INVITED REVIEW

The cell cycle and cancer

Gareth H Williams[1,2]* and Kai Stoeber[1,2]

[1] Department of Pathology and Cancer Institute, University College London, UK
[2] Wolfson Institute for Biomedical Research, University College London, UK

*Correspondence to: Gareth H Williams, Department of Pathology, University College London, Rockefeller Building, 21 University Street, London WC1E 6JJ, UK. e-mail: gareth.williams@ucl.ac.uk

Abstract

Deregulation of the cell cycle underlies the aberrant cell proliferation that characterizes cancer and loss of cell cycle checkpoint control promotes genetic instability. During the past two decades, cancer genetics has shown that hyperactivating mutations in growth signalling networks, coupled to loss of function of tumour suppressor proteins, drives oncogenic proliferation. Gene expression profiling of these complex and redundant mitogenic pathways to identify prognostic and predictive signatures and their therapeutic targeting has, however, proved challenging. The cell cycle machinery, which acts as an integration point for information transduced through upstream signalling networks, represents an alternative target for diagnostic and therapeutic interventions. Analysis of the DNA replication initiation machinery and mitotic engine proteins in human tissues is now leading to the identification of novel biomarkers for cancer detection and prognostication, and is providing target validation for cell cycle–directed therapies.

Keywords: biomarker; cell cycle; cancer; DNA replication; MCM; geminin; phosphohistone; mitosis; therapy

Received 17 August 2011; Revised 30 September 2011; Accepted 1 October 2011

No conflicts of interest were declared.

Introduction

The majority of cells in the human body are not cycling and instead reside in 'out-of-cycle' states. A minority of cells are actively cycling (proliferating) and these are located mainly in the stem-transit amplifying compartments of self-renewing tissues, such as epithelia and bone marrow [1]. In contrast, most functional cells have irreversibly withdrawn from the cell division cycle into terminally differentiated states (eg neurones, myocytes or surface epithelial cells of skin/mucosa) or have reversibly withdrawn into a quiescent (G_0) state (eg glial cells, thyroid follicular cells or hepatocytes) [2,3].

The cell cycle has four sequential phases. Arguably the most important phases are S phase, when DNA replication occurs, and M phase, when the cell divides into two daughter cells. Separating S and M phase are two gap phases referred to as G_1 and G_2. G_1 follows on from mitosis and is a time when the cell is sensitive to positive and negative cues from growth signalling networks. G_2 is the gap after S phase when the cell prepares for entry into mitosis [4]. G_0 represents a state when cells have reversibly withdrawn from the cell division cycle in response to high cell density or mitogen deprivation [5]. Alternatively, cells may irreversibly withdraw from the cell cycle into terminally differentiated or senescent out-of-cycle states. Progression through the cell cycle is driven by the cyclin-dependent kinase (CDK) family of serine/threonine kinases and their regulatory partners the cyclins [6]. Cyclin D-CDK4, cyclin D-CDK6 and cyclin E-CDK2 drive G_1 progression through the restriction point, which commits the cell to complete the cycle [7]. S phase is initiated by cyclin A-CDK2, and cyclin B-CDK1 regulates progression through G_2 and entry into mitosis [8].

Progression through each cell cycle phase and transition from one phase to the next are monitored by sensor mechanisms, called checkpoints, which maintain the correct order of events [9]. If the sensor mechanisms detect aberrant or incomplete cell cycle events (eg DNA damage), checkpoint pathways carry the signal to effectors that can trigger cell cycle arrest until the problem is resolved [10,11]. Effector proteins include the CDK inhibitors (CDKIs), which can reversibly halt cell cycle progression. For example, G_1 arrest can be induced through the action of the Ink4 family [INK4A (p16), INK4B (p15), INK4C (p18) and INK4D (p19)] of CDKIs, which inhibit CDK4 and CDK6, or, alternatively, via the Cip/Kip family of inhibitors (p21, p27, p57), which suppress CDK2 activity [12,13].

Deregulation of the cell cycle engine underlies the uncontrolled cell proliferation that characterizes the malignant phenotype. Mitogens release the brakes of cell cycle progression by stimulating G_1–S CDK activities, which trigger the phosphorylation of pRB proteins, leading to disruption of their interaction with

the E2F family of transcription factors. In cancer cells, the pRB brakes are often defective, resulting in E2F-dependent G_1–S gene expression even in the absence of mitogens [14]. This may arise as a result of activating tumourigenic mutations which have been identified in diverse tumours at all levels in the mitogenic signalling pathways from ligands and receptors (eg HER2/ErbB2/neu receptor mutations or *HER2* gene amplification) to downstream signalling networks (eg Ras–Raf–MAPK or PI3K–Akt signalling pathways) and also for the cell cycle-regulated genes themselves (eg *CYCLIND1* and *CDK4* gene amplification) [15–17]. Aberrant signalling promotes activation of CDK–cyclin complexes, which phosphorylate Rb and attenuate its capacity to induce transcriptional repression. The notion that Rb phosphorylation is a convergence point for these oncogenic signalling pathways is consistent with the fact that inactivation of the RB gene by mutation or methylation is a common occurrence in cancer [18]. The inactivation of tumour suppressor genes that encode CDKIs (eg p15, p16 and p27) are also common events in diverse tumour types. This releases the brakes on cell cycle progression, and further abrogation of checkpoint control mechanisms leads to the acquisition of genomic instability, which drives tumour evolution [12].

The cell cycle machinery—a convergence point for oncogenic signalling pathways?

Analysis of the complex and partly redundant upstream signalling networks that control processes such as cell proliferation, differentiation and invasion by genome-wide analysis remains to be proven as a routine tool for clinicopathological assessment. The early studies using microarray-based gene expression profiling led to the identification of potentially powerful prognostic and predictive signatures, suggesting that this technology might soon replace traditional clinicopathological parameters [19–21]. However, subsequent studies have shown that the prognostic and predictive power of microarrays provides only complementary information and cannot be used as a replacement for traditional clinicopathological variables. Disappointingly, the actual performance of prediction rules using gene expression has not been as informative as hoped for many tumour types and the list of genes identified can be highly unstable [22,23]. For instance, assignment of molecular subtype classes of breast cancer based on the analysis of dendrograms obtained with hierarchical cluster analysis has proven subjective, with modest interobserver reproducibility [24]. Whether prognostic signatures will reduce the number of patients undergoing toxic chemotherapy remains unclear. For example, although the MammaPrint 70-gene signature is expected to identify 10–15% of patients who might be spared chemotherapy, results of a recent finalized feasibility study suggest this outcome is overly optimistic [25]. Transcriptomic profiling has also proved constrained as a predictor of therapeutic response. Predictive signatures do not consistently correlate with treatment response and their predictive value has been significantly reduced when applied to validation cohorts [26,27]. Moreover, retrieval of surgical material for microarray analysis, particularly for small tumours, presents a formidable challenge in the routine clinical setting, together with associated cost implications.

The complex nature of these signalling networks also compromises the approach of using targeted therapies. The 'oncogene addiction' theory suggests a tumour will have unyielding dependence on a particular perturbation of a single gene [28]. The reality is that cancer cells are unstable and have many alterations [29]. Hence the cancer circumnavigates the 'specific' target and eludes the targeted therapy [15,30]. This phenomenon is reflected in the largely disappointing results of targeted therapy clinical trials in cancer, where at best there is a change to the natural history of the tumour, but there is still no cure [31–38].

An alternative approach is to focus on the cell cycle machinery, which acts as an integration point for information transduced through upstream signalling networks [3,39,40]. Notably, many of the current most effective neoadjuvant and adjuvant therapeutic interventions in the clinic are cell cycle directed agents (Table 1). A core component of the cell division cycle, the DNA replication initiation pathway has emerged as a target of particular interest over the last decade [3,41–43]. The DNA replication initiation machinery can be regarded as a final and critical step in growth control positioned at the convergence point of complex and branched upstream signalling networks [40]. This component of the cell cycle engine acts as a relay station, connecting growth signalling networks with the initiation of DNA synthesis, and is therefore a potentially attractive diagnostic and therapeutic target [3].

Here we review the recent literature on cell cycle proteins as cancer biomarkers, with particular emphasis on DNA replication initiation factors and mitotic engine proteins. We also discuss the emerging concept of targeting the replication initiation machinery for cancer therapy. We apologize to the many authors whose important contributions we could not cite due to space limitations.

The DNA replication initiation pathway

DNA synthesis is tightly controlled to ensure that replication origins are not 'fired' more than once per cell cycle [44]. This is achieved through a replication licensing system that coordinates DNA replication initiation events at chromosomal origins with cell cycle progression. The licensing machinery is composed of a complex of initiator proteins that bind

Table 1. Cell cycle-targeted therapeutic agents

Agent	Class	Target	Phase affected
5-Fluorouracil	Antimetabolite	Thymidylate synthase	S
Gemcitabine	Antimetabolite	Nucleoside analogue and ribonucleotide reductase	S
Methotrexate	Antimetabolite	Dihydrofolate reductase	S
Irinotecan	Camptothecin	Topoisomerase I	S
Cisplatin	Alkylating agent	DNA interstand crosslinks	S/G_2
Docetaxel	Taxane	Tubulin	M
Paclitaxel	Taxane	Tubulin	M
Vincristine	Vinca alkaloids	Tubulin	M

to, and unwind, the DNA helix at origins prior to the formation of bidirectional replication forks. During late M and early G_1 phase, the licensing proteins ORC, Cdc6, Cdt1 and Mcm2–7 assemble into pre-replicative complexes (pre-RCs), thereby rendering origins 'licensed' for DNA synthesis during S phase [45,46]. The six MCM proteins (Mcm2–7) function as a replicative helicase, unwinding the template DNA, with Cdc6 and Cdt1 acting as clamp-loaders for this ring-shaped heterohexameric complex [47–49]. At the transition from G_1 into S phase, licensed replication origins are 'fired' by the concerted action of CDKs and the Dbf4-dependent Cdc7 kinase [50]. Cdc7 phosphorylates the Mcm2, 4 and 6 subunits, thereby inducing a conformational change that stimulates MCM helicase activity [51–53]. The formation of an active helicase leads to the recruitment of additional factors, including Cdc45 and the four subunit GINS complex, which is also dependent on Cdc7 kinase activity [54–56]. Once activated, the MCM helicase unwinds double-stranded DNA at origins to generate a single-stranded DNA template required to recruit the DNA synthesis machinery containing RPA, PCNA and DNA polymerase α-primase [46]. Following entry into S phase, the licensing system is shut down to prevent re-initiation events at origins that have already been 'fired'. The key event in suppressing relicensing of origins is the inactivation of the MCM loading factor Cdt1 through two mechanisms [57]. First, Cdt1 undergoes cell cycle-dependent proteolysis during S and G_2 [58]. Second, residual Cdt1 is inhibited by the binding of a small regulatory protein called geminin, which is expressed at high levels during the S, G_2 and M phases [59–61].

Defining the proliferative state

Investigation of the DNA replication initiation machinery in different organisms, tissues and cell types has revealed that cell cycle withdrawal and loss of proliferative capacity are linked to a 'shut-down' of the licensing system [3,43,62–64]. During the proliferation–differentiation switch, the MCM clamp loaders Cdc6 and Cdt1 are rapidly down-regulated as cells migrate from the transit amplifying compartment to the functionally differentiated compartment of self-renewing tissues. There is a more gradual down-regulation of Mcm2–7 proteins as cells mature and adopt a fully differentiated phenotype. The conversion of replication origins into an unlicensed state also characterizes the quiescent (G_0) and senescent out-of-cycle states and therefore appears to be a common mechanism by which proliferation is restrained in multicellular organisms [3,63] (Figure 1A, B). Interestingly, the regulation of Cdc6 protein levels appears to coordinate the proliferative capacity of cells during cell cycle withdrawal and re-entry. Its down-regulation triggers loss of proliferative capacity during early engagement of the somatic differentiation programme, while during cell cycle re-entry (G_0–S), CDK phosphorylation of Cdc6 prevents its destruction by the anaphase-promoting complex (APC), thus facilitating the licensing of origins [65,66]. Proliferating cells are characterized by high expression levels of the MCM proteins throughout the cell division cycle, with cyclical binding to origins occurring in late M/early G_1 and displacement from chromatin during S phase [62,67]. Consequently, Mcm2–7 have emerged as novel biomarkers of proliferation. Unlicensed replication origins and absence of CDK activity, on the contrary, characterize the differentiated and G_0 out-of-cycle states and therefore allow such cells to be clearly distinguished from cycling cells in complex and dynamic heterogeneous cell populations [43,62].

DNA replication licensing and cancer

Identification of MCM proteins in pathological specimens using immunodetection methods has been shown to be an accurate and simple method for determining the growth fraction in dynamic tumour cell populations [39,68–70] (Figure 1B). Moreover, Mcm2–7 expression levels are powerful prognostic indicators in diverse tumour types, including cancers of the lung, breast, kidney, bladder, prostate and ovary [71–77]. This finding is consistent with large-scale meta-analysis of cancer microarray data, which identified up-regulation of the MCM2–6 genes as a component of poor prognostic signatures [78]. In most tumour types, the up-regulation of MCM and other licensing proteins is likely to reflect oncogene-driven engagement of the cell division cycle. Indeed, many components of the DNA replication initiation machinery are under E2F transcriptional control (eg Cdc6,

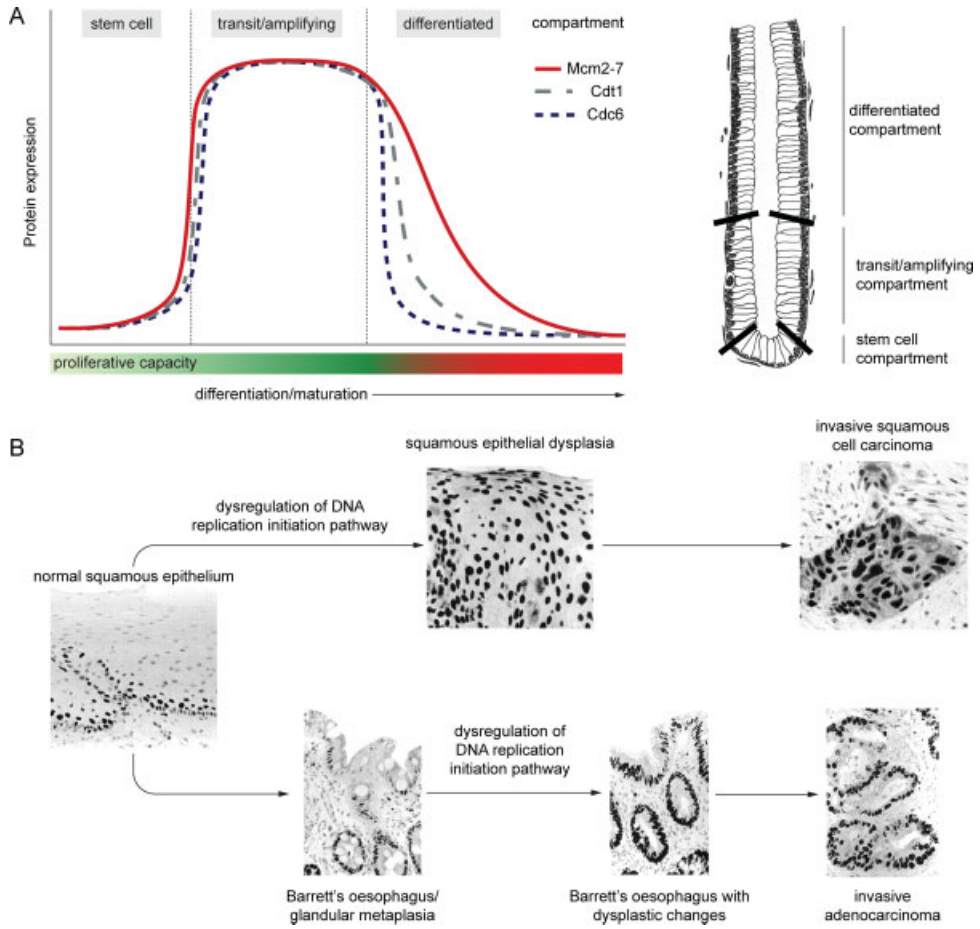

Figure 1. Expression of DNA replication initiation proteins in self-renewing tissues. (A) Schematic showing relative protein expression levels for the initiation proteins Cdc6, Cdt1 (required for loading the Mcm2–7 complex onto chromatin) and Mcm2–7 (replicative helicase) in stem cell, transit/amplifying and differentiated compartments. The schematic drawing of a colonic crypt illustrates this hierarchal organization of self-renewing tissues. The flux of cells through these compartments is continuous; new cells are supplied from the stem cell compartment and their number is multiplied in the transit/amplifying compartment. Cells become functionally competent as they enter the fully differentiated compartment. The stem cell compartment is characterized by low expression of initiation proteins. Cdc6, Cdt1 and Mcm2–7 levels rapidly increase as cells enter the transit/amplifying compartment. There is a gradual down-regulation of Mcm2–7 as cells mature and adopt a fully differentiated functional phenotype. Proliferative capacity, however, is lost at an earlier point in the differentiation programme as cells exit the transit/amplifying compartment and is linked to down-regulation of the loading factors Cdc6 and Cdt1 [62,63,66]. Notably, the arrested differentiation that characterizes cancer, particularly in high-grade tumours, is associated with failure to down-regulate the replication initiation proteins. (B) Spatial organization of Mcm2–7 protein expression in normal oesophageal squamous epithelium and non-dysplastic Barrett's mucosa and disruption of this highly organized spatial arrangement in premalignant dysplasia and invasive cancer [69,96]. In normal squamous epithelium, high expression levels of Mcm2 above a well-defined basal layer fall to undetectable levels in the upper third. In squamous epithelial dysplasia, Mcm2 expression persists to the luminal surface. Invasive squamous cell carcinoma shows high levels of Mcm2 expression. Non-dysplastic intestinal type Barrett's mucosa shows Mcm2 expression in cells of the proliferative zone beneath the mucosal surface. Expression falls away markedly on the mucosal surface. In Barrett's mucosa showing mild dysplasia Mcm2 down-regulation does not occur. Invasive adenocarcinoma shows high levels of Mcm2 expression.

Cdt1, Mcm2–7, Mcm10 and Dbf4) [79–83]. However, deregulation of the licensing system may also be a primary driver of oncogenesis, at least in some tumour types. For example, over-expression of Cdc6 or Cdt1 have been shown to be oncogenic, and deregulated Mcm7 expression has been linked to tumour formation, progression and malignant transformation in animal models [84–90]. Oncogenic mutations in genes upstream of the licensing machinery (eg *RAS, CYCLINE* and *CYCLIND1*) can also impact on tumourigenesis by causing deregulation of the licensing machinery. This may either result in relicensing events or allow cells to enter S phase with insufficient licensed origins (see below), both of which can lead to genomic instability [42].

Replication initiation proteins in cancer detection and screening

In normal self-renewing epithelia (eg found in bladder, cervix, skin, gut and the airways), movement of cells from the stem-transit amplifying to the functionally differentiated compartment is coupled to a shut-down of the licensing system and loss of proliferative capacity [3,43]. Thus, licensing proteins are normally restricted to the proliferative compartment

and are absent from the functional compartment [63] (Figure 1A, B). The important role that repression of the licensing system plays in proliferation control is highlighted by the finding that Cdc6 over-expression sustains the proliferative capacity of differentiating cells [66].

The normal somatic differentiation programme is disrupted in premalignant epithelial lesions, referred to pathologically as dysplasia or intraepithelial neoplasia. These early, non-invasive lesions are characterized by the emergence of cytologically abnormal cells, abrogation of the normal differentiation programme with loss of epithelial polarity, and an increase in the size of the proliferative compartment. Intriguingly, the switch to the dysplastic state is associated with a failure to down-regulate the licensing system, resulting in high MCM protein expression in all epithelial layers, including the surface cells [3,39,68,69]. This expression pattern indicates that the majority of cells in premalignant lesions are locked into the cell division cycle. Notably, only a small proportion of these cells express geminin, a marker for cells in S, G_2 and M phases, showing that the majority of neoplastic cells fail to progress through the cell cycle and therefore reside in a G_1 extended or arrested state.

Deregulation of the Mcm2–7 licensing factors in premalignant and malignant lesions has been exploited in the development of a number of cancer-diagnostic applications. The detection of exfoliated MCM positive cells in body fluids (eg in urine, prostatic secretions, stool samples or gastro-oesophageal aspirates) or active sampling by swabbing or brushing (eg cervical smears or ERCP brushings for pancreaticobiliary tract sampling) provides a sensitive and specific method for the detection of premalignant and malignant lesions in a range of organ systems [39,91–98]. For example, the immunostaining of cervical Pap smears for Mcm2–7 has potential to increase both the sensitivity and specificity of this error-prone test [39,99–101]. Clinical trials are ongoing, combining MCM biomarkers with liquid-based cervical cytology and automated microscopy platforms (eg BD ProEx C/FocalPoint GS Imaging System). Notably, a recent study has shown that primary hrHPV DNA-based screening followed by BD ProEx C triage (antibodies to Mcm2 and Topo2A proteins) represents the optimal cervical screening strategy, resulting in 55% fewer referrals for colposcopy [93]. An alternative method to detect MCM-positive tumour cells in patient samples is to use liquid-phase assays such as ELISA or DELFIA. Clinical studies using this approach have generated encouraging results for diverse tumour types, including the screening of urine sediments for detection of transitional cell carcinoma of the bladder and prostate cancer, and bile aspirates for pancreaticobiliary tract cancer [94,95,97,98]. An MCM-based cancer test (Figure 2) has therefore broad potential clinical utility in cancer detection, tumour surveillance, population screening, monitoring of therapeutic response and prognostication.

Tumour cell cycle phase analysis

Tumour cell cycle kinetics not only impacts on prognostic assessment, but is also of potential importance for predicting response to cell cycle phase specific agents. Prognostic algorithms for many tumour types include a crude measure of their proliferative state, often based on mitotic index and/or Ki67 count (eg Nottingham Prognostic Index for breast cancer, Federation Nationale des Centres de Lutte le Cancer grading system for soft tissue sarcoma) [102,103]. Notably, many of the neoadjuvant and adjuvant chemotherapeutic interventions approved for clinical use include agents targeting either replicating cells (in S phase) or dividing cells (M phase), and will therefore only be effective against cells progressing through the cell cycle. In support of this concept, in breast cancer, high Ki67 levels appear to predict benefit for an adjuvant taxane regime (M phase agent docetaxel) compared with non-taxane regimes [104,105]. However, although Ki67 has emerged as a prognostic marker of potential interest, its introduction into routine clinical practice has been compromised by conflicting data from meta-analysis studies [106]. Moreover, harmonization in methods used to quantify Ki67 levels between laboratories has also proved to be problematic and reported cut-points are highly variable.

Geminin, mitotic kinases and phosphohistone H3 can be used to determine cell cycle position

The analysis of core constituents of the cell cycle machinery provides an alternative method to assess the proliferative state of dynamic tumour cell populations (Figure 3A). As discussed above, expression of the Mcm2–7 proteins allows tumour cells engaged in the cell division cycle to be clearly distinguished from cells residing in out-of-cycle states. Geminin, which prevents relicensing of replication origins after the initiation of DNA synthesis, is only present in cells progressing through S, G_2 and M phases, as are the mitotic engine kinases Plk1, Aurora A and Aurora B [74,107,108]. These three kinases control most mitotic events, including centrosome maturation and separation, chromosome orientation and segregation [8]. Notably, histone H3 is a substrate for the Aurora kinases and is phosphorylated at serine 10 only during the length of M phase [108,109]. Phosphohistone H3 (H3S10ph) is therefore an M phase marker. Hence, multiparameter analysis of these G_1–S and G_2–M regulators, using immunodetection methods, provides a detailed readout of the cell cycle state in complex dynamic tumour cell populations in human tissues [3]. Using this approach to study breast cancer, a complex and highly heterogeneous tumour type with respect to cancer genetics and clinicopathological parameters, has revealed three discrete cell cycle phenotypes [110,111] (Figure 3B). These include: (a) an out-of-cycle state composed predominantly of MCM-negative cells; (b) a G_1-delayed/arrested state composed of cells

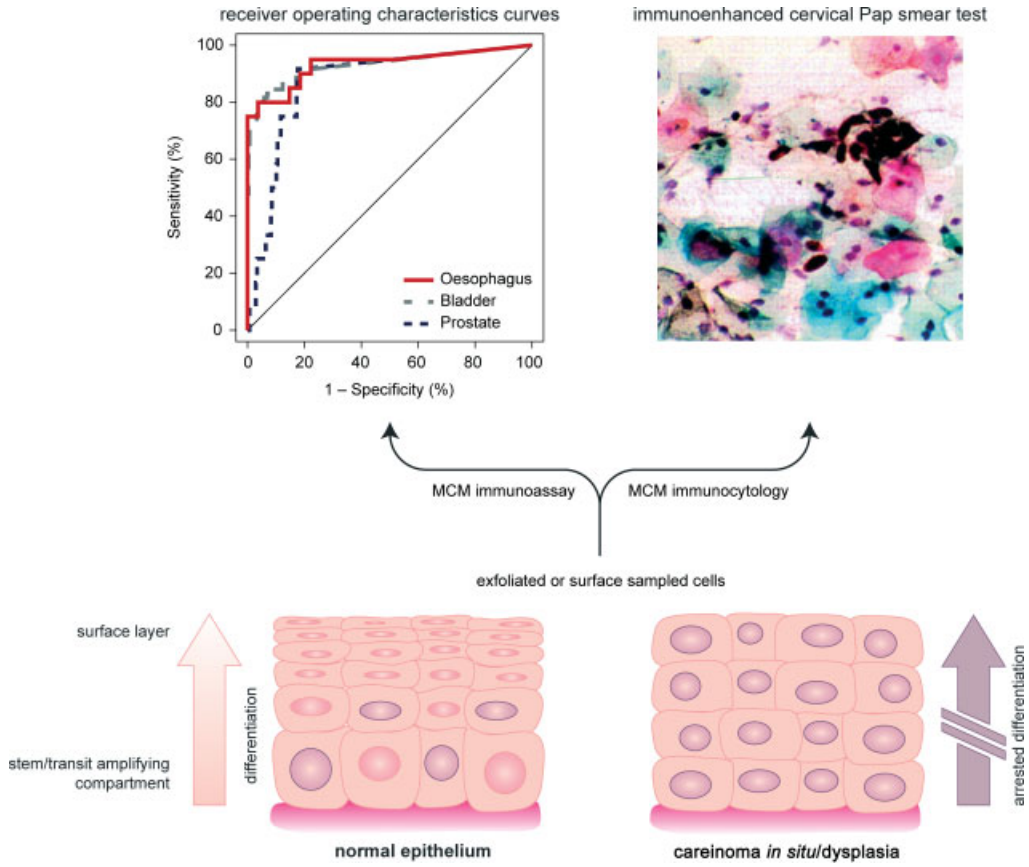

Figure 2. Schematic of the rationale for MCM-based cancer detection tests. Mcm2–7 protein expression in normal epithelium is restricted to the basal stem/transit amplifying compartments and is absent from surface layers as cells adopt a fully differentiated phenotype (MCM expression shown in purple). Superficial cells obtained either through exfoliation or by surface sampling should therefore be negative for Mcm2–7 proteins. In premalignant (dysplastic) epithelial lesions and in malignancy there is an expansion of the proliferative compartment coupled to arrested differentiation, resulting in the appearance of proliferating, MCM-positive cells in superficial layers. Immunodetection of Mcm2–7 protein in exfoliated or surface-sampled cells is thus indicative of an underlying premalignant/dysplastic lesion or malignancy. The receiver operating characteristics curves show the high sensitivity and specificity of MCM-based tests for detection of oesophageal, bladder and prostate cancer [95,96,98], while the detection of (pre)malignant cells in cervical smears is illustrative of the potential for cytology-based testing [39,93].

with high MCM expression but low S–G_2–M phase marker expression (geminin, Plk1, Aurora A) and low M phase marker expression (H3S10ph); and (c) an accelerated cell cycle progression phenotype with cells showing high MCM, S–G_2–M and M phase biomarker expression [110,111].

The cell cycle phenotype is a powerful independent prognosticator in breast cancer and out-performs the gold standard proliferation marker Ki67. In a study of 182 breast cancers, the accelerated cell cycle phenotype had a much higher risk of relapse when compared with the out-of-cycle and G_1-delayed/arrested phenotypes (HR = 3.90, $p < 0.001$) [110,111]. These early proof-of-concept studies, applying the cell cycle phenotype test, are consistent with published gene expression profiling studies showing that conserved tumour expression patterns include many proliferation-associated genes and that increased expression of these so-called 'proliferation signatures' is associated with enhanced malignancy [78,112,113]. It will be of major interest to determine how this simple cell cycle biomarker test, which is highly suited to routine surgical biopsy material, compares with expensive multigene tests such as Oncotype DX [114]. Notably, we have discovered that these discrete cell cycle phenotypes appear to be common to many other tumour types, indicating that the cell cycle biomarker test could be used as a prognosticator for diverse cancer types [115 and unpublished data].

DNA replication initiation factors appear to hold some advantages over the use of the gold standard marker Ki67 for determining the proliferative state of dynamic cell populations in tissue samples. First, the variability in cut-points for Ki67 prognostic scoring partly reflects uncertainty about its function, leading to threshold values being determined empirically. In contrast, the combined use of MCM, geminin and H3S10ph biomarkers provides a cell cycle profile based on the well-characterized biological function of these proteins during the cell division cycle. Second, in our proof-of-principle study the cell cycle biomarker algorithm was able to clearly separate breast cancer into three discrete cell cycle phenotypes, whereas the Ki67 labelling index did not clearly separate between these cell cycle states [110]. Third, these cell cycle biomarkers generate robust, strong nuclear signals in

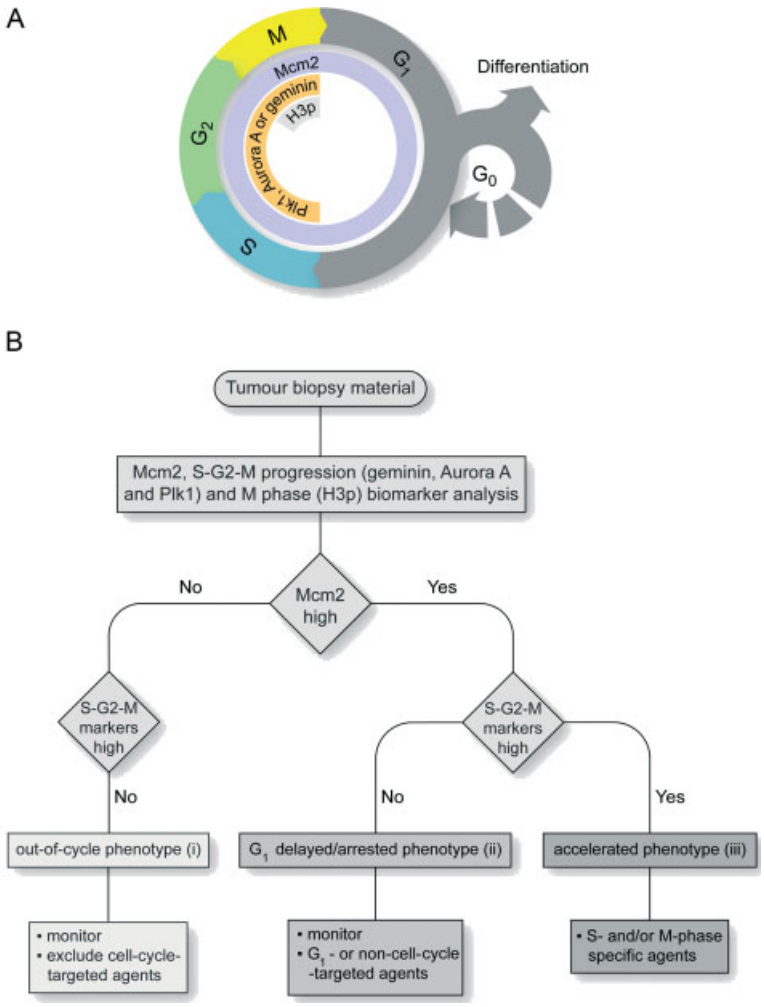

Figure 3. Tumour cell cycle phase analysis. (A) Schematic showing cell cycle phase-specific expression of the biomarkers Mcm2, Plk1, Aurora A, geminin and histone H3 phosphorylated on serine 10 (H3p) in proliferating cells and out-of-cycle states (G_0, differentiation). (B) Three distinct cell cycle phenotypes characterized by differential expression of Mcm2, geminin, Plk1, Aurora A and H3p were found in breast cancer [110,111] and other cancer types [115, and unpublished data]. Prognosis and prediction of response to treatment can be derived from the distinct immunoexpression profile displayed by each tumour. Patients with tumours comprised predominantly of cells with an accelerated cell cycle progression phenotype are more likely to derive benefit from S- and/or M-phase-directed chemotherapeutic agents.

formalin-fixed surgical biopsy material, making them particularly amenable to scoring algorithms developed for digital pathology platforms.

Cell cycle phase analysis as a predictor of therapeutic response

Whether cell cycle biomarker analysis might be used as a predictor of therapeutic response to cell cycle phase-specific agents is an interesting question. The disappointing intent-to-treat analyses of large, conventionally designed trials, such as TACT and tAnGo, suggests that further improvements in adjuvant treatment will require individualized therapeutic decisions [116,117]. Cell cycle phase analysis of breast and ovarian cancers has shown that it is tumours displaying the accelerated cell cycle phenotype that are most likely to show a clinically relevant response to S- or M-phase-directed agents (Table 1, Figure 3B) [3,74,110,111,118]. As non-proliferating cells are radiation-resistant, whereas cycling cells are most sensitive to radiation insult during transit through G_2 and M phase, tumours displaying the accelerated cell cycle phenotype may also represent those that are most radiation-sensitive.

During the last two decades, molecular genetics research has shown that cancer arises as a result of a complex and unique set of mutations that drive oncogenic proliferation. Many of these mutations have been identified in growth signalling pathways, leading to the development of small-molecule inhibitors (SMIs) targeting cell-surface receptors and signalling molecules (eg EGFR, VEGF, KRAS, BRAF, PI3K, MEK, ERK) [15,30,119–121]. This is driving the concept that, rather than describing cancers according to their site of origin and clinicopathological parameters, tumours might alternatively be classified in terms of the main pathways that drive tumour cell proliferation (eg PI3K–PTEN–mTOR-driven cancer, Wnt-driven cancer, etc.) [122–124]. However, molecular tools to measure activation of the signalling pathways in tumour

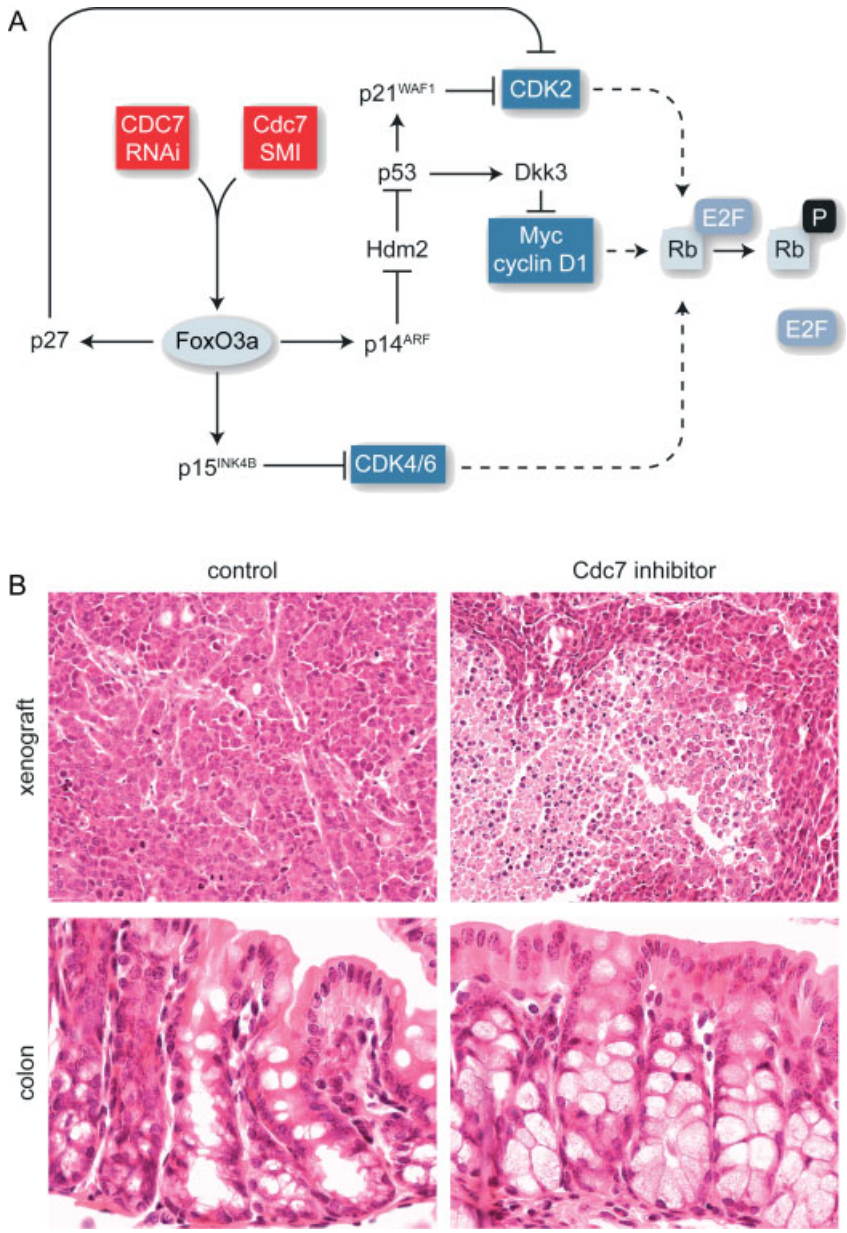

Figure 4. Targeting DNA replication before it starts—exploitation of the DNA replication origin activation checkpoint for cancer therapy. (A) Model to illustrate the molecular architecture of the origin activation checkpoint [129]. CDC7 knock-down by RNAi or, alternatively, inhibition of Cdc7 kinase activity with small molecule inhibitors (SMIs) triggers a cellular response that is dependent on three checkpoint axes coordinated through the cell stress transcription factor FoxO3a. Stalling of the cell cycle in G_1 is initiated by FoxO3a through activation of the ARF–Hdm2–p53–21 pathway and up-regulation of p15(INK4B) and p27(CDKN1B). P53 in turn activates expression of the Wnt/β-catenin signalling antagonists Dkk3, leading to Myc and cyclin D1 down-regulation. The resulting loss of CDK activity inactivates the Rb–E2F pathway and overrides the G_1–S transcriptional programme. A lack of redundancy between the checkpoint axes and reliance on several tumour suppressor proteins commonly inactivated in human tumours provides a mechanistic basis for the cancer cell-specific killing observed with Cdc7 SMIs in preclinical studies (see also B). The exact molecular mechanism by which blocked origin firing activates FoxO3a remains to be determined. (B) A Colo205 tumour xenograft (top right) treated with a Cdc7 SMI shows an extensive area of cell death indicative of a strong cytotoxic effect, while the control tumour xenograft (top left) shows a dense and compact architecture with high mitotic activity. Mouse colonic mucosa (bottom panels) shows normal morphology in both control and inhibitor-treated mice, with no apparent toxic effects.

biopsy material have been lacking and it is not clear which pathways are driving oncogenic proliferation.

Importantly, all these pathways converge at the level of the cell cycle machinery, driving cells through the restriction point in G_1 and culminating in activation of the G_1–S transcriptional programme. It is therefore interesting to speculate whether cell cycle phenotype analysis, as described above, might also be used as a predictor of response to SMIs targeting upstream growth signalling networks that modulate cell cycle progression. Tumours comprised predominantly of cells displaying cell cycle phenotypes (a) and (b) appear not to be receiving sufficient mitogenic signalling to drive them through the cycle and cell division, and therefore should be refractory to such inhibitors [110,111]. In contrast, receptor-signalling

pathways do appear to be exerting an effect on those tumours displaying the accelerated cell cycle phenotype (c), although in a proportion of cases this may be the result of the development of autonomous cancer cell cycles following mutations in critical regulators of the cell cycle machinery itself. In any case, it is only tumours displaying the accelerated cell cycle phenotype (c) that are likely to show significant response to upstream growth signalling inhibitors now entering the clinic [110,111]. Cell cycle phase analysis therefore has potential to stratify patients likely to respond to such inhibitors in clinical trials [3,110,111]. Importantly, following this rationale, cell cycle biomarker analysis might be used to salvage the many reported disappointing intent-to-treat trials using SMIs [35–38]. Retrospective cell cycle phase analysis of surgical biopsy material linked to patients participating in these trials could be performed through utilization of hospital tissue archives.

The DNA replication initiation machinery—a promising anti-cancer target?

The targeting of upstream growth signalling pathways is often constrained by pathway redundancy [40] or the development of growth-independent (autonomous) cancer cell cycles [3]. Efficacy can be compromised through a variety of mechanisms, eg through overexpression of alternative receptor tyrosine kinases or development of new signalling pathways [30]. Therapeutic targeting of the DNA replication initiation machinery, which lies at the convergence point of growth signalling networks, is now emerging as a new concept promising to overcome the limitation of targeting more upstream pathways. A concern is that therapeutic intervention at this level will not discriminate between rapidly dividing normal cells and tumour cells, thus leading to severe systemic side-effects while attempting to reduce tumour mass. This would translate into low therapeutic indices often found for conventional chemotherapeutic drugs targeting the cell cycle. However, potent cancer cell-specific killing has been demonstrated in preclinical models after inhibition of origin licensing [125] or, alternatively, origin activation through targeting Cdc7 kinase [111,118,126,127]. Tumour cell specificity is thought to result from transformed cells entering S phase with inadequate numbers of competent origins to complete chromosomal replication. This results in an abortive S phase with incompletely and/or abnormally replicated DNA. Tumour cells with a functional intra-S phase checkpoint appear to undergo rapid death after replication fork stalling/collapse, whereas more transformed cancer cells appear to survive longer but eventually face mitotic catastrophe as a result of partially replicated chromosomes [125,126,128]. In striking contrast, normal cells avoid entering S phase with a reduced number of replication-competent origins by engaging a recently described cell cycle checkpoint, the 'origin activation checkpoint'. Several studies have shown that following impairment of the DNA replication initiation machinery, normal cells arrest at the G_1–S boundary with unreplicated DNA, elevated p53 levels and induction of CDKI p21 [111,125,126]. We discovered that the molecular architecture of the underlying cell cycle checkpoint is critically dependent on several tumour suppressor proteins, including p53, p21, Dkk3, ARF, Hdm2, FoxO3a, p15, p27 and RB [129] (Figure 4A). This suggests that loss of the protective checkpoint mechanism through inactivating mutations in checkpoint proteins will render most common solid tumours sensitive to anti-cancer agents targeting the DNA replication initiation machinery [129].

Cdc7 kinase has emerged as a particularly attractive anti-cancer target in the DNA replication initiation pathway because it can be readily inhibited using ATP-competitive SMIs. Several biopharma companies have initiated Cdc7 drug development programmes, some of which have reached early-stage clinical trials [130,131]. First-in-class Cdc7 inhibitors have broad tumour spectrum activity in preclinical models, consistent with loss of the protective checkpoint mechanism in most tumour types. Apoptotic cancer cell death in response to Cdc7 inhibition is p53-independent and, at least in some cancer cell lines, is mediated via the stress-activated protein p38MAPK in an ATM- and Rad3-related (ATR)-dependent manner [132]. Interestingly, in addition to its function in origin firing, Cdc7 kinase has been shown to play an essential role in mediating the ATR–Chk1 pathway by phosphorylating the Chk1 activator Claspin [133,134]. Hence, the dual effect of Cdc7 inhibitors on DNA replication and DNA damage response pathways may further potentiate cancer cell killing.

Importantly, expression profile analysis of cell cycle biomarkers in surgical resection specimens provides further target validation for Cdc7 inhibitors and has shown that deregulation of this kinase is linked to aggressive disease. For example, increased Cdc7 expression in breast cancer is associated with Her2 and triple-receptor negative subtypes, the accelerated cell cycle phenotype (c), arrested tumour differentiation, genomic instability, increasing NPI score and reduced disease-free survival [111]. Similarly, increased Cdc7 expression has been linked with arrested tumour differentiation, advanced clinical stage, genomic instability, accelerated cell cycle progression and reduced disease-free survival in ovarian cancer [118]. We postulate that it will be tumours showing the accelerated cell cycle phenotype (c), high Cdc7 levels and harbouring lesions in the origin activation checkpoint axes that are likely to show optimal response to Cdc7 inhibition. Thus, Cdc7 inhibitors may significantly broaden the therapeutic armamentarium available for the treatment of aggressive primary and metastatic disease in which treatment options are limited. This supposition is

supported by preclinical data showing that Cdc7 knockdown by RNAi in p53-mutant Her2 and triple receptor–negative breast cancer cell lines induces potent cancer cell killing [111]. *In vivo*, these breast cancer subtypes are characterized by the accelerated cell cycle phenotype (c), high Cdc7 expression levels, mutation of checkpoint effector proteins, and therefore are potentially sensitive to Cdc7 inhibitors.

The DNA origin activation checkpoint and cyclotherapy

Nucleotide incorporation experiments have demonstrated that the G_1 arrest triggered by Cdc7 knockdown in normal cells is fully reversible on recovery of Cdc7 protein levels and that the arrested cells remain viable [111]. This finding suggests that inhibitors targeting the DNA replication initiation machinery are likely to have limited toxicity in self-renewing tissues with high turnover (eg gut, skin or the haemopoietic system) compared to conventional cell cycle-phase-specific agents. Indeed, in a mouse xenograft Colo-205 tumour model (p53-mutant cell line), we observed potent tumour cell killing after treatment with a Cdc7 SMI, while no overt toxic effects were observed in normal mouse gut mucosa (Figure 4B).

Many of the most effective neoadjuvant and adjuvant systemic chemotherapeutic regimes utilize S- and M-phase agents (Table 1). The Achilles heel of these regimes is that S- and M-phase agents also affect normal cycling cells, in particular those located in the transit amplifying compartments of self-renewing tissues, resulting in marrow suppression (neutropenia), hair loss and gut toxicity. Following the original cyclotherapy concept as proposed by Arthur Pardee and colleagues, treatment with a Cdc7 inhibitor prior to administration of S- and/or M-phase cell cycle phase-specific agents might provide a powerful method for shielding normal somatic cycling cells from the toxic effects of chemotherapy. Such combinatorial treatment regimes may allow increased dosage and frequency of cell cycle phase-specific agents, thus increasing the therapeutic window and thereby reducing the likelihood of drug-resistant clones [135]. The availability of Cdc7 SMIs now provides an opportunity for this novel treatment paradigm to be tested.

Conclusions and implications

The cell cycle engine is a promising diagnostic and therapeutic target in cancer because it lies downstream at the convergence point of complex oncogenic signalling networks and its deregulation is central to the aberrant cell proliferation that characterizes all cancers. Moreover, structurally and mechanistically, many of its components are evolutionarily conserved and therefore clinical applications are likely to be suited to diverse tumour types. This is in stark contrast to the targeting of cancer-specific mutations. Many of the fundamental discoveries in the cell cycle field have come not from mammalian cells, but from genetic and biochemical studies in yeast, *Drosophila*, zebrafish and *Xenopus* model systems. With pathologists traditionally placed at the interface of basic and clinical sciences, the study of candidate cell cycle biomarkers in human tissues with linked clinical outcome measures now provides a crucial bridge to translate fundamental discoveries in the cell cycle field into front-line diagnostic and therapeutic applications.

Acknowledgment

We thank past and present members of the UCL Chromosomal Replication Group, and in particular Marco Loddo and Alex Wollenschlaeger, for their help with the preparation of the manuscript.

Author contributions

GHW and KS wrote the paper and had final approval of the submitted and published versions.

References