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# Morphological Changes Induced by Extracellular Matrix Are Correlated with Maturation of Rat Small Hepatocytes

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**Abstract** Small hepatocytes (SHs), which are known to be hepatic progenitor cells, were isolated from an adult rat liver. SHs in a colony sometimes change their shape from small to large and from flat to rising/piled-up. The aim of the present study is to clarify whether the alteration of cell shape is correlated with the maturation of SHs and whether extracellular matrix (ECM) can induce the morphological changes of SHs. We used liver-enriched transcription factors (LETFs) such as hepatocyte nuclear factor (HNF) 4α, HNF6, CCAAT/enhancer binding proteins (C/EBP) α, and C/EBPβ, tryptophan 2,3-dioxygenase (TO), and serine dehydratase (SDH) as markers of hepatic maturation. To enrich the number of SH colonies, the colonies were isolated from dishes and replated. Replated colonies proliferated and the average number of cells per colony was about five times larger at day 9 than at day 1. When the cells were treated with laminin, type IV collagen, a mixture of laminin and type IV collagen, Matrigel™ or collagen gel (CG), only the cells treated with Matrigel dramatically changed their shape within several days and had reduced growth activity, whereas the cells treated with other ECM did not. HNF4α, HNF6, C/EBPα, C/EBPβ, and TO were well expressed in the cells treated with Matrigel. Furthermore, addition of both glucagon and dexamethasone dramatically induced the expression of SDH mRNA and protein in the cells treated with Matrigel. In conclusion, morphological changes of SHs may be correlated with hepatic maturation and basement membrane (BM)-like structure may induce the morphological changes of SHs. J. Cell. Biochem. 87: 16–28, 2002. © 2002 Wiley-Liss, Inc.

**Key words:** liver-enriched transcription factors; Matrigel; serum proteins; growth; hepatic nonparenchymal cells

Small hepatocytes (SHs) have been identified as proliferating cells with hepatic characteristics [Mitaka et al., 1992, 1993; Tateno and Yoshizato, 1996]. Recently, we showed that a single SH could clonally proliferate and form a large colony [Mitaka et al., 1995, 1999]. Some SH colonies changed their shapes from flat to rising/piled-up cells with time in culture. The rising/piled-up cells were large and tall, possessed many mitochondria, peroxisomes with a crystalline nucleoid, and glycogen granules [Mitaka et al., 1999]. In such colonies nonparenchymal cells (NPCs) invaded under the colony and an accumulation of extracellular matrix

Abbreviations used: AP, alkaline phosphatase; Asc2P, ascorbic acid 2-phosphate; BC, bile canaliculi; BM, basement membrane; C/EBPα, CCAAT/enhancer binding protein α; Cx, connexin; DAB, 3′-diaminobenzidine; DAPI, 6-diamino-2-phenylindole; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethylsulfoxide; ECM, extracellular matrix; EGF, epidermal growth factor; EHS, Engelbreth–Holm–Swarm; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; HNF, hepatocyte nuclear factor; LECs, liver epithelial cells; LETFs, liver-enriched transcription factors; MHs, mature hepatocytes; NPCs, nonparenchymal cells; PAGE, polyacrylamide gel electrophoresis; PCNA, proliferating cell nuclear antigen; SDH, serine dehydratase; SHs, small hepatocytes; TEM, transmission electron microscopy; TO, tryptophan 2,3-dioxygenase.



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(ECM) between hepatocytyes and NPCs was observed. Therefore, we suspected that SHs could differentiate into mature hepatocytes (MHs) that interacted with hepatic NPCs and ECM [Mitaka et al., 1999].

For the purpose of maintaining the differentiated functions, many researchers have used various substances and changed culture conditions through the use of nicotinamide [Inoue et al., 1989; Mitaka et al., 1991], phenobarbital [Miyazaki et al., 1985], dimethylsulfoxide [DMSO; Isom et al., 1985], ECM [Rojkind et al., 1980; Bissell et al., 1987; Ben-Ze'ev et al., 1988; Schetz et al., 1988; Dunn et al., 1992], coculture with NPCs [Guguen-Guillouzo, 1986], and spheroid formation [Koide et al., 1989; Ingber, 1993; Iredale and Arthur, 1994; Rojkind and Greenwel, 1994]. In such experiments, the maintenance of liver-specific functions of the cells was evaluated by expression of mRNAs and/or proteins such as serum proteins, gap junctional proteins like connexin 32 (Cx32) and Cx26, tryptophan 2,3-dioxygenase (TO), and serine dehydratase (SDH). Many genes of those liver-specific proteins are known to be mainly regulated by liver-enriched transcription factors (LETFs) such as CCAAT/enhancer binding protein (C/EBP) $\alpha$ and C/EBP$\beta$, and hepatocyte nuclear factor (HNF) 1$\alpha$, HNF3$\alpha$, HNF4$\alpha$, and HNF6 [Tian and Schibler, 1991; Kuo et al., 1992; Cereghini, 1996; Uzma and Costa, 1996]. Primary hepatocytes cultured on Matrigel$^{TM}$, which is Engelbreth–Holm–Swarm (EHS) sarcoma-derived matrix, maintained some differentiated functions such as albumin, transthyretin, and apolipoprotein A-I production and kept the transcription of HNF1$\alpha$ and HNF4 mRNAs [Nagaki et al., 1995; Oda et al., 1995]. It has been emphasized that cell shape is a key factor to regulate the growth, differentiation, and survival of hepatocytes [Walt, 1986; Maher, 1988]. ECM was reported to be able to modulate the shapes of cultured hepatocytes [Bissell et al., 1987; Maher, 1988; Koide et al., 1989]. Ben-Ze'ev et al. [1988] suggested that cell shape might be a primary regulator of tissue-specific gene expression and that cytoskeletal components might interact directly with the nuclear matrix to affect gene transcriptional rates.

In the present study, we showed that the addition of Matrigel could dramatically change the shapes of the cells as well as the structure of the colonies. In addition, the changes of cell shape were correlated with the expression of hepatic differentiated proteins. To clarify why Matrigel could induce the morphological changes of SHs, we examined the effects of various ECM and growth factors, which are included in Matrigel, on SHs in the colonies. Not only laminin, type IV collagen, a mixture of laminin and type IV collagen, and collagen gel (CG) but also basic fibroblast growth factor (bFGF), pletelet-derived growth factor (PDGF), nerve growth factor (NGF), and transforming growth factor $\beta$ (TGF$\beta$) did not affect the alteration of cellular morphology. Thus, we hypothesize that morphological changes of SHs may be correlated with hepatic maturation and that the formation of the basement membrane (BM)-like structure may be responsible in part for the beneficial effect of those morphological changes of the cells.

## MATERIALS AND METHODS

### Isolation and Culture of Hepatic Cells

Male Sprague–Dawley rats (Shizuoka Laboratory Animal Center, Hamamatsu, Japan), weighing 250–400 g, were used to isolate SHs. All animals received humane care and the experimental protocol was approved by the Committee of Laboratory Animals according to University guidelines. Details of the isolation and culture procedure of the cells were previously described [Mitaka et al., 1999]. Finally, $2 \times 10^5$ viable cells/ml were plated on dishes (1.5 ml/35-mm dish; 4 ml/60-mm dish; 10 ml/100-mm dish; Corning Glass Works, Corning, NY) and cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO Laboratories, Grand Island, NY) supplemented with 20 mM HEPES, 25 mM $NaHCO_3$, 30 mg/L L-proline, 10% fetal bovine serum (HyClone, Logan, UT), 10 mM nicotinamide (Katayama Chemical Co., Osaka, Japan), 1 mM ascorbic acid 2-phosphate (Asc2P; Wako Pure Chem, Tokyo, Japan), 10 ng/ml epidermal growth factor (EGF; Collaborative Research, Inc., Lexington, MA), hormone, and antibiotics. After 4 days of culture, 1% DMSO (Aldrich Chem Co., Milwaukee, WI) was added to the medium. Medium was replaced every other day.

### Replating of Small Hepatocyte Colonies

When SHs proliferated and formed colonies consisting of 15–40 cells (8–12 days after plating), the colonies were detached from dishes and replated on new dishes. Cells were rinsed

with PBS and then treated with 0.02% EDTA/PBS for 1 min. The cells were then treated with cell dissociation solution (Sigma Chem Co., St. Louis, MO) for 5 min at 37°C. After addition of DMEM supplemented with 10% FBS in the dish, SH colonies were collected into conical tubes and the cell suspension was centrifuged at 50*g* for 5 min. The pellet was resuspended in the medium. The number of viable colonies was counted and the colonies were plated on rat tail collagen-coated dishes [Michalopoulos and Pitot, 1975]. Four to five hours after plating, the medium was replaced with the serum-free medium.

### Addition of ECM or Growth Factors

At 11 days after replating, the cells were treated with various ECM such as laminin, type IV collagen, a mixture of laminin and type IV collagen, fibronectin, CG, or growth factor-reduced Matrigel (Becton Dickinson Labware, Bedford, MA). The concentrations of individual ECM components used were similar to those in Matrigel (the manufacturer's data). Forty-eight hours after the treatment, the medium was replaced with fresh medium without ECM. On the other hand, growth factors such as TGF-β, PDGF (Genzyme/Techne, Minneapolis, MN), βNGF (PeproTech EC Ltd., London, United Kingdom), and bFGF (Dainippon Pharm Ltd., Osaka, Japan) were added to the medium at day 11. The concentrations of the growth factors were maximally 10 times larger than in Matrigel. Fresh growth factors were added to the medium at the time of medium change.

### Photographs of Cells

The same fields of dishes identified by needle marks were digitally recorded by using a phase-contrast microscope (Olympus Optical Co., Tokyo, Japan) equipped with a CCD camera (Roper Scientific, Trenton, NJ).

### Immunostaining of Cultured Cells

Cells were fixed with cold absolute ethanol. Mouse anti-proliferating cell nuclear antigen (PCNA; DAKO, Copenhagen, Denmark) and anti-cytokeratin (CK) 8 antibodies (Amersham Corp., Buckinghamshire, United Kingdom) were used as the primary antibodies, followed by the avitin–biotin peroxidase complex method (Vectastain ABC Elite Kit; Vector Laboratories, Inc., Burlingame, CA). 3′-Diaminobenzidine (DAB; Tokyo Kasei Industries, Tokyo, Japan) was used as a substrate. The cells were then counter-stained with hematoxylin. For counting the number of the cells in a colony, immunocytochemistry procedures for CK8 and PCNA were used to identify SHs and to examine the growth activity of the cells, respectively.

For triple immunofluorescent staining, we used a rabbit anti-C/EBPα, a goat anti-HNF4α, or a goat anti-HNF6 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and a mouse anti-E-cadherin antibody (Transduction Laboratory, Lexington, KY) as the primary antibody. Alexa$^{488}$-conjugated anti-rabbit and goat IgG antibodies or Alexa$^{594}$-conjugated anti-mouse IgG (Mol Probe, Eugene, OR) as the secondary antibody were also used. 6-Diamino-2-phenylindole (DAPI) was used as a marker of nuclei. The samples were analyzed using the CELL-Scan system (Scanalytics, Billerica, MA). The details of the procedure were previously described [Mitaka et al., 1999].

### Enzyme-Linked Immunosorbent Assay (ELISA) for Rat Albumin

The medium was collected every 48 h at the time of medium replacement and centrifuged at $1 \times 10^4 g$ for 10 min. The supernatant was kept at −35°C until use. Secreted albumin was measured by ELISA as previously described [Mitaka et al., 1995].

### Western Blot Analysis

The dishes were washed with PBS and then treated with MatriSperse$^{TM}$ Cell Release Solution (Becton Dickinson) for 15 min at 37°C. Thereafter, 300 μl of buffer solution (10 mM HEPES [pH 7.2], 0.25 M sucrose, 0.5 mM $MgCl_2$) was added to the dish. The cells were scraped and collected into microcentrifuge tubes. After pipetting several times with a microsyringe (Hamilton Com, Reno, NV), homogenates were centrifuged at 500*g* for 5 min at 4°C. The supernatants (microsomal fraction) were kept at −35°C until use. The pellets were resuspended in 50 μl of buffer (20 mM Tris-HCl [pH 7.4], 150 mM NaCl, 0.5% deoxycholate, 1 mM EDTA, 2 mM phenylmethylsulfonic acid, 1% NP-40, 200 KIU/ml aprotinin, 20% glycerol, and 0.4 M KCl) and gently mixed for 30 min at 4°C. After centrifugation at 13,000*g* for 15 min, the supernatant was stored at −35°C (nuclear protein fraction). Concentrations of the protein

were measured using a BCA Protein Assay kit (Pierce, Rockford, IL). Samples (medium: 1 μl/lane; microsomal and nuclear protein fractions: 10 or 20 μg/lane) were separated by SDS–polyacrylamide gel electrophoresis (PAGE) and then transferred electrophoretically to an Immobilon-P membrane (Millipore Corp., Bedford, MA) with a semi-dry transfer cell (BioRad, Richmond, CA). Rabbit anti-albumin, anti-transferrin, anti-$\alpha_1$-antitrypsin, anti-fibrinogen (Cappel, Costa Mesa, CA), anti-TO (a gift from T. Nakamura), anti-SDH (a gift from R. Kanamoto), anti-C/EBPα, anti-C/EBPβ, goat anti-HNF1α, anti-HNF3α, anti-HNF4α, anti-HNF6, and mouse anti-PCNA antibodies were used. Horseradish peroxidase-conjugated anti-rabbit IgG, anti-goat IgG, and anti-mouse IgG antibodies (DAKO) were applied and positive bands were detected by incubation in Super-Signal West Dura Extended Duration substrate (Pierce). To induce the expression of TO and SDH, the cells were treated with both $10^{-5}$ M dexamethasone and $10^{-7}$ M glucagon.

### Northern Blot Analysis

Total RNA was extracted from the cells using the single-step thiocyanate–phenol–chloroform extraction method [Chomczynski and Sacchi, 1987] as modified by Xie and Rothblum [1991]. Total RNA (20 μg/lane) was loaded on 1% agarose gel containing 0.5 mg/L of ethidium bromide. Gels were capillary-blotted in 20 × SSPE (3 M NaCl, 173 mM $NaH_2PO_4$, 25 mM EDTA) onto a nylon membrane (Hybond-N, Amersham) and fixed by ultraviolet light. For the detection of TO and SDH mRNAs, alkaline phosphatase (AP)-labeled cDNA probes were prepared from rat TO cDNA (full 1.7 kb *Eco*RI fragment; a gift from T. Nakamura), rat SDH cDNA (full 1.45 kb *Eco*RI fragment; a gift from R. Kanamoto) using an AlkPhos DIRECT Labeling and Detection System with CDP-Star (Amersham). The method used followed the manufacturer's manual (Amersham).

### Perpendicular Sections of Cultured Cells

Perpendicular sections of the colony were examined by using semithin sections of the materials in the process of transmission electron microscopy (TEM). Details of the procedure were previously described [Mitaka et al., 1999].

## RESULTS

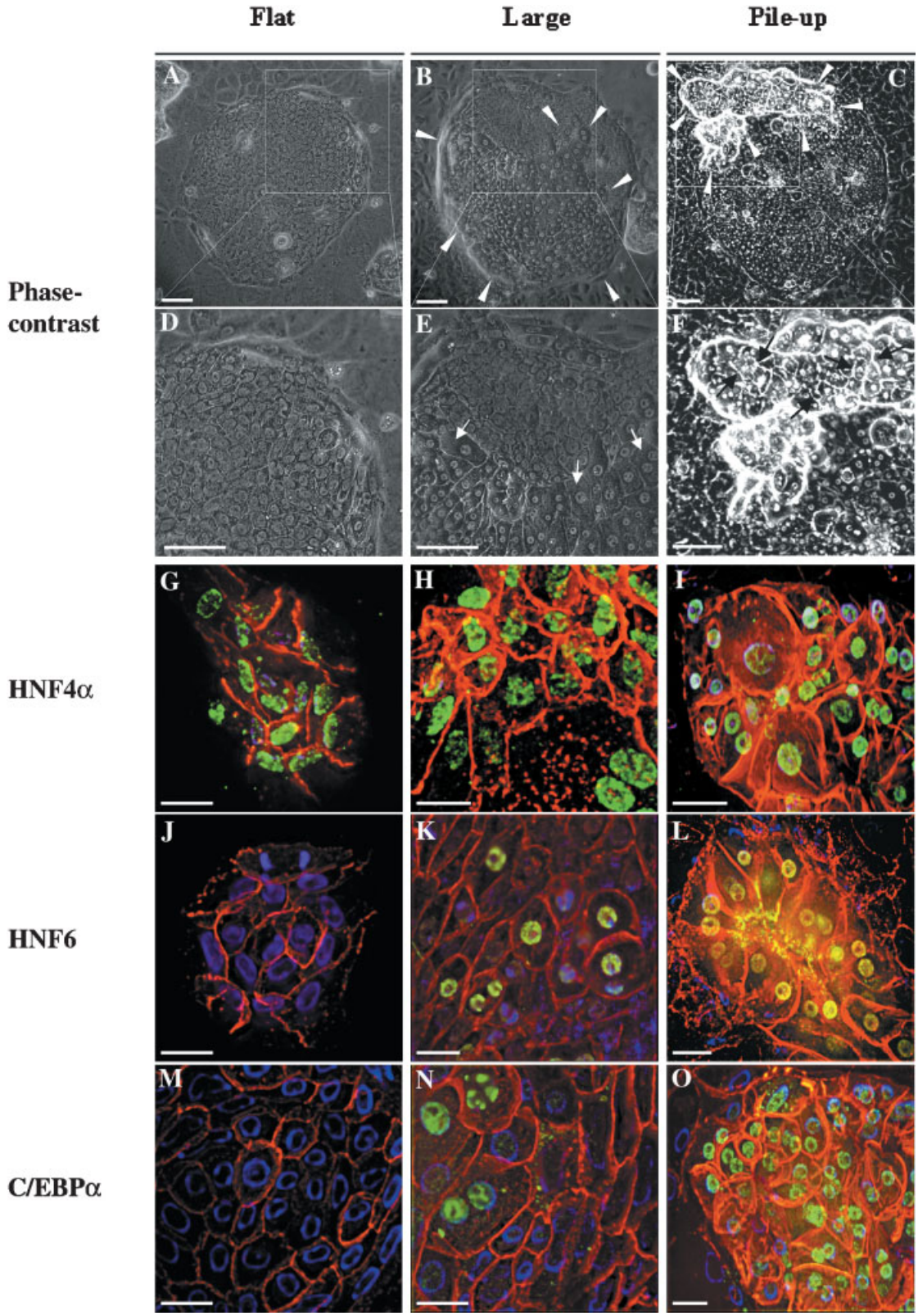
### Morphological Changes of SHs

When hepatic cells, including MHs, SHs, liver epithelial cells (LECs), Kupffer cells, sinusoidal endothelial cells, and stellate cells were cultured in the modified DMEM, SHs rapidly proliferated and formed a colony. Under these culture conditions not only SHs but also LECs and stellate cells proliferated and some SH colonies were gradually surrounded by those cells as previously described [Mitaka et al., 1999]. The colonies not completely surrounded by NPCs continued to expand faster than those surrounded by them and were maintained in a monolayer (Fig. 1A,D). On the other hand, some SHs surrounded and invaded by NPCs gradually changed shape, which looked like rising/piling-up on the colony, and their size became larger (Fig. 1B,E). Their morphology was similar to MHs and they were sometimes binucleate. The piled-up cells formed liver-plate like structures and bile canaliculi (BC) were observed between the cells (Fig. 1C,F).

### Immunocytochemistry for LETFs in SH Colonies

Expression of LETFs such as HNF4α, HNF6, and C/EBPα has been reported to be related to hepatic differentiated functions [Cereghini, 1996; Uzma and Costa, 1996]. Therefore, we carried out immunofluorescent staining for LETFs to examine whether LETF expression correlated with the morphological changes of the cells. As shown in Figure 1, HNF4α was expressed in all hepatocytes, including SHs (Fig. 1G). However, neither HNF6 nor C/EBPα was observed in the nuclei of SHs (Fig. 1J,M).

**Fig. 1.** Expression of HNF4α, HNF6, and C/EBPα proteins in colonies. Triple immunostaining for LETFs (green), E-cadherin (red), and DAPI (blue) shown by digital images analyzed by the CELLScan system. Colonies were classified into three types: those colonies consisting of only SHs and having a flattened shape (flat: **A**, **D**, **G**, **J**, **M**); colonies consisting of SHs and large cells (large: **B**, **E**, **H**, **K**, **N**); colonies consisting of rising and/or piled-up cells (piled-up: **C**, **F**, **I**, **L**, **O**). (A–C) Phase-contrast micrographs of typical colonies of each type are shown. The area indicated by white arrowheads in (B) consists of large, tall cells. The white arrows in (E) show large, binucleate hepatocytes. The area indicated by white arrowheads in (C) shows SHs rising/piled up. The black arrows in (F) show BCs. (D–F) Enlarged photos of the areas surrounded by the squares in (A), (B), and (C), respectively. Scale bars, (A–F), 100 μm. (G–I) Images of cells in each type of colonies for HNF4α. (J–L) Images of cells in each type of colonies for HNF6. (M–O) Images of cells in each type of colonies for C/EBPα. The images are three-dimensionally reconstructed by calculating 30 planes at 0.4-μm intervals. Scale bars, (G–P) 40 μm.


Flat
Large
Pile-up
Phase-contrast
HNF4α
HNF6
C/EBPα
A
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E
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I
J
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L
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N
O

Fig. 1.

**TABLE I. Efficiency of the Recovery and the Ratio of the Attachment of Isolated SH Colonies**

| | Average ± SD (%) |
|---|---|
| The efficiency of the recovery | 81.93 ± 5.78 |
| The ratio of the attached colonies | 78.41 ± 2.43 |

Cells were cultured in DMEM supplemented with 10% FBS, 10 mM nicotinamide, 1 mM Asc2P, and 10 ng/ml EGF. One percent of DMSO was added to the medium from day 4. When SHs proliferated and formed colonies consisting of about 10–40 cells (8–12 days after plating), the number of colonies per viewing area was counted under a light microscope (10 × 10). One hour after replating, the number of colonies per viewing area was counted under a microscope (10 × 10). The efficiency of the recovery of the colonies was calculated and expressed as a percentage of the total number of replated colonies at 1 h per that of recovered colonies. One day after replating, the number of attached colonies per viewing area was counted again. The ratio of the attached colonies was expressed as a percentage of the total number of colonies at 1 h per that of attached colonies at day 1. More than 30 fields per dish were counted, three dishes were examined per experiment were performed, and three independent experiments were carried out.

Nuclei of large and rising/piled-up cells in the colonies were positive for HNF6 (Fig. 1K,L) and C/EBPα (Fig. 1N,O).

### Replating of SH Colonies

To enrich SHs, we collected SH colonies from dishes and replated them on new ones. Table I shows the ratios of the recovery and the attachment on dishes of SH colonies. About 82% of SH colonies were recovered from the dishes and, 1 day after replating, about 78% of the colonies attached on the new dishes. Most cells in the colonies attached on the dish could proliferate and were maintained in a monolayer. As shown in Figure 2, the average area of SH colonies and the number of the cells per colony were about three and five times larger at day 9 after replating than at day 1, respectively.

### Effect of Matrigel Overlay on SH Colonies

In the dishes treated with Matrigel SH colonies dramatically changed shape within several days (Fig. 3D–F). SHs in the colony became large and rose on the colony, and BC-like structures were observed between the cells (Fig. 3E,F). Although the colonies covered with Matrigel did not rapidly expand, piled-up cells gradually formed plate-like structures and slowly extended to the gels. However, in the region that Matrigel did not cover them, SHs continued to proliferate. Parts of colonies covered with Matrigel sometimes formed spheroid-like structures (Fig. 7l). Perpendicular sections of the colony treated with Matrigel are shown in Figure 3G,H. The photos show the moment that a flattened leading edge of a cell may extend upward along Matrigel. The colony covered with Matrigel consisted of large, tall cells and had a multilayered structure. Examination of the cells by TEM showed that they were cuboidal and/or rectangular and appeared to be MHs that possessed many mitochondria, peroxisomes with a crystalline nucleoid, and glycogen granules (Fig. 3I). In addition, BC structures were well developed between the cells.

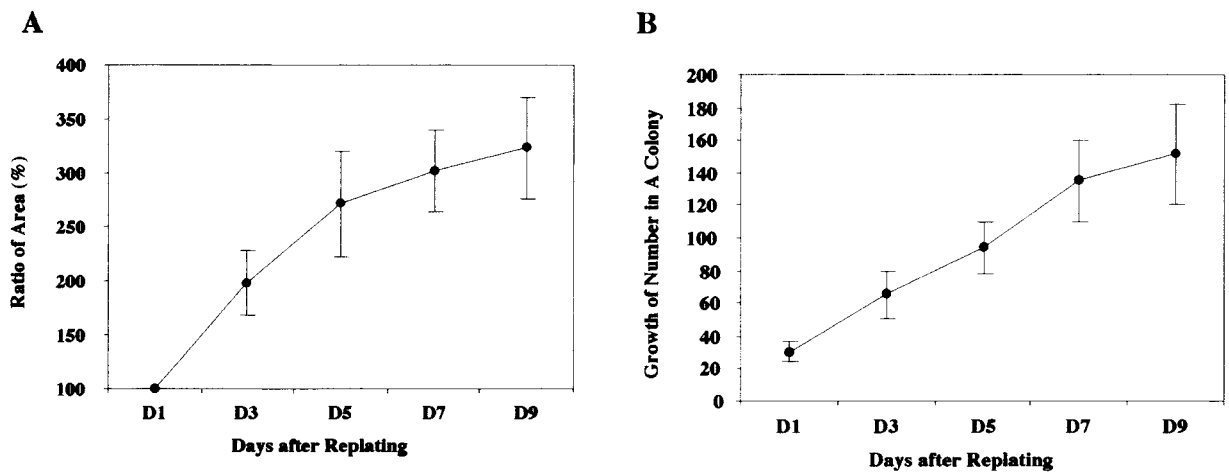


**Fig. 2.** Growth capacity of replated colonies. Growth of colonies replated on the dishes (**A**) and the numbers of the cells in colonies (**B**). The area of the colonies digitally recorded was measured and the number of cells in the colony was counted. More than 20 colonies per dish and three dishes per experiment were analyzed. The area of the colonies at day 1 after replating is shown as 100%. Bars show the average ± SD of three independent experiments.

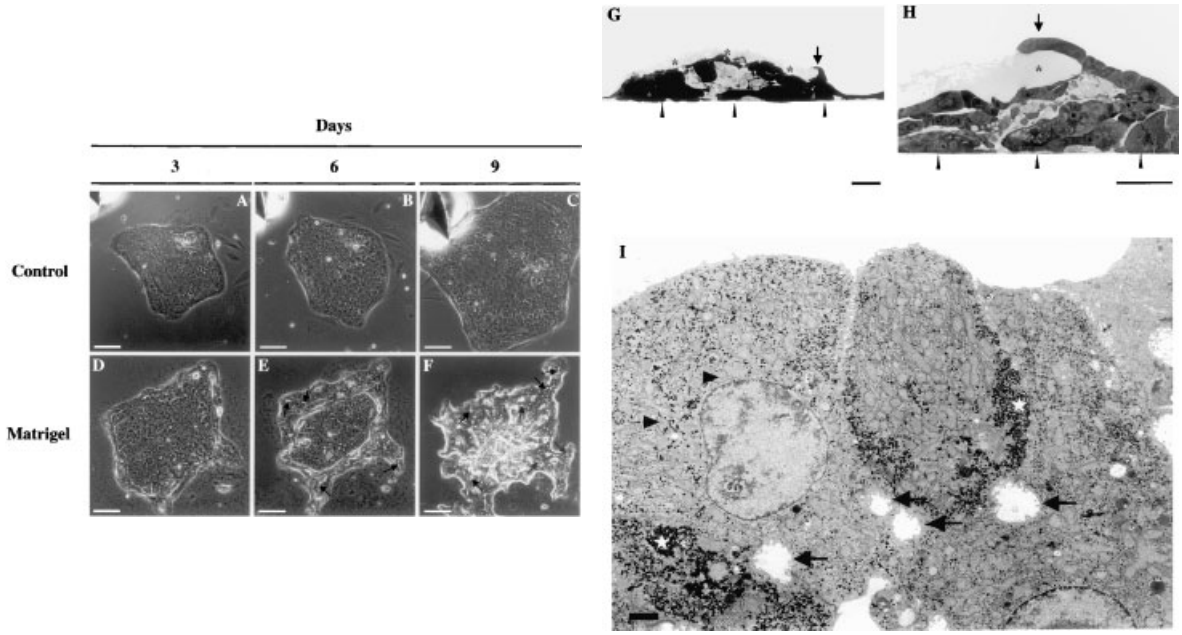


**Fig. 3.** Morphological changes of the colonies treated with Matrigel. Isolated colonies were plated on dishes and cultured in the medium without 10% FBS. Eleven days after replating, the cells were treated with 500 μg/ml of Matrigel (**D**–**F**). Control, no treatment (**A**–**C**). Arrows in (E) and (F) show BCs reformed between the cells. All photos are of the same magnification. Scale bar, 50 μm. Perpendicular views of the colony 4 days after Matrigel treatment. The semithin sections were stained with toluidin blue (**G**,**H**). Arrowheads show the dish surface. Amorphous materials (asterisks) are Matrigel. The flattened leading edge of a cell extends upward along the Matrigel (arrow). A colony covered with Matrigel consists of mutilayered large cells. Scale bars, 20 μm. The electron micrograph of a perpendicular section of a colony 8 days after Matrigel treatment (I). The cells possess many mitochondria, rough endoplasmic reticulum, peroxisomes with a crystalline nucleoid (arrowheads), and glycogen granules (white stars). Well developed BCs are observed between the cells (arrows). Scale bar, 200 nm.

### Production of Serum Proteins by Matrigel Overlay

We examined the production of several hepatic proteins in the cells treated with or without Matrigel. As shown in Figure 4A, albumin secretion into culture medium dramatically increased after the cells were treated with Matrigel. The secretion of albumin by the cells treated with Matrigel reached about 2 μg/ml/h at day 23 and the rate was about six times larger than that in the control. Western blot analysis of the medium (Fig. 4B) and the cells (Fig. 4C) showed that the production of albumin, Tf, $\alpha_1$-AT, and fibrinogen increased in the cells treated with Matrigel.

### Expression of LETFs in the SH Colonies Treated with Matrigel

To examine the expression of HNF1α, HNF3α, HNF4α, HNF6, C/EBPα, and C/EBPβ proteins, we separated nuclei from the cells and the proteins extracted from the nuclei were analyzed by Western blotting (Fig. 4D). When cells were treated with Matrigel, the expression of HNF4α, HNF6, C/EBPα, and C/EBPβ proteins gradually increased, whereas the expression of HNF1α was reduced. In spite of the existence of Matrigel, HNF3α expression gradually increased with time in culture. On the other hand, without Matrigel, HNF1α was strongly expressed in the cells, whereas HNF3α and HNF4α proteins were faintly expressed. Expression of HNF6 and C/EBPα was also detected in the late phase of the culture. However, the amounts of the expression were quite small compared to those in the cells with Matrigel. The expression of HNF4α and C/EBPβ proteins did not change with time in culture.

### TO and SDH Expression in SHs Treated with Matrigel

As shown in Figure 5A, the production of TO protein gradually increased in the cells with Matrigel and its synthesis was not clearly enhanced by the addition of both glucagon and dexamethasone. On the other hand, SDH protein was not detected in the cells throughout the

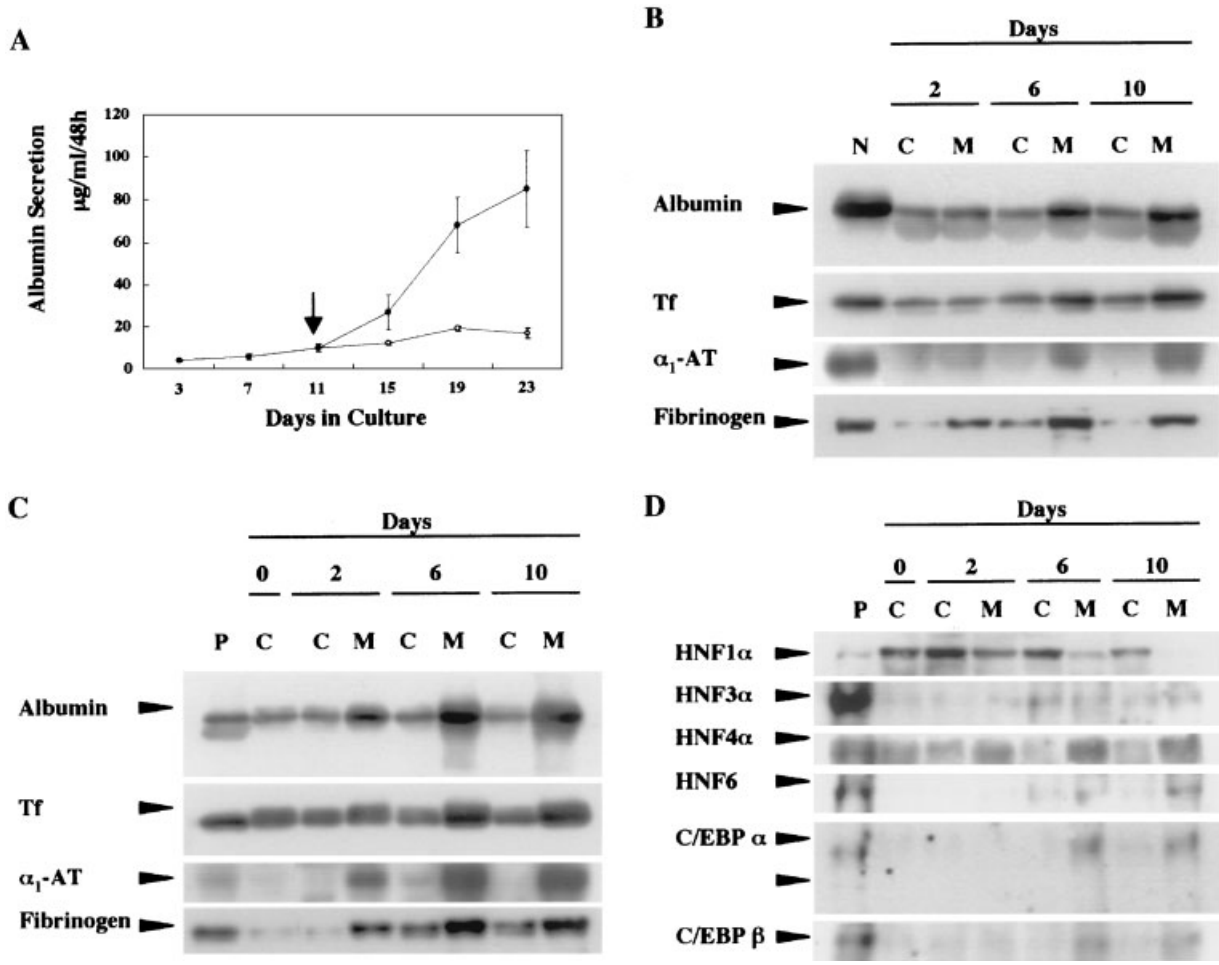


**Fig. 4.** (**A**) Albumin secretion of the cells treated with Matrigel. After replating, the cells were cultured in the medium without 10% FBS for 11 days and treated with (●) or without 500 μg/ml Matrigel (○). An arrow shows the time of the treatment. Albumin concentration in the medium was measured by ELISA. The points show the average ± SD. Western blot analysis for albumin, Tf, $\alpha_1$-AT, and fibrinogen of the medium (**B**) and cells (**C**) treated with Matrigel. Isolated SH colonies were plated on collagen-coated dishes and cultured in the medium without 10% FBS. Eleven days after replating, the cells were treated with (M) or without (C) Matrigel (500 μg/ml). Samples (cells 20 μg/lane; medium, 1 μl/lane) were separated by 7.5% or 10% SDS–PAGE. P, primary MHs at 3 h after plating; N, normal serum or plasma (× 1,000 dilution with PBS). (**D**) Western blot analysis for HNF1α, HNF3α, HNF4α, HNF6, C/EBPα, and C/EBPβ proteins in the cells treated with Matrigel. Isolated SH colonies were plated on collagen-coated dishes cultured in the medium without 10% FBS. Eleven days after replating, the cells were treated with (M) or without (C) Matrigel (500 μg/ml). Samples (20 μg/lane) were separated by 10% SDS–PAGE. P, primary MHs at 3 h after plating.

culture, in spite of the treatment with Matrigel. However, dramatic induction was observed in the cells treated with Matrigel when the hormones were added to the culture medium. As shown in Figure 5B, a small amount of TO mRNA was expressed in the control during the culture, whereas the expression clearly increased in the cells treated with Matrigel. Addition of hormones could not induce the TO mRNA in the cells with or without Matrigel. On the other hand, no expression of SDH mRNA was observed in the cells with or without Matrigel. However, the addition of both glucagon and dexamethasone dramatically induced its expression, especially in the cells treated with Matrigel.

### Effects of Growth Factors and ECM on SHs Colonies

We examined whether similar morphological changes induced by the overlay of Matrigel occurred in the SH colonies when each component of Matrigel was added to the medium. Growth factors such as NGF (Fig. 6j), PDGF (Fig. 6k), and bFGF (Fig. 6l) could not induce alteration of SHs. However, 1 ng/ml TGF-β completely suppressed the growth of SH colonies

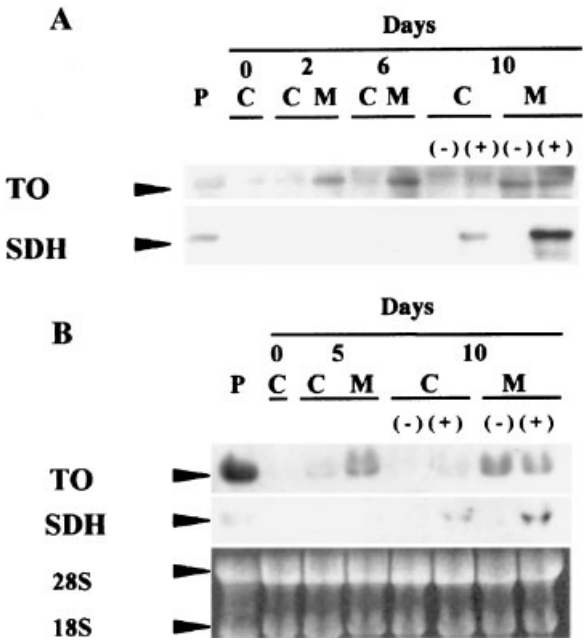


**Fig. 5.** Western blot (**A**) and Northern blot analyses (**B**) for TO and SDH in the cells treated with Matrigel. Isolated SH colonies were plated on collagen-coated dishes cultured in the medium without 10% FBS. Eleven days after replating, the cells were treated with (M) or without (C) Matrigel (500 μg/ml). Twelve hours before harvest, the cells were treated with (+) or without (−) both $10^{-5}$ M dexamethasone and $10^{-7}$ M glucagon. Proteins (20 μg/lane) were separated by 10% SDS–PAGE and total RNA (20 μg/lane) was loaded on 1% agarose gel. P, Primary MHs at 3 h after plating.

and many cells were detached from dishes (Fig. 6g–i). Although 0.1 ng/ml TGF-β partially suppressed the growth of SH colonies (Fig. 6d–f), less than 0.01 ng/ml TGF-β did not suppress the growth of SH colonies and no cells were detached from dishes (Fig. 6a–c). Addition of TGF-β did not make the cells pile-up and cells remained flat and small. In the dishes treated with laminin (Fig. 7a–c), type IV collagen (Fig. 7d–f), fibronectin (data not shown), the mixture of laminin and type IV collagen (Fig. 7g–i), or CG (Fig. 7j,k), SH colonies did not show any apparent morphological changes either. To examine the effects of ECM and growth factors on hepatic differentiated functions, immunoblotting for TO protein was carried out. As shown in Figure 6B, no apparent difference of TO expression was observed among the cells treated with various growth factors. Although the expression of TO protein was induced in the cells treated with laminin, type IV collagen, the mixture, or CG better than in the control cells, that of TO was much less than the cells treated with Matrigel (Fig. 7B).

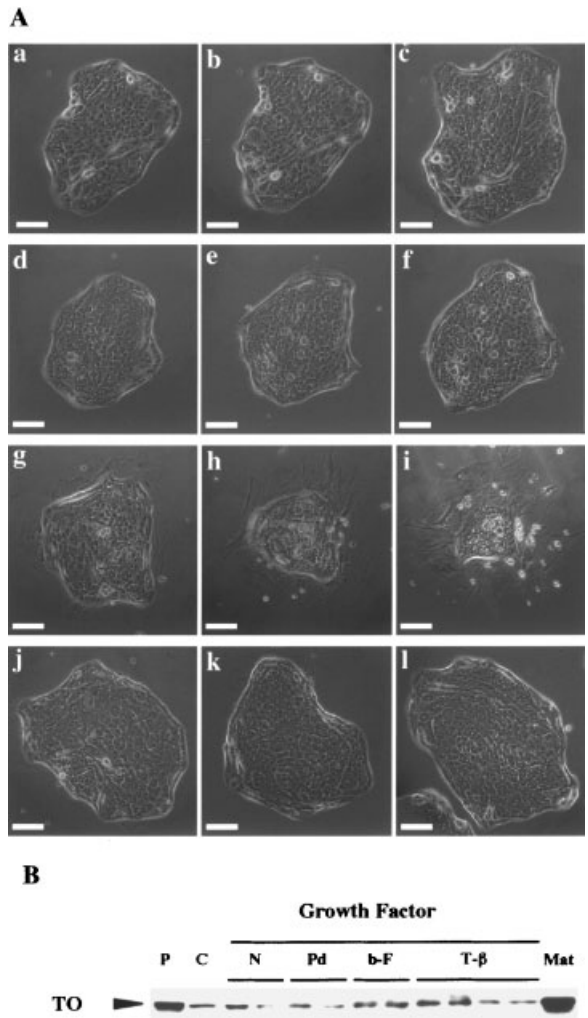


**Fig. 6.** (**A**) Phase-contrast photographs of the colonies treated with growth factors. Isolated colonies were plated on dishes and were cultured in the medium without FBS. Eleven days after replating, the cells were treated with 0.01 ng/ml TGF-β (**a**–**c**), 0.1 ng/ml TGF-β (**d**–**f**), 1 ng/ml TGF-β (**g**–**i**), 0.01 ng/ml NGF (**j**), 0.6 pg/ml PDGF (**k**), or 0.05 pg/ml bFGF (**l**). Fresh growth factors were added to the medium at the time of medium change. The same colony was followed and the images were recorded; at day 3 (a, d, g), day 6 (b, e, h), and day 9 (c, f, i, j, k, l) after the treatment with growth factors. All photos are of the same magnification. Scale bar, 50 μm. (**B**) The expression of TO protein in the cells treated with NGF, PDGF, bFGF, and TGF-β. SHs colonies were replated and cultured in the medium without FBS. Eleven days after replating, the cells were treated with growth factors. Ten days after the treatment, the cells were harvested. Proteins (20 μg/lane) were separated by 10% SDS–PAGE. Lanes show from left: primary hepatocytes at 3 h after plating (P), no treatment (control, c), 0.01 and 0.1ng/ml NGF (N), 0.25 and 2.5 pg/ml PDGF (P), 0.005 and 0.05 pg/ml bFGF (b–F), and 0.001, 0.01, 0.1, 1 ng/ml TGF-β (T-β), and 500 μg/ml Matrigel (Mat).

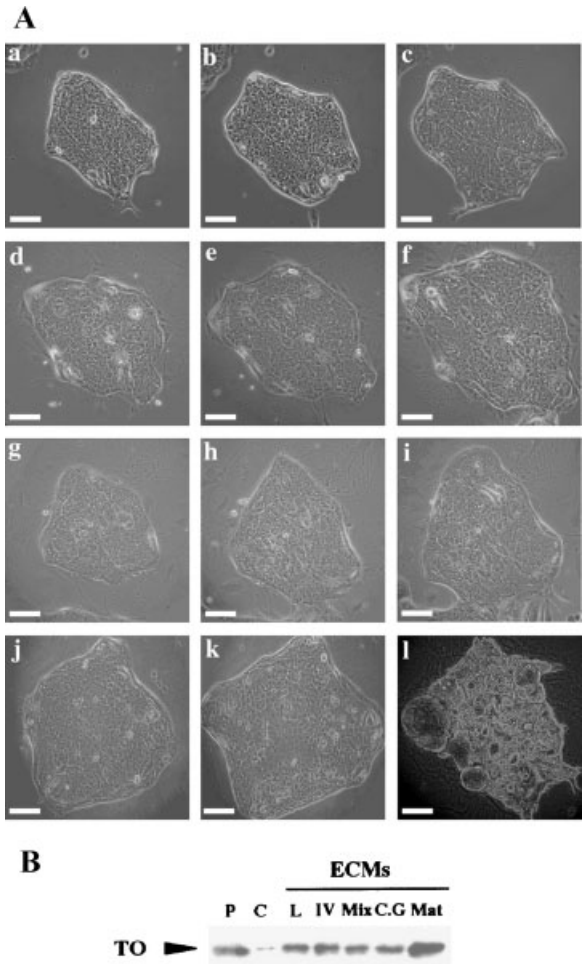


**Fig. 7.** (**A**) Phase-contrast photographs of the colonies treated with laminin, type IV collagen, a mixture of laminin and type IV collagen, and CG. Isolated colonies were plated and cultured in the medium without FBS. Eleven days after replating, the cells were treated with 300 μg/ml laminin (**a**–**c**), 150 μg/ml type IV collagen (**d**–**f**), a mixture of 300 μg/ml laminin and 150 μg/ml type IV collagen (**g**–**i**), CG (**j**, **k**), and 500 μg /ml Matrigel (**l**). The same colony was followed and the images were recorded; at day 3 (a, d, g), day 6 (b, e, h, j), and day 9 (c, f, i, k, l) after the treatment with ECM. All photos are of the same magnification. Scale bar, 50 μm. (**B**) The expression of TO protein in the cells treated with laminin, type IV collagen, a mixture of laminin and type IV collagen, CG, and Matrigel. SHs colonies were replated and cultured in medium without FBS. Eleven days after replating, the cells were treated with 300 μg/ml laminin (L), 150 μg/ml typeIV collagen (IV), a mixture of 300 μg/ml laminin and 150 μg/ml type IV collagen (Mix), CG, and 500 μg/ml Matrigel (M). Ten days after the treatment, the cells were harvested. Proteins (10 μg/lane) were separated by 10% SDS–PAGE. P, primary hepatocytes at 3 h after plating. C, control.

### Growth Activity of SHs Treated with Matrigel

To examine growth activity of SHs treated with Matrigel, immunostaining, and Western blot analysis for PCNA were performed. As shown in Figure 8a, many replated SHs were positive for PCNA. This result meant that many SHs in monolayer colony actively proliferated. However, the ratio of PCNA-positive nuclei to PCNA-negative ones in a colony decreased with the expansion of the colony at the time when some cells became large hepatocytes (Fig. 8b). On the other hand, when the cells were covered with Matrigel, the number of PCNA-positive cells clearly decreased (Fig. 8d). However, the nuclei of the cells in the process of changing their shapes showed the PCNA-positivity,

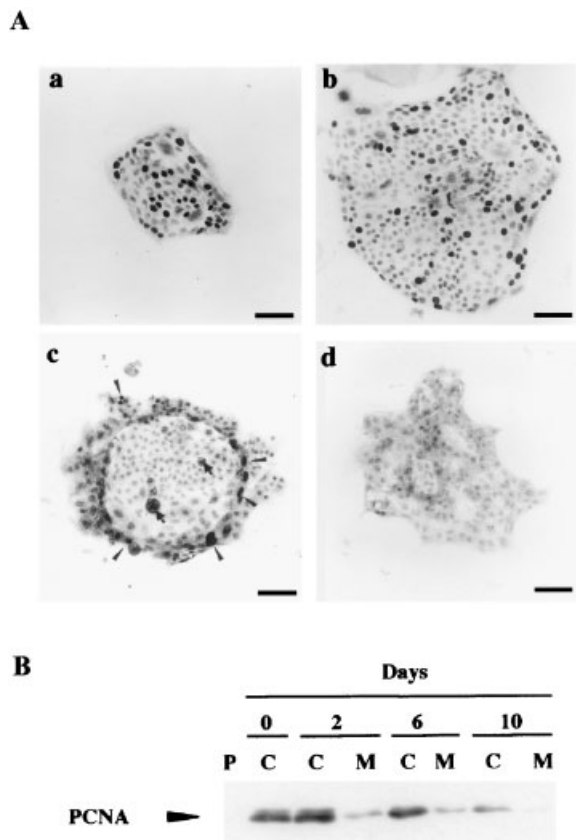


**Fig. 8.** (**A**) Immunocytochemistry for PCNA of the cells in colonies. The colonies were treated with (**a**, **b**) and without Matrigel (**c**, **d**). (a) At day 3 and (b) at day 8 after replating, (c) at day 3, and (d) at day 9 after the treatment with 500 μg of Matrigel. Darkened nuclei are positive for PCNA. The cells were counter-stained with hematoxylin. Arrows in (c) show dead cells and arrowheads show the cells that are rising and positive for PCNA. All photographs show the same magnification. Scale bars, 100 μm. (**B**) Western blot analysis for PCNA proteins in the colonies treated with Matrigel. Nuclear proteins (20 μg/lane) were separated by 10% SDS–PAGE. Isolated SH colonies were plated on collagen-coated dishes cultured in the medium without 10% FBS. Eleven days after replating, the cells were treated with (M) or without (C) Matrigel (500 μg/ml). P, primary hepatocytes at 3 h after plating.

whereas most piled-up cells were not stained (Fig. 8c). As shown in Figure 8B, the expression of PCNA protein was remarkably inhibited in the nuclei of the cells treated with Matrigel. In addition, with time after the treatment, the amount of the protein decreased and the expression was scarcely detected at 10 days after the treatment. This result was similar to that of the immunostaining.

## DISCUSSION

### Morphological Changes and Maturation of SHs

We previously showed that SHs in colonies sometimes changed shape [Mitaka et al., 1999]. In such cases NPCs invaded under the colony and the formation of BM-like structures, which might be reconstituted with ECM produced by NPCs, was observed. On the other hand, many genes of liver-specific proteins are known to be mainly regulated by LETFs and their expression seems to be correlated with hepatic maturation [Birkenmeir et al., 1989; Kuo et al., 1990; Cereghini, 1996; Uzma and Costa, 1996]. Therefore, to investigate whether the alteration of cell shape was correlated with the maturation of SHs and whether BM-like structures could induce the morphological changes of SHs, we first immunocytochemically examined the expressions of LETFs in the primary cultured cells showing various morphologies and, second, examined whether Matrigel could induce the similar morphological changes of SHs. As shown in Figure 1, the sequential expression pattern of LETFs accompanying morphological changes of SHs was observed. When SHs were maintained in a flat monolayer, staining for HNF6, C/EBPα and C/EBPβ proteins was negative in their nuclei, TO expression was quite low, and SDH expression was not induced. On the other hand, when SHs were covered with Matrigel, the cells rapidly changed shape and increased the secretion of serum proteins such as albumin, Tf, $\alpha_1$-AT, and fibrinogen. In addition, the expression of LETFs could be recovered in the cells and the amounts of HNF4α, HNF6, C/EBPα, and C/EBPβ proteins at day 10 after Matrigel addition were near those of proteins in MHs, although the expression of HNF1α and HNF3α was not affected with or without Matrigel. Furthermore, TO was well expressed and SDH could be induced by hormones. Therefore, not only morphological changes from small to large/piled-up but also the synergistic induction of LETFs like HNF4, HNF6, C/EBPα, and C/EBPβ expressions may be necessary for SHs to differentiate into MHs.

### Effects of ECM Components and Growth Factors in Matrigel

We showed that accumulation of ECM could result in morphological changes and the maturation of SHs [Mitaka et al., 1999]. In this experiment Matrigel could induce a change of shape of SHs. To investigate whether induction of those morphological changes was dependent on the individual components of Matrigel or mechanical stress resulting from the overlay of a gel-formed material, we examined the effects of major ECM components, a CG, and growth factors on SH colonies. These individual components did not influence the shape of the colonies shown with Matrigel. Although TGFβ (more than 0.1 ng/ml) had the ability to suppress the growth of SHs as was the case of Matrigel, no differentiation of the cells was induced. On the contrary, the higher concentration of TGFβ resulted in cell death. TGFβ in Matrigel may be not a soluble form but a form binding to ECM. Such different forms may affect the growth and death of SHs. On the other hand, although a large amount of each ECM might possibly induce the morphological changes of SH colonies, the concentration used in the present experiment did not affect the cell shapes. However, ECM could enhance the synthesis of TO proteins compared to the control. Some hepatic differentiated functions may be induced by each ECM, though the degree of the expression was lower than in the cells treated with Matrigel. Furthermore, to exclude the possibility that the morphological changes of SHs resulted from the mechanical stress caused by the overlay of gel-formed materials such as Matrigel, the colonies were covered with a thick CG. Although TO expression was enhanced in the cells, no alteration of the cell shape was observed. However, after the cells were cultured for more than 1 month, they gradually formed cystic structures under the gel (data not shown). Although we have no clear data, the gradual proliferation of NPCs bound to SH colonies and the production of ECM might have caused the cellular changes. These findings suggested that the individual components were insufficient and the combination and/or complex of ECM and growth factors might be required for morphological changes of SHs. The ideal form may be natural

BM. Further experiments will be required to form a compatible bed for the cells similar to BM.

### Formation of Plate-like Structures in Matrigel

It is of interest that SHs and SH-derived large hepatocytes could proliferate and migrate in the Matrigel. As shown in Figure 8, the number of PCNA-positive cells decreased in those colonies and the speed of the colony expansion became slow. However, plate-like structures slowly elongated in Matrigel. Hepatocytes, as is the case with many other cell types, can maintain differentiated functions but do not proliferate in Matrigel [Bissell et al., 1987; Rana et al., 1994]. However, Michalopoulos et al. [1999] showed that MHs isolated from an adult rat liver could form plate-like structures within Matrigel after they were cultured on collagen-coated polystyrene beads in roller bottle for about 2 weeks. They reported that the epithelial cells on beads had characteristics of small MHs and that, after the cells on beads were implanted in Matrigel, the elongation of liver plate-like protrusions was observed. The protrusions consisted of hepatocytes showing cytoplasmic differentiation compared to the cells in the roller bottle. Those phenomena were quite similar to our observations in this experiment. Block et al. [1996] and Michalopoulos et al. [1999] suggested that MHs once lost their differentiated functions to become epithelial cells (they called them "hepatoblasts") and then could redifferentiate to MHs. However, considering our present results, it may be feasible to think that their "hepatoblasts" may be SHs because about 2% of hepatocytes isolated from the adult rat liver are estimated to be SHs [Mitaka et al., 1993].

In summary, although it is unclear how Matrigel can induce the maturation of SHs, our present data suggest that a complex gel-form of ECM like Matrigel and the formation of BM-like structures may result in the morphological changes of SHs that can induce specific LETFs such as HNF4$\alpha$, HNF6, C/EBP$\alpha$, and C/EBP$\beta$. These LETFs may synergistically work for the expression of hepatic differentiated functions. Although we used growth factor-reduced Matrigel, and investigated the effects of the individual major components included in Matrigel, the effects of other possible contaminants can not be ignored. Further experiments will be necessary to examine the exact mechanisms of differentiation/maturation of SHs.

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