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DNA replication licensing and human cell proliferation

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SUMMARY

The convergence point of growth regulatory pathways that control cell proliferation is the initiation of genome replication, the core of which is the assembly of pre-replicative complexes resulting in chromatin being 'licensed' for DNA replication in the subsequent S phase. We have analysed regulation of the pre-replicative complex proteins ORC, Cdc6, and MCM in cycling and non-proliferating quiescent, differentiated and replicative senescent human cells. Moreover, a human cell-free DNA replication system has been exploited to study the replicative capacity of nuclei and cytosolic extracts prepared from these cells. These studies demonstrate that downregulation of the Cdc6 and MCM constituents of the replication initiation pathway is a common downstream mechanism for loss of proliferative capacity in human cells. Furthermore, analysis of MCM protein expression in self-renewing, stable and permanent human tissues shows that the three classes of tissue have developed very different growth control strategies with respect to replication licensing. Notably, in breast tissue we found striking differences between the proportion of mammary acinar cells that express MCM proteins and those labelled with conventional proliferation markers, raising the intriguing possibility that progenitor cells of some tissues are held in a prolonged G1 phase or 'in-cycle arrest'. We conclude that biomarkers for replication-licensed cells detect, in addition to actively proliferating cells, cells with growth potential, a concept that has major implications for developmental and cancer biology.

Key words: ORC, Cdc6, MCM, DNA replication licensing, Cell proliferation, Proliferation marker

INTRODUCTION

Replication of mammalian genomes is an integrated step of the cell division cycle that is strictly regulated by an intricate network of extracellular and intracellular signalling pathways that control cell proliferation, quiescence, differentiation, cellular senescence and apoptosis. The convergence point of these complex growth regulatory pathways is the initiation of genome replication, the core of which is a set of replication initiation factors that sequentially assemble into pre-replicative complexes (pre-RCs) at replication origins resulting in chromatin being 'licensed' for replication in the subsequent S phase (reviewed by Ritzi and Knippers, 2000). The current understanding of how replication initiation is controlled in eukaryotic cells is derived mainly from studies in yeast and *Xenopus* (reviewed by Donaldson and Blow, 1999; Tye, 1999). However, the constituents of the pre-RC have been shown to be conserved from yeast to mammals, suggesting that the basic mechanisms of replication initiation are very similar in all eukaryotic cells (reviewed by Dutta and Bell, 1997). The consensus view that has emerged is that pre-RC assembly begins with the binding of a six-subunit origin recognition complex (ORC) to specific origin sites in the genome (reviewed by Quintana and Dutta, 1999). ORC determines where replication initiation will occur and serves as a landing platform for additional initiation factors during a cascade of protein assembly that finally results in the formation of the pre-RC (Fig. 1). In early G1, the Cdc6 protein functionally interacts with ORC and loads the MCM proteins (Mcm2-7), which were originally discovered and named as factors that support minichromosome maintenance in yeast (reviewed by Tye, 1999), onto chromatin. The assembly of ORC, Cdc6 and MCM proteins into pre-RCs makes chromatin competent or 'licensed' for replication (reviewed by Chevalier and Blow, 1996). Cdc6 is released just before or at the beginning of S phase and replaced by the Cdc45 protein, which, as a first step in establishing replication forks, recruits DNA polymerase α-primase and the single-strand-specific DNA-binding protein RPA to the replication origin (reviewed by Ritzi and Knippers, 2000). At the G1 to S phase transition, DNA replication is initiated by the concerted action of S phase-promoting cyclin-dependent kinases and the Dbf4-dependent Cdc7 kinase (reviewed by Pasero and Schwob, 2000; Sclafani, 2000). The MCM proteins gradually dissociate from chromatin as S phase proceeds (Krude et al., 1996), consistent with their predicted function as a DNA helicase (Ishimi, 1997). Dissociation of Cdc6 and MCM proteins from chromatin ensures that DNA is replicated once and only once during a single cell division cycle, as replicated chromatin is devoid of functional pre-RCs and thus not 'licensed' for replication (reviewed by Stillman, 1996).

Although recent reports confirm the universality of this scheme, they also reveal that the regulation of cell division in

multicellular organisms imposes an additional level of complexity. In higher eukaryotes, cells divide not only to reproduce themselves, but also to ensure the correct formation of tissues and organs, requiring differential regulation of growth. In mammalian tissues the majority of cells have exited the cell division cycle during growth and development into different 'out-of-cycle' states. First, normal somatic cells require the presence of mitogens for continual proliferation. After the removal of mitogens in early/mid G1 prior to progression through the restriction point untransformed cells cease proliferation and reversibly withdraw into a quiescent state (G0) in which macromolecular synthesis is reduced (Pardee, 1989). Second, proliferating precursor and progenitor cells can be induced by both intrinsic and extrinsic signals to stop dividing and to enter the differentiation pathway (reviewed by Hall and Watt, 1989). Often there is mutual antagonism between the cellular circuits that control proliferation and differentiation (reviewed by Myster and Duronio, 2000). Third, normal somatic cells have a finite replicative lifespan that restricts cell division by a process known as replicative or cellular senescence. Normal cells proliferate for a finite number of cell divisions after which they withdraw irreversibly from the cell cycle, entering a senescent state (Hayflick and Moorhead, 1961). Senescent cells remain viable indefinitely but fail to initiate DNA replication in response to mitogens (reviewed by Campisi, 1996). The molecular mechanisms that underlie loss of proliferative capacity as cells withdraw from the cell division cycle are largely unknown. Moreover, the ability to arrest growth in quiescence, terminal differentiation and cellular senescence reflects fundamental growth regulatory mechanisms that operate stringently in untransformed cells but are defective in tumour cells (reviewed by Sherr, 1996). We now report an analysis of replication initiation factor regulation in proliferating, quiescent, differentiated and senescent human cells. These studies have been combined with a functional investigation of the replicative capacity of subcellular components isolated from cycling and non-proliferating cells in a human cell-free DNA replication system, a novel approach for analysing growth regulation in normal human somatic cells. Moreover, the general features of proliferation control emerging from these in vitro studies have been extrapolated into an investigation of the growth regulatory strategies that have evolved in the three classes of human tissue: self-regenerating, stable and permanent.

MATERIALS AND METHODS

Cell culture

Human WI38 lung fibroblasts (American Type Culture Collection, passage 19), NIH3T3 fibroblasts and HeLa S3 cells were cultured as exponentially growing monolayers in Dulbecco's modified Eagle's medium (DMEM, Gibco-BRL) supplemented with 10% fetal calf serum (FCS; Gibco-BRL), 10 U/ml penicillin (Sigma) and 0.1 mg/ml streptomycin (Sigma). Isogenic sets of primary human breast epithelial cells and fibroblasts (populations 48) were derived from reduction mammoplasty samples (a generous gift from Dr M. Stampfer, Lawrence Berkley National Laboratory, Berkley, CA). Epithelial cells were maintained in MM4 media with serum (MEGM, BioWhittaker). Fibroblasts were grown in DMEM with 10% fetal calf serum (Gibco-BRL). Cells were grown at 37°C in 5% CO_2. For routine culture, cells were counted and plated at 2×10^5 cells per 75 cm^2 flask. Attachment efficiency was determined by counting attached cells 15 hours after plating. The number of accumulated populations doublings (PD) per passage was determined using the equation, $\Delta PD=\log(A/(B\times C))/\log 2$, where A is the number of harvested cells, B is the number of plated cells and C the attachment efficiency.

Cell synchronisation

To prepare nuclear templates, cytosolic and total cell extracts from quiescent WI38 and NIH3T3 fibroblasts, cells were driven into quiescence (G0) by density-dependent growth arrest, leaving them to accumulate in G0 for up to 21 days. G1 nuclear templates were obtained by releasing confluent cells through subculturing and replating as previously described (Stoeber et al., 1998). To prepare S phase HeLa cytosolic extracts, cells were synchronised in S phase by a single block in culture medium containing 2.5 mM thymidine (Sigma) for 25 hours (Rao and Johnson, 1970), followed by release into culture medium for 2 hours, as previously described (Stoeber et al., 1998). Cells at various stages of S and G2 phases were obtained by releasing HeLa cells arrested at G1/S by a double thymidine block into culture medium for the times indicated in the Results. Transit into G1 phase was inhibited by adding 40 ng/ml nocodazole (Sigma) to the culture medium. Cells in G1 phase were obtained by releasing thymidine-arrested HeLa cells into culture medium for 3 hours, followed by adding 40 ng/ml nocodazole (Sigma) for an additional 12 hours to arrest them in mitosis. Cells at various stages of G1 phase were obtained by releasing HeLa cells arrested in mitosis into fresh culture medium for the times indicated in the Results. Cell cycle synchronisation was verified by flow cytometry of isolated nuclei as described previously (Krude et al., 1997). To prepare nuclear templates, cytosolic and total cell extracts from senescent WI38 fibroblasts, cells were replicatively senesced by culturing from passage 19 to 33. The late passage fibroblasts were replicatively senescent with very low proliferative capacity as described previously (Helenius et al., 1999).

Preparation of extracts from cultured cells

Total, nuclear and cytoplasmic extracts from synchronised HeLa S3 cells were essentially prepared as described (Heintz and Stillman, 1988) with the following minor modifications. Nuclei and cytosolic extracts were produced by hypotonically swelling, scrape-harvesting and Douncing. Triton X-100 was added to a final concentration of 0.5% (v/v) and total cell extract was incubated on a rotating wheel for 5 minutes at 4°C. Total cell extract was spun at 2,000 ***g*** for 3 minutes in an Eppendorf 5415C centrifuge and cytoplasmic extract was transferred to a new tube. The nuclear pellet was washed twice with PBS and extracted with an equal volume of PBS/0.5 M NaCl. Nuclear proteins solublised in high salt were separated from the pellet by centrifugation at 10,000 ***g*** for 30 minutes, and the soluble nuclear extract was removed.

To prepare total cell extracts from synchronised NIH3T3 and WI38 cells, medium was decanted from 10 cm dishes of adherent cells and plates rinsed rapidly with PBS. Excess PBS was aspirated and 1 ml boiling 2× concentrated electrophoresis sample buffer (250 mM Tris pH 6.8, 4% SDS, 10% glycerol, 0.006% bromophenol blue, 2% β-mercaptoethanol) added to each plate. Cells were scraped from plates, transferred to microcentrifuge tubes, and boiled for an additional 5 minutes. To reduce viscosity, samples were passed several times through a 26 gauge needle and insoluble material was pelleted by centrifugation at 10,000 ***g*** for 5 minutes.

Preparation of nuclear templates and cytosolic extracts

Nuclear templates and cytosolic extracts for in vitro replication reactions were produced by hypotonically swelling, scrape-harvesting and Douncing as previously described (Stoeber et al., 1998).

Assaying for DNA replication

In vitro DNA replication reactions containing 30 μl of cytosolic

extract or elongation buffer, a buffered mix of NTPs, dNTPs, an energy regeneration system and approximately 1×10^5 nuclei were performed as previously described (Stoeber et al., 1998). After incubation for 3 hours, nuclei were washed, fixed and stained with propidium iodide (red) to reveal DNA and with fluorescein-streptavidin (green) to detect biotin incorporation resulting from in vitro DNA synthesis as previously described (Stoeber et al., 1998). Results are expressed as the percentage of nuclei replicating and summarised in the histograms (mean±s.d.).

Immunoblotting

Immunoblots of HeLa, NIH3T3 and WI38 cell extracts were performed as described (Stoeber et al., 1998) using purified rabbit polyclonal antibodies generated in our laboratory against human Orc2 (Romanowski et al., 2000), Cdc6 (Stoeber et al., 1998), MCM conserved oligopeptide (Hu et al., 1993), Mcm5 (Stoeber et al., 1999) and *Xenopus* Mcm7 (Romanowski et al., 1996), or other antibodies obtained from commercial sources (Cdc6 (Santa Cruz Biotechnology), BM28 (Transduction Laboratories), p21 (PharMingen), Ki67 (Dako), neurofilament (NF, ZYMED)). Total cell extracts from isogenic sets of human breast epithelial cells and fibroblasts were separated by gradient (4%-20%) PAA gels (FMC), before transfer to Hybond-P (Amersham) membranes, incubation with primary (BM28 (Transduction Laboratories), anti-β-actin (Sigma)) and secondary (HRP-conjugated goat-anti-mouse antibody (Gibco)) antibodies, and visualisation of immune complexes using enhanced chemiluminescence (Amersham).

Immunofluorescence

Cells were grown in RS-treated glass chambers (Corning), fixed with 100% methanol (–20°C for 1 hour) and kept under 75% ethanol at +4ºC. A blocking solution and a dilution buffer of 4% goat serum (Sigma) in phosphate-buffered saline (PBS) was used. BM28 (Transduction Laboratories), Texas Red- or FITC-conjugated goat anti-mouse antibodies was used (Jackson ImmunoResearch). Microscopic images were acquired by a CCD camera attached to a Nikon TE300 microscope.

Immunocytochemistry

Formalin fixed archival blocks were selected from the files at Addenbrookes Hospital, Cambridge, UK. Sections (4 μm) were cut using a sledge microtome and placed onto Dako ChemMate capillary gap microscope slides. After drying the slides overnight at 60°C, sections were de-paraffinized in xylene and re-hydrated in water. Antigen retrieval was performed by heating the sections in 10 mmol/l citrate buffer pH 6.0 for 2 minutes. Slides were stained on a Dako Techmate™ 500. Briefly, sections were incubated with primary antibody (BM28 (Transduction Laboratories), anti-Mcm5 polyclonal antibody (PAb) (Stoeber et al., 1999), anti-Ki67 (Dako) or anti-NF PAb (ZYMED)) for 45 minutes. Biotinylated secondary antibody and streptavidin peroxidase complex were added consecutively for 30 minutes each at room temperature. The peroxidase activity was visualised using diaminobenzidine tetrahydrochloride to produce a permanent end product. Slides were counterstained, dehydrated and mounted. Appropriate tissue sections as positive and negative controls were included. Microscopic images were acquired by a CCD camera attached to a Leica light microscope.

RESULTS

Regulation of ORC, Cdc6 and MCM proteins in cycling human cells and following reversible withdrawal into the replicative quiescent state

ORC, Cdc6 and Mcm2-7 assemble into pre-RCs as part of the eukaryotic replication licensing mechanism (reviewed by Chevalier and Blow, 1996). It has been demonstrated in yeast and *Xenopus* that pre-RC assembly is sequential, with ORC recruiting Cdc6, which in turn promotes loading of the MCM proteins onto chromatin (Coleman et al., 1996; Ogawa et al., 1999; Perkins and Diffley, 1998; Weinreich et al., 1999). To investigate whether the order of events is similar in human cells, homogenates prepared from synchronised HeLa cells were fractionated into soluble and chromatin-bound factions, and immunoblotted with antibodies against human Orc2, Cdc6 and Mcm5. Orc2 is present exclusively in the chromatin-bound fraction and its levels do not vary during passage through the cell cycle (Fig. 2A). Cdc6 is associated with chromatin in late mitosis and remains chromatin-bound throughout G1 (Fig. 2A). During S phase progression, there is a small decrease in the proportion of chromatin-bound Cdc6 and a slower migrating form becomes detectable in the soluble fraction (Fig. 2A), consistent with cyclin A/CDK2-dependent phosphorylation and *trans*-location of Cdc6 to the cytoplasm (Fujita et al., 1999; Jiang et al., 1999; Petersen et al., 1999). Mcm5 is soluble in mitotic cells and binds to chromatin within 2 hours of release from mitotic arrest – the chromatin-bound fraction increasing with progression through G1, consistent with pre-RC assembly (Fig. 2A). Mcm5 gradually dissociates

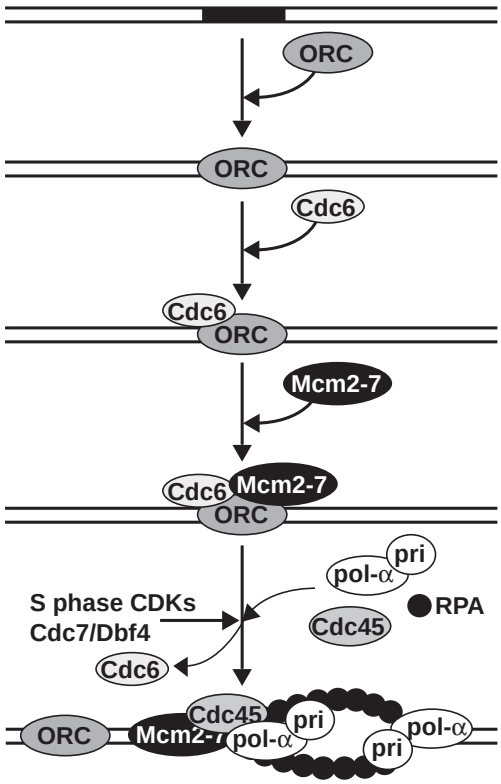

Fig. 1. Assembly of the pre-replicative complex renders chromatin competent for replication. In early G1 phase, the origin recognition complex (ORC) recruits Cdc6, which in turn promotes loading of MCM proteins onto chromatin. Activation of the Cdc7/Dbf4 kinase and S phase-promoting cyclin-dependent kinases (CDKs) induces a conformational change in the MCM complex that is required for unwinding of origin DNA and recruits Cdc45 to the pre-RC. Initiation of DNA replication occurs when replication protein A (RPA) and DNA polymerase α-primase are recruited to the unwound replication origin.

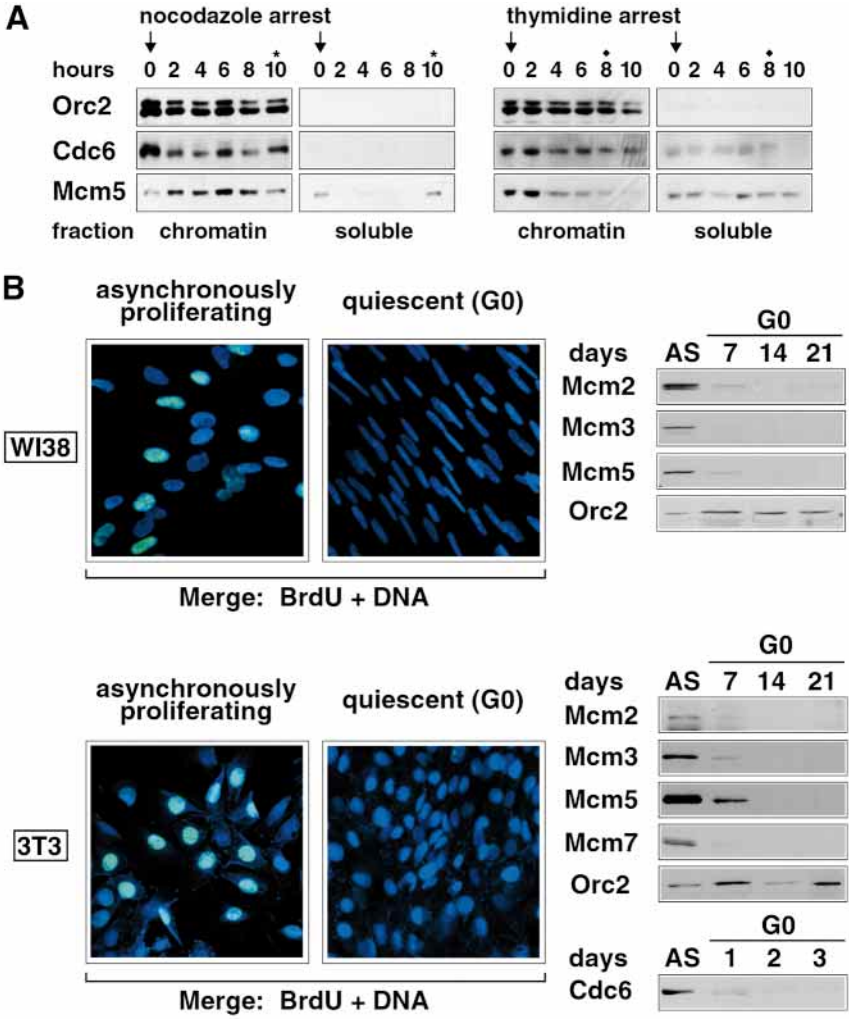

Fig. 2. Regulation of ORC, Cdc6 and MCM proteins in cycling human cells and following reversible withdrawal into the quiescent state. (A) Immunoblot analysis of Orc2, Cdc6 and Mcm5 in chromatin-bound and soluble fractions of HeLa S3 cells during the indicated times after release from nocodazole arrest in metaphase (left panels) or double thymidine block at G1/S (right panels). Cell cycle synchronisation was verified by flow cytometry of isolated nuclei. Cells began to enter S phase 10 hours (*) after release from nocodazole arrest, and the majority of cells reached G2 phase by 8 hours (◆) after release from a double thymidine block. The soluble fraction contains cytoplasmic and soluble nuclear proteins. (B) Immunoblot analysis of ORC, Cdc6 and MCM proteins in total cell extracts of asynchronously proliferating (AS) and contact-inhibited (G0) WI38 and NIH3T3 cells. Entry into quiescence was confirmed by BrdU labelling and antibody staining of asynchronously proliferating and contact-inhibited cultures (left panels; merged images of FITC (BrdU) and TOTO-3 (DNA) channels). (C) MCM protein expression in liver, an example of a stable tissue. The upper and lower panels show indirect immunoperoxidase staining of liver biopsies obtained from two patients undergoing liver transplantation with an anti-Mcm2 MAb (×160).

from chromatin during S phase progression and becomes detectable in the soluble fraction, consistent with the concept of pre-RC disassembly after origin firing (Fig. 2A). Taken together, these data demonstrate that the essential replication initiation factors ORC, Cdc6 and MCM are present throughout the human proliferative cell cycle.

During vertebrate development, cells can reversibly withdraw from the proliferative cycle in early G1 phase and enter an ‘out-of-cycle’ quiescent state referred to as G0 (Pardee, 1989). Generation of stable quiescent fibroblasts through density-dependent growth arrest or serum starvation is a widely used in vitro model for replicative quiescence. To investigate coupling of the replication initiation pathway with loss of proliferative capacity in quiescence, total cell extracts prepared from asynchronously proliferating and contact-inhibited mouse NIH3T3 and WI38 human diploid fibroblasts (HDF) were immunoblotted with antibodies against ORC, Cdc6 and MCM proteins (Fig. 2B). Entry into quiescence was confirmed by BrdU labelling and antibody staining of asynchronously proliferating and contact-inhibited cultures (Fig. 2B). Both fibroblast strains entered quiescence between 3 and 5 days after reaching confluency. The percentage of BrdU-incorporating cells decreased from 40-45% in asynchronously proliferating NIH3T3 cultures to 1-3% in quiescent cultures (WI38, 30-35% to 1%). Orc2 protein levels do not undergo quiescence-dependent changes in cells from either species during density-dependent growth arrest for up to 21 days (Fig. 2B). The regulation of Cdc6 in quiescent

mouse fibroblasts is a controversial issue, as published evidence for Cdc6 protein levels is conflicting. Saha et al. have reported a significant amount of total cellular Cdc6 protein in serum-starved NIH3T3 fibroblasts (Saha et al., 1998). In contrast, our previous findings and a recent study by Berger et al. demonstrate suppression of Cdc6 mRNA and protein in NIH3T3 fibroblasts made quiescent by either serum starvation or contact inhibition (Berger et al., 1999; Stoeber et al., 1998). To clarify this issue, we have analysed Cdc6 total cellular protein at 1 day intervals after density-dependent growth arrest in NIH3T3 fibroblasts. Our data show complete downregulation of Cdc6 protein within 48 hours of exit from cycle into G0, suggesting that Cdc6 expression is strictly linked to cell proliferation (Fig. 2B). Furthermore, it has been recently shown that total Mcm2 protein levels decline with the onset of quiescence in Swiss 3T3 cells (Sun et al., 2000), but this report did not address the important question of whether regulation of Mcm2 expression during G0 is representative of the MCM complex in general. We found similar kinetics of Mcm2, Mcm3, Mcm5 and Mcm7 downregulation in both mouse and human quiescent fibroblasts with total protein levels rapidly declining within the first 7 days of density-dependent growth arrest and falling to less than 1% of those in asynchronously proliferating cells by day 14 (Fig. 2B). These data, together with our previous observation that Mcm5 protein is detectable in the soluble but not chromatin-bound fraction of homogenates prepared from NIH3T3 fibroblasts arrested in G0 for 3 days (Stoeber et al., 1998), suggest that the six MCM proteins (Mcm2-7) are first displaced from chromatin on entry into G0 and then simultaneously downregulated as a gradual response to prolonged arrest in quiescence.

When quiescent human cells are stimulated to re-enter the proliferative cell cycle, transcript and protein levels of Cdc6 and MCMs increase during progression through G1, peaking on S phase entry (Tsuruga et al., 1997; Yan et al., 1998). These studies have been performed in various in vitro systems, but it is not known whether the replication initiation pathway is coupled to reversible growth arrest in human tissues. To address this question, we have analysed regulation of MCM protein expression in liver and thyroid (data not shown), stable tissues that show little growth in adult life but retain capacity for further growth (Leblond, 1963). After hepatocyte loss as a result of surgery/transplantation, viruses or toxins, quiescent hepatocytes re-enter the mitotic cell division cycle and proliferate to restore liver function and mass. Re-population studies have demonstrated that hepatocytes can undergo greater than 10^{20}-fold expansion of the original population following serial transplantations (Overturf et al., 1997), emphasising the fact that the proliferative capacity of hepatocytes is strictly regulated and normally repressed. Tru-cut liver biopsies (five cases) obtained from the donor liver at the time of transplantation (t_0) were immunostained with anti-Mcm2 and anti-Mcm5 antibodies. Liver biopsies were subsequently obtained from the same patients between 1 and 6 days post-transplantation, when hepatocytes regain proliferative capacity and liver regeneration begins. Only a small population of MCM-positive hepatocytes were immunostained in tissue sections from t_0 biopsies (1-3%; Fig. 2C, upper and lower left panels), consistent with a high proportion of cells being in a

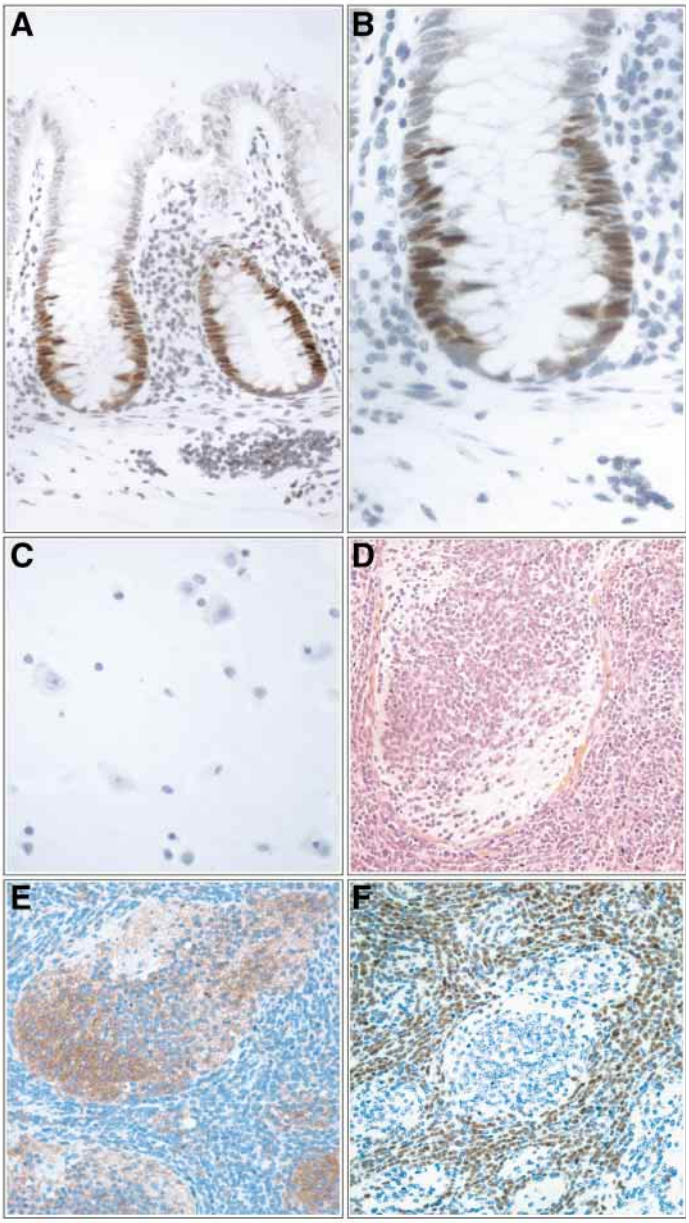

Fig. 3. MCM protein expression in differentiating and terminally differentiated tissues. (A,B) Indirect immunoperoxidase staining of colon with an anti-Mcm2 MAb. (A) The majority of Mcm2-expressing cells are located in the lower third of the crypt, which corresponds to the proliferative zone of the mucosa (×87). B, Notably, it appears to be the anatomical location corresponding to the transit amplifying population of cells that shows the highest levels of Mcm2 expression. The proportion of Mcm2-positive cells and the levels of Mcm2 expression decline in the middle third of the crypt, becoming undetectable in surface terminally differentiated cells (×156). (C) Indirect immunoperoxidase staining of adult brain with an anti-Mcm5 rabbit PAb. Neurones and glial cells lack Mcm5 expression (×170). (D) Hematoxylin and Eosin stained medulloblastoma showing sheets of undifferentiated mononuclear tumour cells containing well demarcated islands of glial differentiation (×68). (E,F) Indirect immunoperoxidase staining of medulloblastoma with an anti-neurofilament (NF) PAb (E) and anti-Mcm5 rabbit PAb (F) (×68). Mcm5 expression is strictly confined to the undifferentiated tumour cells with downregulation occurring in areas of differentiation.

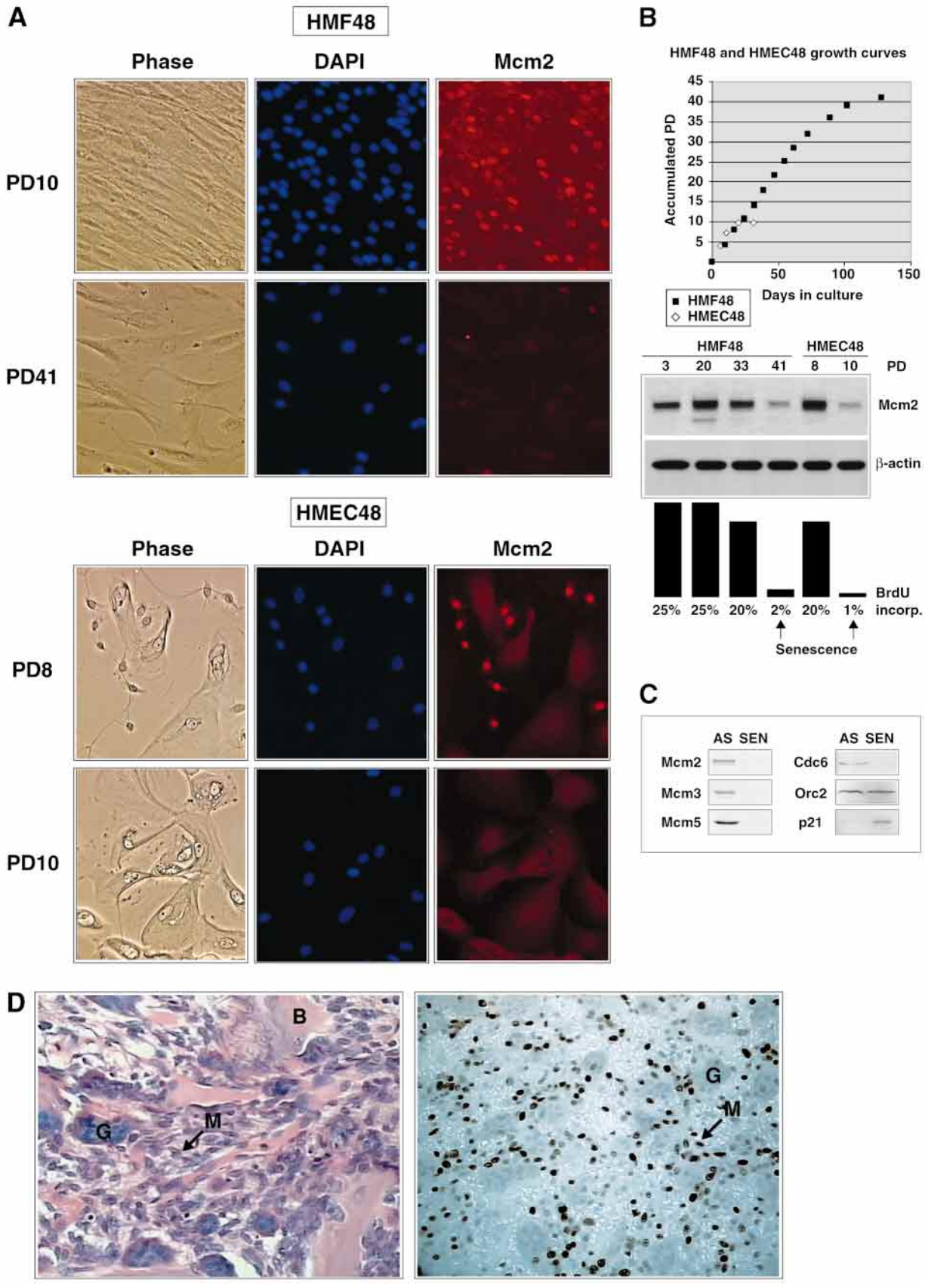
A
HMF48
Phase
DAPI
Mcm2
PD10
PD41
HMEC48
Phase
DAPI
Mcm2
PD8
PD10
B
HMF48 and HMEC48 growth curves
Accumulated PD
45
40
35
30
25
20
15
10
5
0
50
100
150
Days in culture
HMF48
HMEC48
HMF48
HMEC48
3
20
33
41
8
10
PD
Mcm2
β-actin
25%
25%
20%
2%
20%
1%
BrdU incorp.
Senescence
C
AS SEN
AS SEN
Mcm2
Mcm3
Mcm5
Cdc6
Orc2
p21
D
B
G
M
G
M

Fig. 4. (A) Phase-contrast microscopy and immunofluorescence staining of primary human breast fibroblast (HMF48) and epithelial (HMEC48) cells derived from reduction mammoplasty specimens with anti-Mcm2 MAb. Nuclei are counterstained with DNA stain 4,6-diamidino-2-phenylindole (DAPI). (B) Immunoblots of Mcm2 and β-actin in total cell extracts from isogenic sets of human breast fibroblasts (HMF48) and epithelial cells (HMEC48). HMF and HMEC growth curves and BrdU labelling indices indicate the proliferative state. (C) Immunoblot analysis of ORC, Cdc6, MCM and p21 proteins in total cell extracts of asynchronously proliferating (AS) and replicative senescent (SEN) WI38 HDF. (D) Giant cell tumour of bone (B) stained with Haematoxylin and Eosin (left panel) and with rabbit anti-Mcm5 polyclonal antibody (indirect immunoperoxidase staining, right panel) (×130). The tumour is composed of multinucleate giant cells (G) regularly dispersed in a background of small mononuclear (M) neoplastic cells. Mcm5 expression is restricted to the mononuclear neoplastic cell population.

replication-incompetent quiescent state. Immunostaining of tissue sections from post-transplantation biopsies showed increasing numbers of MCM-positive hepatocytes, with 46% of hepatocytes expressing Mcm2 within 24 hours rising to 87% by day 4 (Fig. 2C, upper and lower right-hand panels). Notably, replication licensing, as determined by MCM protein expression, coincides with re-entry of hepatocytes into the proliferative cycle, as indicated by appearance of mitotic figures (data not shown). Similar proportions of hepatocytes were immunostained with the anti-Mcm5 antibody (day 1, 54%; day 4, 92%) suggesting that, as observed for downregulation in quiescence (Fig. 2B), the kinetics of MCM protein expression following re-entry into the proliferative cycle in vivo are similar for the six MCM proteins. Taken together, the in vitro and in vivo data demonstrate that downregulation of the Cdc6 and MCM constituents of the replication initiation pathway is a powerful growth regulatory mechanism for maintenance of the replicative quiescent state in human cells and stable tissues.

Regulation of ORC, Cdc6 and MCM proteins following irreversible withdrawal from the cell division cycle into the terminally differentiated state

Differentiation is defined as a qualitative change in the cellular phenotype that is the consequence of the onset of synthesis of new gene products (reviewed by Lajtha, 1979). Differentiating cells normally mature over the passage of time to form functionally competent cells for a particular tissue, culminating in terminal maturation or differentiation, the step in which the final functional role of the cell is achieved (reviewed by Lajtha, 1979). Self-regenerating stem cell systems such as skin, colon or uterine cervix normally show growth and differentiation throughout life (Leblond, 1963). The prevailing view is that stem cells in the proliferative compartment of these tissues have the capacity for unlimited or prolonged self-renewal that can produce highly differentiated descendants. However, between the stem cell and its terminally differentiated progeny is an intermediate population of committed progenitors with limited proliferative capacity and restricted differentiation potential (reviewed by Watt and Hogan, 2000). The end products of differentiation pathways are cells that have lost proliferative capacity (reviewed by Myster and Duronio, 2000). Recent studies have identified links between differentiation, loss of proliferative capacity and replication licensing in several species, including mouse and human. Expression of Mcm5 protein appears to be negatively regulated by the developmental regulator Pax3 in mouse embryos (Hill et al., 1998). Similarly, loss of proliferative capacity during terminal differentiation of cultured promyelocytic HL60 cells to the macrophage phenotype is linked to downregulation of Mcm3 within 3 days, whereas Orc2 protein levels remain unchanged (Musahl et al., 1998). Furthermore, we and others have previously demonstrated in a range of self-regenerating stem cell systems, including skin, uterine cervix, intestines and bladder, that Cdc6 and MCM expression is restricted to the basal proliferative compartment with downregulation of these replication licensing factors occurring as epithelial cells undergo terminal differentiation (Freeman et al., 1999; Stoeber et al., 1999; Williams et al., 1998; Yan et al., 1998).

To further clarify the inverse relationship between differentiation and replication licensing, we have analysed regulation of Mcm2 and Mcm5 protein expression in histological sections of human colon, brain and myocardium. Immunostaining of colon shows MCM protein expression to be strictly confined to the proliferative compartment of the colonic crypt. Downregulation of the MCM licensing factors occurs as colonocytes undergo transition to a mature differentiated phenotype (Fig. 3A). Interestingly, MCM protein expression levels are highest in the anatomical zone corresponding to the transit amplifying cell population (Fig. 3B). This pattern may relate to the fact that the primary function of the transit cell population is to increase the number of differentiated cells produced by each stem cell division, and that although the stem cell has high capacity for self-renewal, it may divide relatively infrequently (reviewed by Potten and Loeffler, 1990). Adult neurones and cardiac muscle represent examples of permanent tissues that do not divide in adult life and have no reserve capacity for growth (Leblond, 1963). The absence of MCM protein expression in neurones (Fig. 3C) and cardiac myocytes (data not shown) is consistent with their terminally differentiated state.

To determine whether the link between cell proliferation, differentiation and replication licensing also applies to human tumours, histological sections of medulloblastoma (Fig. 3D), a childhood brain tumour, were immunostained with anti-Mcm2 and anti-Mcm5 antibodies. This is a tumour of precursor neuronal origin composed of clearly demarcated undifferentiated and differentiated elements (Coffin et al., 1983). Cells in the differentiated area expressing neurofilament (NF) protein, a marker for glial differentiation, lack MCM proteins (Fig. 3E). In contrast, the undifferentiated component contains a high proportion of MCM-expressing primitive pleomorphic cells (Fig. 3F). Taken together, these data show that loss of proliferative capacity as mammalian cells undergo differentiation is associated with downregulation of the Cdc6 and MCM constituents of the replication initiation pathway, suggesting that differentiation and replication licensing are often mutually exclusive processes.

Regulation of ORC, Cdc6 and MCM proteins following irreversible withdrawal from the cell division cycle into the replicative senescent state

Normal somatic cells have a finite replicative lifespan that restricts cell division by a process known as replicative or cellular senescence (Hayflick and Moorhead, 1961). Senescent

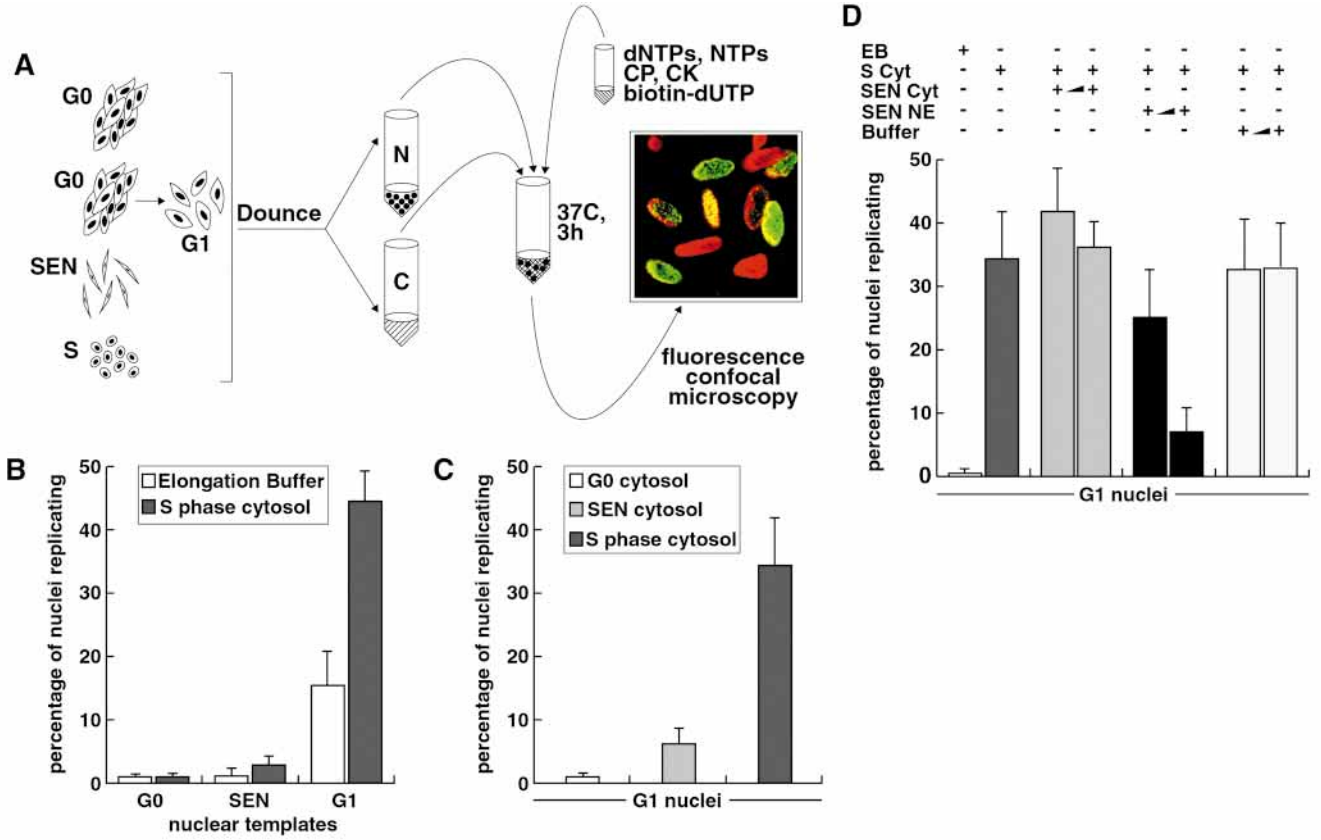

Fig. 5. (A) Cell-free DNA replication system. Nuclei (N) prepared from G1 phase WI38 fibroblasts, synchronised by release from quiescence (G0), initiate a single round of semi-conservative DNA replication in cytosolic extracts (C) from S phase HeLa cells substituted with ribonucleoside and deoxyribonucleoside triphosphates (NTPs, dNTPs) and an energy regeneration system (creatine phosphate (CP) and phosphocreatine kinase (CK)). Nuclei are stained with propidium iodide to reveal DNA (red) and with fluorescein-streptavidin (green) to detect biotin-16-dUTP incorporation resulting from in vitro DNA synthesis (Stoeber et al., 1998). Results are expressed as the percentage of nuclei replicating and summarised in the histograms (mean+s.d.). Substitution of nuclear templates and/or extracts with subcellular components from quiescent (G0), terminally differentiated (not shown) or replicative senescent (SEN) cells provides a functional assay for analysis of the mechanisms that establish and/or maintain loss of replicative capacity in 'out-of-cycle' cells (B-D). (B) In vitro analysis of the replicative capacity of WI38 G1, quiescent and replicative senescent nuclear templates in either physiological buffer supporting elongation or S phase cytosol. Initiation of DNA replication in vitro is restricted to G1 phase nuclei. (C) In vitro analysis of the replicative capacity of WI38 G1 nuclear templates in quiescent, replicative senescent and S phase cytosolic extracts. S phase cytosolic extracts, but not quiescent or replicative senescent extracts, support efficient in vitro replication. (D) In vitro analysis of the replicative capacity of WI38 G1 nuclear templates in elongation buffer (EB), S phase cytosolic extracts and after titration of senescent cytosolic or nuclear extract into S phase cytosol. Titration of senescent nuclear extract but not nuclear extraction buffer (Buffer) alone resulted in striking inhibition of DNA replication initiation in vitro. Note that different proportions of S phase contaminants in WI38 G1 nuclear preparations in (B, 15%) and (D, <1%) relate to the use of two different batches of G1 nuclei.

cells irreversibly arrest proliferation with a G1 phase DNA content, become resistant to apoptotic death, and show selected changes in differentiated cell functions (reviewed by Campisi, 1996). Senescence can occur through both telomere-dependent and telomere-independent mechanisms (Bodnar et al., 1998; Carman et al., 1998; Jones et al., 2000) and is mediated and maintained through the function of negative growth regulators including p53, $p21^{CIP1}$, and $p16^{INK4a}$ (Brown et al., 1997; Gire and Wynford-Thomas, 1998; Zhu et al., 1998). To investigate the linkage of the replication initiation pathway to loss of proliferative capacity in cellular senescence, we have correlated Mcm2 protein expression in human breast fibroblasts (HMF48) and epithelial cells (HMEC48) that are derived from reduction mammoplasty specimens with the number of population doublings in culture. Immunofluorescence studies on mammary fibroblasts and epithelial cells show that the proportion of Mcm2-positive cells decreases with increasing population doublings (PD) (Fig. 4A), coinciding with transition to the characteristic senescent phenotype (reviewed by Stanulis-Praeger, 1987). These findings are further reinforced by a decrease in Mcm2 protein levels with increasing population doublings in both breast fibroblasts and epithelial cells, coinciding with a dramatic decrease in the percentage of BrdU-incorporating cells as cultures reach the end of their replicative life span (Fig. 4B). These data are in agreement with previous kinetic studies of replicative senescence that demonstrated a gradual decline in the proportion of proliferating cells and not an abrupt arrest of the whole cell population (Thomas et al., 1997). To determine whether other constituents of the DNA replication licensing machinery are also downregulated in cellular senescence, we have investigated Orc2, Cdc6, Mcm2, Mcm3 and Mcm5

protein levels in replicative senescent WI38 HDF. Immunoblot analysis of total cell extracts shows Cdc6 and MCM but not Orc2 downregulation in senescent fibroblasts (Fig. 4C). The mechanism responsible for repression of at least some of the G1/S genes in senescent human fibroblasts has been linked to a marked deficiency in the activity of E2F (Dimri et al., 1994; Good et al., 1996). E2F-binding sites have been found in the promoter regions of a number of mammalian MCM genes (Tsuruga et al., 1997) and in the human *CDC6* gene (Yan et al., 1998) suggesting that repression of Cdc6 and MCM proteins may be a downstream consequence of the deficiency in E2F activity in senescent cells. Notably, protein levels of the cyclin-dependent kinase inhibitor (CDKI) $p21^{CIP1}$, originally identified as an overexpressed gene in senescent human fibroblasts (reviewed by Stanulis-Praeger, 1987), are upregulated in senescent cell extracts (Fig. 4C).

To determine whether downregulation of the MCM replication licensing factors also occurs in senescent cells in vivo, we studied Mcm2, Mcm5 and Mcm7 expression in 'giant cell tumour' of bone. This neoplasm affects the epiphysis of bone and is composed of two main components, mononuclear stromal-like cells and multi-nucleate giant cells (Fig. 4D, left panel) (Aparisi et al., 1979). Previous enzyme histochemical and electron microscopy studies on this biphasic tumour suggest that the mononuclear cells are the basic tumour element, and not the multinucleate giant cells after which the neoplasm is named (Athanasou et al., 1985; Hanaoka et al., 1970). Interestingly, mitotic figures and tritiated thymidine incorporation are restricted to the mononuclear cells, suggesting that in this biphasic tumour, proliferation is restricted to the mononuclear population of cells (Roessner et al., 1984). These data are in agreement with the finding that biological evolution of this tumour correlates with number and morphological characteristics of the stromal mononuclear cells (reviewed by Sanerkin, 1980). The giant cells, which are most likely of monocyte/macrophage derivation (Regezi et al., 1987; Yoshida et al., 1982) and that show no morphological evidence of differentiation, thus appear to be in a replicative senescent state. Immunostaining of histological sections prepared from these osseous neoplasms (12 cases) revealed high levels of MCM proteins in the mononuclear stromal-like tumour cells (Fig. 4D, right panel). Strikingly, the multinucleate giant cells lack MCM proteins (Fig. 4D, right panel), thus confirming our in vitro findings of MCM downregulation in senescent human cells (Fig. 4A-C). The observed coupling of the replication initiation pathway with loss of proliferative capacity in senescent epithelial cells, fibroblasts and macrophages is indicative of a general rather than cell type-specific growth regulatory mechanism.

Initiation of DNA replication is suppressed during exit from the proliferative cell cycle

Numerous changes in gene expression, including repression of growth stimulatory genes, overexpression of growth inhibitory genes and interference with downstream pathways occur during withdrawal of human cells from the proliferative cycle into the quiescent, differentiated or senescent states (reviewed by Coffman and Studzinski, 1999; Smith and Pereira-Smith, 1996). The in vitro and in vivo data (Figs 2-4) suggest that downregulation of Cdc6 and MCM replication licensing factors is a powerful downstream mechanism

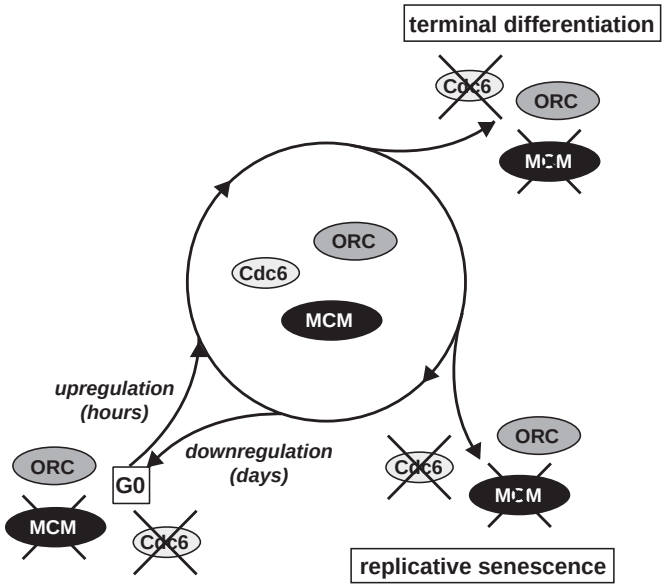

Fig. 6. Three ways out of the cell cycle. The replication initiation factors ORC, Cdc6 and MCM are present throughout all phases of the proliferative cell cycle. In contrast, the Cdc6 and MCM components of the replication initiation pathway are downregulated in quiescent, terminally differentiated and replicative senescent 'out-of-cycle' states. Thus, Cdc6 and MCM proteins comply with the theoretical definition of a proliferation marker.

contributing to loss of proliferative capacity in these 'out-of-cycle' states.

To provide functional evidence for a link between replication licensing and loss of proliferative capacity in human cells, we have compared the replication competence of nuclear templates prepared from quiescent, senescent and G1 phase WI38 HDF in our recently established mammalian cell-free DNA replication system (Fig. 5A; Stoeber et al., 1998). Incubation of G1 nuclei, which contain functional pre-RCs, in a physiological buffer that supports elongation but not initiation of replication resulted in 15% of the nuclei replicating DNA (Fig. 5B). These nuclei represent a small proportion of contaminating S phase nuclei present in the G1 nuclear preparation that continue semi-conservative DNA replication at replication forks established in vivo prior to their isolation (Stoeber et al., 1998). In comparison, 45% of the G1 nuclei replicate DNA upon incubation in S phase cytosol, the difference to the elongation control representing true initiation in vitro (Fig. 5B). In contrast, both quiescent and replicative senescent nuclear templates, lacking functional pre-RCs, fail to show significant levels of replication initiation in vitro (Fig. 5B). The low percentage of senescent nuclei undergoing initiation (2%) is in agreement with a gradual decline in the proportion of proliferating cells during cellular senescence (Thomas et al., 1997), replication competence being restricted to a residual population of 'licensed' cells that have not yet reached their Hayflick limit.

To determine whether an additional soluble initiation activity upstream of replication licensing is essential for initiation of DNA replication, we have compared the in vitro replication potential of 'licensed' G1 nuclear templates in quiescent, senescent and S phase cytosolic extracts (Fig. 5C).

Incubation of WI38 G1 nuclei in S phase cytosolic extract resulted in 34% of the nuclei replicating with less than 1% representing S phase contaminants (Fig. 5C). Notably, substitution of the replication-competent S phase cytosolic extract by either quiescent- or senescent cytosolic extracts led to a marked decrease in the percentage of replicating nuclei (Fig. 5C). These data suggest that quiescent and senescent cytosolic extracts lack a soluble initiation activity present in S phase cytosol and/or contain *trans*-dominant negative regulators of DNA replication.

The growth arrest associated with cellular senescence is a dominant trait in somatic cell fusion experiments. When proliferating pre-senescent cells are fused to senescent cells, they fail to replicate their DNA in response to mitogens (Norwood et al., 1974). Moreover, DNA replication is inhibited in immortal tumour cells when they are fused to senescent cells (Muggleton-Harris and DeSimone, 1980). These findings indicate that growth stimulatory gene or oncogene products do not overcome the growth arrest associated with cellular senescence. Thus, senescent cells must express one or more factors that act in a *trans*-dominant fashion to inhibit progression into S phase. To determine whether in senescent human cells mechanisms upstream of replication licensing contribute to the suppression of cell proliferation through inhibition of DNA replication, we have titrated nuclear and cytosolic extracts from senescent WI38 HDF into co-incubations of replication-competent WI38 G1 nuclei and HeLa S phase cytosolic tumour cell extract (Fig. 5D). Incubation of G1 nuclei in S phase cytosolic tumour cell extract resulted in 34% of nuclei replicating with less than 1% representing S phase contaminants (Fig. 5D). Titration of senescent cytosolic extract had no significant effect on the percentage of nuclei replicating in vitro (Fig. 5D). In contrast, titration of senescent nuclear extract resulted in striking inhibition of DNA replication initiation (Fig. 5D). Notably, titration of nuclear extraction buffer alone had no significant inhibitory effect (Fig. 5D).

Taken together, four main conclusions can be drawn from the in vitro and in vivo studies (Figs 2-5). First, downregulation of the Cdc6 and MCM constituents of the replication initiation pathway results in loss of replication competence and appears to be a common growth regulatory mechanism contributing to the suppression of proliferation in quiescent, terminal differentiated and replicative senescent human cells and tissues. Second, the presence of Cdc6 and MCM proteins in all phases of the proliferative cell cycle, but their absence in quiescence, terminal differentiation and cellular senescence, defines a novel class of proliferation marker (Fig. 6). Third, replication licensing alone is not sufficient for initiation of DNA replication but requires a replication-permissive cytosolic environment. Fourth, replicative senescent cells contain *trans*-dominant negative nuclear regulators of cellular proliferation that may induce an immediate growth arrest by inhibiting initiation of DNA replication.

Identification of an extended G1 phase in mammary progenitor cells

Breast and prostate represent self-renewing glandular tissues that are under hormonal growth regulation. Cell kinetic studies with a range of proliferation markers including Ki67, proliferating cell nuclear antigen (PCNA), mitotic index and in vitro bromodeoxyuridine (BrdU) or tritiated thymidine incorporation have demonstrated low proliferative activity in both tissues. The proportion of cycling epithelial cells in prostate varies from less than 1% to 4% (Carroll et al., 1993; Gallee et al., 1989). In pre-menopausal breast proliferation indices for lobular cells vary from 0% to 11.5%, the higher values corresponding to the secretory phase of the menstrual cycle (Potten et al., 1988). From our in vitro and in vivo studies (Figs 2-5) it would be expected that immunohistochemical staining of MCM proteins in breast and prostate detects the same proportion of cycling cells as those identified by conventional proliferation markers. Indeed, this is the case for normal prostatic tissue with 2% of basal glandular epithelial cells showing positive immunostaining for Mcm2, Mcm5 and Ki67 (Fig. 7A,B; M. V. Meng et al., unpublished). However, immunohistochemical staining of MCM proteins in normal pre-menopausal breast obtained from reduction mammoplasty specimens (ten cases) produced unexpected results. Strikingly high proportions of lobular cells express Mcm2 (22-78%; mean, 47%) and Mcm5 (35-94%; mean, 65%) (Fig. 7C), a much higher labelling index than found for Ki67 (4-10%; mean, 6%) (Fig. 7D) and other conventional proliferation markers (Potten et al., 1988). Interestingly, it is predominantly the luminal epithelial cells that express MCM proteins (Fig. 7C). Notably, these cells have been shown to contain progenitors to the myoepithelial cell population (Pechoux et al., 1999). The proportion of MCM-expressing lobular cells increases to greater than 95% during pregnancy, coinciding with cell expansion, and now also includes the myoepithelial cells (Fig. 7E). The MCM proteins finally undergo downregulation as lobular cells adopt a secretory phenotype that is characteristic of lactating breast (Fig. 7F; <3% of MCM-expressing lobular cells), consistent with differentiation-dependent loss of replication licensing (Fig. 3). The striking discrepancy between MCM expression and conventional proliferation markers suggests the existence of a population of luminal cells in the lobules of adult pre-menopausal breast that although ‘licensed’ for DNA replication are in fact rarely synthesising DNA.

DISCUSSION

Development and maintenance of tissues in multicellular organisms requires precise spatial and temporal control of cell proliferation. Initiation of DNA replication is a crucial decision point during cell proliferation and lies at the point of convergence of complex networks of signalling molecules that have evolved to specify when and where cells divide in an organism. Recent studies in yeast and *Xenopus* have identified ORC, Cdc6, and MCM proteins as essential factors for initiation of DNA replication in eukaryotic cells. These highly conserved replication initiation factors assemble sequentially into pre-replicative complexes (pre-RCs) at origins during the late M to G1 transition (reviewed by Donaldson and Blow, 1999; Tye, 1999), thereby ‘licensing’ chromatin for replication in the subsequent S phase (reviewed by Chevalier and Blow, 1996). The constituents of the pre-RC can therefore be regarded as relay stations coupling growth regulatory pathways with DNA replication. Although the functions of ORC, Cdc6, and MCM proteins in cell cycle regulated DNA replication

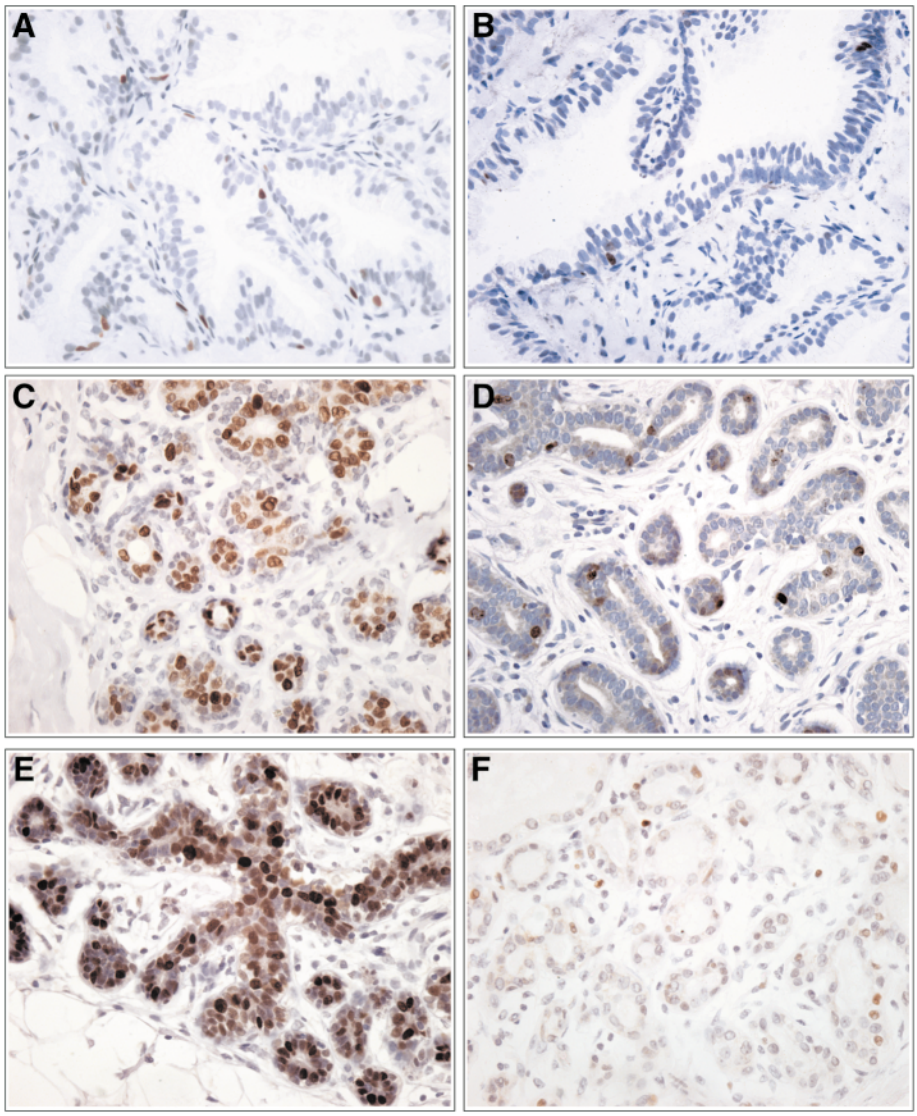

Fig. 7. Indirect immunoperoxidase staining of prostate and breast with anti-Ki67 and anti-Mcm2 monoclonal antibodies. (A,B) In prostate, only a small number of glandular epithelial cells express Mcm2 (A) and Ki67 (B), which are restricted to the basal cell population (×158). (C) A high proportion of mammary luminal epithelial acinar cells in resting pre-menopausal breast show Mcm2 expression (×158). (D) In contrast to Mcm2, Ki67 is only expressed in a small number of mammary luminal epithelial acinar cells in pre-menopausal breast (×158). (E) In breast tissue from pregnant females the proportion of Mcm2 expressing acinar cells increases and now also includes the outer myoepithlial cell population including some specialised stromal cells (×158). (F) In breast tissue from lactating females the majority of mammary acinar cells lack Mcm2 expression (×158).

have been well characterised in yeast and *Xenopus*, their potentially important role in growth regulation of human tissues and their respective tumours remains to be elucidated. For the first time, we have combined studies in tissue culture systems with a reductionist human in vitro DNA replication assay to characterise regulation and function of the pre-RC in human somatic cells. Furthermore, we have integrated our in vitro findings with an analysis of the regulation of pre-RC constituents in self-renewing, stable and permanent tissues to determine coupling of replication licensing, cell proliferation and co-ordination of growth in the three classes of human tissue.

Loss of proliferative capacity during exit of mammalian cells from the mitotic cell division cycle has been linked to growth inhibition mediated by tumour suppressor proteins and CDKIs (reviewed by Campisi, 1996; Sherr and Roberts, 1999; Zhang, 1999). Our studies suggest that downregulation of the Cdc6 and MCM constituents of the replication initiation pathway represents a powerful downstream mechanism that contributes to loss of proliferative capacity in quiescent, differentiating and replicative senescent human cells. In vitro studies in quiescent cultured cells suggest that loss of Cdc6 and MCM expression is most likely enforced at both transcriptional and protein levels (Schulte et al., 1996; Williams et al., 1997). Absence of replication licensing in stable tissues, as defined by downregulation of MCM proteins, is a potentially important mechanism for suppression of cell proliferation in organs with dormant growth potential such as liver and thyroid (Fig. 2C). Interestingly, a similar growth regulatory mechanism may operate in haemopoietic cells. Absence of replication licensing is characteristic of the quiescent state in circulating peripheral human T cells. Stimulation with phytohaemagglutinin (PHA) results in induction of Cdc6 expression and pre-RC assembly coinciding with re-entry into the proliferative cycle (N. C. Lea et al., unpublished). Our immunohistochemical study of the colonic mucosa shows that in self-renewing tissues with a single differentiation pathway, the transition from progenitor cells to the non-mitotic terminally differentiated phenotype is associated with downregulation of the MCM replication licensing factors (Fig. 3A,B). The ‘license to replicate’ is also withdrawn in terminally differentiated permanent tissues such as neurones (Fig. 3C), myocardial and skeletal muscle cells (data not shown). Thus, differentiation and DNA replication licensing appear to be mutually exclusive processes in self-renewing and permanent human tissues, consistent with the concept of antagonism between the cellular circuits that control proliferation and differentiation (reviewed by Olson and Spiegelman, 1999; Olson, 1992). The differential MCM protein expression found in medulloblastoma further reinforces this concept, and suggests that loss of replication licensing coinciding with commitment of precursor cells to a specific lineage of differentiation also applies to tumour cell populations (Fig. 3D-F). The inverse relationship between replication licensing and differentiation is also emphasised by

our recent findings in epithelial dysplasia, a premalignant pathological state characterised by failure of epithelial stem cells to correctly engage the terminal differentiation programme (reviewed by Sell and Pierce, 1994). The resulting disordered growth is associated with persistent and ectopic expression of Cdc6 and MCM replication licensing factors, findings that we have exploited for novel diagnostic cancer screening strategies (Stoeber et al., 1999; Williams et al., 1998).

Although Cdc6 and MCM proteins undergo complete downregulation in quiescent, differentiated and senescent human cells, our immunoblot data for Orc2 suggest that the origin recognition complex associates with chromatin throughout the proliferative cell cycle (Fig. 2A) and remains stably bound in 'out-of-cycle' states (Figs 2B, 4C). Persistence of chromatin-bound ORC in non-cycling human cells raises the possibility that ORC may be involved in functions beyond cell proliferation. In addition to expression in proliferating tissues like testis or colonic mucosa, Orc2 and Orc5 expression has also been reported in tissues without significant proliferation, such as ovary and prostate (Quintana et al., 1998; Takahara et al., 1996). Furthermore, ORC is possibly involved in gene silencing from yeast (Bell et al., 1993) to higher eukaryotes (Pak et al., 1997). However, despite being in agreement with a previous report (Musahl et al., 1998), our data do not explicitly address the question of whether regulation of Orc2 can be taken as typical of ORC in general. Indeed, expression of Orc1 but not Orc2-5 is cell cycle regulated in human cells (reviewed by Quintana and Dutta, 1999), and concomitantly with its hyper-phosphorylation Orc1 is less stably associated with chromatin in M phase (Natale et al., 2000; Tatsumi et al., 2000). It therefore remains to be elucidated whether, in addition to its putative role in resetting of the next replication cycle in M phase, human Orc1 may also be involved in ORC modifications associated with exit from the proliferative cell cycle.

Reversible growth arrest in replicative quiescence is linked to downregulation of the Cdc6 and MCM replication licensing factors but not Orc2 (Fig. 2B). Consequently, quiescent nuclear templates fail to initiate replication in vitro even when exposed to replication-competent S phase cytosolic extract (Fig. 5B). Nuclear templates prepared from mouse or human fibroblasts during re-entry into the proliferative cycle gain competence to replicate in vitro during a defined 'window of opportunity' 16-18 hours after release from G0, concomitantly with Cdc6 and MCM expression, and the step-wise recruitment of these licensing factors onto chromatin (Fig. 5B; Stoeber et al., 1998). In contrast, in cycling human epithelial cells, Cdc6 and Mcm5 proteins are recruited to chromatin within 2 hours of the release from nocodazole-induced mitotic arrest, gradually accumulating on chromatin throughout G1 phase (Fig. 2A). Replication licensing of chromatin at this early time point in the proliferative G1 phase is consistent with the finding that nuclear templates prepared from all stages of G1 phase in HeLa cells (2-9 hours after release from mitotic arrest) can initiate DNA replication in S phase cytosolic extracts (Krude et al., 1997). Thus, although the G0 to S and M to S transitions of the cell cycle are often both referred to as 'G1', we suggest that these transitions represent strikingly different molecular states with respect to replication licensing.

Two crucial questions related to the molecular control of replicative senescence are just emerging. First, why do senescent cells fail to initiate DNA synthesis? In this study we have demonstrated for the first time that withdrawal of the 'license to replicate' (Fig. 4A-D) contributes, at least in part, to the failure of replicative senescent cells to initiate chromosomal replication (Fig. 5B). Second, what dominant inhibitors are expressed by senescent cells? Titration of senescent nuclear extract into in vitro replication reactions containing initiation-competent G1 nuclei and S phase cytosolic extracts indicates that in addition to downregulation of the licensing machinery, senescent cells indeed express dominant negative regulators of replication initiation (Fig. 5D). Candidates for such trans-dominant acting suppressors of growth in senescent cells include p53, pRb, $p16^{Ink4a}$ and $p21^{Cip1}$ (Alcorta et al., 1996; Brown et al., 1997; Hara et al., 1991). However, somatic cell fusion experiments suggest the existence of many more, as yet unidentified, gene products that achieve and maintain the arrested senescent state (reviewed by Smith and Pereira-Smith, 1996). Thus, exploitation of our in vitro replication assay, using subcellular components isolated from replication-competent and senescent cells, for the first time offers a functional approach for identification and direct biochemical analysis of dominant negative regulators operating in cellular senescence.

Identification of growth fractions in tumour cell populations has high prognostic value for predicting survival and tumour recurrence in a range of human cancers, including prostate and breast cancer, and lymphoma (reviewed by Hall and Levison, 1990; Quinn and Wright, 1990). Established methodologies for assessment of growth fractions in human and animal tissues include mitotic counting (reviewed by Donhuijesen, 1986) and labelling of newly synthesised DNA with tritiated thymidine or bromodeoxyuridine (BrdU) (reviewed by Quinn and Wright, 1990). The major limitation of these methodologies, however, is that S phase labelling will underestimate the growth fraction if a considerable fraction of cells have intermitotic times that exceed the labelling interval (reviewed by Alison, 1995). The use of proliferation markers is therefore regarded as a more sensitive method for determining growth fractions in dynamic cell populations. These are antigens, which are present throughout the cell cycle, expressed in all cell types and absent from cells in non-proliferative states (reviewed by Scholzen and Gerdes, 2000). Whereas PCNA has found more limited use as a proliferation marker, owing to its involvement in DNA repair (reviewed by Hall et al., 1990), the Ki67 nuclear antigen, exclusively expressed in proliferating cells, has been exploited in many tumours types for assessing prognosis (reviewed by Brown and Gatter, 1990; Scholzen and Gerdes, 2000). Our findings in human culture cells demonstrate that Cdc6 and MCM proteins are present throughout all phases of the proliferative cell cycle (Fig. 2A) but are absent in 'out-of-cycle' states (Figs 2B,C, 3, 4), suggesting that these replication licensing factors comply with the theoretical definition of a proliferation marker (Fig. 6). In support of this hypothesis, analysis of MCM expression in liver (Fig. 2C), colon (Fig. 3A,B) and brain (Fig. 3C), which represent the three classes of human tissue (Leblond, 1963), demonstrates tight correlation between replication licensing and cell proliferation. However, apparently conflicting evidence arises from our immunohistochemical studies of human pre-menopausal breast where mitotic index (Longacre and Bartow, 1986), tritiated

thymidine labelling (Potten et al., 1988) and Ki67 (Fig. 7D and Pavelic et al., 1992) all indicate low proliferative activity (0-11% cycling cells), whereas MCM immunostaining detects a significantly higher proportion of luminal cells in cycle (40-60%) (Fig. 7C,E,F). Notably, in prostate we found a tight correlation between the proportion of cycling cells identified by MCM labelling (Fig. 7A) and conventional proliferation markers (Fig. 7B; Carroll et al., 1993; Gallee et al., 1989), suggesting that the intriguing discrepancy in breast is not linked to hormonal regulation. However, this discrepancy can be explained by a model in which populations of cells in certain tissues are licensed to replicate and thus have proliferative potential but are rarely synthesising DNA. Intriguingly, this replication-licensed but resting state is restricted to stem-like luminal cells of the acini (Fig. 7C), suggesting a special adaptation related to the unusual biology of this particular epithelium, which undergoes rapid and periodic cell expansion and loss. Importantly, our studies on breast demonstrate that MCM replication licensing factors should be regarded as novel biomarkers of growth that, in addition to actively proliferating cells in dynamic cell populations, also allow detection of cells with proliferative potential.

Tumour cells are frequently observed to proliferate at a slow rate, particularly in the early stages of tumourigenesis (Tannock and Hill, 1998). We have previously observed marked differences between the proportion of premalignant cells that express MCM proteins (80-100%) and the conventional proliferation markers Ki67 and PCNA (3-8%) in dysplastic lesions of the uterine cervix, findings that we have clinically exploited for the development of an immunoenhanced cervical (Pap) smear test (Williams et al., 1998). Furthermore, in comparison to Ki67 or PCNA, antibodies against MCM replication licensing factors consistently label a higher proportion of tumour cells in a range of common malignancies including those of colon, skin and bladder (Freeman et al., 1999). These observations indicate that, as observed in breast (Fig. 7C), neoplastic dynamic cell populations contain both cells with proliferative potential and actively proliferating cells. We therefore suggest that this concept provides a rational for the exceptional diagnostic sensitivity of MCM labelling for detection of dysplastic and malignant changes in mucosal epithelia (Stoeber et al., 1999; Williams et al., 1998; K.S. et al., unpublished). Furthermore, identification in tumours of actively proliferating, replication-licensed with proliferative potential and non-proliferating cells without a license may be of importance in predicting prognosis. Indeed, we have recently shown that MCM protein expression is of prognotic importance in assessment of cerebral oligodendrogliomas (S. B. Wharton et al., unpublished) and as an independent predictor of disease-free survival in prostate cancer (M. V. Meng et al., unpublished). Moreover, it has recently been reported that the mechanism underlying potent anti-tumour properties of adozelesin and related drugs involves disruption of origin licensing (Weinberger et al., 1999). Thus, multi-parameter analysis of tumour cell populations using a combination of conventional proliferation markers and biomarkers for replication licensing may allow more accurate prediction of the efficacy of anti-tumour drugs that target DNA replication.

In summary, the initiation of DNA replication is a crucial decision point in human cell proliferation, and represents an evolutionary conserved mechanism positioned downstream of complex networks of oncogenic signalling and transduction pathways that control cell growth. We have shown that regulation of the Cdc6 and MCM constituents of the replication initiation pathway plays an important role in co-ordinating growth in human tissues. Further characterisation of pre-RC regulation and function in human somatic cells will provide important new insights into the growth regulatory mechanisms operating in development and maintenance of mammalian tissues and how these mechanisms become deregulated in neoplasia.

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