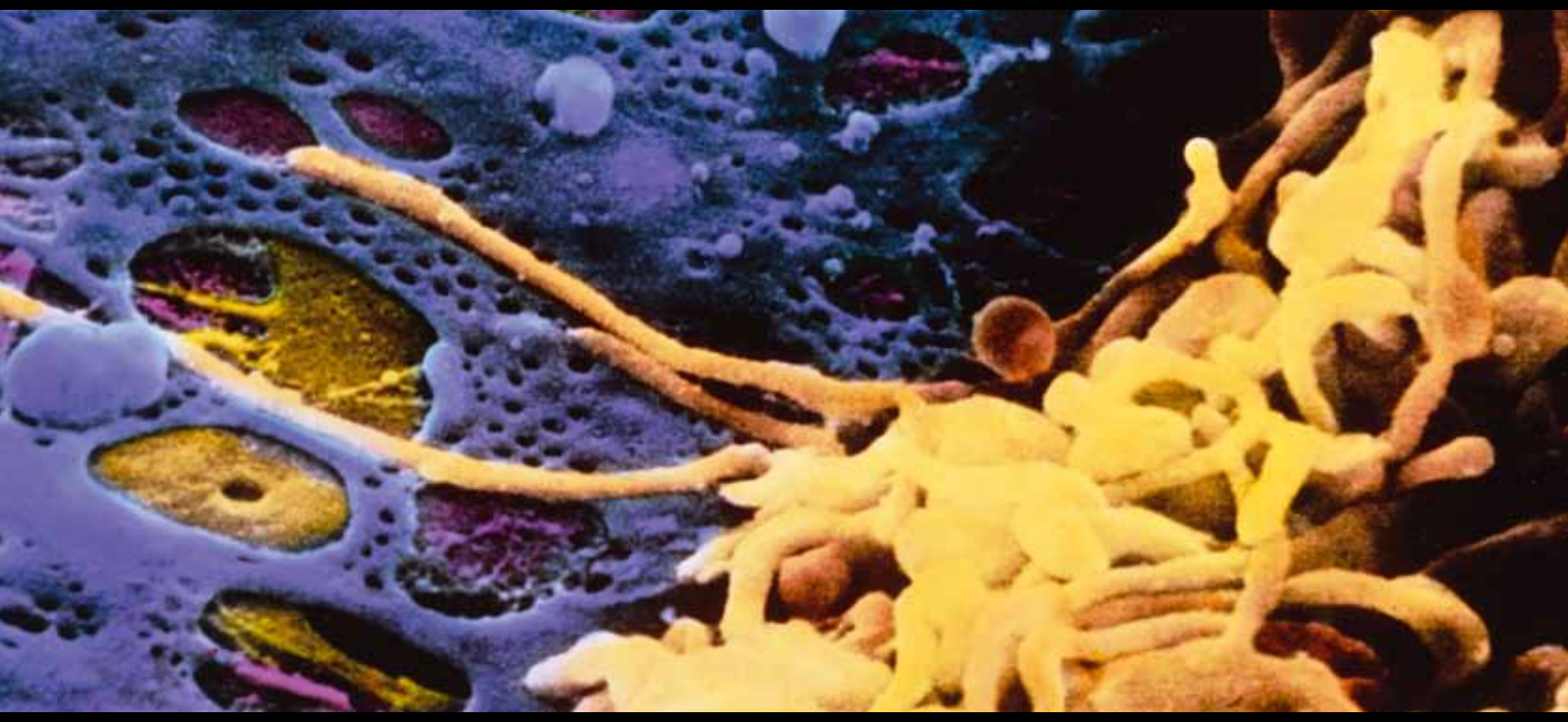
International Journal of Hepatology

Regulation of the Hepatocyte Cell Cycle: Signaling Pathways and Protein Kinases

Guest Editors: Pascal Loyer, Anne Corlu, and Chantal Desdouets



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Editorial Board

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Hindawi Publishing Corporation
International Journal of Hepatology
Volume 2012, Article ID 592354, 3 pages
doi:10.1155/2012/592354

Editorial

Regulation of the Hepatocyte Cell Cycle: Signaling Pathways and Protein Kinases

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Received 5 December 2012; Accepted 5 December 2012

The adult liver exhibits the remarkable ability to "regenerate" following surgical resection or toxic liver injuries. In normal liver restoration of hepatic tissue homeostasis occurs through rapid and partially synchronous proliferation of adult mature hepatocytes. The hepatocytes expressing the liver-specific functions responsible for the crucial hepatic metabolic pathways are quiescent cells that keep the ability to reenter the cell cycle. The fact that liver regeneration is supported by the active proliferation of highly differentiated hepatocytes rather than an expansion of progenitor cells is a unique situation among adult solid tissues. The hepatocytes, which exit quiescence and proliferate for a limited number of divisions present specific proliferation signaling pathways and a peculiar cell cycle regulation. In addition, polyploidy is another characteristic feature of mammalian adult hepatocytes that contributes to the specific molecular mechanisms underlying the cell cycle in hepatocytes. The entry into and progression through G1 phase of the cell cycle are orchestrated by complex networks of extracellular stimuli and intracellular signaling pathways inducing profound modifications of the gene expression required for the exit from quiescence and the cell cycle completion of the differentiated hepatocytes. Several lines of evidences also indicate that cell cycle regulators such as the Cyclin Dependent protein Kinases (CDKs) and their functional partners the cyclins and CDK inhibitors (CDKIs) show specific expression and/or activation patterns compared to the cell cycle in others cell types.

The special issue collected ten original research articles and reviews that present an update of various biochemical pathways underlying the cell cycle regulation of the adult hepatocytes. All the articles of this special issue explore in various extend the signaling pathways and the cell cycle protein kinases controlling the proliferation of adult mammalian hepatocytes. They also illustrate that many discoveries in this field benefited from the combined use of *in vivo* models of liver regeneration in rodents and *in vitro* models of both primary cultures of hepatocytes and in some extent established hepatoma cell lines.

A first topic of interest of this special issue was the role of the liver microenvironment in the initiation of liver regeneration and more precisely the early stimuli leading to the reentry of the hepatocytes into the cell cycle also called "priming". In that context, the release of cytokines and growth factors produced by nonparenchymal cells and the hepatocytes themselves, the early activations of downstream signaling pathways, signaling pathways, and the extracellular matrix remodeling were covered by several manuscripts. T. Nowatari et al. "*Regulation of signal transduction and role of platelets in liver regeneration*", and in a less extent A. Corlu and P. Loyer "*Regulation of the G1/S transition in hepatocytes: involvement of the cyclin-dependent kinase cdk1 in the DNA replication*" provide a detailed update of the considerable literature published over the last 20 years that describes the productions of cytokines and growth factors by Kupffer and endothelial cells that orchestrate the proliferation of hepatocytes to counteract a loss of the liver mass. Notably, T. Nowatari et al. "*Regulation of signal transduction and role of platelets in liver regeneration*" focus on the more recently identified role of the platelets in liver regeneration. In an original article, F. Finot et al. "*Combined stimulation with the tumor necrosis factor alpha and the epidermal*

growth factor promotes the proliferation of hepatocytes in rat liver cultured slices" provide the first description of hepatocyte proliferation in cultured rat liver slices. They further demonstrate in this *in vitro* cell system that the combined stimulation by proinflammatory cytokines such as the Tumor Necrosis Factor alpha (TNFα) and growth factor such the Epidermal Growth Factor (EGF) is required for the sequential activation of cell cycle regulators and the commitment to DNA replication. In their review, A. Corlu and P. Loyer "*Regulation of the G1/S transition in hepatocytes: involvement of the cyclin-dependent kinase Cdk1 in the DNA replication*" further detail the role of TNFα in the extracellular matrix remodeling occurring in regenerating liver and cocultured rat hepatocytes prior any DNA replication. Conversely, the active proliferation of hepatocyte is greatly impaired during cirrhosis. V. C. Sanchez et al. "*Recovery of the cell cycle inhibition in* CCl_4*-induced cirrhosis by the adenosine derivative IFC-305*" report data showing a marked reduction of several cell cycle and mitochondrial regulators during CCl_4-induced cirrhosis in rats. They further demonstrate that the cell cycle activity is partially restored by the adenosine derivate IFC-305 and suggest that the preservation of mitochondrial function may contribute to the recovery of proliferation in cirrhotic livers.

The second and major theme of this issue is dedicated to the activation of intracellular signaling pathways and gene profile modifications controlling the priming of hepatocytes and progression in early G1 phase of the cell cycle. T. Garcin and T. Tordjmann "*Calcium signalling and liver regeneration*" reviewed the latest knowledge regarding the calcium signaling in liver regeneration following stimulation of the hepatocytes by calcium mobilizing agonists and activation of tyrosine kinase receptors, receptor channels, and G-protein-coupled receptors. This review emphasizes that calcium movement both in the cytoplasm and nucleus are clearly important events in the proliferative signaling through the transcriptional activation of immediate early genes and the control of the G1/S and G2/M transitions. Four other reviews develop distinct aspects of the signaling pathways especially phosphorylation events regulating the progression throughout the G1 phase and the commitment to DNA replication. The G1 phase of the cell cycle is *per se* under the control of extracellular growth factors activating a cascade of phosphorylation/dephosphorylation events that ultimately lead to the commitment to DNA replication. Beyond the G1/S transition, committed cells will proceed to DNA replication and mitosis regardless of the presence of growth factors in the extracellular microenvironment. The review by A. C. de l'Hortet and co-workers "*EGFR: A master piece in G1/S phase transition of liver regeneration*" reports in details the crucial role of the EGF receptors and its ligands in the G1/S transition and describes the activation of downstream phosphorylation events trigger by the EGFR. The authors also discuss the potential implication of this receptor in liver diseases including cancer but also metabolic disorders such as steatosis. Following stimulation by growth factors, it is now well established that the MAPK MEK/ERK pathway is crucial for both survival and proliferation in hepatocytes. J. P. Guégan et al. "*The MAPK MEK1/2-ERK1/2 pathway and its implication in hepatocyte cell cycle control*" provide an exhaustive overview of their own work and the literature regarding the role of this pathway in the hepatocyte cell cycle control. Furthermore, they emphasize the specific roles of ERK1 versus ERK2 both in normal and transformed hepatocytes. A. Gougelet and S. Colnot "*A complex interplay between Wnt/β-Catenin signalling and the cell cycle in the adult liver*" present in this issue another important pathway: the Wnt/β-catenin signaling pathway. Following the demonstration on the involvement of Wnt and its functional partner β-catenin in the liver carcinogenesis and the determination of the metabolic zonation in quiescent liver lobule, numerous publications have demonstrated the implication of the Wnt/β-catenin signaling pathway in the regulation of cell cycle genes such as the cyclin D1. Once again, two protein kinases play an important role in this pathway: the casein kinase 1 and glycogen synthase kinase 3. However, the authors have extended the discussion far beyond the induction of limited subset of genes by the activation of β-catenin and conclude with the unresolved dual role of this pathway in the balance between quiescence and proliferation in hepatocytes. Besides these canonical pathways, J. Pajaud et al. "*Regulation of signal transduction by glutathione transferases*" introduce the regulation of signal transduction by glutathione transferases (GSTs). The GSTs are well-known drug metabolizing enzymes but over the last decade a role of GSTs in the modulation of protein kinase activities has emerged. It is now well admitted that GSTs regulated protein kinases either by direct binding and/or by S-glutathionylation of catalytic subunits. In this article, the authors review the literature that link the GSTs to the regulation of apoptosis and cell cycle through the functional interactions with protein kinases such as c-jun terminal kinase (JNK) and the apoptosis signal-regulating kinase 1 (ASK1).

A peculiar feature of the adult liver is the polyploidy of a large fraction of the hepatocytes. The polyploidization of the hepatocytes occurring during postnatal development is a noncanonical cell cycle that relies on an abnormal cytokinesis. G. Gentric et al. "*Hepatocytes polyploidization and cell cycle control in liver physiopathology*" review the molecular mechanisms and functional consequences of hepatocytes polyploidy during normal and pathological liver growth. They emphasize the role of insulin and the PI3K/Akt signaling pathway in the control of this process that they have largely contributed to decipher.

All these contributions to our special issue deal with signaling pathways and protein kinases activating terminal executioner such as transcription factors and cyclin dependent kinases (cdks) and their regulatory subunits the cyclins. In a general review, A. Corlu and P. Loyer "*Regulation of the G1/S transition in hepatocytes: involvement of the cyclin-dependent kinase Cdk1 in the DNA replication*" summarize the latest discoveries on the cdks and cyclins involved in cell cycle control and discuss the emerging differences concerning the expression and regulation of the catalytic activity of cdk1 among the different mammalian cells. They also present the recent data demonstrating the involvement of cdk1 during the DNA replication in hepatocytes and

the possible transcriptional control of the cdk1 gene by the TNFα dependent signaling pathway.

Acknowledgments

The editors are particularly proud to publish this special issue and would like to deeply thank all the contributors. All reviews and original articles summarize the most recent data in the field on the hepatocyte proliferation and we strongly believe that the scientists interested in the liver biology will find a large panel of information for their own research. The editors would like to dedicate the special issue to two contributors who have passed away during the preparation of this issue: Dr. Fabrice Morel (Inserm UMR-S 991, Université de Rennes 1, France) and Dr. Joan Albert Vericat (Noscira SA, Tres Cantos, Spain). Fabrice Morel and Joan Vericat were exceptional scientists and friends whose enthusiasm for life and belief in science will continue to inspire all of us who were privileged to know them.

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Hindawi Publishing Corporation
International Journal of Hepatology
Volume 2012, Article ID 328372, 13 pages
doi:10.1155/2012/328372

Review Article

The MAPK MEK1/2-ERK1/2 Pathway and Its Implication in Hepatocyte Cell Cycle Control

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Received 29 May 2012; Revised 6 September 2012; Accepted 10 September 2012

Academic Editor: Chantal Desdouets

Primary cultures of hepatocytes are powerful models in studying the sequence of events that are necessary for cell progression from a G0-like state to S phase. The models mimic the physiological process of hepatic regeneration after liver injury or partial hepatectomy. Many reports suggest that the mitogen-activated protein kinase (MAPK) ERK1/2 can support hepatocyte proliferation *in vitro* and *in vivo* and the MEK/ERK cascade acts as an essential element in hepatocyte responses induced by the EGF. Moreover, its disregulation has been associated with the promotion of tumor cell growth of a variety of tumors, including hepatocellular carcinoma. Whereas the strict specificity of action of ERK1 and ERK2 is still debated, the MAPKs may have specific biological functions under certain contexts and according to the differentiation status of the cells, notably hepatocytes. In this paper, we will focus on MEK1/2-ERK1/2 activations and roles in normal rodent hepatocytes *in vitro* and in vivo after partial hepatectomy and in human hepatocarcinoma cells. The possible specificity of ERK1 and ERK2 in normal and transformed hepatocyte will be discussed in regard to other differentiated and undifferentiated cellular models.

1. Introduction

Adult hepatocyte has a long lasting life and rarely divides in normal conditions. However, under certain situations of stress as viral infection, toxic injury, and partial hepatectomy, they can divide in reaction to the loss of liver mass. Among these different situations, the regeneration of liver after partial hepatectomy (PHT) provides an *in vivo* model to dissect the mechanisms of control of a highly differentiated normal cell growth. Indeed, surgical removal of 70% of the liver synchronized most hepatocytes and the cell cycle is characterized by a fast G0/G1 phase transition of the cell cycle after PHT, followed by a well-synchronized long G1 phase [1–3]. There is an initial step priming phase, in which the activation of IL6 and TNF alpha pathways allows hepatocytes to undergo the transition from G0 to G1 *in vivo* leading to activation of NF-kB, AP-1, and STAT3. Then, hepatocytes proliferation is regulated by different mitogens including HGF, IGF1, ligands of the EGF, and FGF receptors [4, 5]. *In vitro*, hepatocytes can also proliferate after growth factor stimulations and *in vitro* rat hepatocyte cell cycle progression highly mimicked the kinetic of cell proliferation during liver regeneration after PHT [6, 7]. In response to mitogens (i.e., EGF, HGF, PDGF, TGF alpha), hepatocytes maintained in short-term culture can undergo one or two rounds of replication (for reviews see [4, 8, 9]). This model has been extensively used by many laboratories illustrating that primary culture of hepatocytes can be a powerful model to study the precise sequences of events which are necessary for hepatocyte cell cycle progression from a G0-like state to S phase.

There are four MAPK families categorized by sequence homology and functions: ERK1/2, p38, JNK, and ERK5. Mostly, JNK and p38 are more activated in response to cellular stress and cytokines. Numerous studies have shown that growth factor could enhance cell proliferation and

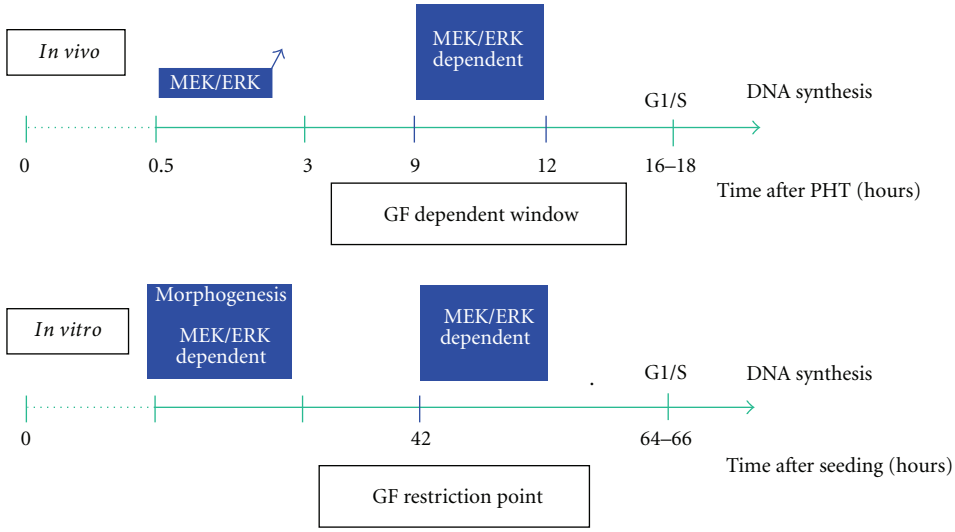
survival through the activation of the MEK1/2-ERK1/2 pathway, including hepatocytes in primary culture. Indeed, the ERK1/2 are activated in response to external and internal stimuli in numerous cell types and play a central role in many signal transduction pathways. The Ras-Raf-MEK1/2-ERK1/2 pathway couples signal from the cell surface receptors to cytoplasmic substrates and transcription factors, which regulate gene expression [10–12]. Following binding of growth factors, cytokines, or extracellular matrix proteins to their receptors, activation of the cascade can occur. The pathway involves the activation of the MEK1/2, by c-Raf which in turn, activates ERK1/2. ERK1/2 can directly phosphorylate many targets (over 160) including transcription factors (e.g., Ets-1, c-Jun, c-Myc, P53) which leads to the induction of many cell cycle proteins (e.g., p21, Cyclin D1, cdk1). ERK1/2 can also phosphorylate and activate cytoplasmic substrates like the 90 KDa ribosomal S6 kinase (P90 RSK) which leads to the activation of the CREB transcription factor, apoptotic factors (e.g., caspase 9, bad, Bim), and also contribute to a mechanism of retrocontrol of the cascade by phosphorylation of the EGFr, Sos, and Raf. In addition to proliferation, the Ras-Raf-MEK1/2-ERK1/2 cascade can antagonize cell death and activate survival signals. Aberrant activation of this pathway is frequently observed in human HCC [13–16]. The MEK-ERK pathway has been implicated in the regulation of both G1/S and G2/M transitions and mitosis in somatic cells. Whereas the possible specificity of MEK1 and 2, ERK1 and 2 isoforms are still in debate, and disruption of ERK2 leads to embryonic lethality early in mouse development after the implantation stage [17]. Conversely, ERK1 Knockout mice are viable and fertile [18], arguing for possible different roles of each kinase or/and that ERK gene dosage is essential and could drive their apparent biological differences.

2. Mechanisms in the Sequential Control of Cell Morphology and G1 Phase Progression Involve MEK-ERK Activations in Normal Hepatocytes

There is an agreement that during liver regeneration, JNK activation is an early event [19] while activation of ERK1/2 occurs in early and mid-late G1. P38 is present in normal liver and rapidly inactivated after PHT suggesting a permissive role in DNA replication [20]. These last ten years, our laboratory has studied the role of the MEK1/2-ERK1/2 pathway in the regulation of the cell cycle and survival of hepatocytes stimulated by the EGF. We looked at long-term survival, control of multiple cell cycles, apoptosis engagement of normal rodent hepatocytes, and rat and human hepatocarcinoma cell lines, *in vitro* and *in vivo*. The MEK1/2-ERK1/2 cascade is activated at two points of the G1 progression in mature rat hepatocytes [21]: the first one occurs in early G1 after PHT; the second one occurs in mid-late G1 phase and is associated with the induction of cyclin D1 [22], a cyclin associated to late G1 phase progression of many cells including hepatocytes [23, 24]. *In vitro*, during tissue disruption by collagenase, hepatocytes can enter into the G1 phase and undergo, depending on the culture conditions in primary culture, at least one round of division [6, 7, 25]. In the absence of growth factor, rat hepatocytes are blocked at 2/3 of G1 phase and rapidly progress through apoptosis [26, 27]. The growth factor (i.e., EGF) is a morphogen in early G1 phase by inducing controlled spreading of hepatocytes via a MEK/ERK-integrin β1 regulation, *in vitro* [28]. During hepatocyte spreading, Rac1 trough NADPH oxidase is part of the signalling pathway constituted by FAK-Rac1-ERK that regulates focal adhesion disassembly important for the turnover of adhesion sites that leads to cell spread [29]. The growth factor-induced nuclear translocation of ERK is an adhesion-dependent event and requires signalling from Rac1 [30]. Cell spread and migration are dynamic processes involving the focal adhesion assembly/disassembly and ERK1/2 are activated downstream of FAK while ERK1/2 can mediate its phosphorylation [31].

A mitogenic effect occurs in mid-late G1 phase and allows hepatocytes to progress through a growth factor restriction point at two thirds of the G1 phase [32]. MEK signaling cascade is essential for progression to late G1 phase *in vitro* as well as *in vivo* after PHT [21]. Indeed, a growth factor-MEK dependency could be defined in mid-late G1 phase in regenerating liver between 9 and 12 h after PHT. This activation controls expression of cyclin D1 and cdk1 which are upregulated in the prereplicative phase of liver regeneration and in proliferating hepatocytes *in vitro*. Very interesting results from the Hansen's lab have demonstrated that adhesion to polymerized collagen could induce growth arrest by inhibiting the Ras/ERK pathway to cyclin D1 required in late G1 [33, 34]. Moreover, the involvement of the cell shape/motility via an ERK-MLCK-P70S6 K-dependent regulation of G1/S was specified in proliferating hepatocytes [35] and in other cell types [31, 36, 37]. All these results highlight the mechanisms by which a growth factor can temporally control morphogenic and mitogenic signals during G1 phase progression (see Scheme 1). A precise location in the cell cycle appears determinant for the regulation of ERK1/2 pathway and sequential checkpoints in early G1, mid-late G1, and G1/S transition control hepatocyte cell cycle progression, making them permissive for DNA replication.

The signaling crosstalk is an important aspect of the regulation of liver regeneration and other pathways (i.e., HGF/c-MET, IGF1/IGF-R, GH) are activated and required for efficient liver regeneration. Indeed, GH receptor KO impaired regeneration with a downregulation of ERK1/2 activation [38]. Liver regeneration and ERK pathway are also impaired in mice with liver-specific knockouts of IGF-1R or IGF binding protein 1 [39, 40]. HGF and IGF-1 strongly activated AKT and ERK1/2 *in vitro* [41]. *In vivo*, EGF and HGF have been implicated in liver regeneration, but specific deletion of EGF receptor in hepatocyte led to liver regeneration deficiencies after 2/3 PHT in mice without activation defect of ERK1/2 while p38 MAPK and NF-kB activation was reduced in regenerating mutant livers, indicating an impaired stress response after hepatectomy [42]. Indeed, p38 MAPK could play a permissive role in



Scheme 1: *In vivo* and *in vitro* MEK1/2-ERK1/2 dependencies during G1 phase progression.

DNA replication during liver regeneration consistent with a role in the maintenance of hepatocyte cell cycle arrest in adult liver [20], while JNK could be involved in the G0/G1 transition [19]. Interestingly, hepatocyte deletion of c-Met which led to liver regeneration defect was associated with MEK-ERK pathway inhibition highlighting that HGF contributes dominantly to ERK1/2 activation *in vivo* [43, 44]. A persistent EGF supplementation *in vitro* only partially rescues the effect of ERK1/2 downregulation in c-MET depleted hepatocytes and restores to some extent DNA synthesis and protein levels of cdk1, Aurora A and B, and Mad2 [44].

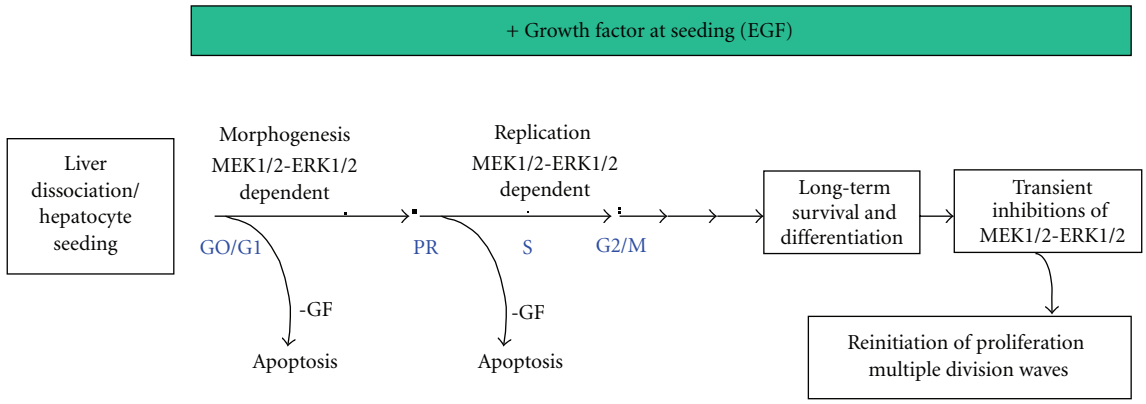
3. Transient Blockade of the MEK/ERK Pathway Using Allows Multiple Cell Cycles

Different *in vitro* models have previously described that hepatocytes can undergo several cell cycles in primary cultures and long-term survival when appropriate culture conditions are provided [45–52]. Indeed, removal of EGF in long-term survival DMSO culture conditions followed by readdition of the growth factor was accompanied with an increase in DNA synthesis, and multiple round of replication could be observed by alternating addition/removal [53–55]. In coculture with liver biliary cell [56–60], EGF alone prolonged cell progression up to late G1 phase, whereas TNFα mediated extracellular remodeling is required for multiple division cycles [51]. Interestingly, TNFα promoted an extracellular matrix degradation required for initiating a new hepatocyte division wave. Furthermore, a network of ECM or polymerized collagen type I gel induces a highly differentiated but growth-arrested phenotype in primary cultures, whereas a film of collagen promotes cell cycle progression and dedifferentiation [34, 61, 62]. Hepatocytes dedifferentiation is reversible in consequence of a specific network triggered by the extracellular matrix, an active process driven by FAK-mediated AKT and ERK1/2 signaling [63]. As well, in hepatocellular carcinoma cells, increasing matrix stiffness promotes proliferation whereas soft environment induces cellular dormancy [64].

All these experiments and others indicated that adult hepatocytes could undergo long-term survival and multiple cell divisions. In this context, our group have demonstrated that rat hepatocytes seeded in the presence of EGF (in the absence of FCS) increased cell spreading [28] and greatly enhanced cell survival [65]. However, only one peak of BrdU incorporation was obtained in EGF-seeded cultured whereas nearly 100% of the hepatocytes accomplished a complete cell cycle. Time-lapse cinematography showed that both mononuclear and binuclear hepatocytes underwent mitosis [66]. Some reports have suggested that sustained activation of ERK inhibits hepatocyte DNA replication and that transient activations of this pathway could stimulate DNA synthesis [67, 68]. We therefore hypothesized that maintained MEK1/2-ERK1/2 stimulation of hepatocytes by EGF could lead to a sustained activation of ERK responsible for the negative control of the progression in a second cell cycle. Indeed, when the MEK/ERK pathway is transiently inhibited with the MEK inhibitor U0126 about 60% of hepatocytes did replicate their DNA showing that primary hepatocytes are able to perform 2 cell cycles when a break of the MEK/ERK signalling pathway activity is done [66]. In addition, cyclin D1, E, A2, cdk1, P21, and P27 were downregulated in MEK-inhibited cells and induced after the U0126 removal. A third peak of DNA synthesis in EGF-seeded hepatocytes by performing another 2 days-break of MEK1/2-ERK1/2 activity could be obtained demonstrating that EGF-seeded hepatocytes were able to perform multiple division waves after sequential MEK1/2-ERK1/2 pathway inhibitions (see Scheme 2).

4. Early Sustained EGF Stimulation and MEK Inhibition Maintain Hepatocytes in a Long-Term Survival and Differentiated States

In hepatocytes, in addition to its proliferating properties, EGF could induce survival. *In vitro*, in the absence of serum



Scheme 2: Long-term survival/differentiation in EGF stimulated cells and sequential MEK/ERK inhibitions allowing multiple divisions waves. Hepatocytes are cultured in presence or absence (-GF) of EGF and after one week of culture, transient inhibitions of MEK/ERK by U0126 allow EGF-cultured hepatocytes to re-enter in new cell cycles.

and growth factor stimulation, hepatocytes in primary culture adhered to the plastic support but underwent spreading with a very low efficiency and die as observed by rapid caspase3/7 activations evaluated using a DEVD-AMC assay [65]. At the opposite, hepatocytes seeded with EGF alone and cultured with the growth factor all along the culture time present a high level of differentiation. Cell survival can be maintained at least 15 to 20 days in this culture condition. Albumin expression reached a level closed to half of freshly isolated cells, and CYP450s can be induced by 3MC or PB showing that the detoxification machinery is still fully operative. In these cells, ERK localization could be determinant for the cell phenotype since Rosseland et al. showed that the cytoplasmic retention of transient peroxide-activated ERK provides survival in primary cultures of rat hepatocytes [69]. Indeed, MEK1 and MEK2 could regulate distinct functions by sorting ERK2 to different intracellular compartments in response to growth factor and ERK2 intracellular localization could determine whether growth factors mediate hepatocyte proliferation or survival in an adhesion-dependent manner [70–72].

Surprisingly, an improvement of the survival of hepatocytes continuously treated with the MEK inhibitor U0126 can be obtained [65]. Indeed, a permanent treatment with U0126 keeps hepatocytes for more than 2 weeks in survival. All the genes of detoxification analyzed (Cyp 1A1, 1A2, 2B2, 3A23, and GSTa2) as well as the aldolase B gene are induced all along the period of treatment. U0126 removal from the culture medium is accompanied with a fast decrease of the expression of these markers related to the reentry of the cells in a new cycle.

In summary, early and sustained EGF stimulation, in the absence of serum, could be a good compromise between "classical monolayers" with limited survival/differentiation, and long-term sophisticated and labor intensive cultures. This model emphasizes that early EGF stimulation of hepatocytes in the absence of FCS and transient or sustained inhibition of the MEK/ERK pathway represent serum-free models (Scheme 2) that will be very helpful for pharmacological studies on drug metabolism and toxicity.

5. Specificity of the MAPK ERK1 and ERK2 Signaling: ERK2 Controls S Phase ERK2 Entry whereas ERK1 Regulates Survival in Hepatocyte

The strict specificity of action of the MAPKs is still debated and today, no one can affirm with certitude the full redundancy of ERK1 and ERK2 or at the contrary the specificity of action of each protein. On one hand, the simple observation of the phenotypes of knockout animals for ERK1 and ERK2 fuels the idea that each ERK isoform could regulate specific and non overlapping functions. Invalidating ERK1 has no strong and lethal impact on animals: mice are viable, fertile, and of normal size [18]. Actually, only a few defects affecting different cell lineages have been related. Thereby, ERK1 was associated with maturation of thymocytes [18], development of adipose tissue [73], or osteoclast formation and differentiation [74, 75]. ERK2 knockout is much more severe as embryos die very early during development [17, 76], because of major defects in the establishment of extraembryonic tissues [17, 76, 77]. To counteract this embryonic lethality and ascertain the roles of ERK2 in embryo or adult tissues, conditional expressions have been used allowing the invalidation of ERK2 in specific sites. Invalidation of *Erk2* gene in the neural crest induces craniofacial and cardiac defects [78]. ERK2 also regulates multiple stages of T-cells development [79, 80]. Invalidation of ERK2 in the central neural system (CNS) leads to anomalies in multiple aspects of social behaviors, decreased anxiety-related attitude, and impaired long-term memory [81]. ERK2 also protects the myocardium from ischemia-reperfusion injury *in vivo* as $Erk2^{+/-}$ gene-targeted mice showed enhanced infarction areas [82]. Based on the strict observation of these phenotypes, one could easily conclude that ERK1 and ERK2 regulate specific functions.

Indeed, studies performed on animal and which attributed to ERK1 or ERK2 unique functions did not really took into account the expression level of each isoform in the tissues or cell types analyzed. In other words, the

lethality of ERK2 embryos could reflect a specific role of the isoform in the establishment of extraembryonic tissues or could be due to a higher expression of the ERK2 isoform (compared to ERK1) in these tissues. We must be careful when drawing some conclusions about specific roles for ERK1 and ERK2. Besides, on a purely biochemical point of view, it has been difficult to associate ERK1 or ERK2 to cellular specific functions. They share a 84% homology at the protein level, seem to be activated in response to similar stimuli and to date no specific substrate for each kinase has been characterized. Actually, only a few papers have reported biochemical differences between both proteins. Thus, preferential activation of ERK1 versus ERK2 was reported in NFB4 cells after LPA stimulation [83]. At the contrary, activation of ERK2 rather than ERK1 occurs during thrombin-stimulated platelet activation [84]. Another biochemical difference concerns the identification of a specific scaffolding protein of ERK1 called MP1 (MEK partner 1). This protein interacts exclusively with MEK1 and ERK1 at the surface of late endosome [85, 86]. Finally, despite the fact that both kinases are simultaneously expressed in all cell types and tissues analyzed, the ERK1 : ERK2 ratio is quite variable. One of the best examples that illustrates this is the quite heterogenous expression profile of ERK1 and ERK2 mRNA in brain [87]. These are essentially the more pronounced biochemical differences that have been reported until today and finally ERK1 and ERK2 appear as tightly close enzymes.

Interestingly, ERK is highly activated in ectoplacental cone and extraembryonic ectoderm, which both give rise to these extraembryonic tissues [88]. Even if the elevated ERK activity in these tissues has not been attributed to ERK1 or ERK2, it is likely to be mainly carried by ERK2 isoform, which would explain the phenotype of ERK2 knockout. It is indeed assumed that ERK2 is more expressed than ERK1 in nearly all tissues examined so far and, as a consequence invalidation of ERK2, is supposed to have a stronger impact on the global ERK activity compared to ERK1 knockout. Actually, the only one way to compare the expression levels of ERK1 and ERK2 in cells is the use of a phosphospecific antibody, which recognizes the phosphorylation of activation loop residues Thr202/Tyr204 and Thr185/Tyr187 of ERK1 and ERK2, respectively. This motif is recognized with the same affinity by the antibody. In a recent report, Lefloch et al. have established a clear correlation between the expression ratio of ERK1 and ERK2 and their activation ratio [89]. In this work, the authors have demonstrated that ERK1 and ERK2 are fully redundant kinases regarding the regulation of cell proliferation in NIH3T3 cells. A similar observation was done on embryonic fibroblast genetically deficient for ERK1 and/or ERK2 [90]. In this study, a strong correlation was drawn between the quantity of ERK proteins inside the cell and the intensity of proliferation.

In addition to these works based on the use of genetically deficient animals and riding the wave following RNAi discovery, a sustained number of studies have emerged in the literature in order to decipher the roles of ERK1 and ERK2. In skeletal myoblasts, proliferation requires one of the two isoforms, whatever it is, but terminal differentiation is strictly dependent on ERK2 [91]. According to Liu et al., if ERK1 and ERK2 silencing would both affect cell proliferation, each kinase would be involved at different phases of the cell cycle: ERK1 would regulate G2/M transition while ERK2 could be essential in G1 phase [92]. A recent study done by John Blenis' group has shown that the ERK2 isoform induces epithelial-to-mesenchymal transformation when overexpressed [93]. Interestingly, ERK1 is not able to reach the same effect despite high expression levels.

What about the roles of ERK1 and ERK2 in the physiology of normal hepatocytes? Interestingly, contrary to other cell types in which ERK2 is much more expressed compared to ERK1, normal hepatocytes harbor closely similar levels of ERK1 and ERK2, according to P-ERK1 : P-ERK2 ratio. We have shown that ERK1$^{-/-}$ hepatocytes proliferate with similar kinetics as wild-type hepatocytes after *in vivo* PHT and *in vitro* [94]. At the contrary, silencing ERK2 has a strong impact on cell proliferation [66, 94]. These results are in accordance with several papers listed above that place ERK2 as a positive regulator of cell proliferation. We went further in the analysis of putative roles of ERK1 and ERK2 in hepatic processes and established a link between ERK1 and the cell survival of hepatocytes. Indeed, silencing ERK1 using RNAi decreases susceptibility to apoptosis as it is observed in ERK1-deficient hepatocytes. This is in accordance with the report from Bourcier et al. which has shown that ERK1$^{-/-}$ keratinocytes are resistant to apoptosis induced by different agents or stress [95]. Interestingly inhibiting ERK2 did not reach this effect. But this is probably not so simple and associating one isoform to the regulation of one specific cellular function could be a dangerous shortcut. As an example, in ovarian cells, the silencing of ERK1 triggers the opposite phenotype to that observed in hepatocytes since cells become more sensitive to apoptosis [96]. How the inhibition of the same protein could have radically opposite effects? The localization of the isoforms inside the cell could be a response element. In that sense, MEK1 and MEK2 were proposed to regulate distinct cellular functions in hepatocytes by localizing ERK2 to different regions of the cell [97]. Following activation by MEK1, ERK2 translocates to the nucleus where it would trigger a proliferative response. At the opposite, when activated by MEK2, ERK2 retains a cytoplasmic localization to mediate survival. This could explain why one isoform would regulate distinct functions according to the cell type.

Finally and in order to reconcile all these data, one can easily imagine that ERK1 and ERK2 regulate overlapping fundamental functions with regard to the most fundamental processes such as proliferation in nonspecialized cells. Results obtained on MEFs cells and which explicit a dose-response effect of ERK on the intensity of cell proliferation argue in that sense [98]. Interestingly, all the major differences that were observed between ERK isoforms have been made on more differentiated cells, and notably hepatocytes. This could suggest a specialization of ERK1 and ERK2 in the regulation of unique biological functions in differentiated cells. ERK1 and ERK2 also share dozen of substrates so one could speculate that potential differences in substrate affinity could explain the predominant role of one isoform in a

function due to the presence of a particular set of partners, in other words in a particular cellular context.

6. Roles of the MEK1/2-ERK1/2 Pathway in Hepatocellular Carcinoma

The MEK/ERK signaling pathway plays a central role in the regulation of various physiological processes such as proliferation, survival or cell motility. Thus, its disregulation has often been associated with the promotion or development of tumor cell growth. Indeed, the chemical inhibition of MEK1/2 kinase activities blocks *in vitro* as well as *in vivo* proliferation of a variety of tumor models, including hepatocellular carcinoma (HCC) [99–103]. Moreover, active mutant forms of Raf or MEK have been shown to transform different cell types [90, 104–106]. The best illustration of MAPK pathway importance in oncogenesis lies in the observation of an ERK1/2 overactivation in 50 out of 138 human tumor cell lines [107]. Indeed, an increased expression and activation of the MEK1/2, and ERK1/2 kinases has been reported in human and mice primary liver tumors [108–111]. Active forms of the MEK/ERK pathway components including pRAF1, pMEK1/2 and pERK1/2 were also associated with poor prognosis in patients with HCC [112, 113]. However, in HCC, the overactivation of the MEK/ERK pathway did not result (or rarely) from an activating mutation of an upstream protein, namely, the GTPase Ras or the Raf protein kinase. Generally, the protein Ras is found mutated in about 30% of all human cancers with a high prevalence in the pancreas (90%) and colon (50%) adenocarcinomas [114]. The BRAF V600E mutation is on the other hand found in about 20% of tumors and especially in melanoma ($\sim$ 50%) [115]. In HCC, Ras and BRAF mutations are rare in humans or could be related to some etiologic factors or genetic backgrounds [116–119]. Overactivation of the MEK/ERK pathway is rather a consequence of a disinhibition, an upregulation of upstream activators, or an oncogenic stimulation.

For example, Calvisi et al. have shown from 80 surgical resections of HCC that 100% of tested malignant tissues had a constitutive activation of Ras and this was linked to the loss or reduction of the Ras inhibitory proteins NORE1A and RASSF1A [120]. Different inhibitors of the MEK/ERK pathway like RKIP, Sprouty-2, Spred-1, or Spred-2 are also frequently downregulated in human tissues of HCC. These decreases, or losses of activity, are supposed to have an important impact on HCC development and progression since ectopic expression of these different inhibitors is sufficient to inhibit the MAPK pathway but, most of all, to suppress tumor cell proliferation both *in vitro* and *in vivo* [121–124]. The downregulation of the previously cited inhibitors would provide an increased activation of the kinases ERK1/2. Moreover, in poor prognosis HCC, ERK1/2 activity should also be unrestrained given the weakened expression of the MAPK phosphatase DUSP-1 found in those patients [125].

In addition, the ERK1/2 kinases could also undergo a more intense stimulation through the overexpression of various components of the MEK/ERK pathway. For instance, the protein c-Raf could be an important source of ERK overactivation. Indeed, Hwang and colleagues have shown that the c-Raf kinase is upregulated in almost all cirrhosis and tumor tissues analyzed. A significantly higher level of expression of c-Raf was also reported in hepatocellular carcinomas when compared to cirrhosis [126]. Numerous tyrosine kinase receptors and their ligands also accuse an increased expression in HCC [127]. The signaling via these receptors will thus activate the MAPK pathway cascade and lead to a sustained activation of ERK1/2. For example, the EGF receptor is overexpressed in about 40% to 70% of HCC [128–130]. Importantly, the transforming growth factor-α which is one of the EGFR ligands presents in parallel an increased expression in cirrhosis and early HCC. Since the gene encoding for TGF-α is also a target of the Ras pathway, an autoamplification loop could be established ensuring, by the way, a persistent activation of the MEK/ERK pathway [131–133]. Hepatocellular carcinoma is also one of the most vascularized solid tumors links to a strong angiogenesis. Therefore, it is not surprising to find an upregulation of various proangiogenic factors such as VEGF, its receptor VEGFR, or the PDGF. All of these proteins will provide activator signals for the MEK/ERK pathway [134–136]. In HCC, a significant deregulation of the IGF signaling has also been reported, and notably a significant increase in IGF2 bioavailability. This was mediated by the upregulation of IGF2 via epigenetic mechanisms and by the downregulation of the IGF2R receptor, which normally lead to the lysosomal degradation of IGFs [79, 137, 138]. In addition, an overexpression of c-MET is observed in approximately 50% of HCC and this was associated with poor prognosis [139].

Finally, ERK1/2 overactivation in HCC could be due to Hepatitis-B virus (HBV) or C virus (HCV) infections, the two major etiologic factors of primary liver cancers. Indeed HCV carrier patients have higher rates of pERK1/2 than other HCC patients [113]. Thus, the core protein and the envelope protein E2 of the HCV and the HBX protein and preS2-activator large surface protein of HBV have been shown to directly activate the MEK/ERK pathway but by different mechanisms [140–144]. For instance, the HBX protein could upregulate the EGFR and interacts with the protein PIN1 to facilitate the dephosphorylation of c-Raf while activation by the preS2-activator large surface protein used PKC-dependent mechanisms [142, 143, 145, 146]. The envelope protein E2 of HCV stimulates the MEK/ERK pathway by binding to the CD81 receptor or to the low density lipoprotein receptor (LDLR). Anyway, MEK/ERK activation by viruses is postulated to promote hepatocarcinogenesis by facilitating the proliferation and survival of infected cells [147].

The MEK/ERK overactivation in hepatocarcinoma cells will promote various cellular processes. First, the proliferation of neoplastic cells would be obviously improved. Indeed, MEK1/2 inhibition, conducted by the use of different chemical inhibitors, could abolish the *in vitro* proliferation of numerous human and rat hepatocarcinoma cell lines. Growth of xenograft tumors in mice is also severely impaired

in a context of MEK/ERK inhibition [94, 99, 104, 111, 148–152]. Using RNAi, we have specified the molecular mechanism involved in tumor hepatocyte proliferation. We have shown that MEK1 deficiency suppressed both *in vitro* and *in vivo* proliferation of Huh7 cells. On the other side, MEK2 silencing did not affect the proliferation capacity of transformed cells [151]. Similar to normal hepatocytes, tumor growth is also supported by the kinase ERK2 but not by ERK1. Indeed, ERK2 targeting by stable chemically modified siRNA altered the *in vitro* proliferation as well as the *in vivo* growth of the highly tumorigenic F1 cells. We have also demonstrated that ERK2 was primordial for the *in vivo* proliferation of the Huh-7 cell line [99, 151]. Interestingly, it is noteworthy that hepatoma cells exhibit a higher expression of ERK2 than ERK1 while normal hepatocytes have a more balanced ERK1 : ERK2 ratio. This could reflect the difference of functions carried by these kinases in the particular context of liver. Indeed, we showed that ERK2 favored the proliferation of normal and transformed hepatocytes as opposed to ERK1 which could promote a death signal [66] (Guegan, personal data). Given the permanent and sustained MEK/ERK activation in HCC, one could speculate that the newly transformed hepatocyte should thus prime ERK2 functions while diminishing ERK1 prodeath activity.

Moreover and besides its role in cell proliferation, we have shown that ERK2 but not ERK1 was involved in hepatoma cell motility and invasiveness by an uPAR and P70S6 K dependent mechanism. RNAi-mediated inhibition of ERK2 or P70S6 K led to strongly reduced cell motility [153]. However, this is not the unique mechanism by which the MAPK pathway regulates HCC invasion. Indeed Honma et al. have shown that an active mutant form of MEK1 could suppress the E-cadherin mediated homotypic adhesion and thus potentiate cell migration [154]. ERK1/2 activity was also involved in the migration of three metastatic HCC cell lines but in a PKC-β dependent mechanism. Interestingly, ERK activation status was shown to increase following the metastatic potential of the cell lines analyzed [155]. Hence, the MEK/ERK overactivation found in tumor cell could support the HCC progression and metastasis.

Finally, this overactivation could also promote the survival of transformed hepatocytes. Indeed it has been shown that treatment of HepG2 or Hep3B cells by MEK1/2 inhibitors led to an apoptosis engagement [111]. Inhibition of MEK1/2 could also sensitize hepatoma cells to the death induced by ER-stress [156]. Moreover, active form of MEK1 prevented serum deprivation-induced death of hepatocarcinoma cells [157] and in HepG2, MEK/ERK activity has been reported to contribute to cisplatin induced death [158]. The MEK/ERK pathway has also been shown to protect transformed hepatocytes from TGF-β-induced apoptosis, a natural inducer of apoptosis in hepatocytes, produced in the liver by hepatic stellate cells [159]. The escape from TGF-β suppressive effects is an important step in hepatocarcinogenesis. Liver tumor bearing late TGF-β gene signature is indeed more aggressive than those expressing early gene signature [160]. Hence the MEK/ERK overactivation might play an important role in the initiation or development of HCC. The prosurvival effects of the MEK/ERK pathway in tumor cells have been shown to pass through the upregulation or stimulation of different antiapoptotic factors such as Bcl-2, Bim, or Bad (for review see [161]). For instance, it has been shown in hepatocarcinoma cells that ERK1/2 could phosphorylate the antiapoptotic factor Mcl-1 on thr163 in order to stabilize it and to thus enhance its prosurvival function [162].

The critical involvement of the MEK/ERK pathway in HCC tumorigenesis strongly suggests that the kinases MEK1/2 or ERK1/2 could be promising therapeutic targets. Sorafenib advent in therapy has also clearly demonstrated the potential of targeting signaling pathways in HCC. Given the predominant role of ERK2 in transformed hepatocyte proliferation, survival, and motility and given the prodeath role of ERK1, it could be preferential to specifically target ERK2 without affecting ERK1 activity. By this way, this might have different effects compared to a nonspecific chemical inhibition of both kinases, what could ultimately improve therapeutic benefits.

Abbreviations

ECM:	Extracellular matrix
ERK:	Extracellular signal-regulated kinase
JNK:	c-Jun N-terminal kinase
PHT:	Partial hepatectomy
HCC:	Hepatocellular carcinoma
MAPK:	Mitogen-activated protein kinase
MEK:	MAPK/ERK kinase
DEVD-AMC:	Asp-Glu-Val-Asp-7-amino-4-methylcoumarin
FCS:	Fetal calf serum
CYP:	Cytochrome P450
PB:	Phenobarbital
3MC:	3-methylcholanthrene
EGF:	Edidermal growth factor
GH:	Growth Hormone.

Authors' Contribution

J.-P. Guégan and C. Frémin contributed equally to this work.

Acknowledgments

This research was supported by the Institut National de la Santé et de la Recherche Médicale (Inserm), the Université de Rennes 1, Rennes Métropole, and by the Ligue contre le cancer (Ligue Grand-Ouest). J.-P. Guégan and C. Frémin were recipients of fellowships from the Ministère de l'Education Nationale, de la Recherche et de la Technologie and from the ARC (Association pour la recherche contre le cancer).

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Hindawi Publishing Corporation
International Journal of Hepatology
Volume 2012, Article ID 282430, 8 pages
doi:10.1155/2012/282430

Review Article

Hepatocytes Polyploidization and Cell Cycle Control in Liver Physiopathology

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Received 21 May 2012; Accepted 10 September 2012

Academic Editor: Pascal Loyer

Most cells in mammalian tissues usually contain a diploid complement of chromosomes. However, numerous studies have demonstrated a major role of "diploid-polyploid conversion" during physiopathological processes in several tissues. In the liver parenchyma, progressive polyploidization of hepatocytes takes place during postnatal growth. Indeed, at the suckling-weaning transition, cytokinesis failure events induce the genesis of binucleated tetraploid liver cells. Insulin signalling, through regulation of the PI3K/Akt signalling pathway, is essential in the establishment of liver tetraploidization by controlling cytoskeletal organisation and consequently mitosis progression. Liver cell polyploidy is generally considered to indicate terminal differentiation and senescence, and both lead to a progressive loss of cell pluripotency associated to a markedly decreased replication capacity. Although adult liver is a quiescent organ, it retains a capacity to proliferate and to modulate its ploidy in response to various stimuli or aggression (partial hepatectomy, metabolic overload (i.e., high copper and iron hepatic levels), oxidative stress, toxic insult, and chronic hepatitis etc.). Here we review the mechanisms and functional consequences of hepatocytes polyploidization during normal and pathological liver growth.

1. Introduction

Polyploidy, the state of having an increase in the number of chromosomes sets, is a widespread physiological phenomenon observed particularly in plants, fungi, insects, fishes, and amphibians [1]. The additional set (or sets) of chromosomes may originate from the same individual ("autopolyploid") or from the hybridization of two different species ("allopolyploid"). Although diploid is the normal status for mammalian cells, various studies have demonstrated during last decades a major role of "diploid-polyploid conversion" during physiopathological processes in several tissues. Indeed, polyploidy seems to be part of a developmental program resulting in the formation of highly differentiated cells, as it has been reported for megacaryocytes (16n–128n) [2], cardiomyocytes (4n) [3], trophoblast giant cells (8n–64n) [4], Purkinje neurons, [5] and retinal ganglion cells (both 4n) [6] or hepatocytes (4n–8n) in the liver parenchyma [7]. Furthermore, in response to stress or injury, genesis of polyploid contingent can be also observed. Uterine smooth muscle during pregnancy [8], heart muscle and vascular smooth muscle cells (VSMC) during hypertension [9, 10], and thyroid cells in hyperthyroidism [11] are prone to switch to polyploid state. Finally, genesis of polyploid cells by unscheduled whole-genome duplications can also participate to carcinogenesis process, by inducing establishment of chromosomal instability (CIN). Indeed, in many human carcinomas (breast, lung, colon, pancreas, oesophagus) emergence of tetraploid cells has been observed in early steps of tumorigenesis and precede the genesis of cells with intermediate DNA content values (aneuploid cells) [12, 13].

Several mechanisms have been involved in the physiopathological emergence of polyploid cells in mammals.

During *cell-cell fusion*, genesis of polyploid cells may occur independently of cell proliferation, as it has been observed during physiological development in osteoclasts [14] and skeletal muscle cells [15] or after pathological viral infection [16]. In this process, cells fuse their nuclei and/or membranes, leading to the genesis of mononuclear or multinucleate cells, respectively.

Other mechanisms are directly associated with proliferative state of the cells.

(1) Endoreduplication. During this process, cells alternate S (DNA replication) and G phases, without performing mitosis and give rise to the genesis of autopolyploid cells (i.e., trophoblastic giant cells).

(2) Endomitosis. Cells can reach metaphase or anaphase A, but nuclear (karyokinesis) and cytoplasmic (cytokinesis) divisions are never observed; the best-studied example being polyploid megakaryocytes [17]. These cells enter mitosis but never fully separate sister chromatids or undergo cytokinesis, resulting in globulated polyploid nuclei [18, 19]. The regulatory mechanisms that control megakaryocytes polyploidization have been explored by different groups with a major focus on the regulation of mitotic phase and cytokinesis. Endomitosis appears to be due to a complex regulation of Cdk1/Cyclin B levels [20]. Studies of different megakaryoblastic cell lines suggest that endomitosis is promoted by the downregulation of Cyclin B/Cdk1 mitotic kinase activity [21, 22]; differently, in primary polyploid megakaryocytes, levels of cyclin B are reported to be upregulated [18, 23, 24]. Moreover, other studies have reported a reduction in the duration of the G1 phase correlated with overexpression of cyclin E [21, 25, 26]. Recent data have shown that cyclin E mediates its effect by promoting the expression of components of the prereplication complex (Cdc6 and MCM2). Overexpression of cyclin E can favor progression to S phase and cell cycling, thus promoting endomitosis and polyploidization of megakaryocytes [24].

(3) Mitotic Slippage. During this pathological process, cells present an altered Spindle-Assembly-Checkpoint (SAC). The SAC prolongs mitosis until all kinetochores are stably attached to spindle microtubules; when the SAC cannot be satisfied, cells exit mitosis without undergoing anaphase or cytokinesis (genesis of mononucleated tetraploid cells). Mitotic slippage has been observed for example in cells after prolonged mitotic arrest in response to spindle toxins [27] or in APC-deficient cells (adenomatous polyposis coli, gene frequently mutated in colon cancers) [28].

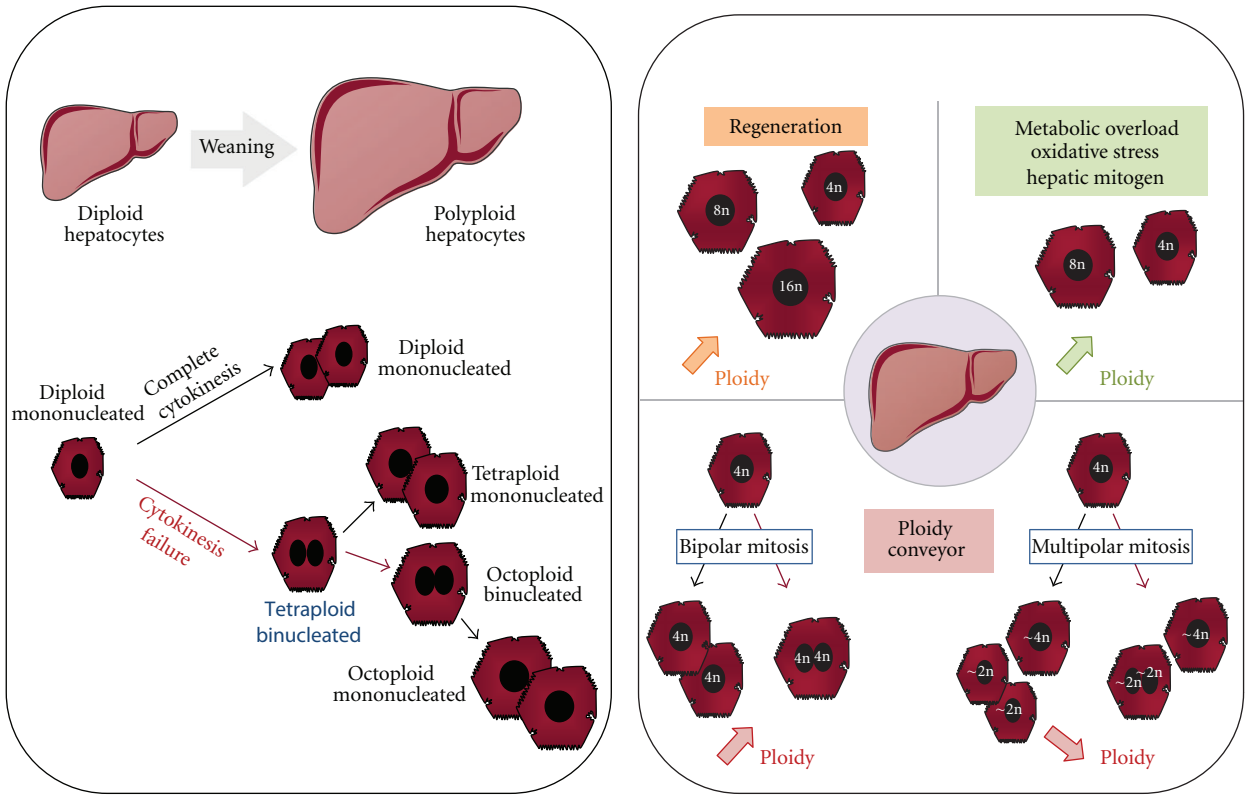
(4) Incomplete Cytokinesis. This process has been extensively described during pathological division and leads to the genesis of binucleated tetraploid cells. These cells can appear following dysfunction of any of a large number of different proteins controlling cytokinesis process [29]. In addition, bulk chromatin or even a single lagging chromosome trapped in the cleavage furrow can induce cytokinesis failure and tetraploidization [30, 31]. Remarkably, recent studies demonstrated that cytokinesis failure process is also a programmed step in normal development (as example: liver and heart tissues; see Section 3.1 for more details) producing differentiated binucleated tetraploid progenies [32–35]. Finally, it is important to note that whatever the mechanism of polyploidization, the increase in cellular DNA content will be associated with centrosomes amplification, which in certain cases could lead to the genesis of aneuploid progenies and CIN (see [13, 36] for reviews).

2. Hepatocytes Polyploidy and Liver Growth

2.1. Postnatal Development. Hepatic polyploidy is a characteristic feature of mammalian liver and accompanies late fetal development and postnatal maturation [7, 37]. In rodents, through 14th embryonic development day (e.g., E14), most hepatoblasts are bipotent with the ability to differentiate into hepatocytes or into biliary cells; by E15 most hepatoblasts are committed to the hepatocyte lineage [38, 39]. During the remaining period of gestation and the first four postnatal weeks, hepatoblasts acquire functions of differentiated hepatocytes, and this period is correlated with a severe decline in proliferative state [40, 41]. During previous studies, we have observed that the liver is almost exclusively made up of diploid hepatocytes for the first three weeks after birth. After weaning (day 21), the proportion of diploid hepatocytes started to fall significantly, with the successive appearance of binucleated tetraploid ($2 \times 2n$) and mononucleated tetraploid (4n) hepatocytes [32, 33] (see Figure 1). The hepatocyte ploidy level effectively reaches a plateau at maturity, octoploid (binucleated $2 \times 4n$ and mononucleated 8n) hepatocytes appearing in significant numbers during the second and third months after birth [42]. Interestingly, a second wave of high ploidization has been also observed at senescence in different species [43]. For example, in humans, polyploid hepatocytes begin to appear during postnatal liver development; their accumulation rate stays stable during the maturity period, and finally a significant increase of polyploid cells is observed during ageing process [44]. In adults, 70% of all hepatocytes in rodents and 40% in humans are tetraploid [42, 45]. It has to be noticed that a negative correlation exists between mitotic index in the liver and the level of hepatocyte polyploidization found in different species [46, 47]. As example, mouse liver has a much lower mitotic index than rat liver and accordingly the highest level of hepatocytes polyploidization was found in the mouse liver.

2.2. Adult Liver. Interestingly, in the adult liver, the genesis of polyploid cells can be reinduced following a variety of signals (see Figure 1).

Indeed, after two-thirds hepatectomy, mitogenic signals (cytokines and growth factors; for review, see [48]) induce exit of quiescence ("priming") of hepatocytes. These hepatocytes undergo one or two rounds of one or two rounds of cell division to restore the hepatic liver mass and this process is associated with a pronounced increase of polyploid hepatocytes [37, 46, 49, 50]. Several reports indicated that



(a) Postnatal development (b) Adult

Figure 1: Hepatocytes polyploidization during development and in challenging circumstances: (A) polyploidization during postnatal liver growth. Hepatocytes in newborn are exclusively diploid (mononucleated 2n). At the weaning period, diploid hepatocytes can engage either into normal cell division cycle (black arrow) giving rise to two diploid hepatocytes or follow an adaptive cell cycle with cytokinesis failure (red arrow) giving rise to one binucleated tetraploid hepatocyte. By this process, progressive polyploidization takes place in the liver parenchyma and tetraploid and octoploid cell classes with one or two nuclei are formed. (B) Ploidy modification during physiopathological processes in adult liver. In adult, liver modulates its ploidy in response to different signals. Liver regeneration induced by partial hepatectomy leads to the disappearance of binucleated hepatocytes and the formation of mononucleated tetraploid and octoploid hepatocytes or even 16n contingent. DNA synthesis induced by chemicals or following oxidative damage and metabolic overload (copper/iron) is associated with a pronounced increase in the proportion of polyploid hepatocytes. Furthermore, in response to different unknown signals, hepatocytes can both increase (bipolar mitosis followed by cytokinesis failure) or decrease their ploidy (multipolar mitosis). In that case, near-diploid/near-polyploid contingents will be generated, leading to the genesis of genetically distinct daughter cells; black arrow: complete cytokinesis, red arrow: cytokinesis failure.

liver regeneration depends mainly on the proliferation of hepatocytes [48, 51]. However, it has to be noticed that hypertrophy of hepatocytes in the regenerated liver has also been described [52–54]. A recent study has conciliated with these two pathways by revealing that hypertrophy precedes proliferation in the regenerating liver [55]. Furthermore, this work also established that preexisting diploid and tetraploid mononucleated hepatocytes generate cells with tetraploid and octoploid nuclei, respectively, by an unconventional cell cycle, probably by endoreplication as hepatocytes entering into S phase and skipping mitosis. Finally, they also described that binuclear hepatocytes undergo reductive divisions to generate two mononuclear daughter hepatocytes of higher ploidy. All these processes lead to an increase in both size and ploidy of hepatocytes during liver regeneration. It is interesting to note, that Sigal and Coll have also observed that 16n populations are found in the hepatic tissue, during the second day of liver regeneration. At the end of the regenerative process, 16n contingent is no more present in liver parenchyma. The authors suggested that the disappearance of these highly polyploid hepatocytes is associated with the establishment of apoptotic mechanisms that target preferentially hepatocytes of advanced ploidy [37].

In adult rodents, DNA synthesis induced by chemical compounds is associated with modifications of polyploid status in the liver. For example, lead nitrate induces the genesis of binucleated hepatocytes [56]. Adjunction of hepatic mitogens such as sodium phenobarbitone [57], 1, 4-dichlorobenzene [58], or peroxisome proliferators [59] are known to favor octoploid mononucleated hepatocytes genesis.

Hepatic polyploidy can be also modified by pathological overload that induce liver lesions. Different studies have

described that liver of Long-Evans Cinnamon (LEC) rat (animal model of human Wilson's disease), which exhibits abnormal hepatic copper and iron concentration due to the deletion of the p-type copper transport ATPase gene (Atp7b), possesses a feature of increase in polyploidy (enlarged hepatocytes with huge nuclei) and a delay in mitotic progression. Interestingly, injection of irondextran in normal mice induces liver polyploidization; this effect is inhibited by the oral intake of iron chelator [60, 61].

Hepatocytes submitted to oxidative stressors develop pronounced increase in their polyploid status. Gorla et al. have demonstrated that subsequent to radiation, hepatocytes exhibit evidence for oxidative injury with deletion of intracellular antioxidants (as glutathione and catalase) and for increase of polyploidy [62]. Furthermore, a study on rats indicates that the rate of reactive oxygen species generation exceeds the induced antioxidant ability with aging, generating a situation that favors oxidative stress and peroxidation. This state is correlated with changes in the proliferative potential of hepatocytes and an increase in the genesis of octoploid contingent [63]. Further evidence for the role of oxidative injury in polyploidy is provided by studies showing that in transgenic mice overexpressing copper-zinc-superoxide dismutase and glutathione peroxidase, which are antioxidants, PH-induced hepatic polyploidization is decreased [64]. Similarly, treatment with aminoguanidine, which attenuates oxidative stress, decreased polyploidy [65]. It is interesting to note that in others polyploid cell types, such as VSMC, a crucial role of oxidative stress in polyploidization process has been underlined. Indeed, McCrann and Coll have described that increased expression of an ROS-producing enzyme, Nox4 (member of the NADPH oxidase family) results in VSMC polyploidy [66]. A role of Nox proteins in megakaryocytes endomitosis has been also suggested. Treatment of mouse bone-marrow cultures with Nox inhibitors resulted in accumulation of MKs with low DNA content levels and significant reduction of higher ploidy MKs. Further examination indicated that Nox-inhibited MKs showed a notable decrease in the level of the G1 phase cyclin E, a cyclin associated with MK polyploidy, and its upregulation restored most of the effect of Nox inhibitors [67].

All together, these results underline an extensive correlation between the generation of polyploid hepatocytes and a variety of cellular stress in the adult liver; however, cellular and molecular mechanisms involved in ploidization modification during pathological state are not well characterized.

3. Signalling and Mechanism Controlling Physiological Hepatocytes Polyploidy

3.1. Cellular Mechanism. Our team has focused on the understanding of polyploidy hepatocytes lineage. We previously unveiled that during postnatal development and more precisely after weaning, diploid hepatocytes (mononucleated 2N) can engage either into a normal cell cycle and give rise to two diploid hepatocytes or follow an adaptive cell cycle with incomplete cytokinesis and give rise to one tetraploid hepatocyte (binucleated $2 \times 2n$) [32, 33]. In these hepatocytes, karyogenesis is achieved but these cells are not able to establish the cleavage plane. Several studies have revealed that RhoA GTPase is a key player to ensure a successful cytokinesis, by regulating the organization of the actin cytoskeleton and myosin II activity at the cleavage plane [68, 69]. We revealed that in hepatocytes, deficiencies in cytoskeleton reorganization inhibit Rho-A GTPase recruitment to the cleavage plane; consequently the cytokinesis ring is never formed [32]. The genesis of such binucleated tetraploid cells is the crucial step for the establishment of gradual polyploidization during postnatal liver growth. Indeed these cells are capable to proliferate and to give rise to two mononucleated 4n cells, which, if they divide, can generate $2 \times 4n$ binucleated or 8n mononucleated hepatocytes.

In the heart, incomplete cytokinesis has been also implicated in the genesis of binucleated tetraploid cardiomyocytes. In mammals, the growth of embryonic heart results in proliferation of cardiomyocytes (hyperplasia) [70]. After birth, ventricular cardiomyocytes respond to an amplification of blood flow by an adaptive increase in volume (hypertrophy). This transition from hyperplasia to hypertrophy is correlated to a tetraploidization process [35, 71]. In this system, a drastic reduction of RhoA and its effector ROCK after birth could account for defects in the process of cytokinesis [72]. Indeed, in some diploid cardiomyocytes, a cytokinetic ring is formed but as it is not at all functional, cytokinesis is never achieved, and tetraploid binucleated cell is consequently generated [35]. Differently from hepatocytes, adult 4N cardiomyocytes are in a postmitotic state and display a low proliferative potential (for review, see [73]). Recently, Gao et al. have shown that RhoA regulation is also a key target in MKs polyploidization and differentiation. Indeed, they have demonstrated that downregulation of the guanine exchange factor ECT2 prevents RhoA activation and cleavage furrow ingression during endomitosis cycle, allowing the formation of $\geq$4N MKs [74].

Interestingly, even if failed cytokinesis is the major event for liver polyploidization during postnatal development, some studies reveal that under certain circumstances, cell fusion can also contribute to this process. Experiments on stem cells and therapeutic applications have discovered that polyploid hepatocytes can be generated following cell fusion between exogenous bone marrow cells and mature hepatocytes [75, 76]. Furthermore, Faggioli and Coll have shown that in adult liver, genesis of binucleated hepatocyte could be directly promoted by homotypic fusion but with a rare occurrence [77].

3.2. Molecular Mechanism. Recently, we discovered that the suckling-to-weaning transition strictly controls the establishment of the cytokinesis failure process in the liver. Using a specific immunocytochemistry approach to detect mitotic events in liver tissue, we showed that cytokinesis failure events never occurred in 19-day-old suckling rat (<3%); whereas in 19-day-old rats weaned early (at 15 days), such events were frequent (>35%), and numerous binucleated tetraploid hepatocytes were generated. It is interesting to note that while suckling is prolonged to 25 days, hepatocytes

mostly enter into complete cytokinesis events (>95%—data not published). Moreover, we reported there was a new wave of proliferation in the liver associated with the establishment of these specific adaptive cell cycles [41, 78]. We pointed out that insulin signalling triggers incomplete cytokinesis cell cycle program. If the physiological rise in insulin after weaning was inhibited in rats (by destroying pancreatic beta cells with streptozotocin drug), hepatocytes did not undergo cytokinesis failure, whereas if this rise was further accentuated (by injecting insulin), cytokinesis failure was even more frequent and an increase in the genesis of binucleated tetraploid hepatocytes was observed. By investigating how insulin controls polyploidy program, we discovered that PI3 K/Akt pathway (signaling pathway regulating cellular homeostasis through its role in regulation of apoptosis, cell growth, cell cycle, cytoskeleton organization and angiogenesis; see [79] for, review) is a key regulator of cytokinesis through the control of cytoskeleton networks. Indeed, direct inhibition ok Akt by chemical compound (iAKT) in hepatocytes primary culture prevents the appearance of incomplete cytokinesis process. We also examined the cytoskeleton organization in treated cells; iAkt-treated cells that completed cytokinesis reorganized the actin cytoskeleton and recruited RhoA to the equatorial cortex by contrast to cells that did not complete cytokinesis. In the past, several studies in yeast, metazoans, and mammals underlined a role of insulin in the regulation of cell proliferation and growth, by controlling G1/S-and G2/M-specific checkpoints [80–82]. However, we have demonstrated for the first time in mammals that this hormone, through the PI3 K-Akt pathway, can also regulate late mitosis progression and tightly control physiological polyploidization process during liver development.

Interestingly, a role of PI3 K-Akt pathway has been also described during a pathological polyploidization process. By overexpressing Akt1, Hixon et al. have demonstrated that VSMCs are able to override the activity of the mitotic spindle checkpoint, facilitating unscheduled degradation of cyclin B, cell-cycle reentry (endoreduplication), and polyploidization process. The same results were obtained by incubating VSMCs isolated from normotensive animals with angiotensin II (regulator of hypertrophic signals during hypertension), which is a key activator of Akt1 in VSMCs. These results demonstrate that Akt1 regulates ploidy levels in VSMCs and contributes to vascular smooth muscle polyploidization and hypertrophy during hypertension [83].

4. Functions of Polyploid Hepatocytes

Many examples from the literature illustrate that the acquisition of a polyploid status confers specific biological properties of cells. In the yeast *Saccharomyces cerevisiae*, polyploidization alters the expression profile of specific genes and regulates certain aspects of physiology and cell morphology [84]. In plants, high polyploidy is correlated with epigenetic changes associated with hybrid vigor (stronger and taller plants) [85]. In mammals, polyploidization of megakaryocytes is associated with terminal differentiation and regulation of platelets formation and function [86]. Indeed, polyploidization process increases the overall MK mass, resulting in an increase in platelet formation. Furthermore, a study suggested that MKs from different ploidy levels produce platelets with different functions: platelets originating from high-ploidy MKs are thought to be more easily activated than platelets generated from MKs with a lower ploidy [87].

In the liver our understanding of the consequences for hepatocytes polyploidization still remains enigmatic. (1) Polyploidy could protect hepatocytes of genotoxic damage by increasing the number of copies of functional genes; this might be especially important for the liver that has a primary function in metabolizing and eliminating toxic compounds. (2) Polyploidy could be an economical solution to growth problems that occur when an organ work within its capabilities, avoiding the great demand in energy that represents cell division. (3) Finally, polyploidy could alter the expression profile of specific genes. Recently, two studies using multitest approach of modular biology underline alteration in a wide range of functional gene groups between diploid and polyploidy hepatocytes. The authors suggest a link between genome multiplication and emergence of specific pathways (increase in metabolic plasticity and for the protection of replicating DNA from oxidative damage) that could promote hepatocyte cell survival and tissue regeneration under stressful conditions [88, 89].

5. Perspectives

Cellular polyploidization is now well known to be correlated to chromosomal instability appearance and carcinogenesis process development. Indeed, in some tumor types, there is direct evidence for the development of aneuploidy from a transient 4n state [13]. However, the impact of polyploid hepatocytes status on hepatocarcinoma (HCC) is still in debate. Recently, Grompe and Coll have shown that hepatocytes can increase (failed cytokinesis) and reduce (multipolar mitosis) their ploidy, thus resulting in the concept of a "ploidy conveyor." In their works, authors showed that this dynamic mechanism can induce the genesis of "near-diploid/polyploid", that is, aneuploid hepatocytes in rodents and humans livers [90, 91]. Given the high tumoral potential of aneuploid cells in tissue, these data are quite surprising as spontaneous tumor in the liver is rarely observed. The genesis of such "near polyploid" cells could then finally represent a source of genetic diversity, providing a strong selective advantage in response to multiple environmental stressors, as it has been demonstrated in yeast. Further investigations on this topic promise to increase our understanding of the mechanisms and functional consequences of hepatocytes polyploidization and could offer insights into hepatic physiopathology.

Abbreviations

APC: Adenomatous polyposis coli
CIN: Chromosomal instability
HCC: Hepatocarcinoma
iAKT: Inhibitor of AKT

LEC: Long-Evans Cinnamon
MK: Megakaryocyte
NOX: NADPH oxidase
PH: Posthepatectomy
PI3K: PI-3 kinase
SAC: Spindle assembly checkpoint
VSMC: Vascular smooth muscle cells.

Acknowledgments

G. Gentric is a recipient of CORDDIM Région Ile de France "CardioVasculaire, Obésité, Rein, Diabète". This study was supported by Grants from Institut National de la Santé et de la Recherche Médicale (INSERM), ANR-FORM-090601-01-01 and INCa-CHC (2009-1-CHC-03-INSERM), and Fondation ARC (SFI 20111203568).

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Hindawi Publishing Corporation
International Journal of Hepatology
Volume 2012, Article ID 785786, 14 pages
doi:10.1155/2012/785786

Research Article

Combined Stimulation with the Tumor Necrosis Factor α and the Epidermal Growth Factor Promotes the Proliferation of Hepatocytes in Rat Liver Cultured Slices

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Received 1 June 2012; Revised 6 September 2012; Accepted 7 September 2012

Academic Editor: Anne Corlu

The culture liver slices are mainly used to investigate drug metabolism and xenobiotic-mediated liver injuries while apoptosis and proliferation remain unexplored in this culture model. Here, we show a transient increase in LDH release and caspase activities indicating an ischemic injury during the slicing procedure. Then, caspase activities decrease and remain low in cultured slices demonstrating a low level of apoptosis. The slicing procedure is also associated with the G0/G1 transition of hepatocytes demonstrated by the activation of stress and proliferation signalling pathways including the ERK1/2 and JNK1/2/3 MAPKinases and the transient upregulation of c-fos. The cells further progress up to mid-G1 phase as indicated by the sequential induction of c-myc and p53 mRNA levels after the slicing procedure and at 24 h of culture, respectively. The stimulation by epidermal growth factor induces the ERK1/2 phosphorylation but fails to activate expression of late G1 and S phase markers such as cyclin D1 and Cdk1 indicating that hepatocytes are arrested in mid-G1 phase of the cell cycle. However, we found that combined stimulation by the proinflammatory cytokine tumor necrosis factor α and the epidermal growth factor promotes the commitment to DNA replication as observed *in vivo* during the liver regeneration.

1. Introduction

Isolation of hepatocytes from normal liver and establishment of *in vitro* culture systems have provided powerful experimental *in vitro* models to identify extracellular signals and to study intracellular signalling pathways regulating differentiation and controlling the ratio between proliferation and apoptosis in liver [1]. Enzymatic liver dissociation triggers G0/G1 transition of *in vivo*-quiescent hepatocytes, which progress up to and arrest in mid-G1 phase in absence of growth factors in primary culture [2]. In primary culture, expression of liver specific functions progressively decreases and apoptosis eventually occurs within a week through the activation of caspases 3, 8, and 9 in hepatocytes [3, 4]. Nevertheless, this *in vitro* culture model has been very useful to identify apoptotic inducers, survival factors and mitogens based on their ability to increase or reduce apoptosis and induce DNA replication, respectively. For instance, supplementation of culture medium with soluble factors such as insulin and glucocorticoids improves cell stability while epidermal growth factor induces cell proliferation [1].

More complex culture systems were further developed in which hepatocytes survive and remain differentiated for several weeks: (1) combination of additives to culture medium, including hormones, nicotinamide, phenobarbital, or dimethylsulfoxide (Me_2SO) [5], (2) cocultures associating hepatocytes and nonparenchymal liver epithelial cells [6], (3) extracellular matrices such as Vitrogen [7], collagen type I, and Matrigel [8]. In Me_2SO-treated cultures [9], coculture [10] and monolayers or sandwich of collagen I [11, 12], hepatocytes are arrested in G1 phase of the cell cycle and do not replicate DNA upon stimulation by growth factors

while initiator caspases 8 and/or 9 are processed into cleaved mature forms but remain inactive, preventing maturation of terminal caspases, execution of apoptosis, and allowing longer survival of hepatocytes [4, 12].

An alternative model to the culture of isolated hepatocytes is the use of precision-cut liver slices [13]. A 250 μm thick liver slice contains about ten intact cells layers maintaining normal tissue architecture with all liver cell types represented. This *in vitro* model is particularly suitable to evaluate selective intralobular hepatic toxicity of endogenous compounds [14, 15] and drugs [16–18], assess functional interaction between hepatocytes and nonparenchymal hepatic cells [19–21], study drug-induced hematologic disorders in whole blood cells cocultured with liver slices [22], and to study the mechanisms of HCV life cycle and new antiviral compounds [23]. Interest in liver slices as a drug evaluation system was reinforced by the demonstration that phase I and II enzymes were inducible by drugs [24, 25] and the establishment of cryopreservation methods [26–28]. As observed for isolated hepatocytes in primary culture, cultured slices progressively undergo a loss of cellular integrity evidenced by release of cytosolic enzymes [29], reduction in ATP content and decrease in expression of some of the liver specific functions [30, 31]. Optimization of slice preparation procedures [32, 33], culture conditions [34, 35], and culture devices [36, 37] with improved air-fluid interface for better cell oxygenation [38, 39] has allowed to significantly increase viability of adult liver slices for up to 10 days.

To the best of our knowledge, little is known about proliferation and apoptosis in cultured liver slices. It has been reported that a limited number of cells, mostly hepatic stellate cells [39], replicate DNA within the cultured slices [40]. However, it is still unknown whether hepatocytes remain quiescent or enter the cell cycle and can actively proliferate in cultured slices. Moreover, the expression and activity of caspases in liver slices during slicing procedure and in culture have not been studied yet.

In this report, we study the cell cycle entry and induction of apoptosis in hepatocytes in precision-cut slices. We demonstrate that liver slicing procedure induces proliferation signalling pathways, which trigger entry into and progression through G1 phase of the cell cycle similar to that observed in isolated hepatocytes after liver dissociation. In addition, hepatocytes in cultured slices undergo apoptosis at very low rates even after treatment with apoptotic factors TGFβ and TNFα, and do not proliferate upon EGF stimulation suggesting that cell-cell and/or cell-ECM interactions protect from apoptosis and inhibit G1/S transition. However, costimulation by TNFα and EGF overrides this G1 phase arrest demonstrating that proliferation of hepatocytes can be induced in cultured rat liver slices by proinflammatory cytokines and growth factors as observed *in vivo* during liver regeneration.

2. Material and Methods

Chemicals and Reagents. Bovine serum albumin (BSA) fraction V (Boehringer, Mannheim biochemicals), recombinant human (rHu) epidermal growth factor (EGF, Promega), rHu TNFalpha (Promocell, Heidelberg, Gremany), transforming growth factor 1 (R and D Systems, Abingdon); bovine insulin, dimethyl sulfoxide (Me_2SO), PIPES, CHAPS, orthovanadate, benzamide, aprotinin, leupeptin, and soybean trypsin inhibitor were purchased from Sigma Chemical Company (USA). Rediprime II DNA labelling kit, DNA herring sperm, [α-^{32}P]dCTP (3000 Ci/mmol), and [H^3]-methyl-thymidine (25 Ci/mmol) were purchased from Amersham Life Sciences. Dulbecco's modified Eagles medium (DMEM) with 4.5 mg/mL glucose and L-glutamine came from B.I. BioWithacker fetal calf serum was from Gibco BRL. The detection of cyclin D1, p53, Cdk4, c-fos, and c-myc mRNAs by Northern blotting was performed as previously reported [2]. *Antibodies:* Anticaspase-3 (H-277, Santa-Cruz Biotechnology), anticaspase-8 (APP-108) and anticaspase-9 (APP-109) were from StressGen Biotechnologies Corp. (Tebu, France); antialbumin and -transferrin (Kent Laboratories, Redmond, WA, USA); CYP3A1/2 and CYP2B (Daiichi Pure Chemicals Co., Ltd., Tokyo, Japan); CYP2E1 (Oxford Biomedical, USA); GSTA1 and GSTP1 were from Biotrin (Dublin, Ireland); anti-Cdk1 and -GSTA4 antibodies were previously described [2, 41]; anti-cyclin D1 (Ab-3, Neomarkers); anti-phospho-JNK (sc6254) and total JNK (sc571) and HSC70 (sc7298) were from Santa-Cruz Biotechnology. Rabbit polyclonal anti-Phospho-Histone H3 (ser10), anti-STAT3 (#9132) and anti-phospho-STAT3 (Tyr705, #9131) were from Cell Signalling. The secondary antibodies conjugated to horseradish peroxidase were purchased from DAKO (France). Fluororimetric substrates Ac-DEVD-AMC, Ac-IETD-AMC, and Ac-LEHD-AMC were from BACHEM (BACHEM, Voisins-Le-Bretonneux). Supersignal came from Pierce Chemical Co. (Rockford, IL, USA).

Animals. Male Sprague-Dawley rats (13 weeks old) were obtained from IFFA CREDO (L'Arbresle, France). They were kept under controlled environmental conditions (12 hr light-dark cycle) and fed a standard diet (Animalabo A 04, water *ad libidum*). Procedures for housing the rats, isolation, and culture rat hepatocytes were in agreement with the French regulation.

Preparation and Culture of Liver Slices. The liver was rinsed *in situ* (20 mL/min) with cold oxygenated (95% O_2 and 5% CO_2, 0.4 L/min) Krebs-Henseleit Bicarbonate buffer, pH 7.4, for 4 to 6 min until the appearance of a homogenous brown color. The liver was then perfused with Viaspan (Belzer's University of Wisconsin solution, Dupont Pharma). Liver slices (250 μm) were prepared according the method of Smith et al. [42], then, preincubated for 90 min in Waymouth medium (supplemented with 10% FCS, 100 IU/mL penicillin, 50 mg/mL streptomycin and 1.7 mM insulin) at 37°C in 95% O_2 and 5% CO_2 atmosphere (0.2 L/mL) in a dynamic culture system (Vitron Incubator). After the preincubation, culture medium was replaced by serum free medium.

Isolation and Primary Culture of Hepatocytes. Primary rat hepatocytes were isolated and purified from male Sprague-Dawley rats as described previously described [6]. Hepatocytes were seeded at $7 \cdot 10^4$ cells/cm^2 on plastic dishes in

a mixture of 75% minimum essential medium and 25% medium 199, supplemented with 10% fetal calf serum (FCS), and per mL: 100 IU of penicillin, 100 μg of streptomycin, 1 mg of bovine serum albumin (BSA), 2 μmol L-glutamine, and 5 μg of bovine insulin. Four hours after plating, the medium was removed and cultures were maintained in the same FCS free medium supplemented with $1.4 \cdot 10^{-6}$ M hydrocortisone hemisuccinate (Roussel-Uclaf).

Treatments with Apoptotic Inducers and Growth Factors. In primary culture of isolated hepatocytes (6-well plates, $5 \cdot 10^5$ cells per well), TGFβ1 (2.5 ng/mL), cycloheximide (5 μg/mL), TNFα (20 ng/mL), or both cycloheximide and TNFα began 24 hours after plating and were carried out for 24 (TNFα and/or cycloheximide) or 48 (TGFβ and TNFα) hours. In cultured slices ($\sim 1.5 \cdot 10^6$ cells/slice), concentrations of compounds were adjusted to obtain similar amounts per cell number. Treatments began at 4 hours and were carried out for 24 hours with TNFα (55 ng/mL) and/or cycloheximide (25 μg/mL) or 48 hours with TGFβ (15 ng/mL) and TNFα. For inducing proliferation, EGF (50 ng/mL for isolated hepatocytes and 250 ng/mL for cultured slices) was added during all the culture time.

Biochemical Endpoints. In the incubation medium collected at 3, 24, 48 and 96 hours, LDH contents were measured using Boehringer Mannheim MPR2 kit according manufacturer's instructions. LDH release was calculated with the ratio: extracellular LDH/total LDH (intra + extracellular). Intracellular ATP was measured by the luciferin/luciferase reaction using HSII kit (Roche) and a luminometer Microlumat-Plus EGG-Berthold.

RNA Extraction and Northern Blot Analysis. Liver slices were harvested and stored at -80°C. Total RNA was extracted using SVRNA extraction Kit from Qiagen (Courtaboeuf, France) and quantified by ultraviolet absorption at 260 nm. Integrity of the RNA samples was confirmed by formaldehyde agarose gel electrophoresis and visualisation by ethidium bromide staining of 18S and 28S ribosomal RNAs. The RNA samples (20 μg) were resolved by electrophoresis in a 1% agarose gel containing 1.85% formaldehyde and transferred to a nylon membrane (Hybond-N+, Amersham Life Science, The Netherlands). Hybridization was carried out using ^{32}P-labeled cDNA probes at 65°C overnight.

Fluorimetric Caspase Activity Assay. Liver slices and cultured hepatocytes were harvested and washed with PBS and lysed in the caspase activity buffer containing 20 mM piperazine-N,N′-bis-(2-ethanesulfonic acid) (PIPES) pH 7.2, 100 mM NaCl, 10 mM DTT, 1 mM EDTA, 0.1% 3-(3-cholamidopropyl-dimethylammonio)-2-hydroxy-1-propanesulfonic acid (CHAPS), 10% sucrose as previously described [4]. 100 μg of crude cell lysates were incubated with 80 μM substrate-AMC at 37°C for 1 hour. Caspase mediated cleavage of peptide-AMC was measured by spectrofluorometry (Molecular Device) at the excitation/emission wavelength pair (ex/em) of 380/440 nm. The caspase activity was presented in arbitrary units of fluorescence (per 100 μg of total proteins).

Histology and Immunostaining of Ki67. For semi-thin sections, liver slices were collected, fixed with 25% glutaraldehyde in 0.4 M Cacodylate buffer, pH 7.2), postfixed in 2% osmium tetroxide and embedded in Epon-Araldite resin. Semithin section (1 μm) were cut, stained with azur blue and examined with a Leitz DMRB light microscope. Hematoxylin and eosin staining was performed on paraffin sections while the immunodetection of Ki67 was performed on frozen sections (Histopathology H2P2 core facility, Fédération de Recherche, Biosit, University of Rennes 1).

Immunoblotting Analysis. Liver slices and cultured hepatocytes were lysed by sonication in lysis buffer containing 50 mM HEPES pH 7.5, 150 mM NaCl, 15 mM $MgCl_2$, 1 mM EDTA, 2.5 mM EGTA, 1 mM DTT, 0.1% Tween 20%, 0.1 mM sodium orthovanadate, 1 mM NaF, 10 mM β-glycerophosphate, 0.1 mM phenylmethylsulfonyl fluoride, 100 μg/mL benzamidine and 5 μg/mL aprotinin, leupeptin, and soybean trypsin inhibitor. Protein concentrations were quantified using the Biorad protein assay (Bio-Rad, France). 100 μg of proteins were resolved on SDS-PAGE and transferred onto polyvinylidene difluoride membranes (PVDF, Biorad). Nonspecific binding sites were blocked with Tris Buffer Saline (TBS) containing 4% BSA, for 1 hour at room temperature. Then, filters were incubated overnight at 4°C with primary antibody diluted at 1 : 250 for anticaspase 3, 1 : 1500 for anticaspase 8, 1 : 600 for anticaspase 9, and 1 : 2000 for other antibodies in TBS containing 4% BSA. Filters were washed three times with TBS and incubated with appropriate secondary antibody conjugated to horseradish peroxidase, for 1 hour at room temperature. Proteins were visualized with Supersignal (Pierce Chemical Co., Rockford, IL).

Statistics. The data presented in this manuscript were obtained from 3 to 6 independent experiments. In each experiment, one rat was killed and all the slices were prepared from the same liver. For each time point 1 to 3 slices were used. For each condition and time-point, the experiment was repeated 2 to 6 times. In figure legend, detailed information was given on the number of experiments performed. Results in tables and figures are expressed as mean ± SD. In some experiments, statistical significance between control and treated hepatocytes was tested by a paired Student's t-test. A P value of < 0.05 was considered to be statistically significant.

3. Results

3.1. Viability and Expression of Specific Liver Functions in Cultured Liver Slices. In order to evaluate cell viability of cultured liver slices, histological integrity was studied (Figure 1) and correlated with ATP content, LDH release, and expression of specific liver functions (Figure 2). Histology of freshly prepared slices after a 90 min preincubation in medium, indicated a normal liver architecture despite a few

damaged cells and dilated sinusoids on the edge of the slices (Figure 1(a)). After 24 hours (h) of culture, the slices exhibited normal liver histology although a necrotic zone restricted to 1 or 2 layers of hepatocytes at or near the center of the slice, could be observed (Figure 1(b)). In viable midzone areas, hepatocyte clarification corresponding to glycogen content eliminated during the fixation and dehydration steps of semi-thin section preparation, was clearly evidenced (Figure 1(c)). At 48 h of culture, scattered hepatocytes with microvacuoles were detected while the glycogen content was markedly reduced (Figure 1(d)). In addition, apoptotic cells, characterized by nuclear chromatin condensation and formation of apoptotic bodies, were observed (Figure 1(d), inset). At 72 h, hepatocytes located mostly in periportal area and in a lesser extent in midzonal area exhibited lucent microvacuoles containing dense material (Figure 1(e), inset). Apoptotic figures were no longer detected. At 96 h, light swollen and dark hepatocytes were detected in disorganised cords in periportal zone indicating that necrotic areas had significantly enlarged (Figure 1(f)). The ATP content (Figure 2(a)) was low in freshly prepared slices (1 nmol/g of slice) reflecting a low energy charge immediately after slicing. This content strongly increased during the preincubation step and in culture up to 19 h after plating to reach 3.5 nmol/g and, then, remained stable until 96 h. LDH release was measured in primary culture of isolated hepatocytes and cultured liver slices. In isolated hepatocytes, LDH release is relatively low during the first days of culture but increases with time to reach very high levels at 96 hours concomitantly with the strong increase in caspase activities (Figure 3). In liver slices, the LDH release was consistently high during the first hours of culture (3 to 24 h), decreased at 48 and 72 h of culture and increased again at 96 h (Figure 2(b)).

Expression of several liver specific proteins including albumin and transferrin, phase I enzymes cytochromes P450 (CYP) 3A1/2, 2E1 and 2B and phase II enzymes glutathione S-transferases (GST) A1 and P1, was analyzed by western blot (Figure 2(c)). Protein levels of these liver specific functions were similar in liver, core, freshly prepared slices and cultured slices up to 3 to 10 hours of culture. Then, three groups of functions could be distinguished: in the first one, levels of albumin and CYP3A1/2 and 2E1 progressively decreased with time; in the second group, the expression of transferrine, CYP2B and GSTA1 were maintained compared to normal liver, without significant changes during 4 days. Finally, the expression of GSTP1 was higher in core and freshly prepared slices compared to normal liver, then decreased between 3 and 48 h before increasing again at 72 and 96 h.

3.2. Early and Transient Induction of Caspase Activities in Cultured Liver Slices. In primary culture, isolated hepatocytes undergo apoptosis within 4 days through the activation of caspases 3, 8, and 9, [3, 4]. To determine whether apoptosis also took place in cultured liver slices, western blot analysis were performed to evidence both the pro- and cleaved forms of the initiator caspases 8 and 9 and the executioner caspase 3 (Figure 3(a)).

No changes in the level of procaspase 8 were observed during the 4 days of culture while the cleaved form of this initiator caspase, undetectable in liver and cultured slices at 3, 10, and 24 h, appeared at 48 h and increased with the time of culture thereafter. Levels of procaspase 9 progressively decreased and became very low at 96 hours while the cleaved product was immediately detected after slicing and remained present during 4 days. The expression of procaspase 3 progressively increased with culture time. Very low amounts of cleaved caspase 3 were evidenced in freshly prepared slices and during the first 10 to 24 h of culture but not thereafter (Figure 3(a)).

To determine whether the cleaved form of caspases detected in cultured liver slices were active, caspase 8, 9, and 3 activities were measured using their main fluorogenic tetrapeptide substrates IETD-, LEHD, and DEVD-AMC, respectively (Figures 3(b)–3(d)). These caspase activities were measured in cell lysates from freshly prepared and cultured slices at different times and compared to activities in isolated and cultured hepatocytes. All three activities were very low in freshly prepared slices and isolated hepatocytes although DEVD-AMC was slightly higher in liver slices than in isolated hepatocytes. During the first 10 h of culture, caspase activities sharply increased in slices but not in isolated cultured hepatocytes, then, decreased at 21 and 48 hours to reach the values measured in cultured hepatocytes. A strong induction of these caspase activities was observed at 3 and 4 days in cultured hepatocytes as previously reported [4] but not in liver slices.

We then compared the induction of apoptosis by treatments with the apoptotic factors TGFβ, cycloheximide, TNFα, and TNFα plus cycloheximide in cultured liver slices and primary culture of isolated hepatocytes (Figure 4). In liver slices, neither TGFβ, TNFα, nor cycloheximide increased the DEVD- (Figure 4(a)), IETD- (Figure 4(b)), and LEHD-AMC (Figure 4(c)) caspase activities while the cotreatment with cycloheximide and TNFα led to a strong induction of these activities. In contrast, in pure culture of hepatocytes, all four treatments strongly induced caspase activities. In addition, LDH release was also studied in cultured slices to confirm that these treatments did not affect cell viability (Figure 4(d)). As observed with caspase activities, cycloheximide combined to TNFα led to a strong LDH release. TGFβ and TNFα alone did not induce cell death while cycloheximide triggered a moderate but significant LDH release without detectable induction of caspase activities.

3.3. Hepatocytes in Slices Enter into and Progress through G1 Phase of the Cell Cycle. To determine whether cells in liver slices remained quiescent in G0 or entered the G1 phase of the cell cycle, we analyzed, by northern blotting, the levels of the protooncogenes c-fos and c-myc mRNAs (Figure 5(a)), two hallmarks of G0/G1 transition and early G1 phase [43], respectively, during the slicing procedure and in culture. Neither c-fos nor c-myc mRNAs were detectable in liver after *in situ* liver perfusion or in liver core but were strongly induced in freshly prepared slices. In culture, c-fos mRNA levels rapidly decreased indicating a very transient

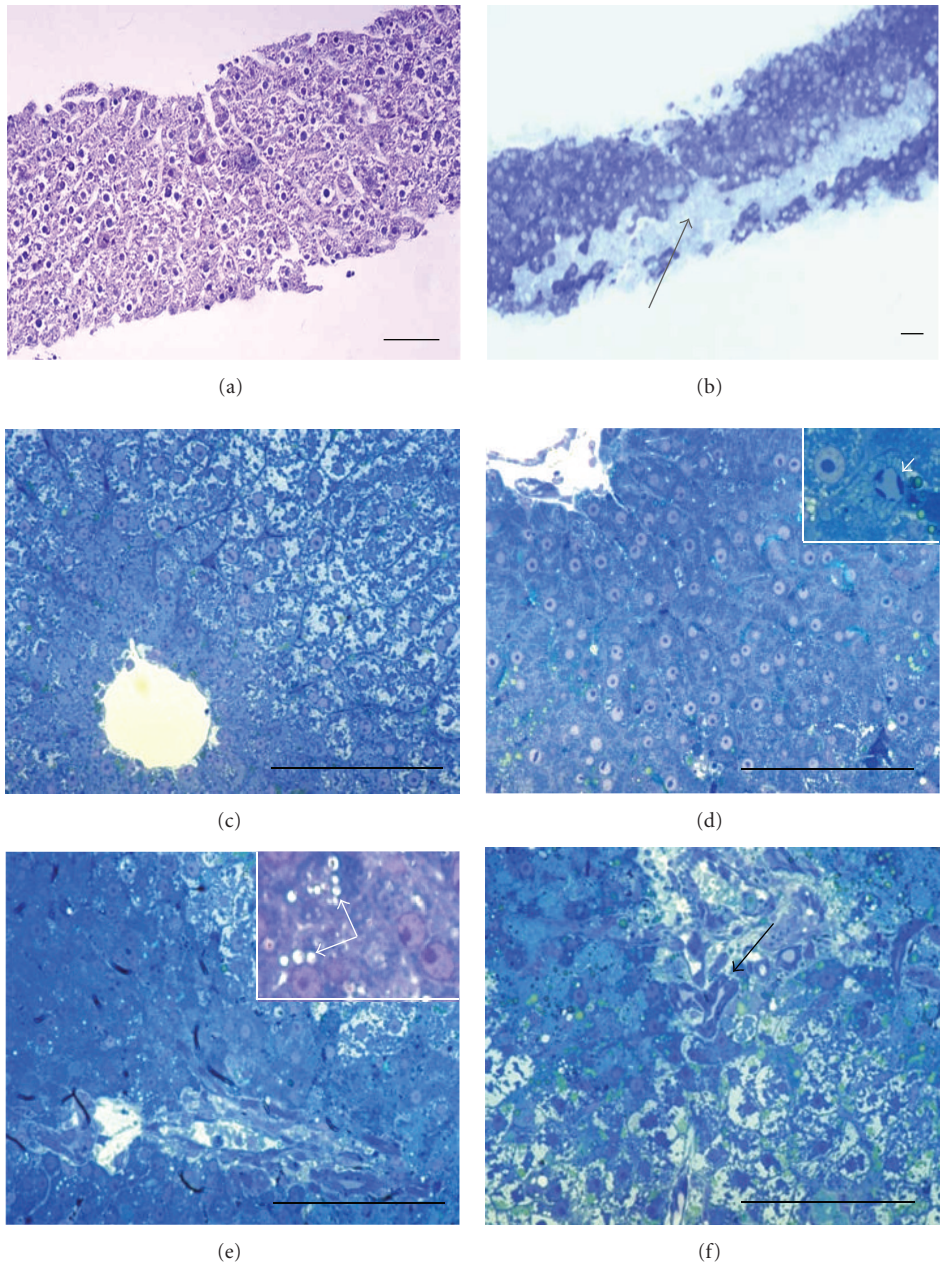

Figure 1: Histology of liver slices in culture. Histology of transversal (a, b) and longitudinal sections (c–f) of rat liver slices immediately after preparation (a) and after 24 (b, c), 48 (d), 72 (e), and 96 h (f) of culture. Hematoxylin and eosin staining illustrates the integrity of liver architecture after slicing (a) and the appearance of a thin necrotic area at the center of the slice ((b), dark arrow). (c–f), semithin sections stained with azur blue evidenced the apoptotic bodies at 48 h ((d), white arrow), the microvacuoles in hepatocytes at 72 h ((e), white arrow) and disorganized periportal zone at 96 h ((f), dark arrow); bar 100 μm.

expression while c-myc expression was stable for at least 21 h of culture. We then investigated the expression of the tumour suppressor gene p53 mRNA, a mid-G1 phase marker. In liver, core, and slices during the first 10 h of culture, p53 mRNAs were not detected. A late induction was found at 21 h. Cdk4 mRNAs, known to be expressed in normal liver and throughout the cell cycle, were detected in all samples with little changes in the expression levels. These results demonstrated that cells in liver slices expressed markers of early and mid-G1 immediately after slicing strongly suggesting G0/G1 transition and progression in early G1 phase of cells in cultured liver slices.

G0/G1 transition is controlled by cytokines and oxidative stress activating intracellular signalling pathways including MEK/ERK, STAT3, and JNK [44, 45]. In order to determine if these pathways were activated in liver slices, we investigated by immunoblotting expression of phosphorylated or total forms of ERK1/2, STAT3, and JNK1/2/3 as well as the GSTA4

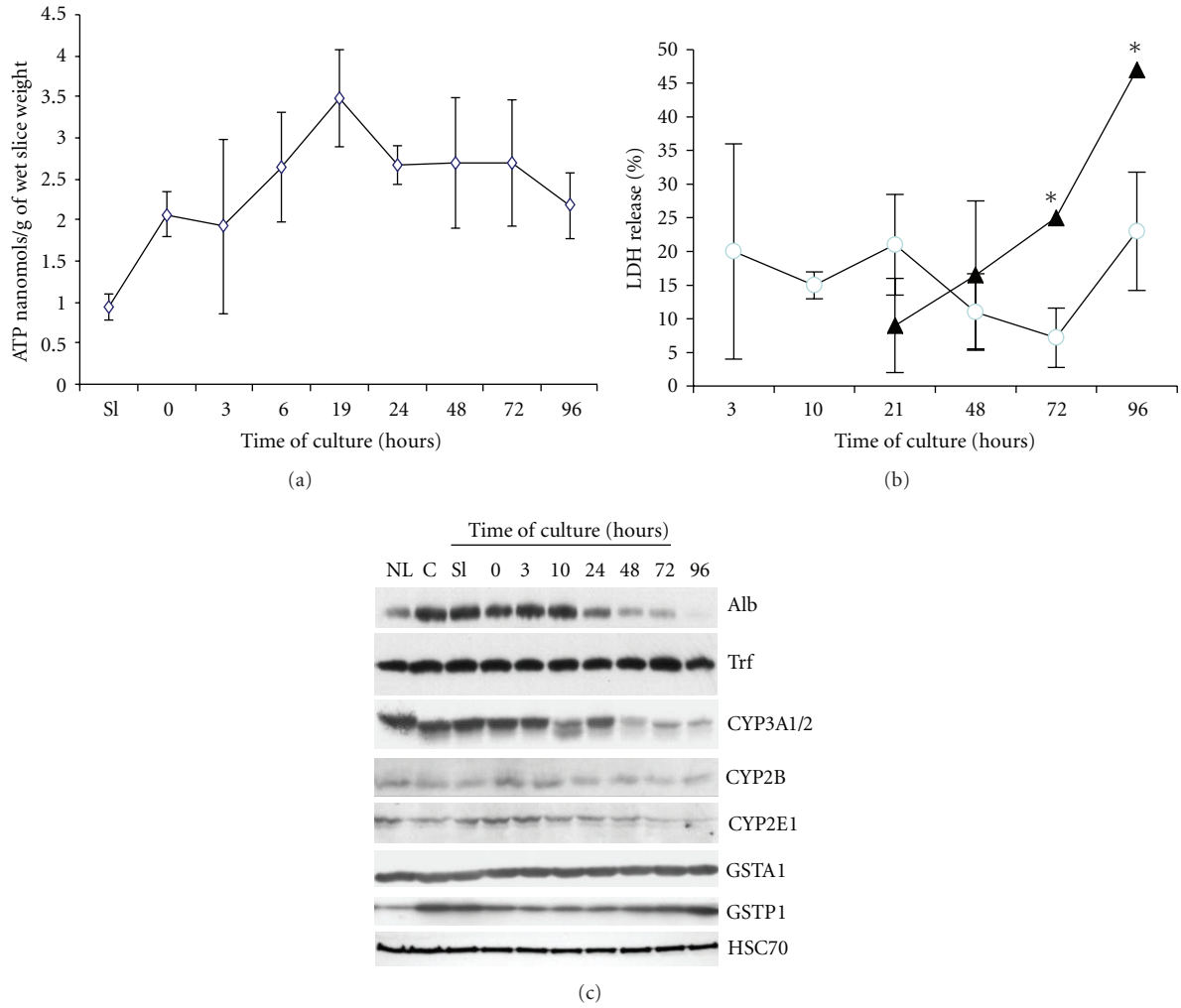

Figure 2: ATP content, LDH release, and expression of liver specific proteins. ATP content, expressed in nanomols/g of wet slices weight (a) and LDH release (b) were measured during the preincubation (Sl) and at the indicated times of culture. LDH release was also measured in primary culture of isolated hepatocytes (dark triangles). Western blot analysis of liver specific proteins (c) in normal liver (NL), in core before slicing (c), freshly prepared slice (Sl), after the preincubation (T0), and in culture at different times. Specific antibodies detecting albumin (Alb), transferrin (Trf), cytochrome P450 (CYP) 3A1/2, 2B, 2E1 subunits, and glutathione S-transferases (GST) A1 and P1 isoforms, were used. The western blot of HSC70 indicated that equal amounts of proteins were loaded in each lane. These experiments were performed on 2 independent experiments with 3 slices in each experiment. $^*P < 0.001$ treatment versus control.

(Figure 5(b)), a GST isoform induced by and involved in metabolism of lipid peroxidation products [41, 46]. Phospho-ERK1/2 and -JNK1/2/3 were strongly induced immediately after liver perfusion and were maintained during at least 48 h of culture demonstrating the early and robust activation of these two signalling pathways. Total STAT3 was detected in all slice extracts but was strongly induced at 1 and 6 hours of culture. Its phosphorylated form was expressed at a low level in normal liver, undetected during the slicing procedure but was induced in cultured slices. GSTA4 was also induced in core and freshly prepared slices but its expression level slowly decreased with time of culture.

Taken together, these data demonstrate the rapid activation of MAPKinase pathways during slicing and induction of downstream genes involved in proliferation such as c-fos, c-myc, and p53 in cultured slices strongly suggesting the entry into and progression through early G1 phase of cells in livers slices.

3.4. G1/S Transition Requires Costimulation by EGF and TNFα in Cultured Liver Slices. In conventional cultures of isolated hepatocytes stimulation by growth factors such as EGF, TGFα and HGF triggers the G1/S transition [47].

To determine whether cells in cultured slices replicated after stimulation by growth factors, slices were maintained in culture for 4 days in absence or presence of EGF and mRNA levels of cyclin D1 and cdk1 known to be induced in late G1 and S phases, respectively [48], were analyzed (Figure 6(a)). Cyclin D1 mRNAs were detected at low levels in liver tissue and during slicing and were undetectable in cultured slices

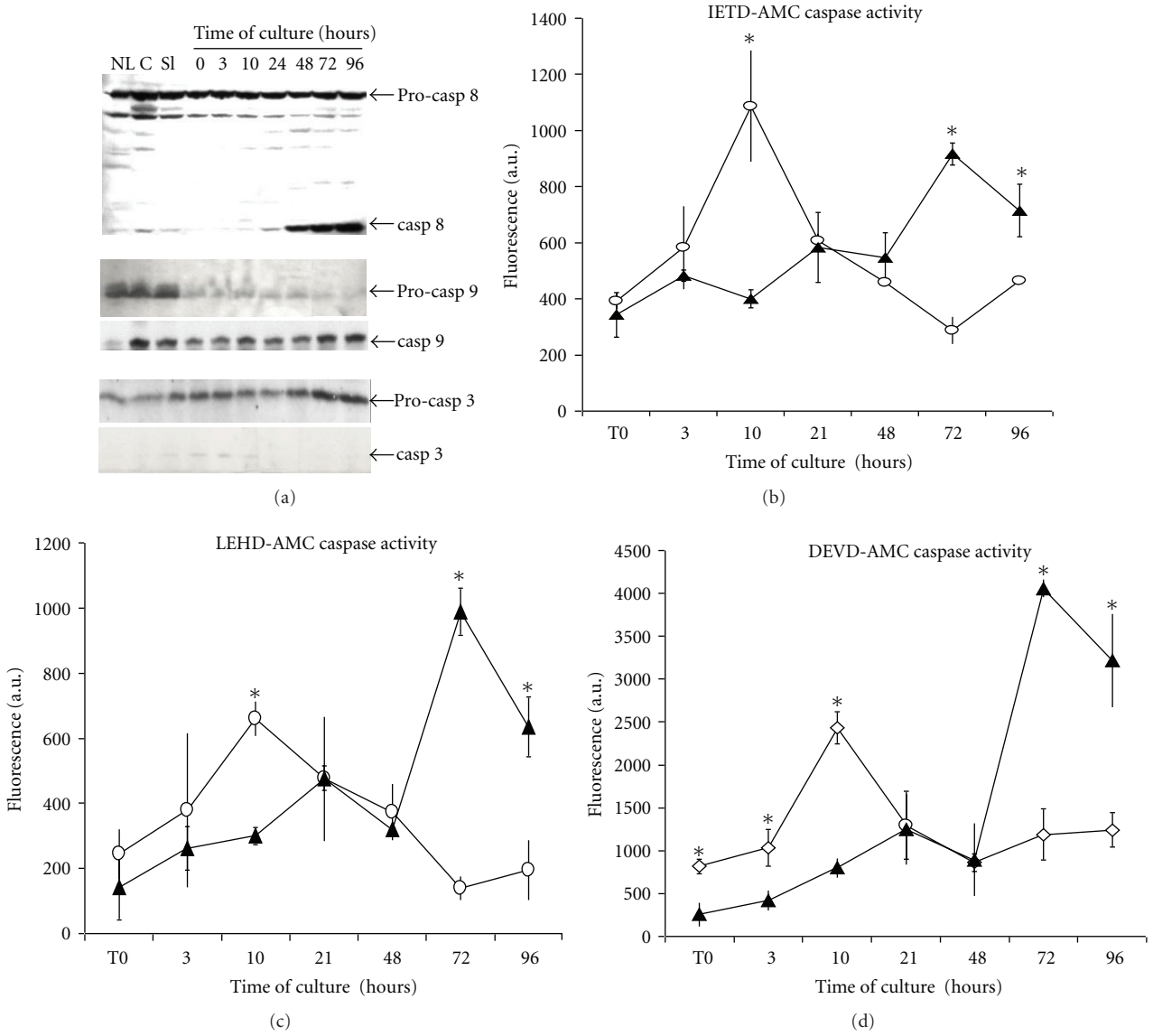

Figure 3: Time course of caspase expression and activation. (a), Western blot analysis of caspase 8, 9 and 3 in normal liver (NL), core (C), after slicing (Sl), after pre-incubation (T0) and at the indicated times of culture. IETD- (b), LEHD- (c) and DEVD-AMC (d) caspase activities were measured in cell lysates from slices (open circles) and isolated hepatocytes (dark triangle) at different times of culture. Activities were expressed in arbitrary units (A.U.) of fluorescence. Caspase activities in isolated hepatocytes were measured in 6 independent experiments while activities in liver slices are the results of 3 independent experiments with 2 or 3 slices in each experiment. $^*P < 0.001$ treatment versus control.

in absence or presence of EGF. Cdk1 mRNAs were never detected in any slice samples. In contrast, cyclin D1 and cdk1 were strongly induced in regenerating liver 24 h post-hepatectomy, used as positive control of proliferation.

We demonstrated that costimulation by TNFα and EGF allow multiple rounds of hepatocyte division in differentiated hepatocytes cocultured with rat liver epithelial cells while the stimulation by EGF alone does induce proliferation [49]. In order to determine if the stimulation with both TNFα and EGF triggers DNA replication in cultured liver slices, expression of cyclin D1 and Cdk1 was investigated by immunoblotting in slices stimulated with TNFα and EGF (Figure 7(b)). In nonstimulated slices, neither cyclin D1 nor Cdk1 proteins were detected (Figure 6(b)). Similarly, in slices stimulated by EGF or TNFα, cyclin D1 was barely detectable despite the expression of P-ERK1 and 2. In EGF-stimulated hepatocytes, used as positive control of proliferation, induction of cyclin D1 protein was observed following EGF stimulation. In contrast, we found that both cyclin D1 and Cdk1 were expressed at 24 and 48 hours of culture in slices stimulated with both EGF and TNFα. *In situ* immunodetection of Ki67 (Figures 7(a)–7(c)) and phosphorylated histone H3 (Figures 7(d)–7(f)) in nonstimulated and EGF-stimulated liver slices indicated that very few hepatocytes were stained

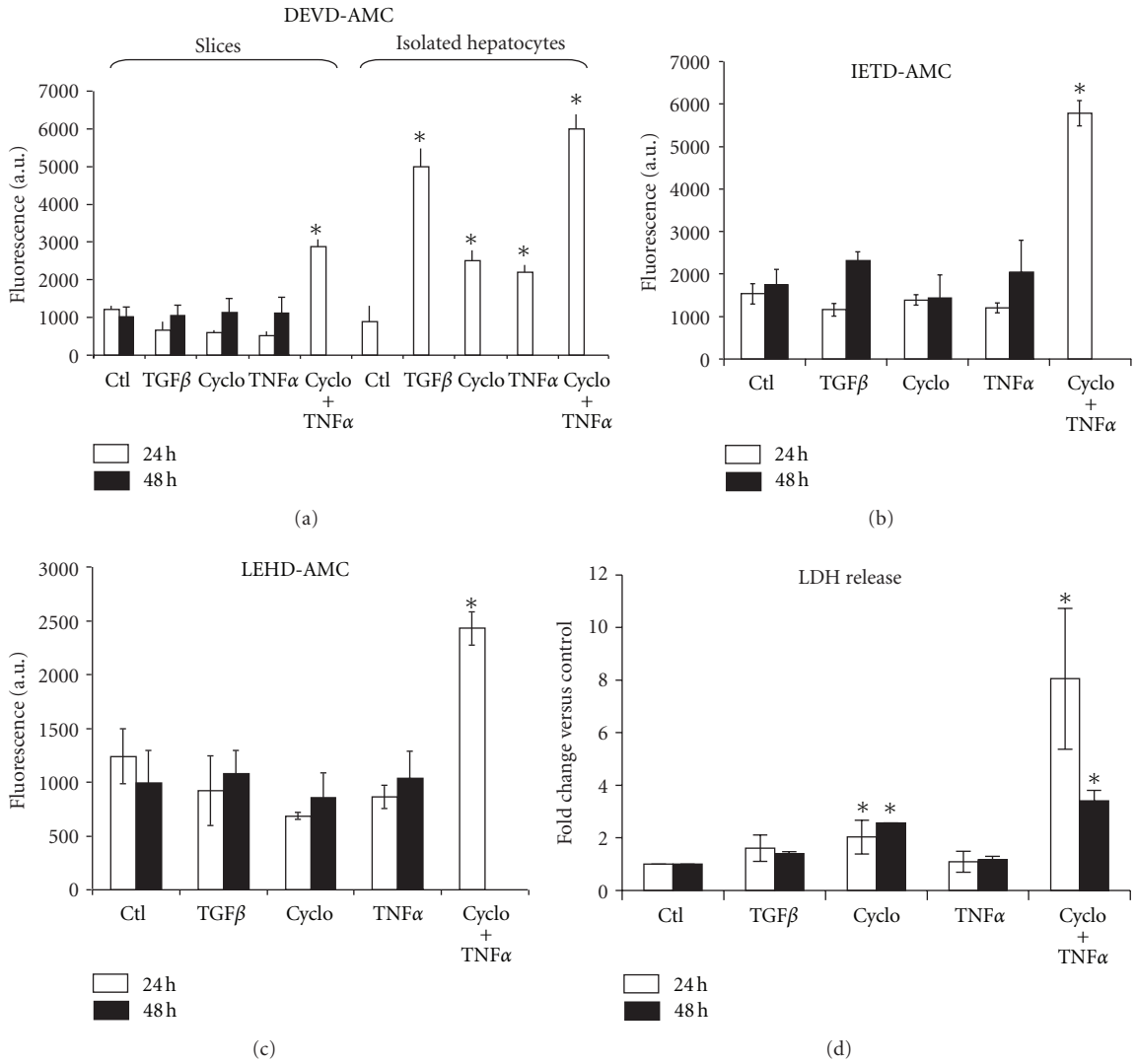

Figure 4: Caspase activities and LDH release in cultured slices and hepatocytes upon treatments with TGFβ, TNFα, and cycloheximide. DEVD- (a), IETD- (b), LEHD-AMC (c), caspase activities, and LDH release (d) in cultured slices at 24 and 48 h in control (Ctl) after treatments with TGFβ, cycloheximide, TNFα, and TNFα + cycloheximide. For slices treated with both TNFα and cycloheximide, caspase activities at 48 h were not presented because of a complete loss of viability between 24 and 48 h. Caspase activities and LDH release in liver slices are the results of 3 independent experiments with 2 or 3 slices in each experiment. $^*P < 0.001$ treatment versus control.

(<0.5%) in both conditions confirming that hepatocytes in cultured slices did not progress in S phase after stimulation by EGF. However, cotreatment with EGF and TNFα induced a strong increase in Ki67 and phosphorylated histone H3 positive cell index reaching ~30% at 48 h (Figure 7(e)).

Altogether, these results demonstrate that hepatocytes had progressed beyond the mitogen-dependent restriction point in mid-G1 phase of the cell cycle in slices stimulated by EGF and TNFα and that TNFα had primed hepatocytes to allow responsiveness to growth factors.

4. Discussion

In normal liver, differentiation and the balance between proliferation and cell death are controlled by complex intercellular communications often referred to as hepatic microenvironment. Alterations of this microenvironment strongly affect differentiation, cell cycle status, and survival. For instance, isolation of hepatocytes by disruption of cell-cell interactions in liver triggers their G0/G1 transition and progression up to mid-G1 phase of the cell cycle [2]. Similarly, *in vitro*, the culture conditions of isolated hepatocytes determine the expression levels of liver specific functions, the capability to proliferate and the cell survival. Hepatocytes cultured in minimal medium are characterized by a rapid decrease in the expression of liver specific functions, the induction of DNA replication upon mitogenic stimulation, a high sensitivity to apoptotic agents, and a 4 to 7 days life-span due to the induction of apoptosis [1]. In contrast, hepatocytes maintained in coculture [10], extracellular matrix sandwiches [12], and Me_2SO-stimulated

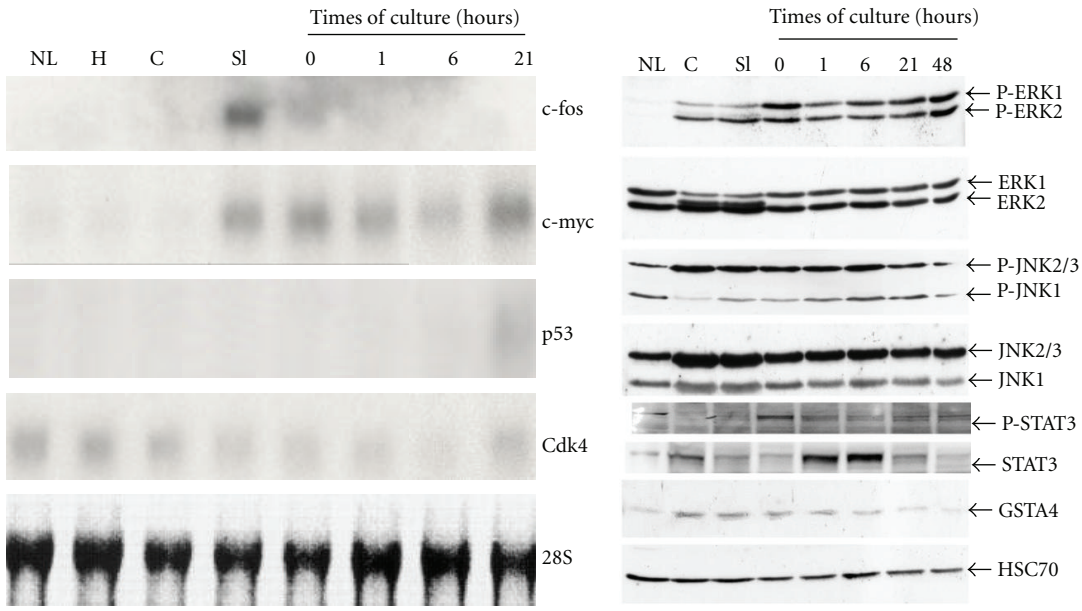

Figure 5: Sequential activation of cell cycle markers and signalling pathways.(a) Northern blot analysis of c-fos, c-myc, and p53 mRNAs. Samples were: livers collected after perfusion by Krebbs buffer (L) and Viaspan solution (H), core (C), freshly cut slices (Sl), after preincubation (0), and at 1, 6, and 21 h of culture. Cdk4, known to be expressed in normal liver and throughout the cell cycle, and 28S ribosomal RNA were used to control equal loading of RNAs in each lane. (b) Western blot analysis of phospho- and total-ERK1/2, STAT3, and -JNK1/2/3. GSTA4, a marker of oxidative stress, was also studied while HSC70 was used as loading control. These data were found similar in 2 independent experiments.

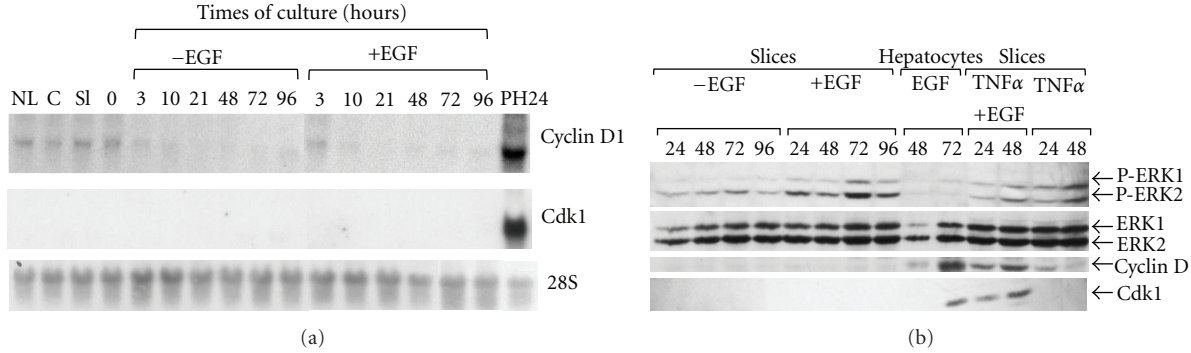

Figure 6: Expression of cyclin D1, Cdk1, and ERK1/2. Northern blot analysis of cyclin D1 and Cdk1 (a) in liver (NL), core (c), freshly prepared slices (Sl), after preincubation (0), and at different times of culture in absence (−EGF) or presence (+EGF) of EGF. Regenerating liver, 24 h after partial hepatectomy (PH24), was used as a positive control of proliferation. Hybridization of 28S ribosomal RNAs was used to control equal loading of RNAs in each lane. Western blot analysis of phospho-(P-)ERK1/2, total ERK1/2, cyclin D1, and Cdk1 (b) in cultured slices at the indicated times of culture in absence (−EGF) or presence (+EGF) of EGF and/or TNFα. Primary cultures of isolated hepatocytes stimulated by EGF (at 48 and 72 h) were used as control of proliferation.

cultures [4] are characterized by higher expression levels of liver specific functions maintained for several weeks, the lack of DNA replication upon stimulation by growth factors, and a much higher resistance to apoptotic agents.

The aim of this study was to address the question whether, in liver slices, the integrity of the tissue architecture, and cell-cell communications allowed proliferation of hepatocytes in response to stimulation by a growth factor and protected from apoptosis in culture. Indeed, liver slices provide a unique *in vitro* hepatic model to assess whether the presence of all the liver cell types keeping their cell-cell interactions and polarity affected cell survival and induction of hepatocyte proliferation. Here, we report that cells in liver slices underwent a G0/G1 transition during slicing and progressed up to mid-G1 phase in culture. Indeed, the slicing procedure induced a strong activation of the MEK/ERK, STAT3, and JNK pathways rapidly followed by the transient upregulation of c-fos and constant expression of

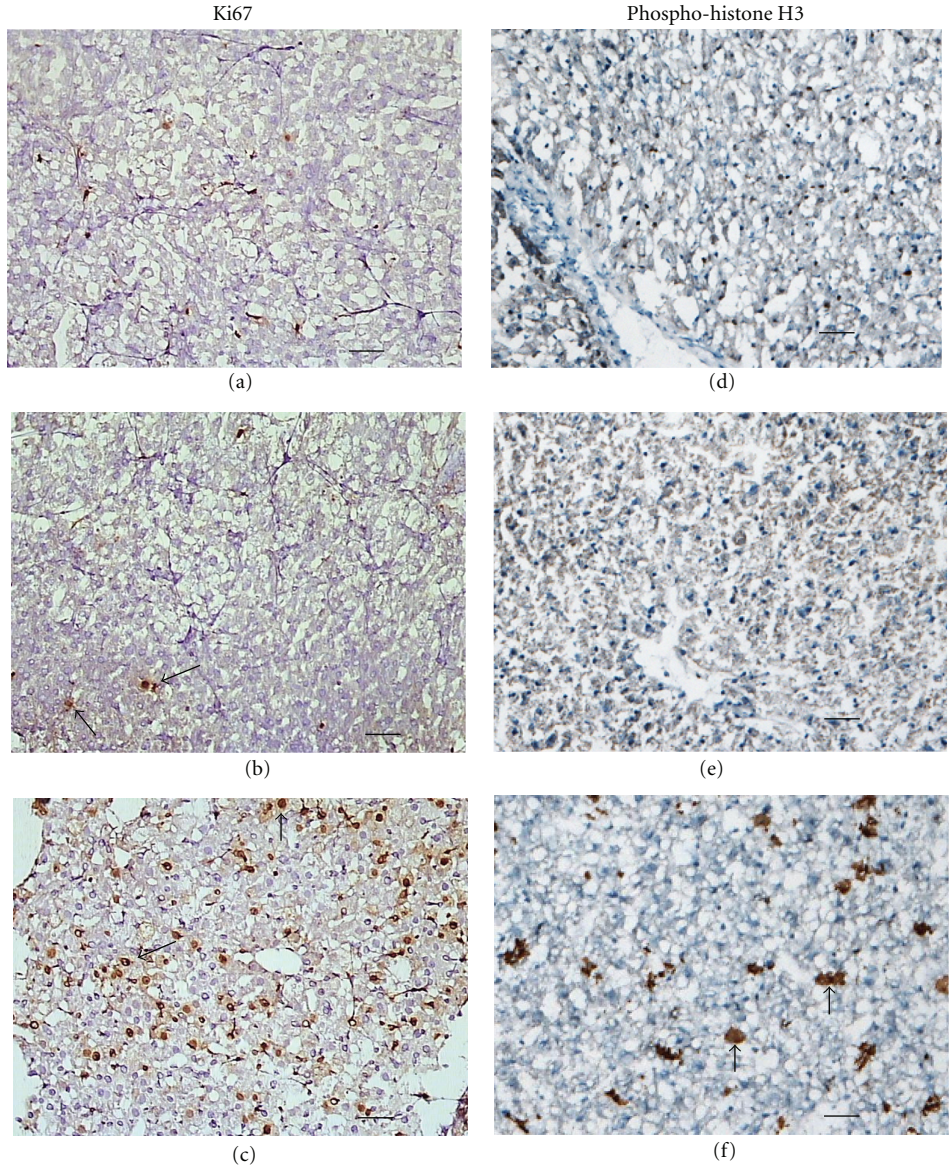

Figure 7: *In situ* immunodetection of Ki67 and phosphohistone H3. *In situ* immunostaining of Ki67 (Figures 7(a–c)) and phosphorylated histone H3 (Figures 7(d–f)) at 48 h of culture in untreated slices (a–d) and after EGF (b–e) or TNFα + EGF (c–f) treatments. Arrows indicate Ki67 or phosphohistone H3 positive cells. Bar: 100 μm.

c-myc mRNA levels, two protooncogenes characterizing the G0/G1, and early G1 phase of the cell cycle, respectively [2]. Concomitantly, expression of GSTA4, a GST isoform induced by lipid peroxydation products and reactive oxygen species, increased as we previously reported during early steps of liver regeneration [41] and during isolation of mouse hepatocytes [50].

Both activation of stress and proliferation signalling pathways and GSTA4 induction most likely result from the cumulated stress signals that occur during the slice preparation procedure including hypoxia, hypothermia, and slicing. This hypothesis is further reinforced by our data evidencing the extracellular LDH release, a decrease in the ATP content, and the transient increase in caspase activities during the first hours of culture. As a consequence, one or two layers of necrotic cells were observed at the center of the slices on the longitudinal histological sections of the liver slices at 24 hours of culture. In addition, the presence of apoptotic bodies was detected at 48 h. These data confirm a postischemic injury following liver perfusion and slicing procedures as previously reported [34, 38] and demonstrate that cell death is heterogeneous within the slices with more necrosis in hypoxic areas. After 24 h, LDH release and caspase activities returned to a basal level, the ATP content went up, and large areas in slices remained viable indicating that the early burst of cell death at 24 h affected only a fraction of cells.

After 24 h of culture, the constant expression of c-myc mRNAs and the induction of p53 mRNAs, two markers of

the G1 phase, strongly suggested that cells progressed up to mid-G1 phase. Interestingly, the stimulation by EGF induced phosphorylation of ERK1/2 proteins demonstrating the activation of the MEK/ERK pathway but failed to induce cyclin D1 and Cdk1 expression and DNA replication confirming that hepatocytes in cultured slices were arrested in mid-G1 phase of the cell cycle in presence of growth factor. A large body of evidences demonstrates that liver regeneration following partial hepatectomy is controlled by two groups of extracellular soluble factors [43]. The proinflammatory cytokines TNFα and IL-6 are the early stimuli that trigger production reactive oxygen species and redox signalling inducing hepatocyte entry into the cell cycle characterized by the rapid activation of MEK/ERK, STAT3 produced by Kupffer cells and JNK signalling pathways, the preexisting NFκB transcription factor, and the transcriptional induction of a large subset of genes called "immediate-early genes" including c-fos and c-jun [51, 52]. The exit from quiescence and progression in early G1 phase of the cell cycle also called "priming" allows the hepatocytes to become sensitive to growth factors such as HFG, EGF, and TGFα that triggers the G1/S transition and the commitment to DNA replication [2, 44].

It is also well-documented that the progression of hepatocytes in late G1 phase during liver regeneration involves the extracellular matrix remodelling [53] and that metalloproteinases MMP-2 and MMP-9 play a crucial role in this remodelling [54]. Similarly, in the coculture model of rat hepatocytes and liver epithelial cells, the induction of the cyclin D1 expression, and the commitment to S phase depends upon the degradation of the extracellular matrix mediated by MMP-9 [49]. Moreover, transcriptional induction of MMP-9 is controlled by TNFα establishing a link between this cytokine and extracellular remodelling.

In cultured slices, the cell cycle arrest in G1 could be due to the maintenance of cell-cell interactions and the absence of extracellular matrix degradation and/or remodelling. Consistently, Vickers et al. [21] recently evidenced an increased expression of collagens in cultured human liver slices that may be linked to activation of stellate cells and/or resident fibroblast. Our data strongly support this hypothesis since the stimulation with both TNFα and EGF led to the induction of the cyclin D1, Cdk1, Ki67, and phosphorylated histone H3 demonstrating a progression through S phase and G2/M transition. Our data also suggest that TNFα may also be involved in extracellular matrix remodelling in cultured liver slices and future investigations would be required to address this hypothesis.

Regarding apoptosis, we showed that liver slices maintained in a basal medium did not undergo massive caspase-dependent apoptosis between days 1 and 4 of culture in contrast with high rates of cell death previously reported in pure culture of isolated heptocytes [3, 4]. These data indicate that maintenance of tissue architecture prevented or delayed massive caspase-dependent apoptosis and that loss of cellular integrity observed at 96 h was most likely due to necrosis or other cell death processes that do not involved executioner caspases. Another striking result was the fact that caspase activities and LDH release were moderately induced in cultured slices by apoptotic agents TGFβ, TNFα, and cycloheximide, suggesting that hepatocytes in slices are more resistant to apoptotic agents than isolated hepatocytes in conventional primary culture conditions [55].

However, in liver slices, procaspases 8 and 9 were cleaved into mature forms but their activity remained very low indicating that survival signal(s) blocked the caspase dependent apoptotic pathway beyond the caspase maturation. Similarly, it has been established that Me_2SO protected hepatocytes from apoptosis in primary culture through the inhibition of both cleaved caspases 8 and 9 and the apoptosis signal-regulating kinase 1 (ASK1), a key element in the cytokine- and stress-induced apoptosis [4]. It was hypothesized that Me_2SO could inhibit ASK1 activity and the downstream activation of caspases 8 and 9 through preservation of high GST expression levels. Interestingly, while the expression of specific liver proteins such as CYP 3A1/2, 2E1, and albumin, progressively decreased during 4 days of culture, the levels of GSTA1 and P1 remained remarkably stable. It would be interesting to determine whether the high levels of GSTA1/2 detected in cultured slices prevented the activation of ASK1 and caspases 8 and 9.

Altogether, our findings led to the conclusion that hepatocytes in cultured liver slices exhibit a complex phenotype characterized by the reentry into the cell cycle and a G1 phase arrest in absence of appropriate mitogenic stimuli, a robust wave of proliferation following combined stimulation by proinflammatory cytokines and growth factors, and a high resistance to apoptotic stimuli. This latter data reinforce the idea that toxicological data obtained in the models of liver slices may be more accurate and reliable that data obtained in culture of isolated hepatocytes maintained in basal conditions [56]. In addition, the demonstration that hepatocytes in liver slices keep the ability to undergo proliferation opens new perspectives for the use of liver slice in the field of liver regeneration [21] as well as genotoxicity.

Abbreviations

H: Hours
LDH: Lactate deshydrogenase
CYP: Cytochrome P450
EGF: Epidermal growth factor
ECM: Extracellular matrix
GST: Glutathione S transferase
ASK1: Apoptosis signalling kinase 1
Me_2SO: Dimethyl sulfoxide
TGFβ: Transforming growth factor beta
TNFα: Tumor necrosis factor.

Acknowledgments

This paper is dedicated to the memory of Dr. Joan Albert Vericat who recently passed away during the review process of this paper. Dr. Vericat was devoted to the field of toxicology and pharmacology. Joan was an exceptional scientist and friend whose enthusiasm for life and belief in science will continue to inspire all of us who were privileged to know him. The authors deeply thank Dr. Annick Prenez for

his clear-sightedness which allowed performing this work. The laboratories localized Z.I. de Limay-Porcheville, 78440 Porcheville (France) were previously the property of Sanofi-Synthélabo. Francis Finot, Régis Masson and Nicole Bichet who contributed to this work were employed by Sanofi-Synthélabo before the transfer to Covance Laboratory SAS. The authors also thank A. Fautrel from the histopathology H2P2 core facility (Fédération de Recherche, Biosit, University of Rennes 1) for the *in situ* detection of Ki67 and phosphorylated histone H3. This work was supported by The Institut National de la Santé et de la Recherche Médicale (Inserm). The authors claim no direct financial relation with the commercial identities mentioned in the paper and declare no conflict of interests.

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Hindawi Publishing Corporation
International Journal of Hepatology
Volume 2012, Article ID 630670, 6 pages
doi:10.1155/2012/630670

Review Article

Calcium Signalling and Liver Regeneration

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Received 29 June 2012; Accepted 12 September 2012

Academic Editor: Chantal Desdouets

After partial hepatectomy (PH) the initial mass of the organ is restored through a complex network of cellular interactions that orchestrate both proliferative and hepatoprotective signalling cascades. Among agonists involved in this network many of them drive Ca^{2+} movements. During liver regeneration in the rat, hepatocyte cytosolic Ca^{2+} signalling has been shown on the one hand to be deeply remodelled and on the other hand to enhance progression of hepatocytes through the cell cycle. Mechanisms through which cytosolic Ca^{2+} signals impact on hepatocyte cell cycle early after PH are not completely understood, but at least they include regulation of immediate early gene transcription and ERK and CREB phosphorylation. In addition to cytosolic Ca^{2+}, there is also evidence that mitochondrial Ca^{2+} and also nuclear Ca^{2+} may be critical for the regulation of liver regeneration. Finally, Ca^{2+} movements in hepatocytes, and possibly in other liver cells, not only impact hepatocyte progression in the cell cycle but more generally may regulate cellular homeostasis after PH.

1. Introduction

After partial hepatic destruction in experimental or clinical context, the initial mass of the organ is restored through compensatory growth of the remnant liver. A complex and yet incompletely elucidated network of cellular interactions (including paracrine, autocrine, endocrine, or nervous pathways) orchestrates the regulation of regeneration, through both proliferative and hepatoprotective signalling cascades [1]. A number of agonists constituting this network drive intracellular Ca^{2+} movements, in particular through the formation of inositol 1,4,5 trisphosphate which binds on its receptor in the membrane of the endoplasmic reticulum, and release the Ca^{2+} stored in this organelle. Such agonists include some of the main comitogenic—as noradrenalin [2], arginine vasopressin (AVP) [3], and adenosine triphosphate (ATP) [4]—and mitogenic factors, as epidermal growth factor (EGF), hepatocyte growth factor (HGF) [5], and insulin [6]. The resulting increase in ionized cytosolic calcium concentration generally consists of a regular succession of Ca^{2+} peaks (oscillations) [7] that can be transmitted to other cells (intercellular calcium waves) which mechanisms and functions are not fully known [8, 9]. The impact of calcium signalling on liver regeneration has, however, only been scarcely studied.

2. Hepatocyte Ca^{2+} Signalling: Mechanisms and Functions

The first calcium oscillations were reported in hepatocytes [10], and numerous studies, both experimental and theoretical, have been conducted after that to decipher, in the hepatocyte, the machinery by which agonists generated cytosolic calcium signals. However, the functions of hepatocyte calcium signalling remain far less understood.

In hepatocytes, as in most nonexcitable cells, Ca^{2+} oscillations originate from the periodic opening of Ca^{2+} channels located in the ER membrane, following activation of the phosphoinositide cascade. The binding of an agonist to a membrane-bound receptor activates the Gα-subunit of a G-protein complex coupled to the receptor. This activated G protein in turn stimulates phospholipase C (PLC) activity. The latter enzyme catalyzes the hydrolysis of the membrane-bound phosphatidyl-inositol bisphosphate (PIP_2) into diacyl -glycerol and inositol trisphosphate ($InsP_3$). Ca^{2+} release

from the internal stores is ensured by the $InsP_3R$, an homotetramer that can bind up to 4 $InsP_3$ molecules, forming a Ca^{2+} channel which equilibrium open probability presents a bell-shaped dependence on cytosolic Ca^{2+} [11]. The decrease of $[Ca^{2+}]_i$ in the cytosol is due to the activity of the Ca^{2+} ATPases (SERCA pumps), which actively transports Ca^{2+} from the cytosol into the ER. Ca^{2+}-regulated $InsP_3Rs$ and Ca^{2+} ATPases are together sufficient to generate Ca^{2+} oscillations [12]. In most cases, hormone-induced Ca^{2+} oscillations in hepatocytes take the form of repetitive, sharp spikes sometimes preceded by a slower, pacemaker-like elevation in the cytosolic Ca^{2+} concentration. These periodic increases in the level of free Ca^{2+} in the cytosol from about 0.1 μM up to 1 μM have been observed in hepatocytes in response to stimulation by a large number of agonists such as noradrenalin, vasopressin, phenylephrin, angiotensin II, adenosine triphosphate (ATP), histamine, and thrombin, the shape of the oscillations being agonist dependent [7]. The oscillation frequency increases with the agonist concentration, a phenomenon known as "frequency encoding", and is affected by external $[Ca^{2+}]$—and thus by the rate of Ca^{2+} entry into the cell through plasma membrane Ca^{2+} channels. Intracellular Ca^{2+} waves do not result from a simple diffusion of Ca^{2+} itself—which is quickly buffered—but from the spreading of $InsP_3$—which is more soluble in the cytosol—that mobilizes Ca^{2+} from storage compartments throughout the cell. A particular spatial pattern of subcellular $InsP_3R$ distribution was reported to support the direction of intracellular Ca^{2+} waves, starting from the canalicular region containing the most abundant and affine $InsP_3R$ isoform (type II $InsP_3R$) and spreading toward the other regions of the cytosol, less sensitive to $InsP_3$ [13]. As in many other cell types, intracellular movements of Ca^{2+} in hepatocytes, induced by hormones and neurotransmitters, may be propagated from cell to cell. Our group demonstrated in multicellular rat hepatocyte systems (couplets and triplets) that agonists such as vasopressin or noradrenalin induce tightly coordinated and sequentially ordered intracellular Ca^{2+} increases [8, 14–16]. Such signals were also observed in the intact perfused liver in which vasopressin elicits waves of $[Ca^{2+}]_i$ increase running along hepatocyte plates across the lobules [17–19]. We demonstrated that unidirectional Ca^{2+} waves resulted from a gradually decreasing cellular sensitivity to hormonal stimuli from the first to the last responding cell, and that this cell to cell heterogeneity was due to a lobular gradual distribution of hormonal receptors density [8]. Moreover, $InsP_3$ has been shown to flow through gap junctions and thereby coordinate Ca^{2+} spiking among adjacent hepatocytes [20]. Such a configuration in which the most responsive hepatocytes drive the response of the less sensitive cells is similar to the cell to cell triggering of cardiac pacemaker cells [8, 21, 22].

In general terms, Ca^{2+} oscillations in hepatocytes optimize the effect of hormonal stimulation, thanks to enzymes decoding [23] their frequency [24, 25], as proposed for glycogen metabolism in hepatocytes [26]. Since the early data showing that production of glucose by the liver was at least in part mediated by hormone-induced intracellular Ca^{2+} increases [27], most recent studies have deciphered the molecular mechanisms linking intracellular Ca^{2+} to glucose metabolism in hepatocytes [28–30]. In particular, the serine-threonine kinase "calcium calmodulin-dependent kinase II" (CaMKII), a major mediator of Ca^{2+} signalling in different cell types, has been found to play essential roles in the regulation of glycogenolysis and gluconeogenesis in hepatocytes, not only during physiological fasting, but also in the pathophysiological setting of obesity [28, 30]. It is moreover well established that Ca^{2+} oscillations in hepatocytes coordinate intramitochondrial ATP synthesis with cellular energy demand, maintaining cell homeostasis and viability [31]. It has also been demonstrated that [32] the temporal pattern of calcium signals was of major impact as to the expression of transcription factors in lymphocytes, but this aspect has never been investigated in hepatocytes. Many events related to bile secretion are also regulated by cytosolic Ca^{2+}, such as vesicular trafficking and canalicular exocytosis of bile acid transporters [33, 34], permeability of tight junctions [35], or canalicular contraction [9, 36]. Intracellular calcium waves, as described above, starting from the canaliculus to the basolateral poles may have physiological impact on secretion, as it has been shown in pancreatic acinar cells [37], although direct evidence in hepatocytes is lacking. Moreover, interhepatocyte calcium waves have been reported to support canalicular peristaltism and thereby to regulate bile flow, in the normal and regenerating rat liver [3, 38]. As emphasized in the following, all these Ca^{2+}-regulated physiological processes may impact the course of liver regeneration.

3. Intracellular Calcium and Hepatocyte Proliferation: Liver Regeneration

It is well established that intracellular Ca^{2+} is crucial for tissue homeostasis through regulation of cell cycle and apoptosis [7]. In particular, intracellular calcium has been reported to regulate cell proliferation at multiple steps of the cell cycle, from immediate early genes activation, toward G1-S and G2-M transitions, as well as during mitosis [39]. Pioneer studies have shown that extracellular calcium was crucial for liver regeneration [40]. Also, modifications of intracellular calcium homeostasis during liver regeneration have been reported, concerning Ca^{2+}-binding proteins [41], membrane Ca^{2+}-ATPases [42], or the $InsP_3$ receptor [43, 44]. It has been also suggested that the alteration of the $InsP_3$ and Ca^{2+} mobilisation pathway could alter liver regeneration in the rat [45]. Subsequently, it has been shown in nonhepatocytic cell lines that the spatiotemporal organisation of Ca^{2+} signals was determined for the activation of transcription factors like CREB, NF-κB, or NF-AT, and for immediate early genes like c-fos or c-jun [46–48]. It is also well established that the activation of the RAS pathway is controlled by $[Ca^{2+}]_i$ oscillations [49]. Moreover, intra-nuclear calcium signals, which have been well documented [50], have a major impact on gene transcription [51–53] and can result either from the diffusion of cytosolic calcium to the nucleus, or from an $InsP_3$-mediated calcium release in the nucleus itself (see below) [6, 54]. Finally, our previous work demonstrated that hepatocyte calcium signalling was deeply remodelled during

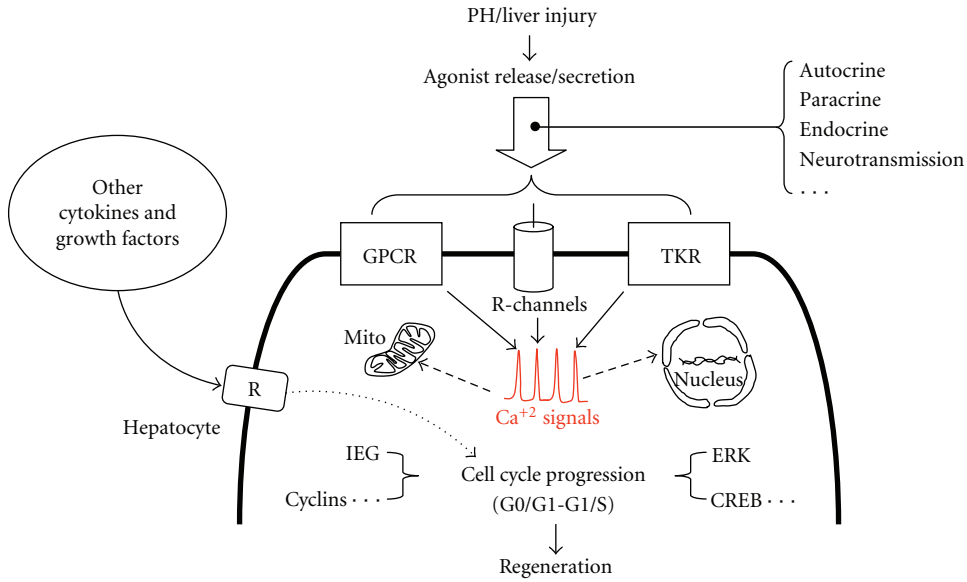

Figure 1: A simplified view of the impact of hepatocyte calcium signals during liver regeneration. After PH or toxic liver injury, a number of calcium mobilizing agonists are released inside or outside the liver, interacting with hepatocytes through autocrine, paracrine, and endocrine pathways. Diverse membrane receptors, either G protein coupled receptors (GPCR), tyrosine kinase receptors (TKR), or receptor channels (e.g., ionotropic purinergic receptors), can be involved in the generation of cytosolic calcium signals. These calcium movements in the cytosol can be transferred to other crucial compartments like the mitochondrion (Mito) or the nucleus, in which they could regulate respectively apoptosis and gene transcription. Previous studies have shown that cytosolic calcium signals regulate cell cycle progression from G0 to G1 and from G1 to S phases in hepatocytes after PH, at least in part through an impact on immediate early genes transcription, cyclin expression, and ERK and CREB phosphorylation.

liver regeneration in the rat, contributing to the regulation of bile flow and cell proliferation [3, 55].

In a recent study, we examined the physiological involvement of cytosolic calcium during liver regeneration in the rat [56]. We interfered with calcium signalling before PH by expressing parvalbumin (PV) in the liver, a calcium-binding protein expressed in muscle cells and neurons but absent from the liver [57], using adenoviruses coding for PV targeted to the cytosol, to selectively buffer Ca^{2+} in this compartment [53, 54]. We found that expression of PV efficiently buffers agonist-induced calcium oscillations in the cytosol and inhibits primary hepatocyte proliferation *in vitro* as well as *in vivo* during liver regeneration.

We found that immediate early gene transcription, early phosphorylation of ERK and CREB, and hepatocyte progression in the cell cycle after PH were inhibited in rats expressing cytosolic PV [56]. These data were in line with previous reports describing these pathways and genes as dependant on cytosolic and/or nuclear calcium signalling [46, 58–60]. We thus suggested that attenuated $[Ca^{2+}]_i$ oscillations in calcium-buffered hepatocytes resulted in impaired activation of these pathways. A potential reduction in CaM-kinase activation, as previously reported [61], or reduced ERK1/2 activation that we observed in PV-NES expressing hepatocytes may have also contributed to altered CREB phosphorylation. Since CaM-kinase II [24], as well as ERK1/2 [60] activity, is sensitive to Ca^{2+} oscillation frequency, an attractive hypothesis would be that cytosolic PV expression, by attenuating agonist-generated Ca^{2+} signals, resulted in impaired phosphorylation of CREB.

Cytosolic calcium signalling impacts most likely the early triggering of hepatocyte progression from G0 to G1 and S phases. In line with this view, a rise in concentration—in the liver and in the plasma—is observed early after PH for several Ca^{2+}-mobilizing agonists, suggesting these agonists might be involved in initiating the regeneration process. In particular, EGF and HGF elicit cytosolic Ca^{2+} oscillations in hepatocytes, the physiological impact of which has never been specifically addressed [5]. Also, extracellular ATP [4], arginine vasopressin [3], and noradrenalin [2], which are comitogenic Ca^{2+}-mobilizing agonists, have been individually reported to contribute to early phases of liver regeneration. Our study thus suggested that buffering hepatocyte calcium signals, potentially generated by these agonists in the minutes after PH, result in delaying hepatocyte cell cycle progression.

There is evidence in the literature for the crucial role of mitochondrial calcium in the regulation of apoptotic processes. It is well known in particular, that mitochondrial calcium overload can be one of the pathways leading to the swelling of mitochondria and to the rupture of the outer membrane, in turn releasing proapoptotic molecules in the cytosol. Mechanisms for excessive calcium transfer to mitochondria are debated and include mainly interactions between proteins of the Bcl2 family and the $InsP_3$-R. Anti-apoptotic members appeared as reducing calcium transfer from the ER to the mitochondria, whereas proapoptotic factors were reported to enhance this flux [62]. In this context, recent data suggest that mitochondrial Ca^{2+}, as well as cytosolic Ca^{2+}, may be critical for the regulation of liver regeneration after PH in the rat [63]. The authors suggested

that buffering calcium in the mitochondria resulted in a shift in the balance between pro- and antiapoptotic factors, thereby protecting hepatocytes from apoptosis, *in vitro* in an hepatoma cell line, as well as *in vivo* in the rat liver after PH.

4. Nuclear Calcium Signalling and Liver Cell Proliferation

Previous studies have established that growth factors important for liver regeneration such as HGF [54] and insulin [6] can differentially affect cytosolic and nuclear calcium in hepatocytes. It has been reported that agonist-induced calcium movements in the nucleus can schematically result from the diffusion of cytosolic calcium to the nucleus and/or from an autonomous $InsP_3$ generation and calcium mobilization from local, intra, or perinuclear Ca^{2+} stores [64, 65]. Nuclear $InsP_3$ may again come from the cytosol or be generated in the nucleus [6]. Indeed, the nucleus, its envelope and the nucleoplasmic reticulum, has been shown to possess several crucial molecules involved in Ca^{2+} storage, $InsP_3$ production, and calcium release, therefore opening the possibility that local nuclear Ca^{2+} signals may occur independently from the cytosol [64, 65]. Although these two views may coexist in the same cells according to circumstances and cell types, it has been shown in SkHep cells that an $InsP_3$-sensitive intranuclear calcium compartment (i.e., the "nucleoplasmic reticulum") exists [50]. PLCβ, PIP_2, and $InsP_3R$ have been found in the nucleus, allowing a local $InsP_3$ production and providing the machinery necessary to generate autonomous Ca^{2+} signals [64, 65]. We also know that calcium signals in these two compartments—cytosol and nucleus—can have different effects [51, 58]. Recent works revealed that buffering calcium in the nucleus, but not in the cytosol, in a hepatoma cell line, resulted in an inhibition of cell proliferation, suggesting that nuclear Ca^{2+} was necessary for centrosome separation and cell progression through early prophase [53]. Gomes et al. further showed that the HGF receptor (c-met) can translocate (upon agonist stimulation) from the plasma membrane to the nucleus and generate an $InsP_3$ production and calcium elevation in the nucleus, independently of cytosolic calcium, in a hepatoma cell line [54]. Very similarly, it was shown by the same group that insulin can induce nuclear calcium signals through a translocation of its receptor to the nucleus, in primary rat hepatocytes [6]. Importantly, the nucleoplasmic reticulum as an intranuclear calcium compartment has not been shown in primary hepato-cytes, and some authors claimed that it was not essential for calcium signalling [66].

Important cellular functions are thought to be regulated by nuclear calcium signals, including nuclear pore permeability, transcription factor activity and protein kinase translocation, thereby controlling gene expression [65]. In particular, the transcriptional activity of CREB [58], NFAT [46], and DREAM [67] has been well described as dependent on nuclear Ca^{2+}. Therefore, agonist-induced nuclear Ca^{2+} movements are potentially expected to impact both hepatocyte progression in the cell cycle and more generally cellular homeostasis after PH.

It is important to realize that liver regeneration, seen as a process involving the whole organ—and even the entire organism—cannot be restrained to the sole hepatocyte division. In that view, after PH, Ca^{2+} signalling may also regulate physiological processes unrelated directly to cell cycle control, although they may ultimately interfere with cell cycle progression, not only in hepatocytes but also in other liver cell types (cholangiocytes, endothelial and Kupffer cells). Whereas we do not have any direct data about the impact of Ca^{2+} signals in nonhepatocytic cells after PH, we can easily anticipate about Ca^{2+}-dependent physiological processes that may be crucial in hepatocytes after PH. For example, glucose homeostasis, which regulation is critical for hepatocyte exit from quiescence [68], may depend on Ca^{2+} signals early after PH. Also, biliary homeostasis, which adaptation after PH is critical for liver regeneration [69, 70], may involve Ca^{2+}-dependent regulation [3, 71].

An integrated full picture of the "liver calcium signalling", which is obviously lacking, may improve our knowledge on the interaction network that regulates liver regeneration processes (Figure 1).

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Hindawi Publishing Corporation
International Journal of Hepatology
Volume 2012, Article ID 137676, 11 pages
doi:10.1155/2012/137676

Review Article

Regulation of Signal Transduction by Glutathione Transferases

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Received 9 July 2012; Revised 13 September 2012; Accepted 13 September 2012

Academic Editor: Pascal Loyer

Glutathione transferases (GST) are essentially known as enzymes that catalyse the conjugation of glutathione to various electrophilic compounds such as chemical carcinogens, environmental pollutants, and antitumor agents. However, this protein family is also involved in the metabolism of endogenous compounds which play critical roles in the regulation of signaling pathways. For example, the lipid peroxidation product 4-hydroxynonenal (4-HNE) and the prostaglandin 15-deoxy-Δ12,14-prostaglandin J_2 (15d-PGJ_2) are metabolized by GSTs and these compounds are known to influence the activity of transcription factors and protein kinases involved in stress response, proliferation, differentiation, or apoptosis. Furthermore, several studies have demonstrated that GSTs are able to interact with different protein partners such as mitogen activated protein kinases (i.e., c-jun N-terminal kinase (JNK) and apoptosis signal-regulating kinase 1 (ASK1)) which are also involved in cell signaling. New functions of GSTs, including S-glutathionylation of proteins by GSTs and ability to be a nitric oxide (NO) carrier have also been described. Taken together, these observations strongly suggest that GST might play a crucial role during normal or cancer cells proliferation or apoptosis.

1. Introduction

Glutathione transferases (GSTs) represent a major cellular defence system; they constitute a multigene family divided in seven families (Alpha, Mu, Pi, Theta, Sigma, Zeta, and Omega) with functions ranging from detoxification to biosynthesis and cell signaling [1, 2]. The most extensively investigated role of GSTs is their function of detoxification enzymes, where they catalyse the nucleophilic attack of glutathione (GSH) on electrophilic substrates. This mechanism allowed to protect a variety of cell components (protein, lipid, DNA) against reactive molecules such as electrophilic metabolites formed after xenobiotics phase I metabolism or endogenous α,β-unsaturated aldehydes and hydroperoxides formed as secondary metabolites during oxidative stress.

GSTs are also involved in metabolism of endogenous lipid mediators which influence diverse-signaling pathways. Among them, the 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 (15d-PGJ_2) regulates the activity of three transcription factors playing a central role in stress response, differentiation and proliferation: the peroxisome proliferator-activated receptor γ (PPARγ), the nuclear factor-erythroid 2 p45-related factor 2 (Nrf2), and the nuclear factor κB (NF-κB) [2]. Another one, the endogenous lipid peroxidation product 4-hydroxynonenal (4-HNE) is also believed to act as an intracellular signaling molecule [3]. Therefore, its conjugation with glutathione by GSTs will influence a number of pathways. Indeed, like 15d-PGJ_2, 4-HNE can stimulate gene expression through Nrf2 and prevent activation of NF-κB by inhibiting IκB phosphorylation. It has also been reported to modulate several cell-surface receptors, to activate epithelial growth factor receptor and platelet-derived growth factor-β receptor, and to upregulate transforming growth factor receptor β1 [4]. Altogether, these observations suggest that GSTs, which are involved in 4-HNE and 15d-PGJ_2 metabolism, will certainly influence many signal transduction pathways and modulate cell survival and proliferation.

During the last decade, research on GSTs has unravelled yet another major function, namely a role in regulating cellular signaling by forming protein-protein interactions with critical proteins involved in controlling stress response, apoptosis, and proliferation. For example, the ligand-binding capacity of GST results in the negative regulation of signaling pathways through sequestration of protein kinases.

Adler et al. [5] published the first study showing that mouse GSTpi interacts with the protein kinase c-jun N-terminal kinase (JNK). Dissociation of this complex by different types of stress leads to the activation of JNK and phosphorylation of its substrate, the transcription factor c-jun. Thereafter, other interactions have been identified and their implication in regulation of different biological processes has been demonstrated.

Another interesting function of GST, and especially of GSTPi, involved the regulation of a posttranslational modification of proteins, the S-glutathionylation and its implication in the protection against oxidative damage and the control of the redox signaling pathway. S-glutathionylation is characterized by the conjugation of GSH to low-cysteine sulfydryl or sulfonic-acid moieties in target proteins. Several studies have shown that various intermediates of signaling pathways controlling the survival/apoptosis mechanisms (p53, caspase 3,...) could be S-glutathionylated [6, 7]. Interestingly, these modifications seemed to modulate their activities.

Last, but not least, a thrilling new concept of NO stockage by GSTs have been brought up [8]. Indeed, several GSTs, and especially GSTP1-1, could bind NO under dinitrosyl iron complexes (DNICs). This binding seems to protect cells against high levels of DNICs, which are known to inhibit glutathione reductase, and to limit the peroxinitrite formation [9].

Taken together, these observations strongly suggest that GST might play a crucial role during normal, or cancer-cells proliferation or apoptosis. In this paper, we will focus on the major findings regarding the different modes of action of GST to regulate cell signaling, and we will give some examples demonstrating the involvement of GSTs in the regulation of hepatocyte proliferation and apoptosis.

2. 4-HNE, Cell Signaling, and GSTA4

4-HNE is a major product of the lipid peroxidation process that is characterized by peroxidative decomposition of polyunsaturated lipids. The mitogen-activated protein kinase (MAPK) pathways involved in cellular stress responses appear to be particularly sensitive to 4-HNE [4]. Indeed, the ability of 4-HNE to initiate increases in tyrosine phosphorylation is involved in the activation of c-jun N-terminal kinases (JNK) and p38 [10]. Both of them can regulate several transcription factors involved in cellular responses including cell proliferation, inflammatory responses, proteasome-mediated protein degradation and apoptosis. Many studies underlined concentration-dependent effect of 4-HNE on cell signaling pathways. A moderately high concentration of 4-HNE can induce apoptosis, differentiation, and affect activation of adenylate cyclase, JNK, protein kinase C, and caspase 3 [11, 12]. In contrast, a low concentration of 4-HNE can induce cell proliferation. Another study confirmed that 4-HNE has a dose-dependent effect, and a distinction could be made between a supraphysiological concentration (100 μM), which was primarily cytotoxic and a physiological range (below 10 μM) modulating cell growth [13]. These effects consist in a transient inhibition of the initial phase of cell growth, which under optimal conditions (in presence of serum) was followed by a period of increased proliferation, compared to untreated control cultures, until confluence was attained [13].

4-HNE also inhibits the expression of cyclin D1, D2 and A and, consequently, the activity of cyclin-dependent kinase 4/6 (Cdk4/6) and Cdk2 [14]. Interestingly, these Cdk-cyclin complexes are involved in the phosphorylation of retinoblastoma proteins, and therefore their partial inactivation, allowing the transcription of E2F-controlled genes and the progression in S phase. Moreover, 4-HNE upregulates the expression of $p21^{waf1}$ which is involved in the negative regulation of cyclin-Cdk complex protein kinase activities [15]. These findings show that 4-HNE can orchestrate the simultaneous expression of many different genes involved in the control of cell proliferation [16].

These observations clearly demonstrate that 4-HNE intracellular amount must be tightly controlled to prevent cellular damages and/or to regulate stress-response signaling. Although different enzymes such as alcohol dehydrogenase, aldolase reductase, or aldehyde dehydrogenase are involved in the metabolism of 4-HNE, the majority of 4-HNE is metabolized by GST, via its conjugation to GSH, which promotes its detoxification [18, 19]. In the liver, Kupffer and stellate cells have the capacity to metabolize 4-HNE, but to varying degrees compared to hepatocytes (100 times less efficiently for Kupffer cells than hepatocytes). The main GST involved in 4-HNE detoxification is GSTA4 [20, 21]. Interestingly, mGSTA4 was induced *in vivo* and in cultured hepatocytes by tumor necrosis factor α (TNFα), interleukin-6 (IL-6), and epidermal growth factor (EGF) [22]. All these factors that play crucial roles in hepatocyte survival and proliferation during liver regeneration. Moreover, *mGsta4* gene expression was increased at 1 and 24 hour post-partial hepatectomy (PH) compared with normal and sham-operated animals while a 3-fold increase in 4-HNE levels was observed 1 hour after PH [22].

Altogether, these studies demonstrate that the intracellular concentration of 4-HNE appears to be crucial for cell cycle signaling and may be a determinant for the signaling during differentiation, proliferation, transformation, or apoptosis. Importantly, the intracellular concentrations of 4-HNE are regulated by the action of GSTA4-4, which conjugates 4-HNE to GSH.

3. Modulation of 15d-PGJ_2 Signaling Pathway by GST

Prostaglandins (PG) are lipid compounds enzymatically derived from arachidonic acid that is released from the cell membrane phospholipids by phospholipase A2. Arachidonic acid is first metabolized by cyclooxygenase in PGG_2, which in turn is transformed in PGH_2 by PGH_2 synthase. PGH_2 is then conversed in other prostagladins (PGE_2, $PGF_2\alpha$, PGI_2, thromboxanes, PGD_2) by several specific synthases. These mediators are autocrine or paracrine molecules with local activities and involved a large panel of functions including inflammation, neuronal plasticity, and platelet aggregation.

Some of them (PGE_2, PGD_2, and $PGF_2\alpha$) are secreted and act by binding to a plasma membrane receptor.

Among the prostaglandin species, 15-deoxy-Δ^{12-14} prostaglandin J_2 (15d-PGJ_2) is a downstream metabolite of PGD_2 that acts by binding to intracellular receptors or transcription factors (Figure 1) [23]. Indeed, this compound owns an electrophilic α,β-unsaturated carbonyl group in its cyclopentenone ring, which can interact with cellular nucleophile groups such as thiols present in glutathione or cysteine. 15d-PGJ_2 biological effects are multiple. For example, it is a natural activating ligand of PPARγ [24]. After activation, PPARγ is heterodimerized with Retinoid X Receptor (RXR) leading to the induction of PPRE-driven gene expression. In the liver, the level of PPARγ is low, however it is implicated in several pathologies and its activation leads to a diminution of hepatocellular cancer growth by induction of cell apoptosis [25, 26]. Interestingly, binding of 15d-PGJ_2 to PPARγ in mouse liver results in the induction of hepatocyte growh factor (HGF) [27] and HGF induction is known to increase apoptosis and to decrease DNA synthesis in HepG2 [28]. A recent study has also linked the antineoplastic role of 15d-PGJ_2 in the HBV-associated HCC (Hepatitis B Virus-associated Hepatocellular Carcinoma) growth and the activation of PPARγ [29].

Furthermore, two different studies have suggested a potential role of 15d-PGJ_2 in hepatic cell proliferation. Cheng et al. [30] reported that the 15d-PGJ_2 was involved in the growth, cell cycle, and differentiation of hepatic oval cells, raising the possibility that the PPARγ ligands may regulate liver regeneration and hepatocarcinogenesis. In a second study, Yamamoto et al. [31] demonstrated that, during rat liver regeneration, the number of PPARγ-stained hepatocytes decreased 24 h after partial hepatectomy and increased in the late phase of liver regeneration compared to the sham-operated group. Moreover, the peaks of serum 15d-PGJ_2 level and hepatic PPARγ expression coincided with the late phase of liver regeneration [31]. These authors concluded that the PPARγ/15d-PGJ_2 system may be one of the key negative regulators of hepatocyte proliferation and may be responsible for the inhibition of liver growth in the late phase of liver regeneration.

15d-PGJ_2 has also been shown to inhibit the NF-κB signaling pathway [32]. In cells, NFκB is associated with IκB proteins in the cytoplasm in an inactive complex. After proinflammatory or growth factor stimuli, phosphorylation of IκB by IκB kinase (IKK) leads to its proteasomal degradation. These conditions allow the release of NF-κB, its phosphorylation and its translocation in the nucleus where, alone or in combination with other transcription factors, it induces target gene expression [33, 34]. The role of NF-κB in controlling cell cycle regulators, and more particularly cyclin D1, has been observed in investigations that used the IκB "super repressor" in order to inhibit NF-κB activity [35]. These findings suggest an important role for NF-κB in the regulation of cell cycle. Furthermore, NF-κB with upstream participation of TNFα, signaling through TNF receptor 1 (TNFR1) together with IL-6 and signal transducers and activators of transcription 3 (STAT3) is required for initiation of liver regeneration [36]. Several studies have shown that 15d-PGJ_2 is able to inhibit the NF-κB, targeting IKK by a covalent binding on a cysteine 179 (Cys-179) [32, 37]. 15d-PGJ_2 also directly inhibits binding of NF-κB to DNA-specific sequences by modifying the NF-κB Cys-38 [32]. Furthermore, Okano et al. [38] have observed that 15d-PGJ_2 suppressed NF-κB activation through independent PPARγ mechanisms in a hepatic cell line (SK-Hep1 cells). Interestingly, the same effects were observed in HepG2 cells, however, in this cell line the mechanism seems to involve the PPARγ activation.

15d-PGJ_2 can also stimulate Nrf2-mediated induction of gene expression through the antioxidant response element [39, 40]. Indeed, 15d-PGJ_2 is able to modify cysteine residues in the cytoskeleton-associated protein Keap1 (Kelchlike ECH-associated protein 1), and thus overcomes the ability of Keap1 to target Nrf2 for proteasomal degradation [41]. Therefore, conjugation of 15d-PGJ_2 with GSH abolishes its ability to modify Keap1. The regulation of Nrf2 by 15d-PGJ_2 might have important consequences in liver regeneration. Indeed, Beyer et al. [42] demonstrated impaired liver regeneration in Nrf2 knockout mice and revealed novel roles of Nrf2 in the regulation of growth factor signaling and in tissue repair. The same group showed that Nrf2 controls insulin receptor signaling in the regenerating liver [43]. Finally, a recent work has demonstrated that Nrf2 recognized a functional ARE (antioxidant responsive element) in the promoter of Notch1 that regulates processes such as proliferation and cell-fate decisions [39]. In this study, the authors have reported a functional role for this cross talk between the two pathways and show a delayed liver regeneration after partial hepatectomy in Nrf2 knockout mice that was rescued by reestablishment of Notch1 signaling. Taken together, these studies suggest that 15d-PGJ_2 could also modulate liver regeneration through the regulation of Nrf2.

Different studies have shown that GSTs are able to regulate the level of 15d-PGJ_2. Indeed, GSTs play a critical role at several levels in the synthesis and the degradation of this compound. GSTS1 has been identified as the prostaglandin synthase implicated in the production of PGD_2 (Figure 1), the metabolic precursor of 15d-PGJ_2 [44]. On the other hand, GSTA1, GSTM1, and GSTP1 have been shown to catalyze the conjugation of PGJ_2 with glutathione [45]. This conjugate is then eliminated by the MRP (Multidrug Resistance Protein) transporter. 15d-PGJ_2 is also metabolized via conjugation with glutathione in HepG2 cells [46], however, this conjugation can be observed in presence or absence of GST suggesting that the level of GSH in cell could modulate the action of 15d-PGJ_2 [47]. Kawamoto et al. [48] have observed that 15d-PGJ_2 is able to induce the GSTP1 in the R34 rat liver epithelial cell line through binding of different proteins, including c-jun, to a responsive element present in the GSTP1 5′-flanking region. On the other hand, 15d-PGJ_2 is able to directly posttraductionally modify GSTP1 to inhibit its activity. This covalent binding implicates alkylation of the Cys-47 and/or 101 [49]. Since GSTP1 is overexpressed in tumor cells and might be involved in anticancer drug resistance, 15d-PGJ_2 binding to GSTP1 could lead to the development of irreversible inhibitors in anticancer therapy. Interestingly, the binding or sequestration of 15d-PGJ_2 to

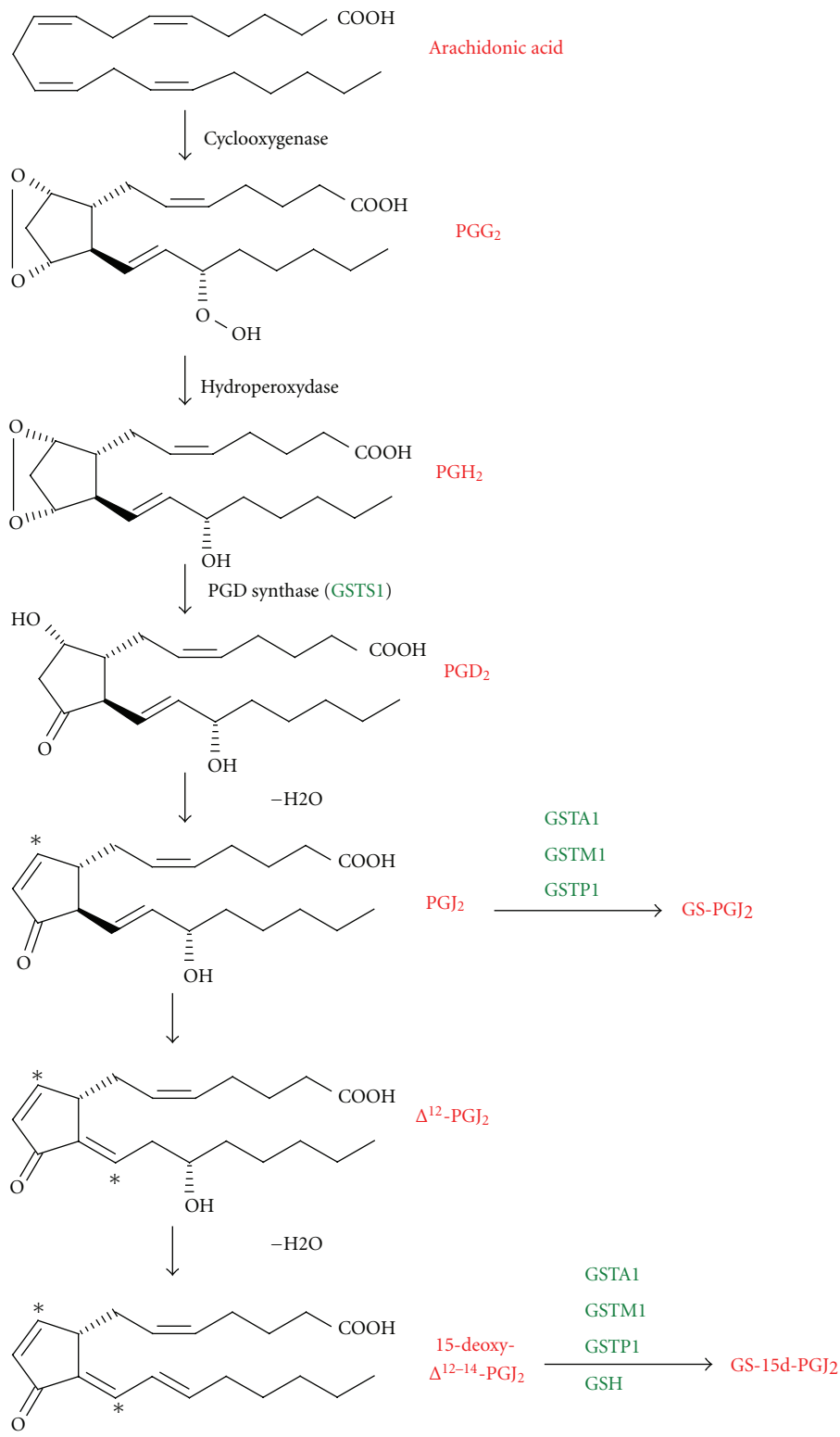

Figure 1: The prostaglandin biosynthetic pathway (adapted from [1]) 15-deoxyΔ^{12-14}-PGJ_2 is a metabolite derived from arachidonic acid. Several GSTs are implicated in the regulation of its formation: GSTS1 metabolized PGH2 in PGD2; GSTA1, GSTM1, and GSTP1 conjugated GSH to PGJ_2 and 15-deoxyΔ^{12-14}-PGJ_2. This conjugation led to the regulation of various transcription factors (PPARγ, NF-κB, and Nrf2).

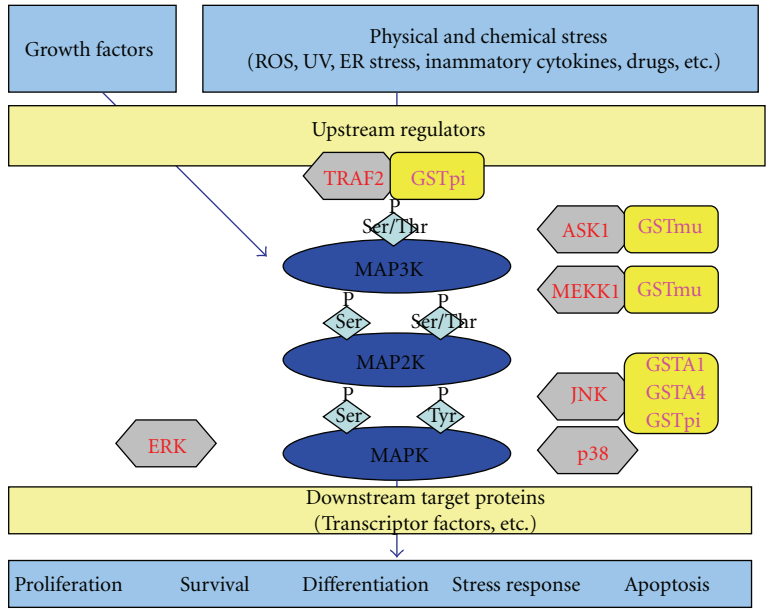

Figure 2: Scheme of the various interactions between GSTs and MAPK implicated in stress-signaling pathway (adapted from [17]). The mitogen-activated protein kinase (MAPK) family is composed of three types of kinases: MAP3K, MAP2K, and MAPK. In mammal, 3 major subgroups of MAPK are found: ERK, JNK, and p38. ERK is activated by proliferation and differentiation stimuli whereas JNK and p38 are preferentially activated by environmental stress. Upstream kinases (MAP3K, MAP2K) initiate activation of MAPK cascade in response to environmental changes and MAPK phosphorylate downstream targets such as transcription factors and generate appropriate biological response. Several GSTs are able to interact with various of these MAPK in nonstress conditions. Environmental stress leads to the disruption of these interactions and the activation of the signaling pathway. ROS: reactive oxygen species; UV: ultraviolet; ER: endoplasmic reticulum; TRAF2; TNF-receptor-associated factor 2; ASK1: apoptosis signal-regulating kinase 1; MEKK1: Mitogen-activated protein kinase kinase kinase 1; JNK: c-Jun N-terminal kinase 1; ERK: extracellular regulated kinase.

GST is also observed with GSTM1a and GSTA1 and inhibits the transactivation of PPARγ [50]. The ability of different GSTs to affect either synthesis, or elimination of 15d-PGJ_2 places GSTs as central regulators in cell signaling mediated by this eicosanoid.

4. GST-Protein Interactions and Cell Signaling

Cells are continuously exposed to external or internal stress which trigger signaling pathways and lead to the activation of several biological processes such as cell proliferation, differentiation, apoptosis or stress response. Control of these different pathways involves upstream activation of three protein kinase families: MAP3K, MAP2K and MAPK. Regulation of these protein kinases is complex and the existence of stress sensors. In the last decade, literature brought up the idea that GSTs could play such a role (Figure 2).

The first evidence for a direct interaction of a GST with another protein has been published by Adler et al. [5]. In this study, the authors demonstrated that mouse Gstpi interacts with JNK in mice 3T3/4A fibroblasts. Under a monomeric state, Gstpi acts as a direct JNK inhibitor in nonstressed cells by forming a complex with JNK and c-jun. Oxidative stress (UV, H_2O_2, etc.) induces the dimerization of GSTpi and activation of c-jun through its phosphorylation on Ser-63 and Ser-73 residues. Residues 194 to 201 (sequence SSPEHVNR) of Gstpi [4] and the C-terminal region of JNK [51] seem to be implicate in this interaction.

Subsequently, several other studies have corroborated this model. For example, Bernardini et al. [52] analyzed the correlation between the modulation of the GSTP1 expression, its dimerization and its catalytic activity following treatment of human leukemia Jurkat cells with agents known to induce apoptosis through a JNK-dependent signaling pathway. Results have shown that hydrogen peroxide (H_2O_2) and, to a lesser extent, etoposide lead to the activation of JNK pathway. This process was concomitant to the apparition of dimerized forms of GSTP1 owning disulphide bound between their Cys-47 and monomeric forms owning intrasubunit disulphide bound between Cys-47 and Cys-101. Furthermore, this dimerization is responsible for an inhibition of the GST activity which could be explained by the localization of these cysteines in the glutathione-binding domain of GSTP1. However, in a recent work, Gildenhuys et al. [53] have criticized this model. Indeed, using equilibrium folding and unfolding kinetic experiments as well as molecular modelling they brought the demonstration that binding with JNK involved the dimeric form of GSTP1-1. Thus, further works are necessary to determine the real mechanisms involved in these interactions. On the other hand, understanding of these processes is also complicated by the fact that different haplotypes of GSTP1 triggered

different effects. Indeed, two common functional variants of GSTP1 have been identified at amino 105 (Ile-Val) and 114 (Ala-Val). These variants lead to the existence of four haplotypes: the wild type GSTP1∗A (Ile^{105} + Ala^{114}), and three variants GSTP1∗B (Val^{105} + Ala^{114}), GSTP1∗C (val^{105} + Val^{114}) and GSTP1∗D (Ile^{105} + Val^{114}). GSTP1∗A has been shown to be able to slowdown cell's proliferation whereas the GSTP1∗C haplotype had no impact on this endpoint [54]. Furthermore, GSTP1∗A seems to be able to protect cells from apoptosis through a JNK-independent pathway while for GSTP1∗C this effect seems to be JNK dependent [54]. More recently, Thévenin et al. [55], have observed a higher inhibitory effect of GSTP1∗C on the phosphorylated isoforms JNK 1 and 2 compared to GSTP1∗A suggesting that these interactions depend on the activation's state of JNK. They have also demonstrated that interaction of phosphorylated JNK is enhanced in presence of ATF2, another substrate of JNK involved in oncogenesis, and that ATF2 is needed for the interaction of inactived JNK with GSTP1.

In vivo studies have also been performed and shown that in GSTpi−/− mice, JNK activity is constitutively enhanced, at least in liver, lung, and fibroblasts, and that, in such conditions, JNK-signalling pathway is upregulated triggering an increase in AP-1 DNA binding and HO-1 mRNA expression [56]. More recently, Castro-Caldas et al. [57] have observed, in a mouse Parkinson's disease model induced by a neurotoxin, that GSTpi−/− mice are more sentitive than wild-type mice to this stress. Indeed, in the midbrain and in the striatum, GSTpi seems to play the role of an endogenous regluator of the JNK signalling pathway by directly interacting with JNK.

Noteworthy, the direct interaction of JNK with GSTs is not limited to the GSTPi family. Indeed, Romero et al. [58] have shown that GSTA1 interacts physically with JNK in caco-2 cells. They showed that GSTA1 levels were lower in preconfluent cells than in postconfluent cells and they observed that response of caco-2 cells to a sodium butyrate JNK-dependent apoptotic stimulus was more important in preconfluent cells. In a different study, Desmots et al. [59] have established a correlation between phosphorylation of JNK and mGSTA4 upregulation under oxidative stress conditions and demonstrated that mouse GSTA4 and JNK coimmunoprecipitate in liver tissue extracts suggesting that mGSTA4 might be also an endogenous regulator of JNK activity by direct binding. Furthermore, these authors showed that hepatic mGSTA4 is strongly increased during oxidative stress possibly via JNK pathway and during proliferation via MEK/extracellular signal-regulated kinase pathway.

In 2001, Cho et al. [60] have shown by yeast two-hybrid technology that mouse GSTM1-1 is able to interact directly with ASK1, a protein kinase belonging to the MAP3K family. This interaction inhibits apoptosis signal regulated kinase 1 (ASK1)-mediated activation of JNK/SAPK signaling pathway induced by several stress stimuli such as H_2O_2 or UV when GSTM1-1 is overexpressed in cells. Therefore, it was suggested that GSTM1-1 has a role as an ASK1-repressor under unstimulated conditions. Furthermore, this role seems to be independent of the GST activity since mutant GSTM1-1 lacking catalytic activity also represses ASK1. The involvement of the C-terminal region of GSTM1-1 and N-terminal region of ASK1 in this interaction has been determined using truncated proteins [60]. Intriguingly, the same region of ASK1 interacts with thioredoxin (Trx) and it has been shown that, depending on the type of stress, ASK1 dissociates from GST or Trx suggesting the presence of a pool of ASK1-GSTM1-1 and ASK1-Trx complexes under unstressed conditions. Indeed, Dorion et al. [61] have observed that heat shock is able to disrupt the interaction between ASK1 and GSTM1 leading to the heat-shock-mediated p38 signaling activation, whereas no dissociations were observed between ASK1 and Trx under the same conditions. Furthermore, they observed that ROS exhibited the opposite effect, triggering dissociation between ASK1 and Trx with an activation of the p38 oxidative stress sensing pathway without any effect on the ASK1-GSTM1 complexes. Interestingly, Gilot et al. [62] have suggested that not only GSTM1, but also GSTA1 and GSTP1, could play a key role in regulation of ASK1 protein kinase activity in rat hepatocytes and thus on apoptosis.

GSTP1 is also able to block ASK1 activation by interacting physically with the Tumor necrosis factor receptor associated factor 2 (TRAF2) [63]. TRAF proteins associate with, and mediate the signal transduction from, members of the TNF receptor superfamily. For example, binding of TNFα on its receptors, TNF Receptor 1 or 2, leads to the homotypic aggregation of these receptors which results in the recruitment of several adaptors in the receptor cytoplasmic N-terminal domain. Among these adaptors, TNF-R1 associated death domain (TRADD) is able to recruit TRAF2 after TNFα-activation of TNF-Receptor 1, while a direct association between TNF-Receptor 2 and TRAF2 is observed. These interactions trigger activation of JNK and p38 signal pathways by a dissociation of the ASK1-Trx complex. Wu et al. [63] demonstrated that the binding of GSTP1 and TRAF2 triggers the suppression of TNFα-TRAF2-ASK1 signaling pathway activation. Similarly to the other interactions described previously, the activity of GSTP1 is not necessary for this binding, and the interaction between GSTP1 and TRAF2 is observed only in unstimulated cells.

Many other studies have confirmed the involvement of GSTs in cell signaling without performing direct-binding experiments: Ishisaki et al. [64] have shown that increasing expression in GSTP1 protects against dopamine-induced apoptosis in dopaminergic neurons by decreasing JNK activity; Elsby et al. [56] have demonstrated an increase in the constitutive JNK signaling in mice lacking GSTpi; and overexpression of hGSTA2-2 protects against apoptosis in K562 cells [65]. More recently, Piaggi et al. [66] suggested that overexpression of GSTO1-1 is associated with activation of survival pathway (Akt, ERK1/2) and inhibition of apoptotic signaling (JNK) as well as protection against cisplatin-induced apoptosis. Among these studies, experiments done by Yin et al. [67] are particularly striking. Using a GSTPi inducible expression vector in 3T3 cells, they have shown that GSTpi allows protection against H_2O_2-induced cell

death by coordinating an ERK/p38/IKK activation and a JNK suppression.

5. S-Glutathionylation

S-glutathionylation is a posttranslational modification of proteins characterized by the conjugation of GSH to a low pKa cysteine residues allowing a protection against oxidative stress. Even if *in vitro* studies have underlined that this process occurs spontaneously, several studies have shown that GSTPi could influence the rate of this reaction [68–70]. Thus, Townsend et al. [68] have observed that, under stress conditions, GSTPi can mediate a self S-glutathionylation on its Cys-47 and Cys-101 and that these modifications by interfering with the GSTPi/JNK complex lead to GSTPi aggregate's formation and JNK activation. Two other papers have reported that GSTpi is able to S-glutathionylate 1-Cys-peroxiredoxin (1-Cys-Prx) [69, 70]. 1-Cys-Prx belongs to the nonselenoperoxidase family and catalyzed the degradation of hydroperoxides to alcohols. The 1-Cys-Prx has a thioredoxin fold in the N-terminal region where a conserved cysteine residue is involved in the peroxidase activity. The oxidized 1-Cys-Prx intermediate must react with another thiol compound to regenerate the sulfydryl cysteine of the active 1-Cys-Prx. In their experiments, Ralat et al. [69] have shown that GSTPi is able to interact with the oxidized form of 1-Cys-Prx and to re-activate this enzyme by glutathionylation. This glutathionylation is followed by the formation of an intermolecular disulfide bond between the two subunits. Then, the GSH-dependent reduction of the disulfide regenerates the reduced active-site thiol.

Interestingly, the number of potential S-glutathionylated protein compared to the proteome is quite low as reported by Fratelli et al. [7] in hepatocyte after induction of an oxidative stress. However, more studies are necessary in order to understand the impact of GSTs on this post-translational modification and their role in the regulation of signaling pathway during oxidative stress.

6. GSTs as a NO Carrier

NO is a short-life messenger playing a role in both physiologic (by activating the soluble guanylate cyclase) and cytotoxic processes (e.g., such as inflammation). Interestingly, many of these effects are linked to its ability to interact with Fe(II). In tumor cells, this mechanism resulted in a rapid diminution of energy and DNA synthesis due to the loss of iron-containing enzymes. Furthermore, several studies have shown that interactions with iron-sulfur cluster in proteins lead to their degradations and to the formation of dinitrosyl dithiol iron-complexes (DNICs). At physiological concentration, these complexes are suspected to play the role of NO carrier, increasing its half life, and suggesting that the concept of NO as a free diffusible compound in cells need to be reevaluated. Furthermore, recent studies have shown that MRP1 transporter is able to release these complexes from the cells [71]. On the other hand, at cytotoxic concentrations, such as during chronic inflammation, these complexes, by sequestrating NO, could prevent its cytotoxic effect. However, when the concentration becomes too important, the system is overhelmed and a toxicity occurs. For example, NO is able to bind iron and 2 glutathiones in order to form the dinitrosyl-diglutathionyl-iron complex (DNDGIC) [8]. This leads to a depletion in glutathion and could represent a key signal trigerring apoptosis.

Several studies have shown that GSTs could bind DNICs. Thus GSTA1-1, GSTM1-1 and GSP1-1 are able to bind DNDGIC *in vitro* [72]. A crystal structure of the GSTP1-1-DNDGIC has even been obtained [73]. Tyr-7, in the active site of the GSTP1-1, coordinated to iron in DNDGIC displacing one of the GSH. More recently, Lok et al. [74] have suggested that GSTP1-1 acts to prevent NO-mediated iron released from MRP1 by sequestring DNICs. Thus a combinating effect of GSTP1-1 (storage of DNDGIC) and MRP1 (efflux of DNDGIC) seems to play a key role in cell protection against cytotoxicty.

7. Conclusion

Altogether, these observations clearly demonstrate that GSTs have roles beyond the simple detoxification reactions and seat themselves as crucial regulators of the stress kinase pathways. Among them, the GSTPi may be the most peculiar GST with its inhibitory role in various signaling pathways implicated in apoptosis or proliferation. Interestingly, GSTP1 is overexpressed in lung, ovary, pancreas, stomach, and colon cancers [1] and this high expression level has been correlated with resistance to several anticancer drugs. [75, 76]. In the light of the more recent works, interactions of GSTs with stress kinases could also be involved in such resistance mechanisms. Recently, Peklak-Scott et al. [77] concluded that the role of GSTP1-1 in cellular detoxification of cisplatin failed to totally explain resistance to this drug and that such mechanism should also involved the modulation of signaling pathways. Thus, strategies to prevent the apparition of multidrug resistance should aim at designing specific inhibitors able to disrupt interactions between GSTs and protein kinases. This approach has already been done by several authors [78–80]. However, in order to obtain these inhibitors, new studies are necessary to define the exact regions implicated in each interaction. On the other hand, GSTs role in the metabolism of endogenous compound such as 4-HNE or 15d-PGJ_2 or in the S-glutathionylation of proteins also indicates that GST levels might be critical in the control of cell signaling.

These specific functions of GSTs could lead to the development of new therapeutic approaches and to the identification of some interesting candidates for preclinical and clinical development.

Abbreviations

4-HNE:	4-Hydroxynonenal
15d-PGJ:	15-Deoxy-Δ-prostaglandin J
ARE:	Antioxidant responsive element
ASK1:	Apoptosis signal-regulated kinase 1
CDK:	Cyclin dependent kinase

DNIC: Dinitrosyl iron-complex
DNDGIC: Dinitrosyl-diglutathionyl-iron complex
GSH: Glutathione
GST: Glutathione transferases
HBV-associated HCC: Hepatitis B Virus-associated Hepatocellular Carcinoma
IL-6: Interleukin 6
JNK: c-jun N-terminal kinase
MAPK: Mitogen-activated protein kinase
MRP: Multidrug Resistance Protein
NF-κB: Nuclear factor κB
NO: Nitric oxide
Nrf2: Nuclear factor-erythroid 2 p45-related factor 2
PPARγ: Peroxisome proliferator-activated receptor γ
PG: Prostaglandin
ROS: Reactive oxygen species
TNFα: Tumor necrosis factor α
TRAF2: Tumor necrosis factor Receptor Associated Factor 2
Trx: Thioredoxin.

Funding

J. Pajaud and S. Kumar were funded by the Conseil Regional and the Ligue Contre le Cancer-Comité d'Ille-et-Vilaine. The team is financially supported by funding through Inserm and the University of Rennes 1.

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Hindawi Publishing Corporation
International Journal of Hepatology
Volume 2012, Article ID 689324, 17 pages
doi:10.1155/2012/689324

Review Article

Regulation of the G1/S Transition in Hepatocytes: Involvement of the Cyclin-Dependent Kinase Cdk1 in the DNA Replication

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Received 29 June 2012; Accepted 29 August 2012

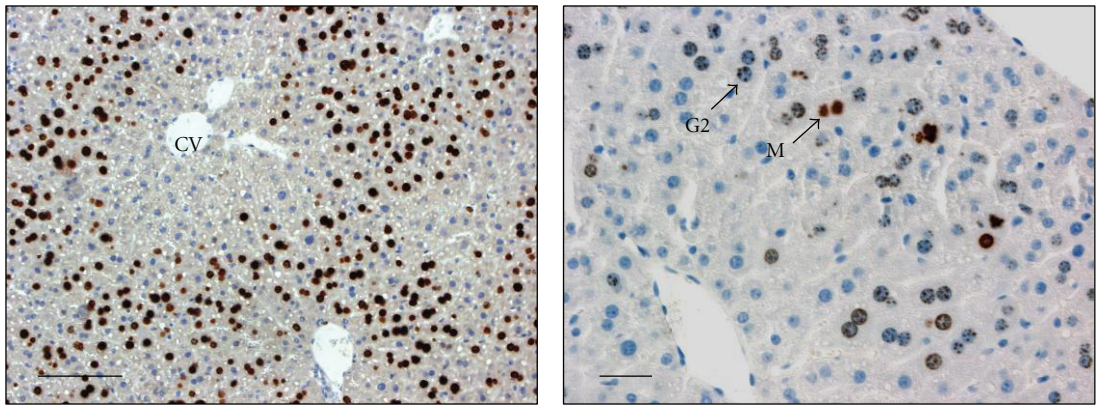
Academic Editor: Chantal Desdouets

A singular feature of adult differentiated hepatocytes is their capacity to proliferate allowing liver regeneration. This review emphasizes the literature published over the last 20 years that established the most important pathways regulating the hepatocyte cell cycle. Our article also aimed at illustrating that many discoveries in this field benefited from the combined use of *in vivo* models of liver regeneration and *in vitro* models of primary cultures of human and rodent hepatocytes. Using these models, our laboratory has contributed to decipher the different steps of the progression into the G1 phase and the commitment to S phase of proliferating hepatocytes. We identified the mitogen dependent restriction point located at the two-thirds of the G1 phase and the concomitant expression and activation of both Cdk1 and Cdk2 at the G1/S transition. Furthermore, we demonstrated that these two Cdks contribute to the DNA replication. Finally, we provided strong evidences that Cdk1 expression and activation is correlated to extracellular matrix degradation upon stimulation by the pro-inflammatory cytokine TNFα leading to the identification of a new signaling pathway regulating Cdk1 expression at the G1/S transition. It also further confirms the well-orchestrated regulation of liver regeneration via multiple extracellular signals and pathways.

1. Introduction

The cell cycle is highly conserved cellular process allowing a cell to divide in two identical daughter cells. Although mammalian cells show a higher degree of complexity, the molecular pathways controlling the progression throughout the cell cycle and both DNA replication and mitosis are relatively well conserved among eukaryotic cells [1]. The most conserved pathways of the cell cycle are probably DNA replication and major check-points for DNA integrity and mitosis. In contrast, more specific pathways control the transition from quiescence to DNA replication in eukaryotic organisms. In mammalian cells, specific combinations of extracellular signal stimuli induce the exit from quiescence, progression throughout G1 phase, and commitment to DNA replication. Proliferation stimuli include a vast superfamily of growth factors and cytokines activating downstream intracellular signaling pathways mainly through a cascade of phosphorylation and dephosphorylation events that ultimately triggers changes in gene expression in order to induce the proteins required for duplication of cellular components including DNA and the subsequent mitosis [2]. Among these protein kinases, the sequential activation of the cyclin-dependent kinases (Cdks) has been extensively characterized and plays a crucial role in regulating the entry into and progression through the cell cycle [3].

The discovery of the first Cdk, Cdk1 initially named cdc2 in yeast, has opened a large field of research leading to the identification of many cell cycle regulators and the pathways they are involved into. The first studies regarding the cell cycle regulation were conducted using cell models such as yeasts and oocytes from amphibians and marine organisms that synchronously progress throughout the different phases of the cell cycle in order to analyze expression and activation of regulators at each step of the cell cycle. From the mid-1970s to the late 1980s, the burst of data obtained in these eukaryotic cells leads to the identification of major cell cycle regulators including the cyclins [4] and their catalytic subunit partners the Cdks [3]. Mammalian homologs of these cell cycle regulators were subsequently isolated and by



(a) (b)

Figure 1: Sections of mouse liver evidencing detection of DNA replication and G2 phase. Mice were hepatectomized, injected at 46 hours after hepatectomy with BrdU, and killed 2 hours later (at 48 h). Livers were fixed for histological studies and detection of BrdU to visualize hepatocytes replicating DNA (a) or phosphohistone H3 (b) to detect cells in G2 phase. (a) This low magnification picture shows the detection of BrdU positive cells replicating DNA, illustrating that replicating hepatocytes are initially localized in the vicinity of the portal vein while around centrolobular veins (CV) only few hepatocytes replicate DNA at 48 h. (b) A higher magnification picture shows nuclei of hepatocytes reaching G2 phase (detection of phosphohistone H3 positive cells with punctuated nuclear signal: G2 and mitosis (M). Bars: 100 μm.

the mid-1990s a network of Cdk/cyclin complexes emerged 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 [5].

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 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 reenter the cell cycle for several rounds of division upon appropriate proliferation stimuli including lymphocytes [6] and fibroblasts [2] 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 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. Although the scoop of this paper 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 was poorly documented for many years. However, recent reports evinced differences between "arrested" cells in various conditions [7, 8]. 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 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. In this paper, we will focus on the peculiar regulation of the Cdk1 expression and activation during the hepatocyte cell cycle.

2. *In Vivo* and *In Vitro* Models of Synchronized Hepatocyte Proliferation

In contrast to other regenerating tissue, the liver regeneration process involves massive proliferation of differentiated hepatocytes in the remnant tissue (Figure 1). The liver regeneration is triggered experimentally by liver resection or by injection of hepatotoxic agent leading to cell death either by necrosis or apoptosis such as the thioacetamide [9] or CCl4 [10]. However, the most commonly used model of liver regeneration is the partial hepatectomy in rat or mouse. After 2/3 hepatectomy, liver regeneration begins with a first synchronous wave of hepatocyte proliferation, followed by sequential proliferation of biliary, kupffer, and endothelial cells [11, 12]. Proliferation of mature hepatocyte first occurs within the parenchyma in the vicinity of the portal triads and proceeds to the pericentral area close to the centolobular veins [13] (Figure 1). The unique ability of differentiated hepatic cells to exit from quiescence and reenter the cell cycle after a tissue loss has aroused numerous studies to identify exogenous factors triggering the liver regeneration and regulators of hepatocyte cell cycle progression. Both *in vivo* and *in vitro* models have been extensively studied for

identifications of the extracellular stimuli regulating cell cycle of mature hepatocytes and downstream signaling pathways.

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 nonhepatectomized animals [14]. Using primary culture of rat hepatocytes, HGF, TGFα, EGF, heparin-binding EGF-like growth factor (HB-EGF), and amphiregulin have been identified as potent hepatocyte growth factors [12]. However, the injection in rat of these growth factors does not induce massive hepatocyte DNA replication since normal hepatocytes *in vivo* are not able to respond to mitogenic signal without priming events allowing hepatocytes to become "sensitive" to growth factors. The proinflammatory cytokines TNFα and IL-6 are the early stimulus during the liver regeneration allowing the exit of hepatocytes from quiescence and the priming of hepatocytes [15, 16]. 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.

Then high levels of HGF are found in plasma around two hours after PH. This initiation phase controlled by proinflammatory 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 extent, 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 [17]. The timing of DNA replication and mitosis is however different between the two species. The peak of DNA synthesis is observed at 22–24 h in rat followed by a peak of mitosis at 28–30 h [18–22] while DNA replication occurs nearly 24 h later in mice. Seven days later, the liver has recovered nearly 70% of its initial mass.

Isolation of hepatocytes from rodent and human liver and establishment of *in vitro* culture systems have provided powerful experimental *in vitro* models to identify extracellular signals and to study intracellular signaling pathways regulating differentiation and controlling the ratio between proliferation and apoptosis in liver. Enzymatic liver dissociation triggers G0/G1 transition of quiescent hepatocytes, which progress up to and arrest in mid-G1 phase in absence of growth factors in primary culture [23, 24]. It has been proposed that rupture of cell-cell interactions [23] and induction of oxidative stress [25] or proinflammatory response [26] during liver dissociation could be responsible of hepatocytes reentry into the cell cycle, mimicking the effect of proinflammatory cytokines TNFα and IL6 which control the G0/G1 transition *in vivo* during liver regeneration [11, 27]. In agreement, we demonstrated that TNFα was released into the perfusion buffers during the *in situ* procedure of hepatocyte isolation by collagenase dissociation of the rat and mouse liver with various amounts ranging from 100 to 500 pg/mL (Corlu A and Loyer P, unpublished data).

In pure culture of hepatocytes, expression of liver specific functions progressively decreases and apoptosis eventually occurs within a week through the activation of caspases 3, 8, and 9 in hepatocytes [28–30]. Nevertheless, this *in vitro* culture model has been very useful to identify survival factors and mitogens based on their ability to induce DNA replication. In pure culture of rat hepatocytes, addition of 25 ng/mL of EGF in the culture induces a robust and partially synchronized DNA replication followed by the mitosis (Figure 2). Using this synchronous primary model, our laboratory and others investigated cell cycle regulation Cdk/cyclin expressions and activations [12, 31–33].

More recently, we used a coculture model associating rat hepatocytes with rat liver epithelial cells (RLEC also called BEC for biliary epithelial cells), in which heterotypic cell-cell contacts are restored and a spontaneous early production and deposition of extracellular matrix are observed [34–36]. This coculture model (Figure 3) compared to the pure culture of hepatocytes exhibits numerous advantages: adult hepatocytes remain highly differentiated for several weeks [37] and are unable to proliferate under EGF or HGF stimulation alone as in liver tissue [38]. Therefore, based on the data obtained *in vivo*, we successfully designed a stimulation procedure allowing multiple hepatocyte division cycles without loss of differentiation [39]. In this coculture system, differentiated and quiescent hepatocytes are able to proliferate under costimulation with TNFα and EGF or HGF.

This co-stimulation with TNFα and EGF leads to proliferation of nearly all the hepatocytes over a week [39]. Three days after TNFα/EGF or TNFα/HGF stimulation, at least 35% of hepatocytes divide whereas no DNA synthesis is observed in presence of HGF or EGF alone. Both mono- and binuclear hepatocytes progressed up to mitosis and cytokinesis allowing the significant expansion of hepatocyte colonies. These results are in agreement with *in vivo* experiments, in which coinjection of TNFα and growth factors induced hepatocyte proliferation in absence of partial hepatectomy [15]. Moreover, TNFα alone did not induce hepatocyte proliferation in coculture as observed *in vivo* [15] and in long-term DMSO cultures [40]. Remarkably, hepatocytes gradually stop synthesizing DNA even under prolonged TNFα/EGF stimulation. We demonstrated that a cell cycle arrest following the first wave of divisions is essential for inducing a second round of proliferation. Although cells do not proliferate in a synchronous manner in this coculture model, this *in vitro* cell system mimics the behavior of the hepatocytes in the whole liver and was used to investigate the involvement of cell-cell and cell-matrix interactions in the regulation of the hepatocyte cell cycle.

3. The G1 Phase and the Mitogen-Dependent Cell Cycle Progression

Nearly three decades ago, the *in vitro* synchronized fibroblasts allowed to distinguish different steps in the G1 phase

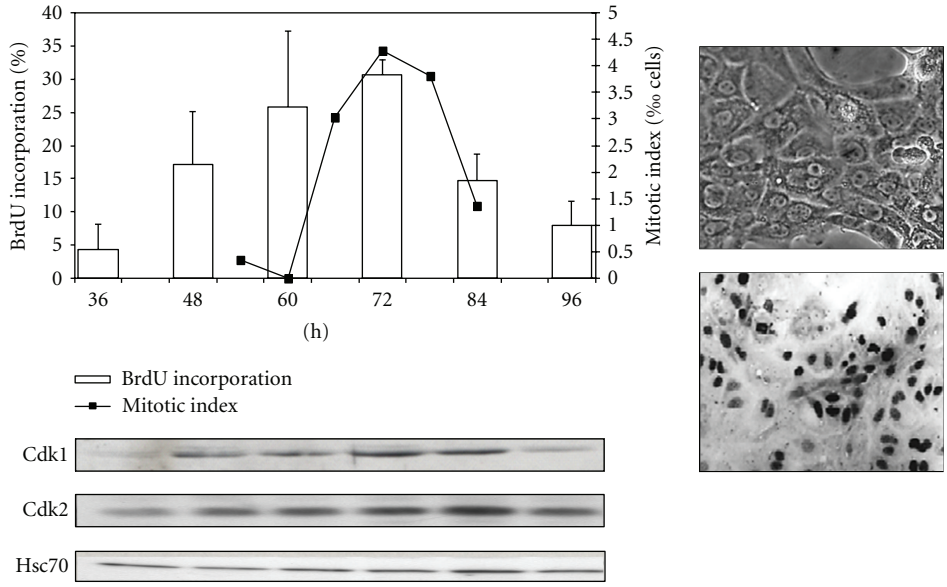


Figure 2: Proliferation of rat hepatocytes in pure culture. Isolated rat hepatocytes (right, picture in phase contrast) seeded at low density ($<5.10^4$ cells/cm^2) and stimulated with EGF (25 ng/mL) commit to DNA synthesis (BrdU incorporation, histogram and *in situ* immunodetection of BrdU positive cells) and complete the cell cycle (mitotic index, right axis). Western blotting of Cdk1 and Cdk2 and the loading control Hsc70.

progression and to define the concept of "restriction point" [41]. The progression through the G1 phase can be divided in several periods, which are different between cell types. For instance, the progression of fibroblasts throughout G1 could be divided in 4 periods: competence, entry, progression, and assembly (Figure 4). The stimulation of starved fibroblasts by PDGF promotes progression in early G1 until the restriction point C, defining the so-called competence, but fails to allow progression in mid- and late G1 [42–45]. Then the progression in late G1 and S phase can be achieved by subsequent stimulation with EGF or insulin [46, 47]. 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 [48] while the progression between point "V" and the mitogen-dependent restriction point (point "R") is called progression. Finally, the period beyond the mitogen-dependent restriction point and before the burst of DNA synthesis is named assembly [49]. A minimal period of stimulation is required to reach the late G1 and, beyond this point, the cell cycle is 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 [50]. It is essential to distinguish the G1 progression between cells that proliferate actively and enter G1 after completion of mitosis and cells reentering the cell cycle after a prolonged quiescence or G0. The transition from G0 to G1 is characterized by a profound modification of the expressed gene profile [2] 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.

Primary cultures of rat and mouse hepatocytes were widely used to analyze hepatocyte cell cycle entry and progression through the G1 phase. Our group has shown that during cell isolation rat hepatocytes expressed immediate early protooncogenes such as c-fos and c-myc suggesting a "spontaneous" G0/G1 transition following cell-cell interaction destruction [23]. On the other hand, it had been also demonstrated that rat hepatocytes in pure culture undergo DNA replication when they were stimulated by growth factors alone [51, 52]. Thus, we hypothesized that hepatocytes were arrested in G1 phase in absence of growth factors and that by comparing unstimulated and stimulated hepatocyte it should be possible to characterize the different steps of G1 phase in hepatocytes [24]. 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 mitogen-dependent restriction point (R point) located to mid-late G1 was shown (Figure 5). Indeed, in absence of growth factor and serum, hepatocytes are able to progress up to mid-late G1 phase as shown by the sequential overexpression 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 Cdk1 are not expressed. Moreover, the progression towards the G1/S is strictly dependent upon the stimulation by growth factor. To further demonstrate the mitogen-dependent restriction point, we hypothesized that if the addition of EGF was performed at any time point before cells had reached the R point, the onset of DNA synthesis would not be affected. In contrast, if the addition of EGF occurred after cells had reached the R point, a delay in the onset of DNA synthesis should be observed. The hypothesis was experimentally confirmed: when addition of EGF occurred at different times but prior to 42 h after hepatocyte seeding, DNA replication

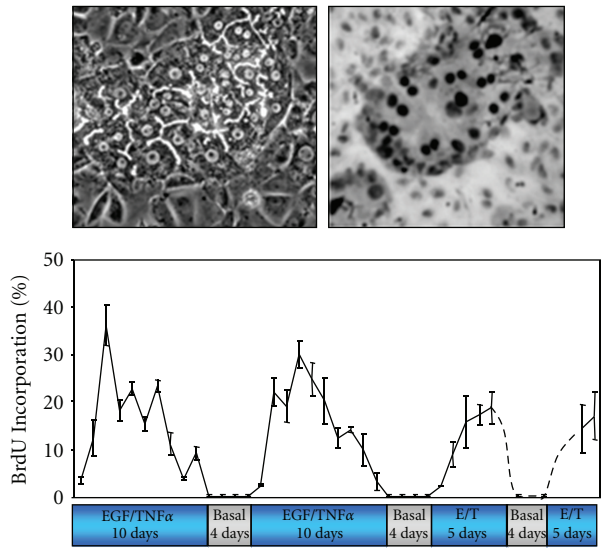


Figure 3: Proliferation of rat hepatocytes in coculture. Upper left: phase contrast picture of a colony of rat hepatocytes surrounded by Rat Liver Epithelia Cells (RLECs). Upper right: indirect immunodetection of BrdU positive hepatocytes evidencing DNA replication within the hepatocyte colony. Chart: multiple waves of replication in hepatocytes maintained in coculture over 52 days. Four periods of stimulation using EGF and TNFα (E/T) were separated by culturing the cells in basal medium lacking the mitogenic cocktail. The BrdU was incorporated for 24 h and at each time point triplicate cultures were fixed and stained for BrdU detection.

took place at the same time (48–60 h) while for delayed stimulations the onset of DNA synthesis was postponed (Figure 5). A lag phase between the R point and the onset of the DNA synthesis appeared to be approximately 12–18 h.

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 10–15 h later. In accordance with these observations, accumulation of cyclin D1 is also detected when the hepatocytes are stimulated by HGF [53]. 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 rat hepatocytes *in vivo* was also observed [54]. To reach that conclusion, we first analyzed the expression of cyclin D1 during liver regeneration and showed its induction at 12 h after 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 15 h after PH and showed that only those isolated from 12–15 h 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 10.5 h 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 [55]. 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 antiproliferative response related to marked upregulation of $p21^{Cip1}$ represses cyclin D1/cdk4- and cyclin E/cdk2-dependent kinase activities. More recently, using mice carrying a floxed *EGFR* allele to inactive the EGF receptor, Natarajan et al. [56] 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.

Similar studies were performed in many other cell models leading to the conclusion that in all cell types the G1 phase could be divided in subphases corresponding to major steps in the metabolic adaptation required for cells to replicate DNA and divide. However, for each cell types, specific growth factors and signaling pathways are involved. Among the soluble factors inducing proliferation, the "priming" factors promote in early G1 while combination of cytokines and growth factors stimulates progression in late G1 and the G1/S transition. Then, following binding to their receptors, priming and growth factors activate multiple phosphorylation events involving multiple protein kinases especially the MAPKinase pathways [57, 58]. Moreover, multiple crosstalks between these pathways exist and lead ultimately to the activation of transcription factors that sequentially trigger induction of cell cycle regulators such as the cyclins and Cdks.

4. The Cell Cycle Is Regulated through the Sequential Activation of Cdk/Cyclin Complexes

Progression of eukaryotic cells through the cell cycle is regulated by the sequential formation, activation, and subsequent inactivation of structurally related serine/threonine

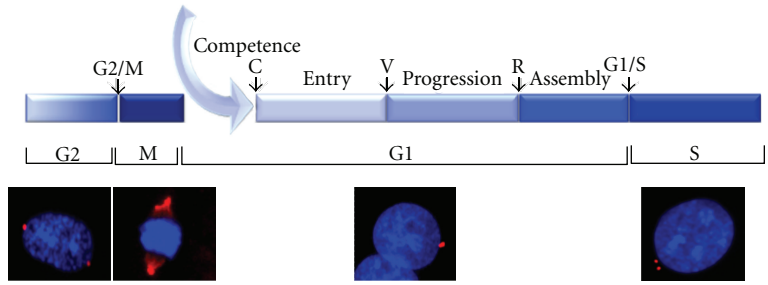


Figure 4: Progression through the G1 phase is divided into several subphases. Photographs illustrate detection of cells in G2, M, G1, and S phases: the cells were stained with DAPI (DNA) and indirect immunofluorescence detection of γ-tubulin in centrioles was used to discriminate between cells in G1 phase (a single centrosome), S phase (two centrosomes side by side), G2 (two centrioles on each side of the nucleus), and M (centrioles pulling apart the chromosomes). Four steps were identified during the G1 phase of the cell cycle: competence, entry, progression, and assembly.

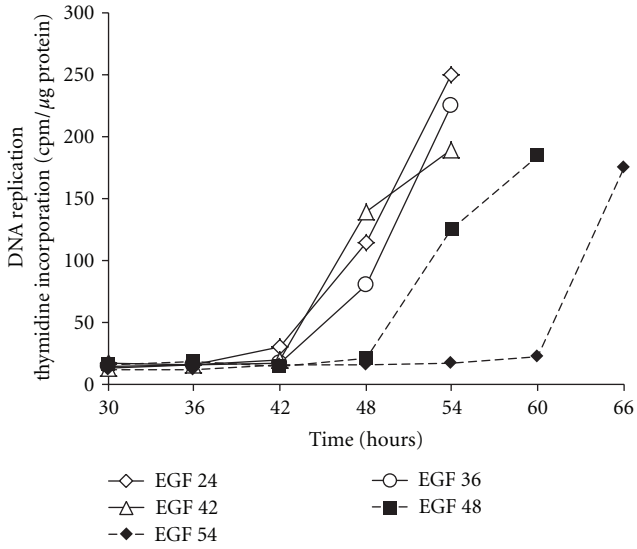


Figure 5: Evidencing the R point in primary rat hepatocytes. In absence of EGF rat hepatocytes do not replicate DNA. However, they sequentially express early G1 phase markers strongly suggesting a cell cycle arrest in midlate G1 phase. The mitogen-dependent restriction point was localized by performing stimulation with EGF at different time points (24, 36, 42, 48, 54 h) after seeding the hepatocytes. Then the DNA replication was monitored by measuring the incorporation of radiolabelled thymidine into the hepatocyte DNA. For stimulations between 24 and 42 h, DNA replication began between 48 and 54 h. When cells were stimulated at 48 or 54 h, DNA replication was significantly delayed demonstrating that the progression in G1 phase regardless of stimulation by EGF ended around 42 h and that the progression beyond this point required a mitogenic stimulation.

protein kinases, the cyclin-dependent kinase or Cdks. In mammalian cells at least 20 Cdks, 5 Cdk-like protein kinases [3], and more than 30 cyclins have been identified which form multiple Cdk/cyclin complexes controlling the cell cycle progression [59] and regulating gene transcription and RNA processing [60]. 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.

For many years, the G0/G1 transition and progression in early G1 phase was thought to occur in a Cdk/cyclin independent manner. Following stimulation by priming factors, immediate early genes are induced at a transcriptional level by preexisting latent transcription factors such as NF-κB [61]. While cells leave quiescence to enter G1, the phosphorylation level of pocket protein family members varies [62] and inactivation of pRb is shown sufficient to induce G0/G1 transition in quiescent cells [63]. Ren and Rollins postulated that hypophosphorylated or unphosphorylated pRb present in glioblastoma T98 G0-arrested cells may be phosphorylated by Cdk3/cyclin C complexes to promote entry into G1 phase [64]. However, most cells lack functional Cdk3 and no conclusive data on the ubiquitous role of Cdk3/cyclin C complex at the G0/G1 transition have been drawn. More recently, it was reported that Cdk2 interacts with cyclin C in early G1 [65, 66] to phosphorylate the transcription factor LSF (late simian virus 40 factor) [67]. Phosphorylation of LSF on serine 291 by the MEK/extracellular signal-regulated kinase (ERK) signaling pathway upon stimulation by growth factors [58, 68, 69] 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*) [70]. In contrast, phosphorylation of LSF on serine 309 inhibits LSF transactivation suggesting the required LSF shutdown in early G1 and its reactivation in late G1 mediated by Cdk/cyclin complexes and ERK, respectively [65]. This work appears important because it suggests a possible involvement 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 late G1 phase (Figure 6).

The signaling pathways essential for the subsequent progression in late G1 are much more documented and clearly involve the Cdk/cyclin complexes [71]. The transition from mid- to late G1 phase is regulated by sequential phosphorylation events of members of the pocket protein

family including the retinoblastoma protein (pRb), p107, and p130 [62] by Cdk/cyclin complexes [4, 72]. In mid-G1, the hypophosphorylated pRb is bound 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 [73]. Upon stimulation by growth factors, D-type cyclins are upregulated [74] and associate with Cdk4 and/or Cdk6 to form active complexes [75, 76] that partially phosphorylate pRb and/or actively phosphorylate a fraction of pRb [72]. In late G1, formation of Cdk2/cyclin E complex triggers additional phosphorylation of pRb to generate the hyperphosphorylated form of pRb (Figure 6) that loses the ability to repress the transactivation domain of E2F's factors [77]. Consequently, the release of E2F proteins promotes transcription of a large set of genes required for the progression in late G1 including Cdk2 and cyclin E [78, 79], S phase entry [80–82] and centrosome duplication [83]. In parallel, Cdk2 phosphorylates the nuclear protein ataxia-telangiectasia implicated in the transcription of histones [84] and the nucleophosmin/B23 regulating centrosome duplication [85]. At this stage of the cell cycle progression cells have committed to DNA replication. Thus, turning on the E2F-dependent transcription coincides with the progression beyond the mitogen-dependent restriction point identified by Pardee and coworkers [49] before the discovery of Cdk/cyclin complexes.

Importantly, single or combined genetic alterations in mice of Cdk4/6-Cyclin D, Cdk2-Cyclin E, p27Kip1, and Rb do not affect early embryogenesis highlighting multiple compensatory mechanisms and overlapping role of these genes introducing the notion of redundancy and flexibility of the cyclin/cdks [86, 87] The analysis of the cell cycle in MEFs derived from these knockout mice indicated compensatory mechanism between positive and negative regulators at the G1/S transition and highlighted a complex network regulating the expression and activation of these cell cycle regulators in the progression from G1 to S phase. For instance, mouse embryos lacking all interphase Cdks (Cdk2, Cdk3, Cdk4, and Cdk6) undergo organogenesis and develop up to midgestation. In these embryos, Cdk1 binds to all cyclins, resulting in the phosphorylation of the retinoblastoma protein pRb and the expression of genes that are regulated by E2F transcription factors [88]. Interestingly, cyclin A ablation in fibroblasts did not affect proliferation but led to prolonged expression of cyclin E whereas its expression is essential for cell cycle progression of hematopoietic cells and embryonic stem cells [89]. Therefore, compensatory mechanisms and overlapping role of Cdks exist but vary between cell types.

The *in vivo* model of regenerating liver 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 [31, 90–92]. Although Cdk2 is

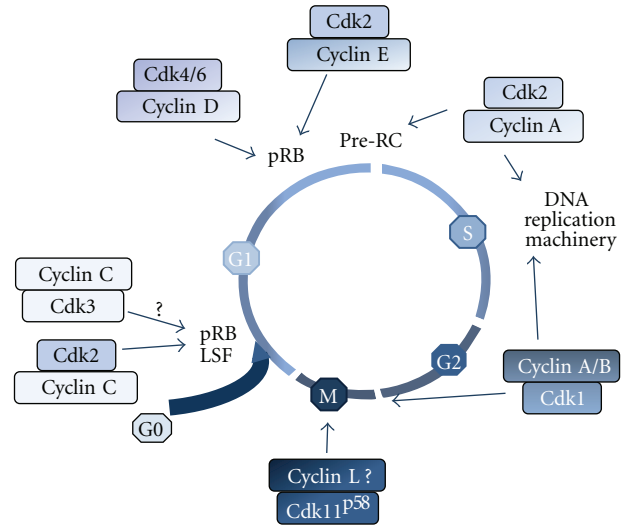


Figure 6: Sequential activation of Cdk/cyclin complexes throughout the cell cycle.

constantly expressed, Cdk1 is completely absent in resting hepatocytes and remains undetectable up to 20 h after PH a time corresponding to late G1 phase and G1/S transition. In quiescent hepatocytes, Jaumot et al. [93] 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 [94]. Around 13 h after PH, cyclins D3 and Cdk4 translocate in the nucleus and significant amounts of cyclin D1/Cdk4 and cyclin D3/Cdk4 complexes are formed but remain inactive whereas at 24 h they are fully active. At 13 and 24 h, cyclin E is detected in both cytoplasm and nuclei. Then, the activity of Cdk4 decreases at 28 h when cyclin D1 translocates to the nuclear matrix and the levels of cyclin D3 diminishes. Similarly, the inactivation of Cdk2 at 28 h 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 13 h after PH while following its strong induction in S phase, cyclin A is present in both cytoplasm and nuclei at 24 and 28 h. Therefore, the specific nuclear localization of the complexes is associated with their activity in liver regeneration. The maximal activity of Cdk2 detected at 24 h comes from cyclin E/Cdk2 and cyclin A/Cdk2 complexes whereas the activity at 28 h is mainly attributable to the Cdk2/cyclin A heterodimer. However, the activity of Cdk2 rapidly decreases after the peak of DNA synthesis at 24 h.

The Cdk inhibitors (Cdki's) are involved in cell cycle regulation following antagonist mitogenic and antimitogenic signals [95, 96]. 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 [96]. The presence of inactive cyclin D/Cdk4 complexes during mid-G1 phase post-PH and Cyclin E/cdk2 at 28 h has led authors to investigate the modulation of

Cdk kinase activities during rat liver regeneration. During rat liver regeneration, $p27^{Kip1}$ is associated with inactive cyclin D/Cdk4 complexes [93]. Furthermore, Pujol et al. [94] have demonstrated that high amounts of $p27^{Kip1}$ bind to Cdk2/cyclin E complexes in early and mid-G1 post-PH concomitantly with low Cdk2 kinase activities. At 24 h, 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 $p21^{Cip}$ associated with these complexes is low during the first 13 h and subsequently increases. At 24 h 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 28 h after the peak of hepatocyte DNA synthesis. Albrecht et al. [97, 98] confirmed these data and showed that expression of $p21^{Cip1}$ is induced during the prereplicative phase and is maximal after the peak of hepatocyte DNA synthesis in mice. In contrast, $p27^{Kip1}$ is present in quiescent liver and slightly induced after PH. Immunodepletion experiments suggested that $p27^{Kip1}$ plays a role in downregulating Cdk2 activity before and after the peak of DNA replication. Interestingly, study of liver regeneration in mice lacking $p21^{Cip1}$ indicated a marked acceleration of hepatocyte progression into the cell cycle. DNA synthesis, upregulation 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 $p21^{Cip1}$ knockout mice. These results demonstrate the role of $p21^{Cip1}$ in the regulation of the hepatocyte progression through G1 phase *in vivo*. Unexpectedly and again in contrast with the current model of mammalian cell cycle regulation, we observed that Cdk1 accumulates in S, G2, and M phase, in proliferating hepatocytes and is active during both S and M phases while one peak of Cdk2 activity is detected in S phase only [90].

5. Involvement of Cdk1 during the S Phase and G2/M Transition

In eukaryotic cells, chromosomal DNA replication is ensured through periodic and tightly controlled assembly and disassembly of prereplication complexes (pre-RC) loaded on DNA replication origins [99, 100]. 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 minichromosome maintenance (MCM) 2–7 subunits to form the pre-RC [100]. Interestingly, loading of the pre-RC components occurs in a low Cdk activity period [101] 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 [102–104]. 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 [100, 105]. These well-documented mechanisms clearly point out the crucial role of Cdk/cyclin complexes in the regulation of pre-RCs formation. Similarly, pre-RC are activated by phosphorylations involving the protein kinase Cdc7 and the Cdk2/cyclin E complex which trigger the recruitment of Cdc45 [106], a crucial docking factor for DNA helicase and polymerases. During S phase, the heterodimer Cdk2/cyclin A also contributes to DNA replication [107–109] 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 in S phase (Figure 6). 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 [83]. Duplication of centrioles is in part regulated through the G1 phase Cdk/cyclin-dependent pRb pathway [110], and there is a large body of evidence for the Cdk2/cyclin E involvement in the activation by phosphorylation of crucial regulators of centriole duplication [83].

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 [111]. The activity of the Cdk1/cyclin B complex, which was the first cyclin-dependent kinase activity detected in sea urchin and in *Xenopus* [112, 113], rapidly appeared to be a master regulator of the G2/M transition, in all eukaryotic cells [111] including in humans cells [114]. Recently, the $Cdk11^{p58}$ protein kinase was also shown to be essential for mitosis [115, 116] most likely associated to the cyclin L's [117]. 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 [106, 118].

This model of cell cycle control has first been challenged by the finding that some cancer cells proliferate despite Cdk2 inhibition [119]. Independently, there was a demonstration that knockout 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 [120–122]. In addition, in the hippocampus of $Cdk2^{-/-}$ mouse, the proliferation of granule neurons of the dentate gyrus which undergo continuous renewal throughout life, is not altered [123]. Similarly, hematopoiesis is not affected in Cdk2 knockout mice [124].These data indicated that Cdk2/Cyclin E complexes were dispensable for commitment to S phase. Along the same line, a Cdk1-dependent compensatory mechanism regulating S phase initiation and progression was also demonstrated in DT40 chicken cells lacking Cdk2 [125]. 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 [118, 126]. Interestingly, it was recently demonstrated that both Cdk1 and Cdk2 were required for efficient DNA replication in *Xenopus* egg extracts [127] suggesting that, at least in some nongenetically modified cell types, Cdk1 could contribute to S phase initiation

and/or DNA replication. This idea was reinforced by the observation that enforced expression of constitutively active Cdk1 mutant in HeLa cells results in abnormal origin firing and premature DNA replication in early S phase and that a loss of Cdk1 activity compromised activation of late origins at late S phase [103]. 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 [71, 128].

In the light of the recent findings showing compensatory involvement of Cdk1 at the G1/S transition in Cdk2 knockout mice and our data showing that Cdk1 was observed *in vivo* and *in vitro* at the G1/S transition in hepatocytes [90, 91], we have further investigated the role of Cdk1 in normal adult rat hepatocytes in the commitment to S phase. Cdk1 is barely detectable in quiescent hepatocytes and during G1 phase but expressed at high levels in S phase while Cdk2 is constantly expressed (Figure 2). Both Cdk1 and Cdk2, associated with cyclins A and/or B, are activated during DNA replication in regenerating rat hepatocytes [33]. We demonstrated that Cdk1 activity is twice higher than Cdk2 activity during S phase in hepatocytes. Then, knockdown experiments of Cdk1 and/or Cdk2 were performed in isolated hepatocytes and human foreskin fibroblasts (HFFs) which express high and low Cdk1 levels during S phase, respectively. SiRNA-mediated repression of Cdk1 and Cdk2 significantly decreased DNA replication in hepatocytes. 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. In hepatocyte, the activation of Cdk1 in early S phase is further demonstrated by showing that hepatocytes arrested after G1/S transition prior to DNA replication by the iron chelator O-Trensox express fully active Cdk1 and Cdk2 [33]. Moreover, the decrease in DNA replication after knocking-down Cdk1 or Cdk2 silencing is not due to impaired formation of the prereplication complex since Mcm7 is localized in the nucleus and loaded onto chromatin. 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 and prior the Cdk-dependent kinase activity taking place in early S phase. Thus, Cdk1 may be involved in the origin firing events downstream the formation of replication complexes in hepatocytes, in agreement with a recent study suggesting that cyclin A2–Cdk1 might function as a transregulator of late origin firing in mammals or Cdk1 is required for proper timing of origin firing [103].

These data further support and extend the conclusion that Cdk1 compensates for Cdk2 gene ablation in genetically modified mice. Indeed, we have shown the involvement of Cdk1 in S phase of normal and nongenetically modified mammalian cells. More precisely, both Cdk1 and Cdk2 play a critical role in hepatocyte cell cycle. Consistent with our observation, Satyanarayana et al. [129] showed that the timing of S phase is not altered in regenerating livers of $Cdk2^{-/-}$. 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 [130]. These data indicated that Cdk2 expressed from the *Cdk1* locus is able to mimic the cell function of endogenous Cdk2 and restore normal regeneration process and that one copy of *Cdk1* is sufficient for a normal liver response after PH. In addition, Hanse et al. [131] showed that 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. Very recently, Diril et al. [132] have shown that the conditional knockout of Cdk1 in adult mouse liver does not impair S phase but results in DNA rereplication and a strong decrease in cytokinesis associated with an increase in Cdk2/cyclin A2 activity. The increase in ploidy and reduced cell number suggest that Cdk1 may not be directly involved in DNA replication but would regulate Cdk2 activity and termination of DNA replication and play a major role in mitosis.

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, cyclin A, and unexpectedly cyclin B1 are localized in the nucleus of replicating cells hepatocytes and form active complexes during S phase in regenerating hepatocytes. In most mammalian cells, Cdk1/Cyclin B1 complexes localize in the cytoplasm during G2 phase [133] and are activated through a positive feedback [134] to phosphorylate cytoplasmic substrates. Then the translocation to the nucleus triggers the breakdown of nuclear envelops and mitosis. The absolute requirement of cytosolic cyclin B1 during initiation of mitosis has been proposed; however, it has also been postulated that relocating cyclin B1 to the nucleus in S phase might compromise entry into mitosis [135]. This would explain why the accumulation of nuclear Cdk1/cyclin B1 complexes during DNA replication does not trigger premature mitosis in hepatocytes. Moreover, P-Tyr15 Cdk1 found in replicating hepatocytes and known to be an inactive form of Cdk1 could also participate to this control. Noteworthy, 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. In addition, several data highlight the role of Cdk2 in hepatocyte progression and survival following acute mitogenic stimulation [131]. Moreover, the role of Cdk2 in proper DNA repair was reported [136] and strongly suggested that Cdk2 could be a sensor able to distinguish between moderate and extensive DNA damage to promote either survival or apoptosis. Several studies have reported that Cdk1 associated with cyclin A2 or cyclin B1 was active during S phase in proliferating hepatocytes. These reports are in disagreement with numerous studies demonstrating the activation and nuclear import of Cdk1 and cyclin B1 at the G2/M transition in most cell types. Further experiments are required to address whether Cdk1 and cyclin B1 exhibit

a specific pattern of expression and activation during the cell cycle of the hepatocytes and to determine their role during S phase.

6. Extracellular Matrix Remodeling and Cdk2 Regulate Cdk1 Expression and Activation

In normal liver, adult hepatocytes quiescent and normally do not undergo cell division but keep the ability to proliferate in response to toxic injury and infection. In regenerating liver, most 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 [12], induction of proangiogenic signals [137], and an important extracellular matrix (ECM) breakdown and remodeling [138] leading to 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 [139].

Some proteins involved in the structural integrity of the liver are also required for normal regeneration. For example, deficiencies in connexin-32, a gap-junction protein [140], and keratin-8, an intermediate filament forming protein [141], lead to extended liver damage after partial hepatectomy. Connexin-32 is also required for normal mitosis by mediating cellular connections during cell division. Loss of 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 [142, 143]. Interestingly, injection or increased expression of collagenase in intact liver, associated with HGF or TGFα, induces hepatocyte proliferation, suggesting that ECM degradation could contribute to hepatocyte priming [144]. Conversely, Issa et al. [145] observed that failure in collagen-I degradation in mouse liver inhibits the hepatocyte proliferation response. In rat, activation of plasminogen to plasmin begins shortly after PH and stays pronounced until 3–6 h. Successive inductions of mRNA levels of the metalloproteinases (MMP)-9, MMP-2, MMP-13, MMP-14, MMP-24, involved in matrix remodeling in both normal and pathological processes, are observed in mouse. Moreover, inhibitors of metalloproteinases (TIMP)-3, TIMP-4, TIMP-1 are also upregulated. In particular, TIMP-1 expression is induced prior the onset of DNA synthesis in rat and mouse models [146, 147]. 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 knockout mice accelerates liver regeneration [147]. Activation of 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 early phases of the liver regeneration, MMP-9 is located in the immediate periportal hepatocytes, then, its localization extends rapidly throughout the lobule before decreasing at 72 h post-PH. In the meantime, MMP-2 expression enhances in the hepatocytes at 24 and 48 h after hepatectomy [148]. 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 through the proteolytic maturation and/or liberation of priming and growth factors during ECM remodeling. In accordance, mature HGF production is delayed by 12 h in the uPA$^{-/-}$ mice along with a delayed DNA synthesis. Loss of uPA results in decreased plasmin levels responsible for activating MMP that in turn digest the ECM and allow release of activated growth factors like HGF from ECM [149]. 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 shortened cell cycle. However, these mice succumbed of liver failure by a TNFα-signaling-dependent cell death demonstrating also the importance of TIMP-3 in controlling TNFα bioavailability [150].

Studies performed *in vitro* have shown that TNFα induces MMP-9 expression in mouse hepatocytes [151] and that MMP-9 transcription involves activation of NF-κB pathway [152]. Cytokine-specific regulation of MMP/TIMP expression in hepatic stellate cells also suggests that the initial matrix degradation during 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 [153]. 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 native collagen I gel [154], collagen sandwich [155], or matrigel [156]. To further address the role of the extracellular matrix degradation to promote cell cycle entry and progression of differentiated adult hepatocytes following stimulation by mitogenic signals, 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 biliary epithelial cells (Figure 7). 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* [36]. Moreover, cytoskeleton organization of hepatocytes is similar in coculture and *in vivo* with a localization of the cytokeratins beneath of the plasma membrane [35, 157] and bile canaliculi structures present between the hepatocytes are

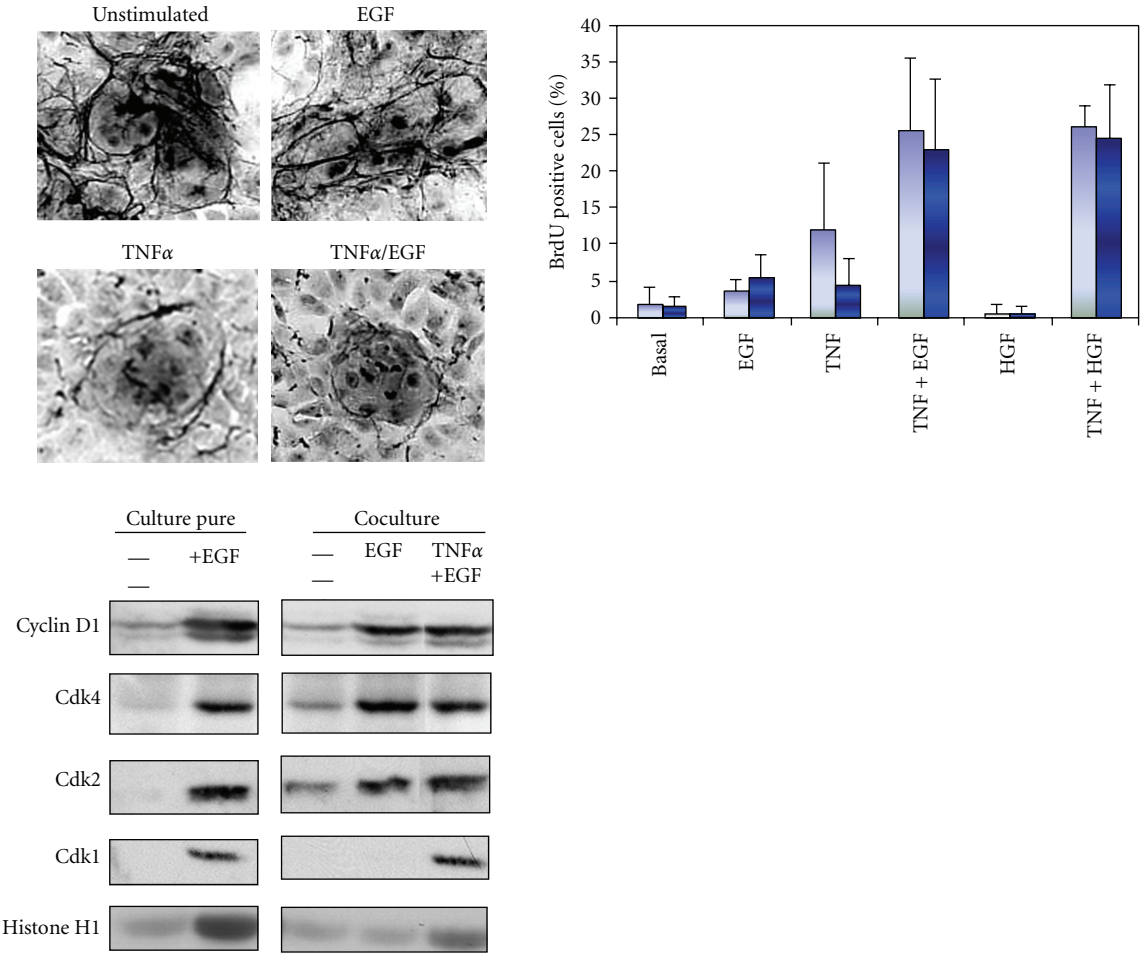


Figure 7: Upper left panel: detection of the extracellular matrix using the reticulin staining in unstimulated and EGF, TNFα or TNFα + EGF stimulated rat hepatocytes. Upper right panel: detection of BrdU positive hepatocytes in cocultured hepatocytes 3 days after stimulation with EGF, TNFα, HGF or combination of TNFα and EGF or HGF. Basal condition: unstimulated cells. Light blue: without fetal calf serum, dark blue: with fetal calf serum. Lower panel: expression of Cdks and cyclins in cultured rat hepatocytes. Cyclin D1, Cdk4, Cdk2, and Cdk1 were analyzed by western blotting. In addition, kinase activities of Cdk1 and Cdk2 were measured using histone H1 as a substrate.

also functional. Using this coculture system, we established new conditions allowing rat hepatocytes to undergo several proliferation waves (Figure 7) without loss of differentiation in presence of the priming cytokine, TNFα, and growth factors, such as HGF, EGF as observed *in vivo* during liver regeneration [39].

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 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 addition, the phenanthroline, a specific inhibitor of MMP activities reduces the TNFα-mediated ECM degradation resulting in the decrease in DNA replication. Additional experiments further demonstrated that the ECM degradation was due to the NF-κB-mediated induction of MMP-9 expression by TNFα [39]. Thus, ECM 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 [39]. These observations have been confirmed by Olle and coworkers using MMP-$9^{-/-}$ mice [158]. 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 post-PH corresponding to hepatocyte DNA synthesis in mice [158]. 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 [159].

Using both pure culture of hepatocytes and the coculture model, we compared expression of cell cycle markers to further investigate the molecular pathways involved in the progression in late G1 phase. In unstimulated cocultures, cyclin D1 and Cdk2 are barely detectable (Figure 7). This pattern of expression, similar to that observed in unstimulated primary pure cultures of hepatocytes, suggested 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 7). Therefore, EGF alone promotes the progression beyond the mitogen restriction point in late G1 although cells arrest before S phase. Our results could be linked to previous reports showing that cyclin E and Cdk2 are present in cells plated on denatured collagen film, while hepatocytes plated on collagen gel do not proliferate and lack the Cdk2 activity [154]. Moreover, both Cdk2 and Cdk1 are active. 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.

7. Conclusion

Altogether, our laboratory and others have demonstrated the concomitant expression and activation of both Cdk1 and Cdk2 during S phase in hepatocytes and their active contribution to the DNA replication. Finally, we show that Cdk1 expression and activation are correlated to ECM degradation via the involvement of the proinflammatory 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. 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. In most cell types, the low levels of expression and activation of Cdk1 in S phase led to the conclusion that Cdk1 and Cdk2 were functionally exclusive with specific functions in G2/M and G1/S transitions, respectively. 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. 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 are correlated to ECM degradation via the involvement of the proinflammatory 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. It also further confirms the well-orchestrated regulation of liver regeneration via multiple extracellular signals and pathways. Several important questions remain unanswered. How does TNFα induce Cdk2 kinase activity? It could be hypothesized that low levels of the Cdk inhibitor $p27^{Kip1}$ following TNFα stimulation favors 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 [160].

Abbreviations

H: Hours;
LDH: Lactate deshydrogenase
EGF: Epidermal growth factor
ECM: Extracellular matrix
TGFα: Transforming growth factor alpha
TNFα: Tumor necrosis factor alpha
HGF: Hepatocyte growth factor
Cdk: Cyclin-dependent kinase.

Acknowledgments

The authors thank Drs. Anne-Laure Serandour and Delphine Garnier for their involvement in some of the experiments described in this paper and all the members of the team "Stress, Defences and Regeneration" from the laboratory Inserm UMR_S 991. Our team is financially supported by federal funding through Inserm, CNRS, and the University of Rennes1 and the European grant, Liv-ES, FP7 program, Health F5-2008-223317, the FEDER (Fonds européen de développement régional) and the "Contrat plan état région" (axe biothérapie).

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Hindawi Publishing Corporation
International Journal of Hepatology
Volume 2012, Article ID 212530, 13 pages
doi:10.1155/2012/212530

Research Article

Recovery of the Cell Cycle Inhibition in CCl_4-Induced Cirrhosis by the Adenosine Derivative IFC-305

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Received 2 May 2012; Revised 1 August 2012; Accepted 21 August 2012

Academic Editor: Anne Corlu

Introduction. Cirrhosis is a chronic degenerative illness characterized by changes in normal liver architecture, failure of hepatic function, and impairment of proliferative activity. The aim of this study is to know how IFC-305 compound induces proliferation of the liver during reversion of cirrhosis. *Methods*. Once cirrhosis has been installed by CCl_4 treatment for 10 weeks in male Wistar rats, they were divided into four groups: two received saline and two received the compound; all were euthanized at 5 and 10 weeks of treatment. Liver homogenate, mitochondria, and nucleus were used to measure cyclins, CDKs, and cell cycle regulatory proteins PCNA, pRb, p53, E2F, p21, p27, HGF, liver ATP, and mitochondrial function. *Results*. Diminution and small changes were observed in the studied proteins in the cirrhotic animals without treatment. The IFC-305-treated rats showed a clear increase in most of the proteins studied mainly in PCNA and CDK6, and a marked increased in ATP and mitochondrial function. *Discussion/Conclusion*. IFC-305 induces a recovery of the cell cycle inhibition promoting recovery of DNA damage through the action of PCNA and p53. The increase in energy and preservation of mitochondrial function contribute to recovering the proliferative function.

1. Introduction

Cirrhosis is one of the most common causes of mortality worldwide and is induced by chronic liver injury, such as that produced by alcoholic hepatitis, viral hepatitis, autoimmune disease against biliary ducts, and metabolic diseases such as hepatic steatosis.

Cirrhosis is a complex process for which no effective treatment has been developed [1]. It is accompanied by a change in the architecture of the liver with loss of function and it is considered irreversible, mainly due to the increased deposition of connective tissue resulting from an increased collagen synthesis accompanied by a deficient degradation of deposited collagen [2]. Another important factor is the well-known impaired capability of the liver to regenerate after hepatic resection [3]. The severity of liver fibrosis is considered to be related with impaired regenerative capacity, suggesting the arrest of cell cycle progression [4]. Cirrhosis development is preceded by inflammation, apoptosis, and fibrosis processes that are accompanied by energetic unbalance as well as oxidative damage induced by reactive oxygen species that could result in chromosomal instability which induces injury in the check points of the cellular cycle causing an impaired regenerative capacity. Our research group has been studying the hepatoprotective effect of adenosine and an adenosine derivative, IFC-305, in the development and in the reversion of established cirrhosis on a rat model of liver cirrhosis induced by CCl_4 [5–7]. Adenosine is a purine nucleoside considered an autocoid, present within and outside the cells that acts as chemical messenger with autocrine, paracrine and endocrine actions. It is mostly formed by *the novo* purine synthesis principally in the liver [8], by phosphohydrolysis of adenine nucleotides via endo- and ecto-5′nucleotidases or by hydrolysis of S-adenosyl homocysteine in the methylation pathway [9]. Extracellular adenosine can exert its function through activation of adenosine receptors (A1, A2a, A2b, and

A3) or can be transported into the cells by the nucleoside transporters (ETN1). Within the cell adenosine can be phosphorylated by adenosine kinase, deaminated to inosine by adenosine deaminase, or transformed in S-adenosyl-homocysteine by S-adenosyl-homocysteine hydrolase [9]. Its metabolism is very active resulting in a short half-life of the nucleoside but with an action as metabolic modulator. The derivative IFC-305 has a longer half-life in the liver potentiating the beneficial effects of adenosine on CCl_4-induced cirrhosis in rats [10]. Adenosine presents interesting effects in hepatic metabolism: it increases the energy charge of the hepatocyte, augments the synthesis of hepatic glycogen, and inhibits fatty acid oxidation [11–13]; it also modulates the redox state of the cell through maintaining the structure and function of the mitochondria [14]. In acute hepatotoxicity, induced by ethanol or CCl_4, adenosine prevents the fatty liver induced by the toxics, restitutes the energetic balance, the redox equilibrium, mitochondrial function, and prevents oxidative stress by diminishing the reactive oxygen species and increasing antioxidant defenses [5, 15–18]. In preestablished cirrhosis, adenosine increases collagen degradation, prevents its accumulation, preserves the energy and functional states of the liver, increases DNA synthesis, the mitotic index, and the expression of proliferating cell nuclear antigen (PCNA) [7]. Recently, we have demonstrated that adenosine administration accelerates progression of the cell cycle during liver regeneration in rats subjected to one-third hepatectomy [19]. Some of these effects, such as prevention of collagen accumulation and increase in DNA synthesis, have been reproduced with the IFC-305 [5, 10]. Moreover, microarray studies showed that 414 genes modified in cirrhosis are decreased to 263 with IFC-305 treatment and the downregulated or upregulated genes showed a tendency to normalize with the compound treatment, among them there are 24 genes involved in the cell cycle [10]. On the other hand, it has been shown that the cell cycle-related molecules play essential roles in hepatocyte proliferation. Specifically, G1-related molecules are important because they are a requisite to enter into the cell cycle from the quiescent state. Although the role of these molecules of the cell cycle has not been studied in detail during cirrhosis development, we hypothesize that the action of adenosine and its derivative IFC-305 must be related with the molecules and regulation points of the cell cycle. Sweet and Singh [20] showed that the progression through the cell cycle is sensitive to changes in mitochondrial-derived ATP and describes two energetic checkpoints at the G_1-S and the G_2-M as borders that prevent the progression of the proliferation cycle.

Numerous studies employing inhibitors of the mitochondrial function have demonstrated that cell division is sensitive to alterations of the energy pool [21–23]. In order to know how the IFC-305 repairs the proliferative function in the cirrhotic liver, we studied the role of cyclins of the cellular cycle (cyclins D, E, A, and B), cyclin-dependent kinase (CDKs), some other proteins such as PCNA, pRb, E2F1, p53, and the hepatic growth factor (HGF) and correlated them with some parameters of the mitochondrial function in experimental cirrhosis induced by CCl_4.

2. Materials and Methods

2.1. Animal's Treatment and Induction of Cirrhosis by CCl_4. Male Wistar rats ($n = 30$) weighing 100 to 110 g were rendered cirrhotic by chronic treatment with CCl_4. Animals were intraperitoneally injected (0.4 g/kg) three times a week during 10 weeks with a solution 1/6 of CCl_4 in vegetable oil. Cirrhosis-induced rats were divided into five groups (six animals per group). At time zero (T0 group), rats were euthanized 24 hours after cessation of CCl_4, two groups were treated with saline solution during 5 (SS5) or 10 weeks (SS10) and two groups were treated with IFC305 at a 50 mg/kg dose, three times weekly, for 5 (IFC5) or 10 weeks (IFC10), all the experiments include rats without treatment (C). Animals were euthanized with a sodium pentobarbital overdose (63 mg/kg animal weight); the liver was recovered, rinsed in saline solution, and frozen with liquid nitrogen. Animals were obtained and housed from the animal facility of the National Autonomous University of Mexico (UNAM). All procedures were conducted according to our institutional guidelines for the care and use of laboratory animals.

2.2. Plasma Collection. Animals were anesthetized as indicated and about 10 mL of whole blood was collected by cardiac puncture into syringes filled with 1ml of 0.25 M EDTA, pH 8.0. Cells were removed from plasma by centrifugation for 10 minutes at 3000 rpm at 4°C. Following centrifugation, the supernatant (plasma) was stored into 0.5 mL aliquots at −70°C until use.

2.3. Liver Subcellular Fractionation. Liver samples were homogenized (1 : 10 w/v) in a medium containing 250 mmol/L sucrose, 10 mmol/L Trizma base, and 1 mmol/L EDTA. The homogenate was centrifuged at 3500 rpm for 5 min at 4°C, the supernatant was used as total liver protein extract, or the supernatant was centrifuged at 8500 rpm for 10 min at 4°C to obtain the mitochondrial pellet. Mitochondrial respiration and phosphorylation were recorded polarographically with a Clark-type oxygen electrode in 3 mL of a medium containing 225 mM sucrose, 10 mM KCl, 5 mM $MgC1_2$, 10 mM potassium phosphate, 10 mM Tris-HCl, and 0.05% fatty-acid-free albumin (pH 7.4). Glutamate and malate (10 and 1 mM, resp.) were used as substrates for site I. Mitochondrial state 3 was initiated by addition of ADP (266 μmol/liter final concentration). The membrane potential ($\Delta\Psi$) was measured by monitoring the movements of tetraphenyl phosphonium $(TPP+)^2$ across the mitochondrial membrane as previously described [24]. Protein concentration was determined by the Lowry method [25].

2.4. Liver Nuclear Protein Extractions. The nuclear fraction was obtained by the method used by Sindić et al. [26]. In brief, livers were homogenized in ice-cold 10 mM HEPES buffer, pH 7.5, containing 5 mM $MgCl_2$, 25 mM KCl, and the protease inhibitor cocktail. Homogenates were spun through a discontinuous sucrose gradient and resuspended

in the indicated medium [26]. Protein quantification was determined with the method of Bradford [27].

2.5. ATP Determination. For adenine nucleotide ATP determination, 300 mg of the liver was extracted with 8% perchloric acid, after centrifugation the sample was neutralized with 4 M K_2CO_3. ATP was quantified by reversed- phase high-performance liquid chromatography [28].

2.6. Western Blot Analysis. Liver nuclear protein or total liver extract (30 μg of protein/well) was electrophoresed in SDS-polyacrylamide gels and transferred to nitrocellulose membranes. Membranes were blocked for 2 h with 5% nonfat dry milk in TBST (50 mM Tris, 150 mM NaCl at pH 7.4, and 0.05% Tween 20). The blots were incubated overnight at 4°C with primary antibodies against PCNA (Upstate, Lake Placid, NY, USA), adenosine Receptors A1, A2a, A2b, A3 (Alpha Diagnostics Intl, San Antonio, TX, USA), cyclin D1, cyclin E, cyclin A, cyclin B1, CDK4, CDK6, p21, p27, phospho-Rb (Ser 795), E2F1, DP1, p53, HGFα, β-actin (Santa Cruz Biotechnology, Santa Cruz, CA), MDM2, and c-Met (Millipore, Temecula, CA, USA). Primary antibody binding was detected with the respective horseradish peroxidase-conjugated secondary antibody. Protein bands were visualized by using chemiluminescence luminol reagent (Santa Cruz Biotechnology). Densitometric analyses of bands were done with the Quantity One software (Bio-Rad, Hercules, CA, USA).

2.7. RNA Isolation and Quantitative RT-PCR Analysis. Frozen liver samples were used for total RNA isolation by Tripure-based extraction (Roche Applied Science). Quantity and purity were determined by measuring the optical density at 260/280 nm in a UV-spectrophotometer. RNA quality was verified by agarose-gel electrophoresis and rRNA 28S/18S > 1.7 ratios were used. cDNA synthesis was performed from 2 μg of total RNA using the high-capacity cDNA Archive Kit (Applied Biosystems, Foster City, CA, USA) following the manufacturer's protocol. PCR reactions were optimized for all genes to obtain one PCR product that corresponded to the size predicted by the primer design. Real-time quantitative PCR reactions (qPCR) were done in the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) using Platinum SYBR Green qPCR Super Mix-UDG with ROX Kit (Invitrogen, Carlsbad, CA USA) according to the manufacturer's instructions. All quantitative PCR assays were performed independently in at least three animals/group in triplicate with the corresponding standard dilution curves. Relative mRNA transcript levels were expressed in arbitrary units as *n*-folds of untreated control after normalization to the acidic ribosomal protein (Arbp) mRNA. The primers used were cyclin D1 forward 5′-GCAAGAATGTGCCAGACTCA-3′ and reverse 5′-ACGGAGATGTGGTCTCCTTG-3′; Rb forward 5′-CACGAAAAAGCAACCCTGAT-3′ and reverse 5′-TCTGATGGCTGATCACTTGC-3′; p53 forward 5′-GCT TCGAGATGTTCCGAGAG-3′ and reverse 5′-CTTCGG GTAGCTGGAGTGAG-3′; Arbp, forward 5′-AGGTGGTGC TGATGGGCA-3′ and reverse 5′-CCTCCGGATGTGAGG CAG-3′.

2.8. PCNA Immunohistochemistry. Sections of liver were fixed in 10% neutral buffered formalin and embedded in paraffin. For PCNA immunohistochemistry, liver sections of 4 μm thick were mounted in lysine-coated slides, deparaffinized in xylene, and passed through graded alcohols. The Dako EnVision+ System-HRP (DAB) (Dako, Carpinteria, CA, USA) was used. Previously, slides were immersed in antigen-retrieval solution using Citrate Buffer (10 mM Citric Acid, 0.05% Tween 20, pH 6.0) in a plastic Coplin jar placed in a microwave oven set on middle power for 10 min. After cooling at room temperature, sections were incubated for 10 min with the peroxidase block, and then incubated 10 min with Protein Block, Serum-Free (DakoCytomation, Denmark). Slides were incubated overnight at 4°C with the primary antibody, monoclonal anti-PCNA clone PC10 (Dako, Carpinteria, CA, USA) at a dilution of 1 : 100. The next day, slides were incubated for 60 min with the labeled polymer-HRP anti-mouse at room temperature. Tissue sections were then incubated with DAB substrate buffer and counterstained with hematoxylin and coverslipped with Entellan (Merck, Darmstadt, Germany). PCNA-positive hepatocytes were counted from images captured with Evolution/QImaging Digital Camera (Media Cybernetics, Bethesda, MD, USA) of five randomly chosen fields by light microscope using a 40x objective magnification from $n = 3$ rats. Counting of PCNA-positive hepatocytes was done with the Image-Pro Plus 7 (Media Cybernetics, Inc., Bethesda, MD, USA). The hepatocytes were considered positive for PCNA when the immunostaining was present in the nuclei or cytoplasm, cells in S + G1 + G2 + M as it has been described by others [29, 30].

2.9. Statistical Analysis. All values were expressed as mean ± SEM of three independent experiments. Statistical analysis was performed using the unpaired, nonparametric Student's *t*-test. Differences with a P value of less than 0.05 were considered statistically significant.

3. Results

3.1. Proliferating Cell Nuclear Antigen (PCNA) in the Cirrhotic Rats Treated with IFC-305. PCNA is an auxiliary protein of the DNA polymerase delta and is an excellent marker of cell proliferation present at the beginning of the S phase. Nuclear cell expression of the protein is presented in Figure 1(a), showing a diminution at 5 and 10 weeks after established cirrhosis in the presence of saline, whereas in the presence of IFC-305, an 8- to 10-fold increase was noticed, supporting the effect of the compound on hepatic proliferation. We also analyzed proliferating cells by immunohistochemical identification of PCNA in liver tissue sections. Although the difference was less pronounced than in the western blot analysis, we also observed an increase in PCNA expression in hepatocytes of liver rats treated with the compound (Figure 1(b)). We observed that in the group of rats at 5 weeks

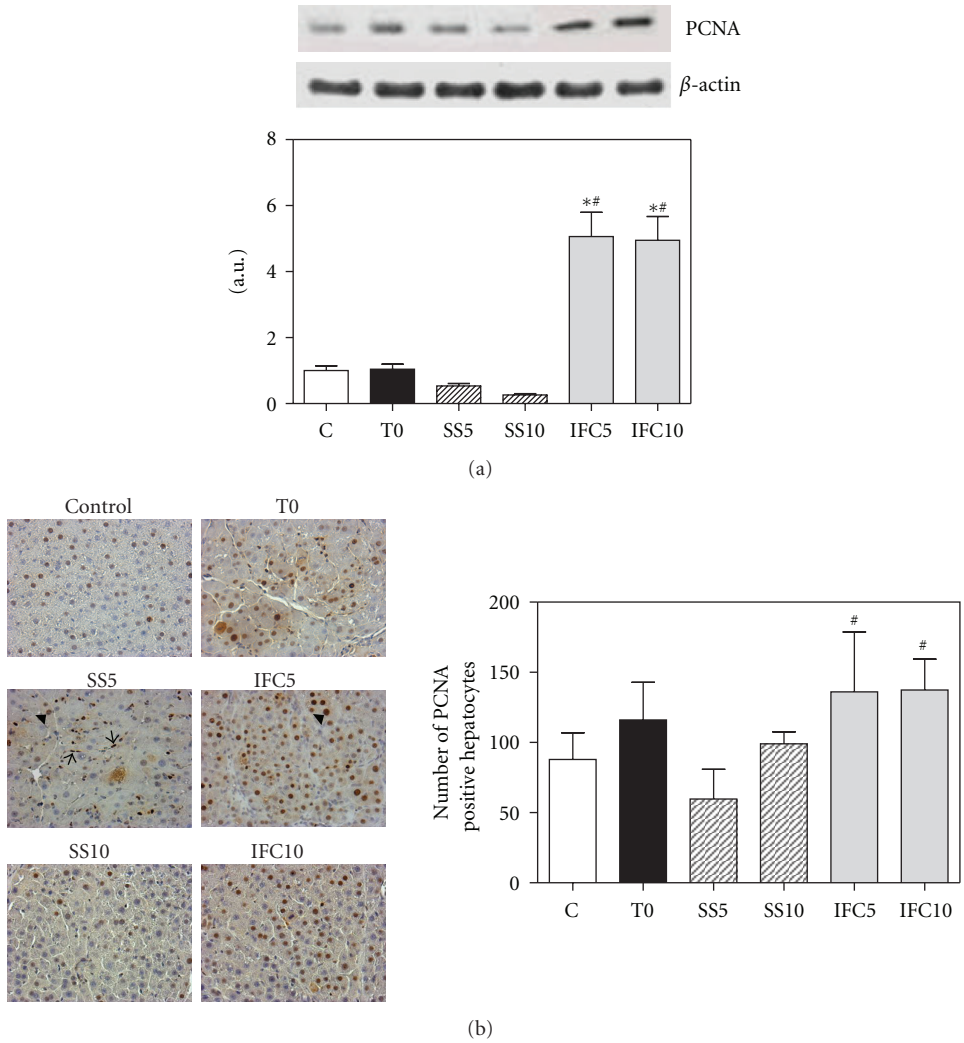

Figure 1: Effect of IFC305 treatment on the liver PCNA protein expression in CCl_4-induced cirrhosis in rats. (a) Protein expression of PCNA and the house keeping gen β-actin from liver nuclear extracts was determined by western blot analysis. A representative western blot image is shown. The bar graph represents the densitometry analysis expressed as arbitrary units of the mean ± SEM from 3 rats/group; values were normalized to β-actin immunodetection. (b) Immunohistochemical analysis of PCNA expression. Hepatocyte (arrowhead) and nonparenchymal cell (arrow) are marked. The number of PCNA positive hepatocytes was quantified as described in Section 2. *Statistical difference ($P < 0.01$) when compared to C group. #Statistical difference ($P < 0.01$) when compared to their respective experimental group SS5 or SS10.

of cirrhosis progress, there was an increase in proliferation of other cells than hepatocytes, nonparenchymal cells, such as hepatic stellate cells, Kupffer or endothelial cells, although the identity of them should be confirmed (Figure 1(b)).

3.2. Cyclins D1, E, A, and B1. Cyclins D and E belong to the G1 phase and are fundamental to initiate the cell cycle, whereas cyclins A and B are mitotic cyclins for the G2 M phase. These molecules are induced by mitogenic signals and extracellular growth factors, they have a short half-life and they are ubiquitinated and destroyed by the proteosome. The level of cyclin D in cirrhotic animals at T0 is similar to that of the control rats, but, at 10 weeks of progress, it increased 35%; animals treated with IFC-305 for 5 weeks showed a 77% increase, and a similar value at 10 weeks of treatment to that observed in nontreated rats (Figure 2(a)), these results are supported by the increase in mRNA of cyclin D1 (Figure 2(b)). Cyclins E and A did not show noticeable changes in the cirrhotic rats treated or not treated with the compound (Figures 2(c) and 2(d)). Cyclin B1 decreased by 30 and 52% at 5 and 10 weeks of IFC-305, respectively, in relation to the cirrhotic animals at T0 and after 10 weeks of progress, the cirrhotic rats treated with saline also decreased cyclin B1 in a similar way to that observed in the rats treated with the compound (Figure 2(e)). It is interesting to keep in mind that the degradation of cyclin B1 is important for metaphase-anaphase transition and progression of the cell cycle [31].

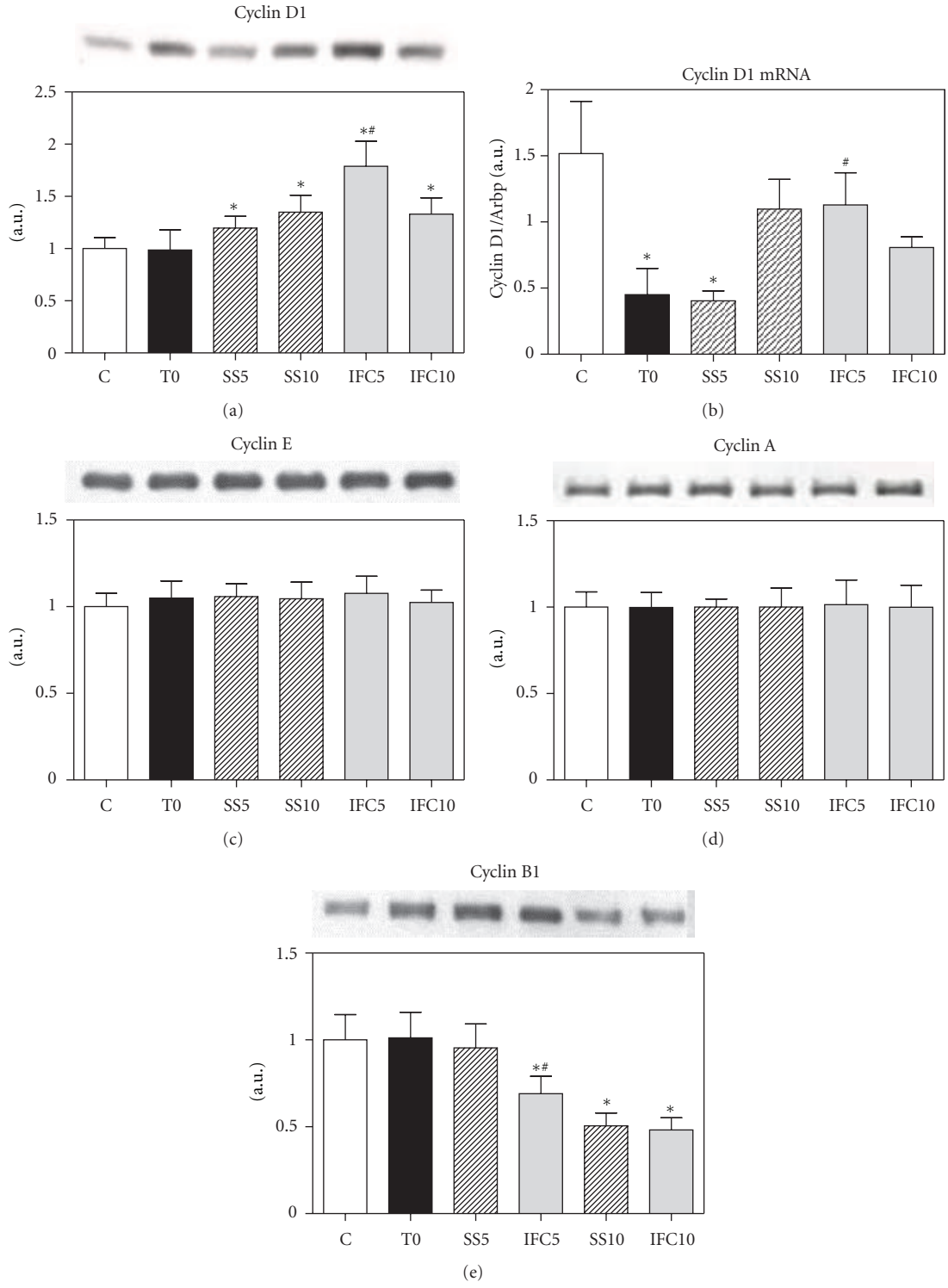

Figure 2: Effect of IFC305 treatment on cyclin D1, cyclin E, cyclin A, and cyclin B1 protein expression in CCl_4-induced cirrhosis in rats. (a), (c), (d), and (e) Expression of the indicated proteins from liver nuclear extracts was determined by western blot analysis. A representative western blot image of each one is shown. The bar graph represents the densitometry analysis expressed as arbitrary units of the mean ± SEM from 3 rats/group, values were normalized to β-actin immunodetection (image, Figure 1(a)). *Statistical difference ($P < 0.05$) when compared to C group. #Statistical difference ($P < 0.05$) when compared to their respective experimental group SS5 or SS10. (b) Effect of IFC305 treatment on cyclin D1 mRNA expression in CCl_4-induced cirrhosis in rats. RNA was isolated from liver and mRNA expression for cyclin D1 was analyzed by quantitative RT-PCR as described in Section 2. Arbitrary units were normalized with Arbp mRNA gene expression level. Data represent mean ± SEM from 3 rats/group. *Statistical difference ($P < 0.05$) compared to C group. #Statistical difference ($P < 0.05$) when compared to their respective experimental group SS5 or SS10.

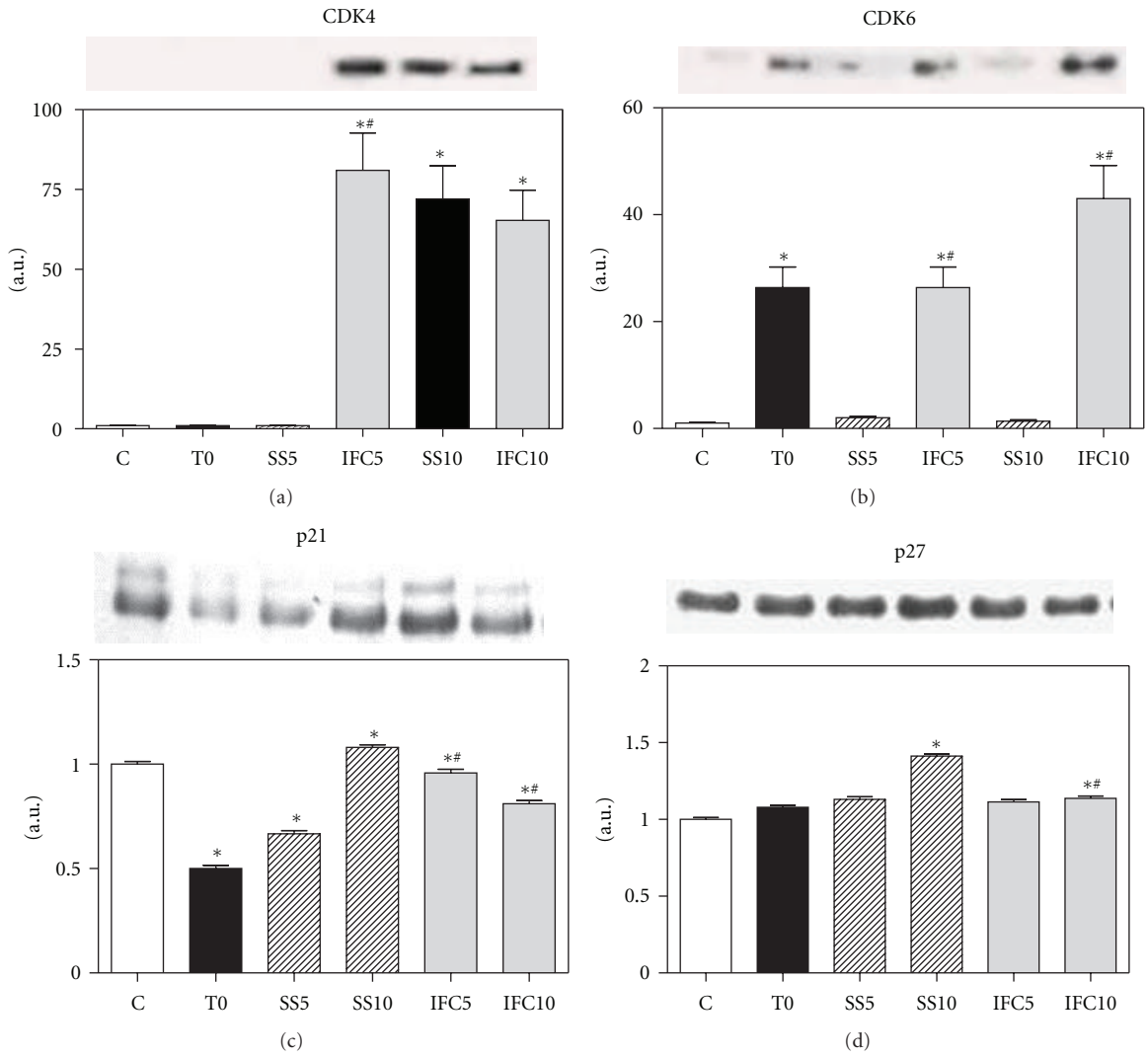

Figure 3: Effect of IFC305 treatment on CDK4, CDK6, p21, and p27 proteins expression in cirrhotic livers. (a), (b), (c), and (d) Expression of the indicated proteins from liver nuclear extracts was done by western blot analysis. A representative western blot image is shown. The bar graph represents the densitometry analysis expressed as arbitrary units of the mean ± SEM from 3 rats/group; values were normalized to β-actin immunodetection (image, Figure 1(a)). *Statistical difference ($P < 0.05$) when compared to C group. #Statistical difference ($P < 0.05$) when compared to their respective experimental group SS5 or SS10.

3.3. Cyclin Dependent Kinases (CDK4, CDK6) and Cell Cycle Inhibitors (p21, p27). For the progression of the cell cycle to S phase, cyclin D1 associates with a cyclin dependent kinase 4 or 6 (CDK4/CDK6) to form an active complex, cyclin-D/CDK4/CDK6 (Figures 3(a) and 3(b)). CDK4 increased at 5 and 10 weeks of IFC-305 treatment but also at 10 weeks of progress without treatment. CDK6 was present in the cirrhotic group at T0 and was maintained at 5 weeks of IFC-305 treatment with an important increase at 10 weeks, a marked decrease was seen in the cirrhotic rats without treatment. Regarding expression of p21 and p27 cell cycle inhibitors (Figures 3(c) and 3(d)), p21 showed a 40% decrease in the cirrhotic animals at T0, recuperating its value after 5 or 10 weeks of progress with and without treatment; p27 did not diminish in cirrhosis, but a 30 and 15% increase was noticed after 10 weeks of progress with and without treatment, respectively.

3.4. Expression of Phospho-Rb and the Transcription Factor E2F-1. An important function of the complex D/CDK4/6 is the phosphorylation of protein Rb, releasing the transcriptional factor E2F1 that activates the DNA synthesis genes and induces the entry of the cell into the S phase. A small increase in the phospho-Rb was observed in the group treated with the compound, but a significant increase in its mRNA at 5 and 10 weeks of IFC-305 treatment was observed. A 70% increase was observed in E2F1 that form a functional heterodimer with DP1 [32] in the nucleus obtained from cirrhotic rats treated for 5 weeks with IFC-305. The expression of DP1 also increased with the

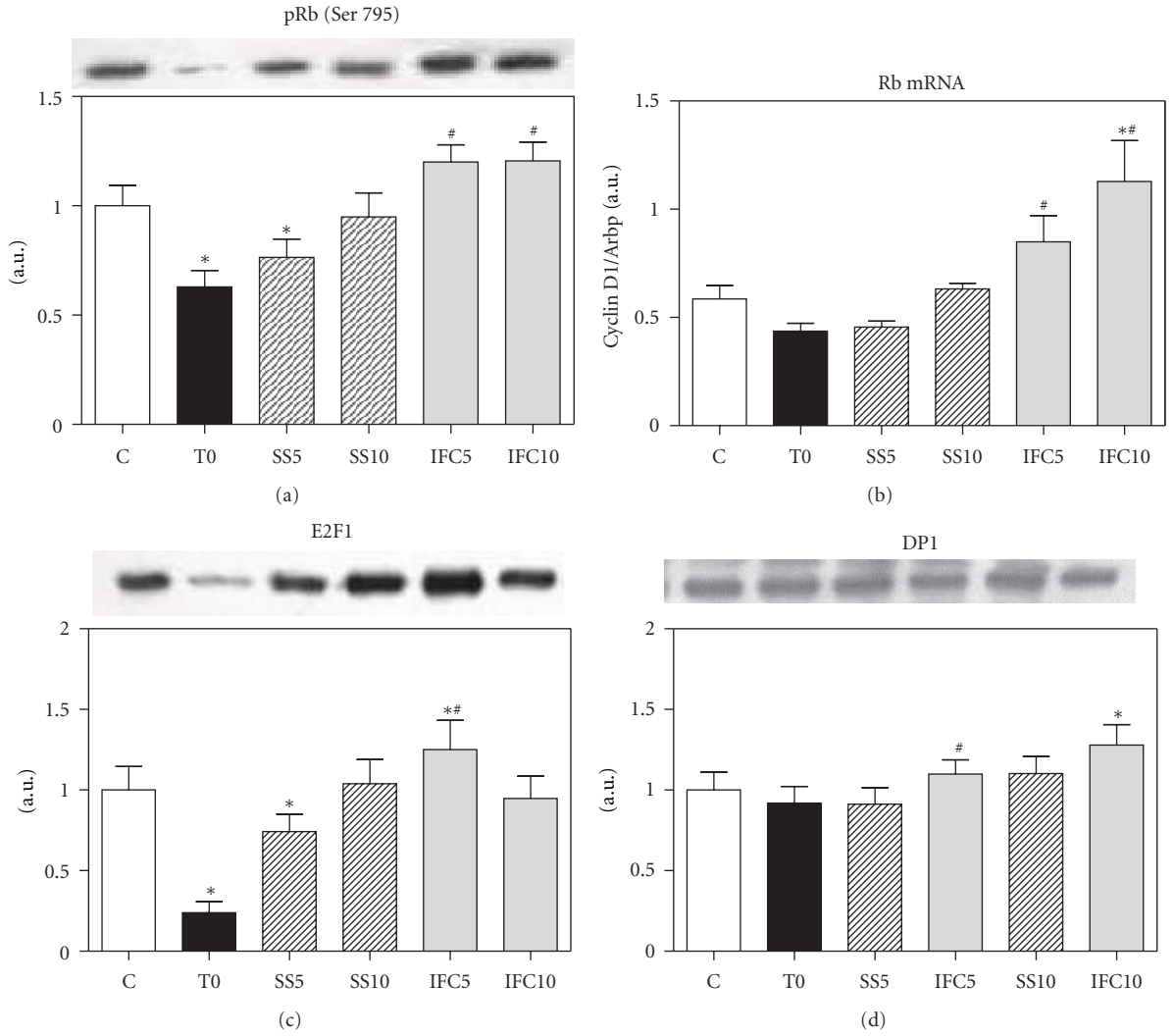

Figure 4: Effect of IFC305 treatment on phospho-Rb (Ser 795), E2F1, and DP1 proteins expression in cirrhotic livers. (a), (c), and (d) Expression of the indicated proteins from liver nuclear extracts was done by western blot analysis. A representative western blot image of each one is shown. The bar graph represents the densitometry analysis expressed as arbitrary units of the mean ± SEM from 3 rats/group; values were normalized to β-actin immunodetection (image, Figure 1(a)). *Statistical difference ($P < 0.05$) when compared to C group. #Statistical difference ($P < 0.05$) when compared to their respective experimental group SS5 or SS10. (b) Effect of IFC305 treatment on Rb mRNA expression in CCl_4-induced cirrhosis in rats. RNA was isolated from liver and mRNA expression for Rb was analyzed by quantitative RT-PCR as described in Section 2. Arbitrary units were normalized with Arbp mRNA gene expression level. Data represent mean ± SEM from 3 rats/group. *Statistical difference ($P < 0.05$) compared to C group. #Statistical difference ($P < 0.05$) when compared to their respective experimental group SS5 or SS10.

compound suggesting that the complex E2F/DP1 induces the S phase (Figures 4(a), 4(b), 4(c), and 4(d)).

3.5. Expression of p53 and the Protein MDM2. p53 has two important functions in the cell cycle, when there is DNA damage it induces arrest of the cycle at the check points of G1/S or G2/M. On the other hand, it induces the DNA repairing enzymes. Once DNA is repaired, it stimulates MDM2 synthesis for p53 degradation in the proteosome. In the cirrhotic T0 animals, there was a 19% increase in p53 expression, which was also observed at 5 and 10 weeks versus control rats; in cirrhotic animals treated with the compound, the increase was of 55 and 40% at 5 and 10 weeks of treatment, respectively (Figure 5(a)). When the gene expression was measured, only the cirrhotic groups presented an increase (Figure 5(b)), suggesting that IFC-305 did not increase the mRNA and that the observed increase in p53 protein could be due to a diminution in its degradation, as observed in the 25% decrease of protein expression of MDM2 in the IFC-305-treated rats indicating a diminution in p53 degradation (Figure 5(c)).

3.6. Hepatocyte Growth Factor/c-Met. The hepatocyte growth factor (HGF) is a mitogenic protein for hepatocytes and it

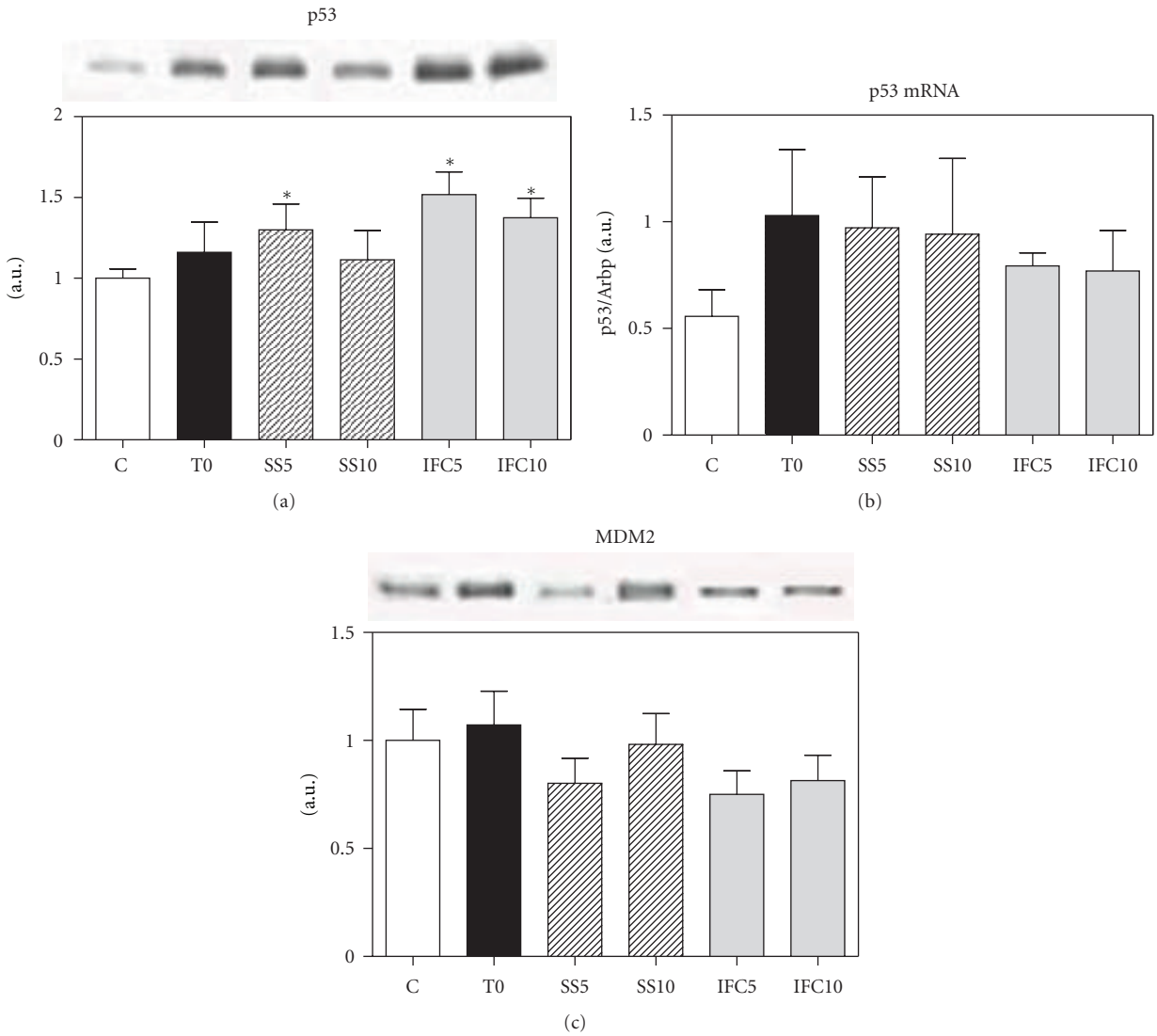

Figure 5: Effect of IFC305 treatment on p53 and MDM2 proteins expression in cirrhotic livers. (a) and (c) Expression of the indicated proteins from liver nuclear extracts was done by western blot analysis. A representative western blot image of each one is shown. The bar graph represents the densitometry analysis expressed as arbitrary units of the mean ± SEM from 3 rats/group; values were normalized to β-actin immunodetection (image, Figure 1(a)). *Statistical difference ($P < 0.05$) when compared to C group. #Statistical difference ($P < 0.05$) when compared to their respective experimental group SS5 or SS10. (b) Effect of IFC305 treatment on p53 mRNA expression in CCl_4-induced cirrhosis in rats. RNA was isolated from liver and mRNA expression for Rb was analyzed by quantitative RT-PCR as described in Section 2. Arbitrary units were normalized with Arbp mRNA gene expression level. Data represent mean ± SEM from 3 rats/group. *Statistical difference ($P < 0.05$) when compared to C group. #Statistical difference ($P < 0.05$) when compared to their respective experimental group SS5 or SS10.

is the ligand of c-Met, which is normally expressed by cells of epithelial origin, whereas HGF is expressed in cells of mesenchymal origin. The cirrhotic animals (T0) presented a small but significant increase of HGF in serum, a further increase after 5 and 10 weeks of progress was observed, whereas the rats treated with IFC-305 showed almost a threefold increase versus control animals and only 35 to 55% versus the cirrhotic animals without treatment (Figure 6(a)). The level of HGF in the liver of cirrhotic rats (Figure 6(b)) did not present significant changes although it showed an increasing tendency in the animals treated with IFC-305. The expression of c-Met increased in cirrhotic animals at T0 (25%) with a further increase after 5 weeks of progress, followed by a decrease after 10 weeks. The treatment with the compound induced a diminution in relation with the untreated cirrhotic animals (Figure 6(c)).

3.7. Adenosine Receptors. The protein expression of adenosine receptors subtypes A1, A2a, A2b, and A3 was evaluated in liver homogenate through western blot assays (Figure 7). A marked (85%) increase in A2a receptor was observed in cirrhotic animals T0 with a less increase after 5 and 10

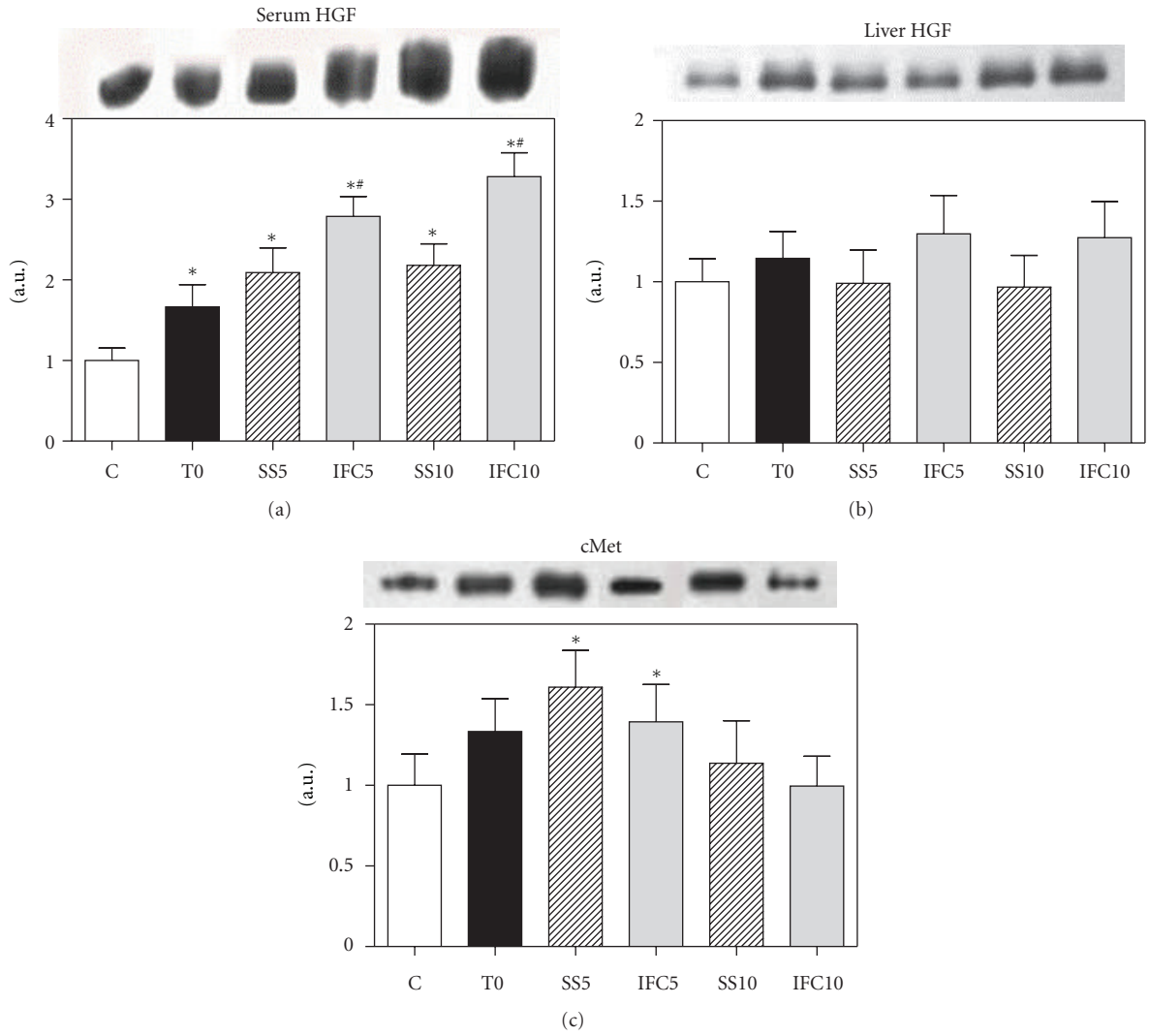

Figure 6: Effect of IFC305 treatment on serum HGF, liver HGF, and cMet proteins expression in CCl_4-induced cirrhosis in rats. (a), (b), and (c) Expression of the indicated proteins from serum or liver extracts was done by western blot analysis. A representative western blot image of each one is shown. The bar graph represents the densitometry analysis expressed as arbitrary units of the mean ± SEM from 3 rats/group; values were normalized to β-actin immunodetection (image, Figure 1(a)). Values from serum (a) were not normalized. *Statistical difference ($P < 0.05$) when compared to C group. #Statistical difference ($P < 0.05$) when compared to their respective experimental group SS5 or SS10.

weeks of cirrhotic progress (43 and 54%, resp.), with IFC-305 administration its expression decreased to 30%. Receptors A1, A2B, and A3 showed no significant changes in the different treatments.

3.8. Mitochondrial Function and Liver ATP Content. The energy state of the liver of the experimental groups was evaluated measuring the ATP level of the hepatic tissue, and some parameters of the mitochondrial function. In Table 1, it can be observed that ATP level decreased in cirrhosis (T0), slowly recovered after 5 and 10 weeks of progress, the groups treated with IFC-305 revealed no significant decrease of ATP at 5 weeks, whereas an important elevation was observed at 10 weeks. Oxygen consumption by mitochondria, using glutamate as substrate, as well as the respiratory control and the mitochondrial potential, presented a similar profile, indicating that IFC-305 restitutes and increases energy in cirrhotic animals.

4. Discussion

Regenerative function of the liver is a very complex process that has been studied after partial hepatectomy and requires a priming process before the growth stimulation factor can progress beyond the restriction points of the cellular cycle. The priming process is mediated by tumor necrosis factor (TNFα) and interleukin-6 (IL-6) resulting in the activation of nuclear factor κ B (NFκ B) before entering the early phase G1 [33]. The main proteins of the cell cycle are cyclins and cyclin-dependent kinases (CDKs) whose concentration is low in the quiescent state of hepatocytes, increasing in the replicative state. Their synthesis and degradation are

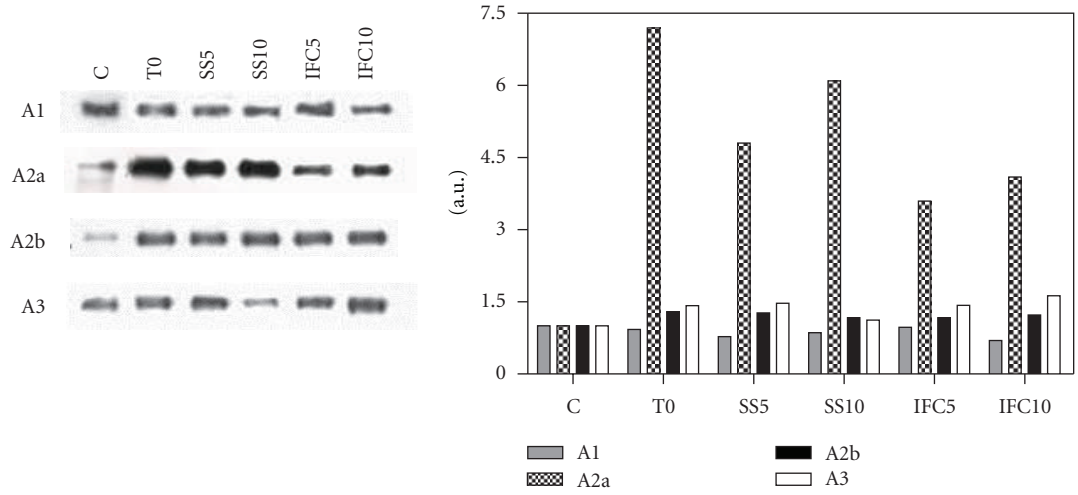

Figure 7: Effect of IFC305 treatment on adenosine receptors expression in CCl_4-induced cirrhosis in rats. Expression of the indicated adenosine receptors from liver extracts was done by western blot analysis. A representative western blot image of each one is shown. The bar graph represents the densitometry analysis expressed as arbitrary units of the mean ± SEM from 3 rats/group; values were normalized to β-actin immunodetection (image, Figure 1(a)).

Table 1: Effect of IFC305 on mitochondrial parameters in cirrhotic livers.

Treatment	(ATP) μmol/gwt	State 3 Glutamate	RC Glutamate	$\Delta\Psi$ ΔDO 540 nm
Controls	3.0 ± 0.7	42 ± 4	5.4 ± 0.5	191 ± 4
Cirrhosis	2.0 ± 0.16*	34 ± 3	2.8 ± 0.4*	164 ± 8*
Plus saline				
After 5 wks	2.2 ± 0.06	29 ± 3*	3.6 ± 0.3*	168 ± 10*
After 10 wks	2.8 ± 0.13	47 ± 6	4.3 ± 0.4	180 ± 6
Plus IFC305				
After 5 wks	2.9 ± 0.3**	38 ± 5	5.2 ± 0.6**	197 ± 12**
After 10 wks	3.7 ± 0.01**	48 ± 6	6.2 ± 0.5**	200 ± 4**

The results are expressed as mean ± SE of seven individual determinations. State 3 is expressed as natoms O_2/min/mg of protein for glutamate-malate (site I) in the presence of ADP. RC: respiratory control (state 3/state 4). Statistics: *$P \leq 0.01$ versus the control group; **$P \leq 0.01$ versus the cirrhotic plus saline group. ATP concentration and $\Delta\Psi$ were determined as described in Section 2. gwt: gram of wet tissue.

important for cell cycle progression and are strictly regulated by the checkpoints, in order to maintain the progression of the cycle in normal conditions. However, the checkpoints are able to arrest cell cycle progression in response to DNA damage. According to these brief considerations, hepatocyte's proliferation requires a cell environment that maintains an energy balance [23], redox equilibrium, with normal synthesis and degradation of proteins, as well as mitochondrial and endoplasmic reticulum function. In cirrhosis, there is a drop in cell ATP content and mitochondrial dysfunction and a decrease in albumin synthesis [6, 24]. In the experimental cirrhosis induced by CCl_4, there is an increase in oxidative stress generated by an increase in reactive oxygen species produced by the toxic and by mitochondrial dysfunction. Moreover, in cirrhotic rats there is a chromosomal instability highly suggestive of DNA damage and arrest of cell cycle and an increased amount of transforming growth factor β, a profibrogenic cytokine and a known inhibitor of liver proliferation [34]. These observations suggest that the regeneration process is a multifactorial event and that in cirrhosis several of those factors are damaged. The profound alterations of the cirrhotic liver predict an inhibition of liver proliferation.

The present results support this possibility; Table 2 depicts a summary of the results. The cirrhotic group (T0) only showed a moderate increase of serum HGF, a marked increase in CDK6, a diminution of the cell cycle inhibitor p21 and phospho-Rb and E2F, the other proteins studied did not show changes. During the 5 and 10 weeks of cirrhosis progress, some changes occurred at 5 weeks, that is, a small increase in cyclin D1, p53, serum HGF, and its receptor c-Met; at 10 weeks of progress, a larger diminution of PCNA, cyclin B1, an increase of CDK4, p27, and serum HGF occurred with no change in the other proteins. Besides, an increase in liver ATP and mitochondrial function was observed at 10 weeks. All these changes are not sufficient to initiate the proliferative process. Clearly, there is a marked increase in cyclins, CDKs, and regulatory proteins of the cell cycle; however, only PCNA and CDK6 are overexpressed in the cirrhotic animals treated with IFC-305. An elevation in liver ATP and mitochondrial functions were found also in the latter.

The proliferating cell nuclear antigen (PCNA) is a cofactor of DNA polymerase delta and appears to be needed for both DNA synthesis and DNA repair and could be associated with cdk6/cyclin D1 [35]. This protein acts as a processivity factor encircling the DNA; thus, by creating a topological link to the genome, PCNA helps in holding DNA polymerase delta to DNA [36]. Considering the chromosomal instability characteristic in cirrhosis, it is possible that PCNA plays an important role in repairing the damaged DNA [37]. Among

Table 2: Summary of the effects of IFC305 treatment on protein expression in CCl_4-induced cirrhosis.

Protein	Cirrhosis T0	Cirrhosis progress		IFC305 treatment	
		SS5 weeks	SS10 weeks	IFC5 weeks	IFC10 weeks
PCNA	NC	↓	↓↓	↑↑↑	↑↑↑
Cyclin D1	NC	↑	↑	↑↑	↑
Cyclins E, A	NC	NC	NC	NC	NC
Cyclin B1	NC	NC	↓	↓	↓
CDK4	NC	NC	↑↑↑	↑↑↑	↑↑↑
CDK6	↑↑↑	NC	NC	↑↑↑	↑↑↑
p21	↓↓	↓↓	NC	NC	↓
p27	NC	NC	↑	NC	NC
pRb (Ser 795)	↓	NC	NC	↑	↑
E2F1	↓↓	↓	NC	↑	NC
DP1	NC	NC	NC	NC	NC
p53	NC	↑	NC	↑↑	↑↑
MDM2	NC	↓	NC	↓	NC
Serum HGF	↑	↑	↑	↑↑	↑↑
Liver HGF	NC	NC	NC	NC	NC
cMet	NC	↑	NC	↑	NC

Each group was compared to the control group. NC: no changes. ↑ or ↓: less than 50% of increase or decrease with respect to the control. ↑↑ or ↓↓: 50 to 100% of increase or decrease. ↑↑↑: more than 100% of increase.

the cellular responses to DNA damage it is the p53-mediated activation of PCNA which binds *in vivo* to p21 and PCNA genes. p21 inhibits DNA replication but not DNA repair, the differential regulation of two DNA damage response effectors, p21/PCNA, by p53 plays an important role in DNA repair that is critical for the maintenance of genomic stability [38, 39].

As mentioned before, IFC-305 showed diverse effects during the reversion process of cirrhosis; microarray studies showed that the genes modified in cirrhosis became normalized after IFC-305 treatment. This study allowed us to visualize that the increase in PCNA and CDK6 expressions is very important to repair the DNA damage in the cirrhotic animals and to recover the genomic stability needed for the restoration of the proliferative capacity. It is possible that this is the pivotal role of action of this compound because once chromosomal instability decreased, proliferation of the liver was recovered, supported by the effect of the compound in regulating energy balance, oxidative stress, recovery of the redox state, and the capacity of protein synthesis. Controversial results of the adenosine actions could be obtained according with the different modes of action of the nucleoside that is mediated by the receptors, transporters, or through its metabolism. Then the results can depend on the experimental model used, producing contradictory results. It has been reported that extracellular adenosine and its receptors promotes fibrosis that could be blocked by the antagonist of adenosine receptors, this experiment considers endogenous extracellular adenosine in nanomolar range and uses antagonist of specific receptors and different temporality [40, 41]. In the experimental model used in this study, the recovery of cell proliferation in CCl_4-induced cirrhosis by the adenosine derivative IFC305 was realized in a whole animal, with millimolar concentrations of adenosine in the compound, and without mitogenic stimulus. In fact, in this study we found that the overexpression of the A2a receptor protein during cirrhosis decreases in the presence of IFC-305 (Figure 7), suggesting a role of this receptor in the described effect. Previously, we showed that the inhibitory effect of IFC305 in hepatic stellate cells activation was not mediated by adenosine receptors, but it was related with adenosine transport and intracellular AMP formation [42], neither in the adenosine acceleration of the cell cycle after one-third hepatectomy [19]. Possibly, a diminution of A2a receptor expression induced by the IFC305 increases adenosine transport into the cell promoting their effects in DNA repair and maintaining the energetic equilibrium. Then, as it has been described by Fredholm and Linden, adenosine can also modulate tissue damage and repair [43, 44]. We cannot discard the participation of adenosine receptors, transporters, or the metabolic effects. Further studies are needed to elucidate the specific mechanisms involved in these processes.

Acknowledgments

The authors thank Susana Vidrio and Rebeca Pérez Cabeza de Vaca for their technical assistance, and Consejo Nacional de Ciencia y Tecnología (CONACYT) as well as Instituto de Ciencia y Tecnología del Distrito Federal (ICyTDF) and Dirección General de Asuntos del Personal Académico (DGAPA), UNAM for financial support.

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Hindawi Publishing Corporation
International Journal of Hepatology
Volume 2012, Article ID 476910, 9 pages
doi:10.1155/2012/476910

Review Article

EGFR: A Master Piece in G1/S Phase Transition of Liver Regeneration

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Received 21 May 2012; Accepted 11 July 2012

Academic Editor: Anne Corlu

Unraveling the molecular clues of liver proliferation has become conceivable thanks to the model of two-third hepatectomy. The synchronicity and the well-scheduled aspect of this process allow scientists to slowly decipher this mystery. During this phenomenon, quiescent hepatocytes of the remnant lobes are able to reenter into the cell cycle initiating the G1-S progression synchronously before completing the cell cycle. The major role played by this step of the cell cycle has been emphasized by loss-of-function studies showing a delay or a lack of coordination in the hepatocytes G1-S progression. Two growth factor receptors, c-Met and EGFR, tightly drive this transition. Due to the level of complexity surrounding EGFR signaling, involving numerous ligands, highly controlled regulations and multiple downstream pathways, we chose to focus on the EGFR pathway for this paper. We will first describe the EGFR pathway in its integrity and then address its essential role in the G1/S phase transition for hepatocyte proliferation. Recently, other levels of control have been discovered to monitor this pathway, which will lead us to discuss regulations of the EGFR pathway and highlight the potential effect of misregulations in pathologies.

1. Introduction

Although mammals have almost completely lost the fascinating regeneration capacities of amphibians, their liver retained this unique ability. This process is evolutionarily conserved, presumably because it is critical to mammals' survival. Two-thirds partial hepatectomy (PH) in rodents has been used extensively to decipher the molecular and cellular clues of liver regeneration. During this process, the liver regenerates through hepatocytes, without the help of a stem cell compartment. A particularly fascinating point about this process is that near all quiescent and differentiated hepatocytes quit the G0 phase in a tightly synchronous manner to progress into the G1/S phase transition and replicate their DNA. This massive coordinated entry into the cell cycle is illustrated by a sharp peak of BrdU (Bromodeoxyuridine) incorporation whose timing differs among species (24 hours in rats and 36 to 42 hours in mice), reflecting the variability in the length of the G1 phase. Even if hepatocyte S phase entry is tightly synchronized, hepatocyte replication starts from periportal area and progresses rapidly towards perivenous area. Other nonparenchymal cells such as stellate cells, biliary and endothelial cells proliferate after hepatocytes, responding potentially to other signals.

This paper will focus on the molecular mechanisms involved in this synchronous entry into the cell cycle, highlighting the specific role of the epidermal growth factor receptor (EGFR) during this process in all its complexity.

2. Growth Factors and the Synchronous Entry of the Hepatocytes into the Cell Cycle

Hepatocyte proliferation is preceded by an inflammatory stimulus, described in the pioneering work of Nelson Fausto as the "priming phase" [1, 2]. This first step is reversible since, without the subsequent involvement of growth factors, hepatocytes do not progress through cell cycle and return to quiescence. It involves the secretion of cytokines by

nonparenchymal cells such as Kupffer cells and poises hepatocytes to become receptive to these growth factors [3, 4]. *In vivo*, this priming stage is required since hepatocytes exhibit only a minimal response to transforming growth factor alpha (TGF-α), epidermal growth factor (EGF), or hepatocyte growth factor (HGF) without it. In contrast, these factors are potent mitogens *in vitro* [5–8]. In primary culture, hepatocytes replicate their DNA synchronously after addition of EGFR ligands, suggesting that isolation of hepatocytes from the liver induces priming [6, 9, 10] and for review [11–13].

After cytokines have triggered the G0 to G1 phase transition, required growth factors for the progression through the cell cycle into the S phase are signaling through two main tyrosine-kinase receptors: EGFR and c-Met.

HGF is the main ligand of c-Met receptor. It is mainly secreted by macrophages and endothelial liver cells [14]. Overexpression of HGF in the liver of transgenic mice increases hepatocyte proliferation during postnatal development and accelerated liver regeneration after PH but has minor effects at adult stage in a quiescent liver [15–17]. On the contrary, conditional deletion of this receptor, as well as studies using RNAi in the liver of mice, caused either a significant decrease in the peak of proliferation [18, 19] or a delay of S-phase entry [20]. Moreover, Thorgeirsson's team indicated that c-Met is required for G2/M progression as well as entering the cell cycle *in vivo* [19].

As opposed to HGF/c-Met axis, EGFR has numerous ligands (EGF, amphiregulin, HB-EGF, TGF-α, epiregulin, betacellulin, epigen). For this reason, the implication of this pathway after PH has been studied extensively throughout the years, as it involves several growth factors and downstream pathways to control the proliferation balance. Interestingly, Mitchell et al. showed that after 1/3 PH in mice, there is a lack of a synchronous wave of DNA replication [21]. They then observed that, among the growth factors induced during liver regeneration, the secretion peak of HB-EGF usually observed 24 h after 2/3 PH was absent after the 1/3 PH [21]. HB-EGF injection in 1/3 hepatectomized mice is then sufficient to restore a peak of BrdU incorporation in hepatocytes [21]. Besides highlighting the robust mitogen potential of HB-EGF, this study indicated the importance of the EGFR pathway in the synchronous induction of DNA replication in a dose-dependent manner after PH.

3. EGFR Pathway

3.1. General Description of EGFR. The Epidermal Growth Factor Receptor (EGFR), also known as ErbB-1, is a plasma membrane glycoprotein, which belongs to the ErbB family of receptor tyrosine kinases (RTKs) jointly with ErbB-2, ErbB-3, and ErbB-4 [22]. It contains an extracellular domain with two cysteine-rich regions, a single transmembrane-spanning region, and a well-conserved cytoplasmic tyrosine kinase domain [23]. Upon ligand binding, ErbB proteins can either homo- or heterodimerize with other members of the ErbB family to activate downstream signaling pathways that regulate proliferation, growth, and differentiation [24].

EGFR was the first member of this family as well as the first RTK to be discovered [25] and plays an essential role in the development of epithelial cells but also in tumors of epithelial cell origin [26]. Ligand induced EGFR dimerization leads to receptor autophosphorylation at tyrosine residues (Figure 1). Some of them can be regulated via other signals like growth hormone [27, 28] or oxidative stress [29, 30]. Phosphotyrosine residues allow the recruitment of specific partners to activate different downstream pathways. EGFR controls a variety of signals ranging from cell proliferation, cell motility, apoptosis decrease, to epithelial-mesenchymal transition, upregulation of matrix metalloproteinases, and has even been proposed to be involved in stem-cell maintenance [31]. Moreover, EGFR has also been shown to regulate downstream targets by directly translocating its internal region into the nucleus, activating cell cycle genes such as Cyclin D1 [32] or genes involved in inflammation like COX-2 [33]. Interestingly, Cox-2-deficient mice showed an impaired liver regeneration [34].

EGFR signaling is regulated in part by endocytic sorting [35, 36]. Upon ligand binding, EGFR is internalized and trafficked to the endosome. Depending on ligand/EGFR complex stability [37] and ubiquitination process by cbl family proteins [38], EGFR is either degraded in the lysosomal compartment or recycled to the plasma membrane [35, 37, 39]. This process may represent an important negative feedback regulatory mechanism to control EGFR signaling [35, 36].

3.2. EGFR Pathway in the Liver. There is a strong expression of EGFR in the adult liver, but also during development and regeneration, suggesting an important role for its function [40]. Disruption of EGFR in mice has led to death from mid-gestation up to third week depending on the genetic background, showing various signs of abnormalities to multiple organs including the skin, kidney, brain, gastrointestinal tract, and the liver with thickened hepatocyte cords, distorted sinusoidal anatomy, and abnormally vacuolized nuclei [41]. Specific deletion of EGFR in hepatocytes did not reveal any phenotypical abnormality apart from a reduction in body weight [42]. It has been shown that EGFR ligands exhibit functional differences in models of paracrine and autocrine signaling [43]. Several ligands, such as amphiregulin, epidermal growth factor (EGF), heparin-binding EGF (HB-EGF), betacellulin, epiregulin, and TGF-α have been shown to be able to activate the EGFR pathway and some of them induced strong mitogens signals in the liver [44]. There is no evidence that these ligands bind specifically to EGFR and not to other ErbB proteins with whom EGFR can dimerize, although their essential role during liver regeneration has been demonstrated for some of them as described below [45].

There are four main downstream pathways usually associated with EGFR activation: Ras/MAPK, PI3K/Akt, signal transducer and activator of transcription (Stats) and phospholipase C-gamma 1 (PLCY1) pathways [46] (Figure 1). However, it has been shown in different epithelial cell types *in vitro* that ligands binding to EGFR induce different

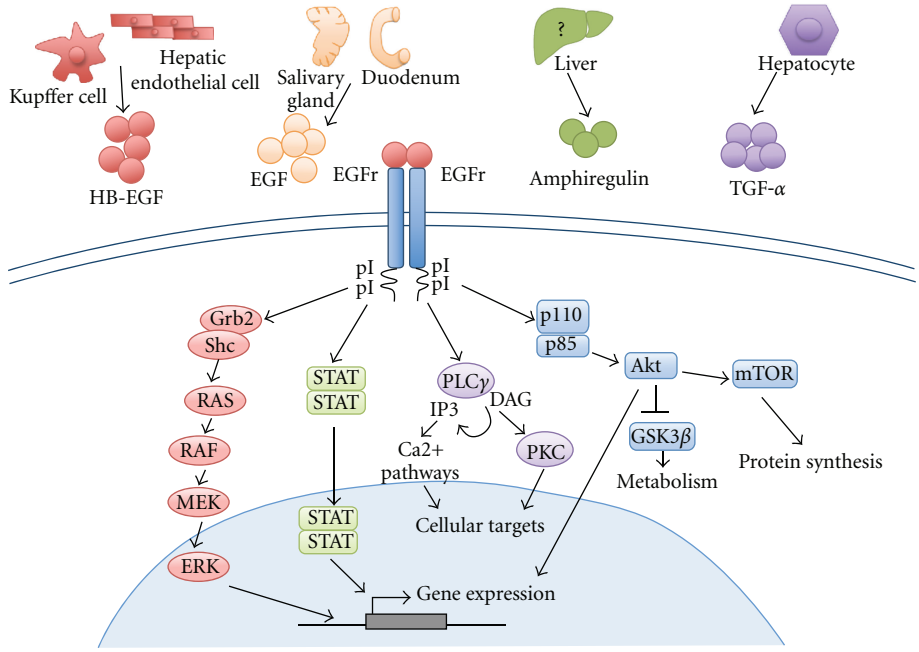

Figure 1: EGFR induced signaling pathways. The major source of each EGFR ligands involved in liver regeneration is schematized. Amphiregulin liver induction right after PH is not sufficient to determine the cellular origin of this secretion. Upon binding of its ligands, EGFR homodimerizes leading to phosphorylation of many tyrosine residues localized in the carboxy-terminal tail of EGFR. Phospho-EGFR is then able to recruit adaptor proteins. They transduce the EGFR signaling by inducing several EGFR-dependent pathways, including the RAS-MAPkinase, PI3K-AKT, PLCγ, and Stat pathways. Collectively these pathways control proliferation, differentiation, migration, and survival of the cell.

downstream signaling pathways according to their affinity. While high affinity ligands (10% of EGFR pool) activate Ras/MAPK and PI3K/Akt pathways, low affinity ligands (90% of EGFR pool) induce Stats and PLCY1 pathways [47]. It is now clear that those different pathways are highly interlinked, but for the following they will be described separately.

Once activated, the internal region of EGFR can serve as a docking site for Src homology 2 domains such as Grb2 and Shc [48, 49]. Grb2 or Shc then interacts with Ras, leading to an interaction with Raf, which will in terms activate the whole MAP kinases pathway [48–50]. The activation of EGFR can also provide a docking site for p85, which is the protein subunit of PI3K. Once activated, it will in turn phosphorylate Akt to promote cell survival and proliferation [51]. In the liver, EGFR-dependent Stats activation does not depend upon JAK kinases activation, as it is usually the case. Instead, Stats have been proposed to be constitutively associated to EGFR, becoming active directly by EGFR phosphorylation [49, 52]. More recently, the Src-kinase has been proposed to activate Stats through EGFR activation [53, 54]. The precise mechanism of PLCY1 activation remains unclear, but it appears that PLCY1 is also directly associated to EGFR but does not need tyrosine phosphorylation [55]. The activation of PLCY1 will yield 1,2-diacylglycerol (DAG) and inositol 1,4,5-triphosphate (IP3). DAG can then activate PKC whereas IP3 can activate Ca^{2+}-dependent pathways [56].

4. EGFR Pathway during the G1-S Phase in Hepatocytes

4.1. The Input of EGFR Ligands in Invalidation Mouse Models. The mitogenic action of the EGFR signal was first determined *in vitro*, on primary culture of hepatocytes. EGFR ligands were added in serum-free medium culture and tested for their capacity to induce hepatocyte proliferation in rodents. Four of them: TGF-α, HB-EGF, EGF, amphiregulin, were determined as hepatocyte growth factors since they allow their synchronized S phase entry [5–8].

After 2/3 PH, the protein level of these EGFR ligands increases rapidly [8, 57, 58]. Their mitogenic role was studied *in vivo* using ligand injection, gene overexpression, RNA interference, and conditional gene knockout strategies.

Loss of HB-EGF expression by knocking-out the gene led to major impairment of liver regeneration characterized by the absence of hepatocytes synchronized S phase entry [21]. Conversely, liver HB-EGF overexpression in transgenic mice induced a drastic increase of proliferating hepatocytes compared to wildtype nontransgenic littermates [59].

Salivary glands ablation in rodent [60–62], which are the main source of EGF, provoke a main liver regenerative defect hepatocytes being blocked in G1 phase, as it is the case in the conditional amphiregulin knockout mice [8]. After salivary glands ablation, EGF injections restore hepatocyte proliferation in rats [60–62].

Thus, misregulation of these three latter EGFR ligands leads to the same profile of liver regenerative defect characterized by a desynchronized S phase entry of hepatocytes. These results then suggest that these ligands are not redundant during liver regeneration. In contrast to these three liver EGFR ligands, gene inactivation of TGF-α in mice had no effect on liver regeneration [63] although it has been demonstrated on hepatocytes primary culture, that TGF-α has the same mitogen capacities as EGF, amphiregulin or HB-EGF [5, 6, 8, 64–66].

Different non-exclusive hypotheses can be proposed to understand the non-redundancy of HB-EGF, EGF and amphiregulin ligands. This can be explained by the importance of ligands sequential binding, by different ligands activating different EGFR downstream pathways or by the necessity to reach a threshold of total ligands quantity, in order to induce proliferative signals. Regarding TGF-α, the paradoxical results obtained *in vitro* and *in vivo,* can be explained if we hypothesize that already expressed EGFR ligands compensate for TGF-α. Genetic replacement of one ligand by another one at the same physiological level using knocking-in strategies, could help to understand these discrepancies.

4.2. The EGF Receptor during Regeneration. The role of EGFR (ErbB1) in the G1 phase of the cell cycle in hepatocytes has also been studied *in vivo* either by RNA interference injection in rats [67] or by conditional gene inactivation in mice [42]. Both experiments induced a major impairment of liver regeneration resulting in an altered progression into the G1 phase. However, mutant livers can finally complete regeneration suggesting that EGFR is a critical regulator of hepatocyte proliferation in the initial phases of this process. As opposed to other tissues, ErbB-2 and ErbB-4 are not expressed in the regenerating liver and thus, cannot heterodimerize with EGFR [40]. However, in contrast with liver regeneration, it has been observed that ErbB-2 is re-expressed in primary culture of hepatocytes, participating to the induction of proliferation *in vitro* [68, 69]. ErbB-3 is induced after PH but its ligands are not known to participate to hepatocyte proliferation [40].

The common molecular mechanism between all these studies consists in a downregulation of cyclin D1 expression, the first cyclin that is activated during progression in G1 phase [70, 71]. However, there is very little information on signaling pathways activated downstream of EGFR in hepatocytes to induce their proliferation. In primary culture of hepatocytes, Erk1/Erk2 and PI3K/AKT cascades have been shown to be activated by EGF to induce hepatocyte proliferation [72, 73]. However, during liver regeneration of EGFR knock outs specifically in hepatocytes in mice, none of these canonical downstream pathways were found dysregulated [42], while livers of rats injected with an EGFR RNAi showed a Stat3 misregulation [67]. Regarding knock outs experiments, the authors only reported a defect in the NF-κB and in p38 activation during the G1 phase [42]. The essential role of NF-κB during liver regeneration is to "prime" hepatocytes and not to participate to the G1 phase progression [4, 10]. However it was suggested that it could control cyclin D1 transcription [74, 75]. *In vitro* studies showed that EGFR could activate Ca^{2+} dependent pathways such as RaI and NF-κB through the phosphorylation of PLCγ. PLCγ is one of the possible downstream pathways activated by EGFR. It has been shown that the increased activity of nuclear PLC in regenerating rat occurs before DNA synthesis peak after PH [76]. Moreover, Farrell's group pointed out the role of the EGFR/PLCγ axis in hepatocyte proliferation in a model of chronic ethanol consumption [77].

In rats injected with RNAi directed against EGFR, Stat3 transcription downregulation was observed by transcriptomic approach [67]. This result may be relevant since Stat3 is a target of EGFR and has the capacity to activate proliferation through cyclin D1 in other cell types. However, it would have been interesting to check both the protein expression and activation level of Stat during the regenerating process, as this hypothesis does not match with cell culture experiments. It was indeed demonstrated that the Stats (Stat3 and Stat5) were not recruited for EGFR dependent hepatocyte proliferation *in vitro* [78].

The discrepancy between the *in vitro* and *in vivo* results makes it difficult to fully understand which are the intracellular targets required to induce the EGFR dependent progression into the S phase.

5. Regulation of the EGFR Pathway during Liver Regeneration

5.1. Regulation of the Ligands. Liver regeneration efficiency could indeed be controlled either by EGFR ligands induction and/or by EGFR activation. While various factors have been shown to regulate EGFR ligands in the quiescent liver, very little is known during the regenerative process when these factors are induced.

The Hippo signaling pathway, well known to be involved in cell proliferation, could also regulate the EGFR pathway through its pivotal effector, YAP (Yes-associated protein). Indeed, it has been shown that YAP regulates amphiregulin at a transcriptional level [79]. Interestingly, one study showed that YAP protein level increases after PH, suggesting a role in liver regeneration [80]. Loss of Hippo signaling in the mouse liver has been shown to lead to YAP induction and liver hyperplasia with hepatocytes progenitors proliferation [81–83]. However, no study has been yet performed after partial hepatectomy in YAP knockout mice to comfort this potential role.

Different members of the ADAM family induce the maturation of EGFR ligands, by cleaving them, and thus increasing their biodisponibility for EGFR binding [84]. ADAM 10 is able to cleave EGF transmembrane precursors [85]. ADAM 17, also known as tumour necrosis factor-α-(TNF-α-)converting, enzyme, or TACE, can shed amphiregulin, TGF-α and HB-EGF precursors [86, 87]. It has been suggested that ADAM17, upon TNF-α addition in hepatocyte cell culture, transactivates EGFR by cleaving TGF-α, increasing hepatocyte proliferation [88]. As for liver regeneration, ADAM 17 expression increases at the late G1-phase corroborating

a potential regulator role of EGFR signaling during this regenerative process [89].

5.2. Regulation of the Receptor. Regarding the receptor, we recently pointed out the major role played by the growth hormone (GH) pathway to control EGFR. GH is a pleiotropic hormone that plays a major role in proliferation, differentiation, and metabolism via its specific receptor. It has been previously suggested that GH signaling pathways are required for normal liver regeneration [90, 91]. Consequently, we recently investigated the mechanism by which GH controls liver regeneration. GH receptor knockout mice (GHrKO) showed a major liver regeneration impairment correlated with a downregulation of ERK1/ERK2 activation [92]. We showed that GH controlled the EGFR expression at the mRNA level in liver from quiescent stage until the mid G1-phase [92]. Most of GH physiological effects are mediated by the Stat5 transcription factor. Interestingly, EGFR expression was drastically down regulated in the liver of mice deleted for Stat5b in their hepatocytes [93]. However, chromatin immunoprecipitation experiments failed to demonstrate that Stat5b binds to EGFR promoter and suggested that it acts indirectly through intermediate proteins [93]. IGF-1, the major target gene of GH/Stat5b axis in the liver could have been an interesting target since it is known to be involved in liver regeneration control and it is drastically downregulated in GHrKO mice [91, 94]. However, we demonstrated that it is not the case, its forced expression in GHrKO mice hepatocytes failing to rescue EGFR expression (personal data). Interestingly, GH has also been described to control EGFR at posttranscriptional level, inducing its phosphorylation in quiescent liver [27, 28]. Accordingly with these data, EGFR failed to be activated by phosphorylation throughout the G1 phase in the hepatectomized GHrKO mice, even when it was reexpressed in mid/late G1-phase [92].

Bile acids that have been shown to contribute to liver regeneration have recently been considered as an intermediate in the interplay between EGFR and the Fas apoptotic pathway. Indeed, CD95L and hydrophobic bile acids are known to transactivate EGFR, but depending on the cell type, CD95-EGFR-mediated signalling ends up in cell apoptosis or cell proliferation. Thus, EGFR activation by CD95L or bile acids can lead to hepatic stellate cell proliferation but hepatocyte apoptosis [95].

Finally, the Wnt/β-Catenin pathway that is activated during the mid G1-phase during liver regeneration process could also participate to EGFR regulation during the liver regeneration process [96, 97]. β-catenin has been proposed to control EGFR in quiescent liver at a transcriptional level [98], but there is no clear evidence for a direct action of β-catenin via the putative Lef/Tcf site present on the EGFR promoter [98]. However, liver regeneration studies on mice deleted for ctnnb1 (the gene coding for β-catenin), although leading to a liver regeneration delay, did not point out an EGFR expression impairment [96, 97, 99]. We can hypothesize that β-catenin pathway compensates for EGFR defect in GHrKO mice from mid G1-phase when EGFR expression was reinduced.

6. Conclusion

Altogether, these data highlight the major role played by growth factors via EGFR in the liver regeneration process. Its activation during the G1 phase controls the cell cycle progression of hepatocytes from the G1 phase until the S phase leading to the synchronized hepatocytes S-phase entry. In liver regeneration, even though ligands have been identified, downstream pathways leading to hepatocytes S phase entry as well as the mechanisms that regulate EGFR pathway activation remain to be determined. In this context, our results point out the major role played by GH to control its expression and activation during the regenerative process [92].

The degree of complexity of hepatocyte proliferation's regulation by growth factors is reinforced by the question of a potential crosstalk and/or redundancy between EGFR and HGF pathways since they both induce hepatocytes G1/S progression and can activate the same downstream gene cascades [18–20, 42, 67]. A relationship between these pathways has been suggested by the observation that loss of c-Met or EGFR both lead to major liver regeneration impairment. This could result from the necessity of two independent pathways or by the existence of an essential interrelation between both pathways, to induce a robust hepatocyte proliferation signal. Since *in vitro* studies led to discordant results, it should be interesting in the future to test the redundancy or independence of c-met and EGFR pathways for liver regeneration in double knockout mice.

Given the importance of EGFR signaling to control hepatocytes division and its regulation by GH, it will be interesting to determine the incidence of misregulations of the GH/EGFR axis on the liver proliferative capacity in hepatic physiopathology. There have been numerous studies in human and in mice reporting defects of the GH signaling in various liver pathologies. For example, liver cirrhosis has been associated with the inhibition of GH signaling in the liver [100, 101]. Obesity, often associated with hepatic steatosis and insulinoresistance, is also characterized by a decrease of GH level in the serum of patients [102, 103]. We found in different mouse models that hepatic steatosis is associated both with a downregulation of GH pathway and a downregulation of EGFR expression (personal data). We therefore hypothesize that the loss of liver proliferation capacity in liver steatosis is related to the GH/EGFR axis misregulation.

In contrast, the EGFR signaling upregulation has been involved in cancer development in many tissues [104]. In hepatocellular carcinoma, its misregulation was found in 60 to 80 percent of patients, depending on studies, leading to the suggestion that EGFR signaling upregulation was associated with the increased proliferative capacity of liver tumoral cells [105].

The complete deciphering of EGFR signaling regulatory pathways resulting in this tricky balance will therefore be crucial in the future to develop appropriate therapeutic strategies allowing stimulation of hepatocyte proliferation in chronic liver diseases if required or in contrast, to reduce it in cases of tumoral progression.

Authors' Contribution

Hélène Gigenkrantz and Jacques-Emmanuel Guidotti contributed equally to this work.

Acknowledgments

The authors' laboratory was supported by grants from AFEF (Association Française pour l'Étude du Foie), Agence de la Biomédecine, ANR (Agence Nationale pour la Recherche) and INSERM.

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Hindawi Publishing Corporation
International Journal of Hepatology
Volume 2012, Article ID 816125, 7 pages
doi:10.1155/2012/816125

Review Article

A Complex Interplay between Wnt/β-Catenin Signalling and the Cell Cycle in the Adult Liver

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Received 26 June 2012; Accepted 2 August 2012

Academic Editor: Pascal Loyer

Canonical Wnt signalling, governed by its effector β-catenin, is known for a long time as playing an important role in development, tissue homeostasis, and cancer. In the liver, it was unravelled as both an oncogenic pathway involved in a subset of liver cancers and a physiological signalling identified as the "zonation-keeper" of the quiescent liver lobule. This duality has encouraged to explore the role of canonical Wnt in liver regeneration and liver-cell proliferation mainly using murine genetic models of β-catenin overactivation or inactivation. These studies definitely integrate Wnt signalling within the hepatic network driving regeneration and proliferation. We will review here the current knowledge concerning the mitogenic effect of Wnt, to switch on its specific role in the liver, which is quiescent but with a great capacity to regenerate. The duality of β-catenin signalling, associated both with liver quiescence and liver-cell proliferation, will be brought forward.

1. Introduction

Since 1982 and the initial discovery of Int1 (Wnt1a) being an oncogene in murine breast cancers, Wnt signalling has been strongly associated with cancer and therefore with cell proliferation [1]. Firstly described as triggering G1 phase progression through Cyclin D1 and c-Myc transcriptional inductions [2–4], it now clearly appears that the interplay between the cell cycle and Wnt signalling is more complex, specific for cell and tissue contexts and not only transcriptional ([5], for review).

The Wnt signalling consists either in a canonical or a noncanonical pathway and only the better characterized canonical pathway will be depicted here ([6], for review). β-catenin is the main effector of the canonical signalling (Figure 1). In cells not submitted to Wnt ligands, the cytosolic β-catenin is continuously ubiquitinylated for degradation through sequential phosphorylations by the caseine kinase 1 (CK1) and the glycogen synthase kinase 3 (GSK3). This occurs within a so-called "destruction complex" scaffolded by the tumor suppressors AXINS. The tumor suppressor APC (Adenomatous polyposis coli) is required within this destruction complex for an efficient degradation of β-catenin. Upon Wnt ligand binding to its frizzled receptor and LRP5/6 coreceptor at the membrane, a cascade of events impedes GSK3 kinase activity, through LRP5/6-dependent sequestration of GSK3 within endosomal vesicles [7]. The ensuing accumulation of β-catenin triggers its nuclear translocation and its association with a Lef/Tcf DNA-binding partner. This leads to the transcription of a genetic program specific for the temporal, spatial, and tissue contexts ([8], for review). Mutations in critical partners of the pathway, that is, β-catenin gene- (CTNNB1-) activating mutations, loss-of-function mutations in APC, AXIN1, or AXIN2 genes, induce a constitutive activation of β-catenin signalling and are found in a large number of human cancers ([9], for review).

2. Wnt and the Cell Cycle: An Overview

How Wnt/β-catenin signalling is mitogenic has been widely explored in many experimental systems and has been shown to occur at distinct levels ([5], for review).

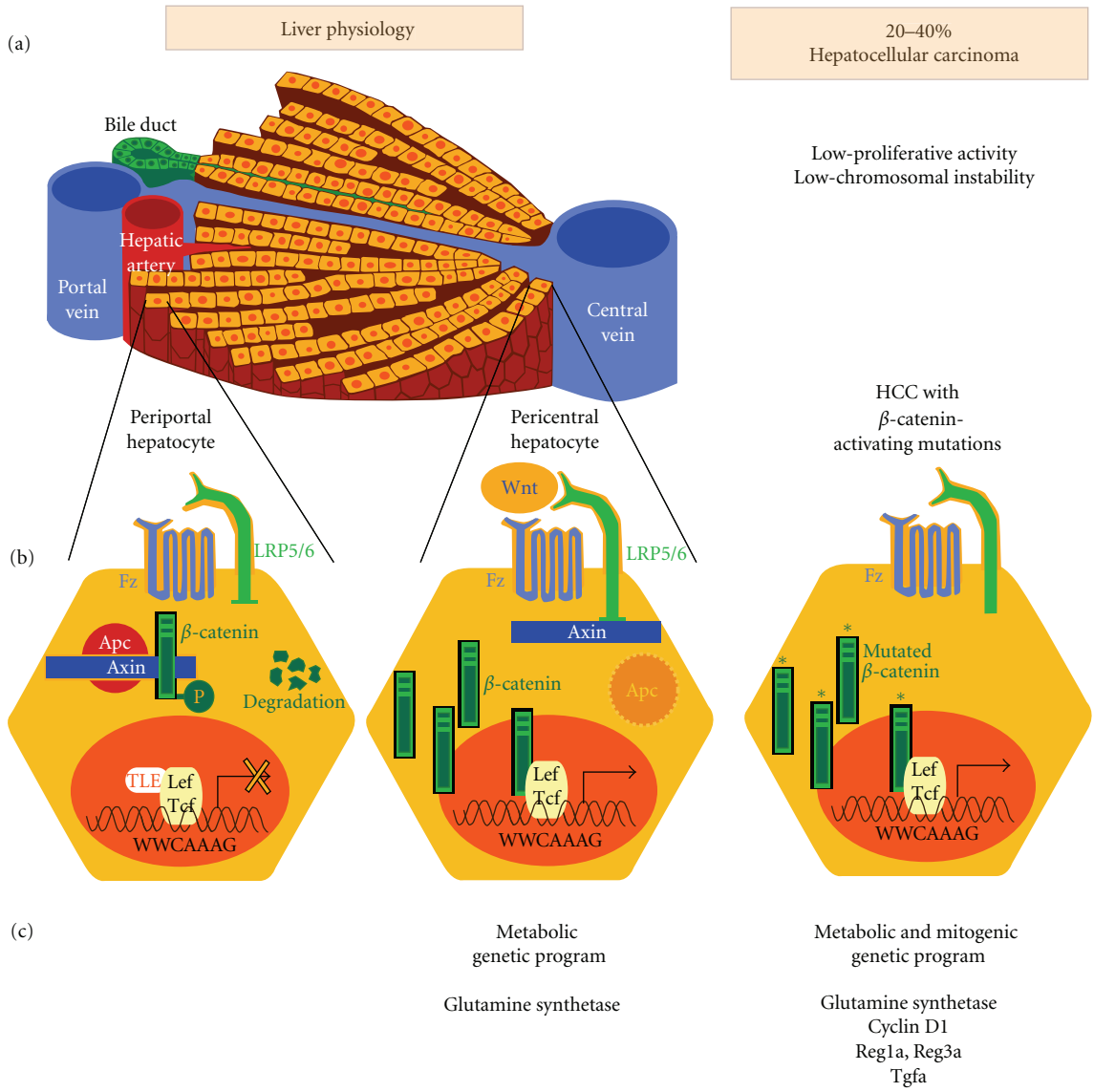

Figure 1: Wnt signalling in the adult quiescent liver and in CTNNB1-mutated HCCs. (a) The liver-cell plate and its portocentral organization; (b) the periportal hepatocyte is deprived of Wnt signalling, due to its high amount of Apc, allowing the destruction complex to be efficient to degrade β-catenin. The pericentral hepatocyte has a Wnt-dependent β-catenin signalling, while in HCCs it is constitutively activated due to mutations in phosphorylation residues in CTNNB1; (c) the output of the transcriptional β-catenin is metabolic in the pericentral hepatocyte, while it is both metabolic and mitogenic in HCCs.

2.1. Wnt Transcriptional and Nontranscriptional Effects during the G1 Phase. Entering S phase and DNA replication is a key decision that forces cells to divide, and it has to be regulated during G1 phase. Indeed, most signalling pathways that regulate cell proliferation exert their effects in G1 ([10], for review). Cyclin D is an important regulator of the checkpoints allowing G1-to-S progression, including the inactivation through phosphorylation of the Retinoblastoma (Rb) complex, increasing Cyclin E levels. At the opposite, growth inhibitory signals inhibit cyclin D (and cyclin E) through p21 and p27 accumulation, thereby leading to entry into quiescence [10].

As expected, Cyclin D1 has been one of the first transcriptional target genes of β-catenin described in colorectal cancer cell lines [3, 4]. But most important was the involvement of c-Myc as a Wnt transcriptional target [2], because this transcription factor has a dual role in G1 phase by promoting Cyclin D [11] and repressing p21 and p27 [12]. This importance has been emphasized by the fact that in the intestine, c-Myc ablation fully rescues Apc loss-driven tumorigenesis [13]. Interestingly, it is not the case in the liver [14], consistent with the fact that c-Myc is surprisingly not a transcriptional target of β-catenin in that tissue [15–17].

But Wnt signalling-mediated GSK3 inhibition not only induces transcriptional changes. GSK3 phosphorylates and destabilizes other substrates than β-catenin, among which are direct regulators of G1 progression, such as cyclin D1, cyclin E1, and c-myc [18–20]. Lastly, GSK3 is also a key

inhibitor of cell growth occurring in G1, during which cells increase their protein levels otherwise they would become smaller after cell division. Similarly to the classical IGF/AKT pathway, Wnt/Gsk3 signalling activates the TOR pathway to stimulate protein translation, including that of Cyclin D1: indeed GSK3 activates TSC2, an inhibitor of the TOR pathway [21].

2.2. Microtubule Dynamics during Mitosis. During mitosis, cells divide both chromosomes and cell components into daughter cells. Thereby it is a phase in which predominate subcellular mechanics, with transcription and translation being dampened [5]. Nevertheless, some aspects of the mitotic program that is, the microtubule (MT) dynamics, spindle formation, and centrosome division can be partly ascribed to Wnt components, even if it is not clearly assessed that these features would be controlled by Wnt/β-catenin signalling (reviewed in [5]).

Briefly, MT dynamics are regulated by Wnt signalling, even without any mitosis [22]. The tumor suppressor APC has been the first component of Wnt signalling to be associated to the mitotic spindle, and this is required for proper chromosome segregation: therefore, Apc loss was shown to lead to chromosomal instability [23, 24], and can also induce polyploidy ([25], this is of interest for the physiologically polyploid liver, reviewed by Gentric et al., in this issue). AXIN2 also associates to the mitotic spindle [26]. Lastly, AXIN2, β-catenin, and GSK3 accumulate at the centrosomes, which align the mitotic spindle. Therein, they regulate MT growth ([5], for review).

2.3. Cell Cycle Impact on Wnt Signalling Amplitude. Wnt signalling influences the cell cycle but conversely the cell cycle has an impact on Wnt signalling. It had been observed that β-catenin levels oscillate with the cell cycle, and peak in mitosis [27]. Similarly, the expression of some β-catenin targets (Lgr5, AXIN2, but not c-myc) peaks at G2/M [28]. This phenomenon has recently found a molecular explanation through LRP6 phosphorylation, required for LRP6 to respond to Wnt ligands. This phosphorylation is primed by the cyclin-dependent kinase 14 (cdk14), which associates with and is regulated by the G2/M cyclin Y. The optimal response of LRP6 coreceptor to Wnts is therefore cell cycle dependent, and consequently the maximal activation of β-catenin occurs at G2/M [29].

3. Wnt Signalling in the Liver

3.1. Wnt in Liver Oncogenesis. In 1998, the link between Wnt signalling and the liver was initially established, through the demonstration that β-catenin activating mutations occur in 20 to 40% of hepatocellular carcinoma (HCC) [30, 31]. HCC is an heterogenous disease that differs both by its risk factors (Viral hepatitis B and C, alcohol abuse, metabolic liver disease, aflatoxin intoxication), and by its mutational profile. Using a cohort enriched in HCCs with alcohol cirrhosis background, a recent extensive study found that mutations in Wnt/β-catenin partners predominate, 32.8% of HCCs being mutated in CTNNB1, 15.2% in AXIN1, and 1.6% in APC genes [32]. A pioneering work showed in 2001 that the HCCs mutated in CTNNB1 belong to a group of HCCs characterized by a low genomic instability and a better prognosis for patient survival [33]. This profile differs from that associated with p53 and AXIN1 mutations, that mainly consists in HCCs with a high chromosomal instability and a poor prognosis [34, 35]. Unexpectedly, AXIN1 mutations in HCCs do not activate efficiently β-catenin pathway, suggesting that its tumor suppressor function is mediated through other partnerships [36]. Interestingly, CTNNB1-mutated HCCs are less proliferative than nonmutated ones, suggesting that the genetic program by which β-catenin signalling triggers hepatocarcinogenesis is somehow different from that which is implemented following P53 mutations [37]. From studies performed in human HCCs and also from murine transgenic models in which Apc loss leads to β-catenin-activated liver tumors, several Wnt target genes have been described with a potential role in hepatocyte proliferation in a cancerous context: these are Cyclin D1, but not c-myc, the regenerating islet-derived 3- and 1-α genes (REG1A and REG3A), Tgf-α [15, 16, 38, 39]. However, the critical transcriptional targets by which β-catenin induces proliferation in liver cancers remain elusive.

3.2. Wnt in Quiescent Pericentral Hepatocytes. After the initial discovery of the oncogenic role for liver β-catenin, an unexpected Wnt signalling was detected in 2006 in a subset of quiescent hepatocytes located within the pericentral area [40]. It should be noted that a role of Wnt signalling in post-mitotic cells has been described not only in hepatocytes, but also in neurons and cardiomyocytes [5].

In mammals, the different metabolic functions of the liver, such as gluconeogenesis, glycolysis, glutamine synthesis, or urea formation are assumed by hepatocytes that differ in their location along the portocentral axis of the liver lobule, either near the portal triad (periportal, PP) or close to the central vein (pericentral, PC). This is the concept of metabolic zonation [41–43]. We found in 2006 that β-catenin is physiologically activated in pericentral hepatocytes [40]. This process is blocked by Dkk1 and is therefore Wnt-dependent even if the Wnt source around the central vein is not clearly identified, but could be of endothelial or stellate cell origin [44–46]. This β-catenin signalling is also due to the low amount in this area of APC, further defined as the "zonation-keeper" of the liver: as a consequence, its liver-specific loss enables a loss of zonation together with hepatocyte hyperproliferation, with a dramatic metabolic phenotype leading to the death of the mice [40]. This zonal patterning is due to β-catenin inducing the transcription of genes encoding metabolic enzymes in the pericentral area, whereas in the same zone it directly represses the transcription of genes encoding periportal enzymes or transporters ([42], for review). The quiescent liver is therefore an attractive model for Wnt research, allowing to decipher the molecular mechanism by which Wnt in G0 hepatocytes controls liver metabolism rather than proliferation.

3.3. Wnt in Liver Regeneration and Hepatocyte Proliferation. Consistent with the oncogenic role of β-catenin in the liver,

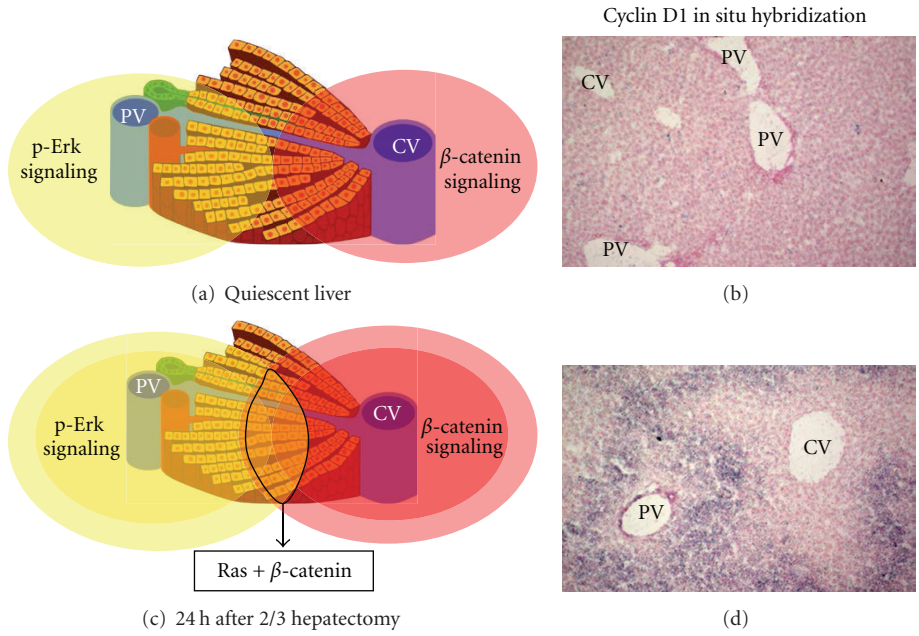

Figure 2: Hypothesis for a crosstalk between β-catenin and Ras signalling pathways to control hepatocyte proliferation. (a) In the quiescent liver, phospho-erk signaling is described as being periportal, while β-catenin is pericentral; (b) no cyclin D1 mRNA is detected in these conditions; (c) 24 h after 2/3 hepatectomy, there is an extension in the β-catenin and the ras activation territories. We now hypothesize that it could generate a common territory in which both which phospho-erk and β-catenin signalings are activated; (d) this can be exemplified by cyclin D1, which is a target of β-catenin, potentiated by ras signaling. Its localization after hepatectomy is restricted to the midlobular region. PV = portal vein; CV = central vein.

the massive activation of β-catenin in more than 70% of hepatocytes in mice leads to hepatomegaly, partly due to hepatocyte proliferation [15, 16, 40, 47]. Conversely, liver weight has been shown to be 20% lower in adult mice with a liver-specific β-catenin inactivation than in wild-type mice [48]. Moreover, such β-catenin inactivation/overactivation murine models were submitted to two-third hepatectomies, and a role for β-catenin in liver regeneration has been firstly reported in 2006 [39, 48–51]. After hepatectomy, β-catenin activation extends from the pericentral area up to the midlobular hepatocytes. It takes place by 24 h after hepatectomy, and this corresponds to progression in G1: it induces at least Cyclin D1 and Tgf-α expressions [39]. But the distribution of proliferating hepatocytes is panlobular, while that of Cyclin D1 begins in the midlobular area, suggesting that β-catenin-dependent hepatocyte proliferation is dictated both by cell-autonomous and non-cell-autonomous mechanisms. Interestingly, the more distal pericentral hepatocytes do not initially express cyclin D1 in response to β-catenin, highlighting the resistance of PC hepatocytes to proliferate [52]. Moreover, we have shown that Ras signalling increased the β-catenin-dependent transcription of cyclin D1 further in hepatoma cells, confirming previous studies in colon cancer cell lines [4, 39]. As Ras/Erk1-2 is the predominant signalling pathway in the periportal area [53], we now hypothesize that it could cooperate with β-catenin in the midlobular hepatocytes to elicit an enhanced cyclin D1 transcription during liver regeneration (Figure 2). This hypothesis is supported by the fact that Ha-Ras and β-catenin signalling cooperate to accelerate liver tumorigenesis [54].

3.4. Wnt and Liver Stem Cells. Wnt signalling has a prominent role in stem cell biology, including self-renewal, pluripotency, and differentiation of both embryonic stem (ES) and somatic stem cells (reviewed in [55]). It was therefore attractive to search for an equivalent role in the liver, and the first publications in that field appeared by 2007.

It must be understood that liver homeostasis is not dictated by its self-renewal, due to the quiescence of the hepatocytes with a lifespan of 300–400 days [56]. Moreover, it is known that the mature quiescent hepatocyte is able to re-enter into the cell cycle and to self-renew without the need for a stem cell. However, some particular cells located in the vicinity of the portal triad within the so-called Herring canal are a reservoir for regeneration in particular contexts of liver diseases or for specific oncogenesis [57]. Several signalling pathways have been shown to play an important role in the emergence, expansion, and differentiation of these transiently amplifying progenitor cells, also referred to as oval cells [58]. An active Wnt signalling has been found during progenitor cell-mediated regeneration of the liver (drug-induced models in which hepatocyte proliferation is blocked, forcing the putative stem cells to engage into the cell cycle) [56, 59, 60].

4. Perspectives: How Hepatic Wnt Signalling Impacts the Cell Cycle?

The role of Wnt in the adult liver is paradoxical, due to its dual role as patterning its metabolic zonation, while

being engaged in liver-cell proliferation both physiologically in regeneration processes, and pathologically during oncogenesis.

In fact, the balance between quiescence and proliferation has to be fine-tuned in order to avoid either a fatal loss or tissue regeneration or neoplasia [61, 62]. So it is attractive that Wnt signalling could be such a sensor, perfectly adapted to liver needs. The dissection of the various modes whereby Wnt signalling impacts G1 in the liver and the identification of the molecular network that shifts Wnt from a metabolic to a mitogenic output may help designing specific cancer therapies.

Abbreviations

CK1:	Caseine kinase 1
GSK3:	Glycogen synthase kinase 3
APC:	Adenomatous polyposis coli
LRP5/6:	Lipoprotein-related protein 5/6
Lef/Tcf:	Lymphoid enhancer factor/T-cell factor
Rb:	Retinoblastoma
c-Myc:	Myelocytomatosis oncogene
IGF/AKT:	Insulin growth factor/Akt
TOR:	Target of rapamycin
TSC2:	Tuberous sclerosis 2
MT:	Microtubule
LGR5:	Leucine rich repeat containing G protein-coupled Receptor 5
CDK14:	Cyclin-dependent kinase 14
HCC:	Hepatocellular carcinoma
REG1A/REG3A:	Regenerating islet-derived 1 alpha/3 alpha
PC:	Pericentral
PP:	Periportal
TGFα:	Transforming growth factor alpha
Ras:	Rat sarcoma viral homolog
ERK:	Extracellular regulated MAP kinase
PV:	Portal vein
CV:	Central vein.

Acknowledgments

A. Gougelet is a recipient of "Agence Nationale de la Recherche" ANR-blanc "WNT-METABOLIV" (2010–2013). This study is financially supported by INSERM (Institut National de la Santé et de la Recherche Médicale), ANR "WNT-METABOLIV," and by LNCC (Ligue Nationale Contre le Cancer) "Equipe labellisée 2011–2013."

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Hindawi Publishing Corporation
International Journal of Hepatology
Volume 2012, Article ID 542479, 8 pages
doi:10.1155/2012/542479

Review Article

Regulation of Signal Transduction and Role of Platelets in Liver Regeneration

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Received 19 April 2012; Accepted 8 June 2012

Academic Editor: Pascal Loyer

Among all organs, the liver has a unique regeneration capability after sustaining injury or the loss of tissue that occurs mainly due to mitosis in the hepatocytes that are quiescent under normal conditions. Liver regeneration is induced through a cascade of various cytokines and growth factors, such as, tumor necrosis factor alpha, interleukin-6, hepatocyte growth factor, and insulin-like growth factor, which activate nuclear factor κB, signal transducer and activator of transcription 3, and phosphatidyl inositol 3-kinase signaling pathways. We previously reported that platelets can play important roles in liver regeneration through a direct effect on hepatocytes and collaborative effects with the nonparenchymal cells of the liver, including Kupffer cells and liver sinusoidal endothelial cells, which participate in liver regeneration through the production of various growth factors and cytokines. In this paper, the roles of platelets and nonparenchymal cells in liver regeneration, including the associated cytokines, growth factors, and signaling pathways, are described.

1. Introduction

Liver regeneration is a physiopathological phenomenon of quantitative recovery from the loss of liver mass to compensate for decreased hepatic volume and impaired function [1, 2]. Although numerous studies have shown that a variety of genes, cytokines, growth factors, and cells are involved in liver regeneration, the exact mechanism of regeneration and the interaction between hepatocytes and cytokines are not fully understood [3]. Liver regeneration is a critical issue related to clinical morbidity and mortality in drug-induced liver injury and after surgery including hepatectomy or living-donor liver transplantation [4, 5]. The temporal development of the signaling pathways specifically activated during liver regeneration may be described in three phases: a priming phase, involving the transition of quiescent hepatocytes from G0 into the G1 phase of the cell cycle; a proliferation phase during which the progression of the entire hepatocyte population occurs; a termination phase during which cell proliferation is suppressed and regeneration is terminated at a defined point [6, 7]. Hepatocytes are not terminally differentiated; rather, the cells are in proliferative quiescence (the G0 phase) but can rapidly enter a cell division cycle upon stimulation [6]. The cytokines/signaling pathways and proteins that are important during the priming phase of regeneration include tumor necrosis factor alpha (TNFα)/nuclear factor κB (NFκB), interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT3), activator protein-1 (AP-1), and mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) [8–14]. Phosphatidyl inositol 3-kinase (PI3K)/Akt is also immediately activated after hepatectomy and plays an important antiapoptotic role during liver regeneration [15]. During the proliferation phase, hepatocytes express various cell cycle proteins that guide the replication process, including hepatocyte growth factor (HGF) and epidermal growth factor (EGF) [16, 17]. Essentially, the major factors involved in the termination phase comprise transforming growth factor beta (TGFβ) and activins [6].

Seventy percent of the cell number or 80% of the liver volume is composed of hepatocytes, and the remaining cells consist of nonparenchymal cells, including Kupffer cells, liver sinusoidal endothelial cells (LSECs), hepatic stellate cells,

and lymphocytes, which are thought to play an important role in cytokine release [18]. Upon activation, Kupffer cells are reported to produce both inflammatory cytokines, such as, TNFα and IL-6, and such growth factors as insulin-like growth factor (IGF)-1 [19]. LSECs have also been reported to produce IL-6 and HGF after hepatectomy and activated hepatic stellate cells mainly produce HGF [20, 21].

Platelets play pivotal roles in thrombosis and hemostasis, but an increasing variety of extrahemostatic functions of platelets have been recognized [22]. Platelets contain many growth factors, such as, platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), HGF, IGF, EGF and TGFβ, and some cytokines [23–29]. Furthermore, platelets play certain roles of stimulation or acceleration during hepatocyte proliferation [30]. We recently reported that platelets play a very important role in liver regeneration after hepatectomy and that HGF and IGF-1 derived from platelets are essential for hepatocyte proliferation [23, 31–33]. Platelets play a direct role in hepatocytes during liver regeneration and also a cooperative role with nonparenchymal cells in the liver [33, 34]. Kupffer cells contribute to the accumulation of platelets in the liver, which can subsequently induce liver regeneration [31, 33, 34]. Furthermore, the direct contact between platelets and LSECs induces sphingosine 1-phosphate (S1P) release from the platelets, which subsequently induces the secretion of IL-6 from LSECs. The LSEC-derived IL-6 promotes DNA synthesis in hepatocytes via the STAT3 pathway [35].

Herein, we describe the cellular and molecular mechanisms of liver regeneration and the functions of some critical signaling pathways involved in hepatocyte proliferation. In particular, we focus on the roles of platelets in liver regeneration.

2. Cytokines, Growth Factors, and Signaling Pathways in Liver Regeneration

Liver regeneration occurs through the proliferation of all of the existing mature cellular populations including the hepatocytes, biliary epithelial cells, LSECs, Kupffer cells, and hepatic stellate cells. All of the cells proliferate to rebuild the lost hepatic tissue, and the hepatocytes are known to be the first cells to proliferate [36]. Hepatocytes exhibit a mitogenic response to various growth factors and cytokines, such as, HGF, IGF-1, IL-6, TNFα, EGF, TGFβ, and PDGF [1, 23]. These growth factors and cytokines lead to the subsequent activation of downstream transcription cascades, which effect the transition of the quiescent hepatocytes into the cell cycle and progression beyond the restriction point in the G1 phase [1]. The cascades also result in the activation of transcription factors and signal transduction pathways, such as, NFκB, STAT3, MAPK/ERK, PI3K/Akt, AP-1, and CCAAT/enhancer-binding protein-β, which subsequently induce hepatocyte proliferation [8–15, 37–41]. Among these transcription factors and corresponding signal transductions, the TNFα/NFκB, IL-6/STAT3, PI3K/Akt, and MAPK/ERK pathways are identified as the major cascades during the process of liver regeneration (Figure 1).

2.1. TNFα/NFκB Signaling Pathway. TNFα is a crucial cytokine during the priming phase of liver regeneration and activates the NFκB transcription factor via the TNF receptor 1 (TNFR1) in hepatocytes [42]. After partial hepatectomy, the production of TNFα is upregulated mainly in Kupffer cells; NFκB is activated within 30 minutes after partial hepatectomy, and the activation usually lasts no longer than 4-5 hours [1, 43]. During hepatocyte proliferation, NFκB is a heterodimer composed of two subunits, p65 and p50, which are assembled in the cytosol; the complex is inactivated by inhibitor of NFκB (IκB), which binds to the p65 subunit. After stimulation with TNFα, NFκB is activated by the removal of IκB from its p65 subunit; the activated NFκB then migrates to the cell nucleus, which regulates the G0/G1-to-S phase transition [44–46]. It was reported that the administration of a TNFα antibody or the knockout of TNFR1 in mice results in delayed liver regeneration after partial hepatectomy [8, 47]. In Kupffer cell-depleted mice, liver regeneration was impaired because of the loss of TNFα and NFκB [48].

2.2. IL-6/STAT3 Signaling Pathway. The STAT3 pathway, which is activated by such cytokines as IL-6, is known to play a crucial role in cell proliferation [49, 50]. Kupffer cells express TNFR1 on their surface and activate themselves in an autocrine fashion. Because the promoter region of the IL-6 gene contains the NFκB-binding site, IL-6 can also be produced by the activated Kupffer cells [51]. Recently, both LSECs and Kupffer cells were reported to produce IL-6 after hepatectomy [20, 33]. IL-6 binding causes the dimerization of gp130, which is the ubiquitously expressed signaling receptor molecule for the IL-6 family, and the activation of the intracellular tyrosine kinase that phosphorylates gp130 and creates the docking site of STAT3 [52]. STAT3 is then phosphorylated and translocates to the nucleus. STAT3 is activated slower than NFκB, becoming detectable at 1 to 2 hours after partial hepatectomy and lasting approximately 4–6 hours [1]. It was reported that hepatocytic mitosis in STAT3-knockout mice was significantly suppressed during liver regeneration after partial hepatectomy [53].

2.3. PI3K/Akt Signaling Pathway. The PI3K/Akt pathway has been known as a survival pathway functioning in antiapoptosis [54–56]. Recently, it was revealed that the PI3K/Akt pathway is responsible for regulating cell growth and determining cell size and functions [57–62]. In addition, Akt and downstream signals cause the compensatory hypertrophy of hepatocytes when cell proliferation is impaired [49]. The pathway is initiated by the activation of receptor tyrosine kinases or G protein-coupled receptors by HGF, IL-6, TNFα, TGFα, and many other signaling molecules [63–67]. HGF is a potent growth factor that is mainly derived from LSECs and activated HSCs and promotes proliferation and DNA synthesis in hepatocytes in a paracrine fashion [20, 68]. C-met is a tyrosine kinase receptor on the surface of hepatocytes that binds to HGF, and HGF/c-met signaling activates PI3K, which then recruits Akt to the site of membranes and subsequently phosphorylates Akt [65, 69, 70].

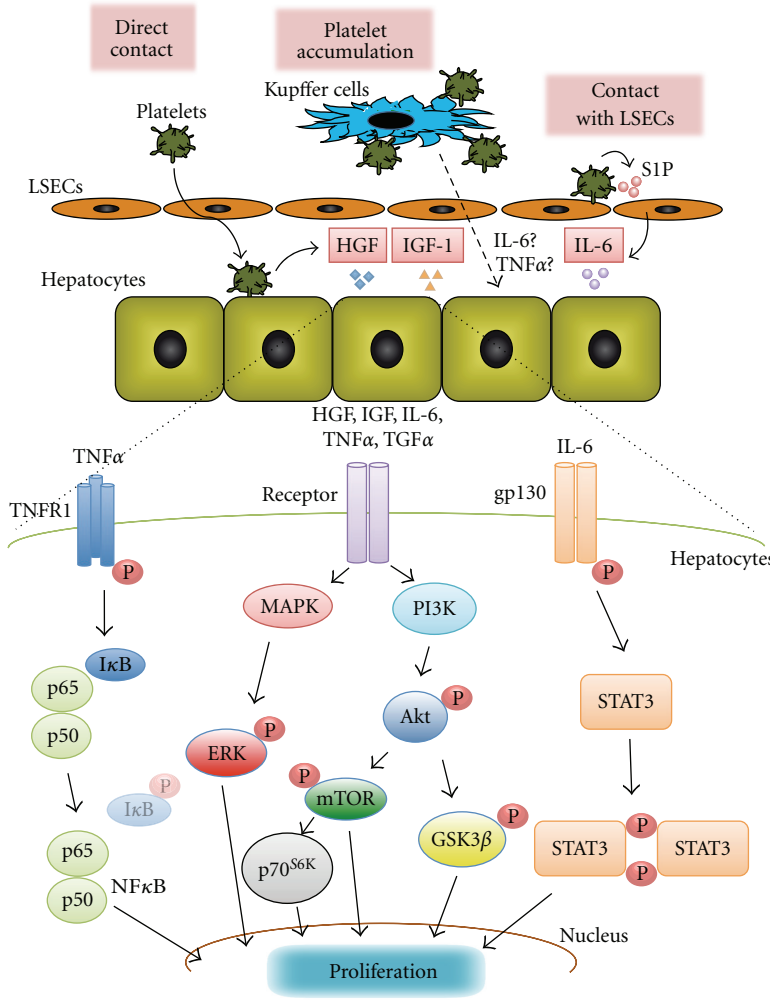

Figure 1: *The Roles of Platelets and the Signal Transductions Identified as the Major Cascades in Hepatocyte Proliferation.* After liver injury, Kupffer cells can play a crucial role in the accumulation of platelets in the liver sinusoids and the production of cytokines, such as, interleukin-6 (IL-6) and tumor necrosis factor alpha (TNFα). Moreover, Kupffer cells induce the translocation of platelets into the space of Disse and the direct contact between platelets and hepatocytes, which trigger the release of growth factors, such as, hepatocyte growth factor (HGF) and insulin-like growth factor (IGF)-1, which are necessary for hepatocyte proliferation. Platelets also have direct contact with liver sinusoidal endothelial cells (LSECs), which trigger the release of sphingosine 1-phosphate (S1P). S1P induces the secretion of IL-6 from LSECs, which promotes hepatocyte proliferation. During hepatocyte proliferation, IL-6 binding induces both the dimerization and the phosphorylation of gp130 and promotes the docking site of signal transducer and activator of transcription 3 (STAT3). STAT3 is then phosphorylated and translocated to the nucleus. Phosphatidyl inositol 3-kinase (PI3K)/Akt signaling pathway is essential for the platelet-induced hepatocyte proliferation and is activated via the receptor tyrosine kinases through the stimulation of HGF, IGF, IL-6, and many other signaling molecules. Phosphorylated Akt activates glycogen synthase kinase 3β (GSK3β), which induces DNA synthesis and cellular mitosis in hepatocytes. Other downstream Akt factors including mTOR and $p70^{S6K}$ also play critical roles in regulating the growth of hepatocytes. Mitogen-activated protein kinase (MAPK)/extracellular signal-regulated protein kinase (ERK) signaling pathway is also immediately activated after hepatectomy and thereafter phosphorylated ERK translocates to the nucleus. TNFα/nuclear factor κB (NFκB) signaling pathway is activated via the TNF receptor 1 (TNFR1). NFκB is a heterodimer composed of two subunits, p65 and p50, with inactivated by inhibitor of NFκB (IκB). After stimulation with TNFα, NFκB is activated by the removal of IκB from its p65 subunit, followed by the migration to the cell nucleus.

Phosphorylated Akt activates glycogen synthase kinase 3β (GSK3β), which induces DNA synthesis and cellular mitosis in hepatocytes [49, 71]. Furthermore, other downstream Akt factors, such as, mTOR and $p70^{S6K}$, play critical roles in liver regeneration by regulating cell growth in addition to activated GSK3β [49, 60, 61]. It was reported that the specific PI3K inhibitor LY294002 abolished DNA synthesis in growth factor-stimulated hepatocytes [72].

2.4. MAPK/ERK Signaling Pathway. Once the MAPK/ERK pathway has been activated, ERK translocates to the nucleus

where it can regulate the transcriptional activity of many immediate early genes [73]. The signaling cascade is activated by growth factors, such as, IGF-1 and EGF and is involved in the regulation of G1 phase progression during liver regeneration *in vivo* and in hepatocyte proliferation *in vitro* [13, 74–76]. In particular, IGF-1 signals activate both the PI3K/Akt and MAPK/ERK pathways, leading to the control of genes involved in hepatocyte proliferation and in protecting against apoptotic cell death [77].

3. Roles of Platelets in Liver Regeneration

It is known that platelets release local mediators and interact with leukocytes and endothelial cells to modulate inflammatory responses [78]. Platelets are involved in wound healing and tissue repair in addition to hemostasis and inflammation and can be associated with liver regeneration or tissue repair in liver injury. Platelets contain both the proteins needed for hemostasis and also many growth factors, such as, PDGF, HGF, IGF, VEGF, EGF, and TGFβ, which are required for tissue regeneration [23–29, 79–81]. In addition, platelets also contain certain cytokines, serotonin and lipid mediators, such as, S1P, ADP, and ATP [79, 82–84]. We previously reported on the relationship between platelets, nonparenchymal cells and hepatocytes during liver regeneration [23, 31, 33, 35] (Figure 1).

3.1. Direct Effect on Hepatocytes. We previously reported that platelets have a potent role in promoting liver regeneration after partial hepatectomy in mice by activating the Akt and ERK signaling pathways and stimulating hepatocyte proliferation *in vitro* [23, 31]. It is clear that the number of platelets affects liver regeneration during the priming phase after hepatectomy [31, 33, 85, 86]. As observed by transmission electron microscopy, some platelets were found to translocate into Disse's spaces through the fenestration of LSECs and had direct contact with hepatocytes [31], which is similar to wound healing in which platelets become activated and release essential growth factors and cytokines by contact with collagen and other extracellular matrixes [79]. We previously reported that, when platelets and hepatocytes were separated by a permeable membrane, the platelets had no proliferative effect on the hepatocytes, suggesting that the direct contact between the cells was essential for inducing hepatocyte proliferation [23]. Growth factors in platelets, such as, IGF-1 and HGF, activated the Akt and ERK1/2 pathways and caused a proliferative effect on hepatocytes [23]. As it was reported that human platelets do not contain a significant amount of HGF, IGF-1 is the most important mediator for liver regeneration in humans [23, 87]. Platelets did not exert a proliferative effect on hepatocytes in the presence of LY294002, which inhibits the activation of the Akt pathway, suggesting that the Akt pathway is an important signal and that the activators of the Akt pathway are key molecules involved in the direct proliferative effect by platelets [23]. Furthermore, even under conditions of Kupffer cell depletion, platelets had a strong effect in hepatocyte proliferation through the phosphorylation of Akt, suggesting that Akt activation could compensate for liver regeneration when the TNFα/NFκB pathway derived from Kupffer cells was impaired [33].

3.2. Relationship between Platelets and Nonparenchymal Cells. Nonparenchymal cells in the liver, including Kupffer cells, LSECs, and hepatic stellate cells, are involved in the process of liver regeneration [36]. Because Kupffer cells are close to hepatocytes, the release of these mediators after partial hepatectomy may initiate the liver regeneration process [8, 88, 89]. Kupffer cells produce inflammatory cytokines, such as, TNFα and IL-6, and such growth factors as IGF-1 are generally presumed to be an important source of hepatic TNFα, which is a key component in the process of hepatocyte proliferation during liver regeneration [42, 90]. The depletion of Kupffer cells was reported to result in delayed liver regeneration because of the loss of TNFα and NFκB and the decrease of IGF-1 [33, 48, 91]. LSECs, which comprise 70% of the sinusoidal cells in the liver, are known to produce immunoregulatory and proinflammatory cytokines, including HGF, IL-6, interleukin-1, and interferon [20, 92, 93]. Under conditions of Kupffer cell depletion, the phosphorylation of STAT3 was detected in the regenerating liver after hepatectomy, suggesting that IL-6 could be produced by LSECs (instead of Kupffer cells) at a sufficient level to activate STAT3 [33]. We previously reported that platelets could function in collaboration with nonparenchymal cells during liver regeneration [33–35].

3.2.1. Relationship between Platelets and Kupffer Cells. Kupffer cells can be associated with the accumulation of platelets in the liver, which induces liver regeneration [33, 34]. It was reported that platelet accumulation in the liver after hepatectomy or other types of liver injury, such as, ischemic reperfusion or lipopolysaccharide administration, depended to some extent on Kupffer cells [31, 34, 94, 95]. Platelets in the liver sinusoids were mostly surrounded by the well-developed cell processes of Kupffer cells without phagocytosis [34]. Furthermore, the depletion of Kupffer cells resulted in the abolition of the accumulation and the migration of platelets in the liver [33, 34]. These results indicated that the cellular interactions between platelets and Kupffer cells play important roles in platelet behavior in the liver. In thrombocytosis, more platelets were recruited into the liver, providing high levels of IGF-1 and HGF, which induced the subsequent activation of downstream signal transductions, such as, the PI3K/Akt and MAPK/ERK pathways and advanced hepatocyte mitosis [33].

3.2.2. Relationship between Platelets and LSECs. We previously reported that platelets induced hepatocyte proliferation through LSEC activation [35]. Direct contact between platelets and LSECs triggered the secretion of S1P from the platelets, which induced the secretion of IL-6 from the LSECs; thereafter, the increase of IL-6 caused the activation of the STAT3 pathway in hepatocytes, Akt and ERK1/2 activation and the promotion of hepatocyte proliferation [35]. In addition, platelets caused the proliferation of LSECs

and induced the secretion VEGF and IL-6 from the LSECs by activating the Akt and ERK1/2 pathways [35].

4. Conclusion

The liver is a vital organ in which the mechanisms of regeneration are orchestrated by a complex network of cytokines and growth factors. Nonparenchymal cells in the liver, such as, Kupffer cells, LSECs, and hepatic stellate cells, participate in liver regeneration with respect to both their own proliferation and effects on hepatocyte proliferation [36]. In particular, the Kupffer cells and LSECs produce various growth factors and cytokines that are involved in liver regeneration [19, 20]. Platelets contain various types of growth factors and cytokines and comprise another important factor involved in liver regeneration [23, 31]. In summary, platelets have a direct effect on stimulating hepatocyte proliferation and cooperative with Kupffer cells and LSECs during liver regeneration.

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