Transcription Factors ETF, E2F, and SP-1 Are Involved in Cytokine-Independent Proliferation of Murine Hepatocytes

Sebastian Zellmer,[1]* Wolfgang Schmidt-Heck,[2]* Patricio Godoy,[3]* Honglei Weng,[4]* Christoph Meyer,[4] Thomas Lehmann,[5] Titus Sparna,[6] Wiebke Schormann,[3] Seddik Hammad,[3] Clemens Kreutz,[7] Jens Timmer,[7] Fritz von Weizsäcker,[8] Petra A. Thürmann,[9] Irmgard Merfort,[6] Reinhard Guthke,[2] Steven Dooley,[4]* Jan G. Hengstler,[3]* and Rolf Gebhardt[1]*

The cellular basis of liver regeneration has been intensely investigated for many years. However, the mechanisms initiating hepatocyte "plasticity" and priming for proliferation are not yet fully clear. We investigated alterations in gene expression patterns during the first 72 hours of C57BL/6N mouse hepatocyte culture on collagen monolayers (CM), which display a high basal frequency of proliferation in the absence of cytokines. Although many metabolic genes were down-regulated, genes related to mitogen-activated protein kinase (MAPK) signaling and cell cycle were up-regulated. The latter genes showed an overrepresentation of transcription factor binding sites (TFBS) for ETF (TEA domain family member 2), E2F1 (E2F transcription factor 1), and SP-1 (Sp1 transcription factor) ($P < 0.001$), all depending on MAPK signaling. Time-dependent increase of ERK1/2 phosphorylation occurred during the first 48 hours (and beyond) in the absence of cytokines, accompanied by an enhanced bromodeoxyuridine labeling index of 20%. The MEK inhibitor PD98059 blunted these effects indicating MAPK signaling as major trigger for this cytokine-independent proliferative response. In line with these *in vitro* findings, liver tissue of mice challenged with CCl_4 displayed hepatocytes with intense p-ERK1/2 staining and nuclear SP-1 and E2F1 expression. Furthermore, differentially expressed genes in mice after partial hepatectomy contained overrepresented TFBS for ETF, E2F1, and SP-1 and displayed increased expression of E2F1. *Conclusion:* Cultivation of murine hepatocytes on CM primes cells for proliferation through cytokine-independent activation of MAPK signaling. The transcription factors ETF, E2F1, and SP-1 seem to play a pronounced role in mediating proliferation-dependent differential gene expression. Similar events, but on a shorter time-scale, occur very early after liver damage *in vivo*. (HEPATOLOGY 2010;52:2127-2136)

In normal liver, hepatocytes remain in the quiescent G_0 phase. Loss of liver mass by partial hepatectomy (PHx) or hepatotoxic chemicals (e.g., CCl_4) induces a complex regeneration process restoring the original liver mass within 5-7 days. Within minutes after partial hepatectomy, signals occur that prime hepatocytes for proliferation. A broad range of cytokines are released and transcription factors are activated (summarized in recent reviews[1-4]). In addition to cytokine signaling, the extracellular matrix (ECM) plays an

Abbreviations: BrdU, 5-bromo-2′-deoxyuridine; CM, collagen monolayer; ECM, extracellular matrix; ETF, embryotrophic factor; LI, labeling index; MAPK, mitogen activated protein kinase; PCA, principal component analysis; PHx, partial hepatectomy; RT, room temperature; TF, transcription factor; TFBS, transcription factor binding site.

From the [1]From the Institute of Biochemistry, Medical Faculty, University of Leipzig, Leipzig, Germany; [2]Leibniz Institute for Natural Product Research and Infection Biology–Hans Knoell Institute, Jena, Germany; [3]Leibniz Research Centre for Working Environment and Human Factors at TU Dortmund University, Dortmund, Germany; [4]Molecular Hepatology–Alcohol Associated Diseases, Department of Medicine II, Faculty of Medicine at Mannheim, University of Heidelberg, Germany; [5]Translational Center for Regenerative Medicine, University of Leipzig, Leipzig, Germany; [6]Institute of Pharmaceutical Sciences, Department of Pharmaceutical Biology and Biotechnology, and [7]Centre for Biological Signalling Studies (BIOSS), Freiburg, Germany; [8]Schlosspark-Klinik Berlin, Humboldt Universität, Berlin, Germany; and [9]Philipp Klee-Institut für Klinische Pharmakologie, HELIOS Klinikum Wuppertal, Wuppertal, Germany.

Received March 5, 2010; accepted August 10, 2010.

**These authors contributed equally to this work.*

Supported by the German Federal Ministry for Education and Research (BMBF) within the program "Systems of Life – Systems Biology". and the Excellence Iniative of the German Federal and state Governments (EXC 294).

important role. Loss of cell-matrix contacts activated c-fos[5] and sensitized rat hepatocytes for cytokine induced proliferation.[6] *In vivo*, degradation of ECM in an experimental mouse model resulted in hepatocyte proliferation.[7] Also, loss of integrin-linked kinase signaling increased hepatocyte proliferation[8] and enhanced liver regeneration.[9]

Hepatocyte cultures are well established and often used in studies on drug metabolism, enzyme induction and toxicity.[10,11] The latter represent applications where a robust hepatocyte physiology, such as a high metabolizing capacity, is required. Recently, we have shown that collagen sandwich cultures preserve polarized mouse hepatocyte morphology and are superior to collagen monolayers (CM), which rapidly and spontaneously lose polarity and transdifferentiate.[12,13] However, CM cultures are resistant to apoptotic stimuli and display an activation of survival pathways. Therefore, we speculated that this type of cultivation may represent an *in vitro* model for hepatocyte priming and entry into G_1/S phase in the absence of cytokine stimulation. This assumption is supported by a higher basal extracellular signal-regulated kinase 1/2 (ERK1/2) and Akt/protein kinase B (PKB) phosphorylation compared to collagen sandwich (CS) cultured hepatocytes.[12]

In the present study, we explored gene expression profiles during the first hours of mouse hepatocyte CM culture and identified signatures describing loss of metabolic functions, increased cell-matrix interactions and transformation into a proliferative state. TF binding sites for upregulated genes were characterized and identified the transcription factors (TFs) ETF (TEA domain family member 2), E2F1 (E2F transcription factor 1), and SP-1 (Sp1 transcription factor) as most important regulators, particularly for genes of MAPK signaling pathways. Similar regulatory events, though more rapidly, occurred *in vivo* after CCl_4 intoxication and after PHx reflecting findings observed in culture on a different time scale. This supports the idea that cytokine-independent MAPK activation contributes to the proliferative response of mouse hepatocytes during regenerative processes.

Materials and Methods

Animal Experiments. Male C57BL/6N mice (8-12 weeks old) were allowed food and water *ad libitum*. All experiments were approved by the ethics committees. Mice were intoxicated with 1.6 g CCl_4/kg body weight, administered intraperitoneally, dissolved in 0.5 mL olive oil. At selected time points, mice were killed by heart puncture and liver sections were snap frozen in liquid nitrogen.

Hepatocyte Isolation and Culture. Hepatocytes were isolated according to a modified method of Seglen[11,14] and cultured in Williams E medium (Lonza Verviers, Verviers, Belgium), supplemented with 10^{-7} M dexamethasone and penicillin/streptomycin (all from Sigma-Aldrich, Taufkirchen, Germany), 2 mM glutamine (Roth, Karlsruhe, Germany) and 10% fetal bovine serum (FBS) in six-well plates (TPP, Trasadingen, Switzerland). After attachment, medium was changed to medium without FBS. Fifty μM PD98059 (Sigma-Aldrich) was added 1 hour before stimulation with 20 ng/mL murine HGF (R&D Systems GmbH, Wiesbaden-Nordenstadt, Germany) and 100 nM insulin (Sigma-Aldrich). Then, cells were cultured for 3 days with inhibitor, cytokines, and BrdU. A wound-like spacing between confluent cultured hepatocytes was generated with an Ibidi culture insert (Applied Biophysics, New York, NY). The device was removed from confluent cultured hepatocytes after 1 day, followed by 3 days culture in the presence of BrdU with and without HGF/insulin.

Hepatocyte Proliferation Assays and BrdU Incorporation. DNA synthesis of hepatocytes was measured by the incorporation of BrdU into nuclei. Briefly, following preculture for 24 hours in the presence of 150 μg/mL BrdU, hepatocytes were incubated with 20 ng/mL HGF and 100 nM insulin for 3 days. Then, cells were washed with PBS, fixed with 4% paraformaldehyde and permeabilized with 0.2% Triton-X 100, followed by 10 minutes incubation with 2 N HCl for DNA denaturation. The slides were blocked with 3% BSA/0.1% Tween-20 in PBS for 1 hour at RT and incubated with rat anti-BrdU antibody (Serotec, Oxford, UK; 1:25) for 1 hour at RT. Detection was achieved using a biotinylated anti-rat (IgG) antibody (Vector Lab, Burlingame, CA; 1:250), followed by peroxidase conjugated streptavidin (Jackson ImmunoResearch, Baltimore, MD; 1:500). Staining was visualized using DAB Substrate Kit (Vector Lab). Mayer's hemalaum solution (Merck KGaA, Darmstadt, Germany; dilution 1:5) was used for counterstaining. Proliferation was expressed as percentage of BrdU positive nuclei, of at least 400 nuclei in four regions per slide.

Address reprint requests to: Sebastian Zellmer, Ph.D., Institute of Biochemistry, Medical Faculty, University of Leipzig, Johannisallee 30, 04103 Leipzig, Germany. E-mail: sebastian.zellmer@medizin.uni-leipzig.de; fax: +49 341 9722109.

View this article online at wileyonlinelibrary.com.

DOI 10.1002/hep.23930

Potential conflict of interest: Nothing to report.

Additional Supporting Information may be found in the online version of this article.

Immunohistochemistry. Liver sections (4 μm) were deparaffinised. Sections were incubated in peroxidase blocking reagent (Dako Deutschland GmbH, Hamburg, Germany) for 1 hour and subsequently with phospho-Erk (mouse IgG2$_\alpha$, sc-7383) or SP-1 antibodies (rabbit polyclonal, sc-14027), both from Santa Cruz Biotechnology (Heidelberg, Germany) overnight at 4°C. EnVision peroxidase (Dako Deutschland GmbH) was applied for 1 hour at RT. E2F1 was stained with polyclonal rabbit anti-E2F1 antibody (Atlas Antibodies, Stockholm, Sweden) overnight followed by incubations with a biotinylated anti-rabbit antibody (AP132B, Chemicon) and POD-extravidin (E2886, Sigma, Taufkirchen, Germany) for 60 and 30 minutes, respectively. Staining was performed in 0.1 M Tris buffer (pH 7.6), composed of 0.05% diaminobenzidine, 2% aminotriazole, and 0.033% H_2O_2. Counterstaining was performed as described above.

Further Applied Methods. RNA isolation, quantitative real-time PCR, preprocessing of microarray data and the subsequent Multivariate Data analysis, promoter analysis, chromatin immunoprecipitation, and preparation of immunoblots are described in the Supporting Information.

Results

Isolation of Hepatocytes Alters Gene Expression Within the First 24 Hours. To understand how isolation and subsequent cultivation of primary mouse hepatocytes on collagen monolayers (CM) alters the physiology of hepatocytes, we studied alterations in gene expression patterns using microarrays. Two different experimental designs were performed in different laboratories to obtain robust data. The "Leipzig" experiment (Supporting Fig. 1) included fresh liver tissue, freshly isolated hepatocytes, hepatocytes after attachment to CM and after cultivation periods between 1 and 2 days. Samples were analyzed using an Affymetrix platform. The second study ("Freiburg" experiment) included cultivation periods of up to 6 days and a cDNA platform for analysis.

Alterations in the gene expression pattern between liver and freshly isolated hepatocytes were small (Fig. 1A). The most prominent changes (>1.5-fold) occurred between attachment of hepatocytes on CM and cultivation from day zero to day one. Between days one and two, alterations in gene expression were less pronounced.

The observed changes in gene expression were visualized by principal component analysis (PCA). Hepatocytes in both data sets change their expression pattern in a similar "direction" (Fig. 1B; dashed arrows) indicating that cultivation on CM induces equal adaptation of hepatocytes, regardless of interlaboratory differences in array platform usage and analysis.

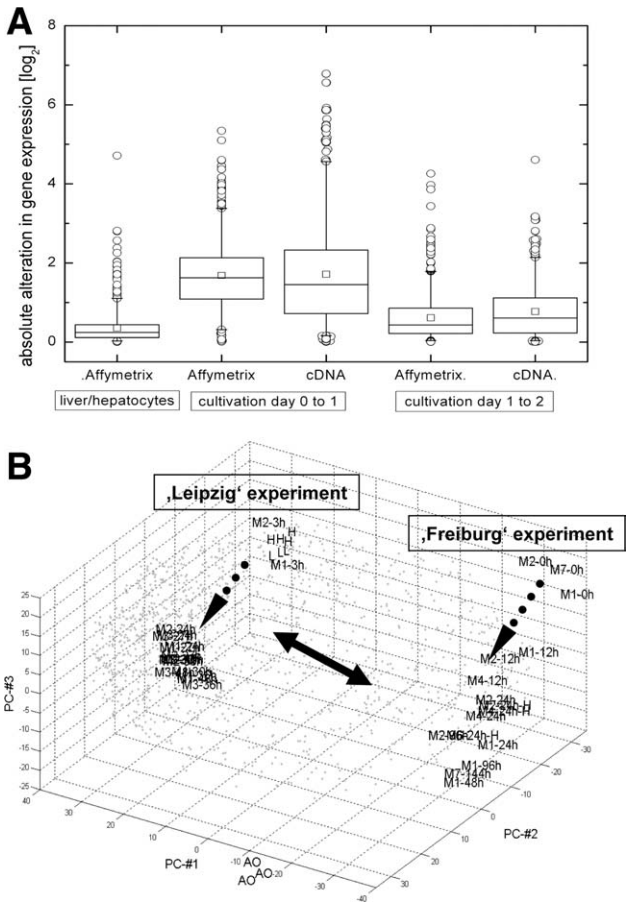

Fig. 1. (A) Time-dependent absolute changes of 678 differentially expressed genes during the first 48 hours of mouse hepatocyte collagen monolayer (CM) cultures. The largest changes occur between days 0 and 1 in both experiments ("Leipzig"-Affymetrix platform and "Freiburg"-cDNA platform). (B) Alterations in gene expression patterns of (A) illustrated by principle component analysis. For comparison, a completely different cell type, aortal cells, were included (AO). The "Leipzig" experiment also shows that relatively small alterations are based on the hepatocyte isolation procedure (H), when compared with liver tissue (L). The use of different gene array platforms results in two separate data sets, but with similar information indicated by the double sided arrow.

Detailed analysis of the four-fold overexpressed genes of both platforms using ANOVA (Supporting Table 1) revealed that the overall pattern of altered gene expression was reproducible between both laboratories and that alterations in gene expression patterns can be attributed mainly to culture time on CM. Thus, cultivation of primary mouse hepatocytes on CM causes major changes of hepatocyte physiology within the first 24 hours.

CM Culture Conditions Decrease Expression of Metabolic Genes and Induces Cell Cycle and Cytoskeleton Associated Genes. Gene set enrichment analysis based on 'KEGG Pathway' definitions (http://

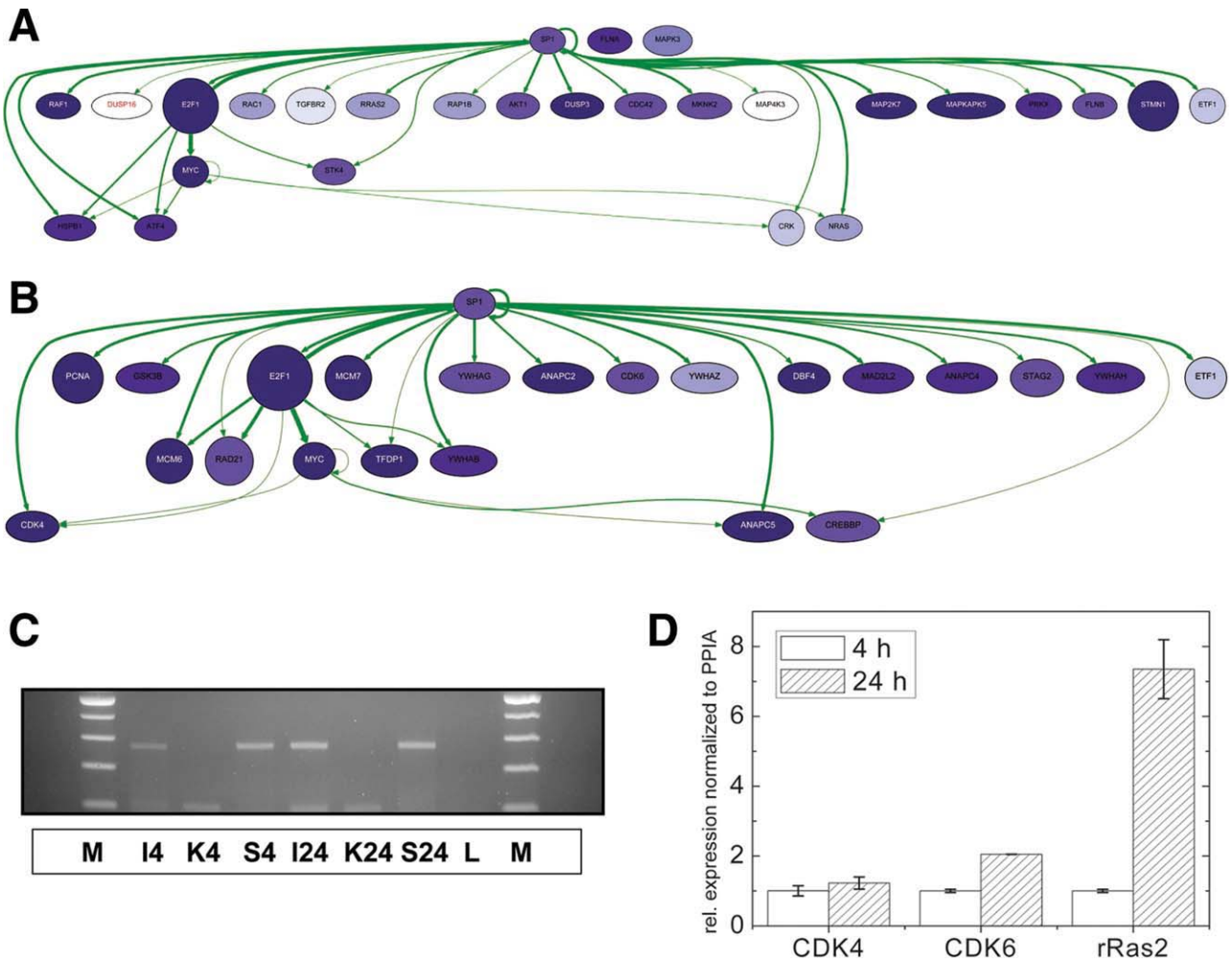

Fig. 2. Analysis of TF-gene interactions. FANTOM 4 (a ChIP-chip database) was used to validate the interaction of E2F1 and SP-1 with (A) 23 of 26 differentially expressed genes of the MAPK pathway and (B) all differentially expressed genes related to cell cycle (Supporting Table 3). Thickness of the arrow indicates how often the interaction was detected experimentally. The color of the node represents the level of expression from light (low) to dark (high). (C) Validation of the *in silico* prediction of the E2F1-PNCR2 interaction using ChIP, where M is the 100 bp ladder, I4 and I24 are input DNA after 4 and 24 hours, K4/K24 are controls without antibody, S4/S24 are ChIP-DNA after 4 and 24 hours with the 275 bp fragment. L is the DNA-free control. (D) Gene expression analysis of selected genes of MAPK pathway and cell-cycle by quantitative RT-PCR validated Affymetrix measurements. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

www.genome.jp/kegg/) was performed and identified down-regulation of genes related to metabolism on both platforms (Supporting Table 2). In more detail, almost 50% of genes related to fatty acid metabolism ($P = 4.2 \times 10^{-16}$), 30% of genes related to bile acid biosynthesis ($P = 2.2 \times 10^{-8}$) and approximately 50% of genes related to ketone bodies metabolism ($P = 6.6 \times 10^{-6}$) were down-regulated. In contrast, genes related to regulation of the actin cytoskeleton ($P = 9.76 \times 10^{-10}$) or involved in cell-cell communication, like focal adhesions, tight junctions and adherence junctions were up-regulated. Additionally, 16% of genes related to cell cycle ($P = 1.9 \times 10^{-6}$), 18% related to ribosomes ($P = 3.0 \times 10^{-5}$) and 10% related to MAPK signaling pathway ($P = 4.5 \times 10^{-4}$) were up-regulated (Supporting Table 3). Up-regulation of three selected genes CDK4, CDK6 and rRAS2, representing members of either the MAPK pathway or the cell cycle, was validated with quantitative rtPCR (Fig. 2D). These results show that murine hepatocytes cultured in CM for 1 day lose many of their metabolic functions, but increase gene expression signatures related to cell cycle control and cell-cell/cell-matrix interactions.

Overrepresentation of ETF, E2F1, and SP-1 Transcription Factor Binding Sites in Differentially Expressed Genes. We were interested whether changes in gene expression are related to the action of specific transcription factors (TF). Using PRIMA (PRomoter Integration in Microarray Analysis),[15] we identified

Table 1. Transcription factor binding sites (TFBS) overrepresented in differentially expressed genes during the first 24 hours of cultivation on collagen monolayers and after partial hepatectomy (raw data from Otu and coworkers[17]), identified by the PRIMA algorithm.

TF binding site (ID)	*P* Value (Bonferroni Adjusted)	Frequency in Set	Frequency Ratio	Direction of Regulation
First 24 hours of cultivation				
ETF (M00695)	5.92 × 10–17	0.46	1.67	Up
E2F (M00803)	9.56 × 10–14	0.57	1.45	Up
E2F1 (M00938)	5.89 × 10–08	0.40	1.44	Up
E2F1 (M00940)	3.16 × 10–03	0.19	1.52	Up
SP1 (M00196)	9.78 × 10–14	0.74	1.28	Up
SP1 (M00196)	2.21 × 10–05	0.74	1.28	Down
After partial hepatectomy				
E2F (M00803)	8.03 × 10–11	0.32	1.28	Up
SP1 (M00196)	4.97 × 10–09	0.44	1.21	Up
ZF5 (M00716)	1.38 × 10–07	0.29	1.27	Up
ETF (M00695)	1.67 × 10–06	0.23	1.31	Up
AP-2 (M00189)	8.71 × 10–06	0.27	1.23	Up

Frequency in set: the frequency of TFBS within the examined set; frequency ratio: frequency in set divided by frequency in background set.

ETF, E2F1, and SP-1 binding sites enriched in differentially expressed genes of CM cultured mouse hepatocytes (Table 1; for a complete list, see Supporting Tables 4 and 5). In order to validate this *in silico* approach, FUNCTIONAL ANNOTATION OF THE MAMMALIAN GENOME (FANTOM 4; http://fantom.gsc.riken.jp/4/) was used, a database containing experimental data (e.g., ChIP-chip data) of mouse and human genomes, in a database query (Fig. 2A). As listed in Supporting Table 3, E2F1 and SP-1 are ChIP-chip proven regulators of most of the up-regulated genes downstream of the MAPK pathway. In addition, E2F1 and SP-1 are regulators for all overexpressed "cell cycle"-related genes (Fig. 2B). To confirm that this validation is applicable to our data, PNCR2 was chosen as one overexpressed gene with an E2F1 TFBS (Supporting Table 5). Chromatin immunoprecipitation (ChIP) demonstrated E2F1 binding close to transcriptional start site of PNCR2 (Fig. 2C), as predicted by the *in silico* approach.

Finally, changes in expression of E2F1 and SP-1 during CM culture were quantified using qRT-PCR. Expression of these TFs was significantly up-regulated in cultured murine hepatocytes in the absence of cytokine stimulation within 48 hours (Fig. 3A). Furthermore, nuclear TFs were isolated from hepatocytes after cultivation for 4 hours and 24 hours. Within that cultivation period a selected nuclear enrichment of E2F1 and SP-1 compared to some other TFs (especially some STAT factors involved in cytokine signaling) could be demonstrated (Fig. 3B). Based on these data, we conclude that changes in gene expression occurring during the first 2 days of mouse hepatocyte CM culture in the absence of cytokines are mediated by E2F1, SP-1 and ETF transcription factors.

CM Culture Primes Hepatocytes for Proliferation. E2F1 is controlled by MAP kinase signaling.[16] Therefore, we asked whether the Ras-ERK1/2 pathway is activated during hepatocyte CM culture without cytokine stimulation. Indeed, we observed that the amount of phosphorylated ERK1/2 started to increase

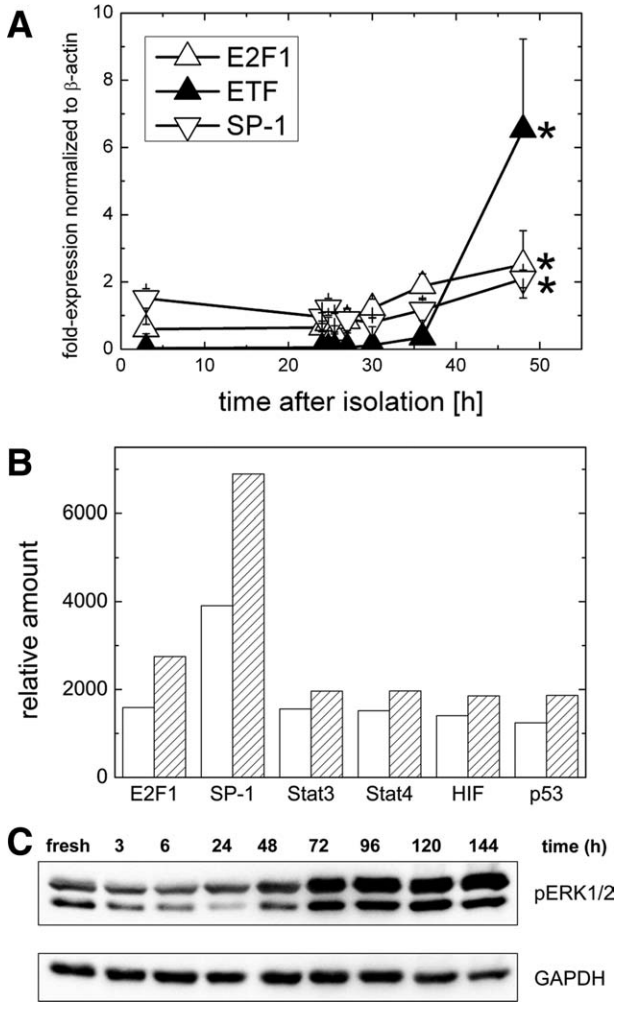

Fig. 3. (A) QRT-PCR quantification of transcription factors E2F1, ETF and SP-1 in murine hepatocytes cultivated on collagen monolayers (CM). Zero corresponds to the time-point of seeding. Symbols represent mean values ± SD obtained from four mice. Significance level, $*P < 0.05$ (Mann-Whitney test). (B) Changes in the concentration of selected transcription factors in the nucleus between the first 4 hours (blank) and 24 hours (hatched bars) of cultivation on CM in the absence of cytokines. E2F1 and SP-1 increase, whereas cytokine-dependent TF like Stat increase only moderately. (C) Phosphorylation of ERK1/2 indicates activation of the MAPK signaling pathway in hepatocytes after a cultivation of 24 to 48 hours on CM. GAPDH was used as loading control (bottom line). Representative image from three independent experiments are shown

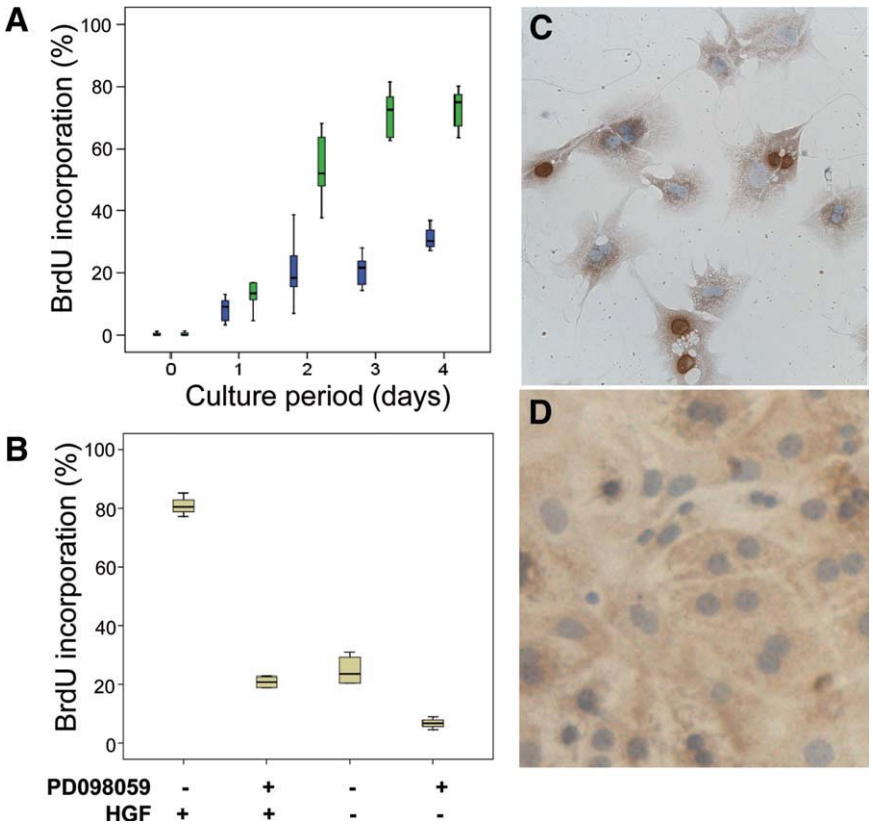

Fig. 4. (A) Basal (blue boxes) and HGF/insulin stimulated (green boxes) BrdU labeling index (LI) in mouse hepatocytes cultivated on CM. Mean ± SD of three independent experiments. (B) The MEK inhibitor PD98059 decreases basal and HGF/insulin mediated LI during 3 days of cultivation. Mean ± SD of three independent experiments. (C) Representative image of a subconfluent hepatocyte culture showing a basal BrdU LI of approximately 20%. (D) Representative image of BrdU incorporation in confluent cultures, not exceeding a LI of 6%. We assume that the low LI of ≤6% could occur only at positions where the dish was not perfectly confluent.

after 48 hours and reached a maximum after 72 hours of cultivation (Fig. 3C).

Next we sought to determine whether spontaneously activated MAP-kinase signaling leads to increased hepatocyte proliferation by analyzing BrdU incorporation in unstimulated mouse hepatocytes on CM. Subconfluent cultures (125,000 hepatocytes/9 cm^2) showed a basal BrdU labeling index (LI) of 20% (Fig. 4A,C), which is clearly enhanced compared to *in situ* conditions in normal mouse liver. The initial 4 hours cultivation of hepatocytes in the presence of 10% FCS does not induce a proliferative state, because the LI in the presence and absence of FCS was equal (Supporting Fig. 3). In order to test whether BrdU incorporation can

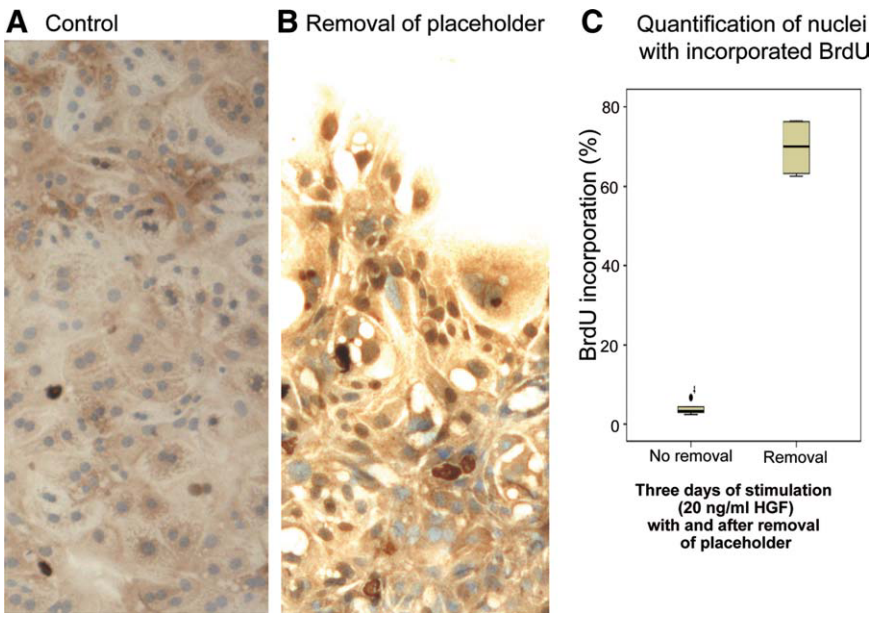

Fig. 5. Basal and HGF/insulin stimulated BrdU incorporation in relation to cell density. Images are representative examples before (A) and after (B) removal of a placeholder device from confluent hepatocyte cultures at day one followed by a 3 days culture with BrdU and cytokines. In the upper third of (B), a free space has been created by removal of the placeholder and BrdU incorporation preferentially occurs at that area. (C) Quantification of BrdU labeling index after 3 days of cultivation with and without removal of the placeholder. Mean ± SD of three independent experiments are shown.

be enhanced further by the presence of cytokines, 20 ng/mL HGF was added in combination with 0.1 μM insulin. This induced a rapid and transient activation of the MAPK pathway as indicated by rapid ERK1/2 phosphorylation (Supporting Fig. 2) and a further increase of the LI to 80% (Fig. 4B).

Blunting MAP kinase signaling with MEK inhibitor PD98059 blunted HGF stimulated and basal BrdU incorporation (Fig. 4B), indicating the requirement of this pathway for induced and spontaneous hepatocyte proliferation in CM culture.

Of note, hepatocyte proliferation was much lower in confluent hepatocyte CM cultures (LI < 6%; Fig. 4D), demonstrating that the state of confluency is critical for proliferation. However, after generating an empty space in a confluent layer by "wounding," hepatocytes again started to incorporate BrdU (Fig. 5C) preferentially at the "wound" margin, although hepatocytes distant from the "wound" also were able to incorporate BrdU to some extent (Fig. 5B).

We conclude that cultivation of murine hepatocytes on CM for 48 hours induces a proliferative cell state, characterized by an activated MAP kinase pathway. MAP kinase downstream signaling induces cell cycle associated genes and a LI of 20% in subconfluent cultures. In confluent cultures proliferation events are prevented by high cell densities, probably through contact inhibition.

Activation of ERK1/2 Signaling Is an Early Event in CCl4 Intoxication and Precedes SP-1 and ETF Activation. Next, we were interested whether activation of the MAPK pathway and overrepresentation of binding sites for SP-1, ETF and E2F in differentially expressed genes observed in CM cultured mouse hepatocytes may be relevant for *in vivo* models of hepatocyte priming.

Intoxication with CCl_4 transiently increased p-ERK1/2 levels in mice. Elevated levels were found after 20 minutes (Fig. 6A), which decreased again after 60 minutes (Fig. 6B). In addition, E2F1 levels remained up-regulated both in the cytoplasm and in the nucleus until 1 hour after CCl_4 challenge (Fig. 6D) and decreased within the next 6 hours (Fig. 6E). SP-1 expression showed a similar time course (Fig. 6G,H). To the best of our knowledge, these data show for the first time that CCl_4 intoxication results in a rapid activation of the MAPK pathway *in vivo*, which then triggers up-regulation of transcription factors E2F1 and SP-1, thus very rapidly directing murine hepatocytes into a proliferative state. The mechanisms seem to have much in common with hepatocyte culture on CM, but occur on a much shorter time-scale.

Partial Hepatectomy Results in an Overrepresentation of E2F, SP-1 and ETF Transcription Factors. Liver regeneration can be induced by PHx. This prompted us to explore, whether the TFBS for E2F, SP-1 and ETF are also overrepresented in differentially expressed genes under these conditions. Published gene expression data[17] were used for a promoter analysis using PRIMA. Indeed, differentially expressed genes following PHx also showed an overrepresentation of the three TFBS (Table 1). In line, elevated levels of E2F1 in liver after PHx were detected by immunohistochemistry (Fig. 7).

Discussion

Cultured primary hepatocytes represent a valuable tool for studying molecular mechanisms of hepatocyte physiology, including liver regeneration and the response of the liver to cytokines.[11,12,18] Here, we show by a genome-wide analysis that primary mouse hepatocytes change their gene expression pattern within the first 24 hours after cultivation on CM. Changes comprised the down-regulation of many metabolic genes and the up-regulation of cytoskeleton-associated and cell-cell interaction-associated genes (Supporting Tables 2 and 3). Similar data have been reported before for primary rat hepatocytes[19,20] cultured on CM. However, whereas prominent up-regulation of cell cycle genes indicative of a preproliferative state was found herein for murine hepatocytes, no such alterations was reported for rat hepatocytes. In line with this, the BrdU labeling index (LI) in unstimulated rat hepatocytes was very low[21,22] but reached approximately 20% in murine hepatocytes (Fig. 4A). Likewise, cytokine stimulation increased the LI in rat hepatocytes by approximately 30%, whereas murine hepatocytes reached a LI of 70%-80%. These differences demonstrate that murine hepatocytes show a higher basal proliferative potential than rat hepatocytes, which is reflected also in their gene expression pattern. The lower basal proliferation of rat hepatocytes might be due to the presence of a growth factor dependent restriction point at the second third of the G_1 phase in rat hepatocytes.[23]

The high proliferative potential of unstimulated murine hepatocytes cultivated on CM is also reflected in elevated levels of p-ERK1/2 that last for several days which are sensitive to inhibition of the MAPK pathway. Although BrdU incorporation is indicative only for progression of S-phase, a certain number of murine hepatocytes complete the cell cycle and undergo cell division, as proven by time lapse video microscopy (data not shown). ERK activation is sensitive to

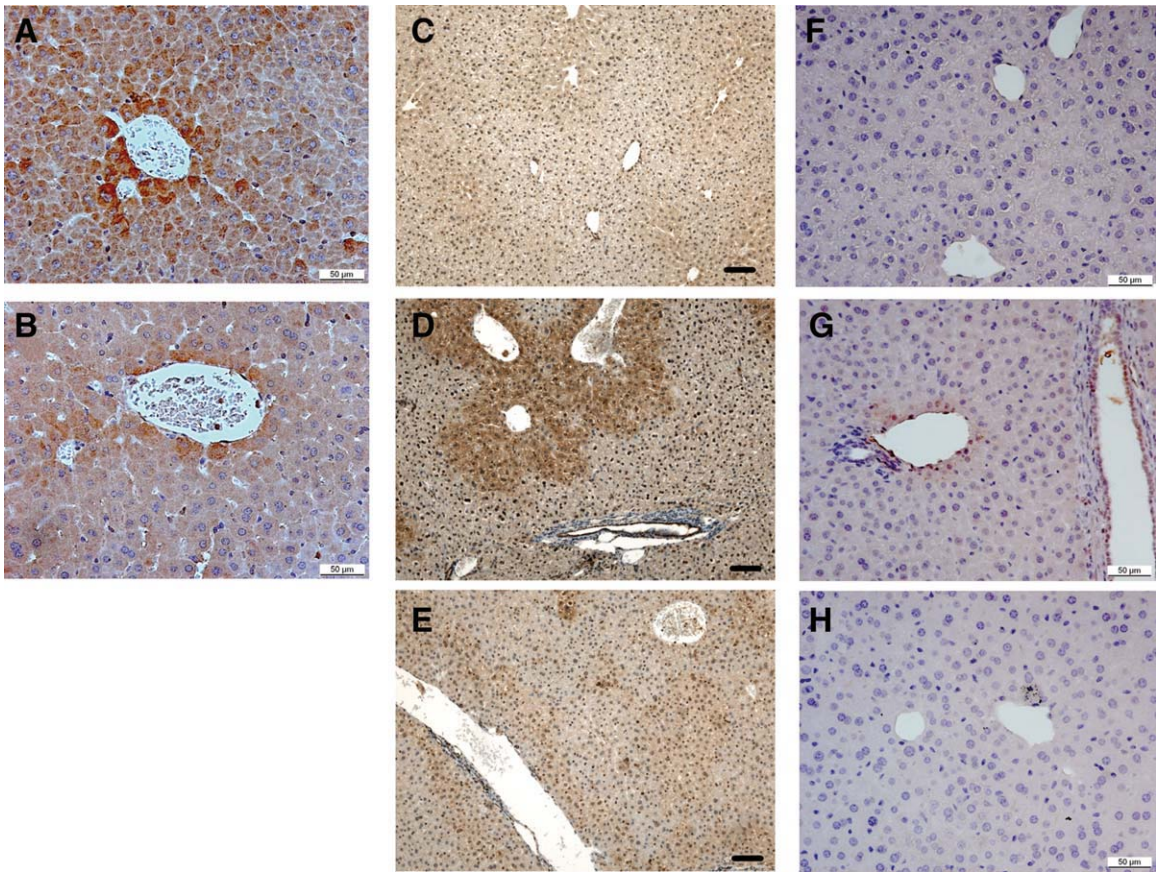

Fig. 6. P-ERK1/2 staining of hepatocytes 20 minutes (A) and 60 minutes (B) after intoxication with CCl_4. Within 20 minutes of intoxication a strong staining occurs around the vessels, which decreases within 1 hour. (C) A low staining of cytoplasmic and nuclear E2F1 occurs in control mice, which strongly increases 60 minutes after intoxication (D) and returns to almost normal levels after 6 hours (E). Staining of SP-1 in control mice (F) and 1 hour (G) and 6 hours (H) after intoxication with CCl_4. A maximum of staining occurs 1 hour after intoxication at the borders of vessels. Representative images of three mice are shown. Control slides without primary antibody showed no staining (Supporting Fig. 4). Size bar represents 50 μm

inhibition of MAPK pathway, but the reason for this continuous up-regulation of MAPK signaling is yet unknown. Again, there is a strong contrast to rat hepatocytes, where elevated levels of p-ERK1/2 are found only transiently within 24 hours in response to EGF.[18]

As shown experimentally for PNCR2 (Fig. 2C) and MAT2A[24] as well as by *in silico* analysis of existing ChIP-chip data (Fig. 2A,B). binding sites of the TFs ETF, E2F1, and SP-1 are highly overrepresented in the set of genes up-regulated during cultivation on CM. Interestingly,

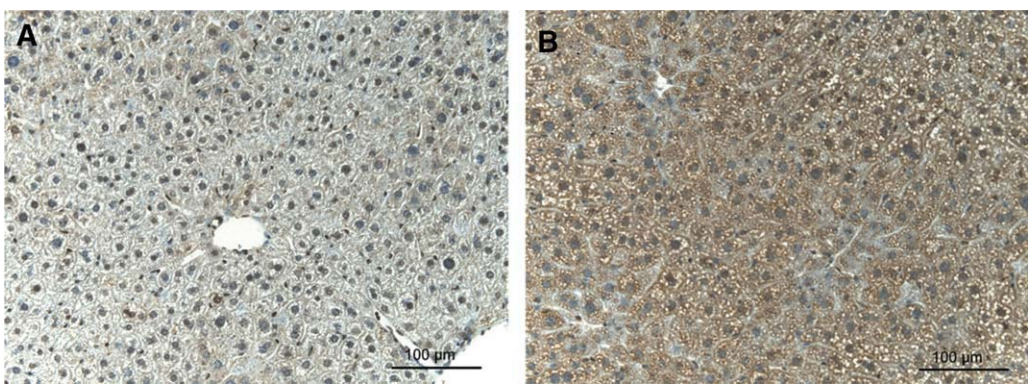

Fig. 7. Staining of E2F in livers after PHx. (A) A weak staining is seen in sham-operated animals. (B) In contrast, most hepatocytes are stained 24 hours after PHx, indicating cell activation. Representative images of three mice. Size bar represents 100 μm.

all three transcription factors are partially involved in proliferation processes. ETF is known as a critical determinant of proliferation.[25] E2F transcription factors are well known as critical regulators of genes required for appropriate progression through the cell cycle.[26,27]

SP-1 can enhance both proliferation[28] as well as cell cycle arrest,[29] depending on the specific context. As shown recently, it interacts with E2F1 thereby stimulating S-phase entry and cell proliferation.[29] Thus, in the context of cultivation of murine hepatocytes on CM, it is likely that all three TFs contribute to a preproliferative state, even in the absence of cytokines. Because these TFs are themselves up-regulated in cultured murine hepatocytes (Fig. 3A), they may be part of a positive feed-back loop contributing to the continuously increasing levels of p-ERK1/2.

To our surprise, increases in MAPK signaling, expression of E2F1 and SP-1 and differential gene expression as described herein for *in vitro* cultures could also be demonstrated *in vivo* in two different models of liver injury. Interestingly, however, all events occurred on a shorter time-scale. To the best of our knowledge, we first demonstrated that intoxication with CCl_4 results in rapid phosphorylation of ERK1/2 within 20 minutes. Because CCl_4-dependent activation of MAPK pathway is accompanied by up-regulation of E2F1 and SP-1, these obvious cytokine-independent events seem to be involved in triggering the early proliferative response of hepatocytes after CCl_4 injury. In this model we were able in identifying further similarities between the *in vivo* and *in vitro* situation. For example, in the present work, down-regulation of genes related to amino acid metabolism was found *in vitro* in line, with the reported reduction of hepatic protein synthesis within 30 minutes after CCl_4 intoxication.[30] The decrease *in vitro* in genes related to oxidative phosphorylation fits with decreased levels of ATP following CCl_4 intoxication.[31] Furthermore, Ca^{2+} homeostasis is disturbed within minutes *in vivo*,[31] due to changes in calcium binding and membrane transport.[32] Altered Ca^{2+} levels may also induce proliferation *in vitro*.[33]

Concerning the second model, PHx, we could show by an *in silico* approach that the differential gene expression induced by PHx is also characterized by an overrepresentation of TFBS for the same TFs—ETF, E2F, and SP-1—indicating that such cytokine-independent events contribute to the initiation of proliferation under conditions where the integrity of parenchyma remains intact. Recent work[34] showing that nuclear E2F1 increases in rat hepatocytes after one-third PHx corroborates the importance of this TF, independent of the species studied.

In conclusion, we have shown that cytokine independent events associated with cell-separation and cultivation prime murine hepatocytes for proliferation by activation of MAPK signaling. The transcription factors E2F1, ETF, and SP-1 seem to be key players in orchestrating the alterations in gene expression during the early culture period. Similar processes occur under *in vivo* conditions indicating that murine hepatocytes cultivated on CM represent a valuable system to study proliferation and regeneration processes.

Acknowledgment: Alexander Bauer, involved in the early work, passed away in 2009. We miss him as a competent, innovative, and valued colleague and friend. We would like to thank J. Amin and E. Bedawy for technical support, C. Kern, S. Lagoutte, D. Mahn, S. Mac Nelly, F. Struck, and especially K. Heise for excellent technical assistance, J. Böttger for providing tissue after PHx as well as K. Krohn and P. Süptitz for microarray analysis.

References

1. Michalopoulos GK. Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. Am J Pathol 2010;176:2-13.
2. Michalopoulos GK. Liver regeneration. J Cell Physiol 2007;213: 286-300.
3. Fausto N, Campbell JS, Riehle KJ. Liver regeneration. HEPATOLOGY 2006;43:S45-S53.
4. Juskeviciute E, Vadigepalli R, Hoek JB. Temporal and functional profile of the transcriptional regulatory network in the early regenerative response to partial hepatectomy in the rat. BMC Genomics 2008;9:527.
5. Loyer P, Cariou S, Glaise D, Bilodeau M, Baffet G, Guguenguillouzo C. Growth factor dependence of progression through G(1) and S phases of adult rat hepatocytes in vitro - Evidence of a mitogen restriction point in mid-late G(1). J Biol Chem 1996;271:11484-11492.
6. Liu ML, Mars WM, Zarnegar R, Michalopoulos GK. Collagenase pretreatment and the mitogenic effects of hepatocyte growth factor and transforming growth factor-alpha in adult rat liver. HEPATOLOGY 1994; 19:1521-1527.
7. Arendt E, Ueberham U, Bittner R, Gebhardt R, Ueberham E. Enhanced matrix degradation after withdrawal of TGF-beta 1 triggers hepatocytes from apoptosis to proliferation and regeneration. Cell Prolif 2005;38:287-299.
8. Gkretsi V, Apte U, Mars WM, Bowen WC, Luo JH, Yang Y, et al. Liver-specific ablation of integrin-linked kinase in mice results in abnormal histology, enhanced cell proliferation, and hepatomegaly. HEPATOLOGY 2008;48:1932-1941.
9. Apte U, Gkretsi V, Bowen WC, Mars WM, Luo JH, Donthamsetty S, et al. Enhanced liver regeneration following changes induced by hepatocyte-specific genetic ablation of integrin-linked kinase. HEPATOLOGY 2009;50:844-851.
10. Gebhardt R, Hengstler JG, Muller D, Glockner R, Buenning P, Laube B, et al. New hepatocyte in vitro systems for drug metabolism: Metabolic capacity and recommendations for application in basic research and drug development, standard operation procedures. Drug Metab Rev 2003;35:145-213.
11. Klingmüller U, Bauer AW, Bohl S, Nickel PJ, Breitkopf K, Dooley S, et al. Primary mouse hepatocytes for systems biology approaches: a standardized in vitro system for modelling of signal transduction pathways. IEE Proc Syst Biol 2006;153:433-447.

12. Godoy P, Hengstler JG, Ilkavets I, Meyer C, Bachmann A, Muller A, et al. Extracellular matrix modulates sensitivity of hepatocytes to fibroblastoid dedifferentiation and transforming growth factor beta-induced apoptosis. HEPATOLOGY 2009;49:2031-2043.
13. Dooley S, Hamzavi J, Ciuclan L, Godoy P, Ilkavets I, Ehnert S, et al. Hepatocyte-specific Smad7 expression attenuates TGF-beta-mediated fibrogenesis and protects against liver damage. Gastroenterology 2008; 135:642-659.
14. Seglen PO. Preparation of rat liver cells: I. effect of Ca2+ on enzymatic dispersion of isolated, perfused liver. Exp Cell Res 1972;74:450-454.
15. Ulitsky I, Maron-Katz A, Shavit S, Sagir D, Linhart C, Elkon R, et al. Expander: from expression microarrays to networks and functions. Nat Protoc 2010;5:303-322.
16. Hershko T, Korotayev K, Polager S, Ginsberg D. E2F1 modulates p38 MAPK phosphorylation via transcriptional regulation of ASK1 and Wip1. J Biol Chem 2006;281:31309-31316.
17. Otu HH, Naxerova K, Ho K, Can H, Nesbitt N, Libermann TA, et al. Restoration of liver mass after injury requires proliferative and not embryonic transcriptional patterns. J Biol Chem 2007;282:11197-11204.
18. Thoresen GH, Guren TK, Christoffersen T. Role of ERK, p38 and Pl3-kinase in EGF receptorMediated mitogenic signalling in cultured rat hepatocytes: requirement for sustained ERK activation. Cell Physiol Biochem 2003;13:229-238.
19. Beigel J, Fella K, Kramer PJ, Kroeger M, Hewitt P. Genomics and proteomics analysis of cultured primary rat hepatocytes. Toxicol In Vitro 2008;22:171-181.
20. Tuschl G, Hrach J, Hewitt PG, Mueller SO. Functional characterisation of long-term primary rat hepatocyte cultures as a model for repeat-dose toxicity. N-S Arch Pharmacol 2007;375:95.
21. Wegner H, Schareck W, Bayerhelms H, Gebhardt R. Different proliferative potential of rat and pig hepatocytes in pure primary culture and coculture. Eur J Cell Biol 1992;58:411-417.
22. Michalopoulos G, Cianciulli HD, Novotny AR, Kligerman AD, Strom SC, Jirtle RL. Liver regeneration studies with rat hepatocytes in primary culture. Cancer Res 1982;42:4673-4682.
23. Talarmin H, Rescan C, Cariou S, Glaise D, Zanninelli G, Bilodeau M, et al. The mitogen-activated protein kinase kinase/extracellular signal-regulated kinase cascade activation is a key signalling pathway involved in the regulation of G(1) phase progression in proliferating hepatocytes. Mol Cell Biol 1999;19:6003-6011.
24. Rodriguez JL, Boukaba A, Sandoval J, Georgieva EI, Latasa MU, Garcia-Trevijano ER, et al. Transcription of the MAT2A gene, coding for methionine adenosyltransferase, is up-regulated by E2F and Sp1 at a chromatin level during proliferation of liver cells. Int J Biochem Cell B 2007;39:842-850.
25. Xu JS, Lee YL, Lee KF, Kwok KL, Lee WM, Luk JM, et al. Embryotrophic factor-3 from human oviductal cells enhances proliferation, suppresses apoptosis and stimulates the expression of the {beta}1 subunit of sodium-potassium ATPase in mouse embryos. Hum Reprod 2004;19:2919-2926.
26. Trimarchi JM, Lees JA. Sibling rivalry in the E2F family. Nat Rev Mol Cell Biol 2002;3:11-20.
27. Hallstrom TC, Nevins JR. Balancing the decision of cell proliferation and cell fate. Cell Cycle 2009;8:532-535.
28. Lu SY, Archer MC. Sp1 coordinately regulates de novo lipogenesis and proliferation in cancer cells. Int J Cancer 2010;126:416-425.
29. Wierstra I. Sp1: Emerging roles - beyond constitutive activation of TATA-less housekeeping genes. Biochem Biophys Res Commun 2008; 372:1-13.
30. Gerhard H, Schultze B, Maurer W. Autoradiographic study of the disorder of protein synthesis in the mouse liver following CCl4 intoxication. Dose-effect graphs for reversible and irreversible damages. Virchows Arch B Cell Pathol 1970;6:38-56.
31. Albano E, Bellomo G, Carini R, Biasi F, Poli G, Dianzani MU. Mechanisms responsible for carbon tetrachloride-induced perturbation of mitochondrial calcium homeostasis. FEBS Lett 1985;192:184-188.
32. Hemmings SJ, Pulga VB, Tran ST, Uwiera RRE. Differential inhibitory effects of carbon tetrachloride on the hepatic plasma membrane, mitochondrial and endoplasmic reticular calcium transport systems: implications to hepatotoxicity. Cell Biochem Funct 2002;20:47-59.
33. Takuwa N, Zhou W, Takuwa Y. Calcium, calmodulin and cell-cycle progression. Cell Signal 1995;7:93-104.
34. Mendieta-Condado E, Pichardo-Olvera M, Sanchez-Sevilla L, de Sanchez VC, Hernandez-Munoz R. Adenosine administration accelerates progression of the cell cycle during rat liver regeneration induced by one-third hepatectomy. J Pharmacol Exp Ther 2009;331:122-132.