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Regulation of the G1/S Transition in Adult Liver: Expression and Activation of the Cyclin Dependent Kinase Cdk1 in Differentiated Hepatocytes is Controlled by Extracellular Signals and is Crucial for Commitment to DNA Replication

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1. Introduction

In eukaryotic cells, DNA replication and mitosis are the two processes allowing cell division and generation of two identical daughter cells without loss or alterations of genetic information. These two crucial steps are separated by the G1 and G2 (the acronym for gap) phases that are essential for metabolic adaptation and checking genome integrity before DNA replication and mitosis. Progression of eukaryotic cells through the cell cycle is regulated by the sequential formation, activation, and subsequent inactivation of structurally related serine/threonine protein kinases, the Cyclin-Dependent Kinase or Cdks (Malumbres et al., 2009). Cdks become active upon binding to their regulatory and periodically expressed subunits, namely, the cyclins. Timing of activation of these complexes is determined by a variety of mechanisms including transcriptional regulation, formation of complexes between Cdks, cyclins and other regulatory partners such as Cdk inhibitors (Cdki). In addition, phosphorylation, subcellular localization and selective proteolysis regulate the catalytic activity of these complexes. The first Cdk to be identified, cdc2, was initially discovered as the protein kinase in complex with several cyclins controlling both G1/S and G2/M transitions of the cell cycle in the yeast *Schyzosaccharomyces pombe* (Nurse and Thuriaux, 1980). In contrast to the yeast in which cdc2 is "the" master cell cycle regulator, in mammalian cells at least 20 Cdks, 5 Cdk-like protein kinases (Malumbres et al., 2009) and more than 30 cyclins have been identified that form multiple Cdk/cyclin complexes controlling the cell cycle progression (Malumbres and Barbacid, 2005) and regulating gene transcription and RNA processing (Loyer et al., 2005). Over the last two decades, it has become apparent that these multiple Cdk/cyclin complexes play specific roles in the regulation of a subset of events in the different phases of the cell cycle. A broadly accepted view of the mammalian cell cycle considers that cyclin D1-bound to either Cdk4 or Cdk6 controls progression in late G1 phase through phosphorylation of the retinoblastoma protein (Rb) allowing activation of the E2F transcription factors and

downstream transcriptional activation of genes involved in G1/S transition and S phase (Sherr, 1994). Cdk2 successively associates with cyclin E and A, completes the hyperphosphorylation of Rb, phosphorylates components of the DNA replication machinery and governs centrosome duplication at the G1/S transition and in S phase. Finally, activity of Cdk1 associated with both A- and B-type cyclins is required for entry and progression through M phase. In this model of mammalian cell cycle, Cdk2 and Cdk1 are thought to function independently at two distinct periods, respectively the G1 to S and G2 to M transitions, without functional redundancy (Bashir and Pagano, 2005).

This model of cell cycle control has first been challenged by the finding that some cancer cells proliferate despite Cdk2 inhibition (Tetsu and McCormick, 2003). Independently, the demonstration was brought that knock-out mice for Cdk2 as well as E-type cyclins are viable and that cell cycle of cultured Cdk2$^{-/-}$ mouse embryonic fibroblasts (MEFs) do not show major alterations besides a delayed commitment to S phase (Berthet et al., 2003; Ortega et al., 2003). These data indicated that Cdk2/cyclin E complexes are dispensable for commitment to S phase and/or that other Cdk(s) would compensate for the loss of Cdk2. In contrast, Cdk1$^{-/-}$ mouse embryo's development is arrested at a very early stage and knock-down of Cdk1 expression by shRNA in synchronized Cdk2$^{-/-}$ MEFs strongly reduces S phase entry (Aleem et al., 2004) demonstrating that other Cdks cannot compensate for Cdk1 ablation. A revisited model of cell cycle regulation in which Cdk1 would compensate for Cdk2 ablation by controlling the G1/S transition and initiation of DNA replication was proposed (Kaldis and Aleem, 2005). Consequently, several new questions have been raised: Does Cdk1 initiate the G1/S transition only in Cdk2$^{-/-}$ cells isolated from genetically modified mice or does Cdk1 generally act as the predominant Cdk in somatic cells? Alternatively, Cdk1 and Cdk2 may act in synergy or redundantly to promote both DNA replication and centrosome duplication during the G1/S transition. It has been proposed that the role of Cdk1 at the G1/S transition may have been overlooked in higher eukaryotic cells since Cdk2 activity appeared higher to that of CDK1 at the onset of DNA replication and because this Cdk1 activity was negligible compared to the peak of Cdk1 activity at the G2/M transition (Bashir and Pagano, 2005). Interestingly, it was also demonstrated that both Cdk1 and Cdk2 were required for efficient DNA replication in *Xenopus* egg extracts (Krasinska et al., 2008) suggesting that at least in some non genetically modified cell types, Cdk1 could contribute to S phase initiation and/or DNA replication. It is clear that Cdks play a pivotal role in orchestrating commitment to S phase and DNA replication but from the most recent publications studying the function of Cdk1 and Cdk2 throughout the cell cycle a large of body of data evidences qualitative and quantitative differences in expression of Cdk/cyclin complexes between mammalian cell types leading to the emerging picture of slightly distinct Cdk-dependent molecular mechanisms during G1 phase that all, however, trigger G1/S transition.

In this review, we briefly discuss some general knowledge of the growth factor dependent entry into and progression through the cell cycle in mammalian cells and the differences observed in the Cdk and cyclin expression between cell types. However, the main goal of this chapter is to highlight the role of Cdk1 in the G1/S transition in differentiated adult hepatocyte. In normal liver, hepatocytes are quiescent differentiated cells that keep the ability to re-enter the cell cycle when liver tissue integrity is challenged. This model has been widely used to study the cell cycle. We will present the *in vivo* and *in vitro* models of normal proliferating hepatocytes and our data showing the involvement of Cdk1 in the G1/S transition in these cells. In addition, our recent data unveiling an unexpected link

between extracellular signals (cytokines, growth factors and extracellular matrix components) and the control of the G1/S transition via the induction of Cdk1 will be developed in this chapter.

2. The cell cycle: a universal cell division process with a large diversity of cell signaling pathways controlling entry into and progression through G1 phase in mammalian cells

The molecular pathways controlling the progression throughout the cell cycle and both DNA replication and mitosis are remarkably conserved among eukaryotic cells (Araki, 2011). Although mammalian cells show a higher degree of complexity, at least some of the molecular mechanisms remain conserved from yeast to humans. A good example of these conserved pathways through evolution is the protein kinase Cdk1: invalidation of Cdk1 also called CDC28 in budding yeast *Saccharomyces cerevisiae* using a thermo-sensitive mutant can be rescued (complementation) by its human homolog Cdk1 (Ninomiya-Tsuji et al., 1991). The most conserved aspects of the cell cycle are probably DNA replication and major check-points for DNA integrity and mitosis. In contrast with these conserved mechanisms, multicellular organisms developed during evolution specific pathways to control the transition from quiescence to DNA replication. In mammalian cells, specific combinations of extracellular signal stimuli for each cell type promote the exit from quiescence, progression throughout G1 phase and commitment to DNA replication. Proliferation stimuli are constituted by a vast panel of growth factors and cytokines activating downstream intracellular signalling pathways after binding to membrane receptors mainly through a cascade of phosphorylation and dephosphorylation events that ultimately triggers changes in gene expression in order to induce the proteins absolutely required for duplication of cellular components including DNA and the subsequent mitosis (Iyer et al., 1999). In this picture, there is a striking contrast between the diversity of proliferation stimuli and early steps of proliferation signalling pathways and, on the other side, the limited number of proteins that control G1/S transition and DNA replication. In another word, cell cycle entry begins with proteins differentially expressed among cell types, which activate the ubiquitous machinery of DNA replication present in all dividing cells. During their journey towards DNA replication and mitosis, cells initially quiescent or exiting mitosis will sequentially activate less and less specific protein complexes throughout the G1 phase to eventually fire the DNA replication machinery identical between cell types. A crucial question, which is currently actively investigated, is how so many distinct proliferation stimuli and signalling pathways can lead to the activation of a limited number of proteins controlling commitment to and progression through DNA replication. To address this question, we will come back on the basis of progression throughout the G1 phase of the cell cycle and how the overall proliferation signalling pathway "narrows down" to activate the DNA replication at the G1/S transition.

2.1 The G1 phase and the mitogen dependent cell cycle progression

Most studies regarding cell cycle are conducted using synchronized cell models in which cells progress synchronously through the different phases of the cell cycle in order to analyze expression and activation of regulators at each step of the cell cycle. Yeasts and ovocytes from amphibians, especially *Xenopus*, and marine organisms such as star fish and sea urchin have provided cell models that proliferate spontaneously in a highly

synchronized manner to investigate cell cycle regulation. In addition, the genesis of thermo-sensitive yeast mutants for the cell cycle progression constituted an extremely powerful technical approach to isolate genes playing crucial roles in the cell cycle regulation. From the mid 70's to the late 80's, the burst of data obtained in these eukaryotic cells lead to the identification of major cell cycle regulators including the cyclins (Sherr, 1995) and their catalytic subunit partners the Cdks (Malumbres et al., 2009). Mammalian homologs of these cell cycle regulators were subsequently isolated and by the mid-90's a network of Cdk/cyclin complexes emerged. Pioneers in this field of investigation, Drs L. Hartwell, P. Nurse and T. Hunt who deciphered the cell cycle regulation in yeast and ovocytes were awarded with the 2001 Nobel Prize of medicine not only for the identification of new pathways regulating cell division (Nurse, 2002) but also for opening a complete new field in cancer research since many of these cell cycle regulators are altered during oncogenesis and/or are potential therapeutic targets for cancer treatments (Knockaert et al., 2002).

In mammals, synchronized cell proliferation *in vivo* is restricted to very few cell types among which proliferation of hepatocytes during liver regeneration following partial hepatectomy has probably been the most used model. Sections 2 and 3 in this chapter will focus on the major findings reported by our groups and others regarding regulation of the G1/S transition in hepatocytes. *In vivo*, cell renewal is mainly achieved through the proliferation of adult stem and progenitor cells that proliferate actively although these cells can probably arrest in G0 before additional rounds of division or entering a program of differentiation. Because stem/progenitor cells are rare cells and cannot be easily purified, there are few data regarding cell cycle regulation in these cell types. There are, however, adult differentiated cell types that remain arrested in G0, which can re-enter the cell cycle for several rounds of division upon appropriate proliferation stimuli including lymphocytes (Ajchenbaum et al., 1993), monocytes (Tushinski and Stanley, 1985) and fibroblasts (Iyer et al., 1999) which can be isolated relatively easily from blood or skin, respectively, plated in culture and used for cell cycle studies. Although these cell types are suitable models for conducting cell cycle studies there have been a limited number of publications reporting cell cycle data using lymphocytes and monocytes mainly because these primary cells need to be renewed for each experiment. The most widely used cell models in the field of cell cycle regulation are the immortalized or transformed cell lines artificially synchronized by drug treatments arresting the cells in G1/S or G2/M transitions and the primary fibroblasts arrested by serum starvation in a G0-like state (Figure 1). Although the scoop of this chapter is to focus on the progression in late G1 and the G1/S transition, it is important to point out that the comparison between these *in vitro* models of G0-like or early G1 arrest and *in vivo* G0 arrested cells were poorly documented for many years. However, recent reports evidenced differences between "arrested" cells in various conditions (Coller et al., 2006; Sang et al., 2008). For instance, the serum starvation of fibroblasts plated at low density obviously provides an experimental condition completely different from G0-arrested cells *in vivo*, which stop dividing for other reasons than the lack of growth factors or nutrients. Nevertheless, these *in vitro* synchronized mammalian cells provided powerful models to investigate cell cycle in mammalian cells and allowed to collect crucial data on the progression from early G1 to the commitment to DNA synthesis.

In the mid-70's, the *in vitro* synchronized mammalian cells allowed to define the concept of "restriction point" during the G1 phase (Pardee, 1974). A major feature of the G1 phase is that cells need a mitogenic stimulation to enter into and progress through G1 phase until

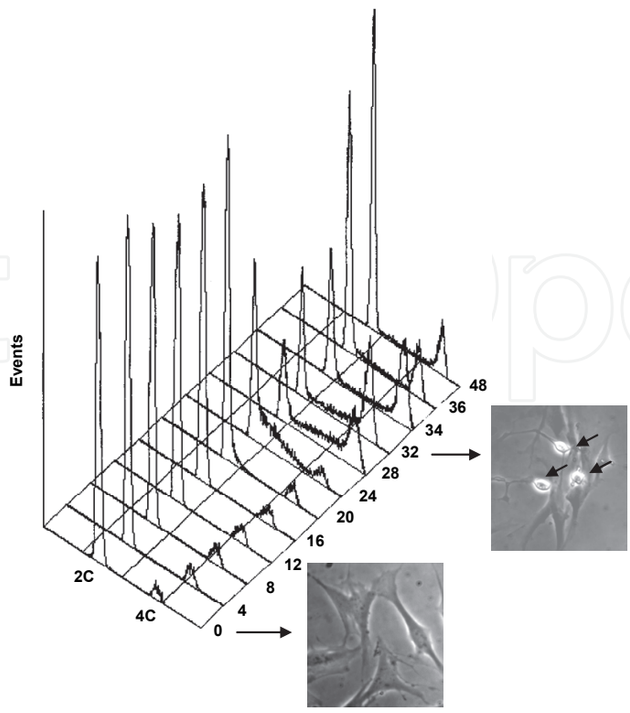

Fig. 1. Flow cytometry analysi of the DNA content in human foreskin fibroblasts plated at low cell density and synchronized by serum starvation. Cells were arrested in G0-like stage by serum starvation for 3 days and cell cycle was re-induced by adding back serum to culture medium. A time-course analysis of the DNA content was performed using propidium iodide staining by flow cytometry. Over 95% of arrested cells (0) in G0-like and cells progressing in G1 phase (from 4 to 20 hours after stimulation) showed a "2C" DNA content. At 24 hours, the number of cells with "2C" decreases as initiation of DNA replication begins (2C< cells<4C). At 24 hours, the peak of replications takes place and the percentage of cells reaching G2/M increases (cells with "4C" DNA content; mitosis : arrows). By 34 hours, cell cycle has been completed and cells initiate a new cell cycle by re-entering G1 phase.

they reach the so-called "mitogen dependent restriction point" in late G1 beyond which completion of the cell cycle becomes independent from extracellular stimuli. A. Pardee and co-workers evidenced this restriction point by using serum-starved fibroblasts stimulated to re-enter the cell cycle by adding back the serum (Pardee, 1974). They were able to show that a minimal period of stimulation was required to reach the late G1 and that, beyond this point, the cell cycle would be completed even after removing growth factors. This restriction point is very similar to the Start point in yeast that controls the commitment to S phase (Reed, 1992). Data from this group and others showed that the G1 phase is the longest phase of the cell cycle in all mammalian cells but the timing varies considerably between cell types. It is essential to distinguish the G1 progression between cells that proliferate actively and enter G1 after completion of mitosis and cells re-entering the cell cycle after a prolonged quiescence or G0. It is now well established that the transition from G0 to G1 is characterized by a profound modification of the expressed gene profile (Iyer et al., 1999) required for metabolic adaptation to cell proliferation and resulting in a longer period of

time for the cells to initiate progression in late G1 compared to the cells exiting mitosis. Following the discovery of the mitogen-dependent restriction point, it was demonstrated that progression through the G1 phase can be divided in several periods. Using purified priming and growth factors to stimulate progression of fibroblasts arrested in G0-like state by serum starvation, it was reported that progression of fibroblasts throughout G1 could be divided in 4 periods: competence, entry, progression and assembly (Figure 2). Stimulation of starved fibroblasts by PDGF is able to promote progression in early G1 in arrested cells until the restriction point C (competence) but fails to allow progression in mid and late G1 (Cross et al., 1989; Denhardt et al., 1986; Pledger et al., 1977; Pledger et al., 1978). This period in early G1 was named competence. Following stimulation by PDGF, further progression in late G1 and S phase is achieved by stimulation with EGF or insulin (Leof et al., 1983; Yang and Pardee, 1986). However, in absence of essential amino acids cells arrest in mid-G1 at a restriction point named "V". The progression between points "C" and "V "defines the period called entry (Pardee, 1986) while the progression between point "V" and the mitogen-dependent restriction point (point "R") was called progression. Finally, the period beyond the mitogen-dependent restriction point and before the burst of DNA synthesis was named assembly (Pardee, 1989).

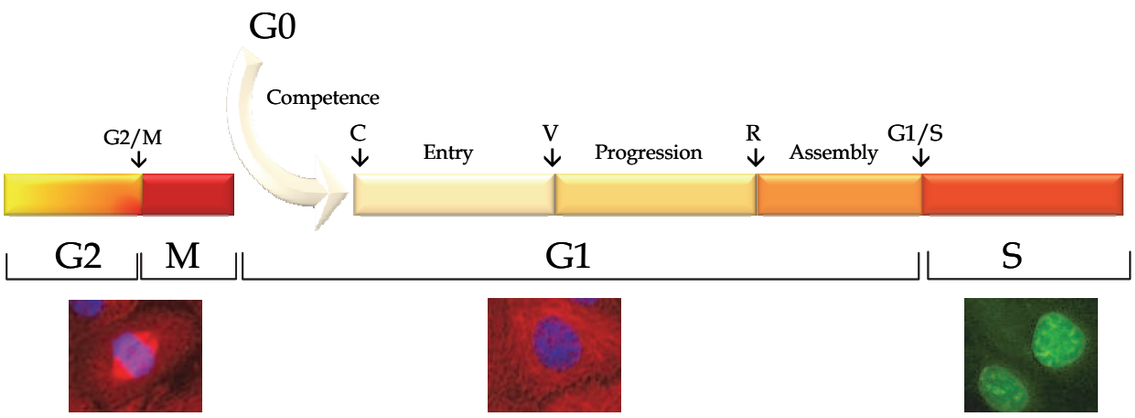

Fig. 2. Progression through the G1 phase is divided in several sub-phases. Photographs illustrate detection of cells in G2/M, G1 and S Phase : the cells in G2/M and G1 were stained with DAPI (DNA) and immuno-fluorescence detecting γ-tubulin while in S phase cells were incubated with BrdU to evidence DNA replication by indirect immunofluorescence. Four steps were identified during the G1 phase of the cell cycle: competence, entry, progression and assembly. Note that the convergence of molecular pathways in early G1 for cells entering G1 after completion of mitosis or re-entry in the cell cycle following stimulation by growth factors remains to be fully characterized in mammalian cells.

Similar studies were performed in other cell models such as mononuclear blood cells (Tushinski and Stanley, 1985) or hepatocytes (Loyer et al., 1996) leading to the same conclusion that G1 phase could be divided in sub-phases corresponding to major steps in the metabolic adaptation required for cells to initiate DNA replication and mitosis. From these data, soluble factors such as the PDGF that promote in early G1 were called priming factors while hormones and cytokines stimulating progression in late G1 and the G1/S transition were considered as mitogens or growth factors.

Following binding to their receptors on plasma membrane, priming and growth factors activate multiple phosphorylation events involving multiple protein kinases especially the MAPKinase pathways (Ruderman, 1993; Ussar and Voss, 2004). There are multiple cross-talks between these pathways but they ultimately control activation of transcription factors that sequentially trigger induction of cell cycle regulators such as the cyclins and Cdks (Talarmin et al., 1999). Cdks are nuclear effectors that play a pivotal role in orchestrating DNA replication, DNA repair, mitosis and centrosome duplication (Harrison et al., ; Muller et al., 2001; Ren et al., 2002). Because of the limited number of Cdk/cyclin complexes expressed in most mammalian cells in late G1 and at the G1/S transition, it was widely accepted until very recently that stimulation by growth factors triggered induction of D- and E--type cyclins in late G1, which associate with Cdk4/6 and Cdk2, respectively, to control entry into S phase. In this view of the cell cycle, by the time cells have reached late G1, the large diversity of growth factors and signaling pathways observed in early G1 is gone to leave the cells with ~four to six Cdk/cyclin complexes to regulate the entry into S phase. This view of the cell cycle has been partially revisited after the demonstration that Cdk2 knock-out mice were viable (Satyanarayana and Kaldis, 2009b; Sherr, 1994; Sherr and Roberts, 2004) and by the re-interpretation of data showing differences in the expression of Cdks and cyclins during G1 in different eukaryotic cells (Furukawa et al., 1990 ; Loyer et al., 1994). In the light of these recent data, it is tempting to ask the question whether additional combinations of Cdk/cyclin complexes can control the G1/S transition and progression in S phase and how such a diversity of complexes can activate the DNA replication machinery and centrosome duplication.

2.2 Expression and roles of Cyclin dependent kinases and cyclins during the G1 phase

Entry into and progression through the G1 phase of the cell cycle and the G1/S transition is associated with a sequential activation of Cdk/cyclin complexes upon the stimulation by priming and growth factors. The catalytic activities of these Cdk/cyclin complexes through the phosphorylation of "specific" protein substrates control the progression in G1 and commitment to DNA replication (Sherr, 1993), the chromosomal DNA replication (Araki, 2011) and centrosome duplication (Harrison et al., 2011). The identification of these substrates over the last 15 years has unveiled the crucial molecular mechanisms regulating the progression through G1 phase that need to be "unlocked" for the cell to proceed in S phase. In this section, we will briefly present the Cdk/cyclin complexes induced and activated during G1 phases and their phosphorylation substrates. Then, we will discuss how the differences in expression and/or activation of Cdk/cyclin complexes observed among mammalian cells may be compatible with the phosphorylation of the proteins controlling the initiation of DNA replication and centrosome duplication.

2.2.1 Sequential activation of Cdk/cyclin complexes during the cell cycle

For many years, the G0/G1 transition and progression in early G1 phase was thought to be associated with the induction of immediate-early and immediate delayed genes in a Cdk/cyclin independent manner. Following stimulation by priming factors a large set of immediate early genes is induced at a transcriptional level by pre-existing latent transcription factors such as NF-κB (FitzGerald et al., 1995). More recently Ren and Rollins made, however, a strong case that Cdk3/cyclin C complex could regulate the G0/G1

transition in human glioblastoma T98G cells (Ren and Rollins, 2004). The phosphorylation status of pocket protein family members including the retinoblastoma protein (pRb) and p130 changes throughout the cell cycle (DeCaprio et al., 1992). While cells leave quiescence to enter G1 this phosphorylation level varies and inactivation of pRb is sufficient to induce G0/G1 transition in quiescent cells (Canhoto et al., 2000). Based on these observations, Ren and Rollins postulated that hypophosphorylated or unphosphorylated pRb present in G0-arrested cells may be phosphorylated by Cdk/cyclin complexes to promote entry into G1 phase. They identified the Cdk3/cyclin C complex mediating pRb phosphorylation during G0/G1 transition (Ren and Rollins, 2004). Interestingly, cyclin C associated with Cdk8 (Tassan et al., 1995) plays a completely different role in transcriptional regulation through the phosphorylation of the C-terminal domain (CTD) of RNA polymerase II (Leclerc et al., 1996; Loyer et al., 2005; Rickert et al., 1999). This report associated for the first time a Cdk/cyclin complex with the G0/G1 transition (Figure 3).

This conclusion has not been generalized to a large panel of mammalian cell types in the past years. Indeed, many cells lack functional Cdk3 or express so little amounts that no conclusive data on the ubiquitous role of Cdk3/cyclin C complex at the G0/G1 boundary could be drawn. It was recently reported that in NIH3T3 cells, the absence of Cdk3 could be compensated by Cdk2 that interacts with cyclin C in early G1 (Hansen et al., 2009; Saxena et al., 2009). In this work, the authors did not investigate the phosphorylation of pRb by the Cdk2/cyclin C complex but rather showed that the transcription factor LSF (late simian virus 40 factor) (Kim et al., 1987) is phosphorylated by Cdk2 associated with cyclins C or E as well as Cdk3/cyclin C predominantly on serine 309. Phosphorylation of LSF on serine 291 by the MEK/extracellular signal-regulated kinase (ERK) signaling pathway upon stimulation by growth factors (Pagon et al., 2003; Ruderman, 1993; Volker et al., 1997) in mid-late G1 phase is essential for the G1/S transition since phospho(S291)-LSF controls the transcriptional activation of the thymidylate synthase (*Tyms*) (Powell et al., 2000). In contrast, phosphorylation of LSF on serine 309 in cells expressing Cdk3 inhibits LSF transactivation suggesting the required LSF shut-down in early G1 and its reactivation in late G1 mediated by Cdk/cyclin complex(es) and ERK, respectively (Hansen et al., 2009). This work appears important because it suggests functional redundancy of Cdk/cyclin complexes in early G1 and identifies LSF as the second known phosphorylation substrates of Cdk/cyclin complexes, in addition to pRb, during progression from quiescence to early G1 phase. There are too few reports describing the signaling pathways regulating the entry into G1 and early progression to draw the final conclusion that Cdk/cyclin complexes are crucial at this stage of the cell cycle but we can expect to learn more the molecular mechanisms regulating exit to quiescence in coming years.

In contrast, signaling pathways essential for the subsequent progression in late G1 are well documented and clearly involve the Cdk/cyclin complexes (Satyanarayana and Kaldis, 2009b). The transition from mid to late G1 phase is regulated by successive phosphorylation events of members of the pocket protein family including the retinoblastoma protein (pRb), p107, and p130 (DeCaprio et al., 1992). Cdk/cyclin complexes are responsible for the changes in pocket proteins phosphorylation status (Kato et al., 1993; Sherr, 1995). In mid-G1, the hypophosphorylated pRb binds to the transcription factor E2F family members thereby preventing active transcription of E2F-regulated genes. The negative regulation of E2F transcription factors mediated by pRb occurs through a conformation structure that prevents E2F's transactivation domain to be active and probably also by recruiting chromatin-modifying enzymes repressing transcription (Trimarchi and Lees, 2002). Upon

stimulation by growth factors, D-type cyclins are up-regulated (Matsushime et al., 1991) and associate with Cdk4 and/or Cdk6 to form active complexes (Matsushime et al., 1992; Matsushime et al., 1994) that partially phosphorylate pRb and/or actively phosphorylate a fraction of pRb (Kato et al., 1993). In late G1, formation of Cdk2/cyclin E complex triggers additional phosphorylation of pRb to generate the hyperphosphorylated form of pRb (Figure 3) that looses the ability to negatively regulate the transactivation domain of E2F's factors (Lundberg and Weinberg, 1998). Consequently, the release of E2F proteins promote transcription of a large set of genes required for the progression in late G1 including Cdk2 and cyclin E (Fan and Bertino, 1997; Geng et al., 1996), S phase entry (DeGregori et al., 1995; Kowalik et al., 1995; Ren et al., 2002) and centrosome duplication (Harrison et al., 2011). In parallel, Cdk2 phosphorylates the Nuclear protein Ataxia-Telangiectasia implicated in the transcription of histones (Zhao et al., 2000) and the nucleophosmin/B23 regulating centrosome duplication (Okuda et al., 2000). At this stage of the cell cycle progression cells have committed to DNA replication and removal of growth factors (in culture cell systems) will not affect either the burst of DNA replication or the mitotic rate. Thus, turning on the E2F-dependent transcription coincides with the progression beyond the mitogen-dependent restriction point identified by Pardee and co-workers (Pardee, 1989) before the discovery of Cdk/cyclin complexes.

In eukaryotic cells, chromosomal DNA replication is ensured through periodic and tightly controlled assembly and disassembly of pre-replication complexes (pre-RC) loaded on DNA replication origins (Diffley, 2004; Fujita, 2006). In mid-late G1, the Origin Recognition Complex (ORC) containing several subunits associated to the proteins CDC6 and Cdt1 is responsible for loading a replicative helicase and the mini-chromosome maintenance (MCM) 2-7 subunits to form the pre-RC (Fujita, 2006). Interestingly, loading of the pre-RC components occurs in a low Cdk activity period (Wheeler et al., 2008) while at the onset of DNA synthesis the increasing Cdk-dependent kinase activities trigger the MCM complex to initiate replication and the degradation of Cdt1 to prevent reassembly of additional pre-RC (Katsuno et al., 2009; Lei and Tye, 2001; Thomson et al.). Recent data from our laboratory demonstrated that in quiescent hepatocytes, MCM7 is not expressed but its expression becomes detectable immediately after the mitogenic stimulation in mid-G1, almost concomitantly with the induction of cyclin D1 (Garnier et al., 2009) and prior the high Cdk-dependent kinase activity taking place in late G1 and early S phase. The induction of MCM7 and the formation of the pre-RC thus occur in a very narrow period of time since in S phase, ORC1 and Cdt1 are degraded through several mechanisms including the phosphorylation by Cdks and downstream ubiquitination by SCFSkp2 ubiquitin Ligase (Fujita, 2006; Fujita et al., 2002). These well documented mechanisms clearly point out the crucial role of Cdk/cyclin complexes in the regulation of pre-RC formation. Similarly, pre-RC are activated by phosphorylations involving the protein kinase Cdc7 and the Cdk2/cyclin E complex that trigger the recruitment of Cdc45 (Woo and Poon, 2003) a crucial docking factor for DNA helicase and polymerases. During S phase, the heterodimer Cdk2/cyclin A also contributes to DNA replication (Cardoso et al., 1993; Rosenblatt et al., 1992; Zindy et al., 1992) by phosphorylating components of the replication machinery including the Proliferating Cell Nuclear Antigen (PCNA) and DNA polymerases. The activity of Cdk2 is thus tightly associated with the entry into and progression through S phase (Figure 3). Following mitosis, daughter cells receive a single centrosome, which, like DNA, must duplicate prior mitosis. In early S phase, centriole duplication begins and by the late G2, two mature centrosomes have been generated to ensure proper chromosome segregation

(Harrison et al., 2011). Duplication of centrioles is in part regulated through the G1 phase Cdk/cyclin-dependent pRb pathway (Adon et al., 2010) and there is a large body of evidences for the Cdk2/cyclin E involvement in the activation by phosphorylation of crucial regulators of centriole duplication (Harrison et al., 2011).

The activity of Cdk1 associated with both A- and B-type cyclins is required for entry and progression through M phase in all eukaryotic cells (Doree and Hunt, 2002). The activity of the Cdk1/cyclin B complex, which was the first cyclin-dependent kinase activity detected in sea urchin and in *Xenopus* (Arion et al., 1988; Gautier et al., 1988), rapidly appeared to be a master regulator of the G2/M transition in all eukaryotic cells (Doree and Hunt, 2002) including in humans cells (Draetta and Beach, 1988). Recently, the Cdk11^{p58} protein kinase was also shown to be essential for mitosis (Hu et al., 2007; Petretti et al., 2006). In humans, the *Cell division control 2 Like* genes *Cdc2L1* and *Cdc2L2* encode two related protein kinases, denoted Cdk11B and A, respectively, which are expressed as two predominant protein isoforms designated by their apparent molecular weight of 110 and 58kDa for the Cdk11$^{p110A/B}$ and Cdk11$^{p58A/B}$ isoforms, respectively (Trembley et al., 2004). The CDK11^{p110} and CDK11^{p58} isoforms are produced from the same mRNAs through the use of an internal ribosome entry site (IRES) and two different AUG codons located in the coding sequence of the CDK11^{p110}A and B mRNAs. The CDK11^{p110} isoform thus contains the entire sequence of CDK11^{p58}, which includes the catalytic domain (Loyer et al., 2005). CDK11^{p110} protein is a nuclear protein present in two macromolecular complexes involved in the regulation of transcription and pre-RNA splicing. Expression of the large CDK11^{p110} isoform is ubiquitous and constant throughout the cell cycle while CDK11^{p58} expression is maximal during G2 and M phases of the cell cycle (Loyer et al., 2011; Loyer et al., 2008; Trembley et al., 2002). CDK11^{p58} is essential during mitosis for centrosome maturation and mitotic spindle formation, sister chromatid cohesion and cytokinesis (Hu et al., 2007; Petretti et al., 2006). Very recently, Franck & al., (Franck et al., 2011) showed that CDK11^{p58} is required for centriole duplication and that it could regulate centriole components such as the protein kinase Plk4 that mediates phosphorylation required for centriole duplication during the subsequent interphase. These data suggest that CDK11^{p58}-dependent kinase activity during mitosis would regulate mitotic events *per se* and downstream molecular pathways during the centriole duplication in S and G2 phases. The cyclin L's, encoded by the *CCNL1* and *CCNL2* genes, are the regulatory partners of CDK11^{p110} and CDK11^{p58} (Loyer et al., 2008) although it was not reported that CDK11^{p58} was associated to cyclin L's for its mitotic role. Nevertheless, CDK11^{p58} probably associated with L-type cyclins plays a pivotal role during mitosis (Figure 3).

Together, these data collected over more than 25 years have demonstrated that the sequential activation of Cdk/cyclin complexes is a hallmark of the cell cycle regulation.

2.2.2 Are Cdk/cyclin complexes functionally redundant to phosphorylate the retinoblastoma protein and components of the DNA replication machinery?

Because the kinase activities of Cdk2 and Cdk1 were mainly detected in G1/S and G2/M transitions respectively, they were thought to function independently at these two distinct periods without functional redundancy (Bashir and Pagano, 2005; Woo and Poon, 2003). This conclusion was reinforced by the experimental inactivation of Cdk1 and Cdk2 in human cell lines: enforced expression of kinase dead Cdk2 caused a G1 arrest while expression of kinase dead Cdk1 result in a G2/M arrest (van den Heuvel and Harlow, 1993). Similarly, cells expressing temperature-sensitive Cdk1 mutant arrest in G2 at the restrictive

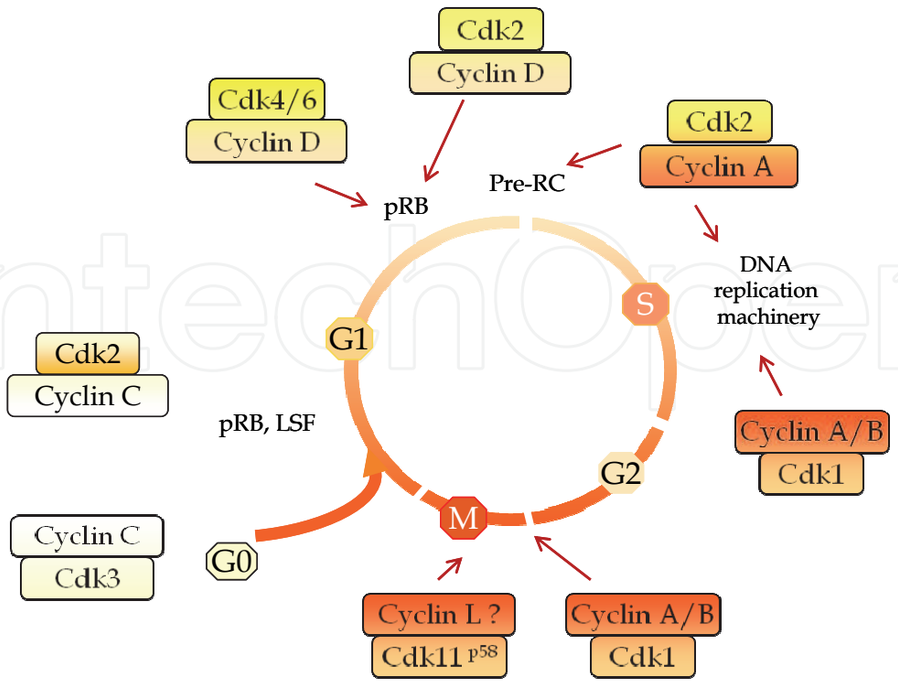

Fig. 3. Sequential activation of Cdk/cyclin complexes throughout the cell cycle.

temperature (Th'ng et al., 1990; Yasuda et al., 1991). This model of cell cycle control has first been challenged by the finding that some cancer cells proliferate despite Cdk2 inhibition (Tetsu and McCormick, 2003). Independently, the demonstration was brought that knock-out mice for Cdk2 as well as for E-type Cyclins are viable and that the cell cycle of cultured Cdk2$^{-/-}$ mouse embryonic fibroblasts (MEFs) did not show major alterations (Berthet et al., 2003; Ortega et al., 2003; Sherr and Roberts, 2004). In addition, in the hippocampus of Cdk2$^{-/-}$ mouse, the proliferation of granule neurons of the dentate gyrus that undergo continuous renewal throughout life, is not altered (Vandenbosch et al., 2007). Similarly, hematopoiesis is not affected in Cdk2 knockout mice (Berthet et al., 2007). These data indicated that Cdk2/Cyclin E complexes were dispensable for commitment to S phase and that other Cdk(s) could compensate for the loss of Cdk2. Along the same line, a Cdk1-dependent compensatory mechanism regulating S phase initiation and progression was also demonstrated in DT40 chicken cells lacking Cdk2 (Hochegger et al., 2007). Together, these data have led authors to propose a revised model of the cell cycle control in which Cdk1 compensates for Cdk2 ablation by controlling the G_1/S transition, initiation of DNA replication and centrosome duplication (Bashir and Pagano, 2005; Kaldis and Aleem, 2005). Interestingly, it was recently demonstrated that both Cdk1 and Cdk2 were required for efficient DNA replication in *Xenopus* egg extracts (Krasinska et al., 2008) suggesting that, at least in some non-genetically modified cell types, Cdk1 could contribute to S phase initiation and/or DNA replication. In mammalian cells, the involvement of Cdk1 in S phase may have been underestimated mainly because the low levels of active Cdk1 compared to the high levels of Cdk2 during DNA replication suggested that Cdk2 was predominant over Cdk1 at this step of the cell cycle (Bashir and Pagano, 2005). In this emerging picture of the cell cycle regulation, these new data probably did not profoundly affect the roles that were initially attributed to the different Cdk/cyclin complexes but rather introduce the notion of redundancy and flexibility (Li et al., 2009b; Satyanarayana and Kaldis, 2009b).

Cdk/cyclin complexes are, at least in part, functionally redundant. Thus, interfering with the activity of one Cdk/cyclin complex does not systematically lead to cell cycle arrest and cell death. This hypothesis was verified by further investigating single and combined alterations in mice of Cdk4/6-Cyclin D, Cdk2-Cyclin E, $p27^{Kip1}$ and Rb (Li et al., 2009a; Malumbres et al., 2004; Satyanarayana and Kaldis, 2009b). These deletions did not affect early embryogenesis demonstrating multiple compensatory mechanisms and overlapping roles of these genes. Moreover, analysis of the cell cycle in MEFs derived from these knock-out mice evidenced compensatory mechanism between positive and negative regulators at the G_1/S transition and highlighted a complex network regulating the expression and activation of these cell cycle regulators in the progression from G_1 to S phase (Malumbres et al., 2009; Satyanarayana and Kaldis, 2009b). This important conclusion needs, however, to be softened since Cdk2 knockout mice exhibit altered self-renewal of neural progenitors in the adult subventricular zone of the brain (Jablonska et al., 2007). In this study, the authors showed that Cdk4 was able to compensate for the loss of Cdk2 during embryogenesis and during 2 weeks post-natal resulting in pRb inactivation. This compensatory pathway declines after a month leading to decreased self-renewal capacity and enhanced differentiation of neural progenitors. These data demonstrated that compensatory mechanisms for Cdk2 loss through the activation of other Cdk family members do not systematically occur in all cell types (Berthet and Kaldis, 2007). These molecular pathways can be silenced in some specific cell types depending upon the differentiation context.

The notion of redundancy between Cdks needs to be also modulated by the demonstration that knocking down certain Cdks in mouse is lethal for the embryos. For instance, $Cdk1^{-/-}$ mouse embryo's development is arrested at a very early stage (Santamaria et al., 2007) and knock-down of Cdk1 expression by shRNA in synchronized $Cdk2^{-/-}$ MEFs strongly reduced S phase entry (Aleem et al., 2005). Similarly, the $Cdk11^{p58}$ is essential for mitosis (Hu et al., 2007; Petretti et al., 2006) and the conventional knock-out of the Cdk11 gene in mouse is lethal as early as the blastocyst stage (Li et al., 2004) demonstrating that some Cdks have specific and essential functions that cannot be rescued by other Cdk family members. Nevertheless, in the experimental situations of genetic alterations of G1-associated Cdks and cyclins, Cdk1 is sufficient to drive the cell cycle in most cell types (Santamaria et al., 2007) illustrating the functional compensations among the Cdks. This conclusion based on models of genetic Cdk gene alterations and/or substitutions is also important to re-analyze some data that evidenced different patterns of cdk and cyclin expression in mammalian cells in absence of engineered genetic modifications. Indeed, both Cdk4 and Cdk6 can associate to Cyclins D1, D2 and D3 to form active complexes phosphorylating pRb (Bates et al., 1994; Kato and Sherr, 1993; Matsushime et al., 1994). However, these six complexes are rarely found expressed in a single cell type and are rather expressed differentially in normal or transformed cells (Alhejaily et al., 2011; Fiaschi-Taesch et al., 2010). In addition, while in cells actively proliferating Cdk1 is expressed constantly throughout the cell cycle, in cells proliferating after a cell cycle arrest in G0, such as lymphocytes and hepatocytes, Cdk1 is barely detectable in G1 but appears at the G1/S transition (Furukawa et al., 1990; Loyer et al., 1994). In fibroblast arrested in G0-like state by serum starvation, cdk1 appears only after the G1/S transition (Garnier et al., 2009) while in cells arrested in late G1 by a double thymidine block, Cdk1 is detected through the cell cycle with modest quantitative variations (Dulic et al., 1992).

The diversity of Cdk expression in G1 phase among mammalian cells may be explained in part by the fact that, in some experimental models, cells initiate the cell cycle from the quiescence in G0 while in others, cells enter G1 immediately after completion of mitosis

without a cell cycle arrest in G0. However, these distinct patterns of expression of the Cdks especially during G1 phase may reflect a diversity of Cdk/cyclin-mediated pathways to promote the G1/S transition in absence of compensatory mechanisms generated by Cdk or cyclin gene invalidation. The diversity of Cdk/cyclin complexes expressed in G1 also raises the question of the phosphorylation events mediated by Cdks during interphase. Assuming that the phosphorylation of "specific" substrate proteins by Cdk/cyclin complexes during G1 and S phases such as pRb, LSF, pre-RC and DNA machinery components, is absolutely required for the cell cycle progression, the different combinations of Cdk and cyclin complexes expressed in interphase must all be capable of phosphorylating these substrates in order to promote cell cycle progression. To the best of our knowledge, detailed studies of all Cdk phosphorylation activities towards a large panel of candidate substrates have not been performed, however, several reports indicated that Cdk1 either associated to D- or A-type cyclins (Joshi et al., 2009; Santamaria et al., 2007) can indeed phsophorylate pRb and, thus, can compensate for the lack of Cdk4 or Cdk6 associated with G1 cyclins.
Recently, our view of the cell cycle regulation has significantly advanced towards a more complex and diversified picture. While in G2/M transition Cdk1/cyclin B complex remains a master regulator of the entry into mitosis, during G1 and in G1/S transition, differential expression of Cdk4, Cdk6 or Cdk2 and C- or D-type cyclins in various cell types obviously allows multiple options of Cdk/cyclin heterodimers that are capable of promoting S phase entry (Satyanarayana and Kaldis, 2009b). Even more surprising was the observation that genetic alterations of these G1/S regulator genes induce the recruitment of Cdk1 to promote S phase. Earlier in this chapter, we pointed out the striking contrast between the large diversity of mitogenic factors and proliferation signaling pathways opposed to the limited number of Cdk/cyclin complexes regulating the commitment to S phase. In the light of the recent findings on the diversity Cdk/cyclin complexes expressed at the G1/S transition in mammalian cells and their functional redundancy this opposition is no longer so evident. In contrast, the diversity of Cdk/cyclin complexes controlling the G1/S transition has emerged leading to the hypothesis that highly similar molecular pathways regulating cell cycle among mammalian cells only occur after the commitment to S phase.

3. Activation and involvement of Cdk1 during S phase in proliferating hepatocytes: synergy and redundancy between the protein kinases Cdk1 and Cdk2

The unique capacity of the liver to regenerate after tissue injury or resection has always fascinated biologists and makes the liver a unique model for studying mammalian adult organ regeneration. Centuries ago, the ancient Greeks recognized the liver regeneration potential in the myth of Prometheus (Fausto et al., 2006; Michalopoulos and DeFrances, 2005). The first "scientific" demonstration that the liver can restore its initial liver mass and its functional status within few days by a compensatory growth process was provided by using the experimental model of 2/3 hepatectomy in rats developed by Higgins and Anderson in 1931 (Fausto et al., 2006).

3.1 The rapid proliferating of differentiated hepatocytes allows the liver regeneration

In contrast to other regenerating tissue, the peculiar feature of the liver regeneration process is to involve massive proliferation of differentiated cells (Figure 4) in the remnant intact

tissue. Hepatic progenitor cells are recruited only when intoxication alters the proliferation of mature cells for instance following administration of drugs targeting hepatocytes such as retrosine (Avril et al., 2004; Laconi et al., 1999).

After 2/3 hepatectomy, liver regeneration begins with a first synchronous wave of hepatocyte proliferation, followed by sequential proliferation waves of biliary, kupffer and endothelial cells (Fausto et al., 2006; Michalopoulos and DeFrances, 2005). Importantly, proliferation of mature hepatocyte occurs within the parenchyma in the vicinity of the portal triads and proceeds to the pericentral area close to the centolobular veins (Rabes et al., 1976) (Figure 4). Since the cell renewal is very low in the normal liver, the unique ability of differentiated cells to exit from quiescence after a tissue loss has aroused numerous studies to identify exogenous factors triggering the liver regeneration and regulators of hepatocyte cell cycle progression. Therefore, *in vivo* and *in vitro* models have been extensively studied for step by step identifications of the extracellular stimuli inducing cell cycle of mature hepatocytes and downstream signaling pathways. These models have also been used to investigate expression and activation of cdk/cyclin complexes throughout cell cycle progression (Fausto et al., 2006; Michalopoulos and DeFrances, 2005).

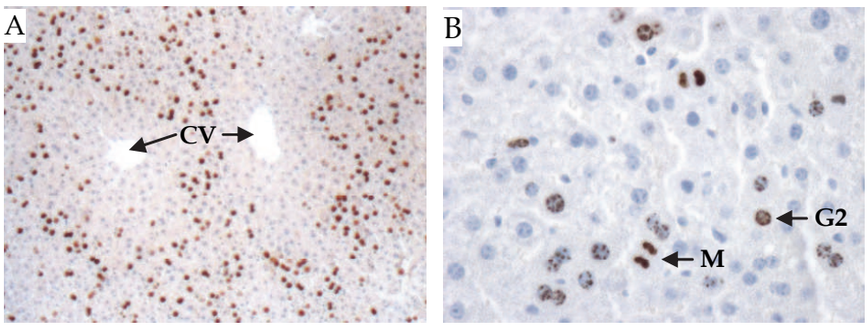

Fig. 4. Sections of mouse liver evidencing detection of DNA replication and G2 phase. Mice were hepatectomized, injected at 46 hours post-hepatectomy with BrdU and killed 2 hours later (at 48h). Livers were fixed for histological studies and detection of BrdU to visualize hepatocytes replicating DNA or phospho-histone H3 to detect cells in G2 and M phases.. A, this low magnification picture shows the detection of BrdU positive cells replicating DNA. It illustrates that replicating hepatocytes are initially localized in the vicinity of the portal vein while around centrolobular veins (CV) only few hepatocytes replicate DNA at 48h. B, a higher magnification picture shows nuclei of hepatocytes reaching G2 phase (detection of phosphor-histone H3 positive cells with punctuated nuclear signal : G2) and mitosis (M).

Using *in vivo* models, Molten and Bucher have shown that circulating growth factors present in the serum of hepatectomized rats induce hepatocyte replication in parabiosed non hepatectomized animals (Moolten and Bucher, 1967). Then using primary culture of rat hepatocytes, HGF, TGFα, and EGF have been identified as potent hepatocyte growth factors. However, the injection in rat of these growth factors does not induce massive hepatocyte DNA replication suggesting that normal hepatocytes *in vivo* are not able to respond to mitogenic signal without priming events allowing hepatocytes to become "sensitive" to growth factors. From different works, evidence was provided that the pro-inflammatory cytokines TNFα and IL-6 are the early stimulus during the liver regeneration allowing the exit of hepatocytes from quiescence (Cressman et al., 1996; Webber et al., 1998). Rapid induction of urokinase activity and urokinase receptor expression appeared within 5 min

followed within 30 min by a rapid activation of NFkB and STAT3. These transcription factors participate to the induction of a subset of genes called "immediate early genes" including c-fos and c-Jun leading to an increase in AP1 activity.
These events are followed by high levels of HGF in plasma around two hours after PH. This initiation phase controlled by pro-inflammatory cytokines thus results in the G0/G1 transition and early G1 progression allowing hepatocytes to become sensitive to growth factors and competent for commitment to DNA replication. Therefore, the complex regenerating process is now divided in three distinct phases: the initiation, proliferation and termination steps. In rat and, to a lesser extend, in mouse the first wave of hepatocyte proliferation following partial hepatectomy (PH) is synchronous. In both rat and mouse, within less than 15 minutes after the PH, hepatocytes exit quiescence and enter in G1-phase (Hsu et al., 1992). The timing of DNA replication and mitosis are however different between the two species. The peak of DNA synthesis is observed at 22-24h in rat followed by a peak of mitosis at 28-30h (Agell et al., 1994; Fabrikant, 1968; Grisham, 1962; Serratosa et al., 1988; Widmann and Fahimi, 1975). Seven days later, the liver has recovered its initial mass. In mouse, the progression in G1 phase of the cell cycle is slower and the peak of DNA and mitosis are delayed of approximately 20h highlighting differences of hepatocyte response between species.

3.2 Liver regeneration: a synchronized in vivo model of proliferation for cell cycle studies

During the 1990's, interest in hepatocyte cell cycle has increased because genes involved in the cell cycle control have been identified. This *in vivo* model of proliferation of non transformed cells was used for cell cycle studies since hepatocyte progression in the cell cycle is naturally synchronous with a long lasting G1-phase. Our group and others investigated Cdk2 and Cdk1 expression and activity as well as cyclin A, B, E and D1 expression during liver regeneration (Albrecht et al., 1993; Loyer et al., 1994; Lu et al., 1992; Zschemisch et al., 2006). Although Cdk2 is constantly expressed, Cdk1 is completely absent in resting hepatocytes and remains undetectable up to 20h after PH a time corresponding to late G1 phase and G1/S transition. Then, Cdk1 accumulates in S, G2 and M phase and both cyclin A/Cdk1 and cyclin B/Cdk1 complexes are formed. During S phase, Cdk2 associates with cyclin A. Additional experiments of kinase activity assays suggested that Cdk1 is active during both S and M phases while one peak of Cdk2 activity is detected in S phase only. At that time, these data contrasted with the dogma that Cdk1 is active only at the G2/M transition while Cdk2 would control G1/S transition. Our study raised the question of the significance of Cdk1 and Cdk2 expression during G1 phase and G1/S transition.
Unexpectedly, cyclins E and D1 are present in resting liver, which again contrasted with the admitted view of the mammalian cell cycle regulation with low D-type cyclin expression in early G1 and its dramatic induction at the mitogen-dependent restriction point in late G1 phase. In quiescent hepatocytes, Jaumot et *al.* (Jaumot et al., 1999) demonstrated that cyclin D3 and Cdk4 were localized in cytoplasm whereas cyclin D1 was nuclear. Low amounts of cyclin E are found in the cytoplasm (Pujol et al., 2000). Thirteen hours after PH cyclins D3 and Cdk4 are mostly located in the nucleus and significant amounts of cyclin D1/Cdk4 and cyclin D3/Cdk4 complexes are formed but remain inactive whereas at 24h they are fully activated. At 13 and 24h, cyclin E is detected in both cytoplasm and nuclei. Thereafter, the activity of Cdk4 decreases at 28h when cyclin D1 translocates to the nuclear matrix and the levels of cyclin D3 diminishes. Similarly, the inactivation of Cdk2 at 28h is associated with a

strong decrease in Cdk2 in the nuclear fraction and a decrease of cyclin E located in the nuclei. During this period, very low amounts of cyclin A are detected in the nuclear fraction at 13h after PH while following its strong induction in S phase, cyclin A is present in both cytoplasm and nuclei at 24 and 28h. Therefore, the specific nuclear localization of the complexes is associated with their activity in liver regeneration. The maximal activity of Cdk2 detected at 24h comes from cyclin E/Cdk2 and cyclin A/Cdk2 complexes whereas the activity at 28h is mainly attributable to the Cdk2/cyclin A heterodimer. However, the activity of Cdk2 rapidly decreases after the peak of DNA synthesis at 24h.

The presence of inactive cyclin D/Cdk4 complexes until 13h post PH and cyclin E/cdk2 at 28h has led authors to question the modulation of Cdk activity during rat liver regeneration. Indeed, reports indicated that Cdk inhibitors (Cdki's) are involved in modulating cell cycle progression following antagonist mitogenic and anti-mitogenic signals (Morgan, 1997; Sherr and Roberts, 1995). Two families of Cdki's were described: the Ink4 family ($p16^{Ink4a}$, $p15^{Ink4b}$, $p18^{Ink4c}$ and $p19^{Ink4d}$) which specifically bind Cdk4 and its homologue Cdk6 and the Cip/Kip family ($p21^{Cip}$, $p27^{Kip1}$, $p27^{Kip2}$) which bind and inhibit the activity of a wide range of Cdk/cyclin complexes including cyclin D/Cdk4/6, cyclin E/Cdk2 and cyclin A/Cdk2 (Sherr and Roberts, 1995). During liver regeneration in rat, Jaumot et *al.* (Jaumot et al., 1999) have observed that $p27^{Kip1}$ is associated with cyclin D/Cdk4 complexes when they are inactive. More precisely, Pujol et *al.* (Pujol et al., 2000) have evidenced that high amounts of $p27^{Kip1}$ bind to Cdk2/cyclin E complexes during the first 13h post-PH when the activity of Cdk2 is very low. At 24h, corresponding to the S phase, the amounts of $p27^{Kip1}$ associated to Cdk2/cyclin E decrease strongly while Cdk2 activity is maximal. Conversely, the amount of $p21^{Cip}$ bound to these complexes is low during the first 13h and subsequently increases. At 24h low levels of both inhibitors associated with the complexes are detected but increase in $p21^{Cip1}$ and $p27^{Kip1}$ proteins associated with Cdk2/cyclin A is observed at 28h after the peak of hepatocyte DNA synthesis. In hepatectomized mice, Albrecht et *al.*, (Albrecht et al., 1997; Albrecht et al., 1998) obtained similar data and showed that expression of $p21^{Cip1}$ is induced during the pre-replicative phase and is maximal after the peak of hepatocyte DNA synthesis. In contrast, $p27^{Kip1}$ is present in quiescent liver and slightly induced after PH. Immuno-depletion experiments suggested that $p27^{Kip1}$ plays a role in down-regulating Cdk2 activity before and after the peak of DNA replication. Interestingly, study of liver regeneration in mice lacking $p21^{Cip1}$ evidenced a marked acceleration of hepatocyte progression into the cell cycle. DNA synthesis, up-regulation of cyclin A and PCNA, induction of cyclin D1- and Cdk2-associated kinase activities, and appearance of the hyperphosphorylated retinoblastoma protein (pRb) occur earlier in the $p21^{Cip1}$ knock-out mice. These results demonstrate the role of $p21^{Cip1}$ in the regulation of the hepatocyte progression through G1 phase *in vivo*.

Primary cultures of rat and mouse hepatocytes were also widely used to analyze hepatocyte cell cycle entry and progression through the G1 phase. It was clearly demonstrated that hepatocytes in culture undergo DNA replication when they were stimulated by growth factors alone (McGowan and Bucher, 1983; Sawada, 1989). Using this model of pure culture of hepatocytes, our group has shown that during cell isolation hepatocytes expressed immediate early proto-oncogenes like c-fos and c-myc suggesting a "spontaneous" G0/G1 transition following cell-cell interaction destruction (Etienne et al., 1988).

Then, Loyer et *al.*, (Loyer et al., 1996) characterized different steps of G1 phase in hepatocytes. Confirmation that collagenase perfusion of the liver induces the G0/G1 transition of quiescent normal rat hepatocytes was provided and we showed that

progression in late G1 triggers hepatocyte ability to respond to growth factor alone. Importantly, demonstration that hepatocytes are able to progress from an early G1 to a restriction point mitogen dependent (R point) located to mid-late G1 was shown. Indeed, in absence of growth factor and serum, hepatocytes are able to progress up to mid-late G1 phase as evidenced by the sequential over expression of c-fos, c-jun, c-myc, jun D and then c-Ki-ras and p53. In addition, low levels of cyclin D1 and D2 are observed while cyclin A and Cdk2 are not expressed. Moreover, the progression towards the G1/S is strictly dependent upon the stimulation by growth factor. Late addition of the EGF at day 2 and 3 of culture induces a sharp peak of DNA synthesis reflecting the high synchrony of the hepatocytes arrested at the R point. A lag phase between the R point and the onset of the DNA synthesis appeared to be approximately 18-20h. In this hepatocyte primary culture, Cdk2 mRNA is detectable throughout the G1 phase but significantly increased after the EGF stimulation. Cyclin A is detected at the entry of S phase and Cdk1 and Cdk2 dependent histone H1 kinase activity is mainly detected in S and M phases. Weak levels of cyclin E mRNA are found in unstimulated cultures but levels of this mRNA greatly increased after growth factor stimulation. Surprisingly, cyclin D3 mRNAs appear to accumulate in absence of EGF stimulation whereas a drastic increase in cyclin D1 expression accompanies the R point overcrossing. The cyclin D1 mRNA accumulation correlates with the R point onset and the cyclin D1 protein is detected 10-15h later. In accordance with these observations, accumulation of cyclin D1 is also detected when the hepatocytes are stimulated by HGF (Albrecht et al., 1995). Importantly, if progression beyond the restriction is delayed by late EGF stimulation, cyclin D1 induction is postponed accordingly demonstrating that cyclin D1 induction is essential for cell cycle progression at the mitogen-dependent restriction point.

The question arises whether this restriction point existed *in vivo*. Nicely, a growth factor dependency in mid-late G1 phase of proliferating hepatocytes *in vivo* was also evidenced (Talarmin et al., 1999). To reach that conclusion, we first analyzed the expression of cyclin D1 during liver regeneration and showed its induction at 12h post-hepatectomy, which is a time coinciding with the 2/3 of G1 progression as previously shown in primary culture of rat hepatocytes. We next isolated rat hepatocytes isolated 5, 7, 9, 12 or 15h after PH, and showed that only those isolated from 12-15h regenerating livers were able to replicate DNA without growth factor stimulation. Moreover, intravenous administration of a MEK inhibitor (PD98059) *in vivo*, before MEK activation at 10.5h post-PH was able to inhibit cyclin D1 mRNA accumulation and hepatocyte DNA replication demonstrating that MEK/ERK signaling pathway was involved in cyclin D1 induction and R point overcrossing. To the best of our knowledge, these data provide the unique evidence that the mitogen-dependent restriction point identified in cultured hepatocytes exists *in vivo* in whole organs and animals. These results were strengthened by Albrecht's observations showing that transient enforced expression of cyclin D1 in hepatocytes stimulates assembly of active cyclin D1/cdk4 complexes, robust hepatocyte proliferation and liver growth in rat liver (Nelsen et al., 2003). However, in this *in vivo* model, after several days, hepatocyte proliferation is inhibited despite the persistence of high levels of cyclin D1 and cyclin E, suggesting that anti-proliferative response related to marked up-regulation of $p21^{Cip1}$ represses cyclin D1/cdk4 and cyclin E/cdk2 dependent kinase actvities. More recently, using mice carrying a floxed *EGFR* allele to inactive the EGF receptor, Natarajan et al., (Natarajan et al., 2007) observed delayed liver regeneration characterized by defective G1/S phase entry, reduced cyclin D1 expression followed by moderate Cdk2 and Cdk1 expression. In parallel, these authors reported an increased mortality after PH associated to high levels of TNFα in the

serum. They also suggested that soluble TNFα, which is a priming agent for hepatocytes, was produced at high levels by liver cells to compensate cell cycle arrest with a subsequent induction of cell death in absence of proliferation.

To evaluate the role of priming agents, another experimental *in vitro* model has been designed. For that purpose, we used a coculture model associating rat hepatocytes with rat liver epithelial cells (RLEC also called LEC for liver epithelial cells), in which heterotypic cell-cell contacts are restored and a spontaneous early production and deposition of extracellular matrix is observed (Clement et al., 1984; Corlu et al., 1991; Guguen-Guillouzo et al., 1983). This coculture model compared to the pure culture of hepatocytes (Figure 5) exhibits numerous advantages: adult hepatocytes remain highly differentiated for several weeks and are unable to proliferate under EGF or HGF stimulation alone as in liver tissue (Corlu et al., 1997; Fraslin et al., 1985). Therefore, based on the data obtained *in vivo*, we successfully designed a stimulation procedure allowing multiple hepatocyte division cycles without loss of differentiation (Serandour et al., 2005). In this coculture system, differentiated and quiescent hepatocytes are able to proliferate under co-stimulation with TNFα and EGF or HGF. Co-stimulation with TNFα and growth factors leads to proliferation of nearly all the hepatocyte population over a week. Peaks of DNA synthesis and mitotic activity occurred day 3 after stimulation. Both mono- and binuclear hepatocytes progressed up to mitosis and cytokinesis leading to significant expansion of hepatocyte colonies. Remarkably, these results are in accordance with *in vivo* experiments, in which co-injection of TNFα and growth factors induced hepatocyte proliferation (Webber et al., 1998). In contrast, TNFα alone does not act as complete mitogen in cocultures. Indeed, it induces DNA synthesis in less than 3% of hepatocytes as observed *in vivo* (Webber et al., 1998) and in long-term DMSO cultures (Iocca and Isom, 2003). In coculture, analysis of cell cycle proteins has revealed that growth factor alone such as EGF, induces cyclin D1 indicating that cells are sensitive to mitogen signal, override the R point in mid-late G1 but fail to reach the G1/S boundary. These observations diverged with Albrecht's results that showed that cyclin D1 expression *in vivo* or *in vitro* was sufficient to induce entry of these cells into S phase (Albrecht and Hansen, 1999; Nelsen et al., 2003). This discrepancy would be explained

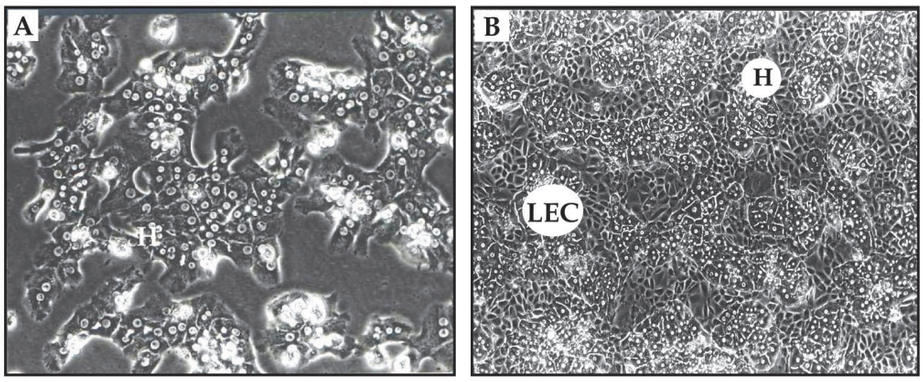

Fig. 5. Phase contrast photographs of A) pure culture of rat hepatocytes and B) co-culture of rat hepatocytes (H) and rat liver epithelial cells (LEC). Differentiated hepatocytes are characterized by a "dark" cytoplasm with one or two round nuclei with a single central nucleolus. In addition, hepatocytes in co-culture maintain a cubical shape and form colonies that remain viable for several weeks.

at least in part, by distinct experimental conditions, mainly the collagen gel system used *in vitro* and the level of cyclin D1 expressed. In contrast, when TNFα was associated with EGF, hepatocytes expressed both Cdk1 and Cdk2 and a progression into S phase was observed. From these data, we postulate that a late G1 checkpoint dependent on Cdk1, not yet described in hepatocyte cell cycle, may regulate entry into S phase independently from the Cdk2-mediated role at the G1/S transition.

3.3 Role of Cdk1 and Cdk2 in proliferating hepatocytes: synergy or redundancy

Most articles reporting data on liver regeneration focused on the G1 phase regulators but not on expression of Cdk1 during G1/S transition or S phase even if Cdk1 was observed *in vivo and in vitro* at this step by us and others (Albrecht et al., 1993; Loyer et al., 1994). Therefore, using *in vivo* and *in vitro* models, we have investigated the role of Cdk1 in normal adult hepatocytes. We have demonstrated that Cdk1 is expressed at high levels in S phase and that both Cdk1 and Cdk2, associated with cyclins A and/or B, are activated prior DNA replication in regenerating hepatocytes (Garnier et al., 2009). Assuming that Cdk1 and Cdk2 kinase activities towards the phosphorylation substrate histone H1 are identical, we provided evidence that Cdk1 activity is twice higher than Cdk2 activity during S phase in hepatocytes (Figure 6). Then, knock-down experiments of Cdk1 and/or Cdk2 were performed in isolated hepatocytes and human foreskin fibroblasts (HFFs) which differed in their ability to express high and low Cdk1 levels during S phase. Indeed, the levels of Cdk1 expression during S phase reached 80 and 15% of the expression levels during mitosis in hepatocytes and HFFs, respectively. Both siRNA-mediated repression of Cdk1 and Cdk2 significantly decreased DNA replication in hepatocytes. In contrast, in HFFs repression of Cdk2 significantly reduced the DNA synthesis while repression of Cdk1 had no effect on the rate of DNA replication but, as expected, reduced the mitotic index. Notably, in accordance with the Cdk1 and Cdk2 kinase activities during S phase in hepatocytes, the greatest decrease in DNA synthesis resulted from Cdk1 rather than Cdk2 silencing. In hepatocyte, the involvement of Cdk1 is evidenced in early S phase by showing that hepatocytes arrested after G1/S transition but prior to DNA replication by the iron chelator O-Trensox, express fully active Cdk1 and Cdk2. Moreover, the decrease in DNA replication after Cdk1 or Cdk2 silencing is not linked to a default in the formation of the pre-replication complex since Mcm7 nuclear localization and loading onto chromatin are not impaired. Therefore, Cdk1 may be involved in the origin firing events downstream the formation of replication complexes in hepatocytes, in agreement with a recent study showing that enforced expression of constitutively active Cdk1 mutant in HeLa cells results in abnormal origin firing and premature DNA replication in early S phase (Katsuno et al., 2009).

These data further support and extend the conclusion that Cdk1 compensates for Cdk2 gene ablation in genetically modified mice. Indeed, we showed for the first time the involvement of Cdk1 in S phase of normal and non-genetically modified mammalian cells. More precisely, both Cdk1 and Cdk2 play a critical role in hepatocyte cell cycle. Consistent with our observation, Satyanarayana et *al*., (Satyanarayana et al., 2008) showed that the timing of S phase is not altered in regenerating livers of $Cdk2^{-/-}$ mice although the percentage of BrdU-positive cells slightly decreases compared to wild type. Interestingly, in $Cdk2^{-/-}$ $Cdk1^{+/cdk2k1}$ mice, in which a Cdk2 cDNA is knocked into the *Cdk1* locus, similar regenerative response and percentage of BrdU–positive cells are obtained compared to $Cdk2^{+/+}$ mice. These data indicated that Cdk2 expressed from the *Cdk1* locus is able to

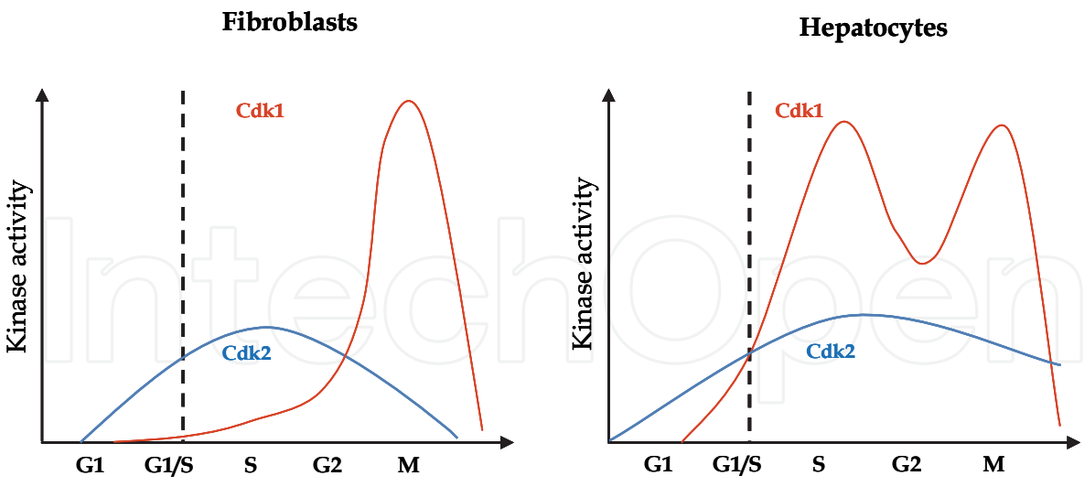

Fig. 6. Schematic representation of Cdk1 (red) and Cdk2 (blue) kinase activities in fibroblasts versus hepatocytes throughout the cell cycle. While in fibroblasts Cdk2 activity is dominant over Cdk1 at the G1/S transition and during S phase, both Cdk1 and Cdk2 are active in hepatocytes during S phase.

mimic the cell function of endogenous Cdk2 and restore normal regeneration process as well as that one copy of *Cdk1* is sufficient for a normal liver response after PH. Later, Hanse et *al*. (Hanse et al., 2009) showed that 42h after PH most hepatocytes enter S phase in wild-type mice whereas their number is diminished significantly in Cdk2$^{-/-}$ mice. In addition, hepatocytes isolated from livers of cdk2$^{-/-}$ mice respond to mitogenic stimulation but to a lower extent than hepatocytes coming from wild-type mice.

Altogether, these results strengthened the conclusion that physiological hepatocyte proliferation is dependent on both Cdk1 and Cdk2. While Cdk1/cyclin E complexes are not detected in normal hepatocytes, Cdk1, cyclins A and, unexpectedly, cyclin B1 are localized in the nucleus of replicating hepatocytes and form active complexes during S phase in regenerating hepatocytes. In addition, Cdk1 is active in all hepatocytes regardless of their ploidy status, excluding a peculiar regulation or role of Cdk1 related to the tetraploidy observed in half of adult hepatocytes in rat. Although the absolute requirement of cytosolic cyclin B1 during initiation of mitosis remains questioned, it has been postulated that relocating cyclin B1 to the nucleus in S phase might compromise entry into mitosis. This could explain why the accumulation of nuclear Cdk1/cyclin B1 complexes during DNA replication does not trigger premature mitosis in hepatocytes. Moreover, Phospho-Tyr15 Cdk1 found in replicating hepatocytes and known to be an inactive form of Cdk1 could also participate to this control. Indeed, recent reports showed that Tyr15 phosphorylation of Cdk1 is important to avoid premature entry into mitosis (Pomerening et al., 2008). Regulation of the ratio between pools of active and inactive Phospho-Tyr15 Cdk1 in hepatocytes might be essential to allow S phase initiation while preventing premature mitosis.

In absence of Cdk2, the induction of cyclin A is diminished consistent with the reduced proportion of hepatocyte in S phase, while Cdk1 is induced to higher levels than in control cells (Hanse et al., 2009). It is located in the nucleus at G1/S transition (Satyanarayana et al., 2008) and remained in the nucleus until the completion of mitosis. Cylin E/Cdk1, cyclin

A/Cdk1 and cylin B1/Cdk1 are successively activated. In this context, it is questioned whether $p21^{Cip1}$ is able to arrest cells at G1/S transition in absence of Cdk2 after DNA damage and if cells are able to repair DNA and resume DNA replication. Indeed, Cdk2 is the primary target of the ATM/ATR, p53, p21 cascade (Bartek and Lukas, 2001). In Cdk2 knock-out mice, activation of p53– $p21^{Cip1}$ pathway is not perturbed in the absence of Cdk2 and $p21^{Cip1}$ can bind Cdk1. However, in Cdk2 knock-out mice DNA repair is delayed and partially impaired. Therefore, cells are more prone to lethal irradiation compared to wild-type although they display resumption of DNA replication in regenerating liver (Satyanarayana et al., 2008). The question of the involvement of Cdk2 in the induction of cyclin D1 has also been asked. Indeed, Albrecht's group has shown that infection with adenovirus leading to enforced expression of cyclin D1 in the liver triggers hepatocyte proliferation (Nelsen et al., 2001). In $Cdk2^{-/-}$ mice, they observed that this response is severely blunted leading to massive hepatocyte and animal death. This data highlights the critical role of Cdk2 in hepatocyte progression and survival after an acute mitogenic stimulation (Hanse et al., 2009). Altogether, these results could point out the emerging role of Cdk2 in proper DNA repair (Satyanarayana and Kaldis, 2009a) and how Cdk2 could be a sensor able to distinguish between moderate and extensive DNA damage to promote either survival or apoptosis.

4. Regulation of the Cdk1 expression and activation under the control of extracellular signals: involvement of extracellular remodeling and Cdk2 kinase activity

Although adult hepatocytes are quiescent and normally do not undergo cell division, they maintain the ability to proliferate in response to toxic injury and infection. Upon regenerative stimulus, 95% of the hepatocytes undergo cell division while maintaining their metabolic function and tissue architecture. This process involved a multitude of cellular processes including at early stage acute-phase reaction (Fausto et al., 2006), induction of pro-angiogenic signals (Ding et al.) and an important extracellular matrix (ECM) breakdown and remodeling (Kim et al., 1997) leading to local and transient changes in the liver architecture. Connective tissue is found around the portal triads whereas reticular fibers and small amounts of basement membrane are present between the sinusoid endothelial cells and the hepatocytes. In the portal areas, mainly type I, III and V collagens are found while type IV collagen, laminin, entactin and nidogen form the basement membrane along the sinusoids. Fibronectin is also present in the space of Disse (Clement et al., 1986).

Some proteins that are involved in the structural integrity of the liver are also required for normal regeneration. For example, deficiencies in connexin-32, a gap-junction protein (Temme et al., 2000) and keratin-8, an intermediate filament forming protein (Loranger et al., 1997) cause extended liver damage after partial hepatectomy. Connexin-32 is also required for normal mitosis by mediating cellular connections during cell division. Loss of certain proteases also results in prolonged liver injury. Mice lacking genes encoding the serine proteases urokinase-type plasminogen activator (uPA) and tissue-type plasminogen activator (tPA) exhibit delayed regeneration whereas the deficiency of the plasminogen inhibitors leads to accelerated liver regeneration (Roselli et al., 1998; Shimizu et al., 2001). Interestingly, injection or increased expression of collagenase in intact liver, associated with HGF or TGFα, induces hepatocyte proliferation, suggesting that ECM degradation may play a role in hepatocyte priming (Liu et al., 1994). Conversely, Issa et *al*. (Issa et al., 2003)

observed that failure in collagen-I degradation in mouse liver inhibits the hepatocyte proliferation response.

Spatial and temporal expression of protease occurs during liver regeneration. In rat, activation of plasminogen to plasmin begins within 15mim after PH and stays pronounced until 3-6h. Successive inductions of mRNA levels of the metalloproteinases (MMP)-9, MMP-2, MMP-13, MMP-14, MMP-24, which constitute a family of zinc-containing neutral proteinases involved in matrix remodeling in both normal and pathological processes, are observed in mouse. Moreover, in parallel, inhibitors of metalloproteinases (TIMP) -3, TIMP-4, TIMP-1 are also up-regulated. In particular, TIPM-1 expression appears just before DNA synthesis in rat and mouse models (Mohammed et al., 2005; Rudolph et al., 1999). After PH, its activation is linked to the hepatocyte cell cycle since experiments based on gain of TIMP-1 function in transgenic mice result in delayed cell cycle progression whereas loss of function in knout-out mice accelerates liver regeneration (Mohammed et al., 2005). Activation of pro-MMP9 in MMP-9 after PH, mediated by plasmin or by plasmin-activated MMP-3, is followed by activation of pro-MMP-2 in MMP-2 probably by the membrane-type 1 MMP. In regenerating liver 3h post-PH MMP-9 is located in the immediate periportal hepatocytes, then, its localization extends rapidly throughout the lobule before it decreases at 72h post-PH. In the meantime, MMP-2 expression enhances in the hepatocytes at 24 and 48h post-hepatectomy (Kim et al., 2000). Interestingly, migration of the MMP's staining pattern correlates with the gradual hepatocyte progression into the cell cycle from the periportal to the pericentral areas. This could be related to an important regulatory mechanism for controlling cell proliferation by the liberation of growth factors after ECM proteolysis. In accordance, mature HGF production is delayed by 12h in the uPA$^{-/-}$ mice along with a delayed DNA synthesis. Loss of uPA results in reduced plasmin levels responsible for activating MMP that in turn digest the ECM and allow release from ECM of activated growth factors like HGF (Schuppan et al., 1998). Deletion of the mouse gene *Timp3* results in the increase in TNF-α converting enzyme activity (TACE), constitutive release of TNFα and activation of TNFα-dependent signaling in the liver. In mice lacking *Timp3* gene, cyclin D1 and PCNA expression as well as hepatocyte division occur earlier than in wild-type mice with a shorter cell cycle time course. However, these mice succumbed of liver failure by a TNFα-signaling dependent cell death demonstrating also the importance of TIMP3 in controlling TNFα bioavailability (Mohammed et al., 2004).

Studies performed *in vitro* have shown that TNFα induces MMP-9 expression in mouse hepatocytes (Haruyama et al., 2000) and that MMP-9 transcription involves activation of NF-κB pathway (Mori et al., 2003). Cytokine-specific regulation of MMP/TIMP expression in hepatic stellate cells also suggests that the initial matrix breakdown following liver injury might be enhanced by TNFα, while diminished matrix degradation during chronic tissue injury might be due to the action of TGF-β1 through TIMP induction (Knittel et al., 1999). Together, these studies clearly demonstrated the importance in matrix remodeling to promote proliferation of adult hepatocytes. This conclusion is reinforced by the observation that normal rat hepatocytes plated on denatured collagen I are able to proliferate following stimulation by EGF while they do not respond to this growth factor when plated on collagen I gel (Hansen and Albrecht, 1999), collagen sandwich (De Smet et al., 2001) or matrigel (Nagaki et al., 2000). Cyclin D1 mRNA and protein expression as well as associated kinase activity are low on collagen gel relative to collagen film. Similar results are obtained when hepatocytes are spread on high fibronectin density (proliferation) or low fibronectin density (cell cycle arrest) coatings (Bhadriraju and Hansen, 2004). In this context, we asked the

question on how mitogen signals and extracellular matrix degradation are linked to promote cell cycle entry and progression of differentiated adult hepatocytes. For that purpose, the primary pure culture of hepatocytes did not appear as a pertinent model since hepatocytes progress regardless of priming factors in this model. In addition, we had previously shown that very low amounts of ECM were synthesized in pure culture. We therefore used quiescent adult rat hepatocytes in coculture with liver epithelial cells. Indeed, as mentioned above, hepatocytes in cocultures are stably differentiated for several weeks and capable of extracellular matrix deposition. This ECM located around the hepatocyte cords contains high amounts of type III, I collagens and fibronectin as *in vivo* (Clement et al., 1984). Moreover, cytoskeleton organization of hepatocytes is similar in coculture and *in vivo* i.e. beneath of the plasma membrane (Baffet et al., 1991; Corlu et al., 1991). Bile canaliculi structures present between the hepatocytes are also functional. Finally, in these *in vitro* conditions hepatocytes are unable to respond to growth factor alone as observed *in vivo* (Corlu et al., 1997).

Using this coculture system, we established new conditions allowing hepatocytes to undergo several proliferation waves (Figure 7) without loss of differentiation in presence of the priming cytokine, TNFα, and growth factors, HGF, EGF as *in vivo* (Serandour et al., 2005). For example, three days after TNFα/EGF or TNFα/HGF stimulation, 35% of hepatocytes divide whereas no DNA synthesis is observed in presence of HGF or EGF alone. Moreover, TNFα alone did not induce hepatocyte proliferation. However, hepatocytes gradually stop to synthesize DNA even under prolonged TNFα/EGF stimulation. Question is thus raised about the requirement of a cell cycle arrest following the first wave of divisions for inducing a second round of proliferation. When the cocktail TNFα/EGF is removed for few days before re-stimulation, induction of a new wave of DNA synthesis is obtained. This model of controlled induction of hepatocyte proliferation has been crucial to define whether the signaling mechanisms induced by TNFα could be linked to ECM remodeling (Figure 7). The quantification of ECM deposition detected using reticulin staining on cells stimulated by EGF alone, TNFα/EGF, or successively by EGF and then TNFα revealed several crucial data: 1) ECM is very abundant in both unstimulated and non proliferating EGF-treated cells, 2) in TNFα/EGF-treated cocultures, ECM deposition is very sparse and most fibers disappear within colonies of proliferating hepatocytes, 3) TNFα stimulation, before or after EGF exposure, induces ECM degradation, 4) during prolonged TNFα/EGF stimulation, DNA synthesis decreases concomitantly with new ECM deposition. In agreement with all these results, the phenanthroline, a specific inhibitor of MMP activities reduces the TNFα-mediated ECM degradation resulting in the decrease in DNA replication. This effect is reversible and after phenanthroline removal, DNA synthesis is completely restored. Among MMPs, MMP-9 expression by hepatocytes is induced by TNFα. Moreover, interferon-γ, described to inhibit TNFα-mediated MMP-9 expression via the Interferon Regulatory Factor-1 binding competition with NF-κB (Sanceau et al., 2002), prevents ECM remodeling and impairs DNA synthesis. Thus, ECM peri-cellular proteolysis controlled by TNFα via activation of the NF-κB pathway and induction of MMP-9 is necessary for S phase entry in hepatocytes. This ECM remodeling signal is also required for initiating any subsequent hepatocyte division wave in presence of mitogen (Serandour et al., 2005). These observations have been confirmed by Olle and coworkers using MMP-9$^{-/-}$ mice (Olle et al., 2006). Indeed, in these animals hepatic regenerative response is delayed compared with wild-type control animals. Moreover, they express significantly less HGF and TNFα at day 2

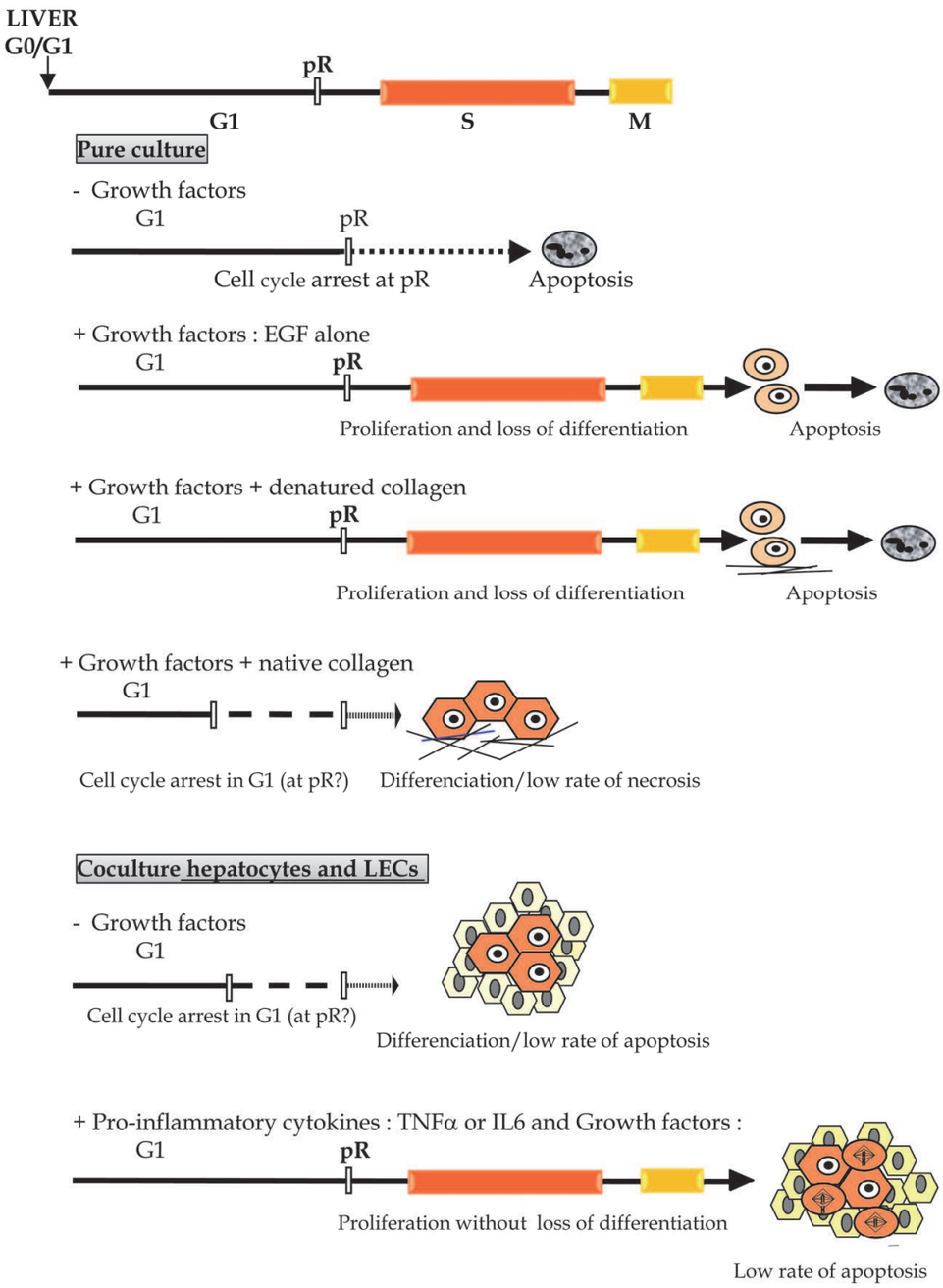

Fig. 7. Schematic representation of the commitment to cell death or proliferation in primary cultures of rat hepatocytes in different *in vitro* conditions. In pure culture, in absence of growth and survival factors, hepatocytes rapidly lose their differentiation and undergo apoptosis. In presence of growth factors they complete a single round of cell cycle before dying. When hepatocytes are maintained on complex bio-matrices (ex: matrigel and native collagens), they arrest in G1 and do not respond to growth factor stimulation. In co-culture, hepatocytes arrest in G1 unless a combination of pro-inflammatory cytokines and growth factors is added to the culture medium, which triggers a complete cell cycle without affecting differentiation and long-term survival.

post-PH corresponding to hepatocyte DNA synthesis in mice (Olle et al., 2006). In addition, in hepatoma cells, TNFα stimulates DNA replication by causing release of TGFα into the culture medium through the metalloproteinase disintegrin TACE. Then, TGFα activates EGFR and multiple downstream intracellular signaling cascades required for DNA replication (Argast et al., 2004).

Unexpectedly, experiments with successive addition of cytokine and growth factors as well as analysis of the expression of cell cycle regulation demonstrate that EGF alone promotes cell progression up to late G1. When we tested whether addition of TNFα before or after EGF stimulation might influence cell cycle progression, we observed that hepatocytes DNA synthesis is rapidly obtained in each situation. In addition, a pause of 2 days following EGF treatment does not affect hepatocyte responsiveness to TNFα, suggesting that these cells integrate a long-lasting mitogenic signal.

Using both pure culture of hepatocytes and the co-culture model, we compared expression of cell cycle markers to further investigate the molecular pathways involved in the progression in late G1 phase. In unstimulated co-cultures, cyclin D1 and Cdk2 are barely detectable (Figure 8). This pattern of expression, similar to that observed in unstimulated primary pure cultures of hepatocytes, suggestsed that they are blocked in G1 upstream the mitogen restriction point. Unexpectedly, although no BrdU-positive hepatocytes are detected in EGF–stimulated co-cultures, cyclin D1, Cdk4 and Cdk2 accumulate in this culture condition. Interestingly, even if Cdk2 was present no histone H1 kinase activity is detected (Figure 8). Therefore, EGF alone promotes the progression beyond the mitogen restriction point in late G1 although cells arrest before S phase.

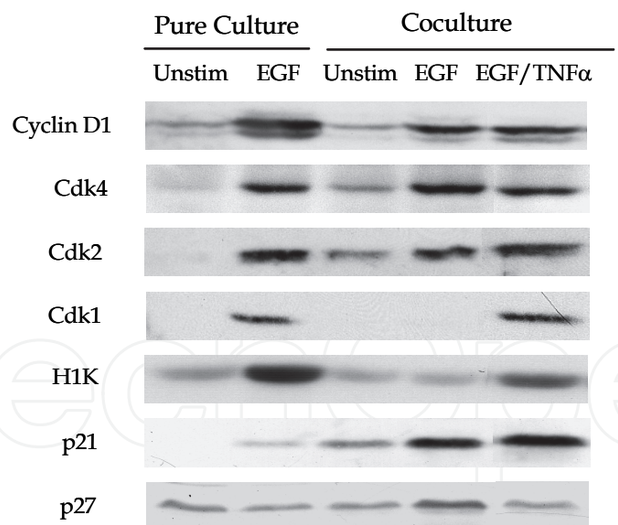

Fig. 8. Expression of Cdks, cyclins and Cdk's inhibitors in cultured rat hepatocytes. Cyclin D1, Cdk4, Cdk2, Cdk1 and the inhibitors $p21^{Cip1}$ and $p27^{Kip1}$ were analyzed by western blotting. In addition, kinase activities of Cdk1 and Cdk2 were measured using histone H1 as a substrate (H1K).

Our results could be linked to previous reports showing that cyclin E and Cdk2 are present in cells plated on collagen gel or film, but on collagen gel, hepatocytes do not proliferate and lack the Cdk2 activity (Hansen and Albrecht, 1999). In these conditions, $p27^{Kip1}$ protein

levels are similar but higher amounts of $p27^{Kip1}$ are associated with Cdk2 in cells plated on collagen gel than to those plated on collagen film. Similarly, $p27^{Kip1}$ $p21^{Cip1}$ are up-regulated in cell cultured on matrigel (Nagaki et al., 2000). In our co-culture condition, $p27^{Kip1}$ $p21^{Cip1}$ are also induced after EGF stimulation while in TNFα/EGF-stimulated co-cultures, expression levels of cyclin D1 and Cdk4 are strongly increased followed by up-regulation of Cdk1 and Cdk2. In contrast, $p27^{Kip1}$ levels are reduced demonstrating that Cdk levels are up-regulated while Cdki's are repressed to favor cell cycle progression. Moreover, both Cdk2 and Cdk1 are active as evidenced by Histone H1 kinase activity. We therefore point out a new cell cycle control in late G1 associated with ECM deposition and overcome by TNFα addition that triggers ECM remodeling and induction of MMP9. Importantly, TNFα stimulation following EGF exposition induces the expression of Cdk1 and the activation of both Cdk2 and Cdk1 kinase activities. Altogether, our results show that induction of Cdk1, correlating with the hepatocyte S phase entry, requires remodeling of the extracellular matrix and induction of the metalloproteinase MMP9 by TNFα stimulation. They also suggest that catalytic activation of Cdk1 may be regulated by Cdk2 kinase activity. This led us to draw the conclusion that Cdk2 and Cdk1 would exhibit a sequential catalytic activation under the control of extracellular signals including cytokines, growth factors as well as extracellular matrix remodeling. TNFα–mediated ECM remodeling is necessary for Cdk2 activity, Cdk1 expression, G1/S transition and completion of the cell cycle of hepatocytes in co-cultures.

Several important questions remain unanswered. How does TNFα induce Cdk2 kinase activity? It could be hypothesized that low levels of $p27^{Kip1}$ following TNFα stimulation favor activation of Cdk2/cyclin E and Cdk2/cyclin A kinase activities. In addition, the mechanism by which TNFα induces Cdk1 expression remains unclear. Does it involve a transcriptional regulation mediated by unidentified signaling pathways and transcription factors? Local remodeling of the ECM could lead to disruption of ECM-cell communications achieved by integrins. Through multiple protein-protein interactions and signaling events, they could activate various signaling cascades regulating transcriptional activities. For example, repression of Integrin-linked kinase (ILK), a cell-ECM-adhesion component implicated in cell–ECM signaling via the integrins, leads to enhanced cell proliferation and hepatomegaly (Gkretsi et al., 2008).

5. Conclusion

The peculiar biphasic pattern of Cdk1 activity during cell cycle of normal hepatocytes and the evenly active Cdk1 and Cdk2 during S phase contrasts with most mammalian cell types in which active Cdk2 is highly predominant over other Cdks in S phase. Indeed, in DT40 chicken cells expressing low levels of active Cdk1 in S phase, elimination of Cdk2 induced a Cdk1-dependent S phase but presence of a single Cdk2 allele rendered the S phase independent of Cdk1 suggesting that Cdk1 and Cdk2 are functionally exclusive at the level of kinase activity. However, in absence of Cdk2, Cdk1 can fully compensate for S phase function of Cdk2 but fails to compensate for Cdk2's DNA repair functions in mammalian cells. Because of its location and function, the liver which is a vital organ, is continuously exposed to a wide range of harmful substances, viral infections which alter the hepatic homeostasis by inducing changes in the balance between proliferation and apoptosis. Despite its efficient defense system, many agents are still able to produce liver damage. Thus, to overcome these damages, liver has to compensate tissue loss. A major feature of

adult hepatocytes is their singular capacity to proliferate despite their high level of differentiation. This ability has been related to the low expression level of $p21^{CIP1}$ Cdk-inhibitors in adult liver and primary hepatocytes *in vitro*, which could explain their rapid exit from quiescence. Based on the data obtained by our laboratory and others, we hypothesize that those high levels of active Cdk1 and Cdk2 following G1/S transition could participate to cellular defense response following stress stimulus in controlling rapid DNA repair and synthesis. We also showed that Cdk1 expression and activation is correlated to ECM degradation via the involvement of the pro-inflammatory cytokine TNFα. We thus identified for the first time a new signaling pathway regulating Cdk1 expression at the G1/S transition upon stimulation by cytokines (Figure 8). It also further confirms the well-orchestrated regulation of liver regeneration via multiple extracellular signals and pathways.

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The study of DNA advanced human knowledge in a way comparable to the major theories in physics, surpassed only by discoveries such as fire or the number zero. However, it also created conceptual shortcuts, beliefs and misunderstandings that obscure the natural phenomena, hindering its better understanding. The deep conviction that no human knowledge is perfect, but only perfectible, should function as a fair safeguard against scientific dogmatism and enable open discussion. With this aim, this book will offer to its readers 30 chapters on current trends in the field of DNA replication. As several contributions in this book show, the study of DNA will continue for a while to be a leading front of scientific activities.

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