Autocrine stimulation of human hepatocytes triggers late DNA synthesis and stabilizes long-term differentiation *in vitro*

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Abstract. Isolated human hepatocytes are of great value in investigating cell transplantation, liver physiology, pathology, and drug metabolism. Though hepatocytes possess a tremendous proliferative capacity *in vivo*, their ability to grow in culture is severely limited. We postulated that repeated medium change, common to most *in vitro* systems, may prevent long-term maintenance of hepato-specific functions and growth capacity. To verify our hypotheses we compared the DNA synthesis and differentiation status of isolated human hepatocytes, cultured in medium which was renewed every day or was not changed for 3 weeks ('autocrine' setting). Daily medium change led to rapid hepatocellular de-differentiation without any signs of DNA replication. In contrast, the autocrine setting allowed hepatocytes to become highly differentiated, demonstrated by an elevated ASGPr expression level, and increased albumin and fibrinogen synthesis and release. Cytokeratin 18 filaments were stably expressed, whereas cytokeratin 19 remained undetectable. Hepatocytes growing in an autocrine fashion were activated in the presence of hepatocyte growth factor (HGF), evidenced by c-Met phosphorylation. However, HGF response was not achieved when the culture medium was renewed daily. Furthermore, the autocrine setting evoked a late but strong interleukin 6 release into the culture supernatant, reaching maximum values after a 10-day cultivation period, and intense BrdU incorporation after a further 5-day period. Our data suggest that preservation of the same medium creates environmental conditions which allow hepatocytes to control their differentiation status and DNA synthesis in an autocrine fashion. Further studies are necessary to identify the key mediators involved in autocrine communication and to design the optimal culture configuration for clinical application.

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Introduction

The demand for clinical and pharmaceutical use of isolated hepatocytes is increasing. Hepatocyte transplantation and extracorporeal liver support systems are both considered alternative strategies to orthotopic liver transplantation for patients suffering from endstage liver disease or congenital hepatic disorders. Since large quantities of healthy, differentiated hepatocytes are required, porcine liver has served as the cell source in most preclinical experiments to date (1-3). However, the potential risk of disease transmission from animals to humans may strongly limit the clinical application of porcine hepatocytes (4,5).

Hepatocytes have also become a powerful tool to examine drug metabolism and toxicity. In most cases, the drug profile is analyzed in relation to hepatocytes derived from rat liver. However, rat hepatocytes are unstable and soon become unresponsive to enzyme inducers. Furthermore, they do not optimally reflect the metabolic activity and specificity of human liver enzymes (6,7).

The existence of important interspecies differences in all aspects of hepatic function has meanwhile become well recognized, and there is no doubt that human hepatocytes are more suited than other mammalian cells to restoring normal

liver function in human patients or performing drug profiling studies. It is, therefore, a major challenge to formulate *in vitro* conditions which allow the long-term cultivation of a sufficient amount of human hepatocytes which both proliferate and maintain functions characteristic of hepatic differentiation. Although hepatocytes exhibit extraordinary replicative capacity *in vivo*, the prolonged *in vitro* culture of primary human hepatocytes has not yet been realised. Rather, isolated human hepatocytes fail to retain their ability to proliferate and to perform the essential functions needed for homeostasis *in vivo*.

Several key issues have been addressed to mimic *in vivo* liver conditions, including three-dimensional tissue architecture, cell-matrix and cell-cell interaction, and a growth factor-enriched environment (8,9). However, none of the *in vitro* assays has permitted the generation of a sufficient mass of physiologically active hepatocytes over an extended time period. Obviously, an important key element has yet to be identified. When different hepatocellular cultivation strategies are critically analyzed, it becomes apparent that they all have a standard procedure in common: the repeated removal of the cell-culture supernatant, replaced by fresh culture medium, which is supposed to guarantee sufficient nutrient supply, remove toxic metabolites and cell debris and, consequently, ensure high cell viability. Based on this common feature, we speculated that medium change may be responsible for the failure to activate the growth and differentiation of human hepatocytes *in vitro*.

Three hypotheses were formulated: i) the capacity to control hepatocellular growth and differentiation is still conserved and not lost under *in vitro* conditions, ii) cell growth and differentiation requires the establishment of an adequate liver-specific milieu, carried out by the hepatocytes themselves in an autocrine fashion and not by external modification, and iii) repeated medium renewal counteracts the establishment of an adequate extracellular environment and prevents autocrine activation.

To verify our hypotheses, we analyzed the DNA synthesis and differentiation status of isolated human hepatocytes, cultured in two opposing conditions, i.e. in medium which was renewed daily or not at all, over a period of 3 weeks.

Materials and methods

Isolation of human hepatocytes. Human liver tissue was obtained from partial hepatectomy due to primary liver cell carcinoma or liver metastases of other tumors. A piece of tumor-free tissue was excised from the edge of the specimen, so that only one surface was cut. Samples from 16 different donors were used for all experiments. The average weight of a liver sample was 19.5±24.6 g (n=16). A perfusion cannula was inserted into several liver veins at the cut surface. Preperfusion with HBSS without Ca^{2+} and Mg^{2+} (Biochrom-Seromed, Berlin, Germany) was performed to locate the most suitable vessel for the following procedure. To ensure optimal perfusion and obtain a high perfusion pressure, the non-selected vessels were sealed with tissue paste (Histoacryl; Braun, Melsungen, Germany). The remaining liver vessel was then flushed for 15-20 min with EGTA-solution (HBSS w/o Ca^{2+} and Mg^{2+}, 0.5 mM EGTA) at 37˚C, at a flow rate which was increased from 20 ml/min to 40 ml/min (Masterflex pump; Novodirekt, Kehl am Main, Germany). It was then perfused under re-circulation with collagenase solution (HBSS w/o Ca^{2+} and Mg^{2+}, 5 mM $CaCl_2$, 0.075% collagenase type IV; Sigma Chemical Co., Munich, Germany) at the same temperature, but at a flow rate which was increased from 40 ml/min to 80 ml/min (incubation, 10-30 min).

At the end of the perfusion, the non-perfused part of the liver tissue was discarded and liver cells from the well-perfused part were scraped out with a scalpel and gently dispersed in cold HBSS, enriched with 10% HEPES buffer and 50 μg/ml gentamycin. The cell suspension was filtered through a 70-μm nylon mesh cell strainer and centrifuged at 28 x g (5 min). The pellet was re-suspended in DMEM/Ham's F12 medium (Invitrogen, Karlsruhe, Germany) and layered over a Percoll gradient, adjusted to a density of 1.065 g/ml. It was then centrifuged at 170 x g for 15 min. The pellet thus obtained was then re-suspended in cold DMEM/Ham's F12 medium and centrifuged for 5 min at 28 x g. The resulting final pellet of purified human hepatocytes was then re-suspended in warm (37˚C) complete culture medium, described below.

The viability and cell yield of hepatocytes were assessed by trypan blue dye exclusion, propidium iodide dsDNA-intercalation (FACscan; Becton Dickinson, Heidelberg, Germany), lactate dehydrogenase activity (LDH Cytotoxicity Detection Kit; Boehringer Mannheim, Mannheim, Germany) using an ELISA reader (490 nm, Ceres UV900 C; Bio-Tek Instruments Inc.), and hemocytometer count. Mean cell viability was >80%. The isolated hepatocytes were seeded at a final density of $6.25x10^4$ cells/cm^2 into multi-well plates.

Preparation of two-dimensional collagen matrix. Each well was coated with 25 μg/cm^2 of collagen type I, derived from bovine skin (Seromed-Biochrom). After incubation for 1 h at 37˚C, each well was washed twice with PBS (Gibco) before the hepatocytes were plated.

Composition of the culture medium and hepatocyte incubation. After coating the plates with collagen I and cell attachment, hepatocytes were cultured in DMEM/Ham's F12 medium (Invitrogen), supplemented with 5% pooled human serum, 20 mM Hepes, 50 μg/ml gentamycin, and 10 ng/ml EGF (Sigma). The hepatocytes were either cultured continuously in the same extracellular environment, i.e. without medium change (autocrine setting), or in medium that was renewed every day.

Analysis of surface EGFr, c-Met and ASGPr expression by flow cytometry. The hepatocytes were washed in PBS without Ca^{2+} or Mg^{2+} followed by a 5-10 min incubation with ice-cold 0.02% EDTA solution (Seromed-Biochrom). Detached hepatocytes were centrifuged at 250 x g for 5 min, cell pellets were incubated with anti-c-Met (rabbit, polyclonal; Santa Cruz Biotechnology, Heidelberg, Germany), anti-EGFr (mouse, monoclonal, clone LA22; Santa Cruz Biotechnology) or anti-ASGPr (rabbit, polyclonal, 1:100; kindly provided by Dr U. Treichel, Essen) in PBS with 0.5% BSA for 60 min at 4˚C. Fluorescence isothiocyanate (FITC)-

conjugated goat anti-rabbit IgG or goat anti-mouse IgG served as the secondary antibody (1:10 in PBS, 0.5% BSA). Fluorescence was then measured using a FACscan [FL-1H (log) channel histogram analysis, $1x10^4$ cells/scan; Becton Dickinson] and expressed as mean fluorescence units (MFU). Unspecific fluorescence was evaluated by mouse- or rabbit-IgG FITC.

Albumin and fibrinogen detection. FITC-conjugated polyclonal rabbit anti-human albumin (Dako, Hamburg, Germany) was used to detect the intracellular protein level. Fluorescence was then measured using a FACscan (as specified above) and expressed as mean fluorescence units (MFU). To allow intracellular detection, cells were fixed with 100 μl fixation medium (Fix & Perm; Biozol-An der Grub Bioresearch, Eching, Germany) and washed twice in blocking solution (PBS, 0.5% BSA). Subsequently, they were incubated for 60 min at 4˚C with 100 μl permeabilization medium (Fix & Perm) before the antibody was added. Protein expression was also examined by Western blotting using horseradish-conjugated rabbit anti-human albumin (1:1000). The collection and lysis of human liver cells for protein analysis is described below. Intracellular fibrinogen was measured by flow cytometry using FITC-conjugated polyclonal rabbit anti-human fibrinogen (Dako). The hepatocytes were fixed and permeabilized before the antibody was added. Albumin and fibrinogen release was explored by enzyme-linked immunosorbent assay (AssayPro, NY, USA). The culture medium was removed, and the cell cultures were washed 3 times followed by incubation with medium without serum supplement. After 3 h the culture supernatant was collected, centrifuged at 400 x g for 5 min to remove remaining cells, and kept frozen at -80˚C until analysis. Analysis was performed according to the manufacturer's instructions.

Interleukin-6 (IL-6) analysis. IL-6 secretion into the culture medium supernatant was performed by enzyme-linked immunosorbent assay (Quantikine human immunoassay; R&D Systems, Wiesbaden, Germany). The culture medium was removed, and the cell cultures were washed 3 times followed by incubation with medium without serum supplement. After 24 h the culture supernatant was collected, centrifuged at 400 x g for 5 min to remove remaining cells, and kept frozen at -80˚C until analysis. Analysis was performed according to the manufacturer's instructions.

Analysis of cytokeratin (CK) expression. The cell culture medium was removed, and the hepatocytes detached as described above. The cells were fixed with 100 μl fixation medium (Fix & Perm) and washed twice in blocking solution (PBS, 0.5% BSA). Subsequently, the cells were incubated for 60 min at 4˚C with 100 μl permeabilization medium (Fix & Perm) together with the monoclonal antibodies anti-cytokeratin 18 (mouse, clone Ks18.04; Progen, Heidelberg, Germany) or anti-cytokeratin 19 (mouse, clone Ks19.1; Progen). Fluorometry was carried out as described above.

Western blot analysis. The hepatocyte cultures were rinsed briefly with ice-cold PBS and lysed for 5 min in lysis buffer [50 mM HEPES, 200 mM NaCl, 0.2 mM $MgSO_4$ (Merck), 0.4 mM PMSF, 2% Triton X-100, 10 μg/ml leupeptine, 10 μg/ml aprotinine, 0.02% soybean-trypsin inhibitor, 0.2 mM orthovanadate (all from Sigma-Aldrich)]. The cell lysates were centrifuged for 10 min at 12,000 U/min at 4˚C. The protein concentration of the supernatant was determined by the Lowry method, and equal amounts of protein (50 μg) in laemmli sample buffer (Bio-Rad Laboratories) with ß-mercaptoethanol were boiled for 5 min. Proteins and molecular weight marker were resolved by 7% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (90 min, 100 V) and then transferred to nitrocellulose membranes. The membranes were blocked for 1 h at RT with 10% dry non-fat milk in Tris-buffered saline, and incubated with the primary antibodies anti-c-Met, anti-EGFr, anti-ASGPr, anti-CK18 or anti-CK19, diluted 1:100 in Tris-buffered saline with 0.5% BSA.

HRP-conjugated goat anti-mouse or goat anti-rabbit IgG (1:5000; Upstate Biotechnology, NY, USA) served as secondary antibodies. The membrane was briefly incubated with ECL detection reagent (ECL™, Amersham) to visualize the proteins and exposed to an X-ray film (Hyperfilm™ ECTM, Amersham). ß-actin (1:1000, mouse; Sigma, Taufkirchen, Germany) served as the internal control.

Receptor phosphorylation. Hepatocytes were maintained in serum and HGF/EGF-free DMEM/Ham's medium F12 for 24 h prior to stimulation in order to avoid any protein phosphorylation artefacts due to serum and growth factors. The hepatocytes were stimulated for 3 min with 1 ng/ml HGF (R&D Systems). Subsequently, the hepatocytes were rinsed with ice-cold PBS, and lysed for 5 min in lysis buffer, after which the Western blot assay was performed as described above. The monoclonal antibody p-Tyr (clone PY99, 1:250 dilution; Santa Cruz Biotechnology) was used to detect ligand-induced tyrosine phosphorylation.

DNA synthesis. The hepatocytes were exposed to 100 μM 5-bromo-2-deoxyuridine (BrdU) for 24 h prior to trypsinization, to specifically label S-phase cells. After washing with PBS, cells were fixed with a 1:1 mixture of ice-cold methanol:glacial acetic acid, and subsequently incubated in 2 N HCl containing 1% Triton X-100 (v/v) for 30 min at room temperature. The cells were then washed three times with PBS, and 20 μl of FITC-conjugated anti-BrdU (Becton Dickinson) monoclonal antibody was added, and they were then incubated in darkness for 20 min at room temperature. The labelled nuclei were then washed twice with 0.1 M PBS and counter-stained with propidium iodide 5 μg/ml for another 10 min in darkness. A flow cytometer (FACscan) was used for data acquisition, and 10,000 events were collected from each sample. Data acquisition was performed using CellQuest software. Cells in S-phase were enumerated for each sample.

Statistical analysis. Data were expressed as mean ± SD. All experiments were performed 3-6 times. Statistical significance was investigated by the Wilcoxon-Mann-Whitney U test. Differences were considered statistically significant at a p-value <0.05.

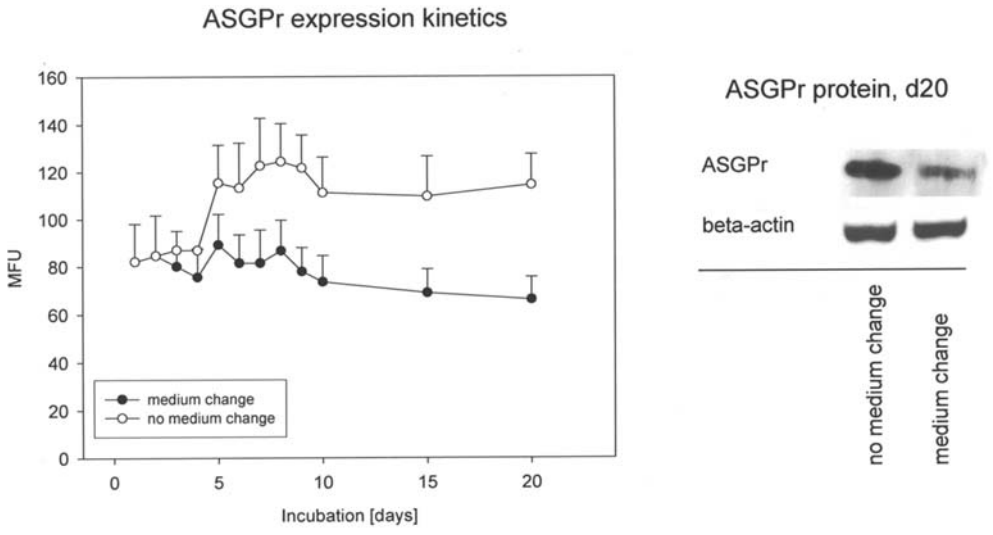

Figure 1. Expression kinetics of ASGPr. Culture conditions were based either on daily medium change or on ongoing cultivation without medium change. To explore the surface expression, anti-ASGPr (rabbit, polyclonal) labeled with FITC-conjugated goat anti-rabbit IgG was used. Fluorescence was then measured using a FACscan [FL-1H (log) channel histogram analysis, $1x10^4$ cells/scan] and expressed as mean fluorescence units (MFU). Unspecific fluorescence was evaluated by rabbit IgG-FITC. The same primary antibody was used for Western blot analysis. HRP-conjugated goat anti-rabbit IgG served as the secondary antibody. ß-actin served as the internal control. A representative experiment is illustrated.

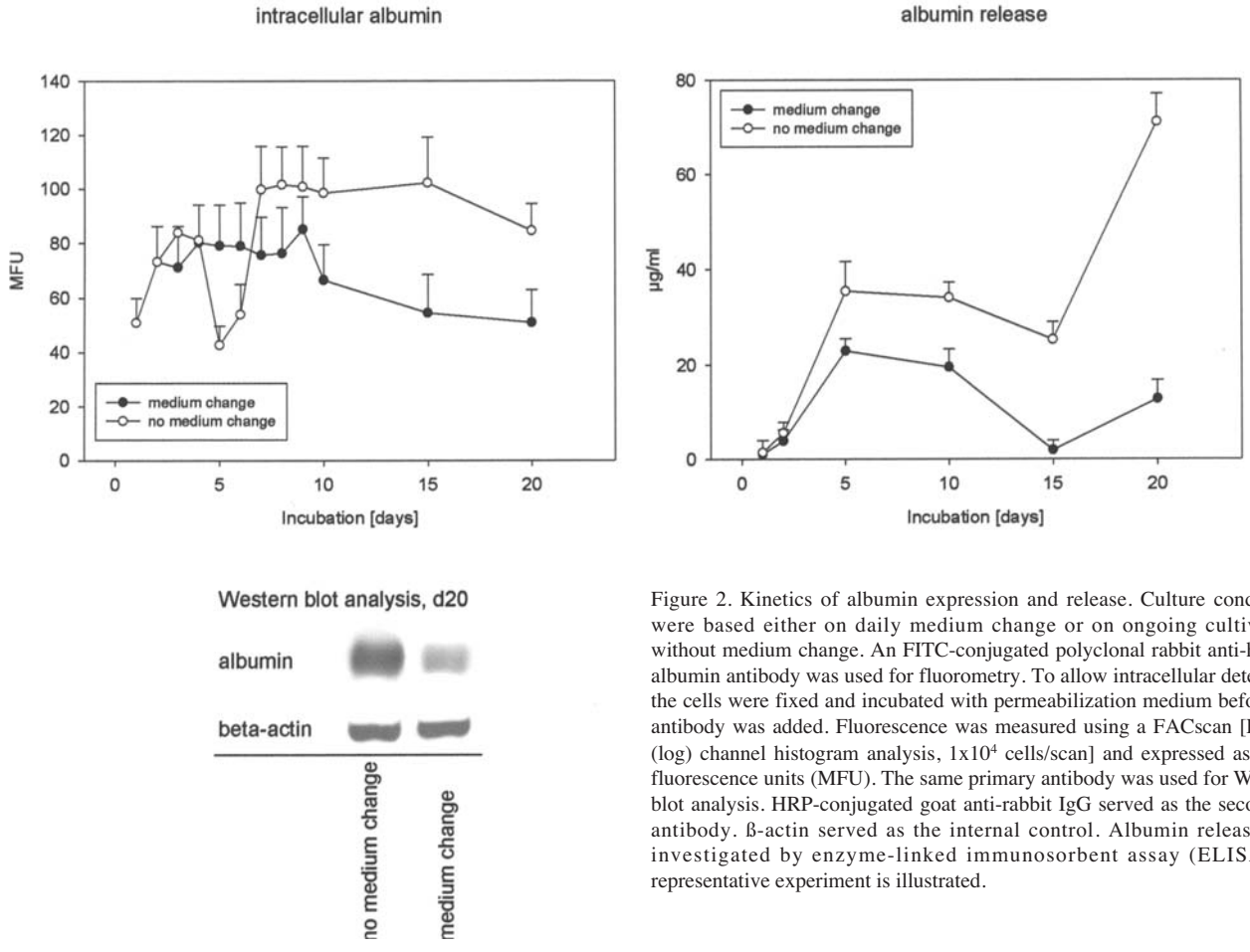

Figure 2. Kinetics of albumin expression and release. Culture conditions were based either on daily medium change or on ongoing cultivation without medium change. An FITC-conjugated polyclonal rabbit anti-human albumin antibody was used for fluorometry. To allow intracellular detection, the cells were fixed and incubated with permeabilization medium before the antibody was added. Fluorescence was measured using a FACscan [FL-1H (log) channel histogram analysis, $1x10^4$ cells/scan] and expressed as mean fluorescence units (MFU). The same primary antibody was used for Western blot analysis. HRP-conjugated goat anti-rabbit IgG served as the secondary antibody. ß-actin served as the internal control. Albumin release was investigated by enzyme-linked immunosorbent assay (ELISA). A representative experiment is illustrated.

Results

Analysis of ASGPr expression level. Previous experiments demonstrated that ASGPr expression correlates with hepatocellular function and differentiation (10). In the present setting, the ASGPr surface level of human hepatocytes remained constant when the culture medium was renewed every day (Fig. 1). However, a striking increase of ASGPr was observed after 5 days in the presence of non-refreshed medium. Western blot analysis revealed enhanced ASGPr

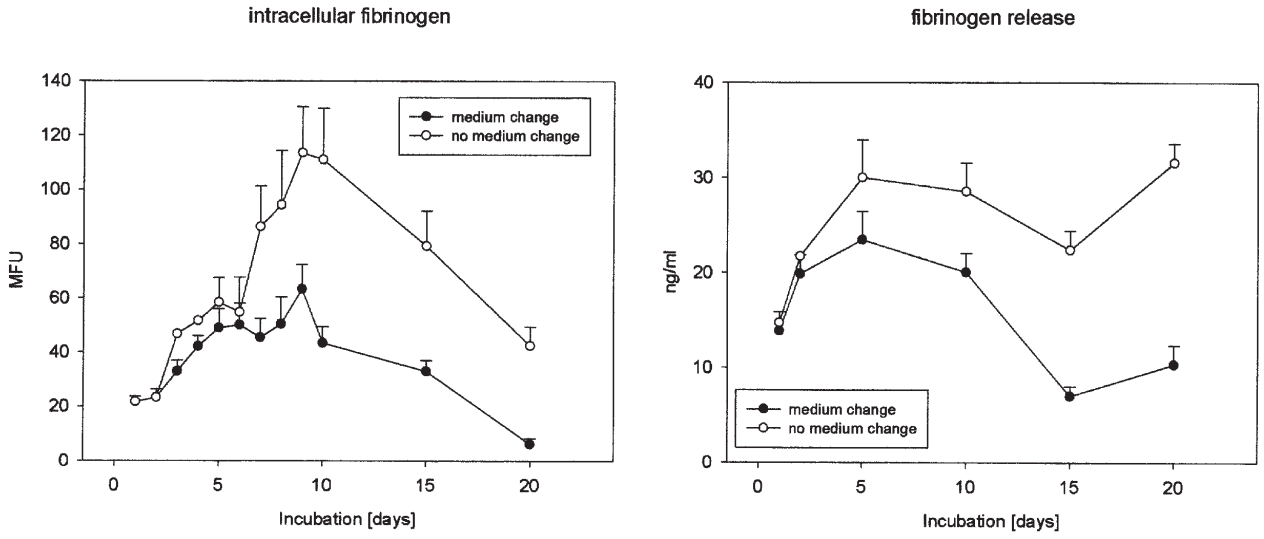

Figure 3. Kinetics of fibrinogen expression and release. Culture conditions were based either on daily medium change or on ongoing cultivation without medium change. Intracellular fibrinogen was measured by flow cytometry [FL-1H (log) channel histogram analysis, $1x10^4$ cells/scan] using FITC-conjugated polyclonal rabbit anti-human fibrinogen. Hepatocytes were fixed and permeabilized before the antibody was added. Fluorescence values are expressed as mean fluorescence units (MFU). The same primary antibody was used for Western blot analysis. HRP-conjugated goat anti-rabbit IgG served as the secondary antibody. ß-actin served as the internal control. Fibrinogen release was investigated by enzyme-linked immunosorbent assay (ELISA). A representative experiment is illustrated.

protein content in hepatocytes growing in the same culture medium, compared to hepatocytes whose cell medium was renewed every day (Fig. 1).

Analysis of hepatocellular function. The physiological activity of human hepatocytes was evaluated in terms of albumin production and synthesis of the haemostasis protein, fibrinogen. Exposing the cells to an extracellular milieu not modified externally (no medium change) evoked biphasic alterations of the intracellular albumin content. Distinct protein down-regulation was seen during the early cultivation phase, and distinct up-regulation during the late cultivation phase, compared to cells grown in renewed medium (Fig. 2). Western blot experiments which were performed in parallel presented evidence of enhanced albumin content in those cell cultures which remained in the initial culture medium. Analysis of the cell-culture supernatant showed that significantly more albumin was secreted by the hepatocytes when initial medium was retained compared to those cultures whose medium was changed daily (Fig. 2).

In good accordance with the albumin data, measurement of intracellular fibrinogen revealed a significant increase of this protein after day 5 of the experiment, when hepatocytes were cultured in non-refreshed medium (Fig. 3). Additionally, higher amounts of fibrinogen were released into the culture supernatant in this setting, compared to cells which were treated with new medium every day.

Cytokeratin expression pattern. Cytokeratins represent specific differentiation markers. Stable expression of CK18 is typically seen in well-differentiated functionally active hepatocytes, whereas increasing CK18 level and *de novo* synthesis of CK7 and CK19 account for a de-differentiated phenotype (11,12). In this context, daily medium change evoked rapid CK18 up-regulation, and *de novo* synthesis of CK19, reaching a plateau phase after 10 and 15 days, respectively. Western blot analysis not only demonstrated distinct amounts of CK18 and CK19 proteins but also of CK7 at day 20 of the experiment (Fig. 4). These changes failed to appear when the extracellular milieu was not modified externally (no medium change). Rather, the CK18 expression level remained stable up to day 10 and increased only slightly thereafter. Very limited amounts of CK7 and CK19 were detected after day 20.

Cell growth analysis. Both c-Met and EGFr have been recognised to stimulate DNA synthesis in human hepatocytes. Fluorometry demonstrated continuously low EGFr expression in hepatocytes treated daily with new culture medium. However, EGFr surface level strongly increased in the autocrine setting (no medium change), reaching peak values after 6 days (Fig. 5). The opposite was true for c-Met. Daily medium change evoked a significant and long-lasting receptor elevation initiated at time periods >day 5. In the presence of non-refreshed medium, only moderate and transient c-Met up-regulation was seen around day 6 of hepatocyte culturing. In good accordance, the intracellular c-Met protein content, analysis of which was carried out at day 20, remained below the detection threshold in the autocrine setting (no medium change), whereas distinct protein amounts were visualized in the modified-culture system. Notably, the quantitative c-Met-receptor enhancement did not correlate with its activation status. Rather, receptor phosphorylation was only inducible when the culture medium was not refreshed (Fig. 5).

Measurement of cytokine levels in the supernatant of both culture systems did not reveal significant alterations of IFNγ, TNFα, IL-2, -4 and -10 release (data not shown). Only slightly elevated IL-6 levels were detected after 20 days in hepatocyte-culture medium which was renewed daily.

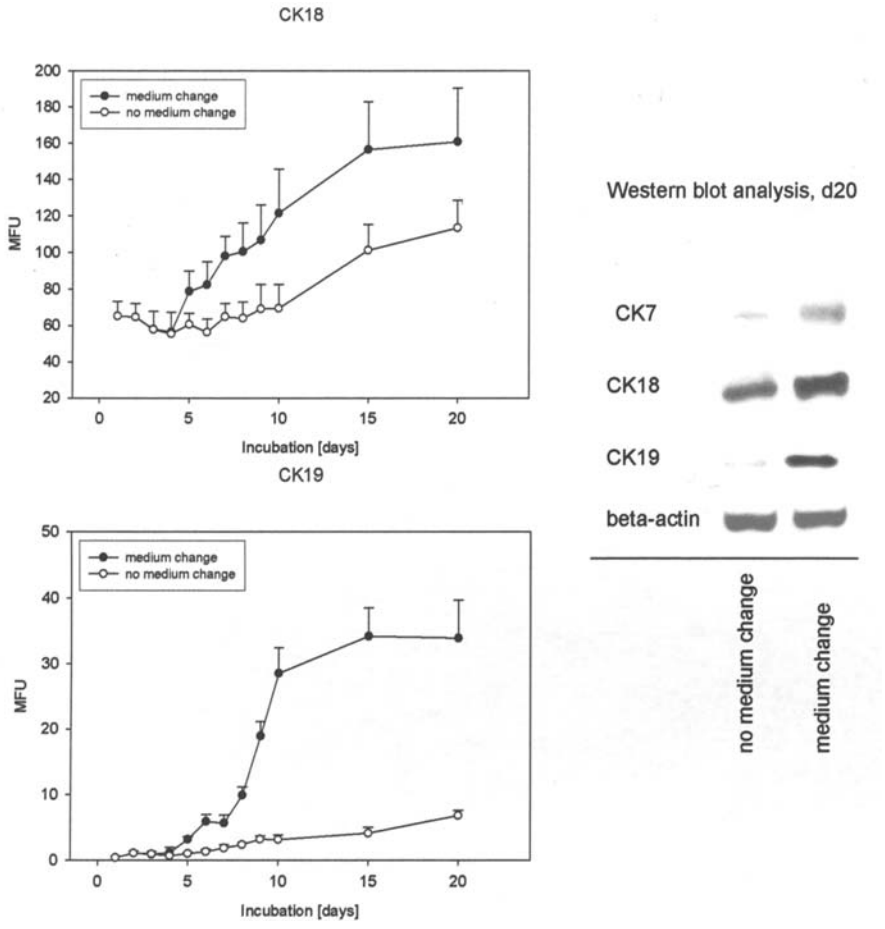

Figure 4. Analysis of cytokeratin (CK) expression level. Culture conditions were based either on daily medium renewal or on ongoing cultivation without medium change. Hepatocytes were fixed and permeabilized before the monoclonal antibodies anti-CK18 (clone Ks18.04) or anti-CK19 (clone Ks19.1) were added. FITC-conjugated goat anti-rabbit IgG was used. Fluorescence was then measured using a FACscan [FL-1H (log) channel histogram analysis, $1x10^4$ cells/scan] and expressed as mean fluorescence units (MFU). Unspecific fluorescence was evaluated by rabbit IgG-FITC. The same primary antibody was used for Western blot analysis. HRP-conjugated goat anti-rabbit IgG served as the secondary antibody. ß-actin served as the internal control. A representative experiment is illustrated.

However, levels of IL-6 in the supernatants of hepatocyte cultures growing in an autocrine fashion (no medium change) increased markedly after 9 days. IL-6 production remained high thereafter, reaching a plateau between days 10 and 20 (Fig. 6).

A similar phenomenon was observed with regard to the BrdU incorporation rate. A moderate increase was detected at day 5 of culture, independent of the experimental setting (Fig. 7A). Interestingly, a second increase of DNA synthesis became obvious between days 10 and 20 after plating out the hepatocytes, showing peak values at day 15. DNA was synthesized to a slight extent in the culture system whose medium was renewed every day. However, the amount of DNA increased greatly in the autocrine system without medium change (Fig. 7A). Morphologic examination revealed high numbers of mononucleated hepatocytes (Fig. 7B). All cell cultures remained negative against human fibroblast antibodies (clone D7-FIB) which excluded a relation between the strong BrdU increase and fibroblast contamination.

Discussion

Researchers have attempted to cultivate differentiated primary hepatocytes with the ability to proliferate, for over 20 years. Although there is no doubt that varying culture conditions may have profound effects on the transcription of liver-specific genes, many differentiated functions are lost, regardless of the culture conditions (13).

We speculated that one basic factor has not been considered in all these culture systems, which is possibly responsible for the non-productive status of the current cultivation techniques. As repeated medium change is common to all *in vitro* protocols, we assumed that this process may be, at least one, key element which prevents the long-term maintenance of hepato-specific functions and growth capacity. Our data present strong evidence that medium renewal negatively affects cell differentiation and DNA synthesis. Conversely, when the extracellular milieu was not modified externally, hepatocytes started to become highly differentiated after a few days, demonstrated by an elevated ASGPr expression level, and increased albumin and fibrinogen synthesis. These events occurred simultaneously, but not earlier than five days after the hepatocytes were plated. We postulated that hepatocytes start to reorganize themselves in an autocrine fashion after they have overcome environmental stress and adapted to the *in vitro* situation. Based on our assay, self-orientation and autocrine organization required at least five days. It is to be expected that such

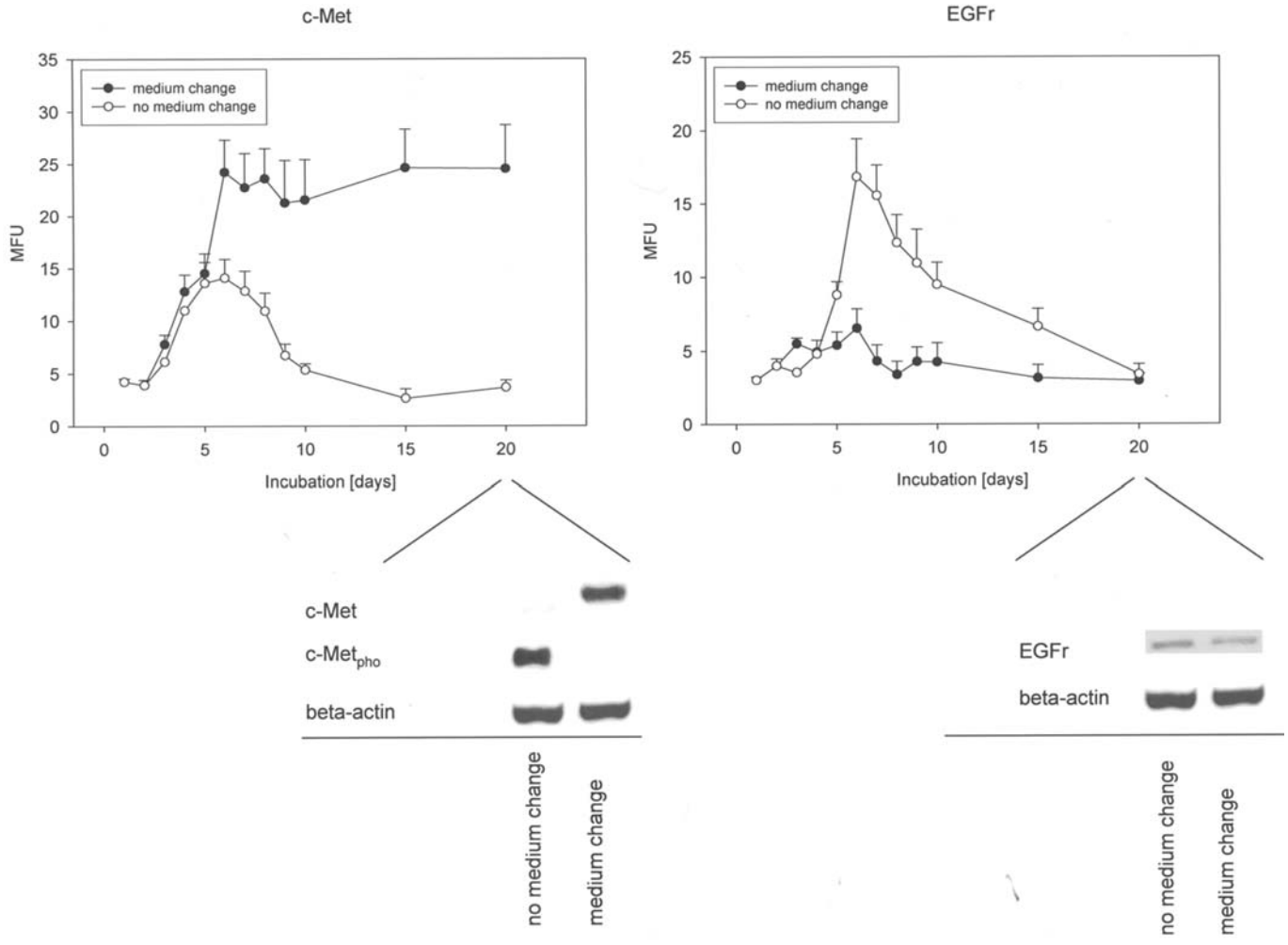

Figure 5. c-Met and EGFr expression levels. Culture conditions were based either on daily medium change or on ongoing cultivation without medium change. Time-dependent receptor expression was measured by flow cytometry [FL-1H (log) channel histogram analysis, $1x10^4$ cells/scan] using FITC-conjugated anti-c-Met (rabbit, polyclonal) or anti-EGFr (mouse, monoclonal, clone LA22) antibodies. Unspecific fluorescence was evaluated by mouse- or rabbit-IgG FITC. The same primary antibodies were used in Western blot assays. The monoclonal antibody p-Tyr (clone PY99) was applied to detect ligand-induced tyrosine phosphorylation. ß-actin served as the internal control. A representative experiment is illustrated.

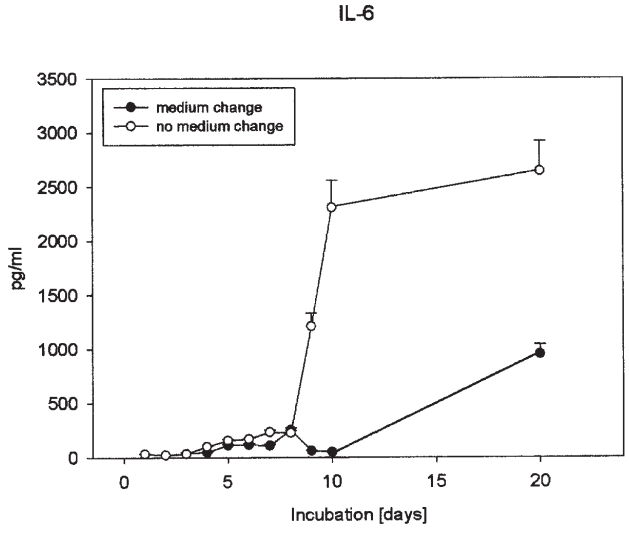

Figure 6. Quantification of IL-6 release. Isolated human hepatocytes were grown in culture medium which was renewed daily or under ongoing conditions without medium change. IL-6 secretion into the cell culture supernatant was measured after different time periods and performed by enzyme-linked immunosorbent assay (ELISA). All experiments were carried out in triplicate.

effects do not occur in well-established culture systems where medium renewal takes place every 2-3 days.

In contrast to the enhanced albumin and fibrinogen content, which indicates an active hepatocellular auto-(re)activation process, the CK levels remained undetectable (CK19) or became only slightly elevated (CK18) in the late cultivation period when medium was not changed. This is of particular importance, as healthy, physiologically intact hepatocytes are characterized by stable CK18 expression and the concomitant non-expression of CK19 (12,14,15). Several reports document the close association of the CK19 expression level with de-differentiation processes of human hepatocytes (11,12). Notably, the loss of albumin production (16-18), as well as the down-regulation of cytochrome P450 isoenzymes was coupled with the induction of CK19 (19,20). Based on a hepatocyte-transplantation model, α-fetoprotein, a marker of hepatocyte de-differentiation, was limited to CK19-positive cells (21). CK19 is not expressed in healthy, mature hepatocytes (22). Concerning CK18 filaments, rapid up-regulation has been ascribed to a distinct loss of liver-specific functions in several culture systems which were based on human hepatocytes (1,12,23). As daily medium renewal

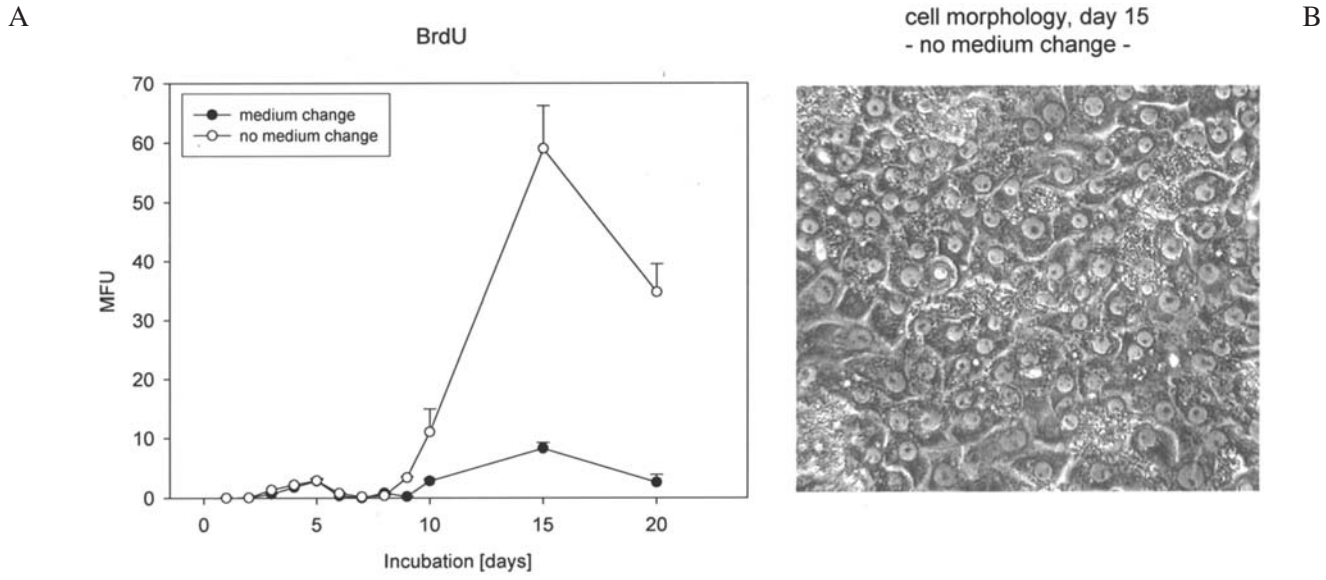

Figure 7. (A) BrdU incorporation, analyzed on hepatocytes either grown in culture medium which was renewed daily or under ongoing conditions without medium change. Hepatocytes were exposed to BrdU for 24 h to specifically label S-phase cells. Subsequently, the cells were treated with FITC-conjugated anti-BrdU monoclonal antibody and counter-stained with propidium iodide. Ten thousand events were collected from each sample by FACS analysis. Data acquisition was carried out using CellQuest software. Mean of three experiments. (B) Cell morphology showing mononucleated hepatocytes (day 15, x20 objective).

significantly altered the filament composition, we assume that this parameter is fundamental to CK expression in human hepatocytes and to CK-triggered differentiation processes.

A similar association may hold true when analyzing growth-related mechanisms. EGFr and c-Met both mediate mitogenic signalling, and contribute to cell-cycle progression (24,25), therefore serving as representative parameters to evaluate the growth activity of human hepatocytes. Notably, distinct c-Met up-regulation was only seen when hepatocytes were treated daily with new culture medium but did not occur in the autocrine system where the medium was not renewed. This reaction may contradict our hypothesis of hepatocellular self-activation. However, previous experiments revealed that c-Met up-regulation does not correlate with c-Met function and the induction of DNA synthesis. In fact, c-Met progressively increased in hepatocytes growing under de-differentiation-inducing conditions. This process was accompanied by the loss of receptor responsiveness to its ligand, hepatocyte growth factor (HGF). Cell cultivation under differentiation-inducing conditions maintained constant receptor responsiveness, allowing c-Met phosphorylation by soluble HGF (10,11,26).

Accordingly, cultivation in an autocrine fashion prevented 'unphysiological' c-Met up-regulation and enabled human hepatocytes to become activated in the presence of HGF. HGF response was not achieved when the culture medium was renewed continuously, which clearly illustrates the consequences that external interference in the culture system may have with regard to sensitizing hepatocytes to growth stimuli.

Unexpectedly, the autocrine-culture system evoked a late but strong IL-6 release into the culture supernatant, reaching maximum values after a 10-day cultivation period, and intense BrdU incorporation after a further 5-day period. IL-6 is closely involved in liver regeneration processes, presumably by priming the hepatocytes to growth stimuli (27-29). It may therefore be concluded that IL-6 release detected in the autocrine assay is, at least in part, responsible for the enhanced DNA synthesis which occurred in the late phase of cultivation. According to our suggestion, Ping and coworkers demonstrated that IL-6 release led to a temporally delayed increase of the PCNA labeling index and thymidine incorporation of hepatocytes in a rat model (28). Nevertheless, IL-6 alone seems to be insufficient to cause hepatic DNA synthesis as i) recombinant IL-6 did not evoke enhanced BrdU labelling when it was added externally to hepatocytes in our culture system, and ii) IL-6-enriched culture supernatant taken from the autocrine assay did not induce DNA synthesis in hepatocytes treated daily with new medium (data not shown). We presume that the autocrine system itself creates the precondition to respond to IL-6. No data are available dealing with this issue. However, it has been demonstrated, that exogenous IL-6 induced only an early and short-term increase in DNA synthesis in human cholangiocarcinoma cells (CC), whereas a continuous growth was induced when CC were allowed to spontaneously produce IL-6 in an autocrine manner (30). A human multiple myeloma cell line started to grow independently of exogenous IL-6 during long-term cultivation *in vitro*. The authors assumed that an autocrine IL-6 loop might have developed which converts the dependence on IL-6 as a paracrine growth factor to a capacity for autonomous growth, dependent on autocrine IL-6 stimulation (31).

We are aware that our data do not necessarily allow a similar interpretation. Nevertheless, we demonstrated for the first time that hepatocyte cultivation without repeated medium exchange may more closely and sufficiently

resemble the *in vivo* liver situation. In contrast to the current culture systems, where proliferation and differentiation programs in hepatocytes are inversely related (32,33), autocrine self-organization enables the cells to maintain differentiated functions while undergoing DNA synthesis. It may not be logical to assume that one single factor is responsible for the autocrine stimulation of the cells. Rather a complete scenario seems to proceed in an environment which stabilizes differentiation and blocks apoptotic or necrotic processes. Indeed, changing the medium of primary cell cultures of central nervous system origin caused severe damage *in vitro* (34), and merely renewing culture medium with fresh medium induced the expression of stress-activated genes in several cell lines, including protein kinases p38, JNK and ERK1/2 and the transcription factor C/EBPß (35). Severe degradation of gap junctions and loss of cell-cell communication were observed in a Chinese hamster cell model after the replacement of culture medium (36). Most significantly, the development of early bovine embryos into hatched blastocytes was reduced in a culture system with medium renewal, compared to a system without medium renewal (37). Hosoya and Marunouchi concluded from their studies that frequent medium renewal accelerates de-differentiation processes by depriving the culture medium of factors produced and secreted by the cells which are necessary for maintenance of the differentiated state (38).

We assume that the sensitive balance between the differentiation and proliferation of hepatocytes, controlled by environmental signals and the extracellular matrix as well as cell-cell interactions, is disrupted upon medium change. External intervention may convert cellular cross-talk from 'physiological' to 'pathological', modify the proximity of neighbouring cells, wash out key mediators involved in intercellular communication, and alter the quantity and quality of released proteins. Nevertheless, our study does not imply the abolishment of medium renewal. In fact, essential nutrients may become limited and possible harmful metabolites enhanced over time. Obviously, a cultivation strategy is required which allows the cells to 'organize themselves', and build up and maintain the optimum environmental milieu but, simultaneously, removes all factors which may negatively affect hepatocellular integrity and function. In this context, it has been demonstrated that as little as 25% of conditioned medium added to fresh culture medium was sufficient to abolish stress response and cellular damage (35). This has not been proven in regard to hepatocytes and, therefore, ongoing studies are necessary to explore whether the use of conditioned medium may be an elegant option to improve hepatocellular culturing.

Our findings are fundamental to the understanding of the self-regulating effects of human hepatocytes *in vitro*, and open novel cultivation strategies. Several unanswered questions yet remain. Is the increase of albumin synthesis related to further liver-specific functions, such as improved urea synthesis, ammonia elimination, and drug metabolization? Does the enhanced BrdU uptake lead to cell proliferation? Further studies are needed to identify the key mediators involved in autocrine communication and to design the optimal culture configuration for clinical application.

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