J. Biochem. 124, 972-979 (1998)

Differentiation and Proliferation of Primary Rat Hepatocytes Cultured as Spheroids[1]

Ryuji Hamamoto,[2] Keisuke Yamada, Masamichi Kamihira,[3] and Shinji Iijima

Department of Biotechnology, Graduate School of Engineering, Nagoya University, Chikusa-ku, Nagoya 464-8603

Received for publication, June 16, 1998

We studied spheroid (multicellular aggregate) formation by hepatocytes and the expression of liver-specific functions such as albumin secretion when hepatocytes were cultured with various extracellular matrices. Hepatocytes cultured on Primaria® and poly-D-lysine coated dishes, and in the presence of a polymer, Eudragit, formed spheroids, and they also exhibited higher liver-specific functions and poor growth compared to monolayer cultures. The results indicated that the cell morphological change and cell-cell interaction caused by the spheroid formation were key factors promoting the expression of the liver-specific functions. To elucidate the mechanism underlying the poor growth in spheroids, we examined the HGF signaling pathway. Phosphorylation and down-regulation of the HGF receptor (*c-Met* proto-oncogene product) were observed for the cells from both monolayer and spheroid cultures, but Ras activation was partly blocked in spheroids. Furthermore, we found that CDK inhibitors, p21 and p27, were highly expressed in spheroids. These results suggested that the reduced Ras signaling and high expression of the CDK inhibitors might cause the lower growth in spheroids. We then examined the relationship between liver-enriched transcription factors (C/EBPα and β) and liver-specific functions. The results revealed that the high expression of C/EBPα was maintained during cultures when hepatocytes formed spheroids. Antisense oligonucleotides of C/EBPα repressed albumin secretion and the expression of p21, suggesting that the transcription factor, C/EBPα, may play a crucial role in the growth and differentiation of hepatocytes in spheroids.

Key words: CDK inhibitor, differentiation, hepatocyte, proliferation, spheroid.

Liver regeneration after the loss of hepatic tissue is a fundamental event in the liver response to injury. By using a regeneration system, many growth factors and cytokines, such as HGF, transforming growth factor-α, insulin, IL-6, and norepinephrine, were shown to play important roles in the growth and differentiation of liver cells (*1-4*). In addition to growth factors, studies on a variety of cell culture systems have suggested that changes in cell morphology are involved in the regulation of growth and gene expression, and also in the maintenance of differentiated functions (*5*). It is considered that not only growth factors but also cell-cell interaction is one of the most important factors that regulate the growth and differentiation of cultured hepatocytes (*6*). In fact, it has been observed that enhanced liver-specific functions could be maintained for a long-term when the cells formed multicellular aggregates called spheroids (*7-9*). However, the molecular mechanism underlying this phenomenon, and the importance of cell morphology and cell-cell interaction for liver differentiation have not been clarified yet.

The liver is composed predominantly of parenchymal cells (hepatocytes) that carry out most of the specialized functions of this organ, including regulation of carbohydrate, urea and lipid metabolism as well as detoxification of exogenous chemicals. The majority of proteins accounting for liver-specific functions are produced almost exclusively by hepatocytes, and their synthesis is regulated primarily at the level of transcription initiation. Transient transfection of the respective genes into hepatoma cell lines led to the identification of four families of liver-enriched transcription factors that participate in the restricted expression of liver-specific genes in adult hepatocytes. These families, classified as to their structurally characterized DNA binding domains, comprise HNF-1 (*10*), the C/EBP family (*11, 12*), HNF-3 (*13*), and HNF-4 (*14*). However, the correlation of these transcription factors in the expression of liver-specific functions is not clear.

The growth ability of differentiated hepatocytes is usually very low. For many cell types, differentiation causes growth arrest. Therefore, it is very important to determine the molecular mechanism which controls the differentiation and growth arrest of hepatocytes. Earlier

[1] This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan (No. 09895023), and in part by the Terumo Life Science Foundation.

[2] R.H. was supported by a Research Fellowship of the Japan Society for the Promotion of Science for Young Scientists.

[3] To whom correspondence should be addressed. Tel: +81-52-789-4277, Fax: +81-52-789-3221, E-mail: kamihira@proc.nubio.nagoya-u.ac.jp

Abbreviations: CDK inhibitor, cycline-dependent protein kinase inhibitor; C/EBP, CCAAT-enhancer binding protein; HGF, hepatocyte growth factor; HNF, hepatocyte nuclear factor; PBS, phosphate-buffered saline.

studies suggested that cell proliferation is regulated by a new class of proteins, CDK inhibitors, including p21 (*15*) and p27 (*16*). These factors form a quaternary complex with CDKs, cyclins and proliferating cell nuclear antigens, and cause inhibition of the kinase activity and DNA synthesis. Furthermore, Liu *et al.* revealed that transcription activation of the CDK inhibitor, p21, by vitamin D_3 leads to the differentiation of a myelomonotic cell line (*17*). Recently, Timchenko *et al.* reported that C/EBPα physically interacts with p21 and thereby increases the stability of the CDK inhibitor in hepatocytes (*18*). These results along with the research on liver-specific gene expression suggest that C/EBPα may play a key role in the control of the growth and differentiation of hepatocytes.

In the present study, we tried to clarify the molecular mechanisms underlying the growth and differentiation of hepatocyte spheroids by investigating the response to HGF, and the roles of C/EBPs and CDK inhibitors.

MATERIALS AND METHODS

Materials—Collagen type IV, fibronectin (Becton Dickinson, Bedford, MA, USA), laminin (Upstate Biotechnology, New York, NY, USA), Eudragit S100 (a copolymer of methacrylic acid and methyl methacrylates; Röhm Pharma GmbH, Darmastadt, Germany), and poly-D-lysine (Sigma Chemical, St. Louis, MO, USA) were used as extracellular matrices. Eudragit S100 was originally developed as an enteric coating polymer and has no cytotoxic effects on cultured cells up to 1% (w/v) (*9*). The surfaces of tissue culture plastic dishes (Iwaki Glass Works, Chiba) were coated with the extracellular matrices. The dishes were also used for cultures in the presence of Eudragit and for control cultures. Collagen type I-coated dishes were obtained from Iwaki Glass Works, and Primaria® dishes (positively charged surface) were purchased from Becton Deckinson.

Isolation and Culture of Hepatocytes—Rat hepatocytes were obtained from male Sprague-Dawley rats (6–7 weeks old) by the collagenase perfusion method (*19*). Williams' medium E (Gibco BRL, New York, NY, USA), supplemented with 0.1 μM $CuSO_4 \cdot 5H_2O$, 25 nM Na_2SeO_3, 1.0 μM dexamethasone, 20 ng/ml EGF (Sigma Chemical), 20 μg/ml egg yolk lipoprotein (Cosmo Bio, Tokyo), 48 μg/ml gentamicin sulfate and 100 μg/ml chloramphenicol, was used as the culture medium. For some spheroid cultures, Eudragit S100 was added to the medium at a concentration of 1.8 mg/ml as an artificial matrix to induce cell aggregation (*9*). Prior to the addition, the polymer was dissolved in water to make a stock solution and the pH was adjusted to 7.4 by adding 2 M NaOH. In some experiments, 10 ng/ml HGF (R&D Systems, Minneapolis, MN, USA) was added to the medium. Hepatocytes were seeded at a density of approximately 5.0×10^5 cells per 35-mm-diameter plastic dish and cultured in a CO_2 incubator.

Assay for DNA Synthesis—DNA synthesis was evaluated as the incorporation of ^{3}H-thymidine. ^{3}H-Thymidine (1 μCi/ml; Amersham, Buckinghamshire, UK) was added to the culture medium and then the cells were incubated for 24 h. The ^{3}H-thymidine labeled cells were collected on a glass microfiber filter (Whatman International, Maidstone, UK) and then rinsed three times with ice-cold 70% ethanol. The filter was dried and then dipped in a scintillation cocktail (Amersham). Radioactivity was counted with a liquid scintillation counter.

Assay for Albumin Secretion—The albumin concentration in the medium was measured by ELISA using an anti-rat albumin antibody (Organon Teknika, Durham, NC, USA) and a peroxidase-conjugated anti-rat albumin antibody (Organon Teknika) for detection, and purified rat albumin (Sigma Chemical) as the standard.

Immunoprecipitation and Western Blotting—For immunoprecipitation of the HGF receptor (*c-Met* proto-oncogene product), membrane fractions of hepatocytes were lysed with a solubilizing buffer (10 mM Tris-HCl, pH 7.8, containing 1% NP-40, 0.15 M NaCl, 1 mM EDTA, 1 mM Na_3VO_4, 10 μg/ml aprotinin, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride) for 30 min at 4°C. Each solution was centrifuged at $24,000 \times g$ for 15 min. One microgram of anti-Met antibodies (Santa Cruz Biotechnology, Santa Cruz, CA, USA) was added to the supernatant containing 500 μg lysate protein, followed by incubation for 1 h at 4°C. The immune complex was adsorbed to protein G-Sepharose beads (Pharmacia Biotech AB, Uppsala, Sweden) for 1 h at 4°C. The beads were then washed four times with the solubilizing buffer and once with PBS (150 mM NaCl, 16 mM Na_2HPO_4, and 4 mM NaH_2PO_4, pH 7.3). After denaturing the protein by mixing with 3×SDS-PAGE sample buffer (195 mM Tris-HCl, pH 8.0, containing 10% glycerol, 0.075% bromophenol blue, 9% SDS, and 15% 2-mercaptoethanol) at 100°C for 5 min, the samples were analyzed by SDS-PAGE (*20*).

The protein expression levels of *Met* receptor, C/EBPα, C/EBPβ, p21, and p27 were determined by Western blot analysis. A nuclear extract was prepared by the procedure of Greenberg and Ziff (*21*) with slight modification. Fifty micrograms of total protein from each sample was applied for Western blot analysis. The samples were boiled in SDS-PAGE sample buffer for 5 min, electrophoresed on 8% (for *Met* receptor) or 12% (for C/EBPα, C/EBPβ, p21, and p27) polyacrylamide gels, and then transferred to Hybond-PVDF membranes (Amersham) using an electroblotter (Atto, Tokyo). The protein-bearing membranes were soaked in a blocking solution (PBS containing 5% skim milk and 0.05% Tween 20). After washing with PBS containing 0.05% Tween 20, the membranes were allowed to react with anti-Met, C/EBPα, C/EBPβ, p21, or p27 antibodies, that were all purchased from Santa Cruz Biotechnology. The specific antibodies were detected with peroxidase-conjugated secondary antibodies using an ECL detection kit (Amersham).

Measurement of the GDP- and GTP-Bound Forms of Ras—The GDP- and GTP-bound forms of Ras were measured according to Muroya *et al.* (*22*) with slight modification. The cells (5×10^5) were plated on 35-mm dishes (2 ml) and then allowed to stand for 48 h for the formation of monolayers (collagen type I-coated dish) or spheroids (Primaria® dish). Then, the cells on the dishes were washed three times with PBS and incubated for 6 h in 2 ml phosphate-free DMEM (Sigma Chemical) containing 100 μCi/ml [^{32}P]orthophosphate (NEN Dupont, Boston, MA, USA), and then stimulated with 10 ng/ml HGF for 5 min. The medium was decanted and the cells were washed twice with an ice-cold buffer (20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl and 1 mM Na_3VO_4). The cells were scraped from the dish surfaces with a rubber scraper

and lysed with an ice-cold lysis buffer (20 mM Tris-HCl, pH 7.5, containing 150 mM NaCl, 20 mM $MgCl_2$, 1 mM Na_3VO_4, 0.5% Triton X-100, 1 μg/ml leupeptin, 1 μg/ml antipain, and 1% aprotinin). Each lysate was centrifuged at 24,000$\times g$ for 15 min to remove insoluble materials. Anti Ras-antibodies (Santa Cruz Biotechnology) were added to the supernatant and then the solution was allowed to stand for 30 min at 4°C. To the solution, protein G-Sepharose beads were added, and then the beads were recovered by centrifugation and washed twice with PBS. The washed beads were resuspended in 12 μl of an elution buffer (20 mM Tris-HCl, pH 7.5, containing 20 mM EDTA, 2% SDS, 1 mM GTP, and 1 mM GDP), and heated at 65°C for 5 min, and then the bound nucleotides were separated on a PEI-cellulose thin-layer chromatography plate. The plate was developed with 0.5 M LiCl-1 M HCOOH, pH 3.4. The ratio of the GTP- to the GTP- and GDP-bound forms of Ras was calculated with the following equation based on the measurement of radioactivity of the GTP and GDP fractions with a liquid scintillation counter: GTP/[(GDP$\times$1.5)+GTP]$\times$100. A factor of 1.5 was used for correction, since three [^{32}P]phosphate molecules were incorporated into GTP and two into GDP (*22*).

Antisense Oligonucleotides—Two different sequences of antisense S-oligonucleotides complementary to the 5′-terminal of C/EBPα mRNA containing an initiation codon (20 mer, 5′-GGGTACCTCAGCCGGCTGAA-3′ and 30 mer, 5′-GGGTACCTCAGCCGGCTGAAGATGCTCCGC-3′) were synthesized. The two antisense oligonucleotides were added independently to the culture medium at the concentration of 5 μM on initiation of a culture. The medium was changed to fresh medium containing the same concentrations of the oligonucleotides every two days. As a control, a sense sequence oligonucleotide (30 mer) derived from the same portion of the C/EBPα mRNA was added to the culture medium.

RESULTS

Cell Morphology and Expression of Liver-Specific Functions under Different Culture Conditions—Figure 1 shows the morphology of cultured adult hepatocytes on various matrices on day 4. When hepatocytes were cultured on collagen type I-, type IV-, laminin-, and fibronectin-coated dishes, the cells were cultured as monolayers (Fig. 1, B-E). On the other hand, the cells formed multicellular aggregates, spheroids, on Primaria® and poly-D-lysine coated dishes. Spheroids were also formed on the addition of a polymer, Eudragit, as reported previously (*9*). Hepatocytes formed cell-aggregates loosely attached to the surfaces of the non-coated dishes (Fig. 1A), but the size of the aggregates was smaller than that of spheroids under other culture conditions.

The albumin secretion by hepatocytes cultured on the various matrices was examined (Fig. 2). When hepatocytes formed spheroids (Primaria® and poly-D-lysine coated dishes, and in the presence of Eudragit), enhanced albumin secretion was observed compared to that by monolayer

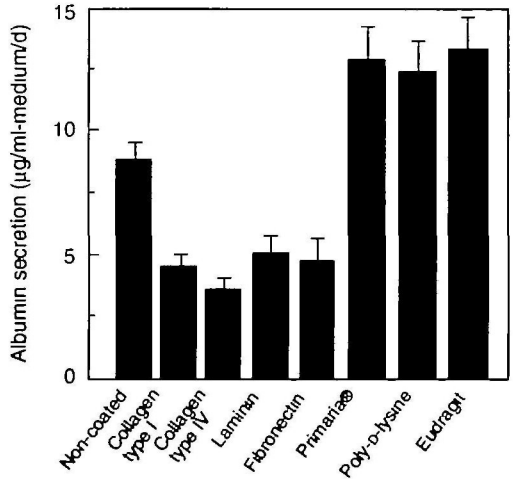

Fig. 2. **Albumin secretion by hepatocytes cultured for 6 d.** Albumin secretion was expressed as μg/(ml-medium)/d, and the data are shown as means±SD for triplicate experiments.

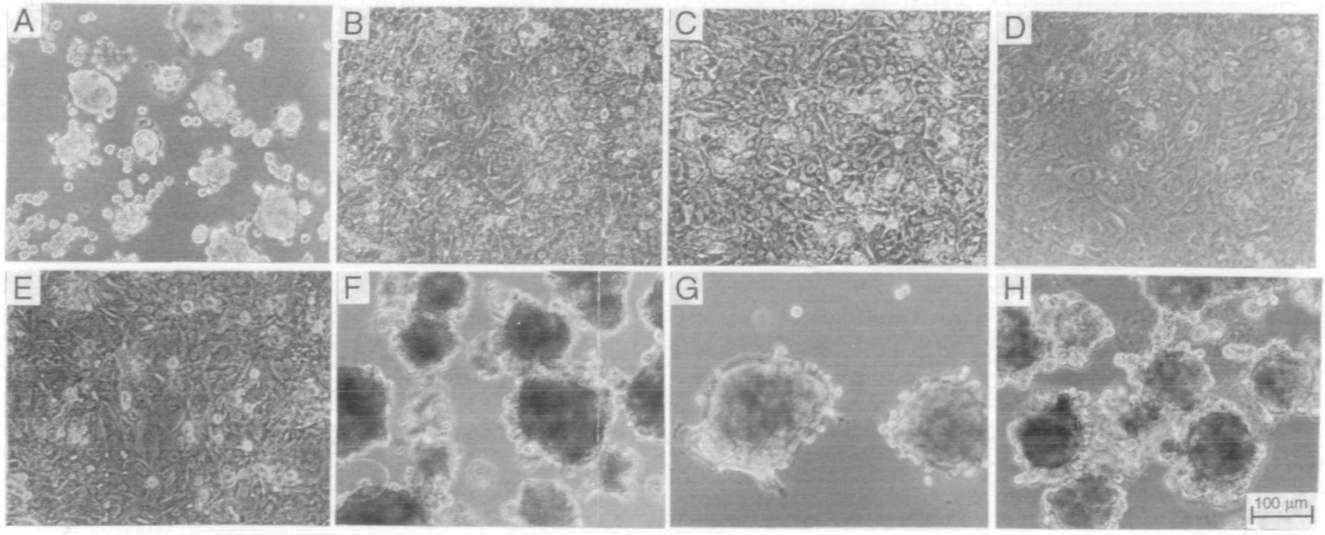

Fig. 1. **Phase-contrast microscopic observation of hepatocytes cultured for 4 d.** (A) Non-coated dish; (B) collagen type I-coated dish; (C) collagen type IV-coated dish; (D) laminin-coated dish; (E) fibronectin-coated dish; (F) Primaria® dish; (G) poly-D-lysine coated dish; (H) in the presence of Eudragit (1.8 mg/ml).

cultures (collagen type I-, type IV-, laminin-, and fibronectin-coated dishes). Even the small cell aggregates that formed on the surfaces of the non-coated dishes produced a higher amount of albumin than the cells in the monolayer cultures. Other liver functions such as ammonia removal and urea synthesis were also enhanced in spheroid cultures (*9*).

It is known that hepatocytes express highly differentiated liver functions in Engelbreth-Horm-Swarm mouse sarcoma tumor gel (EHS gel), which contains laminin and collagen type IV (*5*), and the cells are usually round in shape and form aggregates on the gel. When hepatocytes were cultured on laminin- and collagen type IV-coated dishes, they were cultured as monolayers and albumin secretion was not enhanced, as shown in Figs. 1 and 2. These results indicate that the induction of high liver functions observed in EHS gel cultures is not due to the signals from cell attachment factors such as integrin, but is attributable to the morphological change and/or the cell-cell interaction induced by the aggregate formation.

Response to HGF in Monolayer and Spheroid Cultures— To clarify the roles of the cell morphology and cell-cell interaction in growth and the expression of liver-specific functions, we studied the cellular response to HGF in monolayer and spheroid cultures (Fig. 3). HGF, also known as a scatter factor, was first identified as a blood-derived mitogen for hepatocytes in culture (*2*). HGF and its receptor (*Met* receptor; transmembrane receptor tyrosine kinase) are considered to play key roles in liver regeneration (*23-25*). The growth response to HGF was measured as the ^{3}H-thymidine uptake by hepatocytes. When hepatocytes formed spheroids on a Primaria® dish and in the presence of Eudragit, the uptake of ^{3}H-thymidine after HGF stimulation was drastically repressed compared with that by a monolayer culture on a collagen-coated dish (Fig. 3). In addition, we measured the DNA content by the DAPI method 2 d after HGF stimulation, and the data revealed that the DNA content increased about 1.5 times in monolayer cultures, but no increase was observed in spheroid cultures (data not shown). Then, the expression and phosphorylation of *Met* receptor were investigated (Fig. 4). The levels of expression of *Met* receptor were similar in both monolayer and spheroid cultures, although certain deviation was observed in several experiments (Fig. 4A). Tyrosine phosphorylation of *Met* receptor just after the HGF stimulation was also observed for cells in both monolayer and spheroid cultures (Fig. 4B). Furthermore, *Met* receptor disappeared by 24 h after continuous HGF stimulation from cells in both monolayer and spheroid cultures (Fig. 4C), suggesting that down-regulation of *Met* receptor occurred, as reported previously (*26*). We also obtained the same results (phosphorylation and down-regulation of *Met* receptor in the presence of HGF) for spheroids induced in the presence of Eudragit (data not shown). These results indicate that activation of *Met* receptor occurred in both monolayer and spheroid cultures. In spheroids, however, it is obvious that the signal is modified in the downstream of *Met* receptor, since hepatocytes in spheroids hardly grew upon HGF stimulation.

It is now known that the growth signal from *Met* receptor is transmitted to the nucleus through the Ras-MAP kinase cascade (*27*). Ras is a guanine nucleotide-binding protein associated with the plasma membrane in two states; GDP-bound inactive and GTP-bound active forms. We therefore

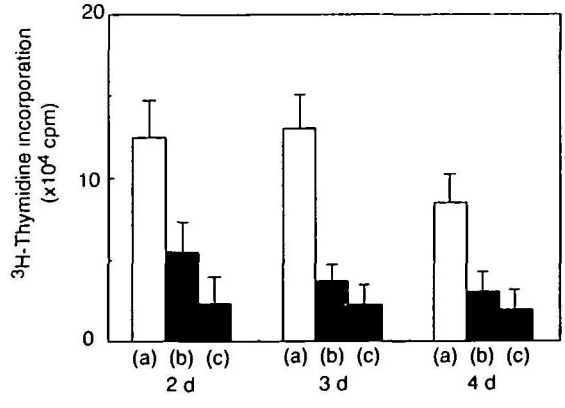

Fig. 3. **^{3}H-Thymidine incorporation by hepatocytes after HGF stimulation.** Hepatocytes were plated on 35 mm dishes at a density of 5×10^5 cells/dish. Cells were cultured for 2, 3, or 4 d with serum-free medium in the presence of HGF (10 ng/ml), and then DNA synthesis was measured as ^{3}H-thymidine incorporation for the following 24 h. The data are shown as means±SD for triplicate experiments. (a) Collagen type I-coated dish; (b) Primaria® dish; (c) in the presence of Eudragit.

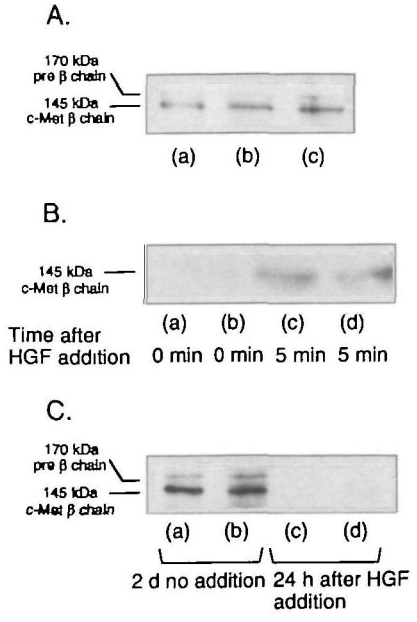

Fig. 4. **Expression and phosphorylation of *Met* receptor.** (A) Western blot analysis of *Met* receptor in monolayer and spheroid cultures. Hepatocyte lysates (500 μg of total protein) were immunoprecipitated with anti-Met antibodies, subjected to SDS-PAGE on 8.0% gel, transferred to PVDF membranes, and detected with anti-Met antibody. (a) Collagen type I-coated dish (monolayer); (b) Primaria® dish (spheroid); (c) in the presence of Eudragit (spheroid). (B) Tyrosine phosphorylation of *Met* receptor. The phosphorylation of *Met* receptor was analyzed before [(a), (b)] and 5 min after [(c), (d)] HGF addition by Western blotting using an anti-phosphotyrosine antibody. (a, c) Monolayer cultures in collagen type I-coated dishes; (b, d) spheroid cultures in Primaria® dishes. (C) Western blot analysis of *Met* receptor in monolayer and spheroid cultures after continuous HGF stimulation. After the cells had been cultured for 2 d in HGF-free medium [(a), (b)], the medium was changed to HGF-containing medium and the cells were incubated for 24 h [(c), (d)]. (a, c) Monolayer cultures in collagen type I-coated dishes; (b, d) spheroid cultures in Primaria® dishes.

measured the ratio of the GDP- and GTP-bound forms of Ras. Figure 5 shows the levels of the GTP-bound form of Ras in hepatocytes in monolayer and spheroid cultures when HGF was added to the medium. About 2-times more of the GTP-bound form of Ras was detected in the cells in monolayer cultures compared to those in spheroid cultures upon HGF stimulation. A low level of the GTP-bound form Ras was also observed for spheroids induced in the presence of Eudragit (data not shown). It was confirmed by immunological analysis that the total amounts of Ras (GTP+GDP bound forms) were almost the same in monolayer and spheroid cultures. Thus, the HGF signaling pathway may be partly modified between *Met* receptor and Ras in spheroid cells.

Expression of CDK Inhibitors in Monolayer and Spheroid Cultures—As the next step, the expression of CDK inhibitors (p21 and p27) in monolayer and spheroid cultures was examined, since CDK inhibitors play important roles in growth regulation. The expression of both p21 and p27 increased in spheroid cultures (Fig. 6). This suggests that these CDK inhibitors may block the growth of hepatocytes in spheroid cultures and that the cell-cell interaction in spheroids may regulate the cell proliferation through an unknown signaling pathway involving expression of these CDK inhibitors.

Expression of C/EBPs in Hepatocytes in Monolayer and Spheroid Cultures—In order to obtain further information on the mechanisms underlying growth regulation and the enhancement of the liver functions in spheroid cells, the expression of liver-enriched transcription factors, C/EBPα and C/EBPβ, was examined (Fig. 7). When hepatocytes formed spheroids, higher expression of C/EBPα was observed compared to in monolayer cultures on Western blotting, especially on day 4 (Fig. 7a). Since C/EBPα regulates the expression of albumin (*28*), the increased expression of albumin in spheroid cultures can be at least partly explained by the increased expression of C/EBPα. The expression of C/EBPβ differed little between the cells in monolayer and spheroid cultures, being slightly higher in spheroid cultures (Fig. 7b).

To clarify the role of C/EBPα in the expression of liver-specific functions and growth regulation, antisense oligonucleotides of C/EBPα were added to the culture medium (Fig. 8). When the antisense oligonucleotides were added

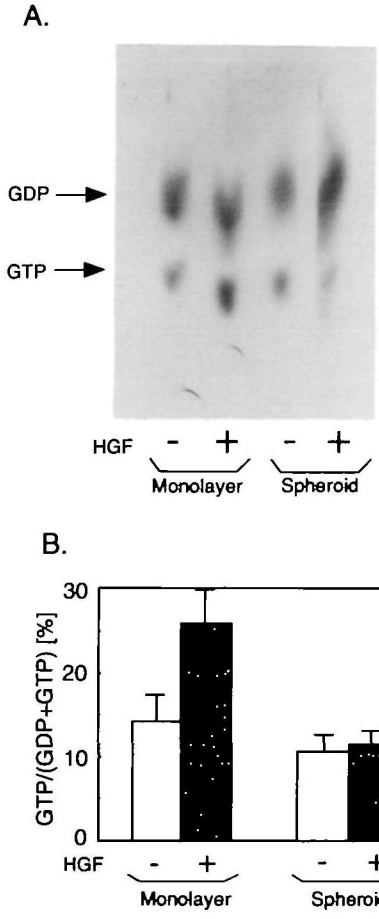

Fig. 5. **The ratio of GTP-form Ras in cells in monolayer and spheroid cultures after HGF stimulation.** (A) TLC analysis of the GTP- and GDP-bound forms of Ras. Hepatocytes were labeled with 100 μCi/ml of [^{32}P]orthophosphate for 6 h and then stimulated with 10 ng/ml HGF for 5 min. Immunoprecipitates obtained with anti-Ras antibodies were subjected to PEI-cellulose thin-layer chromatography. (B) Ratio of GTP-bound form Ras to total Ras. The amounts of the GDP- and GTP-bound form Ras were determined with a liquid scintillation counter, and the ratio was calculated using the following equation: GTP/[(GDP×1.5)+GTP]×100. The data are shown as means±SD for five independent experiments.

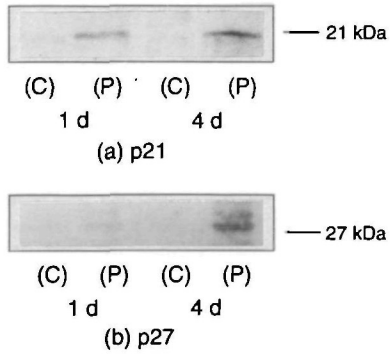

Fig. 6. **Western blot analysis of p21 and p27.** Hepatocytes were cultured on collagen type I-coated (monolayer) (C) or Primaria® dishes (spheroid) (P). The analysis was performed on days 1 and 4. (a) p21; (b) p27.

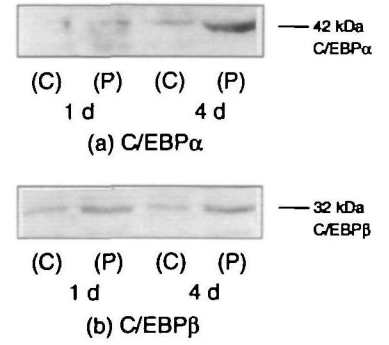

Fig. 7. **Western blot analysis of C/EBPα and C/EBPβ.** Hepatocytes were cultured on collagen type I-coated (monolayer) (C) or Primaria® dishes (spheroid) (P). The analysis was performed on days 1 and 4. (a) C/EBPα; (b) C/EBPβ.

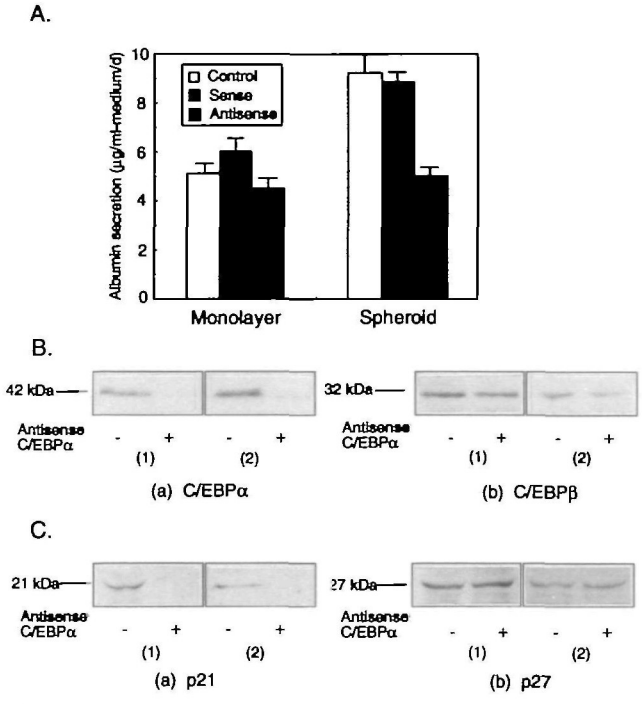

Fig. 8. **Effects of C/EBPα antisense oligonucleotides in hepatocyte cultures on day 4.** Two different sequences of antisense S-oligonucleotides complementary to the 5′-terminal of C/EBPα mRNA were added to the culture medium at the concentration of 5 μM at the beginning of the culture. (A) Albumin secretion by hepatocytes cultured on collagen type I-coated (monolayer) and Primaria® (spheroid) dishes in the presence of C/EBPα antisense oligonucleotides (30 mer) on day 4. Albumin secretion was expressed as μg/(ml-medium)/d and the data are shown as means ± SD for triplicate experiments. (B) Western blot analysis of C/EBPα (a) and C/EBPβ (b) in hepatocytes cultured on Primaria® dishes (spheroid) in the presence of C/EBPα antisense oligonucleotides [20 mer (1) or 30 mer (2)]. (C) Western blot analysis of p21 (a) and p27 (b) in cells cultured on Primaria® dishes (spheroid) in the presence of a C/EBPα antisense oligonucleotides on day 4.

to spheroid cultures, albumin secretion was repressed by about 50%, whereas repression was not observed in monolayer cultures (Fig. 8A). This suggests again that the increased albumin expression by spheroids may be due to the increased expression of C/EBPα. For the Western blot analysis of C/EBPs and CDK inhibitors, antisense oligonucleotides were added to the medium of spheroid cultures, since the expression of C/EBPα, p21, and p27 was very low in cells in monolayer cultures (Figs. 6 and 7). The expression of C/EBPα was certainly repressed by the addition of the antisense oligonucleotides, but the expression of C/EBPβ was not affected (Fig. 8B). Furthermore, we found that the expression of p21 decreased in the presence of the antisense oligonucleotides (Fig. 8C). This indicates the possibility that the level of the p21 protein is regulated by C/EBPα in hepatocytes in spheroid cultures. On the other hand, the antisense oligonucleotides did not affect the expression of p27 (Fig. 8C). In addition, cell cycle analysis of hepatocytes cultured as spheroids in the presence or absence of the C/EBPα antisense oligonucleotide using a flowcytometer revealed that G1-state hepatocytes decreased from 77 to 72%, increasing the proportions of cells in the S and G2/M states. These results suggest that C/EBPα is involved in the growth suppression of hepatocytes cultured as spheroids.

DISCUSSION

Spheroids are multicellular aggregates in which hepatocytes agglomerate and interact with each other through abundant junctional complexes such as gap junctions and desmosomes (*29*). It is well known that cell growth ceases but high liver functions are expressed in spheroid cultures (*7*). In this regard, hepatocyte spheroids are a potentially useful cell system for application as a hybrid-type artificial liver support system (*30*). Judging from the nature of spheroids, we thought that they may also be a good system for analysis of the relationship between cell growth and differentiation.

Our analysis of *Met* receptor suggested that the ligand and receptor formed a proper complex, and that tyrosine phosphorylation of the receptor occurred even in spheroid cells. Proper transmission of the HGF signal was also supported by the fact that *Met* receptor was down-regulated in both monolayer and spheroid cultures. On the other hand, the GTP-bound form of Ras was decreased in spheroids, suggesting that the growth signal was modified between *Met* receptor and Ras. Recently, Adachi *et al.* reported that HGF stimulates the phosphorylation of mitogen-activated protein kinase *via* two different signaling pathways in hepatocytes (*31*). One of them is through Ras, and the other is through phosphatidyl inositol-specific phospholipase Cγ and protein kinase C. Increasing evidence indicates that adaptor Grb2, nucleotide exchange factor Sos, and possibly adaptor Shc are involved in the intercellular transmission of the signal from the tyrosine kinase receptor to Ras (*32*). Grb2 is known to activate the Ras function by recruiting Sos. A similar pathway has been shown for EGF receptor in hepatocytes (*33*). Judging from these facts, the reduced activation of Ras in spheroids may be due to modification of the Shc or Grb2-Sos complex activity. Alternatively, Ras-GTPase activity may change in spheroids (*34*). Similar partial inactivation of Ras after stimulation of the MAP kinase pathway was observed in anergic T cells (*35*). It is generally agreed that Ras activation plays a key role in triggering the cascade of intracellular events which leads to cell proliferation (*36, 37*). Thus, the poor growth of hepatocytes in spheroids may be partly explained by the reduction of GTP-bound Ras. However, the biological significance of the partial inactivation of Ras in spheroid cells is not clear, since *Met* receptor can also interact with pp60$^{c\text{-}src}$, phosphatidylinositol 3-kinase and phospholipase Cγ, in addition to the adapter molecules (*38*). Now we are trying to clarify this point.

Hepatocytes ceased growth, and high level expression of p21 and p27 was observed in spheroids. A CDK inhibitor, p21, was found to be a CDK-associated protein exhibiting inhibitory activity toward several CDKs, a gene product induced by the tumor suppressor, p53 (*39*), and a protein encoded by mRNA which is induced abundantly in senescent cells (*40*). Harper *et al.* reported that p21 inhibits a wide variety of cyclin-CDK complexes, including the cyclin D-CDK4/6 and cyclin A/E-CDK2 complexes (*15*). On the other hand, another CDK inhibitor, p27, is 42% identical to p21 in its N-terminal region (*16*) and inhibits a wide variety of cyclin-CDK complexes like p21. Overexpression

of p27 blocks progression of the cell cycle at the G1 phase. Thus, p21 (or p27) prevents cell proliferation in spheroids.

A spheroid culture is essentially a suspension system. When adherent cells in a submerged culture were transferred to a suspension, the induction of p21 or p27 depending on the cell type along with growth arrest occurred in almost all cases. Therefore, the induction of CDK inhibitors may be a common phenomenon observed in suspended adherent cells (*41*).

In spheroid cultures, we found that the expression level of C/EBPα was higher than that in monolayer cultures. C/EBPα binds specifically to DNA as a homo- or heterodimer, and triggers the transcription of target genes (*11, 42*). High level expression of C/EBPα is restricted to highly differentiated cells such as hepatocytes and adipocytes (*43*). Furthermore, C/EBPα-consensus binding sites are found in several liver-specific genes such as those of albumin, apolipoproteins A1 and A2, alcohol dehydrogenase, cytochrome P450, transferrin and tyrosine aminotransferase (*28, 44*). Therefore, elevated expression of C/EBPα may contribute to the increased liver functions in spheroids, although liver-specific transcription factors HNF-1, 3, and 4 obviously contribute to the expression of the liver-specific functions.

In our experiments, the repression of C/EBPα by antisense oligonucleotides resulted in decreased expression of p21. Recently, Timchenko *et al.* reported that C/EBPα forms a complex with p21 and thereby stabilizes the CDK inhibitor, but does not trigger the expression of the p21 gene in hepatocytes from a newborn mouse (*18*). On the other hand, the same group reported that C/EBPα upregulates the expression of C/EBPα and also stabilizes p21 in adipocytes. They also observed that C/EBPα leads to growth arrest through p21 in a human fibrosarcoma cell line, HT1080 (*45*). Similar growth arrest and terminal differentiation caused by C/EBPα have also been reported for adipocytes (*46*). In our case, the decreased expression of C/EBPα may have caused a decrease in p21 expression at either the transcription or the posttranslation level, or both. Therefore, C/EBPα may be very important for the growth control of hepatocytes.

Since C/EBPα can regulate p21 expression and inhibit growth promotion, and since C/EBPα also directly or indirectly controls liver-specific gene expression, this transcription factor may be a key factor for the growth and differentiation of hepatocytes.

The authors wish to thank Ms. Manami Goto for preparation of the manuscript.

REFERENCES

1. Michalopoulos, G.K. and DeFrances, M.C. (1997) Liver regeneration. *Science* **276**, 60-66
2. Nakamura, T., Nawa, K., and Ichihara, A. (1984) Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem. Biophys. Res. Commun.* **122**, 1450-1459
3. Mead, J.E. and Fausto, N. (1989) Transforming growth factor α may be a physiological regulator of liver regeneration by means of an autocrine mechanism. *Proc. Natl. Acad. Sci. USA* **86**, 1558-1562
4. Cressman, D.E., Greenbaum, L.E., DeAngelis, R.A., Ciliberto, G., Furth, E.E., Poli, V., and Taub, R. (1996) Liver failure and defective hepatocyte regeneration in interleukin-6-deficient mice. *Science* **274**, 1379-1383
5. Bissell, D.M., Arenson, D.M., Maher, J.J., and Roll, F.J. (1987) Support of cultured hepatocytes by a laminin-rich gel. Evidence for a functionally significant subendothelial matrix in normal rat liver. *J. Clin. Invest.* **79**, 801-812
6. Ben-Ze'ev, A., Robinson, G.S., Bucher, N.L.R., and Farmer, S.R. (1988) Cell-cell and cell-matrix interactions differentially regulate the expression of hepatic and cytoskeletal genes in primary cultures of rat hepatocytes. *Proc. Natl. Acad. Sci. USA* **85**, 2161-2165
7. Landry, J., Bernier, D., Ouellet, C., Goyette, R., and Marceau, N. (1985) Spheroidal aggregate culture of rat liver cells: histotypic reorganization, biomatrix deposition, and maintenance of functional activities. *J. Cell Biol.* **101**, 914-923
8. Koide, N., Shinji, T., Tanabe, T., Asano, K., Kawaguchi, M., Sakaguchi, K., Koide, Y., Mori, M., and Tsuji, T. (1989) Continued high albumin production by multicellular spheroids of adult rat hepatocytes formed in the presence of liver-derived proteoglycans. *Biochem. Biophys. Res. Commun.* **161**, 385-391
9. Yamada, K., Kamihira, M., Hamamoto, R., and Iijima, S. (1998) Efficient induction of hepatocyte spheroids in a suspension culture using a water-soluble synthetic polymer as an artificial matrix. *J. Biochem.* **123**, 1017-1023
10. Courtois, G., Morgan, J.G., Campbell, L.A., Fourel, G., and Crabtree, G.R. (1987) Interaction of a liver-specific nuclear factor with the fibrinogen and alpha 1-antitrypsin promoters. *Science* **238**, 688-692
11. Johnson, P.F., Landschulz, W.H., Graves, B.J., and McKnight, S.L. (1987) Identification of a rat liver nuclear protein that binds to the enhancer core element of three animal viruses. *Genes Dev.* **1**, 133-146
12. Cao, Z., Umek, R.M., and McKnight, S.L. (1991) Regulated expression of three C/EBP isoforms during adipose conversion of 3T3-L1 cells. *Genes Dev.* **5**, 1538-1552
13. Vallet, V., Antoine, B., Chafey, P., Vandewalle, A., and Kahn, A. (1995) Overproduction of a truncated hepatocyte nuclear factor 3 protein inhibits expression of liver-specific genes in hepatoma cells. *Mol. Cell Biol.* **15**, 5453-5460
14. Sladek, F.M., Zhong, W.M., Lai, E., and Darnell, J.E., Jr. (1990) Liver-enriched transcription factor HNF-4 is a novel member of the steroid hormone receptor superfamily. *Genes Dev.* **4**, 2353-2365
15. Harper, J.W., Adami, G.R., Wei, N., Keyomarsi, K., and Elledge, S.J. (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. *Cell* **75**, 805-816
16. Polyak, K., Lee, M.-H., Erdjument-Bromage, H., Koff, A., Roberts, J.M., Tempst, P., and Massague, J. (1994) Cloning of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor and a potential mediator of extracellular antimitogenic signals. *Cell* **78**, 59-66
17. Liu, M., Lee, M.H., Cohen, M., Bommakanti, M., and Freedman, L.P. (1996) Transcriptional activation of the Cdk inhibitor p21 by vitamin D_3 leads to the induced differentiation of the myelomonocytic cell line U937. *Genes Dev.* **10**, 142-153
18. Timchenko, N.A., Harris, T.E., Wilde, M., Bilyeu, T.A., Burgess-Beusse, B.L., Finegold, M.J., and Darington, G.J. (1997) CCAAT/Enhancer binding protein α regulates p21 protein and hepatocyte proliferation in newborn mice. *Mol. Cell Biol.* **17**, 7353-7361
19. Seglen, P.O. (1976) Preparation of isolated rat liver cells. *Methods Cell Biol.* **13**, 29-83
20. Laemmli, U.K. (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680-685
21. Greenberg, M.E. and Ziff, E.B. (1984) Stimulation of 3T3 cells induces transcription of the *c-fos* proto-oncogene. *Nature* **311**, 433-438
22. Muroya, K., Hattori, S., and Nakamura, S. (1992) Nerve growth factor induces rapid accumulation of the GTP-bound form of $p21^{ras}$ in rat pheochromocytoma PC12 cells. *Oncogene* **7**, 277-281
23. Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugimura, A., Tashiro, K., and Shimizu, S. (1989) Molecular

cloning and expression of human hepatocyte growth factor. *Nature* **342**, 440-443
24. Kinoshita, T., Tashiro, K., and Nakamura, T. (1989) Marked increase of HGF mRNA in non-parenchymal liver cells of rats treated with hepatotoxins. *Biochem. Biophys. Res. Commun.* **165**, 1229-1234
25. Naldini, L., Vigna, E., Narsimhan, R.P., Gaudino, G., Zarneger, R., Michalopoulos, G.K., and Comoglio, P.M. (1991) Hepatocyte growth factor (HGF) stimulates the tyrosine kinase activity of the receptor encoded by the proto-oncogene *c-MET*. *Oncogene* **6**, 501-504
26. Naka, D., Shimomura, T., Yoshiyama, Y., Sato, M., Sato, M., Ishii, T., and Hara, H. (1993) Internalization and degradation of hepatocyte growth factor in hepatocytes with down-regulation of the receptor/c-Met. *FEBS Lett.* **329**, 147-152
27. Graziani, A., Gramaglia, D., dalla Zonca, P., and Comoglio, P.M. (1993) Hepatocyte growth factor/scatter factor stimulates the Ras-guanine nucleotide exchanger. *J. Biol. Chem.* **268**, 9165-9168
28. Friedman, A.D., Landschulz, W.H., and McKnight, S.L. (1989) CCAAT/enhancer binding protein activates the promoter of the serum albumin gene in cultured hepatoma cells. *Genes Dev.* **3**, 1314-1322
29. Mueller-Klieser, W. (1997) Three-dimensional cell cultures: from molecular mechanisms to clinical applications. *Am. J. Physiol.* **273**, 1109-1123
30. Nyberg, S.L., Peshwa, M.V., Payne, W.D., Hu, W.S., and Cerra, F.B. (1993) Evolution of the bioartificial liver: the need for randomized clinical trials. *Am. J. Surg.* **166**, 512-521
31. Adachi, T., Nakashima, S., Saji, S., Nakamura, T., and Nozawa, Y. (1996) Mitogen-activated protein kinase activation in hepatocyte growth factor-stimulated rat hepatocytes: involvement of protein tyrosine kinase and protein kinase C. *Hepatology* **23**, 1244-1253
32. Pelicci, G., Giordano, S., Zhen, Z., Salcini, A.E., Lanfrancone, L., Bardelli, A., Panayotou, G., Waterfield, M.D., Ponzetto, C., Pelicci, P.G., and Comoglio, P.M. (1995) The motogenic and mitogenic responses to HGF are amplified by the Shc adaptor protein. *Oncogene* **10**, 1631-1638
33. Di Guglielmo, G.M., Baass, P.C., Ou, W.J., Posner, B.I., and Bergeron, J.J. (1994) Compartmentalization of SHC, GRB2 and mSOS, and hyper-phosphorylation of Raf-1 by EGF but not insulin in liver parenchyma. *EMBO J.* **13**, 4269-4277
34. van der Geer, P., Henkemeyer, M., Jacks, T., and Pawson, T. (1997) Aberrant Ras regulation and reduced p190 tyrosine phosphorylation in cells lacking p120-Gap. *Mol. Cell Biol.* **17**, 1840-1847
35. Fields, P.E., Gajewski, T.F., and Fitch, F.W. (1996) Blocked Ras activation in anergic $CD4^+$ T cells. *Science* **271**, 1276-1278
36. Pomerance, M., Schweighoffer, F., Tocque, B., and Pierre, M. (1992) Stimulation of mitogen-activated protein kinase by oncogenic Ras p21 in *Xenopus* oocytes. Requirement for Ras p21-GTPase-activating protein interaction. *J. Biol. Chem.* **267**, 16155-16160
37. Wang, P.L., Nakagawa, Y., and Humphreys-Beher, M.G. (1993) Epidermal growth factor and isoproterenol stimulation of the Ras-guanine nucleotide exchange factor in proliferating rat parotid gland acinar cells. *Biochem. Biophys. Res. Commun.* **192**, 860-866
38. Besser, D., Bardelli, A., Didichenko, S., Thelen, M., Comoglio, P.M., Ponzetto, C., and Nagamine, Y. (1997) Regulation of the urokinase-type plasminogen activator gene by the oncogene Tpr-Met involves GRB2. *Oncogene* **14**, 705-711
39. el-Deiry, W.S., Tokino, T., Velculescu, V.E., Levy, D.B., Parsons, R., Trent, J.M., Lin, D., Mercer, W.E., Kinzler, K.W., and Vogelstein, B. (1993) WAF1, a potential mediator of p53 tumor suppression. *Cell* **75**, 817-825
40. Noda, A., Ning, Y., Venable, S.F., Pereira-Smith, O.M., and Smith, J.R. (1994) Cloning of senescent cell-derived inhibitors of DNA synthesis using an expression screen. *Exp. Cell Res.* **211**, 90-98
41. Carstens, C.P., Kramer, A., and Fahl, W.E. (1996) Adhesion-dependent control of cyclin E/cdk2 activity and cell cycle progression in normal cells but not in Ha-ras transformed NRK cells. *Exp. Cell Res.* **229**, 86-92
42. Landschulz, W.H., Johnson, P.F., and McKnight, S.L. (1988) The leucine zipper: a hypothetical structure common to a new class of DNA binding proteins. *Science* **240**, 1759-1764
43. Umek, R.M., Friedman, A.D., and McKnight, S.L. (1991) CCAAT-enhancer binding protein: a component of a differentiation switch. *Science* **251**, 288-292
44. Cereghini, S. (1996) Liver-enriched transcription factors and hepatocyte differentiation. *FASEB J.* **10**, 267-282
45. Timchenko, N.A., Wilde, M., Nakanishi, M., Smith, J.R., and Darlington, G.J. (1996) CCAAT/enhancer-binding protein α (C/EBPα) inhibits cell proliferation through the p21 (WAF-1/CIP-1/SDI-1) protein. *Genes Dev.* **10**, 804-815
46. Freytag, S.O., Paielli, D.L., and Gilbert, J.D. (1994) Ectopic expression of the CCAAT/enhancer-binding protein α promotes the adipogenic program in a variety of mouse fibroblastic cells. *Genes Dev.* **8**, 1654-1663