^{INK4a}$, have already been described [87].

In addition to its direct activation of genes that drive the cell cycle forward, the already mentioned c-*myc* gene also favours cell-cycle progression indirectly by interfering with the transcription of negative cell-cycle regulators such as *Gadd45*, *Gadd153* or CKIs ($p15^{INK4b}$, $p16^{INK4a}$, $p21^{CIP1}$, and $p27^{KIP1}$) [45, 46, 103].

ATM a protein kinase central to all DNA maintenance responses [1]. Defects in this gene result in the disease ataxia telangiectasia, which is an autosomal recessive disorder characterised by progressive neurological disorders, immunodeficiency and chromosomal instability with a higher predisposition to lymphoid malignancies [92]. Mutation of either *Chk2* or *p53*, both of which act downstream of ATM, results in a genetic disease called Li Fraumeni syndrome, which dramatically predisposes patients to cancer development [5]. The protein product of the breast cancer susceptibility gene *BRCA1* is another substrate of ATM that is involved in cell-cycle checkpoint control and DNA repair [101]. *BRCA1* and its close relative, *BRCA2*, are mutated in approximately half of all familial breast cancer cases [41, 64, 101]. Mutations of these proteins lead to defects in cell-cycle arrest and DNA repair. A reduced expression is also described in sporadic (not inherited) breast cancers [55].

14–3-3σ, one of the seven members of the 14–3-3 family [45], is now considered to represent a new class of CKI. It has been shown to specifically interact with CDK1, CDK2 and CDK4 and can inhibit CDK activities, thereby blocking cell cycle progression [44, 45, 46]. In addition to being a downstream effector of p53, several lines of evidence suggest that loss of 14–3-3σ function correlates with cell transformation. Its expression levels are reduced in several transformed cell lines and primary tumour specimens [44, 45]. Moreover, CpG islands hypermethylation-induced transcriptional silencing of the *14–3-3*σ gene has been reported in breast cancer, gastric cancer and hepatocellular carcinoma [45].

Unequal segregation of chromosomes

Numeric abnormalities in chromosome (aneuploidy) and chromosomal rearrangements are frequently observed in human cancer, and severe karyotypic abnormalities generally are a sign of poor prognosis [30, 43, 75]. Defects in pathways essential for mitotic regulation are likely to be implicated in the cascade of events leading to aneuploidy and neoplasia.

Overexpression of AIM 1 induces defects in cytokinesis and predisposes one to the development of cancer [11]. The Aurora family of serine/threonine kinases is maximally activated in the G2/M phase and comprises key regulators of the mitotic stage of the cell cycle. They are known to be important in centrosome functions, bipolar spindle assembly and chromosomal segregation [36]. All the three members of the mammalian aurora kinase family (Aurora-A, -B, and -C) are reported to be overexpressed in a variety of human cancers. Aurora-A (also known as STK-15, or BTAK) is, for instance, overexpressed in breast, bladder, ovarian, colon and pancreatic cancers and is able to transform cells in culture [6, 36, 59, 84, 97, 110]. The overexpression of Aurora-A correlates with invasiveness and genomic instability in breast cancer and with clinical aggressiveness and aneuploidy in bladder cancer [36]. Centrosome amplification and aneuploidy are also found in cells with ectopic expression of Aurora-A [59]. The chromosomal region in which the Aurora-A gene is located, 20q13.2–13.3, is also often amplified in colon, breast and stomach cancers [83].

Another protein that is expressed in a cell-cycle-dependent manner and could play an important role in chromosomal segregation is survivin. It is a unique member of the inhibitor of apoptosis family, which, in addition to the anti-apoptosis role (as the name implies), also plays a role in the cell cycle. Survivin is specifically expressed at mitosis and forms complexes with various components of the mitotic apparatus, such as centrosomes, microtubules of the metaphase and anaphase spindles and also with the Aurora B [3]. It has also been shown that the kinase activity of Aurora B is stimulated by binding to and phosphorylation of survivin [9]. Dramatic overexpression of survivin has been observed in a wide variety of human tumours, including lung, breast, colon, stomach, oesophagus, pancreas, liver, uterus, ovaries, as well as lymphomas and leukemias [3].

Conclusions and perspectives

The progress in cell-cycle research over the last decade enabled a better understanding of physiological cell-cycle regulation and deregulation in cancer to an extent which only a few could imagine 10 years ago. The cell-cycle field provides a paradigm for the impact of basic research on the development of new diagnostic tests and innovative therapeutic concepts and reagents.

Translation of basic research into the routine practice of histopathological diagnosis has already started. Examples are the demonstration of Cyclin D1 overexpression for the identification of mantle cell lymphoma [19] and the detection of $p16^{INK4a}$ in cervical intraepithelial neoplasia [42]. Both antigens can now readily be detected also in formalin-fixed paraffin-embedded biopsies.

Several proteins with well-defined expression patterns during the cell cycle and well-described molecular function in the cell-cycle machinery are just emerging as new markers for the proliferation status of a given biopsy or even as new prognostic markers. Detection of Cyclin B1 (expressed as a mitotic cyclin only during the G2-M transition) or detection of Mcm2, for instance, seems to be of prognostic significance in squamous cell carcinoma of the oesophagus [37, 62]. Similarly, detection of Cyclin E turned out to be the most important independent predictor of breast cancer outcome, even superior to established proliferation markers [10, 40].

However, the general proliferation marker Ki-67 is still the most widely used and most thoroughly evaluated proliferation marker with proven diagnostic and prognostic power [81]. It labels proliferating cells in all stages of the cell cycle except G0 [22]. Despite the fact that determination of the "proliferation fraction" (percentage of proliferating cells) using this antibody is of uppermost importance for the classification of tumour specimens and prognosis shown in numerous studies, the function of the protein recognised by Ki-67 is not yet well established in molecular terms [17, 80].

Furthermore, immunohistochemical detection of PCNA, an auxiliary protein of DNA polymerase important for DNA synthesis, has been extensively used for quantification of proliferation [18, 49], but its usefulness has been questioned in some studies [81, 106].

As already discussed for some of the cell-cycle regulatory proteins, the cellular localisation as well as phosphorylation status determine biological activity. Therefore, the use of phosphorylation state-specific antibodies will be of great importance in future studies [53].

It should be mentioned that, in the above-cited study of Cyclin E in breast cancer, the occurrence of a low-molecular weight isoform detected by Western blotting was the molecular event with strong predictive power, stressing again that post-transcriptional modifications not detected by many conventional immunohistochemical assays and also not detected by large-scale cDNA microarray screens have to be kept in mind.

Every newly discovered component of the cell-cycle network described above represents a new potential target for therapeutic intervention in case of deregulation. The elucidation of the three-dimensional structure of key components of the cell-cycle regulatory machinery will guide the development of highly specific inhibitors modulating the activity of these molecules. Specific synthetic CDK or proteasome inhibitors represent promising examples [21, 48, 72]. The detailed molecular understanding of the pathways regulating cell-cycle entry and progression will also facilitate the rational design of combination therapies that will maximise therapeutic efficiency and minimise unwanted side effects and development of drug resistance.

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